BIOMEDICAL ENGINEERING: DEVELOPMENT AND CHARACTERIZATION OF BIOMIMETIC STIMULI SENSITIVE THIN FILMS- DRUG DELIVERY AND TISSUE ENGINEERING APPLICATIONS

KALADHAR.K

for the award of the degree
of

DOCTOR OF PHILOSOPHY

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY
THIRUVANANTHAPURAM 695 012
SEPTEMBER 2007

Dedicated to

My Father And My Little Angel

DECLARATION

I, Kaladhar.K, hereby declare that I had personally carried out the work depicted in the thesis entitled **"Biomedical Engineering: Development and Characterization of Biomimetic Stimuli Sensitive Thin Films- Drug Delivery and Tissue Engineering Applications"** under the direct supervision of Dr. Chandra P.Sharma Scientist-G and Head, Biosurface Technology Division, Biomedical Technology wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India, except where external help sought are acknowledged.

Kaladhar. K

Tele: 0471-2340801
Fax : 0471-2341814

Grams: CHITRAMET
Telex : 0435-6290

श्री चित्रा तिरुनाल आयुर्विज्ञान तथा प्रौद्योगिकी संस्थान
बायो मेडिकल टैक्नोलॉजी विंग
पूजापुरा, तिरुवनन्तपुरम-695 012, इन्डिया

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY
BIO MEDICAL TECHNOLOGY WING
POOJAPPURA, THIRUVANANTHAPURAM-695 012, INDIA
(An Institute of National Importance under Govt. of India)

Ref: ***Date:***.....

CERTIFICATE

This is to certify that **Mr. Kaladhar. K** in the Biosurface Technology Division of this institute, has fulfilled the requirements of the regulations relating to the nature and prescribed period of research for the Ph.D degree of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram. The work relating to his thesis entitled **"*Biomedical Engineering:* Development and Characterization of Biomimetic Stimuli Sensitive Thin Films- Drug Delivery and Tissue Engineering Applications"** was carried out under my direct supervision.

Dr. CHANDRA P. SHARMA
(Research Supervisor)

The Thesis

Entitled

BIOMEDICAL ENGINEERING: DEVELOPMENT AND CHARACTERIZATION OF BIOMIMETIC STIMULI SENSITIVE THIN FILMS- DRUG DELIVERY AND TISSUE ENGINEERING APPLICATIONS

Submitted

by

KALADHAR. K

for

Doctor of Philosophy

of

SREE CHITRA TIRUNAL INSTITUTE
FOR
MEDICAL SCIENCES & TECHNOLOGY

TRIVANDRUM

evaluated and approved
by

Dr. Chandra Prakash Sharma

(Names of Thesis Examiner)

Acknowledgement

I express my sincere gratitude to my professor (thesis guide and the doctoral advisory committee chairman) Dr. Chandra P Sharma for his constant encouragement and support through out my research work. His personality, diplomacy, and generousness influenced me quite a bit. I have benefited to a great deal due to his patient instruction during the experiments and the courses. He has offered and shared a lot of knowledge related to biomaterials, biocompatibility, various other subjects, role of scientist, change of personality required to deliver science etc. Studiously, many discussions and clarifications helped me to think much wider than then I was. His continuous interest and advices, given valuable lessons to look challenging problems and situations in an optimistic manner.

I also wish to express my sincere gratitude to the members of the doctoral advisory committee Dr. G. S. Bhuvaneshwar, Head, BMT Wing, SCTIMST, Dr. K. Ramakrishnan Nair, Head, Plastic surgery, KIMS, Dr. T. V. Kumari, Head, Tissue Culture, BMT Wing, SCTIMST for their constant encouragement and support. Their patience and useful suggestions during the teaching courses related to biomaterials and medical devices, Tissue engineering, Surface Characterization, Surface Modification Techniques and Clinical Trials, helped me to understand the above subjects in detail.

My sincere gratitude is also extended to Dr. K. Mohandas, Director, SCTIMST and Dr. G. S. Bhuvaneshwar, Head, BMT Wing, SCTIMST for providing the facilities, administrative helps and encouragement for fulfilling this long term effort.

I also express my sincere gratitude to CSIR for the (CSIR-SRF), DST and DAAD Germany (for the DST-DAAD (PPP) 2006 fellowship), for the financial support.

I am indebted to Mr. Willi Paul and all my colleagues in my lab as well as many others who have worked in various projects for their help suggestions and interests. I also appreciate Dr. P. Umasankar for providing the calf blood during my experiments from the institutes animal facility and Dr. Yakhmi JV and Dr. Sipra Choudhury, BARC, Mumbai, for helping me to initiate the studies using LB apparatus and surface characterization using AFM.

I extend also my sincere thanks to Prof. Dr. Med. Harald Renz, Director, BMFZ, Phillips University, Germany for encouraging me to develop DST-DAAD (PPP) 2006 programme and providing the facilities related to immunological studies. I also express my sincere gratitude to Prof. Garn H and Dr. Bluemer N for the help during the immunological studies. I am indebted to other colleagues in the lab for their constant encouragement throughout the work.

I also acknowledge Dr. Maya Nanda Kumar Head, Microbiology Division, and SCTIMST and Mr. Pradeep for helping me to do the bacterial adhesion studies. Dr. T. V. Kumari, Head, Tissue Culture, BMT Wing, SCTIMST and Mrs. Usha for helping to do the fibroblast adhesion studies, Dr. Annie John, TEM facility, BMT Wing, SCTIMST for helping to do the TEM and Dr. HK Varma, Head, Ceramics Division, BMT Wing SCTIMST & Mr. Sree Kumar for the SEM studies.

Finally I extent my sincere gratitude to the members of my family, especially my wife, mother and brother for their constant support and encouragement through out my graduate career.

Kaladhar.K

CONTENTS

Page No.

1 Introduction

1.1 Regenerative therapeutic strategies: Need of the hour 1
1.1.1 Miniaturisation the "buzz word" 1
1.1.2 Ideal biomaterial 2
1.1.3 Degenerative diseases current therapeutic strategies 2
1.1.4 Future promises in treatment strategies 3
1.1.5 Surface the culprit 4
1.1.5.1 Role of chemical stimuli 4
1.1.5.1.1 Inorganic stimuli 5
1.1.5.1.2 Organic stimuli 5
1.1.5.2 Role of physical stimuli 6
1.1.5.2.1 Role of surface energetics 7
1.1.5.2.2 Role of surface polarity 8
1.1.5.2.3 Role of size and shape of the surfaces 8
1.1.5.2.4 Stability of the interface 8
1.1.5.2.5 Tensegrity of the surface 9
1.1.5.3 Role of biological stimuli 10
1.1.5.3.1 Proteins 10
1.1.5.3.2 Cells 11
1.1.5.3.3 Lipids 11
1.1.6 The environment 12
1.1.6.1 Friction at the interface 12
1.1.6.2 Water at the interface 12
1.1.7 Renewabilty of the interface 13
1.1.8 Mechanical stimuli vs. chemical stimuli 13
1.2 Inflammatory events at the material- tissue interface 14
1.2.1 Implantation of devices: Site of implantation vs size of implant 14
1.2.2 Events in a open wound 15
1.2.3 Wound healing and tissue regeneration 15
1.2.4 Alteration of wound healing and tissueregeneration by the implant 16
1.2.5 Role of agents in the environment in altering the healing process 16
1.2.6 Inflammatory events at cellular level 17
1.2.7 Trans-membrane signaling 18
1.2.8 Cancer cells and embryogenesis, cell response in two different saga 18
1.2.9 Mimicking cell response in cancer metastasis and embryogenesis 19
1.2.10 Cross-talc between growth factor and mechano receptors 19
1.2.11 The challenge of mimicry of embryogenesis in open wounds 20

1.3 Surface modification of materials 20
1.3.1 Modification of surfaces with supramolecular assemblies a lime light for miniaturisation- current strategies 22
1.3.2 Biomimetics of cell membrane – an interesting approach to reduce biofouling using thin films 23
1.3.2.1 Outer cell membrane mimetics 23
1.3.2.2 Inner cell membrane mimetics 24
1.3.2.3 Mimesis of membrane lateral stabilization 24
1.3.2.4 Thin Organic Films 25
1.3.2.4.1 Self-assembly of surfactants 25
1.3.2.4.2 Langmuir-Blodgett Film Deposition 27
1.3.3 Self-assembly of supramolecular architectures; Extension of colloidal knowledge towards development of thin films by supramolecular assembly 29
1.3.4 Self-assembly of proteins 30
1.3.5 Proteins like architectures through self-assembly 30
1.4 Miniaturization of biomaterials and devices and surface modification: Bottom-up vs. Top-down approach in biomaterial field 31

Relevance and objective of the thesis 33
Synopsis 34

2 Materials and methods 37

Materials 37
Development of materials and physico-chemical characterization 38

2.1 Development of substrates for the surface modification 38
2.1.1 Films 38
2.1.1.1 PolyC, PS & PMMA films 38
2.1.1.2 Derivatization of PMMA films for covalent immobilization of laterally stabilized monolayer 39
2.1.1.3 Cross linked PVA (cPVA) 39
2.1.1.4 Preparation of chitosan (CHIT) bare and cross linked films 39
2.1.1.4.1 CHIT films cross linked with the drug 40
2.1.2 Microspheres 40
2.1.2.1 Preparation of porous CHIT microspheres 40
2.1.2.1.1 Ionic cross linking of the microspheres with DIC 40
2.1.3 Nanoparticles 41
2.1.3.1 Nanoparticles of pendant polymer of PVA 41
2.1.3.1.1 Selection of ligand for Pendant PVA 41
2.1.3.1.1.1 Extraction of structures with Arguslab 41

2.1.3.1.1.2 Docking using Hex 42
2.1.3.1.2 Preparation of Pendant PVADIC 42
2.1.3.1.3 Preparation of the nanoparticles out of the pendant polymer by self assembly 42
2.1.3.1.3.1 Preparation of ceramic nanocrystals and drug condensation inside the nanoparticles of pendant polymer 43
2.1.4 Liposomes 43
2.1.4.1 Liposome of mixture of lipids of phospholipids, cholesterol and galactocerebroside 43
2.1.4.2 Liposome of mixture of lipids of phospholipids, cholesterol and galactocerebroside albumin and other anchors 44
2.1.4.3 Preparation of Albumin self- assembled liposomes 44
2.1.5 Preparation of Liposome delivering matrices (Lipomat) 45
2.1.6 Polymeric gels 45
2.1.6.1 Polymeric gels of PVA and Pendant PVA 46
2.2 Development of lipid thin films for surface modification of the substrate 46
2.2.1 Development of Co-solvent system 46
2.2.1.1 Selection of parent solvent 46
2.2.1.2 Solubility studies of lipids 46
2.2.1.3 Incorporation of other macromolecules into lipid systems 47
2.2.2 Interfacial studies of the lipid films using LB trough facility 47
2.2.2.1 Air/water interfacial studies (Intramolecular interactions) 48
2.2.2.1.1 Simple, mixture of lipids incorporated with anchors 48
2.2.2.1.2 Amphiphilic poymer 50
2.2.2.2 Air/solid transfer studies (Intramolecular interactions) 50
2.2.2.2.1 Simple and mixture of lipids 50
2.2.2.2.2 Lateral stabilization by chemical bonding 51
2.2.3 Transfer of the nanoparticle monolayer into the polymer substrates 51
2.2.4 Development of methods to modify different kind of substrates 51
2.2.4.1 Films 52
2.2.4.1.1 In situ method from air/ water interface 52
2.2.4.1.2 Spreading and solvent evaporation method 52
2.2.4.2 Microspheres 52
2.2.4.2.1 Solvent evapouration by freeze drying in the case of Lipomat 53
2.2.4.2.1.1 Biofriction strudies of microspheres by sliding method 53
2.2.4.3 Nanoparticles 53
2.2.4.3.1 Monolayer Coating on Nanoparticles by rehydration 53
2.2.4.4 Polymeric gels 54
2.2.4.4.1 Monolayer coating on polymeric gels by layering, solvent evapouration and vortexing 54

2.2.4.4.1.1 Biofriction studies by protrusion method 54
2.2.5 Stability studies of the monolayer 54
2.2.5.1 Under static conditions 55
2.2.5.2 Under shear conditions 55
2.2.6 Pore volume measurements 55
2.2.7 Sedimentation studies 55
2.2.8 Drug content and drug release studies 56
2.2.8.1 Insulin release studies 56
2.2.8.2 Diclofenac release studies 56
2.2.8.3 Diclofenac content of the supernatant of pendant polymer 56
2.2.9 Optical microscopy 57
2.2.9.1 Liposomes 57
2.2.9.2 Adhered cells 57
2.2.10 Stereo zoom microscopy 57
2.2.11 Goniometry: Contact angle studies 57
2.2.12 UV/ Vis spectroscopy 58
2.2.12.1 Turbidity studies 58
2.2.12.1.1 Turbidity studies indicating the conformational change of proteins 59
2.2.12.2 Protien content evaluation 59
2.2.12.3 Diclofenac content evaluation 59
2.2.12.4 Absorption spectra of nanoparticles 59
2.2.13 Flourimetry 59
2.2.14 FTIR Spectroscopy 60
2.2.15 Dynamic light scattering 60
2.2.15.1 Size of PVA DIC nanoparticles 61
2.2.15.2 Size of the liposomes 61
2.2.15.3 Melting curve of nanoparticles 62
2.2.16 Atomic force microscope (AFM) 62
2.2.17 Scanning electron microscopy 63
2.2.17.1 Microspheres 64
2.2.17.2 Nanoparticles 64
2.2.17.3 Adhered plateletes 64
2.2.18 Transmission electron microscopy (TEM) 64
2.2.18.1 Morphological examination of the liposomes 65
2.2.19 Differential Scanning calorimetry (DSC) 66
2.2.19.1Evaluation of the self-assembled albumin by DSC 66

Biorecognition studies

2.3.1 Calcification studies 67
2.3.2 Protein adsorption studies 68
2.3.2.1 Protein adsorption/desorption studies from mixture of

proteins using SDS- PAGE 68
2.3.2.2 Preparation of samples for conformational studies of proteins using CD spectroscopy 68
2.3.2.2.1 Preparation of samples for CD spectroscopy: 70
2.3.2.2.1.1 Albumin intercalated into the lipid layer 70
2.3.2.2.1.2 Bound protein to the ligand 70
2.3.2.3 Spectral analysis using K2d algorithm 71
2.3.2.4 Quantitative evaluation of the proteins by ELISA 71
2.3.3 Blood cell adhesion studies 71
2.3.3.1 Platelet adhesion studies 71
2.3.3.2 Leukocyte adhesion studies 72
2.3.3.3 Erythrocyte adhesion studies 72
2.3.3.4 Platelet activation studies 72
2.3.3.5 Macrophage adhesion and activation studies 72
2.3.3.5.1 Harvesting and subculturing of the macrophage cell line 73
2.3.3.5.2 Cell seading and experimentation of substrates 73
2.3.3.5.3 Evaluation of adhesion strength after gentle washing 73
2.3.3.6 Polymorphonuclear cell adhesion and activation studies 74
2.3.3.6.1 Cell isolation and harvesting 74
2.3.3.6.2 Cell seading and experimentation 74
2.3.3.6.3 Cell proliferation studies by BrdU uptake assay by ELISA 75
2.3.4 Fibroblast Cell Adhesion Studies 75
2.3.5 Bacterial Adhesion Studies 75

3 Results and Discussion 77

3.1 Developing the co-solvent system 77
3.1.1 Selection of the parent solvent 78
3.1.2 Developing the co-solvent system for heterogeneous lipids 78
3.1.3 Optimization of co-solvent system for the incorporation of macromolecules 80
3.2 Air/water interfacial studies 81
3.2.1 Monolayer behavior of the pure lipids at the air/ water interface 81
3.2.2 Monolayer behavior of mixture of lipids at the air/ water interface: Condensing effect of cholesterol 83
3.2.3 Monolayer behavior with the incorporation of PE: Effect of head group structure 85
3.2.4 Mimesis of lateral stabilisation of membrane lipids using proteins: monolayer behavior of mixture of lipids & macromolecules at the air/ water interface 87
3.2.4.1 Conformation of the incorporated albumin into the lipid layer 89
3.3 Air/solid transfer studies 91
3.3.1 Optimization of monolayer deposition parameters 91

3.3.1.1 Speed vs transfer ratio 92
3.3.1.2 Substrate Vs transfer ratio 93
3.3.1.3 Composition vs transfer ratio 94
3.4 Polarity of the modified surfaces 95
3.4.1 Method Vs Polarity 95
3.4.2 Packing density and orientation Vs Polarity 97
3.5 Stability of the monolayer on substrates 97
3.5.1 Stabilization strategy-1: lateral stabilization with incorporation of proteins 99
3.5.2 Stabilization strategy- 2 effect of physical stabilization using pendant polymer 102
3.5.2.1 Selection of the pendant group 102
3.5.2.1.1 Albumin binding avidity studies 103
3.5.2.1.1.1 In silico docking studies between albumin and free drug 103
3.5.2.2 Pendant group immobilization on polymers by ionic linkage 105
3.5.2.2.1 In vitro binding avidity studies between albumin and bound DIC 108
3.5.2.3 Pendant group immobilization on polymers by covalent linkage 109
3.5.2.3.1 Selection of the polymer to form the backbone 109
3.5.2.3.2 Preparation of PVADIC pendant polymer 109
3.5.2.3.2.1 Liposome binding avidity of the pendant polymer 110
3.5.2.3.2.2 Albumin binding avidity of PVADICa with albumin in presence of insulin 112
3.5.2.3.3 Formation of nanoparticles out of pendant polymer 114
3.5.2.3.4 Air/water interfacial studies of the pendant polymer 115
3.5.2.3.5 Morphology of nanoparticles and their 2D films 115
3.5.2.3.6 Air/ solid transfer studies of nanoparticles and lipid layers 117
3.5.2.3.7 Thermoresponsiveness of the 2D film of nanoparticles 118
3.5.2.3.8 Conformation of the bound albumin to the pendant DIC 119
3.5.2.3.9 Stability of lipid monolayers over the nanoparticles 121
3.6 Surface Profilometry 122
3.6.1 Supported OCMC monolayer 122
3.6.2 Surface profilometry with the incorporation of PE: Effect of head group structure 123
3.6.3 Surface profilometry with the incorporation of macromolecules 125
3.7 Calcification studies 127
3.7.1 Effect of orientation and packing density 127
3.7.2 Effect of incorporation of PE 129
3.7.3 Effect of lateral stabilization 130
3.8 Protein adsorption studies 131
3.8.1 Effect of orientation and packing density 131

3.8.2 Effect of phosphatidylethanolamine 134
3.8.3 Effect of lateral stabilization 135
3.9 Blood Cell Adhesion Studies 137
3.9.1 Effect of orientation of the supported lipid layer 137
3.9.2 Effect of phosphatidylethanolamine 138
3.9.3 Effect of lateral stabilization 139
3.10 Platelet activation studies from PRP 140
3.11 Macrophage behaviour at the material surface 143
3.11.1.I Effect of serum concentration on early cell response 144
3.11.1.2 Effect of surface modification on adhesion strength 147
3.11.1.3 Effect of cell number and serum concentration 149
3.11.1.4 Effect of stimulation with respect to serum concentration 150
3.11.2 Secretory profile of macrophages 152
3.11.2.1.1 Normal conditions 153
3.11.2.1.1 TNF-a production 153
3.11.2.1.2 IL-10 production 154
3.11.2.1.3 IL-6 production 155
3.11.2.2 Effect of stimulation 156
3.11.2.2.1 TNFa production 156
3.11.2.2.2 IL-10 production 157
3.11.2.2.3 IL-6 production 158
3.12 Polymorphonuclear cell behaviour at the material surface 160
3.12.1 PMN adhesion studies 160
3.12.1.1 Effect of stimulation 160
3.12.2 PMN Secretory profile 161
3.12.2.1 IFNg production 161
3.12.2.2 IL10 production 162
3.12.3 PMN proliferation 163
3.13 Fibroblast adhesion studies: Effect of phosphatidylethanolamine 164
3.14 Bacterial adhesion Studies: Effect of phosphatidylethanolamine 167
3.15 Drug release across the monolayers 168
3.15.1 Small molecules loaded into nanoparticles 168
3.15.1.1 Sedimentation dispersibility studies 169
3.15.1.2 Photoresponsiveness of protien like nanoparticles 173
3.15.1.3 Drug Condensation 174
3.15.2 Macromolecular release across the monolayers 176
3.16 Biofriction at the lipid interface 180

4. Conclusion 182

4.1 Development of thin film of cell membrane components 183
4.1.1 Effect of orientation and packing density of the lipid monolayer 183
4.1.2 Effect of head group structure 185

4.1.3 Effect of lateral stabilization 187
4.1.4 Drug release across the monolayers 188
4.1.5 Biofriction at the lipid interface 189

5. Applications 190

5.1 Albumin self assembled liposomes 190
5.1.1Drug immobilization into the polymer 192
5.1.2 Gross structure of the albumin coated liposomes 192
5.2 Thermoresponsiveness of the amphiphilic polymer 193

6. Future plan 195

6.1 Modification of substrates for blood compatible applications 195
6.2 Modification of substrates for tissue compatible applications 196
6.3 Modification of substrates for drug delivery applications 196

LIST OF FIGURES

1.1.5.1	Mechanism of biological interaction with the material surface	4
1.2.1.1	Implantation of devices of different dimension in to the wounds of different length	14
1.2.2.1	Surface activation of cellular responses	15
1.2.3.1	Time based phenomena at an inflammatory site	16
1.2.5.1	Different acute inflammatory cascades during enhanced vascular permeability	17
1.2.9.1	Erk1/2 activation leading to cyclin D1 expression by collaborative cross-talk between integrins and growth factor receptors	19
1.3.1	Gross picture demonstrating the biological interaction at biomaterial surfaces and current surface modification strategies	21
1.3.2.4.2.1	π- A isotherm	27
1.3.2.4.2.2	Deposition of the monolayer into polymer substrates in the X, Y or Z direction	28
2.2.2.1	LB trough facility	48
2.2.11.1	Goniometry	57
2.2.15.1	Dynamic Light Scattering	60
2.2.16.1	The primary components of the light lever atomic force microscope	62
2.2.17.1	SEM	63
2.2.18.1	TEM	65
2.2.19.1	DSC	66
2.3.2.3.2	CD	69

3.1.2.1	Solubility of different lipids in Co-Solvent system	79
3.1.3.1	Solubility of different proteins in the co-solvent system	81
3.2.1.1	π-A isotherm of pure lipids	82
3.2.2.1	π-A isotherm of (PC: Chol) (1:0.7) (A), (PC: Chol) (1:0.35)(B), (PC:Chol:GalC)(1:0.35:0.125) (C)	84
3.2.3.1	π-A isotherm (PC:Chol:GalC:PE) 1:0.35:0.125:0.0725(A), with PE 0.145(B), 0.29 (PCMC) (C)	86
3.2.4.1	π-A isotherm: OCMC- A (A), CMC- AH (B), OCMC -AHP (C)	87
3.2.4.1.1	Structure of the Albumin molecule in lipid bilayer	90
3.3.1.1.1	Speed vs transfer ratio on deposition of the monolayer on hydrophobic polymer substrate	92
3.3.1.3.1	Modified surface Vs Transfer ratio	94
3.4.1.1	Polarity of the lipid monolayers, deposited by different methods on hydrophobic PolyC films	96
3.4.2.1	Polarity of the surfaces after lipid monolayer deposition	97
3.5.1	Stability of the monolayer on substrates under static conditions	98
3.5.1.1	Stability studies under low shear conditions	100
3.5.2.1.1.1.1	Drug docking with Albumin	103
3.5.2.2.1	Modification of the chitosan	106
3.5.2.2.1.1	Densitometric diagram of the protein adsorption- desorption studies using mixture of proteins	108
3.5.2.3.2.1	Immobilization of the drug into the polymer	110
3.5.2.3.2.2.1	DSC of the bound albumin to the PVADICa complex	112
3.5.2.3.3.1	Melting curve of the nanoparticles	114
3.5.2.3.4.1	Thermo responsive behavior of thin solid films of the nanoparticles	115
3.5.2.3.5.1	SEM of the PVADICb nanoparticles (NP) and conformational change with respect to temperature	116
3.5.2.3.5.2	SEM of the nanoparticles (A), 2D film of nanoparticles (B)	116

3.5.2.3.6.1	Transfer of the monolayers on substrates Modified with Thermoresponsive polymer(PVADICc) (TRP) further modified with the lipid monolayer	117
3.5.2.3.7.1	Contact angle of the nanoparticle coated surface with respect to temperature conditions	118
3.5.2.3.8.1	CD spectroscopy of the bound albumin	119
3.5.2.3.9.1	Schematic representation of laterally stabilized monolayer with the pendant polymer	121
3.6.1.1	AFM image of the dried supported OCMC monolayer	123
3.6.2.1	Surface profilometry of lipid films (PC:Chol:GalC:P E) (1:0.35:0.122: 0 (A), 0.0725(B), 0.145(C), 0.29(D) supported using PolyC substrate	124
3.6.3.1	AFM image of supported laterally stabilized bilayers	126
3.7.1.1	Calcification studies	128
3.7.2.1	Calcification studies from metastable solutions [PolyC bare (A), modified with OCMC(B), PCMCc (C)	129
3.7.3.1	Calcification studies.PMMA Bare (A). PMMA modified with, OCMC- AH (B), OCMC- AHP (C)	130
3.8.1.1	Densitometric diagram of the protein adsorption- desorption studies on lipid modified polymer surfaces, using mixture of proteins	132
3.8.2.1	Densitometric diagram of the protein adsorption- desorption studies on lipid modified polymer surfaces, using mixture of proteins: PE	134
3.8.3.1	Densitometric diagram of the protein adsorption- desorption studies on lipid modified polymer surfaces, using mixture of proteins:Lateral stabilization	135
3.9.1.1	In vitro blood cell adhesion studies: orientation	137
3.9.2.1	Blood cell adhesion studies from washed cells:PE	138
3.9.3.1	Blood cell adhesion studies on lipid modified polymer surfaces, using washed cells:Lateral stabilization	139
3.10.1	Platelet adhesion studies on lipid modified polymer surfaces	140
3.10.2	Platelet activation studies on lipid modified polymer surfaces	141

3.11.1.1.1 Macrophage (RAW 264.7) ($1X10^5$ cells /ml) adhesion studies on lipid modified surfaces 144

3.11.1.1.2 Cell spreading (RAW 264.7) ($1X10^5$ cells /ml) on the modified surfaces 145

3.11.1.1.3 Macrophage (RAW 264.7) ($1X10^5$ cells /ml) cell colony formation at different time points 146

3.11.1.2.1 Macrophage (RAW 264.7) ($1X10^5$ cells /ml) adhesion studies on lipid modified surfaces (1% serum) :Effect of washing 147

3.11.1.2.2 Number of macrophage (RAW 264.7) ($1X10^5$ cells /ml) retained after 24hrs 148

3.11.1.3.1 Macrophage (RAW 264.7) phenotype changes with respect to cell number 149

3.11.1.4.1 Macrophage (RAW 264.7) ($1X10^4$ cells/ml) phenotype changes with stimulation (LPS) (100µl of lipopolysaccharide endotoxin (LPS, 1µg/ml)) on modified surfaces (1%serum). 150

3.11.1.4.2 Macrophage (RAW 264.7) ($1X10^4$ cells/ml) phenotype changes with stimulation (LPS) (100µl of lipopolysaccharide endotoxin (LPS, 1µg/ml)) on modified surfaces (10%serum) 152

3.11.2.1.1.1 Macrophage (RAW 264.7) ($1X10^5$ cells/ml) secretory (TNFa) profile(1% serum) 153

3.11.2.1.2.1A Macrophage (RAW 264.7) ($1X10^5$ cells/ml) secretory (IL-10) profile (1% serum) 154

3.11.2.1.2.1B Macrophage (RAW 264.7) ($1X10^5$ cells/ml) secretory (IL-10) profile (10% serum) 154

3.11.2.1.3.1A Macrophage (RAW 264.7) ($1X10^5$ cells/ml) secretory (IL-6) profile (1% serum) 155

3.11.2.2.1.1 Macrophage (RAW 264.7) ($1X10^4$ cells/ml) secretory (TNFa) profile with stimulation (LPS) (100µl of lipopolysaccharide endotoxin (LPS, 1µg/ml)) on modified surfaces (10%serum) 156

3.11.2.2.2.1 Macrophage (RAW 264.7) ($1X10^4$ cells/ml) secretory (IL-10) profile with stimulation (LPS) (100µl of lipopolysaccharide endotoxin (LPS, 1µg/ml)) on modified surfaces (10%serum) 157

3.11.2.2.3.1 Macrophage (RAW 264.7) ($1X10^4$ cells/ml) secretory (IL-6) profile with stimulation (LPS) (100µl of lipopolysaccharide endotoxin (LPS, 1µg/ml)) on modified surfaces (10%serum) 158

3.12.1.1.1 PMN from mouse spleen ($1X10^6$ cells/ml), phenotype changes with stimulation (PMA/ Ionomycin) on modified surfaces (10% serum) 160

3.12.2.1.1	Secretory Profile (IFNg) of PMN from mouse spleen ($1X10^6$ cells/ml), with stimulation (PMA(10μg/ml)/ Ionomycin (10ng/ml)) on modified surfaces (10% serum)	161
3.12.2.2.1	Secretory Profile (IL-10) of PMN from mouse spleen ($1X10^6$ cells/ml), with stimulation (PMA(10μg/ml)/ Ionomycin (10ng/ml)) on modified surfaces (10% serum)	162
3.12.3.1	BrdU uptake by the PMN cells after 24hrs (10% serum)	163
3.13.1	Fibroblast (L929) ($1X10^3$ cells/ml) cell line proliferation studies on lipid modified polymer surfaces, microscopy at different time intervals	165
3.13.2	Fibroblast proliferation studies on supported lipid surfaces	166
3.14.1	Bacterial adhesion studies on supported lipid surfaces. Escherichia coli (I), Staphylococcus aureus (II)	167
3.15.1.1.1	Dispersibility of the nanoparticles	170
3.15.1.2.1	Photochemical evaluation of the nanoparticles	174
3. 15.1.3.1	Drug condensation to the nanodomains of the protein like nanoparticles	176
3.15.2.1	Blend of microspheres which can deliver mixture of stimuli both soluble and insoluble stimuli for tissue engineering applications	178
3.15.2.2	Insulin release profile out of the liposome delivering matrix	179
3.15.2.3	The hydration of microspheres modified with lipid layers	179
3.16.1	Extrudability of polymeric gel	181
3.16.2	Movement of microsphere on wet glass surface	182
5.1.1	TEM of the albumin coated liposomes	193
5.2.1	Macrophage cell removal after changing the temperature from 25 to 10°C for 10min and washing with sterile PBS	195
5.2.2	Macrophage Cell layer pealing off, after changing the temperature from 25 to 10°C for 5min	195

LIST OF TABLES

3.2.4.1.1	Secondary structure of albumin in the lipid layer	90
3.3.1.2.1	Substrate Vs Transfer ratio	93
3.5.2.1.1.1.1	Minimum free energy of the docked albumin molecules	104
3.5.2.3.2.1.1	Effect of polymer coating on size of liposome	111
3.5.2.3.8.1	Secondary structure of the albumin molecule (%)	120
3.14.1	Colony forming assay	168
3.15.2.1	Structure of lipomat	176

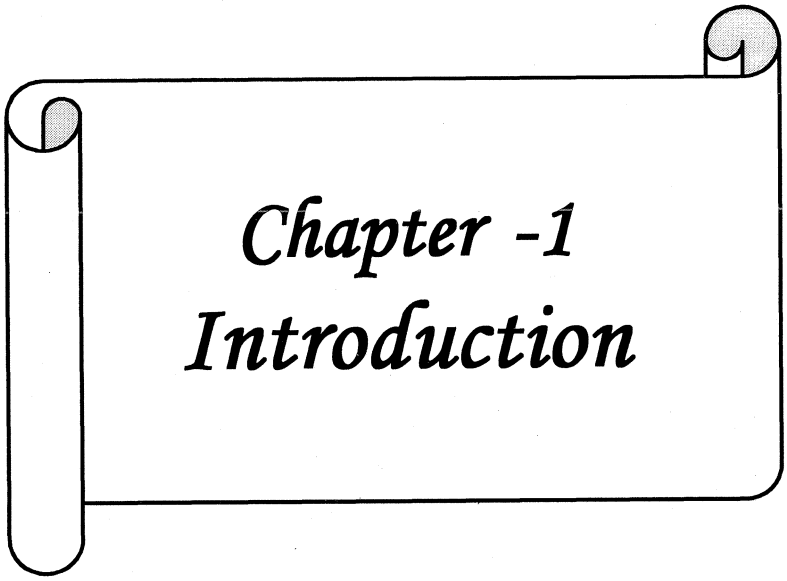
LIST OF SCHEMES

3.5.1.1	Schematic representation of Stabilized supported bilayer	99
3.5.1.2	Reaction scheme of the components, which laterally stabilizes the bilayer on PMMA surface	99
3.15.2.3.1	Macromolecular release by the microspheres	179
4.1.1.1	Schematic representation of the proposed orientation of the deposited mono and bilayers on hydrophobic PC polymer surface and their protein as well as blood cell interaction	184
4.1.2.1	Cell interaction to the phospholipid monolayer supported polymer substrates	186
5.1.1	Schematic representation of the albumin self assembled liposomes	191

ABBREVIATIONS

2D	-	Two Dimensional
3D	-	Three Dimensional
Å	-	Angstrom
Acet	-	Acetone
AFM	-	Atomic Forcee Microscopy
Alb	-	Albumin
CD	-	Circular Dichroism
CF	-	Chloroform
CH	-	Cyclo hexane
CHIT	-	Chitosan
Chol	-	Cholesterol
CMC	-	Critical Micellar Concentration
DAPEG	-	Diaminopolyethylene glycol
DCM	-	Dichloromethane
DIC	-	Diclofenac
DIW	-	Deionised water
DSC	-	Differential Scanning Calorimetry
ECM	-	Extra Cellular Matrix
EDC	-	Ethylene Divinyl carbodiimide
ELISA	-	Enzyme Linked Immuno Sorbent Assay
FTIR	-	Fourier Transform Infra Red
GalC	-	Galactocerebroside
GL	-	Glycolipid
Hep	-	Heparin
IL	-	Interleukin

Indo	-	Indomethacin
Iono	-	Ionomycin
LB	-	Langmuir Blodgett
LPS	-	Lipopolysaccharide
MW	-	Milli Q Water
n- but	-	n-Butanol
NaOH	-	Sodium Hydroxide
n-Hept	-	n- Heptane
PC	-	Phosphatidylcholine
PE	-	Phosphatidylethanolamine
PF	-	Paraformaldehyde
PL	-	Phospholipid
PMA	-	Phorbol Myristic acid
PMMA	-	Poly methyl methacrylate
PMN	-	Polymorphonuclear cells
PolyC	-	Polycarbonate
PS	-	Polystyrene
PTFE	-	Poly Tetra Flouro Ethylene
PVA	-	Poly vinyl alcohol
SDS-PAGE	-	Sodium Dodecyl Sulphate- Poly Acrylamide Gel Electrophoresis
SEM	-	Scanning Electron Microscopy
TEM	-	Transmission Electron Microscopy
TNFa	-	Tissue Necrosis Factor-a
TSF	-	Thin Solid Film
UV-VIS	-	Ultra Violet- Visible
π- A	-	Pressure- Area



Chapter -1
Introduction

INTRODUCTION

1.1 Regenerative therapeutic strategies : Need of the hour

The treatment strategies for degenerative diseases are passing through a transition period, in particular, to address the challenging issues related to patient compliance, socio-economic balance and therapeutic efficiency etc. Recently "miniaturization" has emerged as a buzz word to provide new direction and solve few of the issues related to technology improvement.

1.1.1 Miniaturization the "buzz word"

Miniaturization of devices has the potential benefit of enhancing the treatment efficiency in regenerative therapeutic strategies. The success story of developing miniaturized nano-systems in allied fields like electronics and computing, along with the advances in biological sciences, leads the facet of exploring nano-devices for advanced therapeutic applications. Supramolecular assemblies of biomaterials with certain order and defined functional properties, in 2D and 3D form, play an important role for an optimum performance, through the "intelligent" intervention with altered environmental conditions, which paved the

platform for this facade. Unearthing the potentials of existing biomaterials at nano dimensions and its value addition has incredible prospect in this field. This can be achieved with a bottom up, top down or post synthetic modification of the materials and devices.

1.1.2 Ideal biomaterial

Ideally, biomaterials (for e.g. ceramics, metals, natural products, composites, and polymers) has to be biocompatible (inert or active) but essentially, pharmacologically inert, nontoxic, easily fabricable, sterilisable, stable, non-carcinogenic and economical[1]. This has been explored for blood and tissue compatible applications both internally and externally[2]. Clinically they become part of drug delivery systems, diagnostic agents, prosthetics, tissue engineering scaffolds and combination products needed to treat or diagnose various stages of degenerative diseases and pathological conditions. Many of the degenerative diseases of tissues or organs lead to their functional loss, and further local to systemic, immune and inflammatory communication cascades. Where, age, sex, and other disease conditions cause further complications to this scenario. Therefore "replacing" the diseased tissue with medical devices or artificial organs comprised of biomaterials or tissue counter parts (allogenic and xenogenic) assisted with drug therapy and intensive patient monitoring, have been the current treatment methodology.

1.1.3 Degenerative diseases & current therapeutic strategies

In the case of degenerative diseases affecting blood vessels[3,4] (during aneurismal disease, advanced atherosclerosis, traumatic injury) or heart valves[5] (during symptomatic valvular heart disease), surgical intervention and replacing the tissue with either synthetic or allogenic and xenogenic tissue substitutes (Eg:- cephanous vein and PTFE Grafts as well as allogenic and synthetic valves) is the only alternative. Which have certain disadvantages such as pseudointimal hyperplasia, and early thrombosis in the former and lesser half-life in the later

case. While in the case of functional organs like liver[6,7] (during hepatic encephalopathy), kidney[8,9] (chronic renal failure) or pancreas[10,11] (diabetes) total organ transplantation (liver and kidney) or functional restoration through mechanical means (dialysis of kidney, drug or hormone therapy in the case of pancreas) are the current approaches. This is affected by the scarcity of organs as well as tolerance induced by the prolonged drug therapy. Apart from that substitution is often unsuccessful or impossible at certain regions, where correction is needed than replacement of the organs (urinary bladder, and heart tissue etc.), and the tissue counter parts are the suggested alternative[12]. Tissue engineering and cell therapeutics are considered to be the future prospective in this direction.

1.1.4 Future promises in treatment strategies

The current tissue engineering strategies are either partial functional restoration of tissues as in the case of artificial pancreas or surface modification using endothelialisation. However immune reaction to the xenogenic tissue affects the patency of the implant. In this direction, allogenic or immune protected in vitro cultured tissue grafts are promising. Most interestingly greater success has been achieved with an alternative strategy of tissue regeneration through regenerative medicine with the help of devices at the site of injury. For that the device has to satisfy the following requirements, (1) Minimum invasive intervention of the inflammatory site, (2) Miniaturization of devices with high functional aspect ratio (3) Reduced non-specific biological interaction (4) Progressive and dynamic control of immune and inflammatory cascades. Development of "combination products" of "intelligent materials" and "bioactive agents" with "dynamic controlled ligand supplementation" and "chimeric-morphoneogenesis" with the help of porous scaffolds or injectable nano delivery systems[13] helps in regeneration of the diseased tissue or organ where "repair than replace" has become the slogan of the regenerative therapeutic strategies for degenerative diseases. Genetic engineering is another suggested alternative for reducing the immune reaction to the above mentioned therapeutic agents.

1.1.5 Surface the culprit

The major problem associated with these medical devices in the biological environment is performance failure due to non-specific biological interactions (Figure-1.1.5.1). This is regulated by the adsorbed proteins and the pathological cells at the material surface.

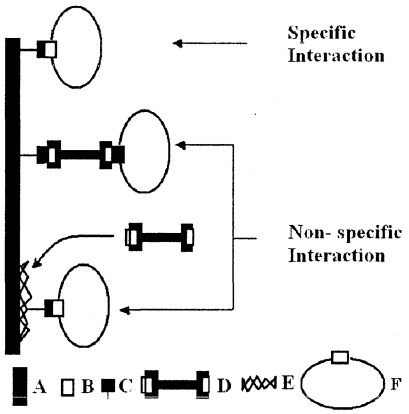


Figure-1.1.5.1: Mechanism of biological interaction with the material surface; Gross cellular activation processes. Substrate (A), Receptor (B), Ligand (C), Protein (D), Protein with altered conformation (E), Cell (F)

The chemical, physical or biological stimuli in the form of, soluble or insoluble ligand induces chemotaxis or mechanotaxis of the cells. The extent of phenotypic and genotypic changes in the cells depends upon the persistence and amplification of this stimulus. Further dynamic environmental changes regulate the structure and sequence of cellular interactions and organ regeneration.

1.1.5.1 Role of chemical stimuli

The chemical stimuli at the surface are due to structural components of the biomaterials or medical devices. For convenience we can classify the chemical stimuli into inorganic and organic stimuli.

1.1.5.1.1 Inorganic stimuli

Many devices have inorganic atoms or ions as their structural components. For eg. In the case of ceramic containing devices highly ionic, inorganic atoms are available in soluble or insoluble form. Many inorganic ions play pivotal role in cell signaling either in the free form or as its derivatives. Soluble calcium and phosphate derivatives are important second messengers for many hormones or enzymes in intracellular signaling processes; at the same time they also form structural component of bone tissue. The calcium ions helps in wound healing, and reduces magnesium and manganese mediated adhesion and proliferation of tumor cells [14], however, their direct action through transcellular receptors or ion channels, on activation of initial pathological events is unknown [15]. Clinically, enhanced vascular calcification during elevated calcium concentration in end stage degenerative diseases has been observed[16]. Calcification of cardiac devices is an important failure mode for heart valve and other cardiac devices. At the same time they form components of bone substitutes and improve hard tissue compatibility. Conclusively ions interact diversely with diverse biological environment and elicit various biological responses[17].

1.1.5.1.2 Organic stimuli

Many devices with polymeric structure have different functional groups. These functional groups are either form structural component of the substrate or formed during degradation, which induces diverse biological interactions in the soluble or insoluble form. The different functional groups immobilized for a beneficial biological response includes carbonyl, hydroxyl, carboxyl[18], amide, amine[19], thiol, sulphate, sulphobetains, and phosphorylcholine[20] or alkyl groups[21], drugs[22] and vitamins[23], etc. The anionic surface inhibits cell activation by "negative cilia effect"[24]. On contrary cationic surface activates cell response to a greater extent as compared to other surfaces[25]. While the nonionic or zwitterionic surface inhibits cell adhesion and activation[26]. The alkyl group interacts with albumin a

non-opsonin, and thus reduces further cellular interactions. The effect of large immobilized groups like drugs and vitamins depends upon their net ligand-receptor interaction, to elicit the biological response. The polarity and the ionic content regulated with the biological environment induces and or inhibits biological interactions based upon the site of implantation.

The functional groups, either associated with a rigid surface or form part of polysaccharides (Leung et. al.[27]) or proteins,[28] or tethered through polymeric chains. The length of the polymeric chain plays an important role in the varied biological response. Apart from the above said specific biological responses, the immune system has the ability to identify any kind of groups through major histocompatibility complex (MHC) proteins. It has been anticipated that zwitterionic groups are an exemption to that, but recent studies by Cobb et. al.[29], indicates that MHCII complex has the ability to identify zwitterionic groups also.

1.1.5.2 Role of physical stimuli

Various physical stimuli of different kind have been currently explored for potentiating different regenerative treatment strategies. In the biological environment shear forces significantly influences many physiological processes. Bain et. al[30] has demonstrated that, under shear conditions the low molecular weight surfactants in sub critical micellar concentrations (CMC) have least affinity for the surface due to kinetic barriers, and under high flow conditions it has been reduced. Under these circumstances, as in biological fluids where proteins and surfactants coexist together, the proteins have a tendency to migrate to the interfaces and adhere[31]. Apart from that under static conditions, above CMC, single tale amphiphiles have least stability at surfaces (due to stable micellar formation), which is further influenced, depending upon number of chains, cholesterol content, and hydrophobicity of the surface etc. In a different environment of plasma membrane, at CMC and above the surfactant layer is stabilized by the intervening protein molecules[32]. At molecular level the structural

features of the lipids and the composite architecture of the lipids and proteins stabilizes the bilayer lipid membrane. The blood flow significantly influences the cellular motility and alignment in the case of endothelial cells as well as adhesion and activation in the case of secretory cells present in the blood. Bruck et. al.[33] reviewed that, the thrombus formed on material surface under low shear conditions is red thrombus, while under high shear conditions it is chiefly of white thrombus. Thus flow, and relative composition of the constituents, and the thermodynamic stability of the components under low and higher concentrations do influence the biological interactions. Apart from that many other forces like, magnetic force for tissue engineering[34], angiogenesis under cyclic strain[35], cell mitogenic activity by ultra-wideband (UWB) technology[36], gravitational forces in musculoskeletal development[37] Hyperosmotic stress induced apoptosis[38], millimeter waves induced reversible phosphatidylserine externalization[39], temperature induced cell layer removal[40] has been explored as part of different treatment strategies. In all these cases the forces are applied to the trans-membrane receptors or contractile proteins, which activate the G protein-coupled systems in the cell and thus influence cell proliferation and differentiation[41].

1.1.5.2.1 Role of surface energetics

Surface energetics plays an important role in non-specific adsorption of proteins and other agents which alter the biological events. Adsorption to hydrophobic and hydrophilic surfaces has been able to explain based on these concepts. Lyman et. al.[42] detailed the interaction of biological environment to the surface in terms of surface free energy. Baier et. al.[43] studied the solid critical surface tension and its role in biological adhesion processes. Andrade et. al.[44] reinforced the role of water in controlling the biological activation processes. Hoffman et. al.[45] Proposed that ratio of polar and nonpolar forces regulates the protein interaction to the material surface. Sharma et. al.[46] proposed that optimum polar and dispersive force components are required for preferential adsorption of proteins to the material surface. Ikada[47] et. al. studied the under water work of

adhesion on hydrophilic polymeric substrates and detailed its role in bioadhesion. However the specific interactions of proteins to surfaces could not be able to explain by these concepts alone.

1.1.5.2.2 Role of surface polarity

Polar surfaces inhibit adsorption of ions, proteins and other amphiphiles and enhance cellular interactions. As the difference in surface free energy between the material-water increases the surface will get adsorbed with ions, other amphiphiles and proteins. However since the protein adsorption is multivalent their interaction is more intense and eventually protein adsorption to the material dictates the cellular response. In the case of highly nonpolar surfaces the adsorbed protein has an altered conformation and the conformational change enhances with the increase in contact area. The blood plasma, serum or other tissue culture liquids has a high content of albumin. Albumin is a non-opsonin therefore albumin adsorption passivates the bioactive processes. However as the hydrophobicity of the surface reduces the affinity of the protein to the surface also reduces, and bigger opsonins will replace the adsorbed albumin. These proteins enhance cell response by exposing hidden functional sequences to the surface.

1.1.5.2.3 Role of size and shape of the surfaces

Recent studies indicate that cell response to different topographies varies with different cells and "contact guidance" may be utilized for specific cell response (For detailed review, Curtis et. al. 1999)[48]. Surfaces with high nano/micro aspect ratio show super- hydrophobic behavior[49], and they interact differently with cells. Apart from that, the concave surfaces[50] with micron-sized diameter show least cell spreading and enhance cell-cell adhesion while convex surfaces[51] with micro sized diameters show enhanced cell spreading and activation.

1.1.5.2.4 Stability of the interface

The mechanical stability of the interface is another important parameter influencing the interfacial adsorption of various amphiphilic molecules[52]. From

adsorption, coalescence, to friction all the physical processes at surface depend upon the mechanical stability of the interface. It is also important in the cell migration over surfaces[53]. Study of ECM mechanical properties and its relation in mechanotransduction in wound healing as well as its reduction in cancer metastasis gives important evidences about the role of mechanical stability of the interface in cell activation processes[54]. Mechanical study of the ECM of different tissues and its modeling will be an important criterion in the development of new generation tissue engineering constructs[55]. The traction force developed by the cells depends very much on the substrate rigidity[56], particularly the surface rigidity. Ladoux et. al. has studied the mechanical activity of a single cell, sub-confluent and confluent monolayer of endothelial cells, and has observed maximum mechanical activity is in the case of sub-confluent layer of endothelial cells[57]. Such studies indicate that the understanding about the minimum mechanical strength of surfaces required for cell adhesion or activation and development of surfaces for controlled ligand supplementation is important in controlling mechanotransduction and related biological events.

1.1.5.2.5 Tensegrity of the surface

In the biological environment, systems exist through tension rather than compression, except in the case of skeletal compartment. It has been understood that cell structure is being stabilized under tension, due to the tensegrity of its structure[58]. In the tissue extension of these tensional force components is extended from cytosketeleton of the cells to ECM proteins to the neighboring cells. The tissue or organ architecture is regulated by the structural advantage of cytoskeletal components in 3D skeleton of the cells as well as regular rearrangement of the components in 2D architecture of the cell membrane and ECM proteins[59]. The system is made dynamic with altering ECM-receptor binding and it plays critical role in cellular responses. Therefore it has been assumed that ECM proteins are physical extension of cytoskeletal proteins[60]. If we look into the structural freedom of the tissues in performing this diverse biological responses the architecture plays

an important role in force transmission two and fro. This indicates that the physical extension of cytoskeletal proteins is extended much beyond ECM to tissue structure and the organ architecture are modulated precisely in the microenvironment by the difference in ECM proteins, indicative from the existence of different stages of cell life in the same microenvironment[61] *in vivo*.

1.1.5.3 Role of biological stimuli

The different biological stimuli are due to the lipids, proteins and cells present in the biological environment. Usually the biological stimuli at material surface are due to an ongoing biological interaction at the material surface.

1.1.5.3.1 Proteins

The interaction of proteins to the material surface may be initiated either through non-specific or specific protein adsorption or being regulated by other pathological processes like calcification. The specific peptide sequences present in the protein molecules are responsible for the receptor mediated signal transduction. The exposure of these adhesion sequences should be energetically favored in the free form or in the bound form for a protein to initiate a receptor- ligand interaction. In the case of growth factors and cytokines these active peptide sequences are either exposed or are exposed suitably during the post-translational modification. In many soluble globular proteins these sequences like RGD[62], YIGSR[63], IKVAV[64], LRE[65], REDV [66], DGEA[67], GXG[68], VGVAPG[69] are hidden, and are exposed when get adsorbed onto the material surface[70]. Apart from that, adsorption and exchange (Vroman effect) of proteins also regulate the transmembrane signal transduction[71]. When adsorption of a combination of proteins like in the plasma to surface is compared, a size related response is observed because more amount of smaller proteins are required to occupy a unit area as compared to larger proteins. These proteins are existing in an equilibrium weight-fraction distribution within the interphase and are identical to bulk solution, however the absolute interphase concentration of any particular protein adsorbing

from a mixture is proportionately smaller to that adsorbed from a pure, single-component solution of that protein due to competition with other constituents. Krishnan[72] et. al. interpreted Vroman effect as a natural outcome of protein reorganization to achieve an equilibrium interphase composition, dictated by a firm set of mixing rules. However under certain conditions, this size discrimination can be amplified by the natural variation in protein-adsorption avidity, depending upon their partition coefficient (P). Here smaller proteins (MW<50kDa) exhibits characteristically higher P than larger proteins (MW>50kDa)[73]. But in the case of ligand based protein adsorption, the protein binding avidity controls the entire scenario of protein adsorption[74].

1.1.5.3.2 Cells

Apart from the proteins, the phenotypic and genotypic pattern of the cells changes, when in contact with different materials. Even though all the different cells are derived from a single cell, and all the properties are conserved, they behave differently depending upon the environment. The literature is flooded with the heterogeneous cell signaling in tissue regeneration and degeneration both in vivo and in vitro. However evidences for homogeneous cell- cell signaling in different architectures like on 2D assay substrates, confluent cell sheet, tissue architecture, spheroids and cancer tissue for a specific material is hardly available[75].

1.1.5.3.3 Lipids

Lipids form one of the major components of the biological building blocks. Apart from their role in membrane structure they also play an important role in signal processing[76].

They are amphiphilic in nature and easily occupy the interface when they are in the free form. In presence of structurally similar molecules they self-assemble to form different 2D &3D structures, which has a lot of significance in the biological milieu (E.g. cell membranes)[77]. They also found to play an important role in the existence of

many micron-sized organisms, under stress[78]. Based on the above said stimuli different kind of interactions happen at the material biology interface.

1.1.6 The environment

The material-biology interface is greatly influenced by the aqueous environment. Here the density of the neighboring solid bodies regulates dynamics of the aqueous environment. Therefore understanding about the physical parameters regulating the environmental dynamics is very important.

1.1.6.1 Friction at the interface

It has been observed that the water layer adjacent to the material surface has maximum friction[79] as compared to the bulk water and is dependent upon the surface viscosity[80]. This increases with increase in interfacial energy differences between the material and the water[81]. Surfactants seem to reduce the friction at the interface[82]. The effect of friction is reduced with increase in flow[83]. In absence of surfactants and flow this surface friction guides the large molecular weight molecules high affinity ions and other molecules to the surface to get adsorbed and thus initiates "surface synthesis". As this reduces, the interfacial energy differences and as a result friction reduces, and the system becomes more thermodynamically stable. However the adsorption of such agents to the surface may create more confined domains and holes in the surface, which induces flow differences in the material surface. These sites eventually act as the site for surface synthesis. But in mono and multi layered surfactant amphiphilic surfaces such enhanced surface synthesis is not observed[84], as compared to surface adsorbed with protein layers[85].

1.1.6.2 Water at the interface

Vogler et. al[86]. has demonstrated that the structural difference of the interfacial water at the material-water interface also determines the biological response to the material surface. Depending upon the polarity of the surface, there exist at least two kinds of water structures one is a relatively less dense water region on hydrophobic surfaces with an open hydrogen bonded network and other

one is relatively denser water region on hydrophilic surfaces with collapsed hydrogen bonded network due to hydrogen bonding with the polar surface. As a result hydrophobic surfaces support adsorption of various surfactants and proteins from water because expulsion of solute from solution into the interphase between bulk solid and solution phases is energetically favorable. Adsorption to hydrophobic surfaces is driven by the reduction of interfacial energetics concomitant with replacement of water molecules at the surface by adsorbed solute (surface dehydration). Hydrophilic surfaces do not support adsorption because this mechanism is energetically unfavorable. Recently Belfort et. al.[87]. demonstrated that the helix and random content (as measures of secondary structure) normalized by the molecular weight of a protein are significant for predicting protein adsorption and are likely related to protein stability at interfaces. This is exceptionally true in the case of hydrophobic surfaces and large molecular weight proteins.

1.1.7 Renewabilty of the interface

Structural components of cells, tissues and biological interfaces are renewable, which intern fine tunes the biological interactions. When an artificial organ is implanted, its surface renewability is lost. Ebberhart et. al. proposed a novel approach to develop a protein based renewable surface[88]. Further Zwaal et al[89] proposed the role lipid composition and its renewability in controlling the pathological interactions at the cell surface. For such cases continuous replenishment of the fresh surface may be relevant for the controlled ligand supplementation at the interfaces.

1.1.8 Mechanical stimuli vs. chemical stimuli

Recent studies indicate that ECM proteins are physical extensions of cytoskeleton and the mechanical transduction plays an important role in all the cell adhesion and activation processes. However here there is enough evidence for cross-talk between the chemical and mechanical stimuli mediated signal transduction. The differences in mechanotransduction in 2D and 3D architecture also indicate the

role of modulating the signal gradience across the substrate. However more understanding is to be generated on the role of mechanotransduction in pathological processes, as this may be helpful in developing new generation of biomaterials for more control of tissue architecture in tissue engineering. As these chemical, physical and biological stimuli and its amplification due to environmental factors regulate the cellular activation processes resulting in implantation or inflammatory events.

1.2 Inflammatory events at the material- tissue interface

The material-biology interaction starts at a very early stage, upon implantation. Bacterial adhesion and bio-burden during manufacturing are an important factor that regulates the early pathological events.

1.2.1 Implantation of devices: Site of implantation vs size of implant

Materials for internal use are being usually implanted in any of the biological cavities or tissues, with or without the surgical assistance. The inflammatory or immune response to these materials varies depending upon the contacting tissue, size of the device and other physical characteristics of the material. The figure-1.2.1.1 below shows the implantation of devices of different dimension into the wounds of different length.

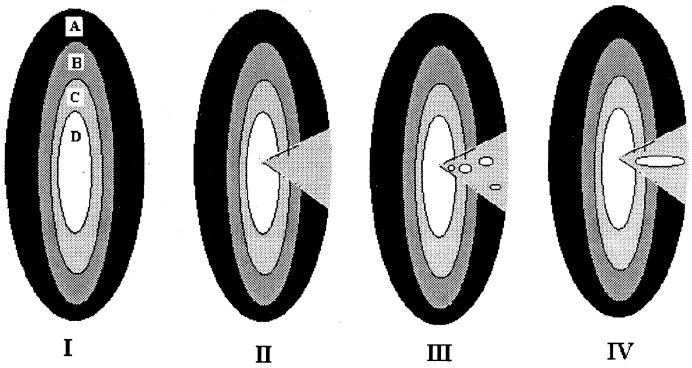


Figure-1.2.1.1: Implantation of devices of different dimension in to the wounds of different length. Tissue architecture (I); Skin (A), Blood (B), Soft Tissue (C), Hard Tissue (D). Injury and wound healing (II), Injury exposed to small devices (III), and Injury exposed to big devices (IV)

1.2.2 Events in an open wound

Coagulation followed by the wound healing and tissue regeneration is the important event after any tissue damage, whether it is due to trauma or implantation of a medical device[90]. Differently, in the case of implantation, the implant surface along with the adjuvant medication regulates the normal wound healing, and tissue regeneration process.

1.2.3 Wound healing and tissue regeneration

In a wound the thrombus stops bleeding and act as a scaffold for the cells to migrate and grow. The platelets and damaged cells deliver bioactive molecules immediately to the site[91]. The neutrophils do initial cleansing and macrophages remove the particulate matter, and continue the synthesis of required growth factors and cytokines. The fibroblasts recruited by the chemotaxis, continue the synthesis of other growth factors and the collagen, which form the basic structural unit of the extra cellular matrix (ECM). ECM formation is followed by tissue regeneration. The figure-1.2.2.1 below shows the surface activation of cellular responses.

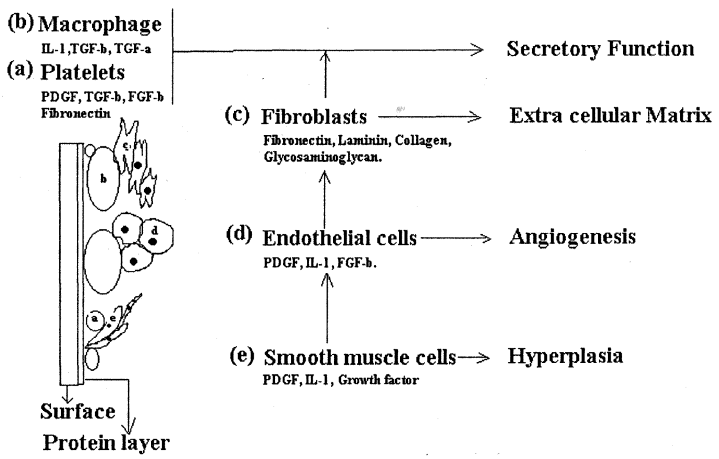


Figure-1.2.2.1: Surface activation of cellular responses

This normal wound-healing pattern is mimicked in many of the therapeutic strategies of structural restoration of the tissues *in vitro* or *in vivo* conditions.

1.2.4 Alteration of wound healing and tissue regeneration by the implant

However for an implant, the blood clotting on the material surface is initiated by the contact activation of the intrinsic coagulation pathway[92]. This thrombus formation is followed by complex acute and chronic inflammatory reactions lead to various kinds of effects like, passivation, capsule formation or tissue induction, integration and rejection. The figure-1.2.3.1 shows the different stages of inflammatory process in a wound.

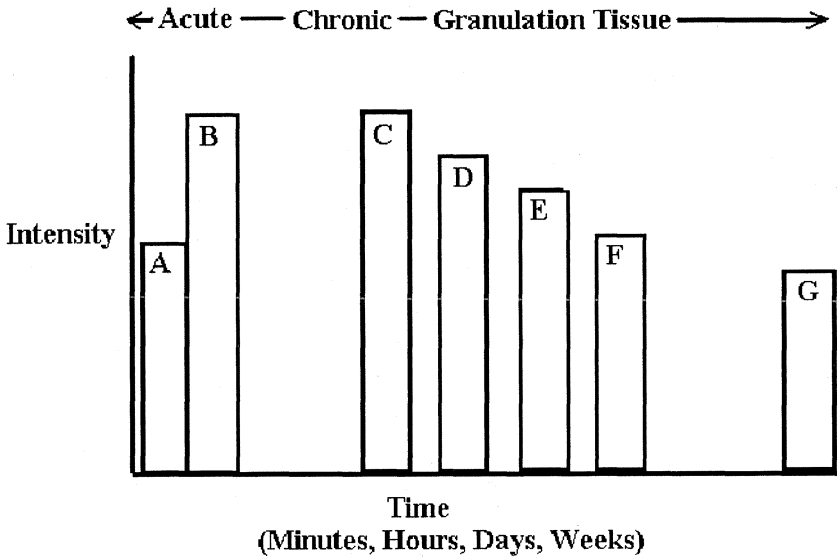


Figure-1.2.3.1: Time based phenomena at an inflammatory site. Mononuclear Leukocytes (A), Neutrophils (B), Macrophages (C), Neovascularisation (D), Foreign Body Giant Cells (E), Fibroblasts (F), Fibrosis (G). Concept conceived[93] and redrawn

1.2.5 Role of agents in the environment in altering the healing process

This is further being modulated by the flow, concentration of ions, growth factors, and other soluble and insoluble bioactive molecules. The extent of biological reaction varies differently with material size, surface properties, extend and duration of implantation, presence of biological fluids, vasculature, physical, chemical and biological stress, and site of implantation as explained earlier. This

deviation in normal wound healing pattern also has been extensively utilized for developing therapeutic strategies in degenerative diseases.

1.2.6 Inflammatory events at cellular level

At cellular level, the initial acute inflammation by the material is very important, in spite of size and shape of implant, as well as site of implantation. This can be considered as the first live cellular response by the material surface, *in vivo,* and is dependent upon the surface properties of the material, stress produced by the material to the surrounding environment, as well as the agents present in the surrounding environment. Therefore a close control of the above said factors gives opportunity for controlling the cellular response, eventually the organ and other in vivo response to the biomaterials.

The competing inflammatory and immune cascades influence the cellular activation process at the material- biology interface, which in turn will causes lysis and immune rejection of the device or biomaterial. Figure- 1.2.5.1 illustrates the contribution of different cascades in the immune processes.

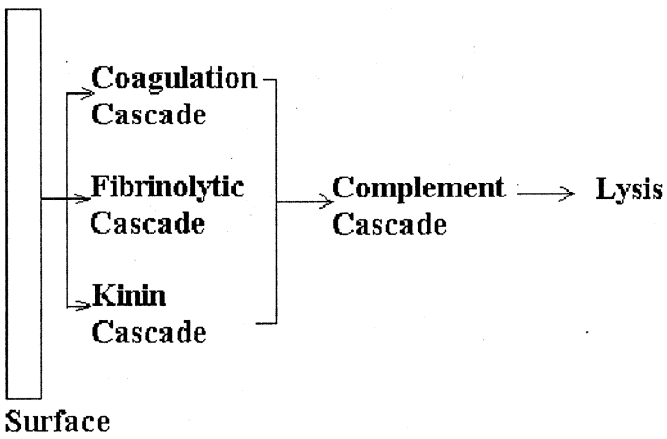


Figure-1.2.5.1: Different acute inflammatory cascades during enhanced vascular permeability

Since the phenotypic and genotypic pattern of the different cells depend upon the transmembrane signaling, which in turn depends upon the ligand- receptor interactions a close understanding about the kind of ligand supplementation is very

important in controlling the cellular responses. Various ligands are available in the bound or unbound form along with bound or unbound receptors. Here monovalent or multivalent interaction can happen between these two. The antigen- antibody, drug-receptor interactions are examples of monovalent interactions while cell- cell, cell-matrix interactions are multivalent interactions. Here the signal transduction is depend upon (1) The relationship between receptor–ligand chemical equilibrium binding parameters (2) The effects of physical forces on bond association and dissociation; and (3) The locus of disruption of adhesion molecules (4) The conformational state and stability of the receptor- ligand complex (5) and the ability of the complex to associate with other molecules. All these interactions will reduce the free energy parameter.

1.2.7 Trans-membrane signaling

In the case of trans-membrane signaling, the association of soluble or bound ligand with the G protein coupled trans-membrane receptors activates the secondary messengers which in turn initiate other precursor enzymes in the cytoplasm and in the nucleus. This determines the phenotypic and genotypic pattern of different cells. Ingber et. al [20] demonstrated the critical role of mechano-transduction in growth, apoptosis, differentiation and motility in the case of adhesion dependent cells. Studies on ECM coated beads demonstrated that the focal adhesion points are formed within few minutes, when rounded, suspended cells are coming in contact with the beads, but they are transient as compared to focal adhesion points (FA) forming on substrates[94], and they will mature on beads only if external force is applied[95]. But mature FA is easily formed on ECM coated flat surfaces due to contractile transmembrane proteins[96]. Therefore it has been concluded that the mechanotransduction plays an important role in many of the phenoty pic factors of the cells in organized tissues.

1.2.8 Cancer cells and embryogenesis, cell response in two different saga

Cancer cells extensively proliferate in absence of ECM stimuli. This is also true in the case of organogenesis along with angiogenesis. Where soluble stimuli

plays an important role. However the cancer cannot perform an organized mechanical function. Indicating that in cancer tissue the tensegrity of the organ architecture is lost. Ingber[97] et. al. redefined cancer as a disease of epithelial-mesenchymal interaction and extra cellular matrix regulation.

1.2.9 Mimicking cell response in cancer metastasis and embryogenesis

Directed cell migration is an important phenomenon in many important biological processes like angiogenesis, tumour metastasis, wound healing as well as in immune response. In this case the cell response is with respect to the gradience of soluble ligands[98]. These factors promote cell migration by activating the members of the Rho family of GTPases, Rac and Cdc- 42[99] guided through ligand receptor interaction or ECM adhesion and integrin mediated directed cell migration, which is further influenced by the mechanical rigidity of cell substrate[100]. It induces actin based lamellopodia, filopodia, and fascin containing microspikes that drive cell extensions.

1.2.10 Cross-talc between growth factor and mechano receptors

Cross-talc (Figure-1.2.9.1) between the growth factor and mechano receptors also happens, which gives rise to a synergetic or antagonistic active response.

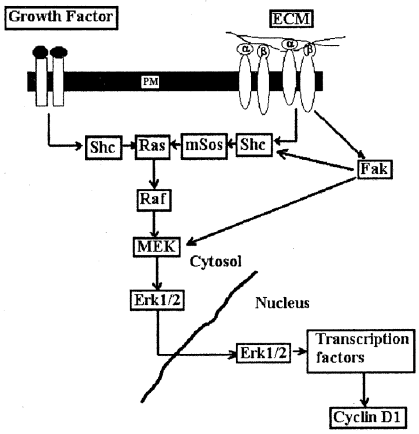


Figure-1.2.9.1: Erk1/2 activation leading to cyclin D1 expression by collaborative cross-talk between integrins and growth factor receptors

In effect it results in macroscopic effects like cell adhesion, spreading, phagocytosis, proliferation and differentiation depending upon the above-mentioned molecular level interactions[101,102].

1.2.11 The challenge of mimicry of embryogenesis in open wounds

In the case of implant in an open wound first blood cells come in contact with the materials followed by pathological tissue counter parts, followed by the invasion of the repair cells, and finally respective tissue cells. Various multiple soluble and insoluble ligands are regulating the diverse biological events. This usual wound-healing model is not similar in tissue formation in the case of embryogenesis and cancer metastasis. Where close control of the signal transduction reduces change in phenotypic and genotypic pattern of the cells as well as reducing heterogeneous cellular migration during organ regeneration. Therefore a dynamic system which can respond with the tissue environment can mimic cell response resembling embryogenesis in open wounds, which has tremendous opportunity in tissue regenerative processes. The major challenge in this scenario is to control the actin mediated cellular activation processes. A detailed understanding about the current surface modification strategies and renewed understanding about the material-biology interface and various miniaturization strategies helps in addressing such challenges.

1.3 Surface modification of materials

Surface modification of the materials is a need when the inherent structural components of the material or the device cannot elicit a satisfactory biological response. This is mainly due to biofouling at the material surface. Biofouling is the undesirable physical adsorption of the various agents onto the substrate, which influences the functionality of an interface. This may be involved in biorecognition processes of the surface of devices like targeted drug delivery systems, biosensors etc. In the bottom up approach of miniaturization strategies, the surfaces with high specific binding avidity or antifouling surfaces are most desirable. Antifouling

surfaces attain special recognition, as it is more important in many biomedical applications like intraocular lens, long circulating drug delivery systems, biosensors, mechanical ventricular assist devices etc. The figure 1.3.1 shows a comprehensive picture of current surface modification strategies

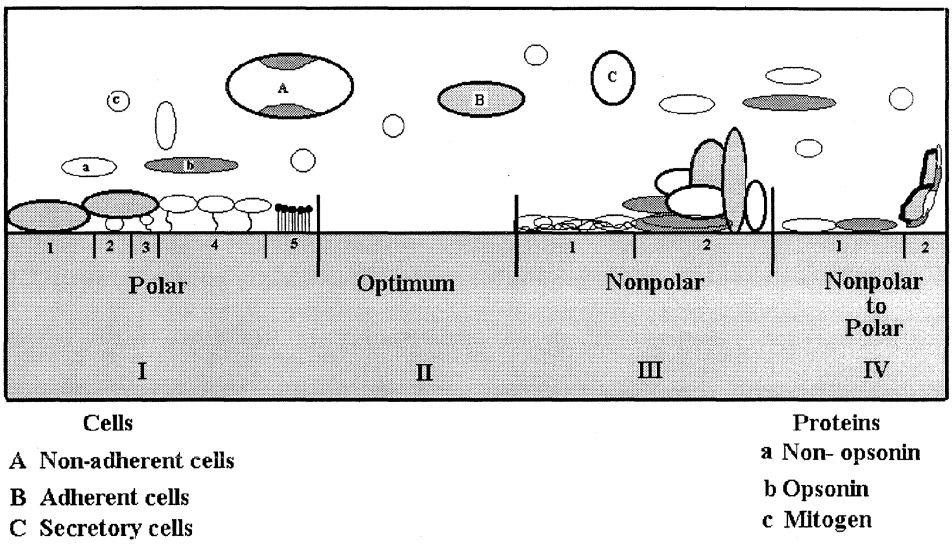


Figure- 1.3.1: Gross picture demonstrating the biological interaction at biomaterial surfaces and current surface modification strategies. The division colored with grey indicates the surface and white indicates the biological fluids (Blood, Inflammatory exudates, Tissue culture, Tissue regeneration fluids etc.). Section-1, Polar; Direct cell interaction (1), Growth factor adsorbed or immobilized on surface (2), Tethered growth factor (3), mediated cell interaction; Non-opsonin (Albumin) binding renewable surfaces, where least cell interaction happens (4), Cell membrane mimetic surfaces (5). Section-II, Optimum; Ideal surface. Section-III, Non-polar, Preferential albumin adsorption (1); Vroman effect, cell adhesion and activation (2). Section-IV Non- polar to Polar (Thermoresponsive), Initial protein adsorption (1), Protein mediated cell adhesion and cell layer removal (2)

Surfaces with specific binding affinity for proteins, is more desirable under special cases like blood contacting nanomaterials for diagnostics, targeted drug delivery systems, guided tissue engineering etc. This acts as a switch between passive to active interface for molecular recognition.

All these processes require an understanding of the interactions that occur at the interface between a solid material surface and the biological environment. The material surface energetics play an important role in regulating the non-specific binding of the molecules. It has been observed that most amphiphilic biological molecules like lipids and proteins have specific affinity to the material-biology interface. The interfacial energy changes between the surface and the surrounding system are the driving force for these molecules to migrate to the interface, as explained earlier.

The specificity and strength of adhesion depends upon the size and area of contact in the case of stable surfaces. As the surface to volume ratio of the system increases, biorecognition also increases. Most hydrophobic surfaces are highly biofouling, while hydrophilic surfaces are antifouling. Thus, the tailoring of surface chemistry by synthetic tools and nanofabrication techniques becomes an important avenue for the production of surfaces of biospecific binding properties with minimized background interferences.

1.3.1 Modification of surfaces with supramolecular assemblies a lime light for miniaturization- current strategies

Development of surfaces with supramolecular assemblies has been identified as one of the important strategy in bottom up synthesis of highly functional nano-scale surfaces. Synthetic amphiphilic molecules of varying dimension, which can self-assemble to varied morphologies such as vesicles, tubes, disks, lamellas are being explored for developing surface coatings[103]. The different supramolecular assemblies can also be formed out of polymeric amphiphiles. For. Eg. Large molecular weight peptide amphiphiles found to form nano-tubes and nano-vesicles[104] in different solvents. Synthetic diblock polymers also shows bundled fibre morphology like colloids. The supramolecular structure depends upon the size and the shape of the amphiphiles. Apart from that peptide-polymer conjugates also found to form colloidal architectures with defined

geometry [105]. Supramolecular membranes has also prepared from hybrid amphiphiles[106]. Catanionic surfactants also show supramolecular self-assembled structures like rods [107]. These structures have been explored for the surface modification of biomaterials.

1.3.2 Biomimetics of cell membrane – an interesting approach to reduce biofouling using thin films

The biological membranes are versatile in circumventing the adsorption-related phenomena and are effectively participating in the biological communication processes. This is attributed by the membrane fluidity, packing, and orientation of the surface groups[108]. The composition of the lipids is different at different regions of the natural bilayer membrane, which affects the packing and fluidity of these membranes and contributes to the functional requirements of the membrane. Cell mimetic approaches have been extensively studied for the surface modification of biomaterials for tissue[109] and blood-compatible applications[110].

1.3.2.1 Outer cell membrane mimetics

Materials modified with phosphoryl choline group, are found to be blood compatible[111]. The protein adsorption as well as conformational changes onto these phosphorylcholine-immobilized surfaces has been found to be low[112]. The fluidity of the amphiphiles of the bilayer membrane is important in the biological scenario, which is hindered during the covalent immobilization of the phosphorylcholine moieties to the surface. In natural membranes, the membrane lipids are closely packed at surface pressures above 20 mN/m [113, 114] while on binary phospholipid/cholesterol (Chol) monolayer model systems[115] the lipids are closely packed at 30-35 mN/m. The stability of the natural bilayer membranes at much lower surface pressures reveals that apart from these two lipids other constituents of the membrane also play a role in the process of membrane stabilization. Glycolipids are found to be an integral part of myelin, which maintains the membrane integrity of the myelin sheath[116]. It is also present in trace amounts, in

the outer plasma membrane of the luminal surface. The phospholipids, glycolipids, and Chol are the three most important classes of membrane lipids. Choline lipids like phosphatidylcholine (PC) and sphingiomyelin (SM)) form the majority of the outer membrane PL[117], are non-thrombogenic, and their different compositions could be explored for the surface modification of blood contacting devices.

1.3.2.2 Inner cell membrane mimetics

The amine PL like phosphatidylethanolamine (PE) and phosphatidylserine (PS) are preferentially residing to the inner side[118], is thrombogenic, contributed by their head group structure. This asymmetry is maintained by ATP- dependent aminophospholipid translocases[119], and is lost during pathological events by scramblases[120]. This is reported for platelets[121], erythrocytes[122] and fibroblasts[123]. It indicates that, the packing of the membrane components is playing an important role in regulating the membrane fluidity, which intern influences the adsorption of ions, proteins, cells etc. to these surfaces. This could be explored for controlled ligand supplementation from material surface.

1.3.2.3 Mimesis of membrane lateral stabilization

The integral membrane proteins, rich in β- sheet exist in the calveolae of the cholesterol rich domains, which anchors the membranes to the cytoskeleton. The hydrophobic interaction between the integral proteins and the core of the bilayer membrane holds these integral proteins in the membrane bilayer. These factors enable these membrane surfaces to retain the fluidity, and exchangeability and thus the renewability of its components. Such a renewable system may interact more actively with the biological environment and gets easily integrated into the system. However, the monolayer behavior of this ternary phospholipid/ glycolipid/ Chol system at the air/water interface as well as on supported systems as well as its lateral stabilization using membrane proteins has seldom studied. The exploration of this lipid mono and bilayers with its structural heterogenecity, immobilized on surfaces may be able to have retained the functional ability of natural membranes.

1.3.2.4 Thin Organic Films

Dense thin organic films of few nanometers (a monolayer) with desired functionalities can significantly influence surface interactions and have many practical and commercial applications like in the case of sensors, detectors, displays and electronic circuit components[124]. These thin organic films can be deposited on a polymer substrate by various techniques, such as thermal evaporation, sputtering, electro deposition, molecular beam epitaxy, adsorption from solution, Langmuir- Blodgett Technique, and Self- assembly[125]. Various agents using for surface modification of materials include, surfactants, polymer brushes (natural & synthetic), cyclodextrins and fullerenes, dendrimers and carbon nano-tubes and polymeric liquid crystals etc. However few of them only qualify for development of it as a thin solid film. Biological membrane lipids are amphiphilic linear molecules which can form thin film at the surface. The surface morphology of these thin solid films can be studied using atomic force microscope (AFM)[126].

1.3.2.4.1 Self-assembly of surfactants

Surface active agents (surfactants) are a large class of molecules which have a significant technological & biological importance. They have a hydrophilic (water soluble) and a hydrophobic (water soluble) part. This amphiphilic nature of surfactants is responsible for their association behavior in solution (micelles, bilayers, vesicles etc). and their accumulation at the interphase (air/ water or oil/ water). During association they follow a programmed [127] or a synkinetic pathway[128]. The programmed aggregation process, self- assembly, is the process by which molecules spontaneously form supramolecular architectures[129]. In the synkinetic aggregation route the monomeric building blocks "synkinons" are held together by non-covalent interactions, which follow the synkinetic plans of the chemists rather than a self-organization process. The hydrophobic effect is the driving force for the self-assembling behavior of amphiphiles in water [130].

According to Kauzmann et. al.[131] the entropy gain upon interaction of apolar compounds in aqueous environment by the release of structured water molecules during a destructive overlap of hydrophobic hydration shells, is the base for the hydrophobic effect. Recently it has been redefined by considering the thermodynamics of solvation as the favorable overlap of the hydrophobic shells of hydrophobic groups[132]. Above a certain concentration called critical aggregatory or micellar concentration (CMC), the formation of the assembly is entropically favored over a solution of individual molecules. Upon dispersal into water, both the natural and synthetic surfactants organize in such a way that the polar head groups become oriented towards the water, while at the same time the hydrophobic tails cluster together. This can lead to various superstructures such as micelles, vesicles, multilayers, and lyotropic liquid crystalline phases (at higher concentrations)[133]. More exotic aggregation morphologies have also been observed in surfactants and involve in the formation of chiral superstructures such as "cigars"[134], twisted ribbons[135], Helices[136], Tubes[137], Braids[138], Boomerangs[139] and super helices[140].

The specific superstructure formed by the amphiphiles is determined by the combination of following factors of free energy. They are a favorable hydrophobic contribution caused by the clustering of the hydrophobic tails within the interior of the aggregates. A surface term that reflects the opposing tendencies of the molecules to crowd closely together minimizing the unfavorable hydrocarbon water interactions and to spread apart as a result of the electrostatic (head group) repulsion, hydration and steric hindrance. A packing term that requires that the hydrophobic core of the aggregate exclude water and polar head groups, which as a consequence, limits the possible geometry of the aggregates. The aggregation process also makes use of molecular information such as the shape, rigidity, and flexibility apart from that mass and magnetic dipole also influences the aggregation process. The interactions between the individual molecules within the aggregates can be hydrogen bonding, dipole- dipole interactions, π- π stacking; non- specific vander Waals repulsive steric forces or electrostatic interactions.

Theoretical models to predict the shape- structure relationship between the monomeric units and their aggregates has also been developed based on statistical mechanics of phospholipids[141]. These surfactant 2D monolayers can be transferred from the air/water interface to polymeric substrates.

1.3.2.4.2 Langmuir-Blodgett Film Deposition

Langmuir-Blodgett film deposition technique is giving considerable advantage in the study of air/ water as well as air/ solid interfacial behavior of the 2D thin films. Irwing Langmuir has been the first to perform systematic studies on floating surfactant monolayers[142]. However the first detailed description of sequential monolayer transfer has been given several years later by Katherine Blodgett[143]. These built up thin solid films are therefore known as Langmuir- Blodgett films.

The most important indicator of the monolayer properties of an amphiphilic material is given by measuring the surface pressure as a function of the area of water surface available for each molecule. This is carried out at constant temperature and is known as surface pressure-area (π-A) isotherm. Figure - 1.3.2.4.2.1; show the typical pattern of surface π-A isotherm.

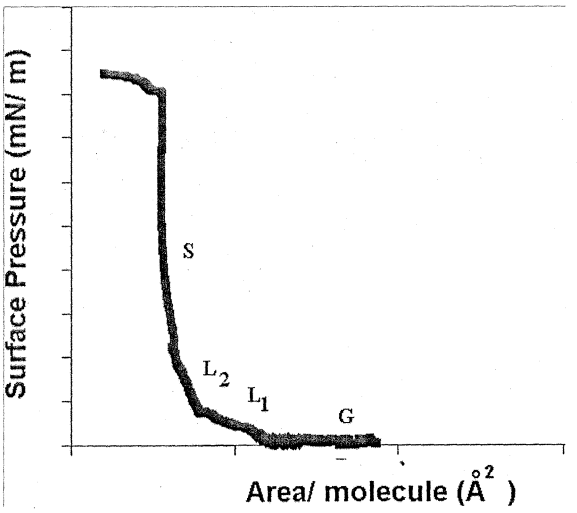


Figure-1.3.2.4.2.1: π- A isotherm. Gaseous state (G), Liquid Expanded (L_1) (LE), Liquid Condensed (L_2) (LC), Solid (S) state

A number of distinct regions are immediately apparent on examining the isotherm. These regions are called phases. A simple terminology used to classify different monolayer phases of fatty acids has been proposed by W. D. Harkins[144] .

Now this is applicable to different kind of molecules at the air/ water interface. At large the monolayers exist in the gaseous state (G) and can on compression undergo a phase transition to the liquid expanded (LE) state (L_1). Upon further compression the L_1 phase undergoes a transition to the Liquid Condensed (LC) state (L_2) and even at higher concentrations the monolayer reaches the Solid (S) state. If the monolayer is further compressed after reaching the S state the 2D layer will collapses into 3D structures.

Apart from these interfacial studies LB apparatus could also be used for transferring the mono and multi layers into solid substrates. The LB deposition is traditionally carried out in the solid phase. Figure -1.3.2.4.2.2 demonstrates the orientation of the deposited monolayers.

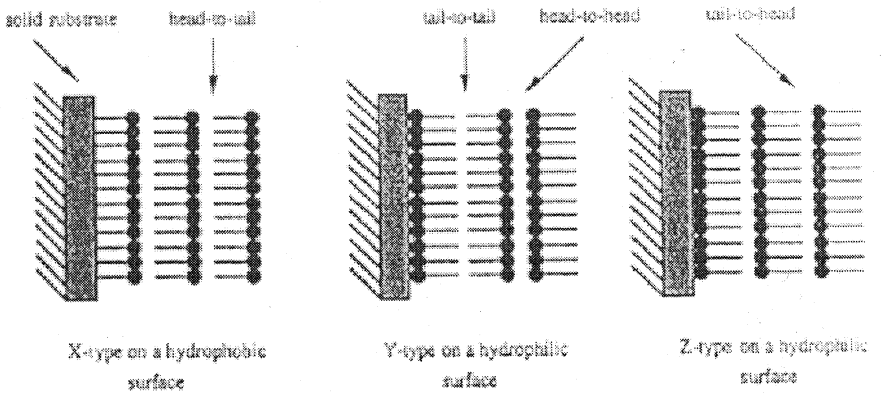


Figure-1.3.2.4.2.2: Deposition of the monolayer into polymer substrates in the X, Y or Z direction

However amphiphiles can seldom be successfully deposited at surface pressures lower than 10mN/m and surface pressures above 40mN/m, due to lower film rigidity related problems. When the solid substrate is hydrophilic, the first layer is deposited by raising the solid substrate from the subphase through the

monolayer, whereas if the solid substrate is hydrophobic the first layer is deposited by lowering the substrate into the subphase through the monolayer. The deposited (supported) monolayers can be characterized by various techniques including Transfer ratio. This is defined as the ratio between the decrease in monolayer area during a deposition stroke A_1 and the area of the substrate A_s. A transfer ratio of 1 and above has been considered as a complete wetting of the polymer surface by the monolayer[145].

An alternative strategy to deposit the monolayer is Langmuir- Shaffer technique. This technique differs from the vertical technique described above only in the sense that the solid substrate is horizontally lowered in contact with the monolayer.

The supported membranes have self-healing power and minimize defects on the surface during hydration[146]. Radler et. al. has studied the spreading kinetics of the self-assembled monolayer over polymer substrates from a large source of lipids using a fluorescent dye. They demonstrated that the spreading continues if the lipid reservoir is available in an air/ water interface[147] and tend to self-limiting fingered edges (due to pinning defects) due to loss of intermolecular interactions within the membrane[148]. This indicates that the monolayer at the edges form such fingered edges due to the above mentioned pinning effects.

These thin solid films offer additional advantages of lateral movement of the molecules within the monolayer. This gives a time dependent renewability of the surfaces under water. Sackman et. al. has immobilized proteins into these monolayers and attempted to evaluate the adhesion induced receptor aggregation and adhesion plaque formation[149].

1.3.3 Self-assembly of supramolecular architectures; Extension of colloidal knowledge towards development of thin films by supramolecular assembly

The close packing of the individual structural elements in colloidal systems, as well as the elasticity and expandability of these colloidal systems can

be mimicked for enhancing the functional units per unit volume. Apart from that the expandability of these colloidal systems to non-uniform surfaces as well as its self-limiting nature could be mimicked for coating thin solid films on non-uniform surfaces. They also impart heterogeneous surface with domains based on the cholesterol concentration. This property could be mimicked for making surfaces with moving receptors. Here these kinds of confluent monolayers could be attained only in the case of linear molecules.

However self-assembling proteins or protein like molecules is different from that of the linear lipid molecules. Albumin is a versatile candidate for self-assembling over the material surface to inhibit cellular activation processes.

1.3.4 Self-assembly of proteins

Albumin adsorbing surfaces are famously anti-thrombogenic. Eberhart et. Al. observed that a confluent layer of conformationally intact albumin will provide a biocompatible, surface lead to the development of albumin self-assembled systems[150]. Further Piskin et al demonstrated that dyes could be used as a ligand for albumin adsorption to material surface[151]. Albumin remains passive in its native as well as altered conformations[152]. However recent studies have observed that albumin with an altered conformation tend to become thrombogenic after getting calcified. Here the strategies to make a self-assembled monolayer of albumin over the material surface in its minimally altered conformation look impressive. We have attempted to self-assemble the albumin molecule over the surface using spatially oriented drug molecules[153]. These strategies are based on the receptor-ligand interaction. One of the important advantages with these systems is the refreshing nature of these biological systems.

1.3.5 Proteins like architectures through self-assembly

Essentially globular proteins have hydrophobic interior surrounded by hydrophilic exterior and does catalysis, signal transduction, synthesis, transport, degradation, imaging and various other activities[154]. The competing physical

forces, like hydrophobic forces balanced by the hydrogen bonding, are regulating the initial self-assembly of these protein molecules. This gives them conformational flexibility, under altered environments. Creating such structures at nano-scopic level is important, as it can overcome many limitations of the proteins, due to high functional aspect ratio. However achieving functional proficiency in the synthesis of protein like globular structures is often difficult and has been found to be rarely successful. Attempts in this direction are progressing. Such thin films prepared on material surface can be explored for the miniaturization of biomaterials and devices.

1.4 Miniaturization of biomaterials and devices and surface modification: Bottom-up vs. Top-down approach in biomaterial field

Two complementary strategies can be used in the fabrication of submicroscopic supramolecular structures biomaterials. In the 'top-down' approach, biomaterials are generated by stripping down a complex entity into its component parts. This contrasts with the 'bottom-up' approach, in which materials are assembled molecule by molecule to produce novel supramolecular architectures. Surface modification of nano-sized devices is difficult in the top-down miniaturization strategy. There fore bottom-up or post synthetic surface modification has been found to be more impressing and it has a lot of biomedical applications. This helps to reduce the signal to noise ratio in the case of biosensors. Ladoux et. al. did the force mapping in epithelial cell migration with a discrete array of vertical micro needles of silicone elastomer used as cantilevers on which cells attach and exert forces[155]. The latter approach is likely to become an integral part of nanomaterials manufacture and requires a deep understanding of individual molecular building blocks and their structures, assembly properties and dynamic behaviors. Two key elements in molecular fabrication are chemical complementarity and structural compatibility, both of which confer the weak and non-covalent interactions that bind building blocks together during self-assembly. Using natural processes as a guide, substantial advances have been achieved at the

interface of nanomaterials and biology, including the fabrication of nano-fiber materials for 3D cell culture and tissue engineering, the assembly of peptide or protein nanotubes and helical ribbons, the creation of living micro-lenses, the synthesis of metal nano-wires on DNA templates, the fabrication of peptide, protein and lipid scaffolds, the assembly of electronic materials by bacterial phage selection, and the use of radiofrequency to regulate molecular behaviors.

Conclusion

In this scenario post-synthetic surface modification of these nano devices need special consideration. The self-assembly of molecules can effectively be utilized for such purposes. Here a dynamic interface of self-assembled architectures offer significant advantage over conventional covalent surface modification strategies and is the subject of this thesis.

Relevance and Objectives of the thesis

At this juncture we understand that, beyond the surface stimuli, how it is being presented is more important. If we look at plasma membrane, all the kind of components or surface stimuli do exist inherently, despite that it offers optimum performance at a said environment.

This thesis is about an attempt to understand how controlled ligand supplementation can be achieved by means of dynamic interfaces of cell mimetic self-assembled thin films physically immobilized at the biomaterial surface.

- We have attempted to understand the mechanism of protein adsorption, conformational change, calcification, cell adhesion, spreading, colony formation and immune response at these surfaces.
- Most interestingly a cross comparison between nonspecific protein adsorption, preferential albumin adsorption vs. antifouling properties of lipid modified surfaces towards biological recognition processes are subjected to study.
- Studies are done on polymeric films and the knowledge has attempted to explore towards drug delivery using micropheres, nanoparticles and liposomes.
- Further its potential for controlled ligand supplementation and temperature dependent cell layer retrieval has been studied for tissue engineering applications.

Synopsis

Treatment strategies of degenerative diseases, has emerged to a new dimension with the powerful tools of tissue engineering, regenerative medicine, cell therapeutics, nanotechnology and targeted drug delivery. Miniaturization has surfaced as the buzz word of the current era. Managing the space with respect to time emerged as the underlying principle. Optimizing biomimetic systems than individual synthetic units has become the methodology.

For an optimum performance in a said biological environment the devices made of synthetic and natural origin has to satisfy safety and efficacy requirements. Safety is affected, due to inflammatory and immune cascades, initiated at the surface. This is due to "uncontrolled ligand (as chemical, physical and biological stimuli) supplementation" from the surface.

In this thesis we have done an attempt to control the ligand supplementation from material surface by biomimetic strategies. For that we have developed thin films for the post synthetic surface modification of materials, by mimicking biological cell membrane lipid components. We have developed cell mimetic monolayers (thin films) of phospholipid/glycolipid/cholesterol ternary lipid systems, and used for the studies. They have been laterally stabilized, chemically by peptide bonding, and physically using pendant polymers. Further mechanism of potential sub acute phase biological recognition processes has been studied by *in vitro* techniques.

The thesis is divided into six chapters. Chapter- 1 gives a brief introduction about different inflammatory responses at materials surface, overall understanding about biological interactions at the surface, current surface modification strategies, biomimicry of cell membrane and other bioactive processes, strategies based on biomimicry towards post synthetic surface modification using thin films and their bottom up synthetic approach of the surface modification of materials.

The Chapter -2 deals with the materials and methods. It gives detailed procedures on development of substrates, development and physical characterization of thin films, post synthetic surface modification of substrates and their characterization, stability studies, improvisation of the stability of these thin films by chemical and physical means. Further methods for important biorecognition studies like calcification, protein adsorption and cell interaction studies are detailed. Calcification studies have been done from metastable salt solutions, whereas protein adsorption studies from mixture of proteins by Sodium Dodecyl Sulphate- Poly Acrylamide Gel Electrophoresis (SDS-PAGE) and densitometry. Cell interaction studies have been done from blood cells, immune cells like macrophages, and polymorphonuclear cells, fibroblasts and bacteria. Further drug delivery and cell layer retrieval and biolubrication studies have been done on these thin films towards drug delivery and tissue engineering applications. The physical characterization have been done by using Langmier Blodgett (LB) Trough, Ultra Violet (UV-VIS), Fourier Transform Infrared (FTIR)., Circular Dichroism (CD) spectroscopy, goniometry, Atomic Force Microscopy (AFM), Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM) and Differential Scanning Calorimetry (DSC).

Chapter-3 deals with the results and discussion. Where the air/water interfacial studies of the lipid monolayers and their air/solid transfer studies has been discussed with respect to substrates. Change in fluidity of the monolayers with the incorporation of phosphatidylethanolamine (PE) has been discussed. The incorporation of soluble globular proteins after the conformational change, and other macromolecules like Di amino Polyethylene Glycol (DAPEG) and heparin as well as their lateral distribution are discussed. Further optimization of deposition parameters with respect to substrates, composition, speed and different methods has been discussed. The lateral stabilization of these monolayers chemically by peptide bonding and physically by pendant polymers has been discussed. Change in surface topography with respect to composition and effect of incorporation of PE and other macro molecules, has been discussed and correlated with air/water

interfacial studies. Further biorecognition studies in terms of calcification, protein adsorption, and cell interactions have been discussed. Cell adhesion, activation spreading, colony formation, proliferation, secretary profile has been discussed with respect to modified surfaces towards controlled ligand supplementation. Cross comparison between blood cells, fibroblasts, immune cells and bacteria has been done to reveal the role of these modified surfaces in controlling the preacute phase cell recognition processes. Further drug delivery and bio lubrication on these surfaces has been discussed.

The Chapter-4 deals with the conclusion. Here a comprehensive understanding about the following parameters has been discussed.

1) The mechanism of protein adsorption, calcification, cell adhesion, spreading, colony formation and immune response at these lipid modified surfaces.
2) Cross comparison between non-specific protein adsorption, preferential albumin adsorption vs antifouling properties of lipid modified surfaces towards biological recognition processes.
3) Relevance of the study towards controlled ligand supplementation for tissue engineering and systemic drug delivery applications.

   In conclusion the cell mimetic lipid thin films could be able to control the ligand supplementation from material surface.

Chapter-5 deals with few immediate applications of the work.

They are

1) Enhancing the blood compatibility of liposomes for systemic drug delivery applications.
2) Temperature depended cell layer retrieval for tissue engineering applications.

   Finally Chapter-6 deals with the future plan of exploration of thin films for various biomedical applications like surface modification of stents, small diameter vascular grafts, nanoparticles for imaging, cell layer tissue engineering, biolubrication, drug targeting and controlled drug delivery etc.

Chapter -2
Materials & Methods

MATERIALS AND METHODS

Materials

The polymers Polycarbonate Bisphenol A based (M.Wt.32,000-36,000) (PolyC), (Poly Science Inc.), Poly methyl methacrylate, (PMMA), (M Wt 1, 00,000), Polyscience Inc., Warrington; Polystyrene (Tissue Culture Grade Wells) (PS) Poly vinyl alcohol (PVA) (SD Fine Chemicals, India) (M. Wt. 1.25kDa) (8%w/v), Chitosan (CHIT) of (Approx. M.Wt. 2.70 kDa) (85% deacetylated) (CIFT, India), Diamino Poly Ethylene Glycol (Fluka) (M.Wt. 1900) was used in the form of films, microspheres, nanoparticles or polymeric gels or anchors in these studies.

Albumin (human fraction V, 96-99%), γ Globulin (Cohn fraction II) Fibrinogen (human fraction I, over 95 % protein clottable), Indomethacin, Aspirin, L-α-phosphatidylcholine (egg yolk) (PC), Ethylene divinyl carbodiimide (EDC) Phosphatidylethanolamine (PE), and galactocerebroside (from bovine brain)

(GalC) were from Sigma Chemicals Co. St. Louis Mo, USA. Cholesterol (Chol) was from (Himedia Pvt. Ltd, Bombay).

Diclofenac Sodium (DIC) (was kindly supplied by Ranbaxy Lab Ltd), Insulin (40IU/ ml) (Human) was from Abbott India Ltd.

All the solvents like, Acetone (SD Fine, India), Dichloromethane, Choloroform, Ethanol, Methanol, Isopropanol, Cyclohexane, n-Butanol, n-Heptane etc., and other reagents like Sodium Hydroxide (NaOH), Paraformaldehyde (PF), Calcium Chloride ($Cacl_2$) etc., were from standard sources. All the cell culture mediums and reagents used were from standard sources.

Development of materials and physicochemical characterization

2.1 Development of substrates for the surface modification

Different kind of substrates like films, microspheres, nanoparticles, liposomes, liposome delivering matrices were prepared for the immobilization of the thin films and for their biorecognition studies.

2.1.1 Films

Polymeric films of polycarbonate (PolyC), poly methyl methacrylate (PMMA), polystyrene (PS), cross linked poly vinyl alcohol (cPVA) and chitosan (CHIT) were prepared by casting and solvent evaporation method.

2.1.1.1 PolyC, PS & PMMA films

The PolyC, PS or PMMA substrates were prepared by, dry solvent casting method in a hot air oven. The PolyC & PS was dissolved in dichloromethane or PMMA in chloroform (10% w/v) and casted on clean glass plates to make films of 0.1mm thickness. The temperature of the surface balanced glass plates were maintained at 40°C, and closed with glass slabs during curing. An area of 5X5 cm^2 were cut and cleaned with 0.1% soap solution (Teepol), rinsed with distilled water and kept in distilled water for 12 hrs under stirring. Finally the PolyC and PS films were rinsed with ethanol, while PMMA in double distilled deionised water. The

clean films were then dried in a vacuum oven at 60°C for 4hrs. The dried films were packed aseptically, sealed in bottles and stored at ambient temperature.

2.1.1.2 Derivatization of PMMA films for covalent immobilization of laterally stabilized monolayer

The cleaned PMMA films as per the procedure 2.1.1.1 were used for the derivatisation. The partial hydrolysis of PMMA films, were done by treating, in 4M sodium hydroxide for 2hrs at 85°C and is then immersed in 10% (w/v) citric acid solution overnight at ambient temperature. The surface hydrolysed PMMA was washed with double distilled water until the pH of the washing reaches 5.6. Further, the polymer substrate was placed in an aqueous solution containing Ethylene divinyl carbodiimide (EDC) (0.5M) at ambient temperature for 48hrs to activate the carboxyl groups until deposition of the lipid monolayer.

2.1.1.3 Cross linked Poly vinyl alcohol (cPVA)

The cPVA films were prepared by dry solvent casting method in a hot air oven. For that a PVA (10gm%w/v) in distilled water and paraformaldehyde (PF) (40gm% w/v) in NaOH (5gm %) in distilled water were prepared separately. PVA and PF solutions were mixed well in the ratio 10:3 (v/v) for 30 min using at stirrer at 2000 rpm and casted onto glass plates and dried at 60°C for 4 days. The dried films were separated by incubating in water and washed for 4 days in distilled water to remove the excess PF. Then washed with ethanol and dried at 60°C for 2 days. The dried films were sealed in bottles and stored at ambient temperature.

2.1.1.4 Preparation of Chitosan (CHIT) bare and cross linked films

The CHIT films were prepared by using 3%w/v CHIT solution in 1.5 M acetic acid. It was casted on to surface balanced glass plates and dried at 40°C in a hot air oven. The acetic acid present in the membrane was removed by washing with 1N NaOH for 48hrs and then washed with plenty of water to remove the alkali. A piece of CHIT film 5X5 cm was swollen at pH 3.0 in Neutralised

Phthalate Buffer (NPB) for 30min and was then neutralized with pH 5.0 Phosphate Buffer (PB) for cross linking with the drug.

2.1.1.4.1 CHIT films cross linked with the drug

This swollen polymer was then washed with copious amount of distilled water and incubated in 25ml of 50mg % diclofenac sodium (DIC) in Distilled water (DW) for 5 hrs. The loosely bound drug was removed by incubating in DW for 24hrs. The dried samples were packed aseptically and stored at ambient temperature, until used. It had been optimized that 50% of the loaded drug was ionically cross-linked with the polymer.

2.1.2 Microspheres

Porous microspheres of CHIT were prepared to develop liposome delivering matrices for studying the drug release across the lipid monolayers.

2.1.2.1 Preparation of porous CHIT microspheres

The microspheres were prepared by using CHIT 2% (w/v) in 1.5% v/v acetic acid solution. A quantity equivalent to 25ml of this solution was added drop wise with the help of a syringe with 15G needle into 200ml of ethanolic NaOH (10% (w/v) solution in 250ml beaker under stirring (@ 2000rpm). The stirring was continued for next two hrs. Thus prepared microspheres were washed extensively with copious amount of water to remove the alkali until the pH comes to 5.6. These microspheres were swollen at pH 5.5 NPB. This swollen microspheres was then washed with copious amount of DW. They were sequentially introduced into Acetone/water mixture (10/90, 30/70, 50/50, 70/30, 100/0) and freeze dried (Labconco) after removing the excess acetone, to get a porous scaffold.

2.1.2.1.1 Ionic cross-linking of the microspheres with DIC

For drug immobilization the above mentioned swollen microspheres were incubated in 100ml of DIC 50mg % (w/v) solution in DW for 16 hrs. The loosely

bound drug was removed by incubating in DW for 24hrs. Further the microspheres were treated similarly (2.1.2.1), to get a porous scaffold.

2.1.3 Nanoparticles

Self-assembling nanoparticles were synthesized out of pendant polymers to study the drug release and immunological response to the monolayers.

2.1.3.1 Nanoparticles of pendant polymer of PVA

Nanoparticles of pendant polymer of PVA-DIC were prepared after the careful selection of the polymeric backbone and the pendant groups.

2.1.3.1.1 Selection of ligand for Pendant-PVA

The selection of the pendant groups for the preparation of the pendant polymer has been done based on the *in silico* docking studies with albumin molecule.

2.1.3.1.1.1 Extraction of structures with Arguslab

The crystal structures of the Albumin molecule (ID No: 2BXI)[156], Aspirin (ID No: 1TGM)[157], Indomethacin (ID No: 2BXK)[158], Diclofenac (ID No: 1DVX)[159] were obtained from the protein data bank (PDB). The bound ligands were extracted with the help of Argus Lab (version 4.0.1)[160] Arguslab can do geometry optimizations using the UFF force field with all elements of the Periodic Table. It also offers geometry optimization using the MNDO, AM1 or PM3 semiempirical levels, as well as single point calculations using these. There are also single point semiempirical calculation facilities using Extended Huckel (for a bigger element coverage) or ZINDO (for excited states for UV/visible absorption prediction). It also has facilities for calculating electron density or orbital surfaces at the semiempirical levels, electrostatic potential, onto a surface etc. The water molecules were removed and hydrogens were added to the PDB structures according to the standard method. All residue deletions were also done at this time.

2.1.3.1.1.2 Docking using Hex

Docking simulations were performed using Hex 3.1 docking software. A docking "run" with Hex 3.1 commences with the random placement of the probe within a search space that includes all or part of the target molecule. Flexible body Fourier correlation based annealing is then performed on the probe-target configuration, according to a fixed annealing schedule. Dockings that fall below a user-specified interaction energy cutoff are written to output.

2.1.3.1.2 Preparation of Pendant Polymer PVADIC

A quantity equivalent to 4gm of PVA was weighed and dissolved in 50ml of Dimethyl sulphoxide (DMSO) and heated at 80°C for 1hr. To this 2ml of pyridine was added as a base catalyst. The dissolved solution was then heated to 80°C for 1 hr. A quantity equivalent to (0.1, 0.5, and 1) gm of DIC was weighed and dissolved in 50 ml of DMSO (Here the drug to polymer ratio of poly vinyl alcohol- Diclofenac (PVADIC) pendant polymer was decided as (0.025:1) (a), (0.125:1)(b), (0.25:1)(c) respectively). To this 3ml of thionyl chloride was added slowly and kept outside for 15 min and the precipitated drug was redissolved by adding 50ml of acetone into the solution. This solution was transferred to the preheated PVA solution, slowly. The heating was continued for next 5hrs at 80°C. This solution was then diluted to 1000 ml with water which is further supersaturated with sodium chloride under stirring. The precipitated polymer formed as froth was separated from the surface with the help of a glass plate and transferred to pure water in which the polymer is insoluble. The polymer is redissolved in ethanol then by (acetone: water) (1:1) and reprecipitated.

2.1.3.1.3 Preparation of the nanoparticles out of the pendant polymer by self- assembly

A 10% (w/v) solution of the PVADIC (b & c) was prepared in ethanol. A quantity equivalent to 1ml of this solution was diluted to 100 ml with water. Thus formed nanoparticles or different structures were utilized for further studies.

2.1.3.1.3.1Preparation of ceramic nanocrystals with drug inside the nanoparticles of pendant polymer

For ceramic nanoparticle synthesis, (0.2M) calcium in the form of calcium chloride dehydrate and (0.12M) phosphate in the form of potassium dihydrogen ortho phosphate was used. All the solutions were prepared in double distilled deionised water under unbuffered conditions at ambient temperature. The calcium to phosphate ratio was maintained as 1.6 in the solution. To a 10ml test tube added 1.5ml of human insulin (40IU/ml) and made up with nanoparticle PVADICb (1%w/v) solution in distilled water to 7.5ml. To that Diclofenac (62.5, 31.25, 15.625 mg/ml) solution was added. To that 2.5ml of ethanol (0.25ml/ml) was added and exactly after 5 minutes 2.5ml of calcium solution (0.25ml/ml) was added and after mixing the 2.5ml of phosphate solution (0.25ml/ml) was added. The formed crystals remain suspended was separated after 24 hrs sedimented and washed three times with distilled water.

2.1.4 Liposomes

Liposomes of different lipid compositions were synthesized for the exploration of the thin solid films for drug delivery applications and to study the immunological response.

2.1.4.1 Liposome of mixture of lipids of phospholipids, cholesterol and galactocerebroside.

The following lipid solutions were used for the preparation of liposomes (1) (PC:Chol))(1:0.35), (2) (PC:Chol))(1:0.7), (3) OCMC (PC: Chol: GalC) (1:0.35:0.125), & (4) PCMCc (PC: Chol: GalC: PE) (1: 0.35: 0.125:0.29), w/w mg wt ratios, with respect to the concentration of PC(as per the procedure 2.2.2.1.1) was prepared in CH/ n- but (5:2 v/v) (71.43/ 28.57) % v/v solvent system. The solution was transferred to a round bottom flask and was rotary evaporated at 40°C and obtained a thin film of lipids. The thin lipid film was dried in a vacuum oven overnight to ensure complete removal of the solvent. The film was rehydrated

using the respective solution in PBS 7.4 and vortex mixed.[161]. They were further extruded using 100nm polycarbonate filters to form unilamellar liposomes.

2.1.4.2 Liposome of mixture of lipids of phospholipids, cholesterol and galactocerebroside, albumin, other anchors

The following lipid solutions were used for the preparation of liposomes (1) OCMC-A (PC: Chol: GalC: Alb) (1:0.35:0.125: 0.008), (2) OCMC-AHP (PC: Chol: GalC: Alb: Hep: PEG) (1:0.35:0.125: 0.008: 0.052: 0.15) w/w mg wt ratios with respect to the concentration of PC (as per the procedure 2.2.2.1.1) was prepared in ((DIW: Acet: CH: n-but) (5.00/45.02/35.72/14.26) % v/v) solvent system (as per the procedure 2.2.1.3). For the incorporation of albumin, its structure was altered as mentioned in the procedure 2.2.1.2 and incorporated into lipid solution as per the procedure 2.2.2.1.1. Vacuum dried along with the respective lipid components. The solution was transferred to a round bottom flask and was rotary evaporated at 40°C and obtained a thin film of lipids. The thin lipid film was dried in a vacuum oven overnight to ensure complete removal of the solvent. This lipid film was reconstituted in PBS 7.4 solution to form the liposomes. They were further extruded using 100nm polycarbonate filters (*liposoft*) to form unilamellar liposomes. The albumin concentration in the final solution was 0.075gm/ L.

2.1.4.3 Preparation of Albumin self- assembled liposomes

The unilamellar liposomal suspension, prepared by using a lipid solution of PC: Chol (1: 0.7 & 0.35) mg% w/ v in 3ml of a mixture of CH/ n- but (5: 2) v/v, as per the procedure 2.1.4.1, was further coated with the pendant polymer by incubation in PVADICa (1%w/v polymer in distilled water) solution at 10°C for 30 min. The PVA coated liposomes are used as control. A quantity equivalent to 2ml of polymeric solution was used for coating 2 ml of 5 gm% w/v of the homogenized liposome suspension, incubated at 10 °C for 2 h. The uncoated polymer from the liposomes was separated by filtration using Sephadex

(G120/200) (Sigma, USA) column and then it is diluted with 2ml of albumin (100 mg% w/v) solution. This solution was stored at 4-8 °C for further analysis.

The final polymer concentration in the liposomal suspension was 3-4%w/w. The coating of the polymer was evaluated by a previously reported procedure[162] after slight modification. In short a small quantity of the polymer coated liposomal suspension (5ml) was added with 5ml of acetone to precipitate the liposomes and was then centrifuged (Remi C24) at 15, 000 rpm for 30 min (Remi centrifuge), The components like lipids in CH/ n- but (5: 2) and polymer in water was separated and evaluated gravimetrically.

2.1.5 Preparation of Liposome delivering matrices (Lipomat)

Our preliminary studies have demonstrated that the liposome formation is directly proportional to the flexibility of the monolayers, Incorporation of cholesterol and other agents found to reduce the liposome formation.

The Lipomat was prepared by loading the model drug insulin and PC together in a porous CHIT microspheres prepared as per the procedure 2.1 .2. 1. For that the required amount of insulin was added to these microspheres and freeze dried for diffusion filling into the microspheres. The incorporation of the PC into the microspheres was done, after precipitating the entrapped drug with acetone. A solution of 50mg% PC in CH/ n- but (5:2 v/v) solution was added into the microspheres and vacuum evaporated until all the solvents were evaporated. Thus formed lipomat was dried completely in a vacuum oven, sealed aseptically in to bottles and stored at 4°C until used for further studies.

2.1.6 Polymeric gels

Polymeric gels of PVA and PVADICa was prepared to study the biofriction of the lipid thin films.

2.1.6.1 Polymeric gels of PVA and Pendant PVA

A 2% solution of PVA and PVADICa was prepared in distilled water at 40°C. They were thoroughly mixed with magnetic stirrer and kept under vacuum to remove any trapped air bubbles. The gels were stored in the refrigerator until used.

2.2 Development of lipid thin films for surface modification of the substrate

Development of lipid thin films by mimicking biological cell membrane was done for the post synthetic surface modification of the substrates.

2.2.1 Development of Co-solvent system

The heterogeneous phospholipid/ glycolipid/ cholesterol with other macromolecules like albumin, heparin and polyethylene glycol were attempted to prepare. For that a common co-solvent system which is not affecting the supporting substrate was developed.

2.2.1.1 Selection of parent solvent

A film of 2X2 cm of different polymers (prepared as per the procedure 2.1.1) was dipped in the various solvents (25ml of DCM, CF, n-hept and CH) for 1min and dried in air under ambient conditions. The solvents which affect the optical clarity of the films were discarded.

2.2.1.2 Solubility studies of lipids

The CH was selected as the parent solvent. The polarity of the CH was altered with Eth, Meth, and n-But, in specific ratios. Solubility studies of the lipid were done by dissolving a quantity equivalent to 25mg of the specific lipids (PC, Chol, GalC) into 3ml of solvent system and stirred well and kept the ampoules for 16hrs. then the supernatant was removed, 1ml of the supernatant was added to tarred ampoules and dried at 40°C to measure the quantity of lipid dissolved. Further mixture of lipids were prepared by dissolving sequentially the individual lipids in the following order GalC > PC> Chol into the co-solvent system.

The following lipid mixtures were prepared for the preliminary air/water interfacial studies (1) (PC: Chol) (1:0.7), (2) (PC: Chol) (1:0.35), (3) (PC: Chol: GalC) (1:0.35:0.125) w/w mg wt ratios with respect to the concentration of PC. Based on the air/water interfacial studies we have chosen (3.2.2) the particular lipid composition (PC: Chol: GalC) (1:0.35:0.125) as the optimized Outer Cell Mimetic lipid Composition (OCMC) containing the minimum essential lipid components for further studies. The concentration of the lipids in mixed lipid systems were expressed in mg wt ratios (w/w), with respect to the concentration of PC.

2.2.1.3 Incorporation of other macromolecules into lipid systems

The macromolecules attempted to incorporate into the lipid monolayer were Alb, Hep and DAPEG. Here we had selected the acetone/ water co-solvent system for the said purpose based on the solubility studies of Alb, Hep, and DAPEG. The optimized concentration of the macromolecules in the final solution was (PC: Chol: GalC: Alb: Hep: DAPEG) (1:0.35:0.125: 0.008: 0.052: 0.15) w/w mg wt ratios with respect to the concentration of PC) (59.34:20.77:7.42:0.46:3.09:8.90)w/w of total solid content). Here the macromolecules were dissolved in DIW then the required quantity of Acet was added. From that 1:1 quantity CH/n-But and Acet/DIW system ((DIW: Acet: CH: n-but) (5.00/45.02/35.72/14.26) % v/v)) was mixed. The lipids were predissolved in the CH/n-but solvent system prior to mixing (as per the procedure 2.2.1.2).

2.2.2 Interfacial studies of the lipid films using LB trough facility

The figure 2.2.2.1 shows the schematic diagram of the Langmuir balance. The equipment works according to the principle of thin solid films by Langmuir and Blodgett (1.3.2.4.2). The trough holding the subphase (Teflon) which was thermostated by circulating water bath. The surface area of the trough can be varied by sweeping movable barriers (Delrin)[163]. The surface pressure and the mean molecular area were continuously monitored during the compression.

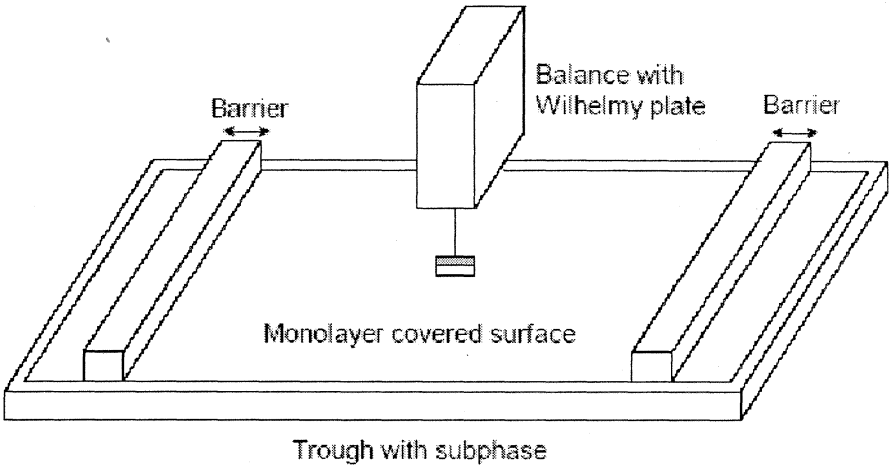


Figure-2.2.2.1: LB trough facility

The surface pressure was measured by the Wilhelmy plate-method. In this method a measurement was made by determining the force due to surface tension on a platinum plate suspended so that it was partially immersed in the subphase (see Figure-2.2.2.1). This force was then converted into surface tension (mN/m or dynes/cm) with the help of the dimensions of the plate.

2.2.2.1 Air/water interfacial studies (Intramolecular interactions)

The air/ water interfacial studies of the amphiphiles were done using KSV 5000 and 2000 (KSV, Finland) LB trough. Studies were done on a subphase of Milli Q water (MW) at ambient temperature conditions.

2.2.2.1.1 Simple, mixture of lipids incorporated with anchors

The individual PC, Chol, and GalC lipids, binary and the ternary lipid combinations with or without macromolecular anchors like Alb, Hep and DAPEG were studied at the air/ water interface at ambient temperature. The lipids were used without further purification. Lipid stock solutions were freshly prepared by dissolving the pre-weighed lipids in a known volume of spreading solvent and mixed in required proportions (CH/n-but) (71.43/ 28.57) (5:2) % v/v (as per the

procedure 2.2.1.2). The working solutions were prepared by mixing appropriate volumes from stock solutions. Apart from the individual lipids, the following lipid solutions were used for the preparation of monolayers (1) (PC: Chol) (1:0.35), (2) (PC:Chol))(1:0.7), (3) OCMC (PC: Chol: GalC) (1:0.35:0.125) w/w mg wt ratios with respect to the concentration of PC. For the incorporation of phosphatidylethanolamine (PE) varying concentrations of PE (a, b& c) (PC: Chol: GalC: PE) (1: 0.35: 0.125: (a) 0.0725, (b) 0.145, (c) 0.29) was added. For macromolecule incorporated lipid systems the co-solvent was (DIW: Acet: CH: n-but) (5.00/45.02/35.72/14.26) % v/v). The following lipid solutions were used for the preparation of monolayers (1) OCMC-A (PC: Chol: GalC: Alb) (1:0.35:0.125: 0.008), (2) (1) OCMC-AH (PC: Chol: GalC: Alb: Hep) (1:0.35:0.125: 0.008: 0.052), (3) OCMC-AHP (PC: Chol: GalC: Alb: Hep: PEG) (1:0.35:0.125: 0.008: 0.052: 0.15) w/w mg wt ratios with respect to the concentration of PC. The Alb, Hep, and DAPEG were dissolved in acetone/ water system and mixed with predissolved lipids the cyclohexane/ n- Butanol system to come to the definite proportions (PC: Chol: GalC: Alb: Hep: PEG) (1:0.35:0.125: 0.008: 0.052: 0.15) (as per the procedure 2.2.1.3). The concentrations of the Alb, Hep, and the DAPEG were selected based on its maximum solubility in the above solvent system. Monolayer experiments were performed on a subphase of Deionised water MW (double distilled and passed through a Milli-Q (Millipore Corp., Bedford, MA, USA) purification system to a resistivity of 18 MΩ/cm) at ambient temperature. Plots of surface pressure vs. molecular area of monolayers were constructed using a computer-controlled wilhelmy balance in LB trough (KSV 5000, Finland). All mixtures were prepared immediately prior to use. Lipids were spread in small aliquots of 10 µl with a Hamilton syringe, which was rinsed with spreading solvent repeatedly before and after use. The compressions were initiated after a 10min delay to allow evaporation of the spreading solvent, as well as the area vs. barrier position comes to the equilibrium (i.e. after congealing of the monolayer). The

compression rate did not exceed 4 $Å^2$/ molecule/ min. Data were collected with KSV Inbuilt software[164] .

2.2.2.1.2 Amphiphilic polymer

The mechanical properties of the thin solid films of the pendant polymer PVADICc (prepared as per the procedure2.1.3.1.2) at the air/ water interface at ambient temperature were studied with the help of a LB trough (KSV 2000, KSV Finland). Polymer stock solutions were prepared by dissolving the pre-weighed polymer (10gm%w/v) in a known volume of spreading solvent (Eth: CH: n-but) (3:5:2 v/v). Monolayer experiments were performed on a subphase of MW (double distilled and passed through a Milli-Q (Millipore Corp., Bedford, MA, USA) purification system to a resistivity of 18 MΩ/cm, at ambient temperature. Plots of surface pressure vs. molecular area of monolayers were constructed using a computer-controlled wilhelmy balance in LB trough. All polymer solutions were prepared immediately before use. Polymer solutions were spread in aliquots of 250 µl with a Hamilton syringe, which was rinsed with spreading solvent repeatedly before and after use. The compressions were initiated after a 10min delay to allow evaporation of the spreading solvent, as well as the area vs. barrier position comes to the equilibrium (i.e. after congealing of the monolayer). The compression rate did not exceed 4 $A^{\circ 2}$/ molecule/ min. Data were collected with a KSV Inbuilt software[164].

2.2.2.2 Air/solid transfer studies (Intramolecular interactions)

Further the compressed monolayers were transferred from air/water interface to polymeric films using LB trough to study the monolayer substrate interactions and for biorecognition studies.

2.2.2.2.1 Simple and mixture of lipids

The compressed monolayers of different lipid compositions (prepared as per the procedure 2.2.2.1.1) were deposited on to the PolyC films (prepared as per the procedure 2.1.1.1) by the vertical dipping method[164] either in the upstroke or

down stroke mode using LB double barrier Teflon trough (KSV 5000) at ambient temperature. The surface pressure was maintained at 30mN/ m during deposition, is higher than the critical bilayer pressure. The surface pressure was continuously monitored using a platinum Wilhelmy plate microbalance. The coated membranes were dried at 40°C for 4hrs in a hot air oven and stored in sealed containers. These surfaces were used for further studies.

2.2.2.2.2 Lateral stabilization by chemical bonding

The compressed monolayers of different lipid compositions (prepared as per the procedure 2.2.2.1.1) were deposited on derivatised PMMA substrates (prepared as per the procedure 2.1.1.2) by the vertical dipping method[164], using LB double barrier Teflon trough (KSV 5000) at ambient temperature. The surface pressure was maintained at 30mN/ m during deposition, used to be higher than the critical bilayer pressure. The surface pressure was continuously monitored using a platinum wilhelmy plate microbalance. The coated membranes were then immersed in an aqueous solution containing EDC (0.5M) at ambient temperature for 48hrs to activate the carboxyl groups of the anchors and to cross-link them[165], [166]. They were then extensively washed using double distilled water under static condition to remove the unreacted EDC for four days. The samples thus prepared were dried at room temperature and stored in sealed containers. These surfaces were used for further studies.

2.2.3 Transfer of the nanoparticle monolayer into the polymer substrates

Transfer ratio of the thin film of nanoparticles of PVADICc into different substrates was studied by LB technique (KSV 2000). The studies were done in up stroke as well as down stroke mode into various substrates (as per the procedure 2.2.2.2.1).

2.2.4 Development of methods to modify different kind of substrates

Methods to transfer the monolayer into different kind of substrates like microspheres and nanoparticles were developed further. This is very important for

the post synthetic surface modification of biomaterials. Since these are self-assembled systems, at confined geometries, they can retain the maximum packing density.

2.2.4.1 Films

Deposition of monolayer onto flat surfaces is important for the surface modification of tissue culture plates, and devices with larger surface area. Here monolayer deposition from the air/water interface is often difficult. Therefore we have developed simple deposition techniques to have a comparison with the model supported lipid layers.

2.2.4.1.1 In situ method from air/ water interface

A quantity equivalent to 1ml of the lipid solution prepared as per the procedure 2.2.2.1.1 was spread on a 250ml beaker containing MW with the help of a pipette. The solvent was allowed to evaporate for next 10 min. Then the monolayer was deposited onto polymeric substrate with vertical dipping either in the down stroke or upstroke mode. The coated surfaces were dried at 40°C for 4hrs in a hot air oven and stored in sealed containers.

2.2.4.1.2 Spreading and solvent evaporation method

For deposition of the monolayer onto films by spreading the lipid solutions were prepared as per the procedure 2.2.2.1.1, and for Pendant polymer ((PVADICc), 2.2.1.2). A quantity equivalent to 1ml of the lipid solution was added in a 24 or 96 well tissue culture plate (or films kept in the plates), then pipetted out immediately. The coated surfaces were dried aseptically under an aseptic hood with the help of a hair blower at 40°C for 20min. For the coating of a second monolayer the process was repeated. The dried surfaces were then sealed aseptically and stored for a week, before the studies.

2.2.4.2 Microspheres

Deposition of the monolayer onto curved surfaces is important for the surface modification of drug delivery systems, small diameter vascular graft etc.

Therefore methods to modify microspheres were developed to study the properties of the monolayer on curved surfaces.

2.2.4.2.1 Solvent evaporation by freeze drying in the case of Lipomat

For deposition of the monolayer (of PC&OCMC) onto lipomat microspheres (prepared as per the procedure 2.1.5) by dipping, (the lipid solutions were prepared as per the procedure 2.2.2.1.1). A quantity equivalent to 100mg of microspheres were weighed to a tarred beaker to that 5ml of the lipid solution is added and allowed to dry by freeze drying and stored in sealed containers.

2.2.4.2.1.1 Biofriction studies of microspheres by sliding method

The studies were done on a 15ml glass test tube placed vertically. One microsphere was placed at the open edge and time required for it to travel 10ml down the tube was measured under wetting with distilled water. This is done for different kind of microspheres and the readings are mean±S.D. of at least six samples.

2.2.4.3 Nanoparticles

Development of strategies for the post synthetic surface modification of nanoparticles is important in the case of drug delivery systems and tissue engineering scaffolds. Therefore methods were developed for modifying the nanoparticles with lipid thin films.

2.2.4.3.1 Monolayer coating on nanoparticles by rehydration

The PVADICb nanoparticles (prepared as per the procedure 2.1.3.1.3 and 2.1.3.1.3.1) were modified with lipid monolayers of OCMC & PCMCc (prepared by using a lipid solution prepared as per the procedure 2.2.2.1.1). The solution was transferred to a round bottom flask and was rotary evaporated at 40°C and obtained a thin film of lipids. The thin lipid film was dried in a vacuum oven overnight to ensure complete removal of the solvent. The film was rehydrated using the nanoparticle suspension in Distilled water and vortex mixed[167]. The PVADICb

nanoparticle coated with the lipid film alone served as the control. These modified nanoparticles were freeze dried and stored at 4°C.

2.2.4.4 Polymeric gels

Polymeric gels have been explored for drug delivery applications. Post synthetic surface modification of polymeric gels for improving the boundary lubrication has tremendous opportunity in the case of synovial fluid substitutes. Therefore strategies were developed for the transfer of the monolayer to polymeric gels.

2.2.4.4.1 Monolayer coating on polymeric gels by Layering, solvent evaporation and vortexing

Coating of the monolayer over the polymeric gels was done by preparing the polymeric gel as per the procedure 2.1.6.1 and lipid solution of PC, as per the procedure 2.2.2.1.1. A quantity equivalent to 5ml of the polymeric gel was spread on a 100ml beaker to that 1ml of the lipid solution was added. The solvent was allowed to evapourate for 10 min under vaccum then the second polymer layer was added and then the lipid layer, which is continued for 20ml of polymeric gels, and the layers were mixed thoroughly with the help of a magnetic stirrer for 16 hrs, followed by sonication (56Hz) for 10min.

2.2.4.4.1.1 Biofriction studies by protrusion method

The biofriction of the polymeric gel was studied by protrusion method using a 5ml syringe. The time required for 1ml of the polymeric gel to pass through 25G needle was measured. Entrapped air bubbles were removed by applying vacuum prior to the test. The results are Mean±S. D. of six samples.

2.2.5 Stability studies of the monolayer

Stability studies of the monolayer was done under static and shear conditions.

2.2.5.1 Under static conditions

Stability studies were done under static conditions at ambient temperature. For that 2X2 cm of the films were cut and incubated in 25ml of Phosphate Buffered Saline (PBS) (7.4) for 24 hrs. The collected samples were washed with distilled water to remove buffer salts, and dried at room temperature. The change in contact angle was studied by using a goniometer[168].

2.2.5.2 Under shear conditions

Stability studies were done under shear conditions using a rotary shaker at ambient temperature. The modified surfaces (2X2cm) were immersed in 25ml of PBS (7.4) and shaken at 150rpm at ambient temperature. Samples were collected at specified intervals, washed with distilled water to remove buffer salts, and dried at room temperature. The change in contact angle was studied by using a goniometer[168].

2.2.6 Pore volume measurements

The pore volume of the microspheres was evaluated by measuring the volume occupied by the organic solvent. For that the swollen microspheres were exchanged with acetone/water mixture (10/90, 30/70, 50/50, 70/30, 100/0) as per the procedure 2.1.2.1, Then with Acetone/ Dichloromethane (50/50) mixture and finally with dichloromethane alone. The volume occupied by the DCM was evaluated gravimetrically after centrifugation at 10,000rpm for 10 min in a sealed test tube.

2.2.7 Sedimentation studies

The pendent polymer form nanoparticles at all the concentrations of PVADIC (b & c), through internal self-assembly. Nanoparticles prepared (as per the procedure 2.1.3.1.3) was further diluted with water at (1:2 and 1:4) concentrations. These samples were centrifuged (Remi R4C Laboratory Centrifuge) at 1000, 2000 and 3000 rpm, were then redispersed with a test tube shaker before the analysis. The samples at 25 & 70° C were used for the studies. They were further characterized by UV/ Vis and DLS experiments.

2.2.8 Drug content and drug release studies

Drug release studies across the monolayers were studies after immobilizing the monolayer over microspheres and nanoparticles. The insulin and diclofenac was used as model drugs in these studies.

2.2.8.1 Insulin release studies

Insulin (Human 40 IU/ ml) was used as a model drug in these studies. The drug was loaded into the microspheres by diffusion filling method. Drug content and drug release studies of the microspheres was done using Lowry method using the Folin-Cu reagent[169]. Briefly, 100mg of the microspheres dispersed in 1ml of the buffer is tied into a dialysis bag tubing (M. Wt. cutoff 33,000) and was incubated in 19 ml (total dissolution medium = 20ml) of the PBS (7.4). A quantity equivalent to 200µl of the samples were taken at specified intervals (replaced with the buffer) and analyzed by Lowry method using UV- Visible spectrophotometer (Model UV 160A) at 750 nm.

2.2.8.2Diclofenac release studies

A quantity equivalent to 10 mg of the nanoparticles (prepared as per the procedure 2.1.3.1.3.1, and monolayer coated nanoparticles as per the procedure 2.2.4.3.1) is weighed and added to 1ml of the PBS 7.4 solution. At regular intervals the samples were centrifuged at 3000rpm (Remi R4C) and 0.2ml of the supernatant is collected and evaluated by UV-Vis Spectroscopy at 284 nm.

For the drug content evaluation a quantity equivalent to 10mg of the nanopartcle was dissolved in1ml of 0.5N Hcl. Further the pH is adjusted to 7.4 with 1N NaOH and the solution was made upto 5ml with distilled water. The solution was sedimented at 10,000rpm for 30 min. 0.2ml of the supernatant is collected and evaluated by UV-Vis Spectroscopy at 284nm.

2.2.8.3 Diclofenac content of the supernatant of pendant polymer

The supernatant solution was collected during preparation of pendant polymer 2.1.3.1.2, and 1ml of the solution was diluted with 1ml of 0.1N NaOH

and the pH is further adjusted to 7.4 with 0.1 N Hcl. This solution was diluted to 10ml with distilled water and evaluated by UV-Vis Spectroscopy at 284 nm.

2.2.9 Optical microscopy

The formation of liposomes from the lipomat and the blood cell adhesion studies were done by optical microscopy using optical microscope (Nikon, Japan).

2.2.9.1 Liposomes

The formation of the liposomes from the Lipomat was studied at different time intervals with the help of an optical microscope at 100X magnification.

2.2.9.2 Adhered cells

The blood cell adhesion studies were done as per the procedure 2.3.3. The number of adhered cells per unit area was counted using an optical microscope at 400X magnification, after staining the leukocytes and platelets with coomassie blue staining solution.

2.2.10 Stereo zoom microscopy

The size distribution of the Lipomat was studied by stereo zoom microscope (Leica MZ 6) at 100x magnification. Ten Lipomat were counted and taken the average in the dried state.

2.2.11 Goniometry: Contact angle studies

The contact angle measurements provide information about of the polarity and surface energy of a substrate. A drop of liquid is placed onto a material (Figure2.2.11.1), and the angle that the drop makes is measured by a goniometer. This angle is used in a force-balance equation in order to determine the surface tension of the material.

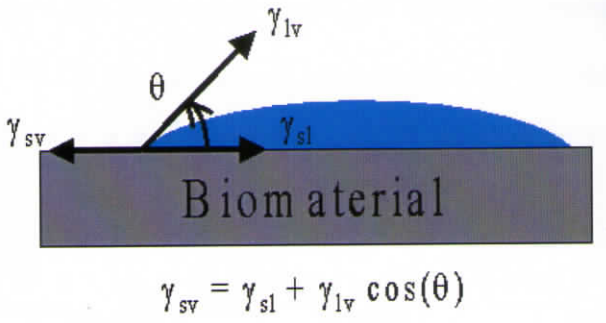


Figure-2.2.11.1 :Goniometry

Method

The Contact angle measurements were done by sessile drop method, using double distilled deionised water, at different temperature conditions. The polymer films were mounted on a glass plate and placed into the stage. One drop of water was placed on the film using Hamilton syringe. Angle on both side of each bubble was measured.

2.2.12 UV/ Vis spectroscopy

Principle

The change in intensity of light passing through a sample is proportional to path length, concentration and intensity of incident light as per Beer –Lambert's Law. It can be explored for the evaluation of turbidity changes and concentration of various molecules in the solution. The UV-Vis spectroscopy measurements were done using a UV-Vis spectrophotometer (UV-160A, Shimadzu).

2.2.12.1 Turbidity studies

Turbidity studies of the different solutions were done, after diluting it suitably with distilled water, at 350nm with the help of a UV/ Vis spectrophotometer with respect to time.

2.2.12.1.1 Turbidity studies indicating the conformational change of proteins

The conformational change of Alb was studied by preparing a 25 mg % w/v albumin solution in DW and from this 1ml of albumin solution is added to different test tubes to this acetone is added in different ratios and finally the volume is made upto 10ml the corresponding solvent system as such was used as the blank for the studies.

2.2.12.2 Protien content evaluation

The protein content evaluation was done by Lowery method. For that to the 200µl of the collected sample (as per the procedure 2.2.8.1) 5ml of sodium potassium tartarate and 200µl of Folin- Cu reagent was added. After 15 min the absorbance was measured with the help of a UV-Vis spectrophotometer at 750nm, using the solvent as a blank. The concentrations of the respective samples were calculated from a standard curve prepared by serial dilution.

2.2.12.3 Diclofenac content evaluation

The diclofenac content was evaluated by UV-Vis Spectroscopy. For that, the 200µl of the sample (collected as per the procedure 2.2.8.2) was made upto 5ml by adding 4.8 ml of distilled water (other cases suitably diluted). The absorbance of the suitably diluted samples were evaluated at 284nm using the distilled water as the blank. The concentrations of the respective samples were measured from a standard curve prepared by serial dilution.

2.2.12.4 Absorption spectra of nanoparticles

The absorption spectra of the nanoparticles were studied by using a UV/Vis spectrophotometer at wavelength 200 to 800nm in overlay mode. Samples were diluted suitably to get the UV-Vis Spectra in the detectable range.

2.2.13 Flourimetry

Fluorescence spectra were created by a, Varian, Cary Eclipse Fluorescence Spectrophotometer. The nanoparticles of PVADICc Sample 0.1(A), 0.05(B),

0.025(C) gm% were studied by this method. The samples were excited at excitation wavelengths 244 and 266 as well as 274, as well as at 310nm and the emission spectra were studied from 300-600nm. The emission peak was observed to be at 497nm when excited at 244nm. Otherwise none of the samples was emitting between the above wavelengths from 300-600nm at any of the above said wavelength, except sample A. The sample A was showing feeble absorption maxima at 290nm and emission peak at 495 and 497nm and it was found varying with concentration. Three concentrations of the sample B nanoparticles were excited at 244nm and emission was checked between 300-600nm.

2.2.14 FTIR Spectroscopy

The FTIR spectra of the samples were done using FTIR Impact 410 spectrometer. Solid samples are milled with potassium bromide (KBr) (IR transparent) to form a very fine powder. This powder is then compressed into a thin pellet which is analyzed.

2.2.15 Dynamic light scattering

When a laser beam having a known frequency is directed, at the moving particles, the light is scattered, but at a different frequency. The shift is termed as Doppler shift or broadening (Figure-2.2.15.1) is related to the size of the smaller particles causing the shift. Due to their higher average velocity, smaller particles cause a greater shift in the light frequency than larger particles.

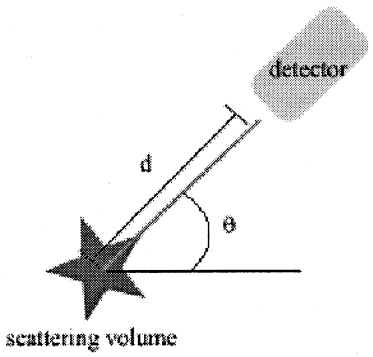


Figure-2.2.15.1 Dynamic Light Scattering

A commercial DLS spectrometer (Zetasizer Nano ZS Red Badge, Model No: ZEN 3600, Malvern) equipped with a multi-row digital time correlator and a He-Ne, 3.0 mW laser (Lamda Zero 633nm) with a automated power attenuator (with a attenuation index of 0-11 with transmission (% normal) from 0-100 with corresponding inward attenuation) was used. The incident beam was vertically polarized with respect to the scattering plane. The measurement position within the system was automatically moved to allow a large range of sample concentrations to be measured. All the measurement positions were above the 4.65mm from the cell wall when a 12mm square cuvette was used (Indicating that all the measurements were done in planes which is far from the cuvette surface). In this instrument the attenuator index and measurement position are automatically adjusted by the software. Standard 12mm square glass cuvettes were used for the studies. All the experiments qualified the size quality criteria. The equipment utilizes Non Invasive Back Scatter (NIBS) Technique using a 173° detection optics. Here the incident beam is not traveled through the entire sample, therefore if offers least multiple scattering, and reduced scattering noise by large particles. The instrument is equipped with a movable lens, which allows a much larger range of sample concentration to be measured.

2.2.15.1 Size of PVA DIC nanoparticles

A 10% (w/v) solution of the polymers PVADICb & c (Prepared as per the procedure 2.1.3.1.2) was dissolved in ethanol was prepared. A quantity equivalent to 1ml of this solution was diluted to 100 ml with DIW. Thus formed nanoparticles were studied by DLS at 25°C.

2.2.15.2 Size of the liposomes

The size of the polymer coated liposomes (Prepared as per the procedure 2.1.4.1) was measured by DLS, after diluting the stock solution with PBS 7.4, to prepare a 1mg/ml solution.

2.2.15.3 Melting curve of nanoparticles

The nanoparticles were prepared at 1mg/mL concentration in distilled water. The melting point is defined as the temperature at which the structure denatures. The change in size that accompanies the denaturation is easily identified using DLS techniques. The temperature dependent Z-Average diameter and scattering intensity for nanoparticles was measured with a Zetasizer Nano ZS, using a 1°C incremental temperature and a 3 minute equilibrium time at each measurement temperature.

2.2.16 Atomic force microscope (AFM)

In an AFM a constant force is maintained between the probe and sample while the probe is raster scanned across the surface (Figure-2.2.16.1). By monitoring the motion of the probe as it is scanned across the surface, a three dimensional image of the surface is constructed.

The constant force is maintained by measuring the force with the "light lever" sensor and using a feedback control electronic circuit to control the position of the Z piezoelectric ceramic. The motion of the probe over the surface is generated by piezoelectric ceramics that move the probe and force sensor across the surface in the X and Y directions.

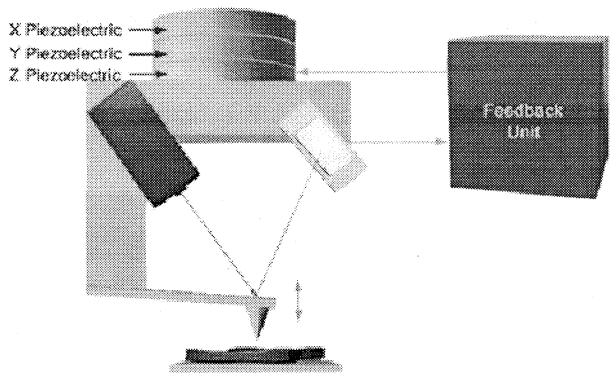


Figure-2.2.16.1 The primary components of the light lever atomic force microscope. The X and Y piezoceramics are used to scan the probe over the surface

Procedure

The modified polymer films using various supported lipid monolayers were studied using AFM (NT-MDT model –SPM solver P47)[164]. in contact mode in air. Scanner with maximum scan area of 40μm X 40μm and rectangular cantilever of silicon nitride (length 200μm, width 40μm) having force constant of 3N\m were employed for the measurement. Several areas were scanned for each sample.

2.2.17 Scanning electron microscopy

The SEM scan the high energy secondary electron beam across a surface to generate highly resolved topographical images. Only those secondary electrons that are produced within a very short distance of the surface are able to escape from the sample enable the SEM mode boasts high resolution topographical images. The figure-2.217.1 shows the assembly of a conventional SEM.

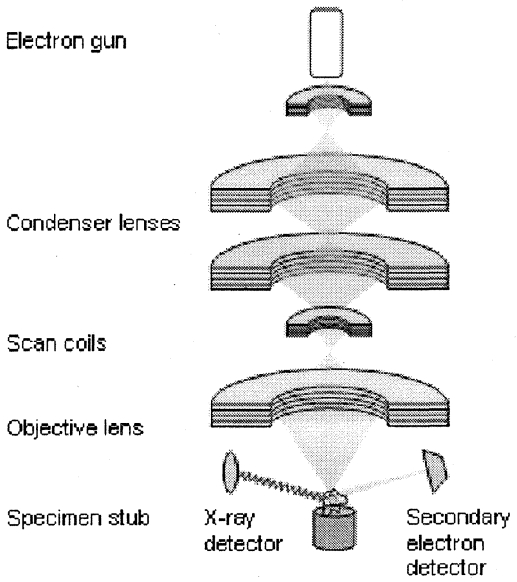


Figure-2.2.17.1 SEM

General Method

The surface or internal morphology exposed samples were examined by SEM. Dehydration of the tissue specimens were carried out with ethanol in a graded series to avoid shrinkage. For dehydration the samples were dipped for 15min each in a series of 30. 50. 70. 80 90, 95 & 100% ethanol. Finally the samples were critical point dried (CPD) in liquid CO_2. The samples were mounted onto aluminium stubs using double sided adhesive tape, further coated with gold film and were observed under SEM (Model S-2400, Hitachi, Japan).

2.2.17.1 Microspheres

The surface morphology as well as the internal structure (cut open) of the Lipomat (prepared as per the procedure 2.1.5) was studied after mounting on an aluminium stub (as mentioned in 2.2.17) using SEM after gold coating.

2.2.17.2 Nanoparticles

A quantity equivalent to 0.5ml of the nanoparticle (10mg/ml) suspension (prepared as per the procedure 2.1.3.1.3) was suspended over a 100nm filter paper, which is pre treated with a sticker. Then the sample was air dried at 25°C for 5 days and these samples were dried by CPD and then gold coated (as mentioned in 2.2.17), and studied by SEM.

2.2.17.3 Adhered platelets

The samples collected after the platelet activation studies (as per the procedure 2.3.3.4) were dehydrated with gradient ethanol method and further dried by CPD method (as mentioned in 2.2.17). The dried films after gold coating were examined with SEM[170].

2.2.18 Transmission electron microscopy (TEM)

Principle

When an electron beam encounters a nucleus in the sample, few of these electrons will be completely backscattered, re-emerging from the incident surface

2.2.19 Differential Scanning Calorimetry (DSC)

Principle

Differential scanning calorimetry (DSC) measures the heat change with respect to temperature. The detection of changes in the heat content (enthalpy) or the specific heat of a sample, for a given energy input, depending upon the specific heat of the sample. The specific heat of a material changes slowly with temperature in a particular physical state, but alters discontinuously at a change of state, accompanied by a change in enthalpy, the latent heat of fusion, heat of reaction etc. Such enthalpy changes may be detected by thermal analysis and related to the processes occurring in the sample.

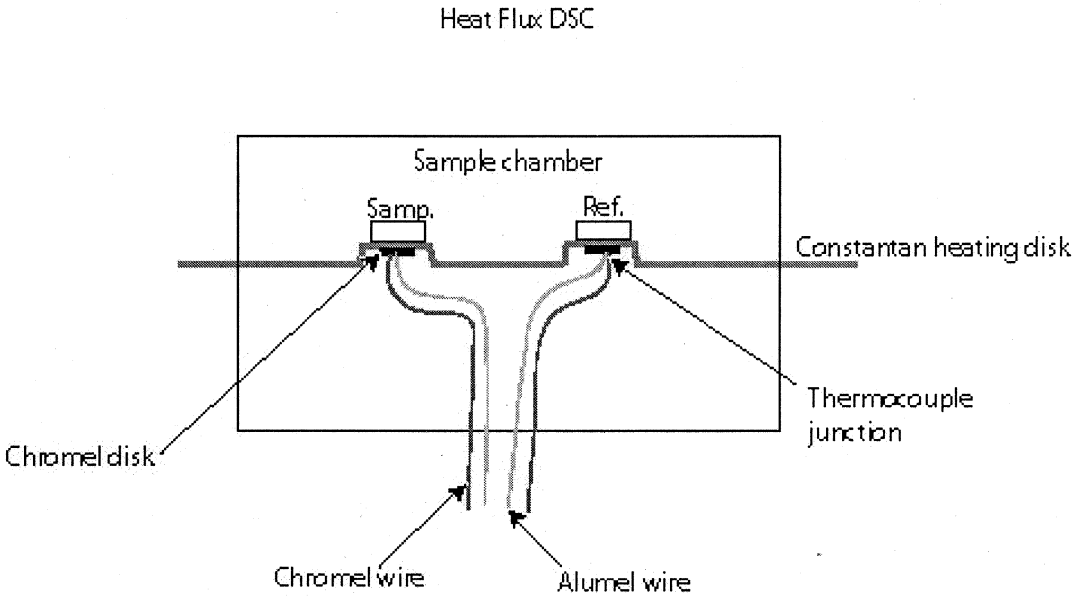


Figure-2.2.19.1 DSC

Method

2.2.19.1 Evaluation of the self-assembled albumin by DSC

Calorimetric measurements were done at a scan rate of 1°C/ min using a DSC 2920 Differential Scanning Calorimeter (DSC) (TA Instruments Inc.). The liposome concentration for the calorimetric study was 2.5 gm% w/v. The insulin

content of the liposome was 0.16 gm %w/v. The albumin concentration of the liposome solution was 0.1 gm % w/v. All the solutions were prepared in PBS pH7.4. The samples were equilibrated at 25°C. The reference solution, sample and the calorimetric cells were kept at ambient temperature at the time of sample introduction and were subsequently cooled to 20 °C. Calorimetric measurements were done from 20- 120°C at a heating rate of 1°C/ min. An external pressure of 1atm was maintained to prevent possible degassing of the solution on heating. Buffer base lines were substracted from each DSC scan. ΔH was calculated from the area under the curve of each calorimetric peak.

Biorecognition studies

2.3.1 Calcification studies

The calcification studies were done using metastable salt solutions[171]. In this system the calcium to phosphate molar ratio was maintained at 1.67 as in hydroxyapatite. The final concentration of calcium (0.20 M) as (calcium chloride dihydrate) and phosphate (0.12 M) as (potassium dihydrogen orthophosphate) was used. During the studies, the pH was maintained at 5.5 using (0.2M) neutralized phathalate buffer (NPB), to reduce the induction of nucleation[172],[173]. The studies were conducted under static conditions at constant volume and ambient temperature. The samples of 2X2cm were taken and vertically placed in the incubation medium. This can avoid the error due to the deposition of nucleates on the surface and related crystal growth. After 48 hrs of incubation period, the substrates were removed and were gently washed with the distilled water to remove the excess solution. The films were then oven dried at 60 °C for 4 hrs and the surface morphology was studied using an optical microscope.

2.3.2 Protein adsorption studies

2.3.2.1 Protein adsorption/desorption studies from mixture of proteins using SDS- PAGE

The SDS-PAGE was used to separate and evaluate the adsorbed proteins from mixture of proteins. The studies were done from a mixture of proteins of albumin, γ globulin, and fibrinogen, after desorbing them using Triton X-100. The polymer substrates (5X5cm) were exposed for 3hrs to a protein mixture. The surface adsorbed proteins were desorbed using 0.1 M Tris- HCl buffer (pH 8.5) containing 1 % Triton X 100. The samples prepared for SDS-PAGE were similar to that prepared by Chiu et al.[174]. (They standardized the procedure using radiolabeled proteins. Chiu et al. pointed out that it is better to use SDS-PAGE to separate the fibrinogen and γ-globulin bands. But in this procedure the peptide chains of the protein molecules are not cleaved. Therefore, The individual proteins were separated based upon their molecular masses). The electrophoresis was carried out with 150 µl of the sample. The SDS-PAGE was performed on a 5.6 % cylindrical gels containing 1 % SDS. Bromophenol blue was added to the samples to a final concentration of 10 mg/ml as a tracking dye, the electrophoretic run was done and analyzed at 4°C at a voltage of 20 V/gel. When the dye front was moved about 0.5cm from the bottom of the gel, the gels were removed, stained with 0.05 % Coomassie brilliant blue, destained in 5 % acetic acid / 10 % methanol and were scanned using a UV-VIS spectrophotometer (UV -160A Shimadzu) at λ max 620nm, attached with gels scanner.

2.3.2.2 Preparation of samples for Conformational studies of proteins[175] using CD spectroscopy

Principle

Linear polarized light can be viewed as a superposition of opposite circular polarized light of equal amplitude and phase. A projection of the combined amplitudes perpendicular to the propagation direction thus yields a line. When this

light passes through an optically active sample with a different absorbance for the two components, the amplitude of the stronger absorbed component will be smaller than that of the less absorbed component. The consequence is that a projection of the resulting amplitude now yields an ellipse instead of the usual line. The occurrence of ellipticity is called Circular Dichroism. The phenomena of Circular Dichroism (CD) is observed when optically active matter absorbs left and right hand circular polarized light slightly differently. It is measured with a CD spectropolarimeter. It is extensively being explored for yielding valuable information about secondary structure of biological macromolecules[176].

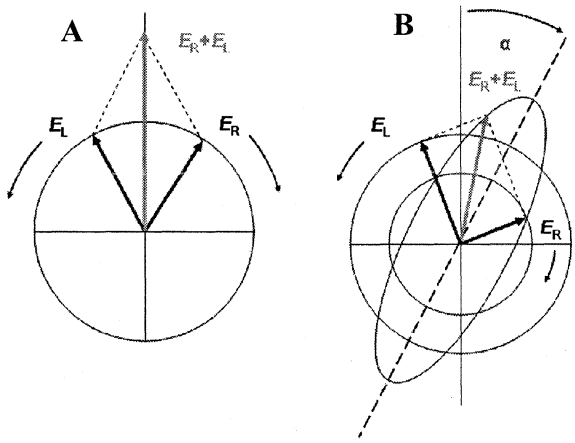


Figure-2.3.2.3.2 CD: Linear polarized light can be viewed as a superposition of opposite circular polarized light of equal amplitude and phase(A). Different absorption of the left- and right hand polarized component leads to ellipticity (CD) and optical rotation (OR). The actual effect is minute and using actual numbers the ellipse would still resemble a line (B)

The CD spectra of the solutions were recorded on a Jasco- 810 spectropolarimeter, using a 0.1cm path length quartz cuvette. Acquisition was performed using a 100nm/ min scan rate, 0.5 nm bandwidth, 2s response, sensitivity 1000mdeg, resolution 0.2nm, measuring range 200- 350nm. Each spectrum was averaged for three runs. The corresponding baseline (buffer, buffer/

acetone or lipid buffer solution) was subtracted from each spectrum. The final spectrum are presented in mean θ residue ellipticity

$$\theta = \theta/100C_r l$$

Where θ is the measured ellipticity (mdeg), l is the path length of the quartz cell (cm), and C_r is the mean residue molar concentration obtained by the following formulae.

$$C_r = 1000nC'/ Mp$$

Where n is the number of amino acids present in the protein molecule (585 for albumin), C' is the protein concentration in the solution (gm/ L) and Mp is the molecular weight of the protein (= 67, 000g/ mol for albumin).

2.3.2.2.1 Preparation of samples for CD spectroscopy

2.3.2.2.1.1 Albumin intercalated into the lipid layer

The structure of the membrane-incorporated albumin was studied by CD spectroscopy (Jasco- 810 spectropolarimeter, using a 0.1cm path length quartz cuvette). For that albumin was phase inverted (as per the procedure 2.2.1.3) and vacuum dried along with the respective lipid components. This mixture is reconstituted in PBS 7.4 solution to form the liposomes, they were further extruded using 100nm polycarbonate filters to form unilamellar (prepared as per the procedure 2.1.4.2) liposomes. The albumin concentration in the final solution was 0.075gm/ L.

2.3.2.2.1.2 Bound protein to the ligand

The structure of the bound albumin to the polymer bound drug (PVADICb) was studied by CD spectroscopy (Jasco- 810 spectropolarimeter, using a 0.1cm path length quartz cuvette). The albumin concentration in the final solution was 0.075gm/ L. The solutions were prepared in PBS 7.4 at ambient temperature prior to the experiment[23].

2.3.2.3 Spectral analysis using K2d algorithm

The spectral analysis was done using the algorithm K2d, which allows the estimation of the percentages of the protein secondary structure using a Kohonen neural network with a two dimensional output layer [177,178]. This algorithm allows calculations in the 200- 240 nm wavelength ranges[179].

2.3.2.4 Quantitative evaluation of the proteins by ELISA

Mouse TNFa, IL6, IL10, and IFNg were determined in pooled cell-free supernatants of stimulated or unstimulated cells by sandwich enzyme-linked immunosorbent assay using coated recombinant mouse IL-10, IL6, TNFa, IFNg antibodies and detected with anti-mouse IL-10, IL6, TNFa, IFNg monoclonal antibodies using standards, and POD as the substrate, and red using a multi plate ELISA reader (TECCAN) analyzed using Megallan 3.0 software, exactly according to the manufacturers protocol (BD bioscience). The data shown are the means +/- S.D. of triplicate samples of one representative experiment.

2.3.3 Blood cell adhesion studies

2.3.3.1 Platelet adhesion studies

The platelets were separated from a freshly drawn citrated (tri-Sodium citrate 3.8% w/v solution) calf blood (at the ratio of (1:9)) by the procedure described elsewhere [180]. In short, the blood was centrifuged (Remi RC4) at 70 g for 10 min to remove the supernatant, which was centrifuged at 140 g for 10 min to remove the leukocyte button. Then the supernatant was again centrifuged at 560 g for 10 min. The supernatant was discarded and the platelet button was washed further three times with the tyrode solution. The washed platelet suspension was spread over the polymer films and incubated for 15 min. Then the films were gently and uniformly washed with 0.1M phosphate buffered saline (PBS) (pH 7.4). The washed films were then fixed with 2.5 % glutaraldehyde solution and stained with coomassie blue staining solution. The fixed films were counted by light microscopy.

2.3.3.2 Leukocyte adhesion studies

The leukocytes from the citrated calf blood was separated according to the already reported procedure[23] The erythrocyte removed (as above mentioned) citrated calf blood was centrifuged at 140g for 10 min, to remove the leukocyte button and the cells were washed and suspended in tyrode solution and studied similarly as mentioned above.

2.3.3.3 Erythrocyte adhesion studies

The erythrocyte adhesion studies, were done by using the procedure described elsewhere [23]. In short, the erythrocyte was separated from the citrated calf blood by centrifuging at 70 g. The supernatant was discarded. The sedimented erythrocyte was washed three times with the 0.1M Phosphate Buffer (pH 7.4), and suspended in phosphate buffer and studied similarly as mentioned above.

2.3.3.4 Platelet activation studies

A sample of 10 ml of fresh calf blood was collected in tri-Sodium citrate 3.8 % w/v solution anticoagulant system at the ratio of 1:9, by standard venipuncture technique. In short, the blood was centrifuged at 140 g for 10 min to get the platelet rich plasma (PRP), after removing the leukocyte and erythrocyte button as mentioned above. The polymer substrates of size 0.5X0.5 cm were cut and were incubated with saline at 37°C for 15min. The saline was replaced with 2ml of PRP. The membranes were incubated with the PRP for 2hrs. After the specified incubation period the PRP was decanted and the membranes were rinsed with the saline. The washed membranes were then treated with 2.5 % glutaraldehyde in saline for 2 days at 4 °C. The activated platelets were evaluated by SEM (as per the procedure 2.2.17.3) All the blood cell adhesion studies were done independently within 2hrs of the collection of the blood.

2.3.3.5 Macrophage adhesion and activation studies

Macrophage plays an important role in regulating the acute phase interactions to the biomaterials. The macrophage adhesion, activation, spreading

and secretory profile was evaluated to understand about the phenotypic changes of the cells on these lipid thin films.

2.3.3.5.1 Harvesting and subculturing of the macrophage cell line

The murine macrophage cells (RAW 264.7) were cultured in DMEM high glucose with L Glutamine (4.5g/l) supplemented with 1% penicillin/streptomycin and 10 % foetal bovine serum at 37°C in a humidified incubator with 5% CO_2. These adherent cells were harvested after gentle scrapping with a cell scrapper, washed with sterile phosphate buffered saline (PBS) (7.4), and viable cells were counted using coulter counter. Macrophage viability was always greater than 98% at the start of the experiment. The cells were harvested on culture flasks. The mouse macrophage cells between 5-25 passage was used for the studies.

2.3.3.5.2 Cell seading and experimentation of substrates

Cells of required number were separated and washed with sterile PBS (7.4), and incubated in DMEM as per the procedure 2.3.3.5.1. Cells of different cell numbers $1X10^{6, 5, 4, 3}$ were introduced onto each well. Modified 24 well PS plates were incubated with 1ml of DMEM high glucose with L Glutamine (4.5g/l) supplemented with 1% penicillin/streptomycin and 1 or 10 % foetal bovine serum at 37°C for 2min. The required number of cells were seeded onto this wells and made upto 1ml. Cells were also added directly to the uncoated wells and served as negative controls, or were additionally stimulated with 100 µl of lipopolysaccharide endotoxin (LPS, 1 mg/ml) to serve as positive controls. Pictures (Phase contrast microscopy) were taken at regular intervals to study the morphological behaviour of cells.

2.3.3.5.3 Evaluation of adhesion strength after gentle washing

The culture medium was separated after the specified time interval. Further plates were washed gently and incubated in PBS (7.4) for the evaluation of adhesion strength.

2.3.3.6 Polymorphonuclear cell adhesion and activation studies

Polymorphonuclear (PMN) cells are primary immune cells change the course of acute inflammatory response on material surface. The PMN adhesion, activation, spreading, secretory profile and proliferative potential were evaluated to understand about the phenotypic changes of the cells on these lipid thin films.

2.3.3.6.1 Cell isolation and harvesting

Two mice were sacrificed (Procedure approved by internal review committee Phillips University, Marburg, Germany) by cervical dislocation and the spleen was collected after lateral dissection of the thoracic cavity. Before the collection of the spleen the blood supply to the spleen is disconnected and in no time the spleen is separated and harvested in RPMI medium with 10%FCS in ice. The spleen is mashed in a Petri dish with the help of a 0.1μ nylon mesh and 0.1ml syringe barrel. A 1:1 proportion of the suspended cells in RPMI with 10%FCS to Pancoll in polypropylene tube were centrifuged at 300g for 20min without break. The interface is harvested in 1ml of fresh RPMI medium and washed two times with fresh PBS (low EU). Then the cells were resuspended in 1ml of RPMI medium. The cell number was counted with the help of a coulter counter after making dilution of 1:2000 with the PB (7.4). The viability of the cells was determined based on the mean size of the cell size which form the major area under the curve of the histogram of the diluted cell suspension. The viability was always greater that 98%.

2.3.3.6.2 Cell seeding and experimentation

The modified wells (as per the procedure 2.2.4.1.2) are pre incubated with the respective medium with either 1 or 10%FCS. The required number of cells / ml were seeded onto the wells and distributed uniformly. The rest of the studies were as like procedure no 2.3.3.5.2.

2.3.3.6.3 Cell proliferation studies by BrdU uptake assay by ELISA

For cell proliferation assays, cells were seeded into 96-well plates (modified or unmodified) at 1000 cells/well, in duplicate. BrdU (BrdU-based Cell Proliferation ELISA kit; Roche Applied Sciences, Indianapolis, IN) assay was performed according to the manufacturers instructions. For the BrdU assay, 10 µl of 100 (lM)BrdU was added, to each well, and the cells were incubated for 4 h. After removal of the medium, cells were fixed and the DNA denatured using the FixDenat reagent (Roche Applied Sciences). After 30 min, the FixDenat reagent was removed, anti-BrdU-POD solution was added and the plate was incubated for 90 min at room temperature. The cells were then washed three times with wash solution, after which 100 µl of the substrate solution was added, and absorbance was measured at 370 and 490 nm using a microplate reader. For the SRB assay, 25 µl of trichloroacetic acid was added to each well.

2.3.4 Fibroblast Cell Adhesion Studies

Primary cultures of mouse blastoma fibroblasts cells (L-929, cell line, NCCS, Pune, India) were grown in eagle's minimum essential medium (EMEM) supplemented with 10% foetal bovine serum with 1% antibiotic, antimicotic solution, (Sigma). Cells were removed by trypsinization, washed once in EMEM, suspended in the culture medium and seeded on sample surface at a cell density of $1X10^3$ cells/ cm^2. Cultures were incubated for 24h and five days at 37°C in a humidified atmosphere with 5% CO_2/ 95% air atmosphere with intermediate exchange of culture medium. The samples were collected after the specified intervals and washed with phosphate buffer and fixed with buffered formalin and stained with haematoxillin and eosin. Stained cells were counted using an optical microscope[181].

2.3.5 Bacterial Adhesion Studies

Gram +ve *Staphylococcus aureus* (ATCC25923) and Gram – ve *Eschericia coli* (ATCC 25922) adhered to the samples and the viable count of the cells

adhered was studied. The test samples were separately placed in tryptone soya broth (inoculum density of about 10^6 (fu/ ml) cells) and incubated with the test bacterial strains. The sample containers were incubated at 35°C overnight. After overnight incubation the samples were collected, rinsed in sterile PBS and viable count was done. For performing viable count the samples were separately placed in sterile PBS and adhered bacteria were collected by sonication (1min) and vortexing (1min) three times. Known volume of the PBS suspension of the adhered bacteria was inoculated on tryptone soya agar incubated overnight at 35°C and the number of colony forming units was counted and the viable count was calculated[182].

Chapter -3
Results & Discussion

RESULTS AND DISCUSSION

In this work we have attempted to immobilize cell mimetic lipids as monolayers onto different kind of polymeric substrates and studied various biorecognition processes. Preliminary experiments and modeling studies have been done on polymeric films and this knowledge is attempted to explore in the development of different miniaturized substrates including microspheres, nanoparticles, liposomes and polymeric gels for drug delivery and tissue engineering applications.

3.1 Developing the co-solvent system

Here different cell mimetic lipids and other components of thin films are solubilized in a solvent system and compressed monolayers of these thin films have been deposited onto different substrates. For solubilizing the components, a co-solvent system had to be developed which meets all the following requirements. (1) It could be able to dissolve all the components aimed to incorporate in the thin film. (2) Should not affect the supporting polymer substrates. (3) It should be

useful for modifying different 2D and 3D substrates. A single solvent can not perform this multitude of functions.

3.1.1 Selection of the parent solvent

As part of developing the co-solvent system, the first task is to bring together the heterogeneous PL, GL and Chol components together. For the development of co-solvent system careful selection of the parent solvent is very important. The selection of parent solvent has been done as per the procedure 2.2.1.1. It is known from the literature that majority of the cell mimetic amphiphilic lipids like PL and Chol are hydrophobic and is soluble in non-polar solvents like DCM and CF[183]. However these solvents also interact with many of the hydrophobic biocompatible polymers in current use today. To avoid this discrepancy a suitable parent non-polar solvent has been selected based on their interaction with polymer substrates and solubility studies of the lipids.

3.1.2 Developing the co-solvent system for heterogeneous lipids

From the preliminary studies for selecting the parent solvent we have observed that among various solvents like DCM, CF, n-Hept and CH, the CH is least disturbing the base hydrophobic substrates, while all the other solvents reduces the translucency of the substrate.

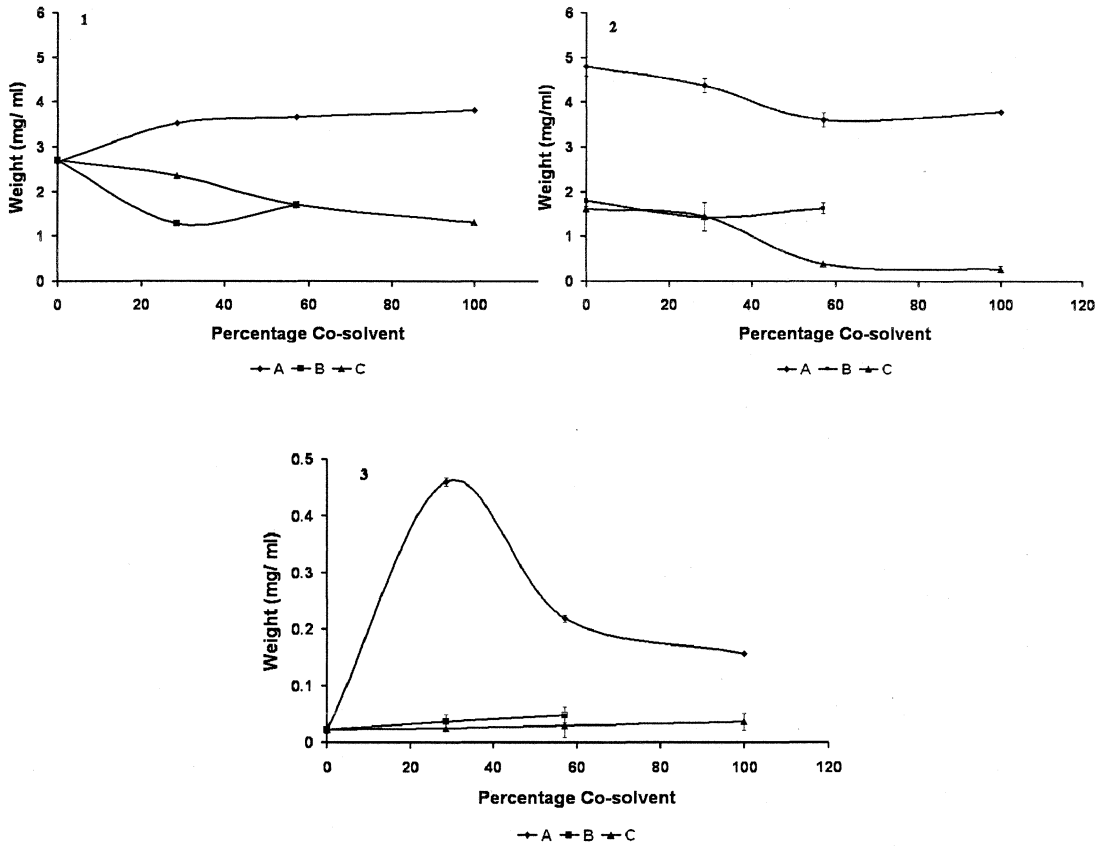


Figure- 3.1.2.1: Solubility of different lipids in Co-Solvent system. PC (1), Chol (2), GalC (3). Methanol (A), Ethanol (B), n-Butanol (C)

The figure- 3.1.2.1 shows the solubility of lipids into the co-solvents of CH. It indicates that PC is highly soluble (figure- 3.1.2.1 (1)) in CH and the solubility is getting reduced with the incorporation of Met and Eth as co-solvents, while it is increasing with the n-but, in all the concentrations. However, differently solubility of cholesterol Figure-3.1.2.1(2) has reduced slightly with all the co-solvents with the increase in co- solvent concentration. In contrast, GalC being hydrophilic is least soluble (Figure-3.1.2.1 (3)) in CH, and is soluble only at an optimum co-solvent concentration between CH and n-butanol, and is being insoluble in all the other co-solvent systems. Among the different ratios of the different co-solvents, the CH/Eth is immiscible beyond 50%v/v.

The co-solvent system where maximum quantity of GalC, PC and Chol dissolves in CH/n-but) (71.43/ 28.57) % v/v and is taken as the optimum co-solvent system. The total lipid concentration needed to form the monolayer is well below the above said maximum concentrations. This gives enough margin for solubilising other components. Apart from that the difference in boiling point of these solvents helps in easy deposition of the monolayers, by various methods.

3.1.3 Optimization of co-solvent system for the incorporation of macromolecules

Further we have attempted to incorporate various macromolecules into the lipid system as per the procedure 2.2.1.3. The macromolecules attempted to incorporate into the lipid monolayer are Alb, Hep and DAPEG. These macromolecules have been selected based on their proven merit, as surface modification agents to improve the haemocompatibility of the polymers. The incorporation of Alb has been found to be affected by its hydrophilic exterior. Therefore the solubility of the albumin has been improved by sequentially introducing the molecule into solvents of decreasing polarity and finally to the lipid environment. We have utilized here the flexible nature of the albumin molecule to integrate this globular protein into the monolayer. The amino acid sequence, dictates the folding of membrane proteins in a said solvent. In the case of soluble proteins, like albumin, the hydrophobic core is buried inside the protein molecules. During the exposure in a hydrophobic environment, for a preferred conformation, the hydrophobic core of the protein is exposed to the exterior[184,185]. Earlier it has been observed that the hydrophobic core of the membrane integral proteins interacts firmly with the hydrophobic core of the bilayer membrane. Acetone has been used here as a co-solvent to change the conformation of albumin. The optimum solvent composition (Water: Acet: CH: n-but) (5.00/45.02/35.72/14.26) % v/v) has been selected based on the miscibility studies, where the total solution shows, minimum turbidity changes upon constitution, in absence or presence of the macromolecules. Here the lipid stabilizes the conformationally changed albumin by occupying the hydrophobic core. Earlier it has been demonstrated that the lipid molecules

are acting as molecular chaperons and stabilize the integral membranes in the bilayer membrane[51], and control the folding or refolding of the proteins. The solubility of albumin and other proteins like γ globulin and fibrinogen that could be able to incorporate into the co-solvent system is shown in figure- 3.1.3.1.

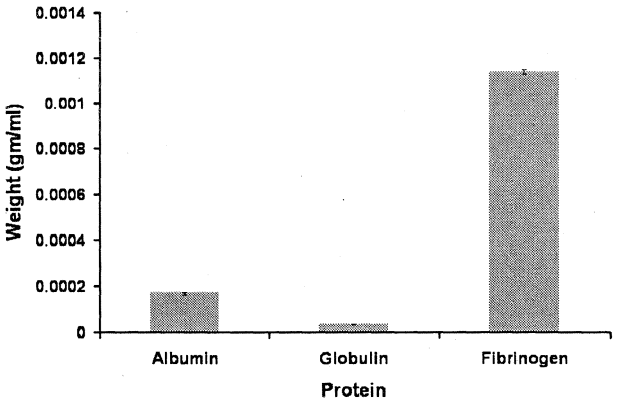


Figure-3.1.3.1: Solubility of different proteins in the co-solvent system

Among albumin, globulin and fibrinogen, fibrinogen has been found to be highly soluble in the solvent system (Figure- 3.1.3.1). Further air/water interfacial studies, of this lipid mixtures have been done with the help of LB trough, to understand how the intermolecular interactions regulate the self-assembly of these components to form a monolayer.

3.2 Air/water interfacial studies

Air/water interfacial studies gives the understanding about the mechanical properties of the monolayers when the amphiphiles are in close proximity (1.3.2.3). The role of different components in regulating the packing density and their self-assembly can be elucidated from these studies.

3.2.1 Monolayer Behavior of Pure Lipids at The Air/ Water Interface

Air/ water interfacial[186] behavior of these lipids is done by using LB trough[164] as per the procedure 2.2.2.1. The (Figure-3.2.1.1A) shows the pressure-area (π-A) isotherm of the PC using deionized water as the subphase at ambient temperature. The liquid expanded (LE) and liquid condensed (LC) phases of the π-A

isotherm of the PC monolayer are coexisting together as observed earlier[187]. This may be due to the unsaturation of the hydrophobic chain of the egg-PC. The double bond in egg-PC is found to be cis[188, 189] that reduces further close packing of the monolayer. The collapse pressure of the isotherm is at 42 mN/m, and the limiting molecular area of the PC monolayer is at 70A^{o2}/molecule.

The π-A isotherm of the Chol monolayer (Figure-3.2.1.1B) shows a sharp entry from gaseous (G) to LC phase during compression, and is similar to that of long chain fatty acids[190]. This is due to the strong interaction between the hydrophobic cyclo pentano perhydro phenanthrene ring of the Chol, when they are in close proximity. The limiting molecular area is at 55 Å^2/ molecule, and the collapse pressure of the cholesterol is near to 42 mN/m.

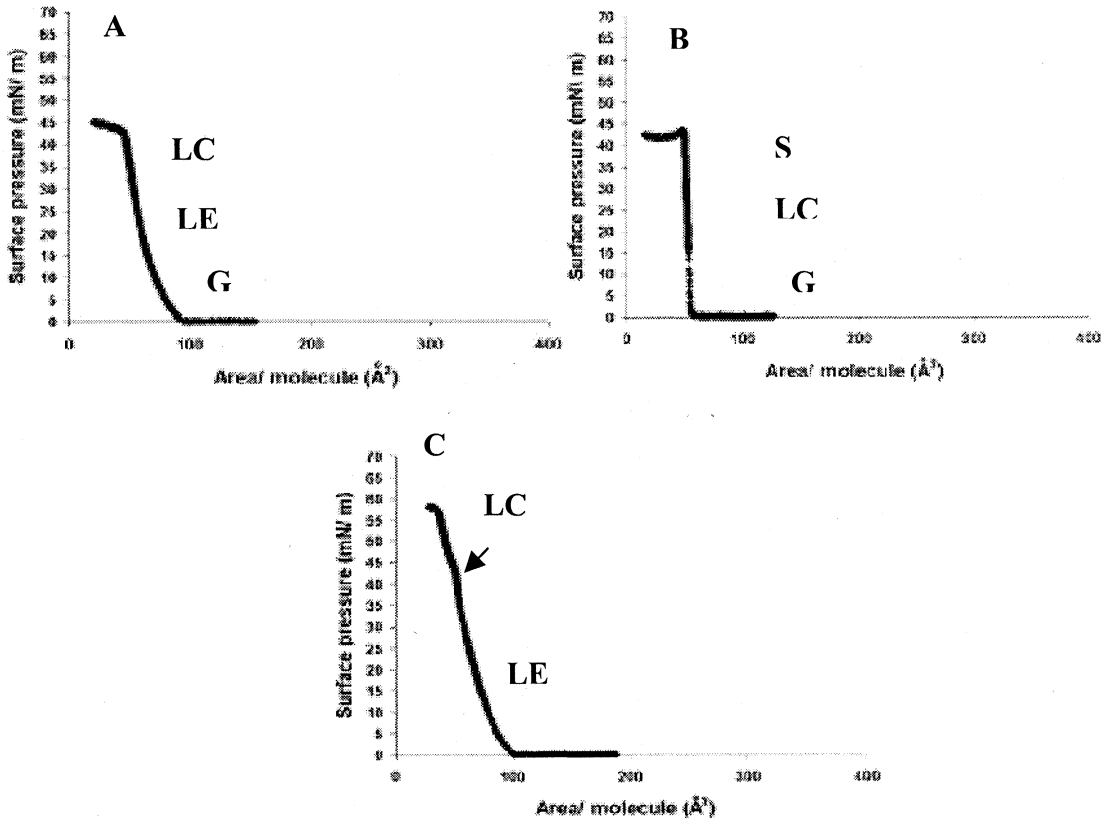


Figure-3.2.1.1: π-A isotherm of pure lipids. PC (A), Chol (B), GalC (C).The different phases observed in the Pressure area isotherm are, G(gaseous)( ) LE(Liquid expanded), (LC (Liquid condensed) ( ) state. Arrow(C) indicates "kink" in isotherm

The π-A isotherm of the GalC (Figure-3.2.1.1C) is also showing extended LE phase similar to PC, but the collapse pressure is near to 60 mN/m. This may be due to the extended stability of the monolayer contributed by the head group of the GalC. The kink observed at 42mN/m is similar to that observed earlier[191], and the limiting molecular area is at 85 $Å^2$/molecule.

3.2.2 Monolayer Behavior of Mixture of Lipids at the Air/ Water Interface: Condensing effect of cholesterol

The air/water interfacial behavior of the mixture of lipids has been studied using LB trough as per the procedure (2.2.2.1.1) They also exists in fluid phase through out the compression. However the packing density has been directly proportional to the concentration of cholesterol. Figure-3.2.2.1 A&B shows that LC phase is extended with increase in Chol concentration. The slope of the Le phase of compressed monolayer increases with the concentration of the Chol may be because of the formation of the cholesterol rich domains, as observed by others[192].

At reduced Chol concentration the LE phase is increased. During the incorporation of GalC under reduced Chol concentrations, LC phase has increased considerably and the LE phase has reduced. In the case of GalC incorporated monolayer, (figure-3.2.2.1C) the mean molecular area has been decreased from 135-75 $Å^2$/ molecules, in the Le phase. Upon compression at the lateral surface pressure of 28mN/m and 75 $Å^2$/ molecule the monolayer has been transferred from the Le to Lc phase. Further the surface pressure sharply increased and a less significant decrease in mean molecular area has (75-67 $Å^2$/ molecule) occurred. The area/ molecule for the said monolayer is at 100 $Å^2$/ molecule. The monolayer collapses at 42 mN/m similar to PC or Chol monolayer. The slope of the curve is shifted to the right, which may be due to the condensing effect of cholesterol and GalC, and improves the stability of the monolayer at the air/ water interface.

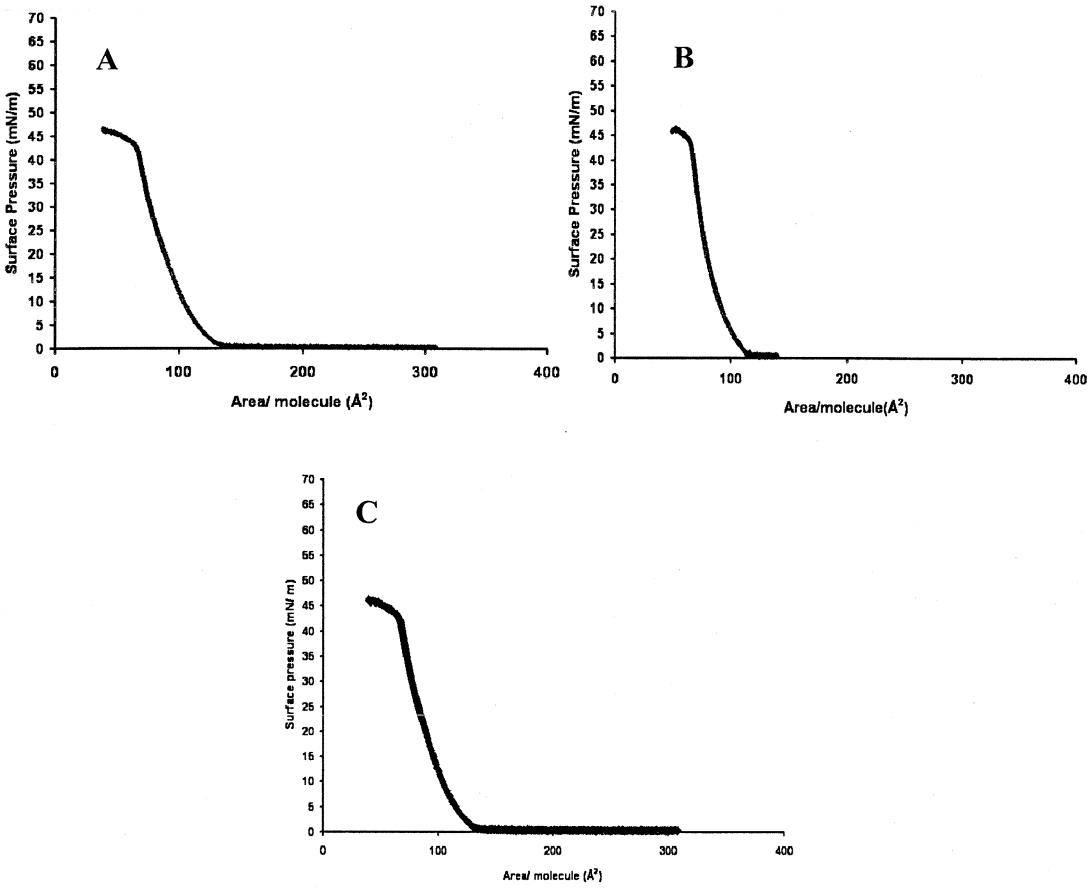


Figure-3.2.2.1: π-A isotherm of (PC: Chol) (1:0.7) (A), (PC: Chol) (1:0.35)(B), (PC:Chol:GalC)(1:0.35:0.125) (C)

Here the GalC incorporated ternary lipid composition (PC: Chol: GalG) (1:0.35:0.125) has been found to have better monolayer stability. This may be contributed by the head group structure of the GalC. This is due to their hydrogen bonding regulated head group association, which may help them to have preferential participation in the Chol rich domain formation. Earlier such preferential organization of sphingiomyelin (SM) into Chol rich domain[193] has been observed in a PC/Chol monolayer. Here the PC and SM differ only in their head group structure. Studies using binary PL/ Chol lipid compositions also indicates that the lipids are closely packed at 30–35 mN/m[194] While in plasma membranes the lipids are closely packed at much smaller surface pressure of

20mN/m [195, 196]. It indicates the role of other constituents in improving the membrane integrity of natural membranes.

However studies using PC/ Chol/GalC ternary lipid combinations are very less in the literature maybe because of the limited solubility of GalC. Here the significant contribution of GalC in improving monolayer stability has been visible from the air/water interfacial studies. Therefore we have selected this particular lipid composition (PC: Chol: GalC) (1:0.35:0.125) as the optimized Outer Cell Mimetic lipid Composition (OCMC) containing the minimum essential lipid components, for further studies. Further to understand about the condensation effect of Chol on regulating the fluidity of the lipid layer and effect of phosphatidylethanolamine(PE) head group structure on monolayer behavior, we have incorporated increasing concentration of PE into these monolayers. This is by mimicking the lipid transfer in biological membranes (1.3.2.2).

3.2.3 Monolayer behavior with the incorporation of PE: Effect of head group structure

For that, to the (OCMC) (PC: Chol: GalC) (1:0.35:0.125) lipid mixture has been incorporated with varying concentration of PE to prepare the lipid compositions of (PCMC a, b& c) (PC: Chol: GalC: PE) (1:0.35: 0.125: (a) 0.0725, (b) 0.145, (c) 0.29) using the same solvent system (CH/n-but) (71.43/ 28.57) (5:2) % v/v (as per the procedure 2.2.1.2). The air/water interfacial studies has been done using LB trough, at ambient temperature as per the procedure (2.2.2.1.1).

The π- A isotherm of the PCMC (a, b & c) lipid compositions also exists in fluid state through out the compression (figure- 3.2.3.1). Here in the case of PCMC a, b, & c, the Le phase of the monolayer has decreased with increase in concentration of the PE (figure- 3.2.3.1A, B & C). The effect is maximum with PCMCc (figure- 3.2.3.1C). The area/ molecule have also been increasing with the increase in concentration of the PE. The π- A isotherm of PCMCc (figure- 3.2.3.1C) shows that the molecular area decreased from 245-132 $Å^2$/ molecules, in

the Le phase. The Le to Lc phase transition happens at 26mN/m and 132A$^{\circ 2}$/molecule. The mean molecular area decreases from 132-106 Å^2/ molecules in the Lc phase during compression. The monolayer collapses at 42 mN/m. The limiting molecular area for the said monolayer has been 180 Å^2/ molecule. After the collapse, the isotherm of the PCMCc also behaves similar to OCMC (figure-3.2.2.1C) monolayer. Here in PCMCc the Chol concentration has reduced with the incorporation of PE. The observed reduction in the slope of the Le phase of the PCMC monolayer may be associated with the reduction in condensing effect of Chol.

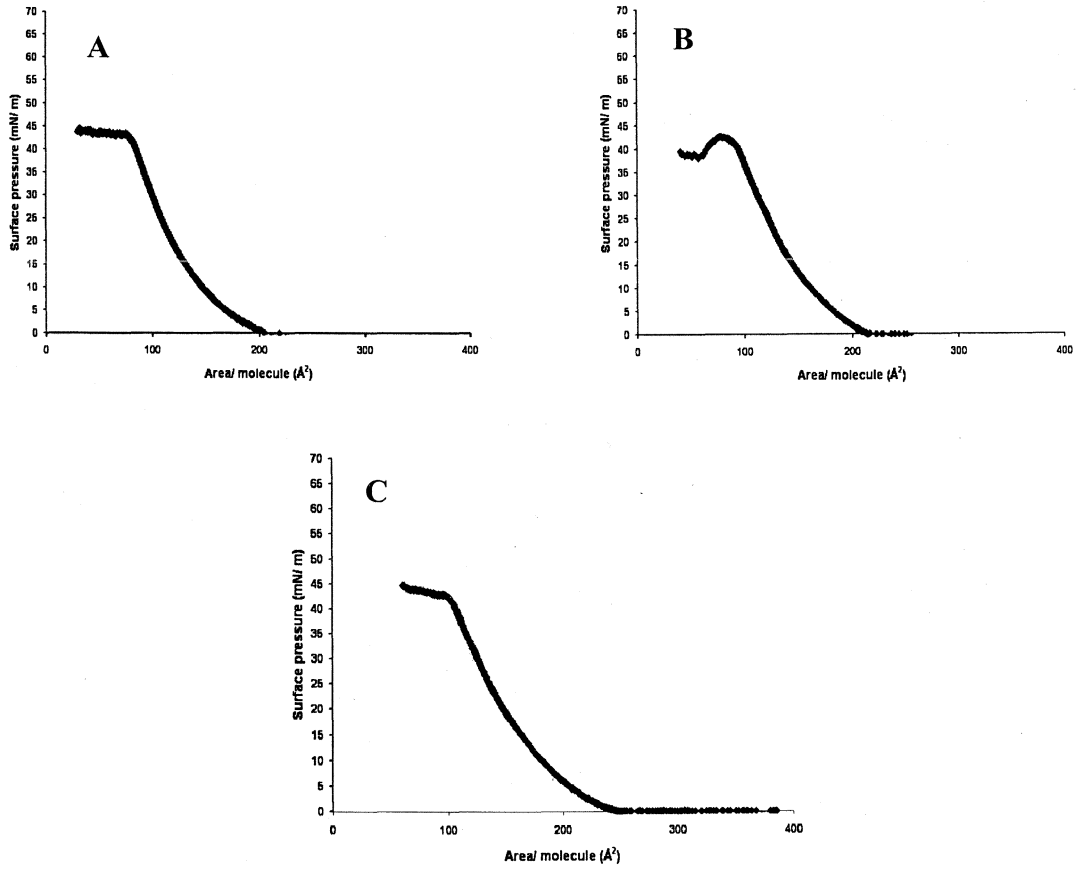


Figure-3.2.3.1: π-A isotherm (PC:Chol:GalC:PE) 1:0.35:0.125:0.0725(A), with PE 0.145(B), 0.29 (PCMC) (C)

From these studies we have selected the PE incorporated PCMCc (PC:Chol:GalC:PE) (1:0.35:0.125: 0.29) lipid composition as the optimum PE

incorporated lipid composition for further studies. Further we have incorporated macromolecules into these lipid monolayers to regulate the monolayer stability, at the air/water and air/solid interface.

3.2.4 Mimesis of lateral stabilization of membrane lipids using proteins: Monolayer behavior of mixture of lipids & macromolecules at the air/ water interface

The π-A isotherm of the anchor incorporated lipid monolayers is shown in figure-3.2.4.1 The optimized OCMC lipid mixture, incorporated with the anchors (Alb, Hep and DAPEG) are studied at the air/water interface using LB trough at ambient temperature (as per the procedure (2.2.2.1.1)).

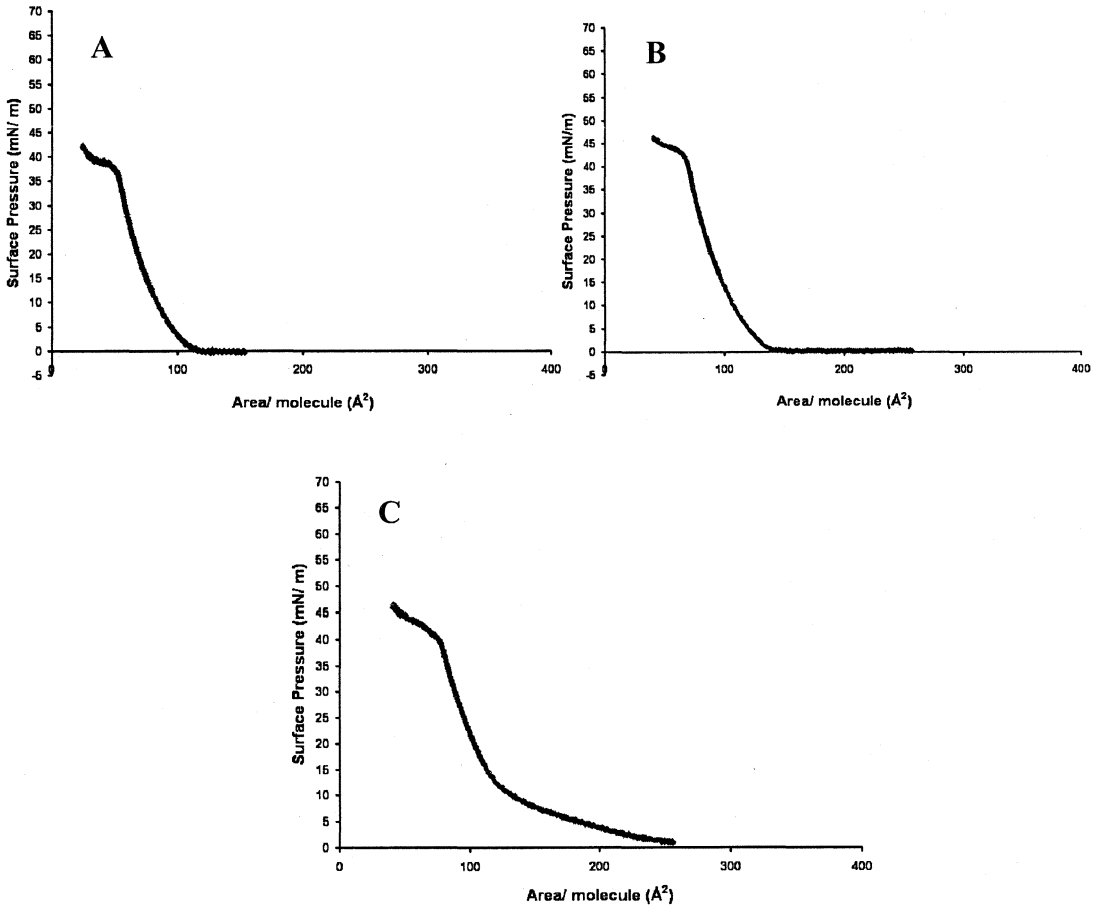


Figure-3.2.4.1: π-A isotherm: OCMC- A (A), CMC- AH (B), OCMC -AHP (C)

These studies are aimed to laterally stabilize these lipid layers onto polymer substrates. For that we have selected Alb, Hep and DAPEG molecules of proven anti-thrombogenecity[197] as the anchors. The collapse pressure of the monolayer is reduced after the incorporation of Alb into the monolayer, OCMC- A, (Figure- 3.2.4.1A), which indicates the interaction between hydrophobic core of the lipid and conformationally changed Alb in the monolayer. However the mean molecular area of the monolayer has not reduced. The π-A isotherm also has not shown any sharp discontinuity of phase transfer from LE to LC phase during compression. Perhaps the LE and LC phases are coexisting together. The reduction in collapse pressure may be due to the induction of strong discontinuity in the lipid monolayer under compression, by the larger albumin molecule at the air/ water interface. The imbalance of the compressive force between the continuous lipid layer and the albumin molecule due to the size difference may be the reason for this discontinuity, resulting in reduction of the monolayer stability. Therefore to further stabilize the monolayer by linking the neighboring albumin molecules we have added Hep also along with Alb into the lipid solutions. Heparin has been found to be interacting ionically with the albumin molecule[198]. The incorporation of Hep along with Alb (Figure- 3.2.4.1B), into the monolayer improves the collapse pressure from 38 to 42 mN/m (retained the normal collapse pressure of PC monolayer). This is a better indication of incorporation of albumin and heparin into the monolayer and partial interconnection of the albumin molecule with heparin, by ionic interactions. This monolayer shows a right shift in the mean molecular area. The figure- 3.2.4.1C shows the π-A isotherm of the monolayer containing Alb, Hep, and DAPEG. The LE phase of the monolayer increases with the incorporation of DAPEG into the monolayer. The isotherm shows a sharp discontinuity at 12mN/m surface pressure, after that the monolayer behaves similar to the other monolayers until the collapse. Here when the hydrophilic DAPEG molecules are introduced into the monolayer along with albumin and heparin there has been a sharp phase change at low surface pressures. This indicates that the

DAPEG is migrating out of the monolayer during compression[199]. However there is no difference in the collapse pressure, indicating the stabilization of Alb into the monolayer by the Hep molecules ((Figure- 3.2.4.1C). However, due to the ionic interactions with the heparin (amino group of DAPEG and carboxy group of heparin), the DAPEG molecules cannot escape into the sub phase. This ensures that the bulk of the DAPEG molecule exists out of the monolayer at 30 mN/m the pressure at which we have transferred the monolayer to the polymer substrates.

In these studies the albumin molecule is mixed with the lipid solution by sequentially treating with less polar solvents. For that albumin has been dissolved in water then mixed with acetone and the solution is quickly introduced into CH/ n-but mixture containing the lipids in definitive proportions. Incorporation of the higher concentration of albumin molecule, into the lipid solution, leads to the precipitation of albumin molecule due to self-aggregation. The self-aggregation is due to hydrophobic interaction between the exposed hydrophobic groups of the albumin molecule[200]. But at this specified concentrations, we did not observed any self-aggregation of the albumin molecule within the time period of processing it. This may be due to the stabilization of the altered confirmation of the albumin molecule, by the lipids. Here the hydrophobic interaction between the albumin and the lipid tail chain may reduce the self-aggregation of the albumin molecule. Further we have studied the conformation of the albumin incorporated into the lipid layers by CD spectroscopy.

3.2.4.1 Conformation of the incorporated albumin into the lipid layer

The far UV CD spectra (as per the procedure (2.3.2.2.1.1)) of the native as well as the lipid stabilized albumin after incorporating into the membranes of liposomes (prepared as per the procedure 2.1.4.2) have been studied. The figure-3.2.4.1.1 shows the CD Spectra of the albumin molecule. The CD spectra demonstrate that the secondary structure of the native albumin is altered during the phase inversion. This altered structure is retained irrespective of the lipid composition in all the protein lipid mixtures.

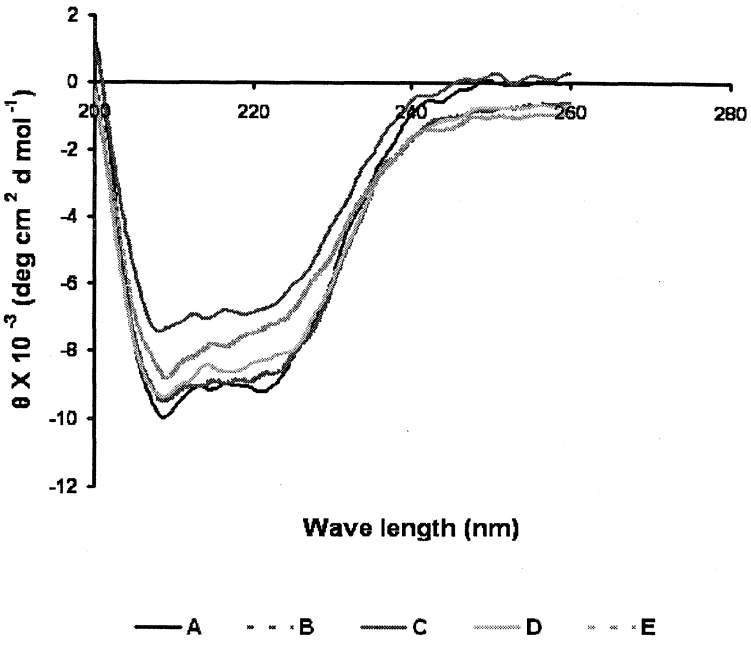


Figure-3.2.4.1.1: Structure of the Albumin molecule in lipid bilayer. CD spectra of albumin in its native state (A) and altered (B), incorporated into PC (C), OCMC- A (D), OCMC- AHP (E)

Table-3.2.4.1.1: Secondary structure of albumin in the lipid layer

Drug	Native	Altered	Incorporated into PC	OCMC-A	OCMC-AHP
β	11	46.33	35	39.74	38
Random	57.5	38	40.27	42	41.21
Sq. Distance (Å)	309.54	734.26	618.56	664.25	667.7

The spectral analysis was done using the K2d neural algothrithm[201,202]. The calculated percentages of the secondary structure are given in Table-3.2.4.1.1. It indicates that the secondary structure of the phase inverted albumin is different from the native albumin. The helical content of the phase-inverted albumin has

decreased and mainly converted to β- sheet during the phase inversion. The denaturation of the native protein structure is reduced in presence of lipids. Especially there has been little difference in the random coil structure. However the total dimension of phase-inverted albumin has increased. This indicates that the albumin exists in molten globular state without entirely loosing its secondary structure in the lipid membranes.

These studies clearly indicates that, the property of the monolayers in terms of packing density as well as lateral stability can be modulated, with the help of different lipid as well as macromolecular components. Further we have attempted to immobilize these monolayers over polymeric substrates to study various monolayer-substrate factors regulating their physical assembly.

3.3 Air/solid transfer studies

The interaction of the compressed monolayers behaves differently with different kind of substrates, when these substrates are introduced at the air/water interface. Air/solid transfer studies gives information about (1) lateral stability of the monolayer during transfer (2) interaction of substrate with the monolayer (3) packing density of the deposited monolayer and (4) orientation of the deposited monolayer. These studies are crucial for understanding the exact mechanism of the deposited monolayers in regulating the surface events.

3.3.1 Optimization of monolayer deposition parameters

In an attempt here, we are developing physically immobilized thin films for regulating the biological interactions. The advantage is that the non-covalently supported monolayers retain membrane fluidity[203]. Therefore the system could actively communicate with the surroundings by maintaining the natural membrane fluidity. For that we have attempted to optimize the speed of deposition in terms of transfer ratio of the monolayer from the air/water interface to the hydrophobic PolyC substrates (prepared as per the procedure 2.1.1.1).

3.3.1.1 Speed vs transfer ratio

To optimize the speed of deposition, binary PC/ Chol (1: 0.7) lipid compositions at different dipping and withdrawal speeds of the PolyC substrate from the air/water interface has been studied with the help of the LB trough. Studies at varying dipping speeds have not shown any difference in the transfer ratio with the speed (deposited as per the procedure 2.2.2.2.1). It indicates the strong hydrophobic interaction between the monolayer and the polymer films[204]. However, the second monolayer deposition during withdrawal of the polymer substrate has been varying with the speed, for the same composition, as shown in Figure 3.3.1.1.1. At 5 mm and 100 mm/min, withdrawal speed there has been no second monolayer deposition.

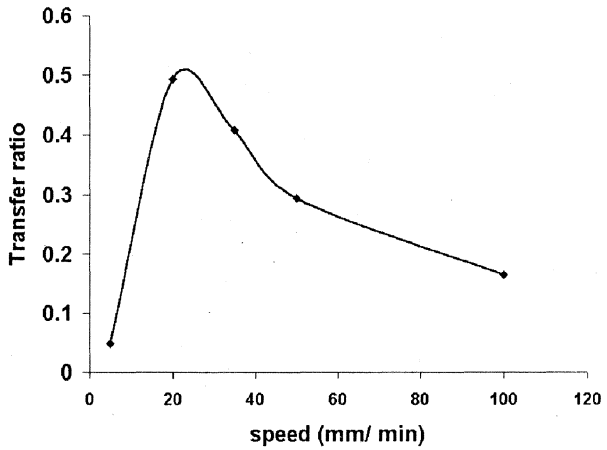


Figure-3.3.1.1.1: Speed vs transfer ratio on deposition of the monolayer on hydrophobic polymer substrate: Upstroke mode, PC/Chol (1:0.7)

At 30mm/min the deposition was optimum. Apart from that most of the transfer studies in literature is done at this transfer rate. Further we have evaluated the transfer of the compressed OCMC monolayer onto different kind of substrates.

3.3.1.2 Substrate Vs Transfer ratio

For that, OCMC monolayers has been transferred from the air/water interface into polymeric films at 30mm/min. The substrates vs transfer ratio is shown in table-3.3.1.1.1.

Table-3.3.1.2.1: Substrate Vs Transfer ratio

Substrates	PolyC	PMMA	Chitosan	cPVA	Glass
Contact angle	77.275+/- 0.953	82.9+/- 3.155	85.833+/- 0.752	38.56+/- 3.12	52.34+/- 2.26
Down stroke	0.976	1.086	0.756	0.224	0.523
Up stroke	0.753	0.725	0.522	0.889	0.896

It indicates that on hydrophobic polymer substrates like PMMA, PolyC ((prepared as per the procedure 2.1.1.1) and chitosan ((prepared as per the procedure 2.1.1.4), maximum deposition happens in the down stroke mode. This is due to strong hydrophobic interaction between the monolayer tail side and the substrate[205]. In the case of hydrophilic substrates like cPVA (prepared as per the procedure 2.1.1.3) and Glass (cleaned with chromic acid and distilled water) maximum deposition happens in the up stroke mode (Table-3.3.1.2.1). This is due to hydrophilic interaction between the monolayer head side and the substrate[206]. This gives the information that depending upon the polarity of the substrate the mode of deposition can be altered to get the maximum monolayer transfer. Apart from that it also gives the idea about the physical stability of these monolayers based on the substrate-monolayer interaction. This could be explored for manipulating surfaces, regioselectively with these monolayers for tailor made surface interactions. The compositions of the monolayer have been varied at this

juncture to see the effect of composition on the interaction and packing of the monolayers.

3.3.1.3 Composition vs Transfer ratio

Further deposition of various PC/ Chol/ GalC lipid combinations have been done at constant deposition parameters (5 mm/min dipping as well as withdrawal speed) to PolyC films at ambient temperature(deposited as per the procedure 2.2.2.2.1) with the help of LB trough. The Figure-3.3.1.3.1 shows the composition effect on transfer ratio of the monolayer to the polymer substrates, from the air/ water interface. The deposition of the PC monolayer on this hydrophobic polymer surface shows a tight packed second monolayer deposition during withdrawal (Figure 3.3.1.3.1B).

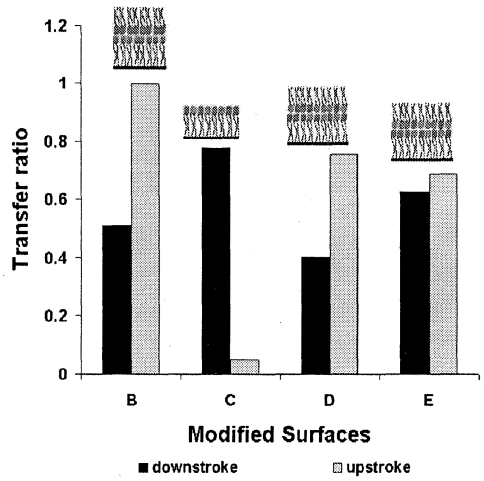


Figure-3.3.1.3.1: Modified surface Vs Transfer ratio, Down stroke followed by Upstroke [PolyC modified with PC (B), (PC:Chol) (1:0.7) (C), (PC: Chol) (1:0.35) (D), (PC:Chol:GalC) (1:0.35:0.125) (E)

This ensures the exposure of the nonpolar face of the monolayer by burying the polar group inside. For the combination of (PC: Chol) (1:0.7) only a single monolayer deposition is seen on the polymer surface, exposing the hydrophilic phase outside as shown in, (Figure 3.3.1.3.1C). This surface resembles the natural

cell membrane where the polar groups are exposed to the aqueous phase. This is due to the condensing effect of Chol, which imparts close packing of the deposited monolayer. When the Chol concentration is reduced to half (PC/Chol) (1:0.35) to its original concentration, the interaction between the two monolayers have been increased during deposition, (Figure 3.3.1.3.1D). This gives a loosely packed second monolayer on the surface, which is oriented inversely exposing the hydrophobic face to the aqueous phase. From this it is evident that the concentration of Chol plays an important role in the second monolayer deposition. The introduction of the GalC into the monolayer (PC: Chol: GalC) (1: 0.35: 0.125) further improves the transfer ratio during dipping as well as withdrawal to give a more optimum deposition under reduced Chol concentrations (Figure 3.3.1.3.1E). Therefore we choose this OCMC monolayer for further detailed characterization, which can form a compact monolayer or bilayer as evident from (Figure 3.3.1.3.1E). Apart from that, the microenvironment of the first deposited monolayer influences the orientation as well as the packing of the second monolayer. Further the polarity of the modified surfaces has been studied.

3.4 Polarity of the modified surfaces

At the material –biology interface, water plays an important role in regulating the surface events. Structuring of water due to restricted hydrogen bonding may happen with the surface depending upon the polarity (1.1.5.2.2). The surface polarity of the modified surfaces has been studied by goniometry (2.2.11).

3.4.1 Method Vs Polarity

To check the effect of method of deposition, lipid modified surfaces have been prepared by different methods as per the procedures (1) LB deposition (2.2.2.2.1), (2) Transfer from air/water interface by in situe method (2.2.4.1.1), (3) Spreading and solvent evaporation (2.2.4.1.2) techniques.

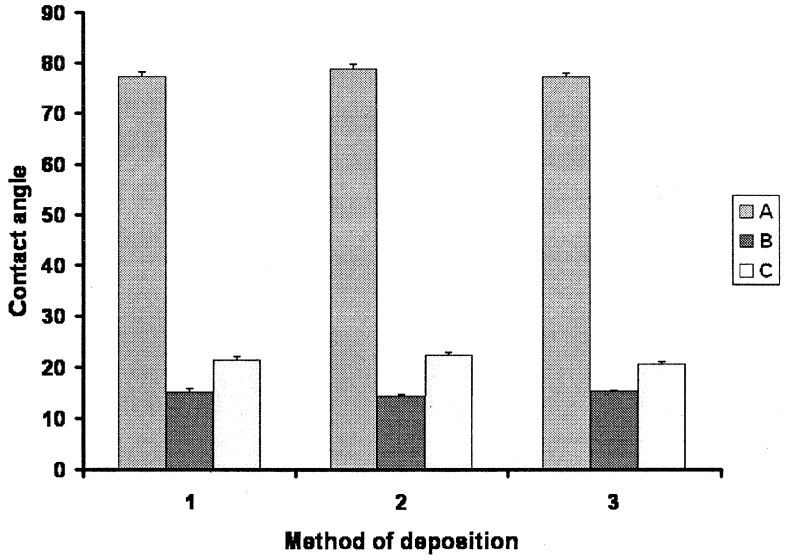


Figure-3.4.1.1: Polarity of the lipid monolayers, deposited by different methods on hydrophobic PolyC films: LB (1), In situe film from air/water interface (2), Solvent evaporation (3), Bare PolyC (A), PC (B), (PC:Chol)(1:0.7) (C)

The studies have been done using PC and (PC:Chol)(1:0.7) lipid monolayers mainly because from our early air/water interfacial studies it has been observed that PC represents a flexible monolayer and the other one represents a more rigid composite monolayer. The contact angle data of the modified substrate is shown in the (Figure 3.4.1.1). The water contact angle (θ) of the polymer reduces from 77 to 10-20 after the monolayer deposition. It indicates that the polarity of the lipid monolayer modified substrates have been reduced, irrespective of mode of deposition. Further we have attempted to evaluate the effect of orientation of the monolayer on surface polarity.

3.4.2 Packing density and orientation Vs Polarity

To check the effect of packing density and orientation of lipid modified surfaces on polarity the surfaces have been prepared as per the procedure 2.2.2.2.1.

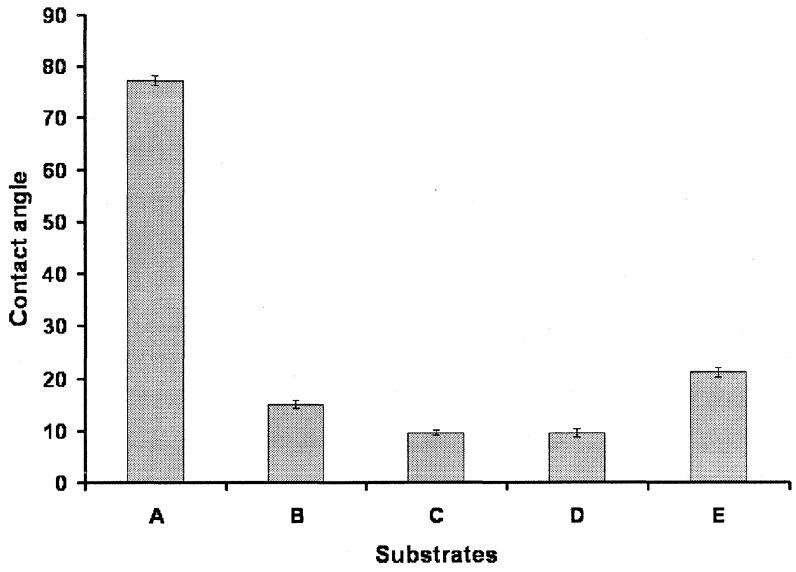


Figure-3.4.2.1: Polarity of the surfaces after lipid monolayer deposition, PolyC (A), PolyC modified with PC(B), (PC:Chol)(1:0.7) (C), (PC:Chol)(1:0.35) (D), (PC:Chol:GalC)(1:0.35:0.125) (E)

The contact angle data of the modified substrate is shown in the figure-3.4.2.1. The water contact angle (θ) of the polymer reduces from 77 to 10-20 after the monolayer deposition. It indicates that the different orientation of the monolayer has no influence in the polarity of the lipid modified substrates.

3.5 Stability of the monolayer on substrates

The stability studies have been done as per the procedure 2.2.5.1 under static conditions. Earlier we have observed that the (table-3.3.1.2.1) transfer ratio of these monolayers into the polymeric surfaces, depend upon the polarity of the substrate. The stability of these monolayers onto the polymeric surfaces has been

evaluated in terms of change in polarity of the surfaces. Figure-3.5.1, shows that these monolayers are quite stable onto the hydrophobic surfaces (PolyC, PMMA, &PS) as compared to hydrophilic surfaces (cPVA and Chitosan).

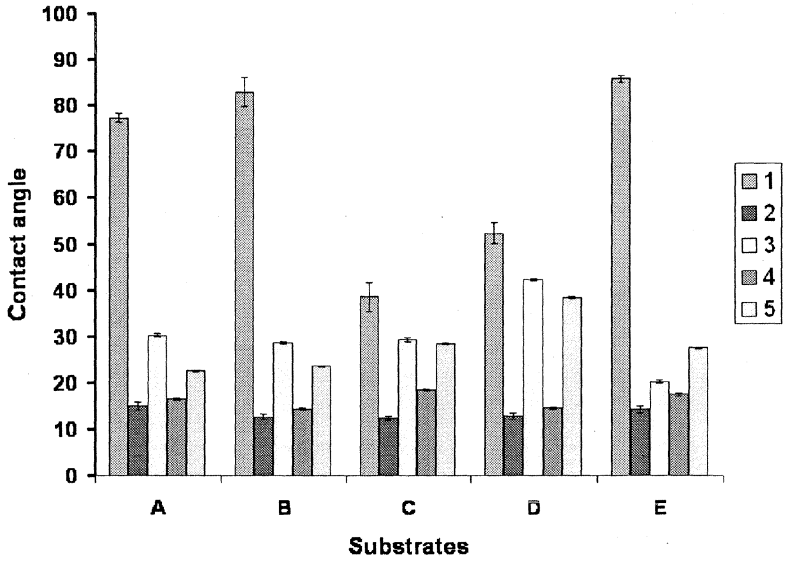


Figure-3.5.1: Stability of the monolayer on substrates under static conditions, PolyC(A), PMMA(B), cPVA(C), Chitosan(D), PS (E) Bare(1), PC(2), PC after incubation under static conditions 24hrs(3), (PC:Chol:GalC)(1:0.35:0.125) (4), after incubation under static conditions 24hrs(5)

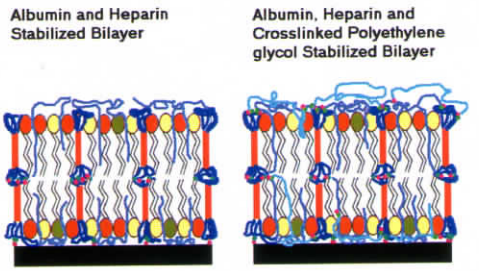
Improving the stability of these monolayers under low shear conditions is important for surface modification of devices, biosensors, targeted drug delivery systems etc. As an attempt to improve their stability we have mimicked bilayer plasma membrane. Here the lipid bilayer membrane is laterally stabilized by the transmembrane proteins intercalated into the cell membrane. Earlier we have been successful in incorporating Alb into the lipid monolayers with the help of Hep and DAPEG. Further we have explored the possibilities of using these macromolecules as anchors for the lateral stabilization of the monolayers.

3.5.1 Stabilization Strategy-1: Lateral Stabilization with incorporation of proteins

Two such anchor incorporated monolayers of respective compositions have been transferred to the functionalized PMMA (as per the procedure 2.2.2.2.2) films in the upstroke mode with the help of LB trough. The anchor network formed on this supported bilayer is stabilized by carbodiimide chemistry. Where the Alb molecule is perfectly incorporated into the lipid layer (3.2.4). These three anchor molecules cross-link to each other to form a 3D network and stabilizes the bilayer (Scheme- 3.5.1.1 & 2).

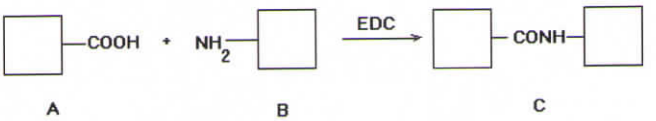
Schematic representation of the modified surfaces and the adsorption phenomena.

Scheme- 3.5.1.1



Schematic representation of Stabilized supported bilayer Alb ( ), Hep ( ), DAPEG ( ) PC ( ), Chol ( ), GalC ( )

Scheme- 3.5.1.2



Reaction scheme of the components, which laterally stabilizes the bilayer on PMMA surface. Carboxyl group of, PMMA, Alb, and Hep (A). Amino group of, Alb and DAPEG (B), The cross-linked network of anchors (C)

The water contact angle studies of the modified surfaces revealed that the surface hydrophobicity of the PMMA membranes have decreased after the surface modifications. The contact angle θ is reduced from 85° to 20° after the surface modification.

The scheme-3.5.1.1 shows the schematic representation of the laterally stabilized bilayer. Stability studies of these laterally stabilized monolayers have been done under low shear conditions. The Figure-3.5.1.1 shows the stability studies under low shear conditions (studied as per the procedure 2.2.5.2) of the modified surfaces represented in terms of the change in contact angle[207].

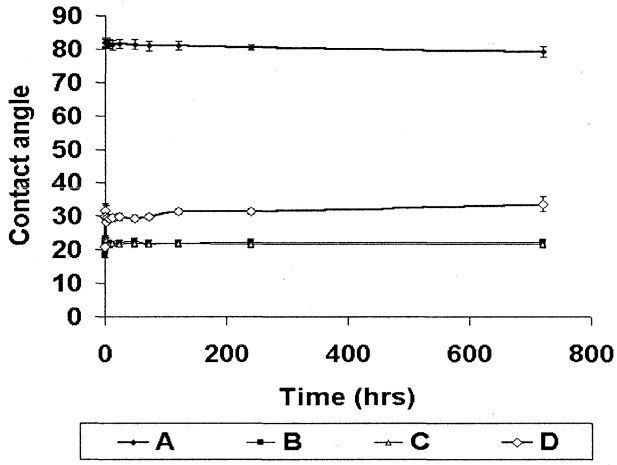


Figure-3.5.1.1: Stability studies under low shear conditions. PMMA Bare (A), modified with OCMC (D). PMMA modified with OCMC- AH (B), OCMC- AHP (C)

The PMMA surface modified with the OCMC lipid layer shows less stability as compared to the laterally stabilized surfaces. In this surface the contact angle have been stabilized after a certain period, may be due to the improved lateral stability contributed by the Chol and GalC (condensation effect) (Figure: - 3.5.1.1D). However, in the case of PMMA modified with OCMC-AH and OCMC-AHP lipids containing anchors (Figure: - 3.5.1.1B and C) the contact angle has not varied significantly with time. This shows that the anchored lipid layers are much more stable on the polymer surface than the unanchored lipid layers.

The biological lipid bilayer membranes are stabilized laterally with the help of integral proteins. The integral membrane proteins, rich in β- sheet exist in the calveolae of the cholesterol rich domains, which anchors the membranes to the cytoskeleton[208]. The hydrophobic interaction between the integral proteins and the core of the bilayer membrane holds these integral proteins in the membrane bilayer[209, 210.] These factors enable these membrane surfaces to retain the fluidity, and exchangeability and thus the renewability of its components[211]. Such a renewable system may interact more actively with the biological environment without sacrificing its specific functions.

Here instead of the integral membrane proteins we have used albumin as lateral stabilizer after changing the structure of albumin to expose its interior hydrophobic core by treating with organic solvent. Albumin is a flexible passive protein, abundantly present in the blood (3.5-4gm %) [212]. This protein has been found to be non-thrombogenic in its native and altered conformations[213]. The membrane proteins are rich in β- sheet with a hydrophobic exterior. This limits the solubility of the integral membrane proteins. Water soluble globular proteins have rich α helical content in its native confirmation, changes to molten globular state with more β pleated structure, and random conformations in its less-soluble state, under adverse conditions of varied pH, solvents, ionic concentration, and temperature[214, 215.] These proteins can be incorporated into the model lipid bilayer membranes after its phase inversion[216,217] to increase its hydrophobicity by treating with organic solvents. Thus integrated membrane proteins can be anchored to linkers with functional groups.

Heparin is a sulfated polysaccharide and is a natural anticoagulant. Immobilization of heparin on material surface for blood contacting applications as linkers is widely explored [218 , 219]. Polyethylene glycol (PEG) is another antithrombogenic molecule, explored as a linker or tethering agent as well as a surface modifying agent due to its antifouling properties[220,221]. Attempts have

been made by using such lateral stabilizers could be used for improving the stability of the supported bilayer systems [222].

This novel strategy of stabilizing lipid films with peptide linkages could be considered as a more natural way of stabilizing these monolayers. Attempts to explore albumin, heparin and PEG as anchors are more economically feasible. Receptors or active proteins after tethering with alkyl groups can be immobilized onto these monolayers as movable entities to perform different functions like in natural cell membrane[223].

From feasibility point of view physical stabilization have more wider application, than the chemical processes. The time, energy and space is minimum in the various physical immobilization processes. Further it gives enough versatility to modify any kind of substrates. Therefore we have further attempted to laterally stabilize these monolayers, by physical means.

3.5.2 Stabilization Strategy- 2 Physical stabilization using pendant polymer

For that we have developed pendant polymers. Our assumption here was that (1) the polymer should be soluble in some less polar organic solvents, but not in water. (2) It should be able to preferentially adsorb albumin and have high interaction with lipid layers. (3) It should form stable film over any kind of substrates.

3.5.2.1 Selection of the pendant group

The selection of pendant group is more crucial as it significantly influences the physical properties of the pendant polymer. We have taken certain assumptions while selecting the pendant groups. From the chemical point of view (1) It should have functional groups which can easily cross link with the PVA, (2) The molecule should have a bulky side chain with benzene rings. From the physical point of view 1) It should have high partition coefficient (So that interaction with lipid membranes will be high) 2) Should be soluble in water or water/ethanol, acetone co-solvent system and in DMSO. From the biological point of view (1) It should have proven therapeutic identity; in terms of safety and efficacy (2) Even the metabolite should have proven safety records.

3.5.2.1.1 Albumin binding avidity studies

3.5.2.1.1.1 In silico docking studies between albumin and free drug

Some of the drug molecules like, Non- Steroidal Anti Inflammatory Drugs (NSAID) have been found to be highly albumin bound[224]. We have selected three Non Steroidal Anti Inflammatory Drug (NSAID) molecules for the said purpose. The selected molecules are Aspirin (ASP), Indomethacin (INDO) and Diclofenac (DIC) which have proven safety records[225] from the literature. Further in silico docking studies (studied as per the procedure 2.1.3.1.1) have been done between these drug molecules and albumin to select the molecule with maximum affinity for albumin.

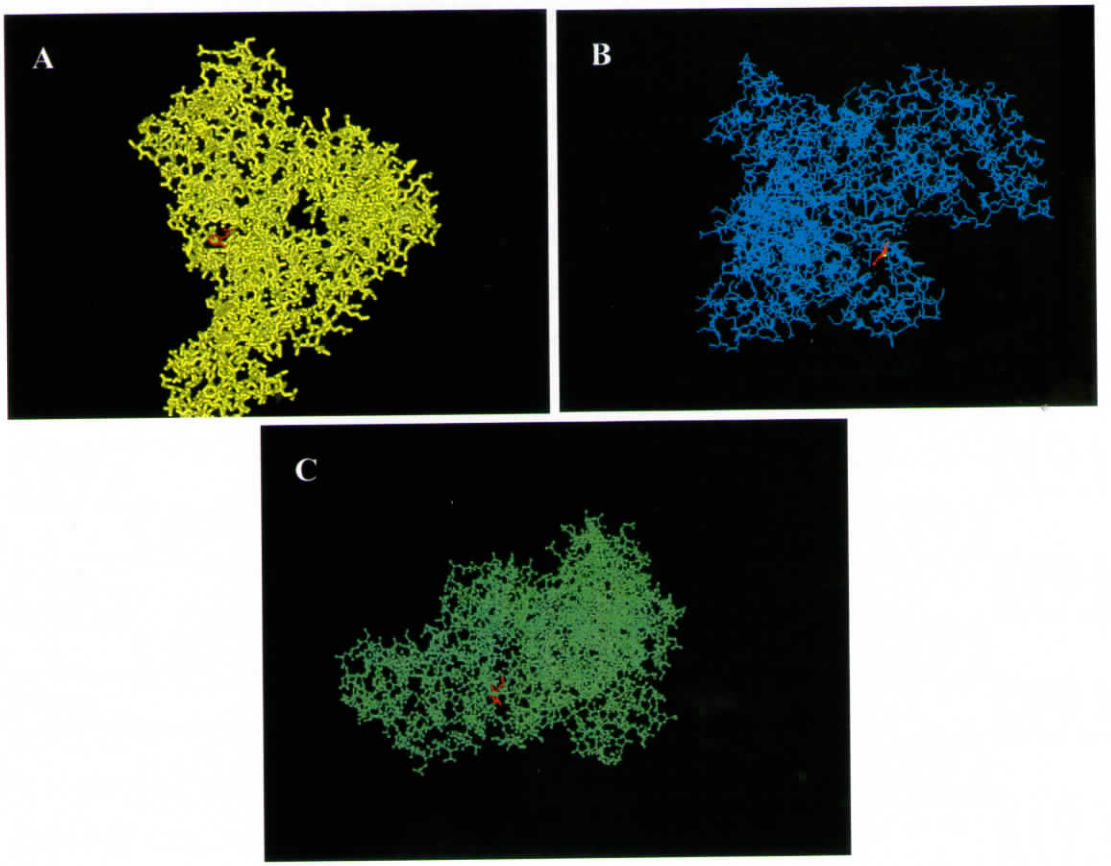


Figure-3.5.2.1.1.1.1: Drug docking with Albumin: Aspirin (A), Indomethacin (B), Diclofenac (DIC) (C), The best fit model of the drugs and albumin

Table-3.5.2.1.1.1.1: Minimum free energy of the docked albumin molecules

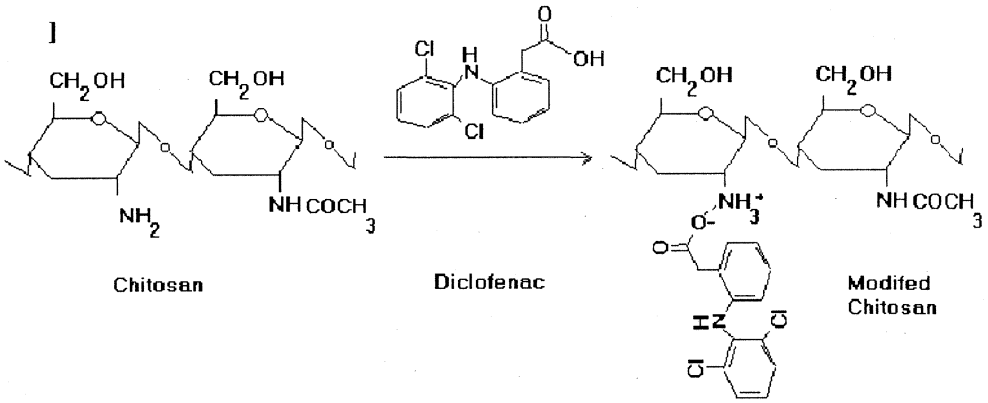
Drug	Emax	Emin	Eavg top 10
Aspirin	-140.95	-33.14	-130.71
Indomethacin	-140.78	-133.57	-130.71
Diclofenac	-174.57	-60.69	-161.05

Among the three drug molecules DIC shows high affinity for albumin. This is evident from the minimum free energy (Table-3.5.2.1.1.1.1) of the best fit model. Diclofenac (DIC) is a highly tolerated NSAID. It acts by potent cyclo-oxygenase inhibition, reduction of arachidonic acid release, and enhancement of arachidonic acid uptake. The cyclooxygenase regulates the production of inflammatory arachidonic acid metabolites in the body[226] (The drug have very high therapeutic index,(LD 50 in mice , rats; 390, 150 (mg/ kg); Orally), compared to (23, 21.5 mg/ kg) for stearic acid and is highly bound (>99.5%) to albumin, In vivo. The drug binds preferentially to a high affinity site in albumin and may get transferred to a low affinity site in presence of other ligands[227]. All these information suggest that this drug molecule could be effectively utilized for the preferential adsorption of albumin to the material surface. The diclofenac has a partition coefficient of 13 in octanol/ phosphate buffer (PB) (7.4)[228] system A linear relationship has been found between the lipophilicity of the drug and binding parameters[229]. Further we have attempted to immobilize the drug by ionic and covalent immobilization strategies.

3.5.2.2 Pendant group immobilization on polymers by ionic linkage

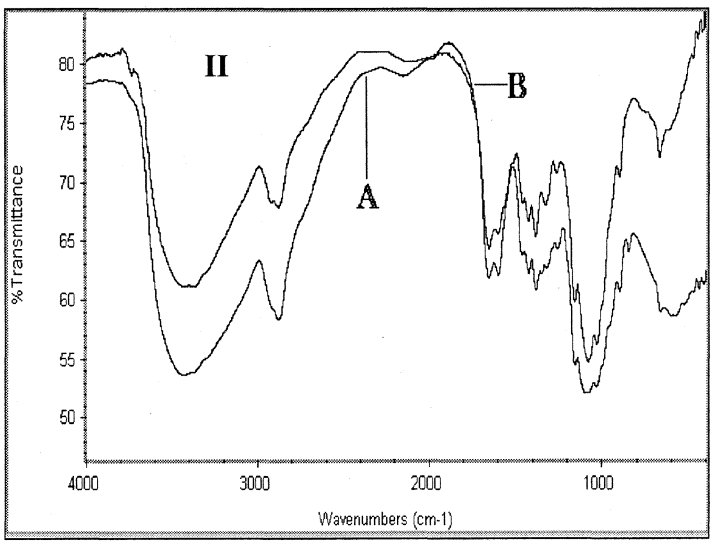
For ionic cross-linking, chitosan has been used as the polymer. The DIC is a weak acid with a P Ka value of 4.0, and for chitosan the P Ka value is 6.5 [230]. This gives opportunity to modify the free amino groups of the chitosan molecule with the drug molecule. Due to the high partition coefficient of the drug, the drug-polymer complex precipitates from solution in the bound form. This may be the reason for the strong ionic cross-linking of the drug with the polymer. The ionic cross-linking of the polymer has been done by swelling-diffusion filling method (as per the procedure 2.1.1.4.1).

I



Chitosan Diclofenac Modifed Chitosan

II



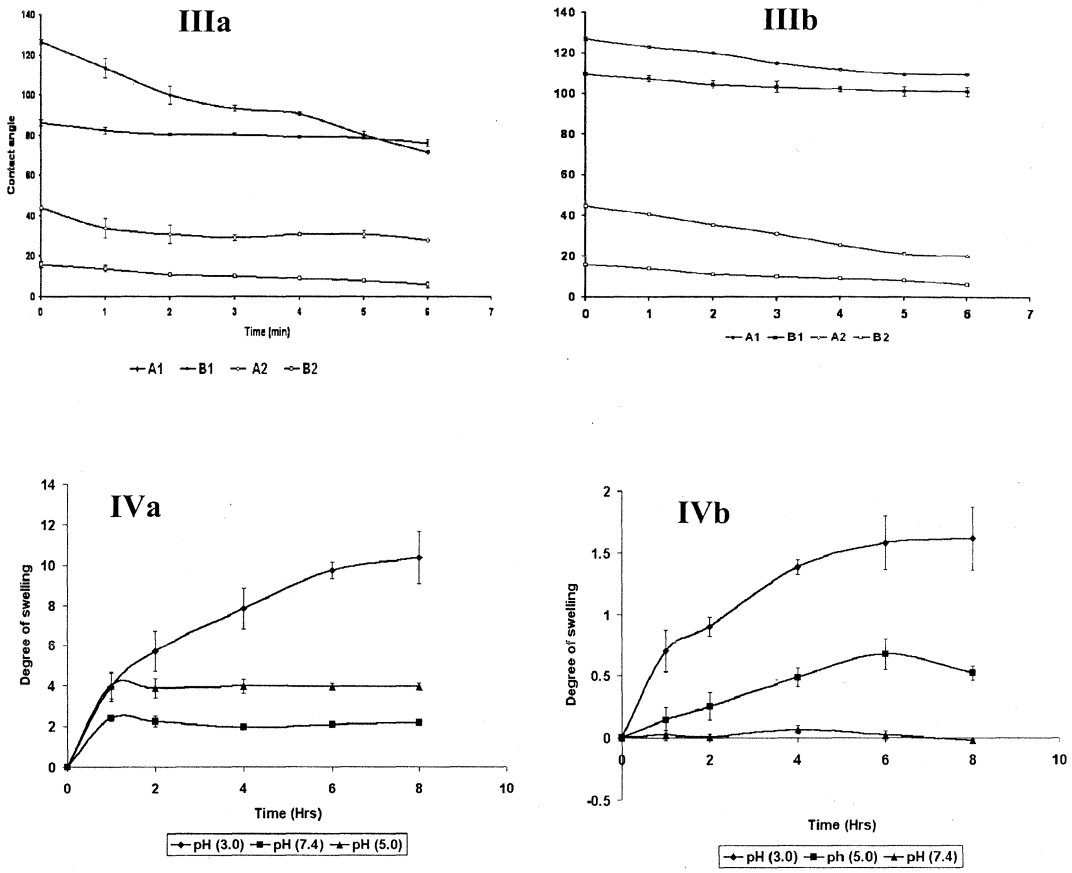


Figure-3.5.2.2.1 Modification of the chitosan. The scheme of the chemical reaction (I), FTIR spectrum of the pendant polymer (II); Chitosan bare (A), Modified chitosan (B). Dynamic contact angle data of polymer surfaces (III); Chitosan (a), Modified Chitosan (b); Dry polymer (A), Pre-swollen (B), Advancing(1), Receding (2) contact angles. Swelling properties of the pendant polymer (IV); Chitosan bare (a), Modified chitosan (b)

The FTIR spectroscopy, figure- 3.5.2.2.1 II A of CHIT shows specific peaks at 1651 cm^{-1} (amide I)[231], 1598 cm^{-1} due to NH bend (hydrogen bonded)[232]. The decrease in peak intensity at 1598 cm^{-1} of the hydrogen bonded NH bond in modified chitosan shows the ionic cross-linking between the carboxyl group of the DIC and the amino group of CHIT. The increase of peak 1093 cm^{-1} (C-Cl aromatic) is

evidence of the drug immobilization and another peak at 1316 cm^{-1}, (C-N and N-H) linkages, also confirms the drug immobilization (figure- 3.5.2.2.1 II B).

The polarity of the surfaces have been studied by sessile drop method using a goniometer is shown in Figure-3.5.2.2.1 III. The contact angle of the CHIT films (70.53+/-0.638) has been increased after the surface modification (80.6+/-0.8825). This is due to the orientation of the hydrophobic groups of the DIC at the film surface. The advancing and receding contact angle of the dry and pre-swollen substrates has studied. The decrease in advancing contact angle of the dry chitosan films indicates that (3.5.2.2.1 IIIa), the polymeric chains at the surface are changing their conformation with respect to time. However this is less significant with the modified chitosan (3.5.2.2.1 IIIb). This is due to hydrophobic interactions between the pendant drug molecules, restricts the chain mobility. However in the case of pre- swollen chitosan films, this is not happening.

The swelling profile of the CHITDIC at pH (3, 5 and 7.4) is shown in figure-3.5.2.2.1 IV. The overall degree of swelling has been reduced considerably at all the pH conditions after the DIC immobilisation. CHIT shows a biphasic swelling profile (figure-3.5.2.2.1 IVa. The initial phase is due to water diffusion. This is followed by a plateau phase. The second phase of swelling is due to chain expansion. The second phase is more predominant at pH 3.0. This is due to the protonation of the free amino groups. The plateau phase shows that a counter acting deswelling mechanism works at this phase. This is observed between 2-3 hr at pH 5 and 7.4, in the case of bare CHIT also. While in the case of CHITDIC (figure-3.5.2.2.1 IVb) it is observed at two stages, at 2hr as well as after 4 hrs. Here the second deswelling phase is varied at different pH conditions. This phase is again depends upon the protonation of the amino groups.

At pH 7.4 when the free amino groups are least protonated the second deswelling phase starts at 4hrs while at pH 5 it is further shifted to 6hrs. At pH 3.0 when all the amino groups are protonated the phase II deswelling is not visible.

This may be due to the entropy introduced into the system by the water diffusion. The deswelling phenomenon observed at two stages in the modified chitosan also suggests that the hydrophobicity of the chains play an important role in the deswelling mechanism[233]. This is also evident from the swelling profile of the bare chitosan at pH5.0 and 7.4, where the free amino groups are less protonated as compared to pH 3. The CHITDIC membranes shows minimum swelling at pH 7.4 as compared to the bare CHIT also suggests that the increase in hydrophobicity of the membranes regulate the swelling profile.

3.5.2.2.1 In vitro binding avidity studies between albumin and bound DIC

The CHITDIC films (prepared as per the procedure 2.1.1.4) were used for the studies. The protein adsorption studies were done as per the procedure 2.3.2.1. The desorbed proteins adsorbed onto the polymeric substrates as well as the supernatant after 3hrs has been evaluated by SDS-PAGE.

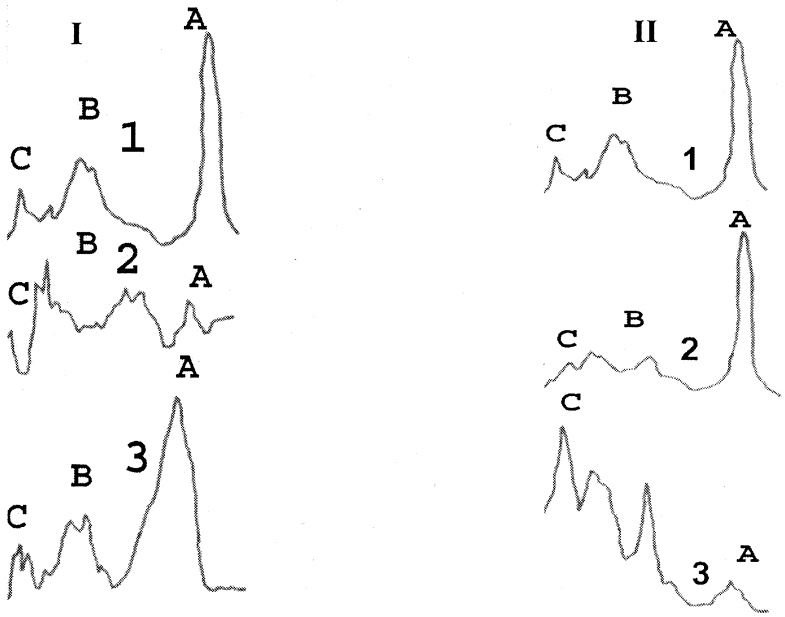


Figure-3.5.2.2.1.1: Densitometric diagram of the protein adsorption-desorption studies using mixture of proteins. X axis length in cm (maximum 6cm), and Y axis absorbance (maximum value = 0.5). Control (1), CHIT(2), CHITDIC(3). The peaks in protein adhesion studies are (A) albumin, (B) γ globulin and (C) fibrinogen Desorbed proteins (I), Supernatant (II)

The CHITDIC modified surfaces has shown preferential adsorption of albumin (Figure-3.5.2.2.1.1). During ionic crosslinking of the drug to the polymer part of the pharmacodynamic phore, which is responsible for the NSAID activity, is engaged with the polymer. This makes the drug molecule to orient the hydrophobic domain (Pharmacokinetic phore) to the aqueous environment. Earlier it has been reported that this hydrophobic domain fit into the (Site II) hydrophobic domain of the albumin molecule with high specificity. The carboxylic group along with the hydrophobic group is necessary for the firm binding of this drug to the albumin molecule. This leads to the reversible change in conformation of the albumin molecule[234]. Since the carboxylic acid group is engaged with the polymer, we propose that the conformational change in the adsorbed albumin molecule may be less in the modified surfaces.

3.5.2.3 Pendant group immobilization on polymers by covalent linkage

3.5.2.3.1 Selection of the polymer to form the backbone

From our preliminary studies using PEG, PVA, Chitosan and alginate we have found that PVA is one of the most appropriate polymer for the said purpose, because of the following reasons. (1) It is soluble in less polar solvent like DMSO in which all the other polymers are less soluble. (2) The hydroxyl group of the PVA can be easily functionalized. (3) Alternating OH groups gives free rotational flexibility around the single bond, which can be temporarily hindered with bulk pendant groups.

3.5.2.3.2 Preparation of PVADIC pendant polymer

Further we have attempted to immobilize this drug molecule into PVA (As per the procedure 2.1.3.1.2). The immobilization of the drug to the polymer has been evaluated by quantifying the drug content of the supernatant solution (as per the procedures 2.2.8.3 and 2.2.12.3). Here we have chosen the composition PVADICa (with drug to polymer ratio of 0.025:1w/w) for the albumin binding avidity studies, where the drug pay load has been evaluated to be 6.58+/-0.12% w/w, and

is water soluble. The FTIR spectra of the PVA, PVA-DIC and DIC are shown in (Figure-3.5.2.3.2.1).

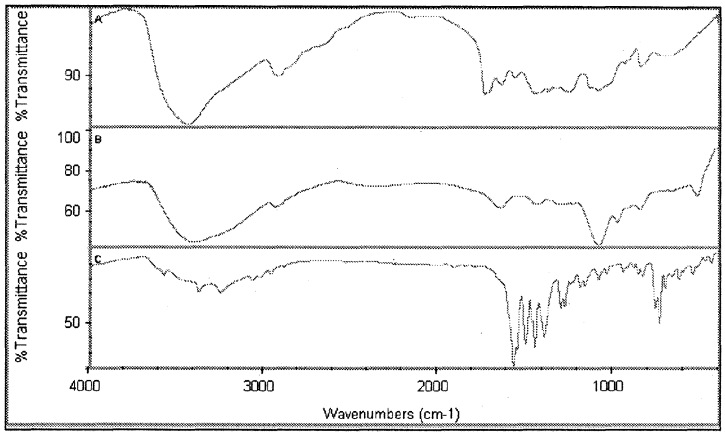


Figure- 3.5.2.3.2.1: Immobilization of the drug into the polymer, FTIR Spectroscopy of PVA- DIC complex, PVA (A), (PVA- DICa (B), DIC (C)

The reduction of the peak at 1238 cm^{-1} of DIC and the increase of peak 1093 cm^{-1} (C-O-C) and (C-Cl aromatic) are evidences of the ligand immobilization onto the polymer by ether linkages (Figure- 3.5.2.3.2.1B). Another peak at 1316 cm^{-1} (C-N and N-H) linkages also confirms the immobilized ligand (Figure-3.5.2.3.2.1B).

3.5.2.3.2.1 Liposome binding affinity of the pendant polymer

The main objective of the development of these pendant polymers is to laterally stabilise the lipid layers. However a renewable surface which can bioactively interact for the optimum performance can be achieved with a surface which can interact with lipid layers and reversibly bound with albumin. For that the interaction of the pendant polymer and the liposomes has been subjected to study. The liposomes has been prepared by using a lipid solution of (PC: Chol) (1: 0, 0.35, 0.7) composition in 3ml of a mixture of CH/ n- But (5: 2)v/v solvent system(As per the procedure 2.1.4.1). The polymer coating studies has been done

as per the procedure 2.1.4.3 using PVADICa (which is water soluble) pendant polymer. The Table-3.5.2.3.2.1.1 shows the effect of cholesterol concentration on the coating of the pendant polymer. Which is evident from the increase in size of the liposomes.

Table-3.5.2.3.2.1.1: Effect of polymer coating on size of liposomes

Lipid composition	Z average size of liposomes before coating (d.nm)	Z average size of liposomes after coating with the polymer (d.nm)	Z average size of liposomes after coating with the polymer and albumin (d.nm)
(PC: Chol) (1:0) with PVA	78+/-1.25	82+/-2.34	84+/-3.69
(PC: Chol) (1:0) with PVA-DICa		96+/-1.24	112+/-5.26
(PC: Chol) (1:0.35) with PVA	96+/-1.48	102+/-1.35	104+/-1.78
(PC: Chol) (1:0.35) with PVA-DICa		135+/-2.21	152+/-1.8
(PC: Chol) (1:0.7) with PVA	112+/-1.64	115+/-1.26	118+/-2.42
(PC: Chol) (1:0.7) with PVA-DICa		120+/-1.52	138+/-2.24

Maximum interaction between liposomes and pendant polymer has been observed with 0.35mg% Chol concentration. This may be due to the flexibility of the bilayer due to reduced Chol concentration. Earlier we have observed that the LE phase of the monolayer is increasing with the reduction of the Chol concentration (figure- 3.2.2). Further coating of the polymer coated liposomes with Alb indicates that this pendant polymer coated liposomes has the ability to self-assemble the albumin also. In contrast in the case of PVA coated liposomes incubation in albumin solution does not change the size. This is an important development because these polymers can be used for variety of surface modification applications like albumin self-assembled liposomes, surface modification of nanoparticles etc.

3.5.2.3.2.2 Albumin binding avidity of PVADICa with albumin in presence of insulin

Preferential binding of albumin to pendant DIC (CHITDIC) had demonstrated earlier in presence of other proteins like γ globulin and fibrinogen (3.5.2.2.1). This has a lot of significance in improving the blood compatibility of advanced drug delivery systems or polymer drug complexes etc. Here we have attempted to study the competition of albumin with other biological proteins which may be delivered under a clinically relevant scenario, like systemic protein delivery applications. For that we have studied the competition between insulin and albumin for binding to the pendant polymer.

Here, the binding avidity of albumin to PVA-DICa (prepared as per the procedure 2.1.3.1.2), which is precoated over insulin loaded liposomes (As per the procedure 2.1.4.3), has been studied by DSC. The liposomes have been prepared by using a lipid solution of PC: Chol (1: 0.35) in 3ml of a mixture of CH/ n- But (5: 2)v/v solvent system(As per the procedure 2.1.4.1). The figure-3.5.2.3.2.2.1 shows the thermal properties of the ligand-protein complex.

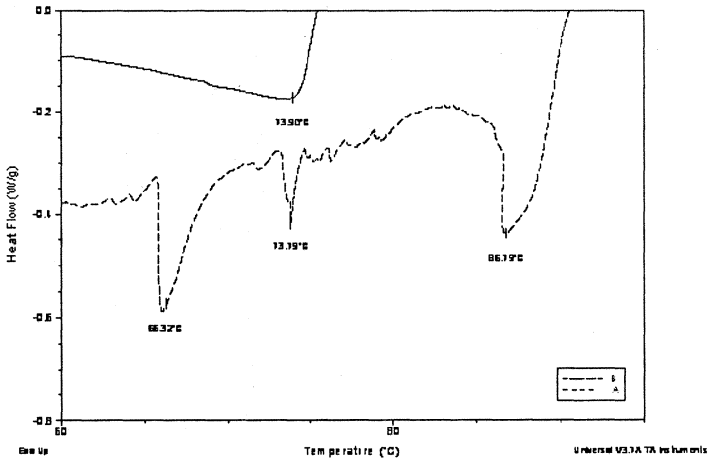


Figure- 3.5.2.3.2.2.1: DSC of the bound albumin to the PVADICa complex. Bound albumin (PVADICa coated liposomes) (A) and unbound albumin (uncoated) (B)

The DSC isotherm of the sample shows an endothermic thermal transition. In the absence of the ligand (figure 3.5.2.3.2.2.1A) the endothermic transition is at 73.90°C. This is an additive endothermic transition due to insulin and albumin. Earlier it has been observed that the mid transition temperature of albumin is between 55- 60 °C [235], while that of the insulin is at 70- 75°C [236]. This is also evident from studies in presence of ligand. The figure 3.5.2.3.2.2.1B shows basically three mid transition points at 66.32°C, 73. 79°C and 86.79 °C. The right shift in endotherms is due to the ligand binding. The ligand binding split the monophasic thermal transition of the albumin molecule to a biphasic one. This is due to increase in thermal stability of ligand bound albumin. Since ligand bound and free albumin are present in the solution that will be observed as shifts in thermal transitions or as multiple peaks[237]. The affinity and concentration of the ligand as well as number of ligand binding domains of the protein do influence the thermal denaturation process. It has been understood that this type of biphasic unfolding process arises from a substantial increase in stability of remaining native protein during denaturation.

This increase in stability derives from the free energy of ligand binding becoming more negative due to the release of high affinity ligand by unfolding protein. The tendency for biphasic denaturation is greatest at low (sub-saturating) levels of ligand where maximum increase in the stability occurs. Biphasic unfolding arising from such ligand redistribution results from denaturation of different kinds of protein molecules, ligand-poor and ligand-rich species, and not from sequential unfolding of domains within the same molecule. To reestablish this hypothesis, in all these studies we have added an excess quantity of albumin, to demonstrate the redistribution of ligand during thermal denaturation process. Apart from that no other peak has been observed indicating the interaction of the insulin with the ligand. This reestablishes the affinity of the ligand with the albumin in presence of other proteins.

3.5.2.3.3 Formation of nanoparticles out of pendant polymer

Among the three pendant polymers optimized, the PVADICb and c formed nanoparticles (prepared as per the procedure 2.1.3.1.3). Both the polymers shows least minimum aggregatory concentration. At all the concentrations they form nanoparticles upon dilution through self-assembly process. The figure-3.5.2.3.3.1 below shows the melting curve (Studied as per the procedure 2.2.15.3) of the nanoparticles. These nanoparticles shows temperature depended size changes. The melting point of the nanoparticles has been found to be 59 °C for PVADICb and 19°C for PVADICc

The nanoparticles also shows shape memory effect below the melting point with a hysterisis in the case of PVADICb. This is not happening in the case of PVADICc.

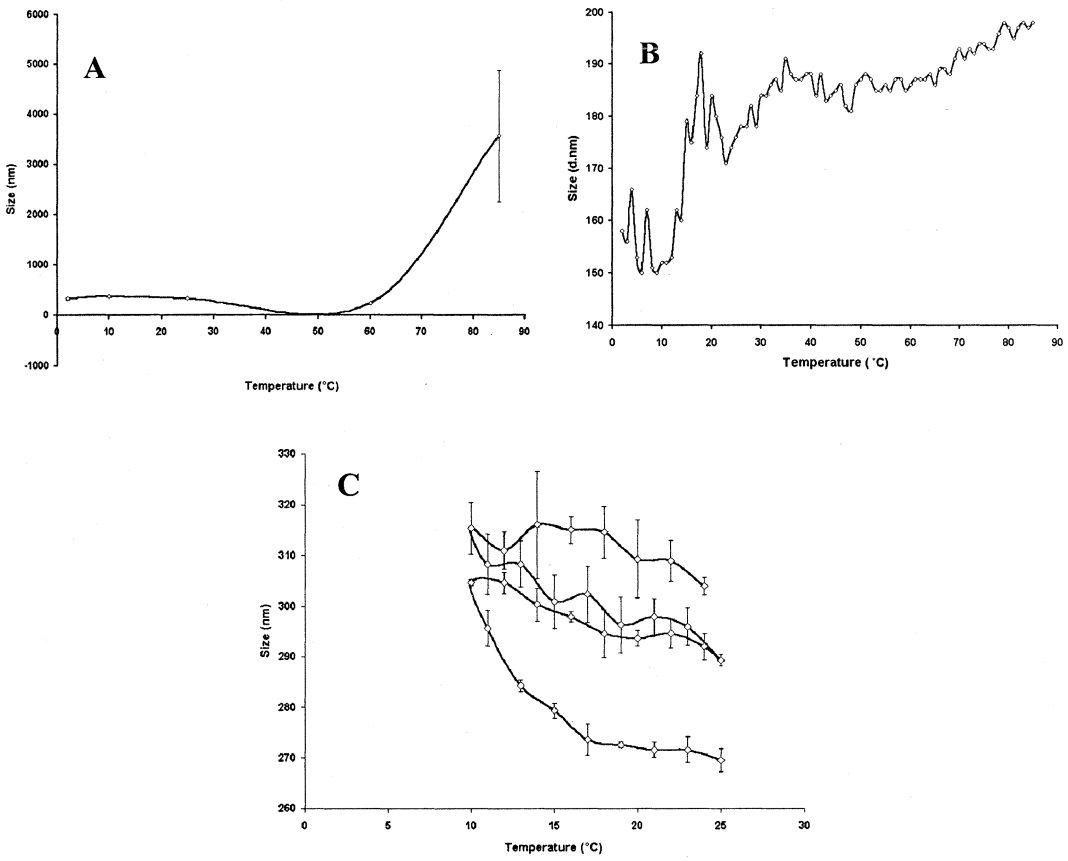


Figure-3.5.2.3.3.1: Melting curve of the nanoparticles. PVADICb (A), PVADICc (B). Shape memory effect of the nanoparticles, PVADICb (C)

The PVADICb shows least aggregation behavior with time, while the PVADICc has shown strong inter nanoparticle aggregation and settling behavior, the settling behaviour has been reduced by adding albumin.

3.5.2.3.4 Air/water interfacial studies of the pendant polymer

Further we have studied the air/water interfacial studies of the pendant polymer (PVADICc) with the help of LB trough (as per the procedure 2.2.2.1.2).

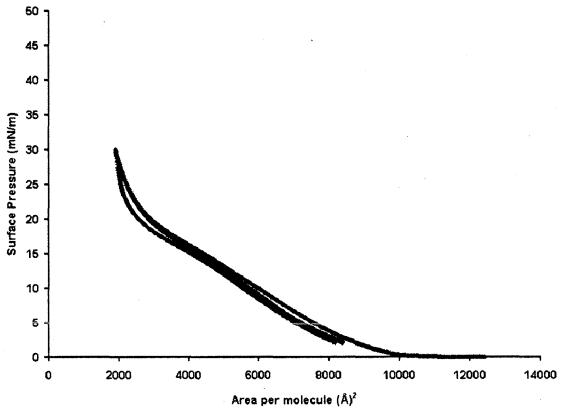


Figure-3.5.2.3.4.1: Thermo responsive behavior of thin solid films of the nanoparticles: Compression-decompression studies of the thin solid films of the nanoparticle in the air/water interface

The PVADICa & b could not be able to form a monolayer at the air/water interface. The PVADICc has formed a monolayer at the interface. Upon multiple compression followed by decompression of the monolayer shows reversibility without hysterisis (Figure-3.5.2.3.4.1). This clearly shows that short range interactions are controlling the formation of the monolayer. Apart from that nanoparticles are behaving like individual molecules at the air/water interface.

3.5.2.3.5 Morphology of nanoparticles and their 2D films

To understand the structure and mechanism behind this diverse physical property, SEM (as per the procedure 2.2.17.2), studies has been done using samples prepared at two different temperature conditions. PVADICb has shown a

globular structure (Figure-3.5.2.3.5.1) at room temperature. The nanopaticles are spherical and shows uniform size distribution. Above the melting point (at 70°C for 5min incubation followed by 24 hrs at room temperature) the nanoparticles are opened up and formed sheets. Further the sheets are grown into spindle structures with defined geometries.

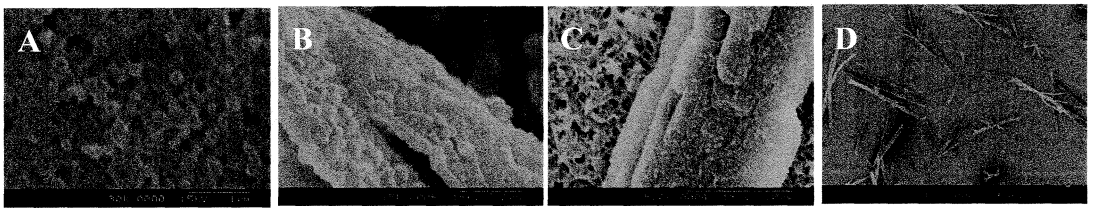


Figure-3.5.2.3.5.1: SEM of the PVADICb nanoparticles (NP) and conformational change with respect to temperature, NP at 25°C (A), NP at 70°C (B-D)

The PVADICc nanoparticles (Figure-3.5.2.3.5.2) are not spherical but oval in shape and shows strong one to one adhesion. The SEM of, peeled and dried thin sheet of the polymer shows nanoparticulate architecture (Figure-3.5.2.3.5.2B) through out its structure. It clearly indicates that this thin films hold the properties of the individual nanoparticles.

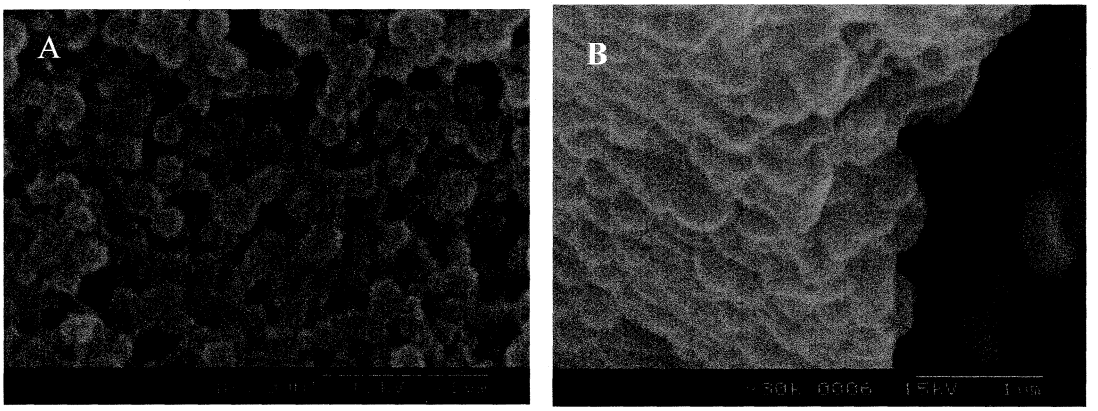


Figure-3.5.2.3.5.2: SEM of the nanoparticles (A), 2D film of nanoparticles (B)

Further we have studied the interaction of these thin films of nanoparticles with substrates of different polarity. For that the pendant polymers have been compressed at an air/water interface and transferred into different substrates (as per the procedure 2.2.3 and further lipid monolayers are transferred as per the procedure 2.2.2.2.1).

3.5.2.3.6 Air/ solid transfer studies of nanoparticles and lipid layers

The figure-3.5.2.3.6.1 shows that the transfer ratio of monolayers of the thermoresponsive polymer is maximum on both hydrophobic (PolyC) and hydrophilic (glass) surfaces. Earlier it has been observed that the hydrophilic monolayer transfer is maximum onto hydrophilic substrates while hydrophobic onto hydrophobic substrates (Table-3.3.1.2.1).

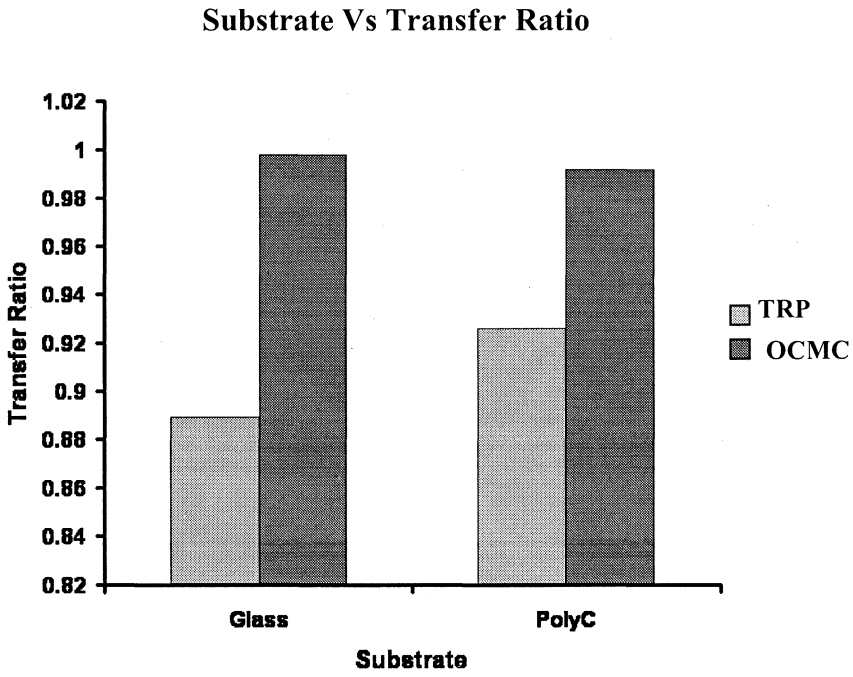


Figure-3.5.2.3.6.1: Transfer of the monolayers on substrates Modified with Thermoresponsive polymer(PVADICc) (TRP) further modified with the lipid monolayer

Interestingly on both the kind of substrates significant transfer of monolayers of the polymer is achieved. This may be due to the hydrophobic/hydrophilic ratio

of the polymer monolayer. After the surface modification with PVADICc we have found that lipid monolayer deposition onto the substrates is similar on both the kind of surfaces with different polarity. Therefore this pendant group strategy could be explored for laterally stabilizing the monolayers independent of substrate polarity.

3.5.2.3.7 Thermoresponsiveness of the 2D film of nanoparticles

Further the thermoresponsiveness of the nanoparticle based 2D thin films, have been done after depositing it over the PolyC substrates (as per the procedure 2.2.3). and studied the polarity using goniometer by sessile drop method(as per the procedure 2.2.11) . The figure-3.5.2.3.7.1 below shows the contact angle of the surfaces by sessile drop method.

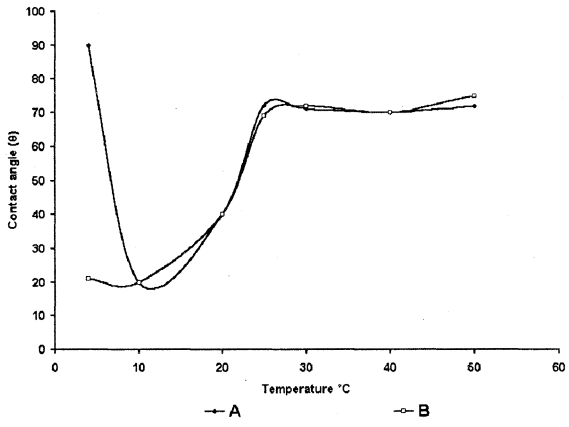


Figure-3.5.2.3.7.1: Contact angle of the nanoparticle coated surface with respect to temperature conditions, (Stored in absence of humidity (A) and in presence of humidity (B))

The surfaces have shown polarity difference at the melting pint of the nanoparticles. At 20°C the contact angle has been nearly 70° as compared to 15° at 10°C. This clearly indicates that the nanoparticles are undergoing phase change with the change in temperature. The interaction of these pendant polymers with albumin and lipid layers has already demonstrated.

3.5.2.3.8 Conformation of the bound albumin to the pendant DIC

For the conformational studies of bound albumin PVADICb nanoparticles (prepared as per the procedure 2.1.3.1.3) have been used as the substrate. The far UV CD spectra (as per the procedure (2.3.2.2.1.1)) of the native as well as the bound albumin is studied. The figure-3.5.2.3.8.1 shows the CD Spectra of the albumin molecule.

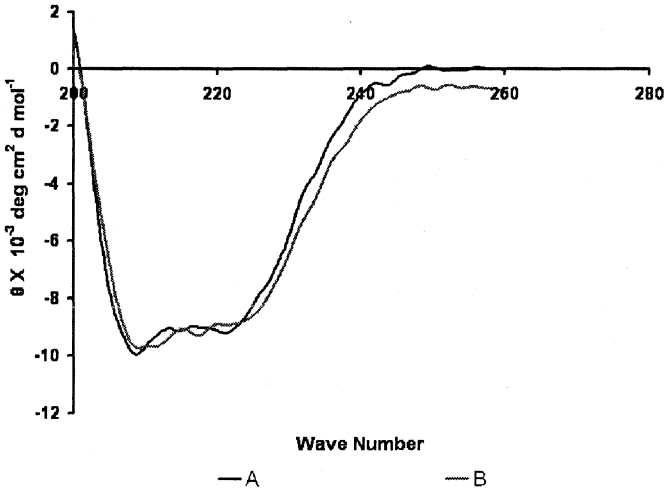


Figure-3.5.2.3.8.1: CD spectroscopy of the bound albumin. Albumin (A), PVA-DICb bound albumin (B)

The figure- 3.5.2.3.8.1 shows the conformation of the bound albumin. Since diclofenac itself is not optically active, and albumin does not give rise to any Cotton effects at the wavelengths used, there can be no doubt that the observed Cotton effects are extrinsic in origin[238]. The near UV CD spectra between 250- 350 nm has been done to study the tertiary structure of the albumin molecule.

The high-affinity binding site for diclofenac is present in site II domain of the albumin molecule. However site II to site I shift in the presence of other ligands is also observed [239]. The Figure-3.5.2.3.8.1 demonstrates that the bound albumin shows least conformational change. During covalent cross-linking of the drug to

the polymer, part of the pharmacodynamic phore, which is responsible for the NSAID activity, is engaged with the polymer.

The carboxylic acid group of the NSAIDs is essential for the pharmacological activity of these drugs. But the albumin binding ability of these drug, is more related to the hydrophobic groups and is reduced in the absence of the carboxylic acid group[239].

The carboxylic group along with the hydrophobic group is necessary for the firm binding of this drug to the albumin molecule[240]. The absence of the carboxylic group reduces the affinity of the ligand to the albumin molecule. However it has been earlier observed that the ligand binding found to improve the conformational stability (3.5.2.3.8.1) of the albumin molecule[241]. Further the spectral analysis of the CD spectrum (table-3.5.2.3.8.1) also suggested the same.

Table-3.5.2.3.8.1: Secondary structure of the albumin molecule (%)

Conformation	Native	Bound to PVADICb
α	31	30
β	10	12
Random	58	58
Square distance (Å)	289.43	274.34

The preferential adsorption of albumin in presence of other opsonins like γ globulin and fibrinogen are quintessential, to better understand the ligand specificity towards albumin. As the ratio of adsorption of albumin to globulin and fibrinogen (non-opsonin to opsonin) regulate the blood compatibility of the materials. In vitro studies using mixture of proteins in the same ratio as in the blood could be used to directly correlate the results from plasma[242]. Eberhart et. al. demonstrated that C18 alkylation significantly improves the resistance to coagulation of polyurethane small diameter vascular grafts up to 20hrs [243]. There

fore this pendant polymer alone could be explored for the surface modification of blood contacting devices.

Further we have done stability studies under static conditions to explore these monolayers for the surface modification of tissue culture plates. Development of systems which can be easily explored for the surface modification of tissue culture plates has potential application in the cell layer tissue engineering.

3.5.2.3.9 Stability of lipid monolayers over the nanoparticles

Further the stability of the laterally stabilized thin lipid films using the nanoparticles of the pendant polymer have been studied under static conditions(studied as per the procedure 2.2.5.1).

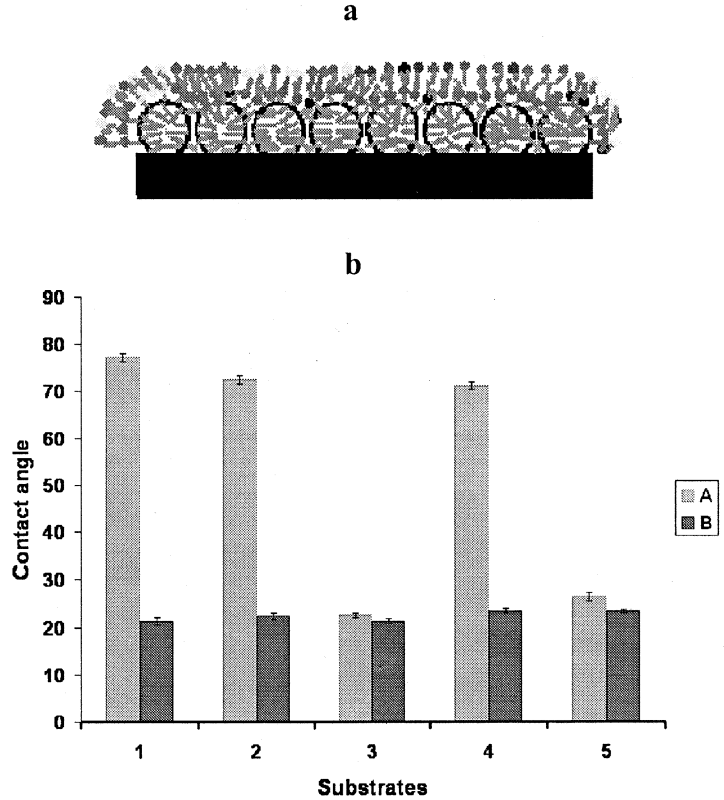


Figure-3.5.2.3.9.1: Schematic representation of laterally stabilized monolayer with the pendant polymer(a) Stability of the monolayers on substrates under static conditions (b), Lipid uncoated (A), Lipid (OCMC) coated (B), Poly C(1), PVA-DICc coated PolyC, 25°C(2), PVA-DICc coated PolyC 10°C(3), (2) after 24hrs incubation (4), (3) after 24hrs incubation (5)

Our studies demonstrate that lateral stabilization of the monolayers with thermoresponsive polymer stabilizes (figure-3.5.2.3.9.1b) the lipid layer even under different biologically relevant thermal conditions.

Biomimicry of ECM proteins have been considered for the development of these thermoresponsive thin films of nanoparticles made of pendant polymers. ECM proteins are secreted in soluble globular form and the post translational modification change the conformation for its preferential alignment to the structural components of the basement membrane. Attempts to synthesize such protein like polymers have rarely been successful, because of the stearic hindrance in the architectural formation. In our studies the formation of nanoparticles out of the polymers shows many physical properties of proteins. Further it intercalates strongly with the lipid layers and albumin preferentially. The slightly increased size of these particles to nanometer level gives tremendous opportunity to improve its functional aspect ratio, while maintaining the properties of the proteins. Its strong interaction gives a lot of opportunity to modulate the material biology interface. Further reversible, preferential adsorption of albumin significantly minimizes antifouling by other agents to these surfaces. Such systems have tremendous opportunity in multiple drug targeting. For example through intestine, or lung alveoli to blood and to the remote site. Apart from that 2D film of these nanoparticles further maintaining the properties of the nanoparticles is very important in the bottom up synthesis of highly functional surfaces.

3.6 Surface Profilometry

Surface profilometry of the lipid modified surfaces have been done with atomic force microscope (AFM)[244].

3.6.1 Supported OCMC monolayer

The surface morphology of the lipid monolayer is being correlated with their biological performance[245]. The contact mode AFM studies (as per the procedure 2.2.16) of the dried PolyC surface modified with OCMC monolayer

have been done after transferring the monolayer from the air/water interface to the solid support (transfer ratio = 1)with the help of LB trough at 30 mN/m(as per the procedure 2.2.2.2.1).

The figure-3.6.1.1 represents the surface morphology of the OCMC monolayer (The figures are representative figures of five different areas of three different samples). The z- axis provides the surface roughness of the supported lipid monolayer.

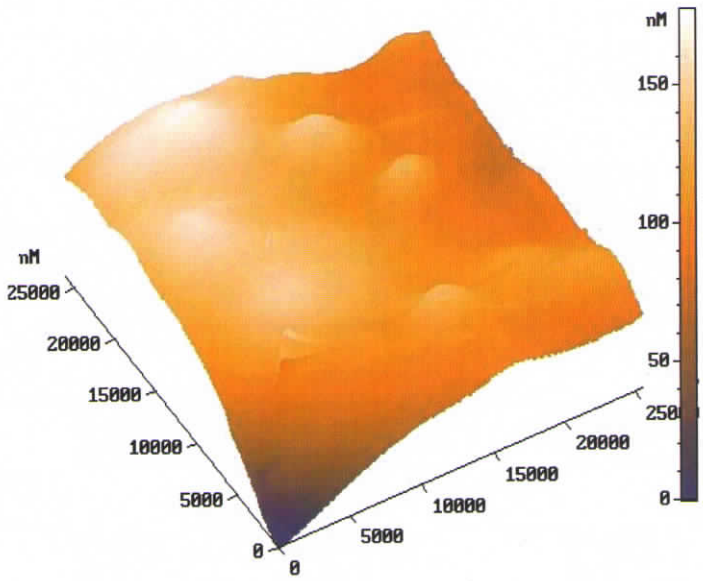


Figure-3.6.1.1: AFM image of the dried supported OCMC monolayer

The surface shows a smooth texture with domains of the size range of 50-100nm. The surface has been appeared without holes or uneven regions. Further the effect of head group structure on the surface morphology of supported monolayers have been done with the incorporation of PE.

3.6.2 Surface Profilometry with the incorporation of PE: Effect of head group structure

The AFM studies have been done as per the procedure 2.2.16. For that OCMC and PCM Ca,b,c monolayers have been deposited (transfer ratio= 1) onto PolyC films as per the procedure 2.2.2.2.1.

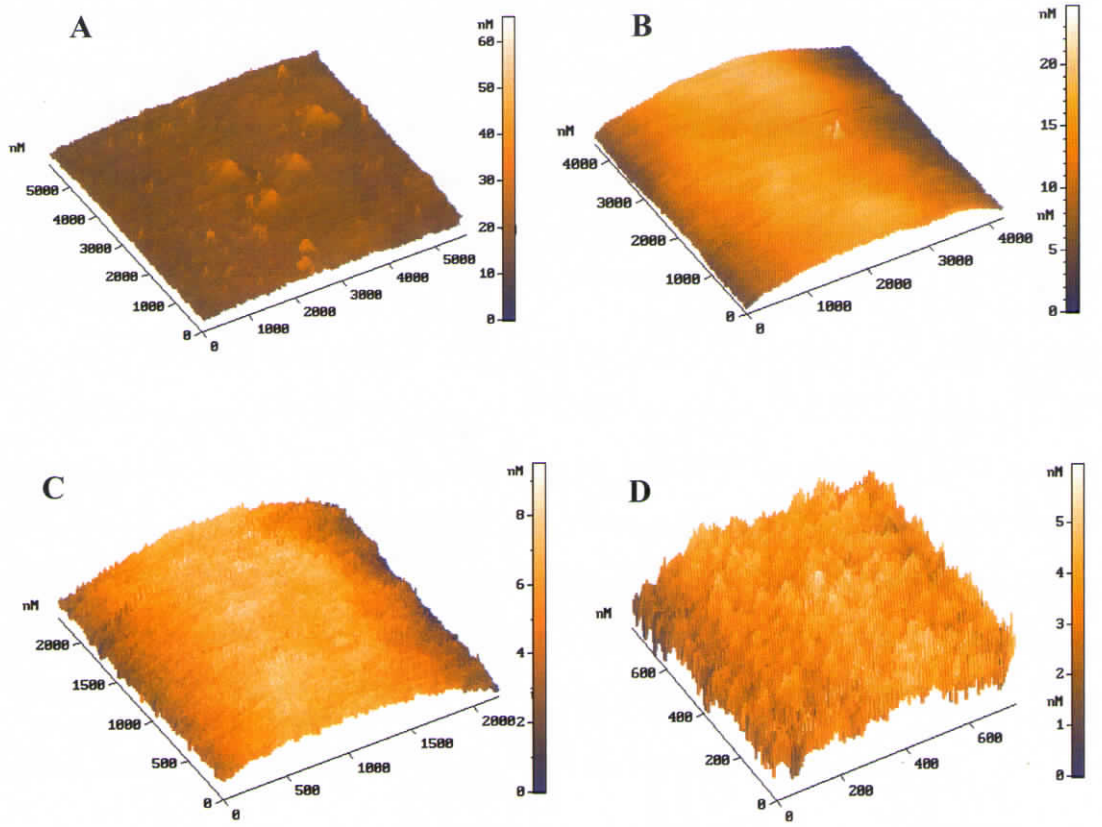


Figure-3.6.2.1: Surface profilometry of lipid films (PC:Chol:GalC:PE)(1:0.35:0.122: 0 (A), 0.0725(B), 0.145(C), 0.29(D) supported using PolyC substrate

The OCMC lipid surface have (Figure-3.6.2.1a) appeared uniform, with randomly distributed domains. The domains is of the size range of 30-50nm and shows a heterogeneous size distribution over the entire membrane surface. Nanoscopic domains of ~30 nm sizes have been randomly distributed than the larger domains. Similar domain border structures had been reported earlier[246, 247]. The size of the domains have decreased with increasing concentration of PE (Figure-3.6.2.1b,c &d). The surface of the PCMC(c) lipid supported monolayer the domain size has reduced to the range of, less than 5nm. This also reinforces that PE reduces the condensing effect of cholesterol.

Usually the lipid domains have been observed to be dynamic in nature in presence of water. They also appeared to change their size and shape frequently,

when studied under water, as observed by others[248]. When studied under dry conditions the mobility of membrane domains appeared to be arrested. The shape and size of these domains have also retained under dry condition due to the hydrophobic interactions of the cholesterol molecule[249]. Therefore we have investigated the surface morphology of the dried supported lipid surfaces with the help of AFM.

From the air/ water interfacial studies (Figure-3.2.3.1) and surface morphology of supported monolayer (Figure-3.6.2.1) systems it is evident that the membrane integrity have been reduced with the increase in concentration of the PE, and it appears maximum with PCMCc composition, Therefore, for further preliminary in vitro biorecognition studies the OCMC and PCMCc supported monolayer surface have been chosen. Further we have studied the surface profilometry of the macromolecule incorporated supported monolayers.

3.6.3 Surface Profilometry with the incorporation of macromolecules

Two layers of anchor immobilized monolayers of respective compositions have been transferred to the functionalized PMMA surface in the upstroke mode with the help of LB trough. The anchor network formed on this supported bilayer have been stabilized by carbodiimide chemistry, where the albumin molecule have perfectly incorporated into the lipid layer (as per the procedure 2.2.2.2.2). These three anchor molecules cross-link to each other to form a 3D network and stabilize the bilayer (Scheme- 3.5.1.1& 2).

The surface morphology of the modified PMMA surfaces are stabilized by anchors has been studied using AFM in contact mode in air (as per the procedure 2.2.16). The Figure-3.6.3.1 shows the contact mode AFM image of the modified PMMA surfaces in air. The surface of the monolayer containing albumin and heparin has (Figure-3.6.3.1A) shown less number of domains and holes. The depth of the holes is found to be 36- 82Å. The size of the smaller holes is found to be nearly half to that of the larger holes. This shows that some of the holes have

continuity through the bilayer to the polymer surface, while the other holes have not. The smaller holes with depth of 36 Å show the thickness of the upper monolayer and the depth of the bigger holes reveal the total thickness of the bilayer (The fully extended state of the PC has been nearly 34Å). The deviation from the standard bilayer thickness may be due to the inclusion of the polymeric chain of the anchors. Standard deduction of the size of the domains contributed by the polymer chains from the depth of the smaller holes suggests that the monolayers are in less condensed state, (26-30 Å). The surface modified with albumin, heparin and PEG (Figure-3.6.3.1B) has also shown least number of holes, Many domains with 5Å have observed on the PEG incorporated lipid surface, while not in the other surfaces. This confirmed that the smaller domains observed here are due to the polymeric chains. The reduction in holes and smaller domains in the PEG incorporated lipid layers may be due to perfect packing of the monolayer inside the anchor network.

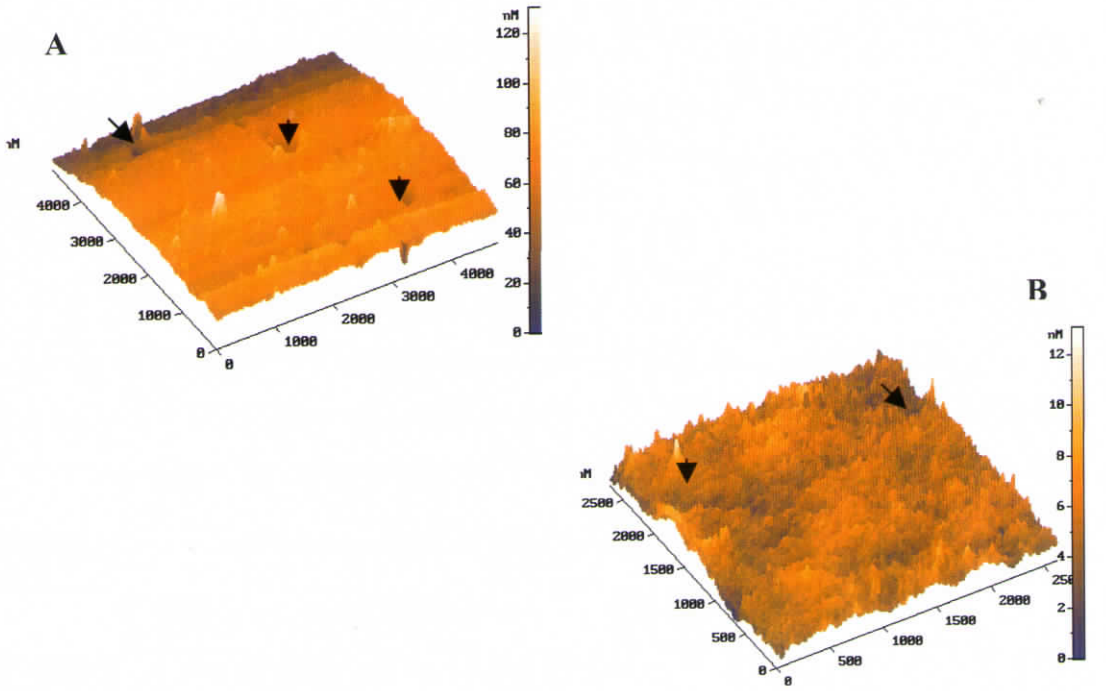


Figure-3.6.3.1: AFM image of supported laterally stabilized bilayers. PMMA modified with, OCMC- AH (A), OCMC- AHP (B). The depth of the bilayers are calculated from the holes (indicated by arrow), visible from the monolayer surface

The lipid-lipid immiscibility between the cholesterol and the phospholipids form cholesterol rich domains in model membranes, and calveolae for the transmembrane proteins in the plasma membrane[250]. Earlier such domains of larger dimension have been observed in binary phospholipids/ cholesterol systems at the air/ water interface, and in unilamellar vesicles, which is correlated with that of the domains or rafts present in biological membranes[251]. Here in the case of model lipid modified surfaces also we have observed similar effects. This phenomenon is related with the condensing effect of cholesterol[252]. Here with the incorporation of PE (Figure-3.6.2.1) and anchors (Figure-3.6.3.1) the condensing effect of Chol is reduced lead to the reduction in number of domains. The depth analysis using AFM in the limited holes available (Figure-3.6.3.1) in the surface revealed the bilayer structure of the deposited lipid layer. It shows slightly higher thickness than the standard bilayer thickness might be due to the entangled hydrophilic chains between the substrate and the bilayer, as well as on the lipid surface.

BIORECOGNITION STUDIES

Further we have attempted to study the adsorption of ions, proteins and adhesion of cells onto these modified surfaces as these initial events strongly regulate the blood and tissue compatibility of the lipid modified surfaces.

3.7 Calcification studies

The calcification at material surface has been observed at organized organic – inorganic interface[253]. This is also an important long-term problem in hydrophobic biomaterials used for cardio vascular devices[254]. However the natural membranes have appeared to be effectively circumventing this adsorption related phenomena.

3.7.1 Effect of orientation and packing density

To check the susceptibility of these cell mimetic supported lipid surfaces towards calcification, studies have been done from metastable salt solutions (as per the procedure 2.3.1). The figure-3.7.1.1 shows the calcification onto the material

surface. (The figures are representative pictures of 5 different fields under same magnification (X100) using a Labophot-2 inverted phase microscope with an attached Nikon FX- 35 DX camera).

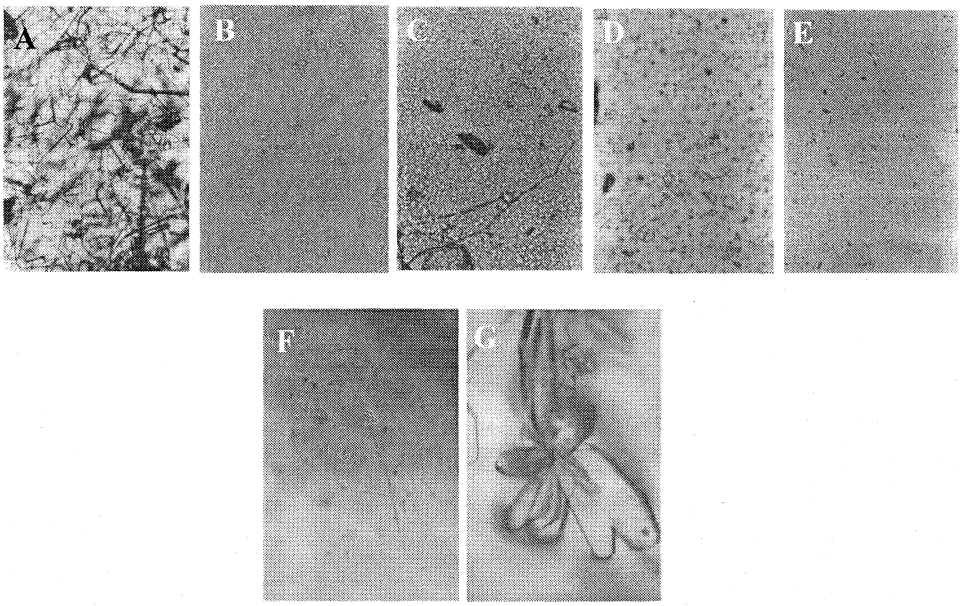


Figure-3.7.1.1: Calcification studies, PolyC bare (A), PolyC modified with PC (B), (PC:Chol) (1:0.7) (C), (PC:Chol) (1:0.35)(D), (PC:Chol:GalC) (1:0.35:0.125) (E), Chitosan bare(F), Chitosan modified with PC (G)

We have found that the overall calcification to the lipid modified surfaces has reduced significantly with respect to the PolyC bare (Figure-3.7.1.1A). But the monolayer of the (PC: Chol) (1: 0.7) have shown certain degree of calcification (Figure-3.7.1.1C). This is due to the nucleation at the phosphate moieties of the monolayer. The phosphate moieties of the cell membrane are found to be acting as the nucleation site for calcification in the natural environment [255]. To compare the influence of packing of the polar head group, we have studied the calcification on chitosan (Figure-3.7.1.1F) and chitosan modified with PC monolayer (Figure-3.7.1.1G) (This is an example for loosely packed polar head group exposed monolayer, due to the swelling of chitosan). The result shows that the calcification to the PC modified chitosan surface is increased significantly as compared to the bare chitosan. The calcification to the hydrophobic phase exposed ordered

monolayer surface (Figure-3.7.1.1 (B, D and E)), has been less than the hydrophilic phase exposed ordered monolayer surface (Figure-3.7.1.1C). While the calcification studies suggest that in hydrophilic phase exposed ordered monolayer surfaces, the packing of the monolayer further influenced the degree of calcification.

3.7.2 Effect of incorporation of PE

Further we have checked the effect of head group structure by incorporating PE into the OCMC monolayer on calcification. For that OCMC and PCMCc monolayer has been deposited onto PolyC films as per the procedure 2.2.2.2.1

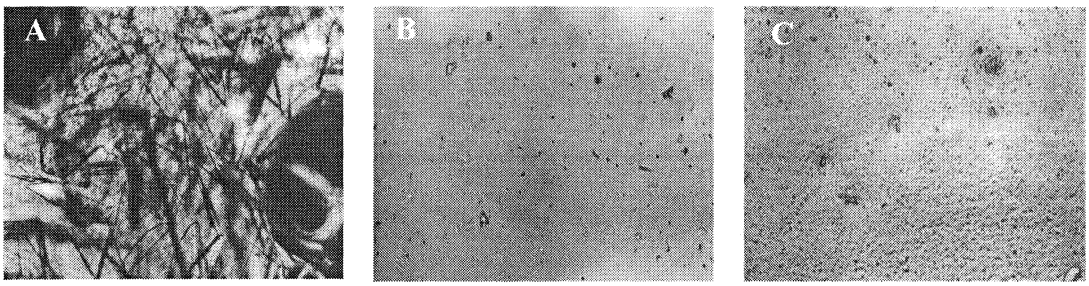


Figure-3.7.2.1: Calcification studies from metastable solutions [PolyC bare (A), modified with OCMC(B), PCMCc (C)

The overall calcification onto the supported lipid surfaces is less (Figure 3.7.2.1A-C) as compared to the bare hydrophobic polymer surface and there is no significant difference in calcification between the lipid surfaces. From our earlier studies it is evident that, when these monolayers have been supported onto hydrophilic expanding surface (like chitosan) figure-3.7.1.1G the surface gets calcified, which is a reverse phenomena of what we have observed on non-expanding hydrophobic surface (like polycarbonate) Figure-3.7.1.1 (B, D and E). This may be related to the movement of the head group of the phospholipids when they are in the self-assembled form, than in the free form. Therefore packing of the monolayer influences the fluidity of the microenvironment, which governs nucleation at the surface.

3.7.3 Effect of lateral stabilization

The laterally stabilized lipid monolayers by peptide bonding have been further subjected to calcification studies.

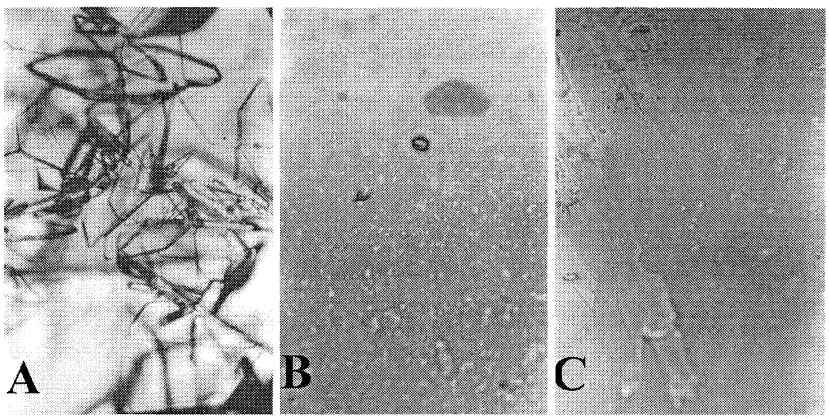


Figure-3.7.3.1: Calcification studies.PMMA Bare (A). PMMA modified with, OCMC- AH (B), OCMC- AHP (C)

Calcification data from metastable salt solutions (as per the procedure 2.3.1) are shown in figure-3.7.3.1. The overall calcification to the modified surfaces, have significantly reduced as compared to the bare PMMA surface (Figure-3.7.3.1A-C). The size and the density of the crystals formed on the modified surface are reduced as compared to the bare polymer. On bare polymer, flower type crystals are formed with its nuclei originating from the material surface. The crystals are uniformly formed throughout the surface, with long petals at the cut edge of the surface. This is totally absent in the case of modified polymers.

The calcification to the organized organic– inorganic interface in biological systems has been reported[256]. The surface induced nucleation is proposed to be the reason for the calcification, on the surfaces. Therefore we have studied the propensity of the surface towards calcification from metastable salt solutions. The pH of the solution is reduced to pH 5.5 to reduce the induction of nucleation[257,258]. The calcification data shows that, the overall calcification to the modified surfaces is significantly reduced as compared to the bare polymer substrate. This is due to the fluidity of the microenvironment. The loose packing of this stabilized

supported bilayer increases the osmotic pressure of the microenvironment and this intern improve the fluid flow in the microenvironment, which leads to the osmotic exclusion of the ions. This has been earlier observed in the case of exclusion of proteins by polyethylene glycol immobilized surface [259]. There also the packing of the polyethylene glycol influences the effective osmotic exclusion of the proteins from the surface. On the contrary, in natural environment we can find that the phosphate moieties of the cell membrane are found to be acting as the nucleation site for calcification[260]. There are evidences of calcification on phosphorylcholine-immobilized surfaces also[261]. However, here we find that the calcification is significantly reduced in these laterally stabilized surfaces. This clearly indicates that the fluidity of the microenvironment plays an important role in the adsorption related phenomena.

3.8 Protein adsorption studies

Protein adsorption onto the material surface greatly influences the cellular interaction to the material surface (1.1.5.3.1). The studies have been done from a mixture of albumin, γ- globulin and fibrinogen under static conditions at ambient temperature. It has been reported that the protein adsorption to any substrates from a mixture of proteins, could be directly correlated to that from plasma[262].

3.8.1 Effect of orientation and packing density

The protein adsorption studies have been done from mixture of proteins for three hrs. The desorbed proteins have been studied by SDS-PAGE. (as per the procedure 2.3.2.1). The figure-3.8.1.1 represents the protein adsorption onto the lipid surfaces of different orientations and packing density.

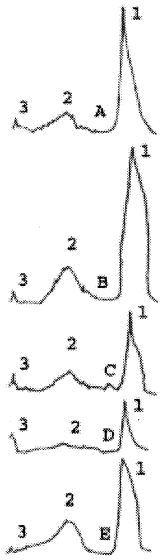


Figure-3.8.1.1: Densitometric diagram of the protein adsorption- desorption studies on lipid modified polymer surfaces, using mixture of proteins. X axis length in cm (maximum 6cm), and Y axis absorbance (maximum value = 0.5). [PolyC bare (A), PolyC modified with PC (B), (PC:Chol) (1:0.7) (C), (PC:Chol) (1:0.35) (D), (PC:Chol:GalC) (1:0.35:0.125) (E). The peaks in protein adhesion studies are (1) albumin, (2) γ-globulin and (3) fibrinogen]

Studies indicates an increased protein adsorption to the hydrophobic PolyC surface (Figure 3.8.1.1A). The increased albumin to fibrinogen ratio of the adsorbed proteins enhances the blood compatibility and reduces the cell adhesion. The modified surfaces with the tightly packed hydrophobic phase exposed ordered monolayer (Figure 3.8.1.1 (B and E)) is also adsorbed more amount of albumin. Earlier it has been observed that the albumin adsorption to phosphatidylcholine immobilized hydrophobic surface is more[263]. Our studies on PC modified PolyC surface also suggested the same. On the contrary, the phosphorylcholine (the head group of the PC) immobilized surfaces has been found to be less susceptible for albumin adsorption[264]. The reason for the contradiction has not been so clear. Our studies revealed that the exposed hydrophobic tail group and their packing strongly influence the albumin adsorption. The albumin molecules have specific receptors for binding to the nonpolar tail group of the fatty acids[265]. The receptor-ligand

interaction is reversible and therefore the conformational change due to the adsorption is negligible[266]. Here we hypothesize that the preferential adsorption of albumin onto the tightly packed hydrophobic phase exposed ordered monolayer surfaces is due to the specific interaction of the exposed nonpolar tail group structure with the albumin molecule. When the packing of these monolayers has reduced, the overall protein adsorption has also reduced (Figure-3.8.1.1D). On the contrary, the protein adsorption to the hydrophilic head group exposed surface has reduced significantly with respect to the unmodified surface (Figure-3.8.1.1C). It clearly indicates that the membrane orientation and fluidity due to the variation in packing observed with the transfer ratio is an important parameter, which influences the adsorption of the proteins to these surfaces. Apart from that, the adsorbed proteins on to these ordered monolayers form a gradient at the surface by retaining the natural conformation and hinder further interaction with the cells. Sackmann et. al. proposed that the protein-functionalized monolayers with the native conformation could be effectively utilized for the specific ligand receptor interaction[267]. Whereas the adsorbed protein molecules on bare hydrophobic polymer, change their conformation based on the interfacial energy parameters. The exposed Arginine-Glycine-Aspartic acid (RGD) functional sequences due to the conformational change of the adsorbed fibrinogen and other opsonins are the first step to wards the thrombosis or events regulating cellular activation. These functional sequences like (RGD sequences interact with the GPIIb/IIIa receptor of the platelets and lead to platelet adhesion, activation and thrombosis[268]. This effect is similar in the case of cell adhesion under cultured in vitro conditions. Here from the protein adsorption studies it has been established that either the lipid-modified surfaces adsorb more amount of albumin, or the overall protein adsorption has reduced, based upon their orientation. Both the situation enhances the bio-compatibility of these surfaces. But on surfaces where the protein interaction to the surface is minimal the cellular activation processes may get mediated through the direct interaction between the exposed surface groups and the receptors of the

cells. Further we have evaluated the effect of head group structure in the protein adsorption

3.8.2 Effect of phosphatidylethanolamine

For that OCMC and PCMCc monolayers have been deposited onto PolyC films as per the procedure 2.2.2.2.1. The protein adsorption studies have been done from mixture of proteins for three hrs (as per the procedure 2.3.2.1).

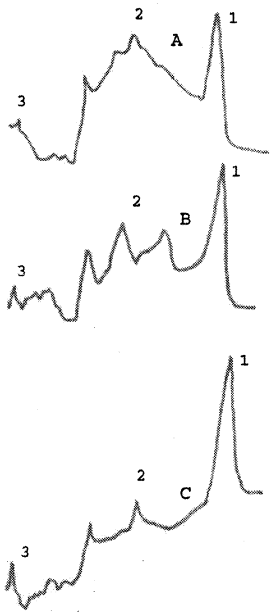


Figure-3.8.2.1: Densitometric diagram of the protein adsorption- desorption studies on lipid modified polymer surfaces, using mixture of proteins. X axis length in cm (maximum 6cm), and Y axis absorbance (maximum value = 0.5). [PC bare (A), OCMC(B), PCMCc (C). The peaks in protein adhesion studies are (1) albumin, (2) γ globulin and (3) fibrinogen]

The figure-3.8.2.1 is a representation of the densitometric data of the polyacrylamide gel. The area of the specific peaks indicated that the overall adhesion of the proteins to the lipid modified surfaces has reduced (Figure-3.8.2.1 B& C). Incorporation of PE has further reduced (Figure-3.8.2.1 C) the protein adsorption to the lipid surface. This may be due to the higher fluidity of the

microenvironment of the lipid surface, imparted by the reduced packing of the membrane as already explained.

3.8.3 Effect of lateral stabilization

For that anchor incorporated OCMC monolayers have been deposited onto PMMA films as per the procedure 2.2.2.2.2. The protein adsorption studies have been done from mixture of proteins for 3 hrs (as per the procedure 2.3.2.1).

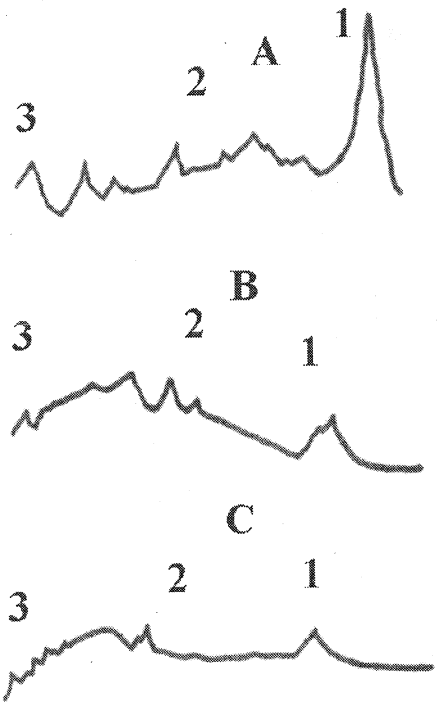


Figure-3.8.3.1: Densitometric diagram of the protein adsorption- desorption studies on lipid modified polymer surfaces, using mixture of proteins. X axis length in cm (maximum 6cm), and Y axis absorbance (maximum value = 0.5). PMMA bare (A). PMMA modified with, OCMC- AH (B), OCMC-AHP (C). The peaks in figure are (1) albumin, (2) γ globulin and (3) fibrinogen

The protein adsorption data using mixture of albumin, γ globulin, and fibrinogen is shown in Figure-3.8.3.1. It shows reduced protein adsorption to all the lipid modified surfaces (Figure-3.8.3.1B and C) as compared to the bare PMMA (Figure 3.8.3.1A). However the ratio of albumin to fibrinogen and γ globulin is

higher in the case of bare PMMA surface. This ratio is not maintained in the case of modified polymer surface.

Protein adsorption is an important phenomenon, which influences the biological events. It has been reported that the protein adsorption to any substrates from a mixture of proteins present in the blood, could be directly correlated to that from plasma[269]. The protein adsorption to all the modified surfaces (Figure-3.8.3.1B and C) is lower than the bare PMMA surface (Figure 3.8.3.1A). However the ratio of albumin to fibrinogen and γ globulin is higher in the case of bare PMMA surface. This ratio is not maintained in the case of modified polymer surface. However different from our earlier studies (Figure- 3.8.1.1 & 2.1), the protein adsorption to the anchor incorporated lipid surface is further (Figure-3.8.3.1) reduced may be due to the surface decorated PEG molecules. The adsorbed protein molecules on bare PMMA change their conformation since it is highly hydrophobic. The increased albumin to fibrinogen ratio of the adsorbed proteins enhances the blood compatibility in other terms reduces cell adhesion, and the surfaces preferentially adsorb fibrinogen are found to be thrombogenic or enhances cell adhesion and activation. The adsorbed fibrinogen changes its conformation to expose the functional RGD sequences, and interact with the receptors in the platelet surface[270], which lead to platelet adhesion, activation, and thrombosis[271, 272]. The phosphorylcholine (the head group of the PC) immobilized surfaces has been found to be less susceptible for protein adsorption[273]. This is due to the inertness of the phosphorylcholine groups in the biological environment. Here we can find that the overall protein adhesion has significantly reduced in the case of modified surfaces as compared to the bare polymer. In addition to the effect of the phosphorylcholine moieties of the bilayer the flexibility of the brushes of polyethylene glycol and heparin also helps to reduce the adsorption phenomena. The resistance to protein adsorption of PEG has been attributed to the stearic stabilization effect, solution properties, and its molecular conformation in aqueous solution. It has been proposed that competition between stearic expulsion forces

and Van der Waals attraction between the protein and PEG control the protein adsorption process.[274] Here in addition to the effect of PEG the fluidity of the microenvironment due to the lipid bilayer also might be enhancing the anti-fouling property of the modified surface.

3.9 Blood Cell Adhesion Studies

From the protein adsorption studies it is evident that on PE incorporated lipid modified surfaces the protein interaction is minimal. When protein interaction to the surface is minimal the cellular activation may get mediated through the direct interaction between the exposed surface groups and the receptors of the cells (1.1.5.3.1 &2) Therefore we have studied the blood cell interaction to these surfaces from washed blood cells.

3.9.1 Effect of orientation of the supported lipid layer

For that lipid deposition onto PolyC substrates have been done according to the procedure 2.2.2.2.1. The blood cell adhesion studies have been done as per the procedure 2.3.3.1,2 &3). The Figure-3.9.1.1 shows the data of blood cell adhesion using washed platelets, erythrocytes and the leukocytes to the bare PolyC and the modified surfaces.

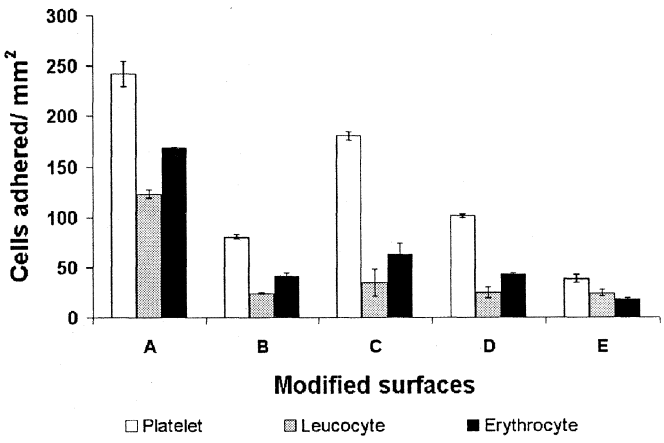


Figure-3.9.1.1: In vitro blood cell adhesion studies. PolyC bare (A), PolyC modified with PC (B), PC:Chol (1:0.7)(C), PC:Chol (1:0.35)(D), PC:Chol:GalC (1:0.35:0.125) (E)

The results show an overall decrease in cell adhesion (Figure-3.9.1.1) on to modified surfaces as compared to the bare PolyC surface. The orientation as well as packing of the exposed monolayer influences the observed difference in cell adhesion. On ordered hydrophobic phase exposed surfaces the reduction in cell adhesion have been more predominant (Figure-3.9.1.1 (B, D and E)), than the surface where the hydrophilic ordered phase is exposed (Figure-3.9.1.1). The PC surface modified by OCMC shows least blood cell adhesion (Figure-3.9.1.1E). This is associated with the packing of the ordered monolayer.

3.9.2 Effect of phosphatidylethanolamine

For that optimized OCMC and PCMC a,b &c lipid compositions have been deposited onto PolyC substrates according to the procedure 2.2.2.2.1. The blood cell adhesion studies have been done as per the procedure 2.3.3.1,2&3).

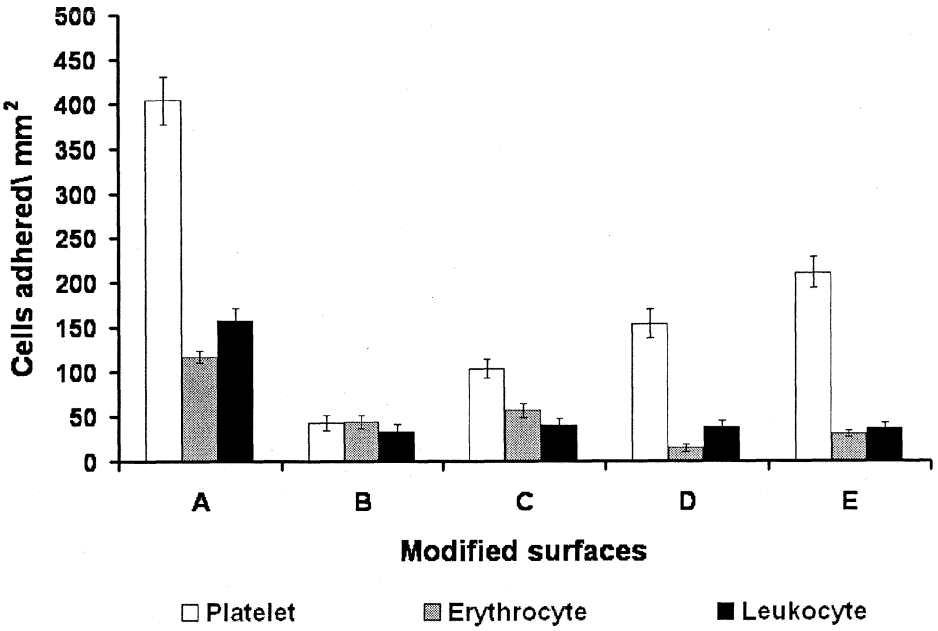


Figure-3.9.2.1: Blood cell adhesion studies from washed cells [PolyC bare (A), OCMC(B), PCMCa (C), b(D), c(E)

The study from washed cells indicates the direct interaction between the surface and the blood cells. The overall blood cell adhesion is less on supported lipid surfaces (Figure-3.9.2.1B-E). However the platelet adhesion have increased

The reduction in protein adsorption and the blood cell adhesion reveal that these modified surfaces could considerably reduce the cellular activation processes. This could be explored for the surface modification of blood contacting materials. However further blood compatibility studies on this surfaces are required for complete understanding of this surfaces. Such modified blood contacting materials could be used for low shear stress applications like modification of surface properties of biosensors or small diameter vascular grafts.

3.10 Platelet activation studies from PRP

The platelet adhesion and activation are the two important steps, which regulate the formation of the thrombus and medical device rejection. In vitro studies using platelet rich plasma gives the information about the propensity of the surface to initiate platelet activation and thereby cellular activation processes. The platelet activation studies have been done as per the procedure 2.3.3.4. For that PolyC substrates have been modified with the OCMC and PCMCc monolayer as per the procedure 2.2.2.2.2

The Figure-3.10.1 represents the platelet adhesion to the lipid surface in presence of plasma proteins using PRP. The samples have been evaluated by SEM. The trend in platelet adhesion from PRP is similar to that from washed platelets (Figure-3.9.2.1). Overall platelet adhesion to the lipid modified surfaces is reduced. However incorporation of PE enhanced the platelet adhesion.

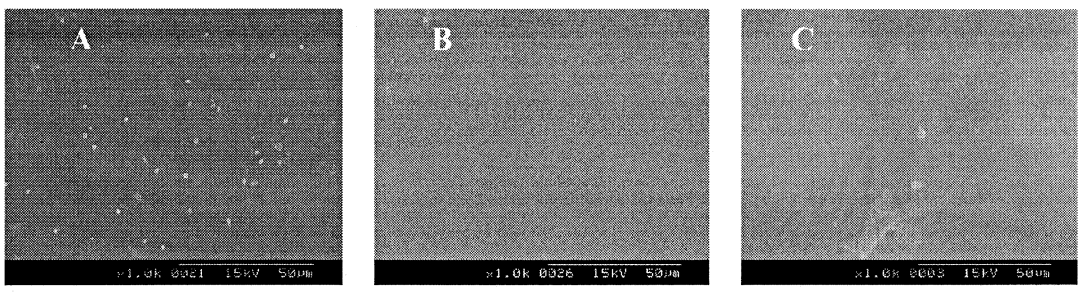


Figure-3.10.1 Platelet adhesion studies on lipid modified polymer surfaces, using Platelet Rich Plasma. Bare polymer (A). OCMC (B), PCMCc (C)

Morphological analysis by SEM shows that, more than half of the platelets adsorbed to the bare PolyC surface (Figure-3.10.2A) are fully spread and the other platelets are on the verge of spreading. Most interestingly the OCMC modified surfaces (Figure-3.10.2B) are devoid of platelet activation even after 2hrs under static conditions. However, incorporation of PE (Figure-3.10.2C) enhances the platelet activation.

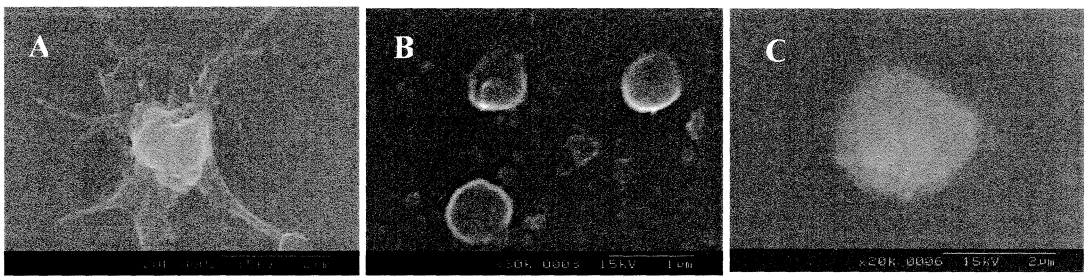


Figure-3.10.2 Platelet activation studies on lipid modified polymer surfaces, using Platelet Rich Plasma. Bare polymer (A). OCMC (B), PCMCc (C)

The extended pseudopods of the activated platelets onto bare polymer surface indicates that these platelets could recruit the other platelets still in suspension and form surface bound aggregates with time. On OCMC surface the platelets are least adhered and activated. But with the incorporation of PE the platelets are slightly activated. This approach can effectively used for the controlled ligand supplementation from material surface.

During the initial stage of surface activation the change in conformation of the adsorbed proteins, exposes various functional sequences like RGD, In the case of platelets the ligand is sensitive to the platelet GPIIb/IIIa receptor. When platelets are surface activated, they progress through a sequence of morphological changes. This can be evaluated by using SEM. The surface activation contributes to the change in the organization of the cytoskeleton, in turn increases the surface area of the platelets by the formation of pseudopods. The platelets thus adhered and activated goes through a sequence of cytoskeletal events and rise in endoplasmic Ca++ concentration, polymerization of actin filaments, thrombin activation, release of the cytoskeletal granule contents, as well as platelet

aggregation. The extent of shape change and the spread area has been related to the surface energetics of the polymer materials [275]. Therefore the platelet activation studies are essential to prove the blood compatibility of the surface. The shape change could be correlated with the activation. The studies have been done for 2 hrs with PRP under static conditions. Usually 1 hr is sufficient to establish the activation of the platelets. Our studies show that the platelet activation to the modified surface has been significantly reduced as compared to the bare polycarbonate surfaces (Figure-3.10.2). The surface mediated shape changes have been related to the physical (interfacial energy) and the chemical (due to specific groups) interactions. More than half of the platelets adsorbed to the bare polycarbonate surface are fully spread and the other platelets are on the verge of spreading. The extended pseudopods of the activated platelets on this surface indicate that these platelets can recruit the other platelets still in suspension, and form surface bound aggregates with time.

Earlier it has been observed that the platelet adhesion and activation is poor on surfaces under high shear conditions[276]. Therefore this bare polymer surface may qualify for applications under high blood flow extracorporeal conditions like blood pump. Since the albumin to fibrinogen ratio of the adsorbed proteins is more here, the platelet adhesion and activation to the bare polycarbonate surfaces get further hindered under flow conditions. But for the low shear stress conditions like small diameter vascular graft applications, this material is not sufficient enough. The occlusive thrombosis under low shear stress conditions is one of the major issues in small diameter vascular grafts. So the platelet activation studies under static conditions can better represent the low shear stress blood compatible applications. The platelet activation is very low in the OCMC monolayer deposited surface. Since the experiment has been conducted from PRP the normal sequence of surface induced activation of the coagulation pathway (protein mediated) is well simulated. Therefore the mechanism of improved blood compatibility of this modified surface can be well correlated to the natural hematological conditions. It

clearly indicates that the surface modification with the lipid composition discussed above is a good candidate for the purpose of low shear stress hematological applications like small diameter vascular grafts. The luminal surface of the vascular grafts can be modified by transferring the lipid monolayer by controlled rotation of the graft at the air/ water interface with the help of LB trough.

Further to evaluate the interaction of immune cells to these surfaces we have done the adhesion activation, secretory profile as well as the proliferation of macrophages and other poly morphonuclear cells. The mouse macrophage cell line(RAW 264.7) for macrophage adhesion studies and the polymorphonuclear cells collected from the mouse spleen have been used for the studies. In these studies we have attempted to understand mainly three parameters. (1) How antifouling properties of cell mimetic monolayers respond to immune cells (2) How it is different with albumin preferentially adsorbing surfaces (3) Effect of controlled ligand supplementation from the surface to initiate cellular activation processes. Apart from that we have also evaluated (1) Role of serum proteins (2) Activation of the cells with bacteria and other bacterial components (3) Effect of substrates of different sizes on the immune cell activation processes.

3.11 Macrophage behavior at the material surface

Macrophages adhering to the material surface play a central role in regulating the inflammatory cascades at an implantation site [277]. Adherent macrophages often goes through a sequence of complex cellular events, including spreading[278] proliferation and change in their normal secretory profile. Such changes in the normal phenotypic behavior lead to the secretion of pro-inflammatory cytokines like TNFa[279]. These pro inflammatory cytokines can invite other inflammatory cells like neutrophils[280] to the implantation site and thus further aggravate the scenario.

3.11.1 Macrophage adhesion studies

The adsorbed opsonins such as fibrinogen has been investigated in monocyte/ macrophage adhesion studies[281] and correlated to the surface polarity. However information on dynamic surfaces like cell mimetic or albumin self assembling surfaces is scarce.

3.11.1.I Effect of serum concentration on early cell response

Macrophage adhesion studies have been done with RAW 264.7 macrophage cell line (as per the procedure 2.3.3.5). For that the 24 well PS tissue culture plates has been modified as per the procedure 2.2.4.1.2 with the lipid monolayers. The samples have been used without any sterilization except it has been kept under UV light in the hood for 30minutes before starting the experiment. The inverted microscope photographs of the cells grown on modified PS tissue culture plates is shown in figure-3.11.1.1.1, at 3hrs and different serum concentrations.

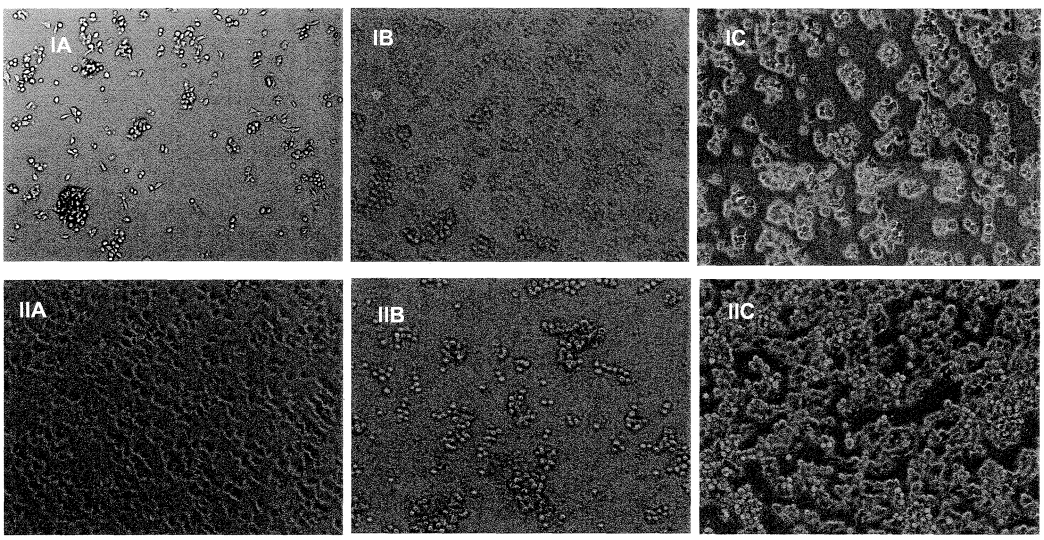


Figure-3.11.1.1.1: Macrophage (RAW 264.7) (1X10^5 cells /ml) adhesion studies on lipid modified surfaces, Phase contrast microscopy image after 3hrs (10X). 1% Serum (I), 10% Serum (II). Bare polymer (PS) (A). PS modified with OCMC (B), PCMCc (C)

The cell adhesion and patterning is different on lipid modified surfaces with respect to the bare PS. In the case of bare polymer cell adhesion and spreading is more Figure-3.11.1.1.1.IA. It is more pronounced with respect to serum concentration Figure-3.11.1.1.1.IIA. OCMC modified surfaces shows (Figure-3.11.1.1.1.IB) less cell adhesion and activation among the three surfaces. In PCMCc modified surface (Figure-3.11.1.1.1.IC) the adhered cells have been grouped together to form colonies. While on lipid modified surfaces under reduced serum concentration Figure-3.11.1.1.1.IIB&C. has less significance on actin mediated cellular activation processes.

Further number of round and spreaded cells have been counted to understand the surface induced change in cell morphology (Figure-3.11.1.1.2).

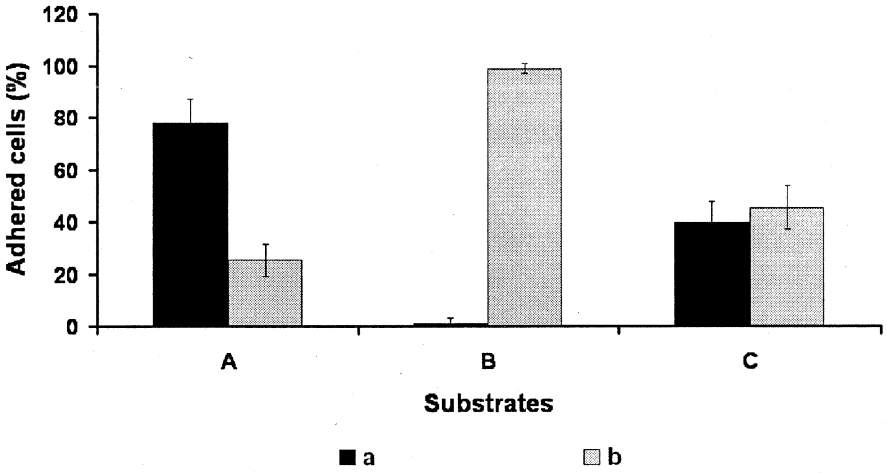


Figure-3.11.1.1.2 Cell spreading (RAW 264.7) (1X10^5 cells /ml)on the modified surfaces, Bare polymer (PS) (A), PS modified with OCMC (B), PCMCc(C), Spreaded cells (a), non-spreaded cells (b)

The majority of cells in the OCMC modified surface remain non-spreaded (Figure-3.11.1.1.2 B) inactive form. On PCMCc modified surface equal number of spreaded and non-spreaded cells (Figure-3.11.1.1.2 C) have been found. However differently in the case of bare surface the number of spreaded cells (Figure-3.11.1.1.2 A) is more. Earlier in the case of platelet adhesion studies we have observed similar trend (Figure-3.10.2). This clearly indicates that the OCMC

modified surface significantly reduces the cellular activation processes. While incorporation of PE into the monolayers do controlled ligand supplementation as compared to the bare surface.

Further we have counted the number of colonies of the cells present in the surfaces, because that was one of the unique activation processes that regulate organotypic multi cellular spheroid formation. This gives the information about early cell-cell interactions in presence of cell-matrix interactions.

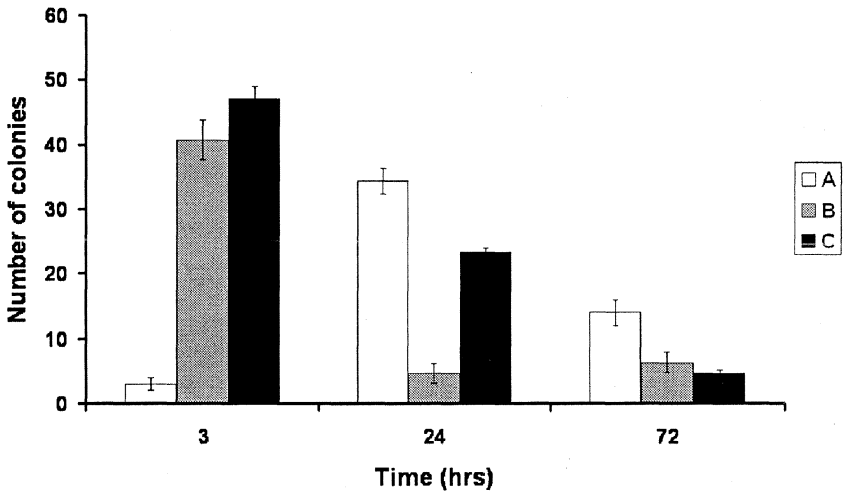


Figure-3.11.1.1.3: Macrophage (RAW 264.7) ($1X10^5$ cells /ml) cell colony formation at different time points: Bare polymer (PS) (A), PS modified with OCMC (B), PCMCc(C)

It has been uniquely observed that on lipid modified surfaces the cells remain in a colonized form at early hours with respect to the bare surface (Figure-3.11.1.1.3). On OCMC (Figure-3.11.1.1.3B). modified surface the size of the colonies are smaller than that on PCMCc surface(Figure-3.11.1.1.3C). At 24hrs on bare and PCMCc modified surface (Figure-3.11.1.1.3C) more colonies have been observed, while it is reduced considerably on OCMC surface (Figure-3.11.1.1.3B). At 72 hrs the number of colonies has been considerably reduced on lipid surfaces and they formed big sheet like structures. The colony or multi cellular spheroid formation is regulated by enhance cell-cell have interactions. Enhanced cell- cell

interactions are observed when cell-matrix interactions are minimal. Here on lipid modified surfaces the cell- matrix interactions are minimal. As the time progresses cells start secreting ECM proteins lead to enhanced cell-matrix interactions. On PS surfaces more colonies have observed. This may be due to increased ECM remodeling with time. Te increased number of cells or polarity of the surface enhances these phenomena with time. However on dynamic or less rigid dynamic lipid modified surfaces the secreted proteins are less adherent and provide enough flexibility for remodeling. This may be the reason for the formation of cell layer like structures. These effects are more prominent on serum depleted studies.

3.11.1.2 Effect of surface modification on adhesion strength

Further we have attempted to evaluate the strength of adhesion of the cells to these surfaces. For that macrophage adhesion studies have been done with RAW 264.7 macrophage cell line (as per the procedure 2.3.3.5) after gentle washing (as per the procedure 2.3.3.5.1,2 &3) . For that the PS tissue culture plates have been modified as per the procedure 2.2.4.1.2). The figure-3.11.1.2.1 shows comparatively, the number of cells retained on the surface after washing.

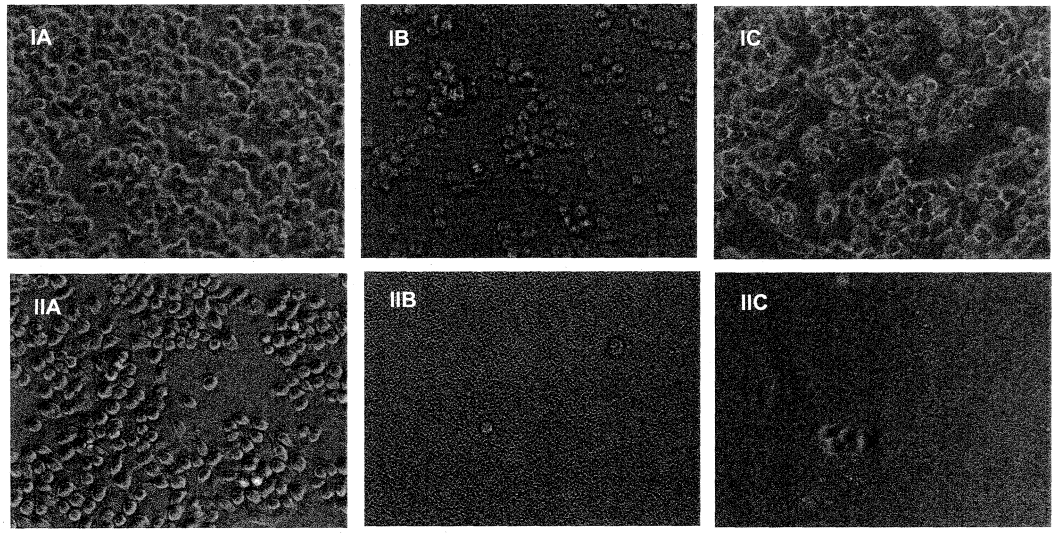


Figure-3.11.1.2.1**: Macrophage (RAW 264.7) (1X10^5 cells /ml) adhesion studies on lipid modified surfaces (1% serum), Phase inverted microscopy image after 3hrs (20X). Before washing (I), After washing (II). Bare polymer (PS) (A). PS modified with OCMC (B), PCMCc (C)**

Number of cells retained on the surface after gentle washing has been considerably reduced (Figure-3.11.1.2.1&2) on lipid modified surfaces. This may be due to less rigidity and renewability of the lipid monolayers during ECM remodeling and cell layer removal upon washing.

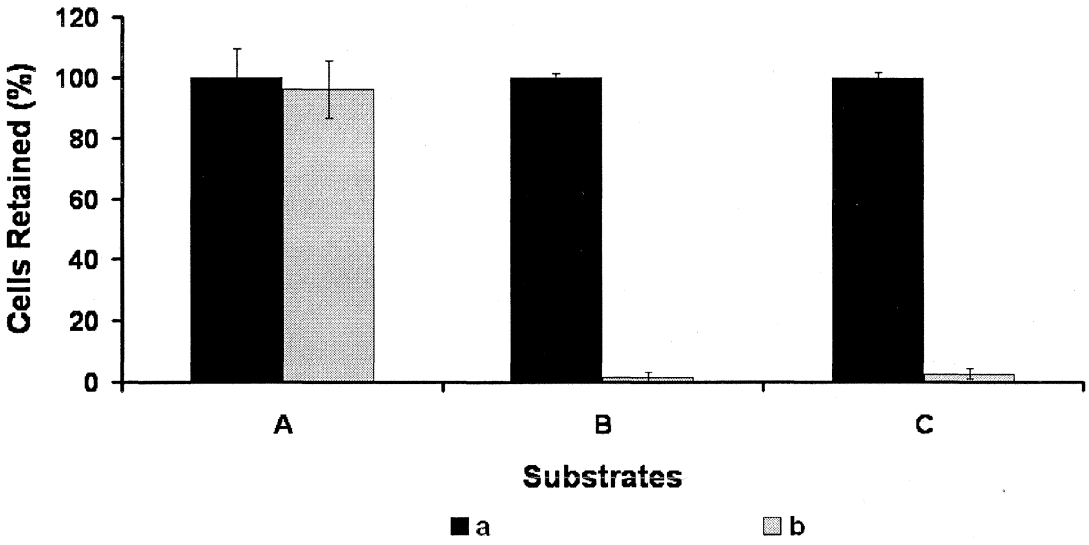


Figure-3.11.1.2.2: Number of macrophage (RAW 264.7) (1X10^5 cells /ml) retained after 24hrs: Bare polymer (PS) (A). PS modified with OCMC (B), PCMCc (C). Before washing (a), After washing (b)

This indicates that the cell adhesion strength is minimal on lipid modified surfaces. Earlier we have also observed that blood cell adhesion is minimal on these modified surfaces (Figure-3.9.2.1). In tissue culture conditions macrophage cells proliferate in 24hrs and secrete various protiens (growth factors, cytokines and ECM) and provide a more dynamic environment. Evaluation of cell adhesion strength is more important under these conditions, as it resembles material-biology interactions under In vivo conditions. Reduction in adhesion strength on lipid modified surface without affecting multi cellular spheroid formation is more important for tissue engineering applications. Enhanced cell-cell interaction under reduced cell-ECM interaction helps in organotypic tissue formation. Further we have attempted to evaluate the morphological changes of the adhered cells with respect to cell number and serum concentration.

3.11.1.3 Effect of cell number and serum concentration

For that macrophage adhesion studies has been done with RAW 264.7 macrophage cell line (as per the procedure 2.3.3.5). The PS tissue culture plates have been modified as per the procedure 2.2.4.1.2). The cell spreading, cell-cell association to form colonies has been subjected to study.

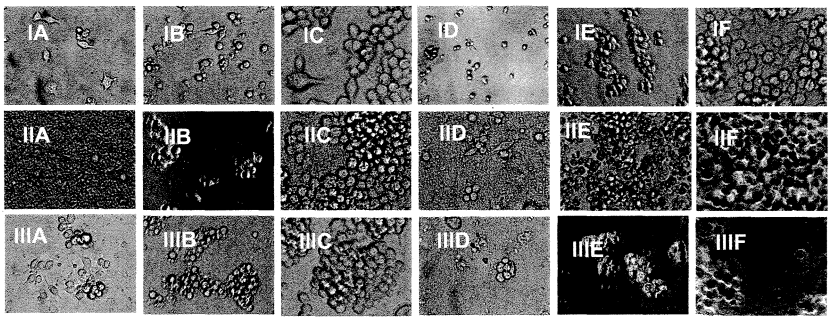


Figure-3.11.1.3.1: Macrophage (RAW 264.7) phenotype changes with respect to cell number (10^3(A &D), 10^4(B&E), 10^6(C&F) cells/ml) and serum concentration (1% serum (AB&C), 10% serum (D,E&F)), on lipid modified surfaces, Phase inverted microscopy image after 24hrs (40X). Bare polymer (PS)(I). PS modified with OCMC (II), PCMCc (III). on lipid modified surface

The effect is similar uniformly with respect to cell number and serum concentration on lipid modified surfaces (figure-3.11.1.3.1), as explained earlier. But at low cell numbers the cell spreading and colonization is more visible on the lipid modified surfaces (figure-3.11.1.3.1II&IIIA), at lower serum concentrations. However, at higher serum concentrations the effect is less significant (figure-3.11.1.3.1II&IIID). This clearly indicates that the serum concentration plays an important role in regulating the morphological and the phenotypic changes of the cells. This may be due to a competition between the soluble and insoluble stimuli towards cellular activation. Further under high cell density lipid modified surfaces (figure-3.11.1.3.1II&IIIB&C), (figure-3.11.1.3.1II&IIIE&F) could not elicit a distinguishable response. It clearly indicates that a competition between the cell-cell adhesion and cell-matrix adhesion happens with time and the cell-cell adhesion dominates with time. At this point the ECM is remodeled to adjust the

contractile forces. This may not be happening in the case of bare surface (figure-3.11.1.3.1IA&D) where the cell-ECM interaction dominates. Reduction in cell spreading and multicellular spheroid (colony) formation is more visible under increased serum concentrations. As the cell density increases the size of the colony enhances to form a continuous layer. Here controlled ligand supplementation from the surface during early hours helps in rapid colony formation in the case of PCMCc surface. However differently in the case of bare substrate due to the uncontrolled ligand supplementation there is less control in the organotypic growth pattern.

3.11.1.4 Effect of stimulation with respect to serum concentration

Further we have attempted to evaluate the effect of stimulation on the cellular response. Macrophage adhesion studies have been done with RAW 264.7 macrophage cell line (as per the procedure 2.3.3.5). For that the PS tissue culture plates have been modified as per the procedure 2.2.4.1.2. The liposomes and nanoparticles of the thin films have added into the respective wells. In these studies effect of LPS on cellular morphological changes have been studied with respect to time & serum concentration on modified surfaces.

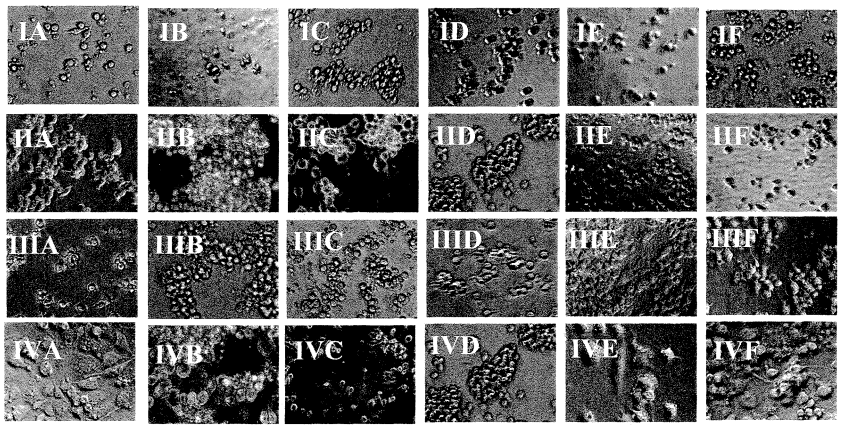


Figure-3.11.1.4.1: Macrophage (RAW 264.7) (1X10^4 cells/ml) phenotype changes with stimulation (LPS) (100µl of lipopolysaccharide endotoxin (LPS, 1µg/ml)) on modified surfaces (1%serum). Row I & II are 24, III & IV are 72hrs Phase contrast microscopy image (40X). I &III are unstimulated & II &IV are stimulated, Bare polymer (PS) (A). PS modified with OCMC (B), PCMCc (C) PVADICc (TRP) (D) TRP+OCMC(E), TRP+PCMCc (F). On A to F all the samples, nanoparticles or liposomes (10 µl/ml) of the respective thin films are added.

At depleted serum concentration, LPS stimulation has treamendous effect on the cellular activation processes (Figure-3.11.1.4.1 II). The effect has been further pronounced with time (Figure-3.11.1.4.1 IV). On bare surface at 24hrs the cells are spread and started colonizing (Figure-3.11.1.4.1 I&IIA). In OCMC and PCMC modified surfaces also cellular colonization process have more pronounced (Figure-3.11.1.4.1I&II B&C), where cell spreading is minimal. While in the case of TRP modified surface (Figure-3.11.1.4.1 1&IID), in contrast to the bare surface, cells have been colonized like in the case of lipid modified surface. Here albumin preferentially self-assembles in its native conformation, whereas in the case of bare surface, nonspecific protein adsorption predominates. Further in the case of OCMC and PCMC stabilized TRP modified surface (Figure-3.11.1.4.1 1&IIE&F) the cellular activation process is more similar to that of unstabilized surface.

At 72 hrs all the cellular activation processes have more pronounced (Figure-3.11.1.4.1 III&IV). In the case of bare substrates (Figure-3.11.1.4.1 IVA) under stimulation cells are spread and do exocytosis. However on OCMC (Figure-3.11.1.4.1IVB), PCMCc (Figure-3.11.1.4.1IVC) and TRP (Figure-3.11.1.4.1IVD) modified surface, cellular activation have less pronounced. In the case of laterally stabilized lipid modified surfaces (Figure-3.11.1.4.1IVE&F) the cell spreading have more pronounced, than the unstabilised, eventhough exocytosis is minimal. This may be due to intercalation of LPS components into the lipid layers. Here due to lateral stabilization the rigidity of the interface has increased may be the reason for the pronounced cellular activation. The minimum cellular activation in the case of lipid modified and albumin self-assembling surfaces may be related to their less rigidity at the interface.

The figure-3.11.1.4.2 below shows the effect of stimulation at 10%serum concentration.

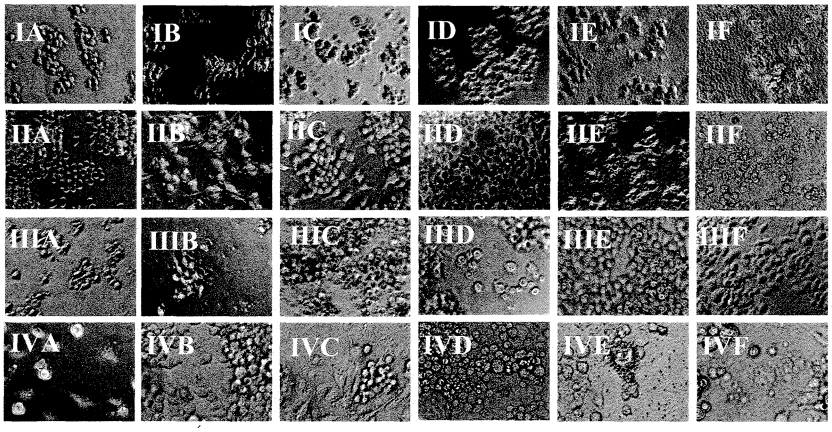


Figure-3.11.1.4.2: Macrophage (RAW 264.7) (1X10^4 cells/ml) phenotype changes with stimulation (LPS) (100µl of lipopolysaccharide endotoxin (LPS, 1µg/ml)) on modified surfaces (10%serum). Row I & II are 24, III & IV are 72hrs Phase contrast microscopy image (40X). I &III are unstimulated & II &IV are stimulated, Bare polymer (PS) (A). PS modified with OCMC (B), PCMCc (C) PVADICc (TRP) (D) TRP+OCMC(E), TRP+PCMCc (F). To A to F all the samples, nanoparticles or liposomes (10 µl/ml)of the respective thin films are added

At higher serum concentrations (figure-3.11.1.4.2) the overall cellular activation process have reduced. Even though the overall trend in cellular activation process remain same as in the case of experiments with depleted serum concentrations. Further secretary profile of the cultured cells is subjected to study with respect to serum concentration and time. The TNFa production have enhanced on bare surfaces with respect to time under depleted serum concentrations. Under higher serum concentrations TNFa production is significantly reduced in bare surfaces also.

3.11.2 Secretory profile of macrophages

'Macrophage secretory profile changes at biomaterial surface due to cellular activation. The activation of the adhered macrophages is different under normal and activated conditions. The cell-cell signaling under pathological conditions has been regulated by the cytokines produced by these macrophages.

3.11.2.1 Normal conditions

Macrophages at the material-biology interface under normal conditions behave with respect to the surface property of the biomaterials. At this point it is interesting to study how biomimetic surface modification strategies control the secretary profile of the macrophages.

3.11.2.1.1 TNF-a production

Macrophages secrete TNFa due to inflammatory cellular process. The acellular supernatant of the cell adhesion studies have been separated and used for the analysis of cytokines by ELISA (as per the procedure 2.3.2.5). The (Figure-3.11.2.1.1.1) shows TNFa production by the cells with respect to serum concentration.

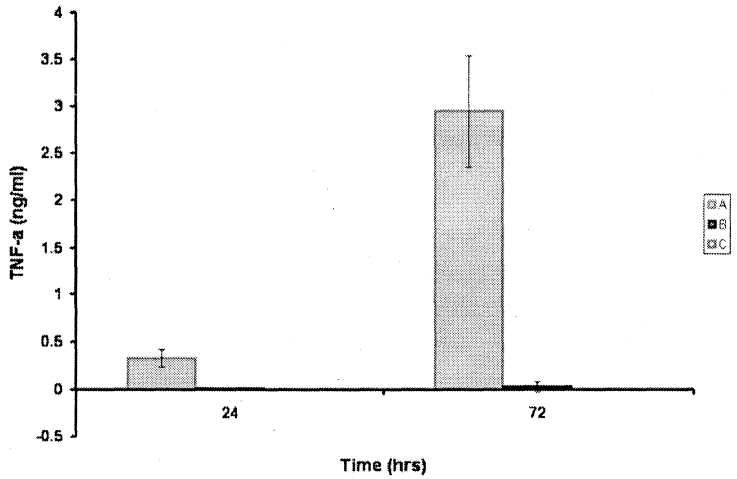


Figure-3.11.2.1.1.1: Macrophage (RAW 264.7) (1X10^5 cells/ml) secretory (TNFa) profile(1% serum), Bare PS substrate (A) PS modified with OCMC (B), PCMCc (C)

The TNFa production Figure-3.11.2.1.1.1 has increased on bare substrates with respect to time under depleted serum concentrations. However on lipid modified substrates the TNFa secretion has not elevated. At higher serum concentrations on bare surface also TNFa secretion has minimized. Earlier we have found excessive cell spreading on bare surfaces with respect to time under depleted serum concentrations (Figure-3.11.1.4.1A). This has direct relation to the proinflammatory TNFa production.

3.11.2.1.2 IL-10 production

Macrophages secretes the proliferative cytokine IL-10 in the culture medium for their survival, is considered as normal phenotypic behavior of the cells. Evaluation of change in IL-10 production indicates about the change in normal phenotypic behavior of the cells. The acellular supernatant of the cell adhesion studies have been separated and used for the analysis of IL-10 by ELISA (as per the procedure 2.3.2.4).

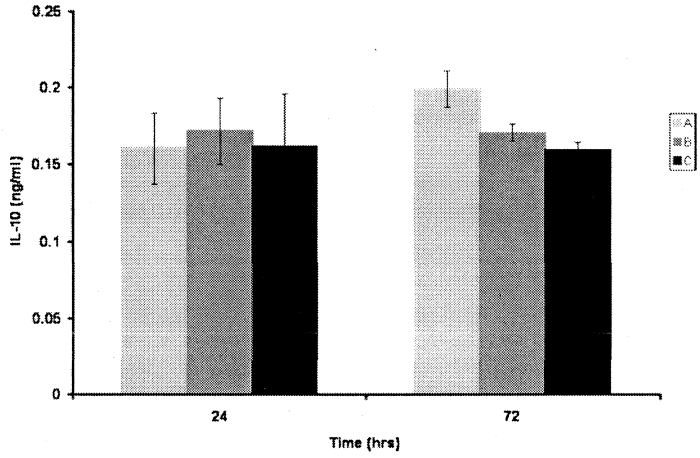


Figure-3.11.2.1.2.1A: Macrophage (RAW 264.7) (1X10^5 cells/ml) secretory (IL-10) profile (1% serum), Bare PS substrate (A) PS modified with OCMC (B), PCMCc (C)

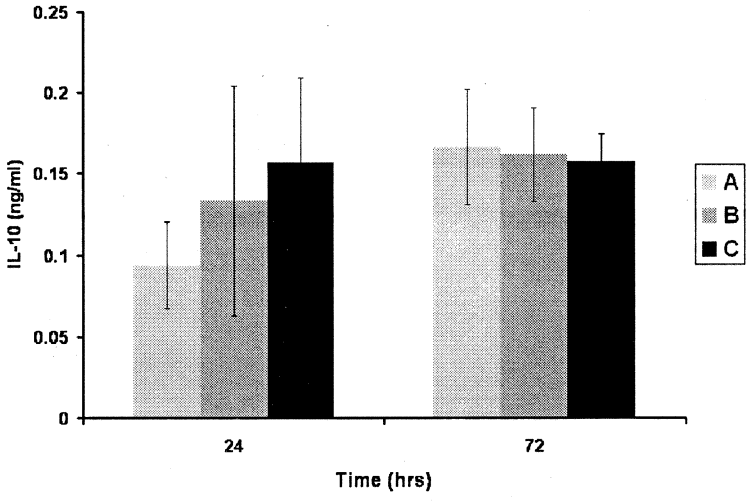


Figure-3.11.2.1.2.1B: Macrophage (RAW 264.7) (1X10^5 cells/ml) secretory (IL-10) profile (10% serum), Bare PS substrate (A) PS modified with OCMC (B), PCMCc (C)

The overall IL-10 production remain unaltered (Figure-3.11.2.1.2.1A & B) on all the surface with respect to time, serum concentration and between different substrates. This indicates that the modified surfaces do not alter the normal secretory profile of the cells.

3.11.2.1.3 IL-6 production

The IL-6 has been considered as a pro immune cytokine secreted by the macrophages. Its evaluation gives the idea about the proimmune response of the surfaces. The acellular supernatant of the cell adhesion studies have been separated and used for the analysis of cytokines by ELISA (as per the procedure 2.3.2.4).

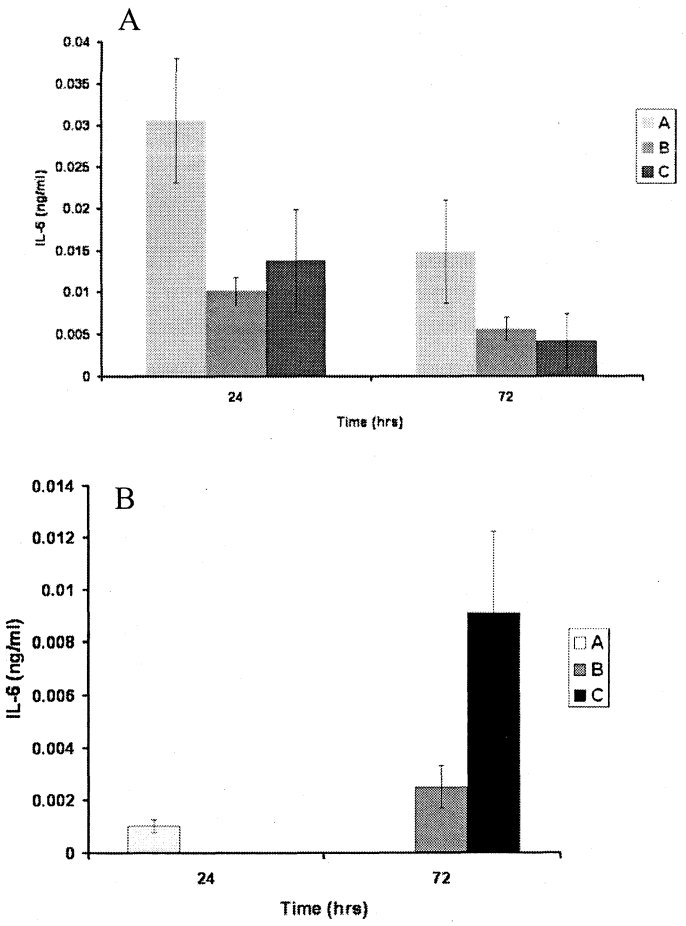


Figure-3.11.2.1.3.1A: Macrophage (RAW 264.7) (1X10^5 cells/ml) secretory (IL-6) profile (1% serum), Bare PS substrate (A) PS modified with OCMC (B), PCMCc (C)

The IL-6 production (Figure-3.11.2.1.3.1A) has been significantly reduced on lipid modified surfaces at 24hrs and further with respect to time under depleted serum concentrations. However differently at higher serum concentrations IL6 production has been slightly increased on lipid modified surface at 72 hrs.

3.11.2.2 Effect of stimulation

Macrophages under stimulation due to bacterial contamination or in presence of other stimulants get activated and initiates pro-inflammatory and pro-immune response. Study of secretary profile under stimulation gives the behavior of activated macrophages on the surface in septic conditions. Therefore studies have been done under activation with respect to time, serum concentration, stimulation between the surfaces with the addition of the nanoparticles of the respective thin films to the corresponding wells where cells grown on the respective surfaces.

3.11.2.2.1 TNFa production

The acellular supernatant of the cell adhesion studies have been separated and used for the analysis of TNFa by ELISA (as per the procedure 2.3.2.4).

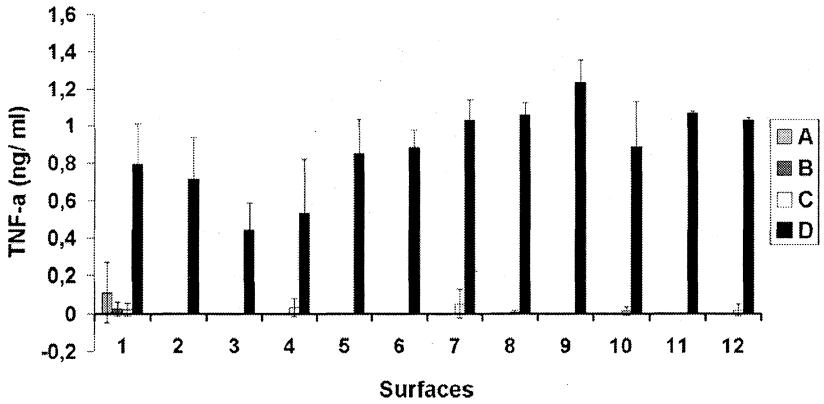


Figure-3.11.2.2.1.1: Macrophage (RAW 264.7) (1X10^4 cells/ml) secretory (TNFa) profile with stimulation (LPS) (100µl of lipopolysaccharide endotoxin (LPS, 1µg/ml)) on modified surfaces (10%serum). A&C are unstimulated, B&D are stimulated, 1-6 are 24hrs and 7-12 are 72hrs. Bare PS substrate (1&7) PS modified with OCMC (2&8), PCMCc (3&9) PVADICc (TRP) (4&10) TRP+OCMC(5&11), TRP+PCMCc (6&12). To A - F all the samples, nanoparticles or liposomes (10 µl/ml) of the respective thin films are added

The TNF-a production (Figure-3.11.2.2.1.1) is uniformly increased on all the surfaces under stimulation at 72hrs under both the serum conditions. However in absence of stimulation under depleted serum conditions the TNFa production is higher, while cells grown on modified surfaces TNFa production is minimal. The results indicate that the nanoparticles or liposomes of the surface or the surface itself did not significantly influence the normal cellular activation process under stimulation.

3.11.2.2.2 IL-10 production

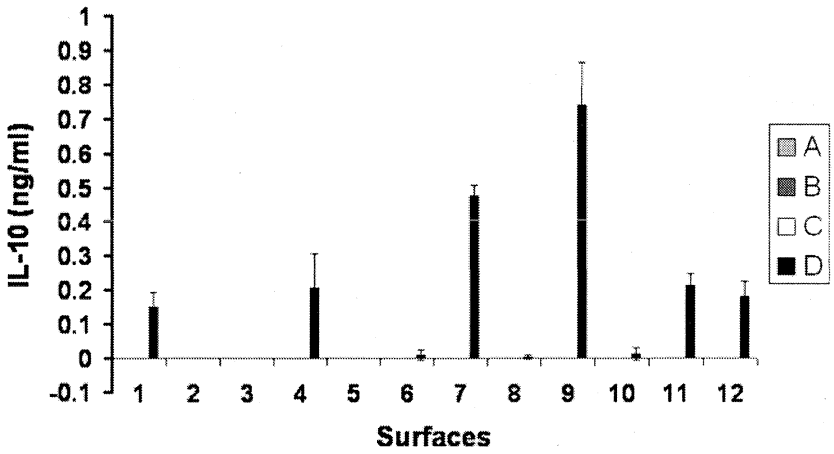


Figure-3.11.2.2.2.1: Macrophage (RAW 264.7) (1X10^4 cells/ml) secretory (IL-10) profile with stimulation (LPS) (100µl of lipopolysaccharide endotoxin (LPS, 1µg/ml)) on modified surfaces (10%serum). A&C are unstimulated, B&D are stimulated, 1-6 are 24hrs and 7-12 are 72hrs. Bare PS substrate (1&7) PS modified with OCMC (2&8), PCMCc (3&9) PVADICc (TRP) (4&10) TRP+OCMC(5&11), TRP+PCMCc (6&12). To A to F all the samples, nanoparticles or liposomes (10 µl/ml) of the respective thin films are added

However differently IL-10 production (figure-3.11.2.2.2.1) is more specific with respect to surface. Here also the results are more evident at 72 hrs under stimulation. At 72hrs on bare and TRP modified surfaces the IL-10 production is higher by the cells grown in depleted serum conditions under stimulation, while all the other lipid modified surfaces remain intact. On the other hand at full serum

conditions on bare and PCMC modified surfaces and OCMC stabilized surfaces the IL-10 production has tremendously enhanced.

3.11.2.2.3 IL-6 production

The acellular supernatant of the cell adhesion studies have been separated and used for the analysis of cytokines by ELISA (as per the procedure 2.3.2.4).

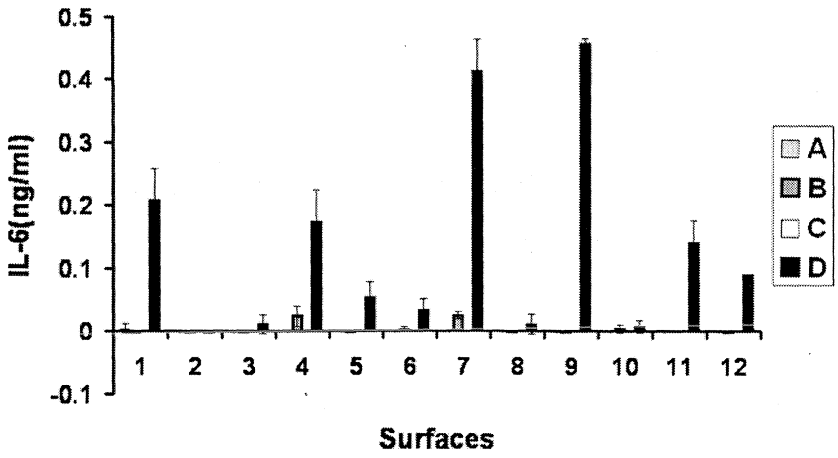


Figure-3.11.2.2.3.1: Macrophage (RAW 264.7) (1X10^4 cells/ml) secretory (IL-6) profile with stimulation (LPS) (100µl of lipopolysaccharide endotoxin (LPS, 1µg/ml)) on modified surfaces (10%serum). A&C are unstimulated, B&D are stimulated, 1-6 are 24hrs and 7-12 are 72hrs. Bare PS substrate (1&7) PS modified with OCMC (2&8), PCMCc (3&9) PVADICc (TRP) (4&10) TRP+OCMC(5&11), TRP+PCMCc (6&12). To A to F all the samples, nanoparticles or liposomes (10 µl/ml) of the respective thin films are added

The IL6 has been differentially secreted (figure-3.11.2.2.3.1) by the cells depending upon the substrate under stimulation. Its effect is more prominent at 72 hrs, but is visible at 24hrs. At 24hrs cells grown on bare polymer, stabilized PCMCc and TRP, and at 72 hrs, cells grown on, bare, PCMCc, TRP stabilized OCMC and PCMCc modified surfaces, secreted IL-6, with stimulation, under depleted serum concentrations. However cells grown under full serum concentrations, on bare, OCMC and TRP modified surfaces, at 24 hrs and Bare, PCMCc stabilized OCMC and PCMCc at 72 hrs, secreted IL6 under stimulation.

The adhesion and activation of macrophages on to the material surface plays an important role in acute inflammatory responses. The activation of these cells leads to change in spreading, and secretory profiles. The cytokines produced by these macrophages play an important role in the cell-cell signaling. The cytokines can be classified based on their source from CD4+ Tcells into Thelper1 (Th1) IL-1, IFN- γ, TNF- β, and TNF- α, and T helper2 (Th2) IL-4, IL-5, IL-6 &IL-10. Th1 cytokines initiates proinflammatory and antiimmune while Th2 cytokines has anti-inflammatory, proimmune responses. Many of these cytokines are also produced by the macrophages at the implant site[282], [283]. The most prominent inflammatory cytokine produced by the macrophages are TNFa. In our studies under depleted serum concentration at 72 hrs high TNFa production is observed on bare surfaces. This is considerably reduced in presence of serum proteins. Most interestingly surfaces modified with lipid monolayers or the albumin self-assembling thermoresponsive surfaces, TNFa production has been not escalated. This clearly indicates that reduced adhesion and activation of the macrophages reduces proinflammatory responses of macrophage cells. However under activation TNFa production have unaltered, irrespective of surface modification. Differently increased IL-6 production has observed on PCMCc modified surface under full serum concentrations. These modified surfaces also does not alter the normal secretary profile (IL10) of the cells both normal and under activation. This gives a Th2 shift at the implant site which can probably reduces the inflammatory responses. Apart from that these adhesion dependent macrophage cell line also gives information about the normal cell behavior on these modified surfaces. As compared to the bare surface, both the lipid modified and albumin self-assembling surfaces encourage cell-cell adhesion. In absence of PE this effect has minimized on lipid modified surfaces. This is an important development, where PE can be explored for the controlled ligand supplementation from the surface for organotypic behavior without altering the normal secretory profile of the cells. This has far reaching implications in tissue engineering using stem cells.

These studies have been done under full serum conditions. In the case of PMN cells (Figure-3.12.1.1.1) on all the surfaces under stimulation the cellular activation is visible at 24 and 72 hrs. On bare surface it is visible, as the destruction of the protein bed, while on lipid modified surfaces, it is liposome formation. The effects are profound at 72 hrs on unstabilised and stabilized lipid surfaces. However differently we have found least effect on TRP modified surfaces.

3.12.2 PMN Secretory profile

Secretary profile of the PMN cells is elevated under stimulation. Here we have attempted to study the influence of the modified surfaces on the secretory profile of PMN cells.

3.12.2.1 IFNg production

The IFNg is a proinflammatory cytokine produced by the PMN cells under stimulation.

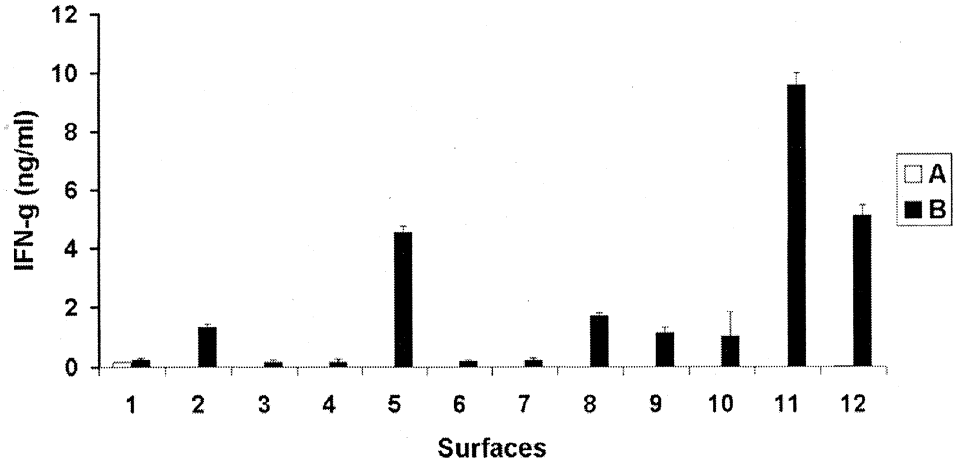


Figure-3.12.2.1.1 Secretory Profile (IFNg) of PMN from mouse spleen ($1X10^6$ cells/ml), with stimulation (PMA(10µg/ml)/ Ionomycin(10ng/ml)) on modified surfaces (10% serum). Unstimulated (A), Stimulated (B), 1-6 are 24hrs and 7-12 are 72hrs. Bare PS substrate (1&7)PS modified with OCMC (2&8), PCMCc (3&9) PVADICc (TRP) (4&10) TRP+OCMC(5&11), TRP+PCMCc (6&12) Respective nanoparticles or liposomes (10 µl/ml) of thin films was added to the all the samples

Under normal conditions, the IFNg production (Figure-3.12.2.1.1) has been shown only by the bare substrates. Upon stimulation all the modified substrates show time depended increase in IFNg production. The profound increase is in the case of laterally stabilized lipid modified surfaces. Here most interestingly OCMC surface enhances IFNg production as compared to PCMCc modified surfaces. TRP modified surfaces also slightly enhances the IFNg production with time. However on bare substrate time depended increase in IFNg production is not visible.

3.12.2.2 IL10 production

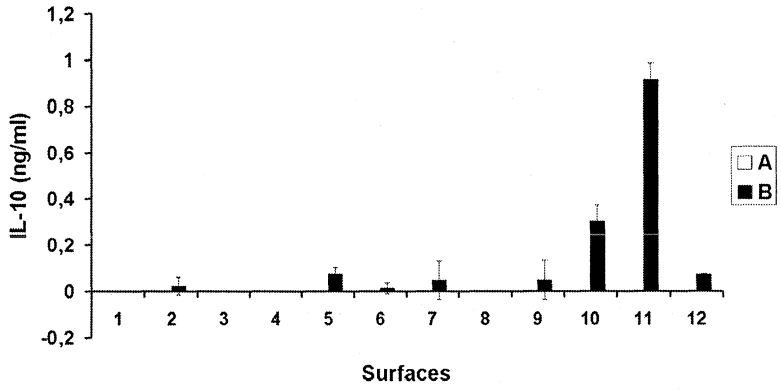


Figure-3.12.2.2.1 Secretory Profile (IL-10) of PMN from mouse spleen ($1X10^6$ cells/ml), with stimulation (PMA(10µg/ml)/ Ionomycin (10ng/ml)) on modified surfaces (10% serum). Unstimulated (A), Stimulated (B), 1-6 are 24hrs and 7-12 are 72hrs. Bare PS substrate (1&7)PS modified with OCMC (2&8), PCMCc (3&9) PVADICc (TRP) (4&10) TRP+OCMC(5&11), TRP+PCMCc (6&12) Respective nanoparticles or liposomes (10 µl/ml) of thin films was added to the all the samples

Under unstimulated conditions, there is no significant production of IL10 (Figure-3.12.2.2.1) by the cells grown on any of the substrates. Upon stimulation all the modified substrates shows time dependent increase in IL10 production. The profound increase is in laterally stabilized lipid modified surfaces. Here most interestingly OCMC surface enhances IL10 production as compared to PCMCc modified surfaces. TRP modified surfaces also slightly enhanced the IL10 production with time. On bare substrate time depended increase in IL10 production is negligible.

3.12.3 PMN proliferation

Further we have studied the proliferation potential of the primary cells on these surfaces. For that the 96 well PS tissue culture plates have been modified with the different coatings as per the procedure, 2.2.4.1.2. Studies are done with primary PMN cells extracted from mouse spleen (as per the procedure 2.3.3.6). The nanoparticles (prepared as per the procedure 2.2.4.3.1) and liposomes (prepared as per the procedure 2.1.4.2) of the respective thin films are added into the cell suspension, grown on bare wells. These studies are done under normal and activated conditions.

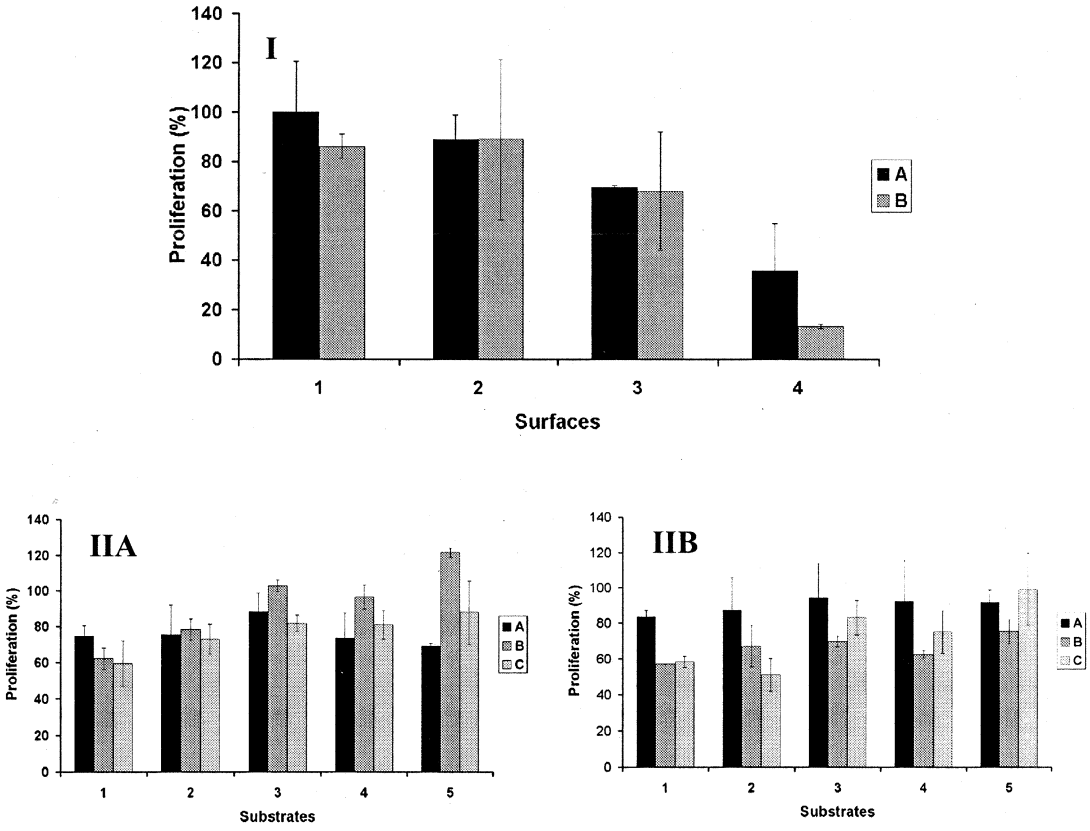


Figure-3.12.3.1: BrdU uptake by the PMN cells after 24hrs (10% serum). The proliferative potential of the modified surfaces (I), Bare PS (1), PS modified with OCMC(2), PCMCc(3), PVADICc (4). The effect of debris (in the form of nanoparticles and liposome in altering the proliferation, nonactivated (IIA) and activated with PMA (10µg/ml)/ Iono(10ng/ml) (IIB), liposomes of OCMC (1), PCMC (2) PVADICc (TRP) (3) TRP+OCMC(4), TRP+PCMCc (5). are administered in three different concentrations 5 (A), 10 (B), 20 (C) µl/ml concentrations, to PMN cultured on bare plates

Our studies indicates that lipid modified surfaces (Figure-3.12.3.1) does not alter the proliferation of the cells. Least proliferation has been observed in the case of PVADICc modified surfaces, this may be due to the preferential adsorption of albumin. Apart from that debris of the thin films has least effect in altering the proliferation.

Polymorphonuclear cells are (PMNs or granulocytes), primarily neutrophils secrete chemotactic cytokines under stimulation. Our studies demonstrate that under normal conditions cytokine secretion is not elevated on all the surfaces. However under activation, on lipid surfaces supported with the TRP, both the IFNg and IL10 are elevated. This may be due to stacking of the activating agent (PMA and ionomycin into the lipid layers. On pendant polymer supported lipid layers, the membrane flexibility has been limited and the cells get enough strength to exert a physiological response. The IFNg/IL10 ratio is higher on these surfaces clearly indicates the Th1 response which is proinflammatory. However there are no significant changes in the proliferation of PMN cells. This effect is not distinguishable in the case of adhered macrophage cells may be because they respond very sensitively with even soluble agents. It clearly distinguishes that as the rigidity of the interface enhances, chances of cellular activation also enhances. This discrepancy in cell signaling has far reaching implications in drug targeting.

3.13 Fibroblast adhesion studies: Effect of phosphatidylethanolamine

For that PolyC substrates has been modified with the OCMC and PCMCc monolayers as per the procedure 2.2.2.2.1. The fibroblast adhesion studies have been done as per the procedure 2.3.4. The in vitro fibroblast cell adhesion and proliferation onto these lipid-modified surfaces have done by studying the morphological changes of the adhered fibroblasts. The Figure -3.13.1 &2 shows the cell adhesion and proliferation of fibroblasts onto these surfaces.

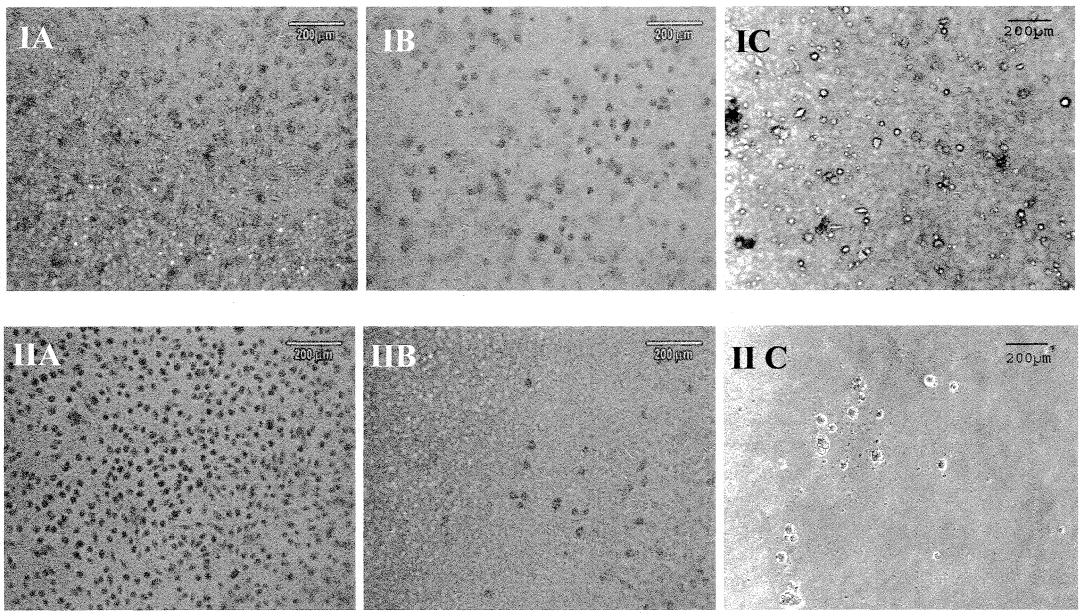


Figure-3.13.1. Fibroblast (L929) (1X10^3 cells/ml) cell line proliferation studies on lipid modified polymer surfaces, microscopy at different time intervals. One day (I), Five day (II). PolyC bare (A). PolyC modified with OCMC (B), PCMCc (C)

The adhered fibroblast cells with a non-spreaded (NSP) and spreaded (SP) morphology are studied from a given area in all the fields. The spread and non-spread cells are defined based on the morphology given by Chandy. et. al.,[284] for platelets.

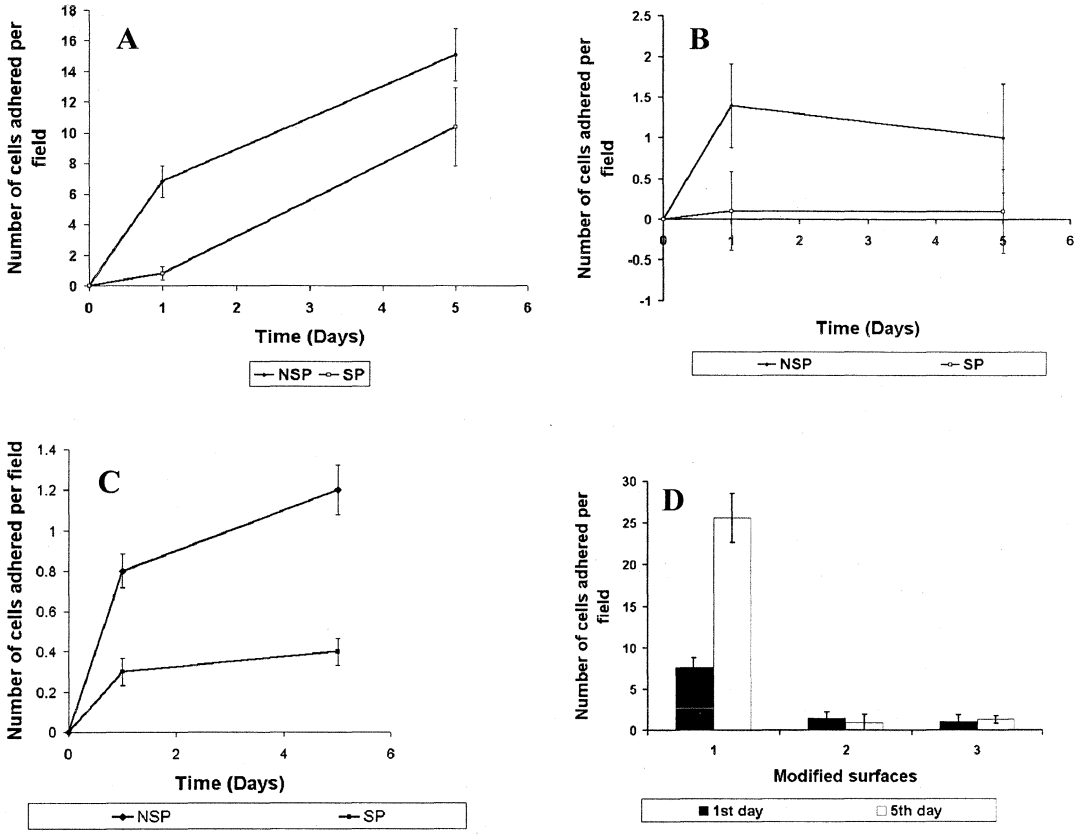


Figure-3.13.2: Fibroblast proliferation studies on supported lipid surfaces. Bare polymer (A). Modified with OCMC (B), PCMCc (C). Total number of fibroblast cells adhered on modified surfaces (D). Fibroblasts with spread (SP) and non-spread (NSP) morphology

The fully spread fibroblast cells with pseudopods are defined as the spread cells, while the cells with a rounded morphology are identified as the non-spread cells. In the case of bare polycarbonate the total number of adhered and spread cells increased with time, (Figure 3.13.2A). On lipid-modified surfaces the initial cell adhesion is lower than that of the bare polymer. The OCMC surface, (Figure 3.13.2B) shows less fibroblast spreading and proliferation with time (Figure 3.13.1 (I, II B)), (Figure 3.13.1D2). While with the incorporation of PE (Figure 3.13.1(I, II C), (Figure 3.13.2C) the initial cell adhesion is similar to that of OCMC surface, and the cell spreading increases with time. Here the cell activation (due to spreading and proliferation increases in presence of PE (Figure 3.13.2D3). Further

we have attempted to evaluate the bacterial adhesion onto this lipid modified surfaces.

3.14 Bacterial adhesion Studies: Effect of phosphatidylethanolamine

For that PolyC substrates have been modified with the OCMC and PCMCc monolayers as per the procedure 2.2.2.2.1. The bacterial adhesion studies have been done as per the procedure 2.3.5. The Figure-3.14.1 (Fluorescent image) and Table-3.14.1 represents the bacterial adhesion studies onto the lipid-modified surfaces. The overall bacterial adhesion (G+ve and G-ve) reduces after the surface modification.

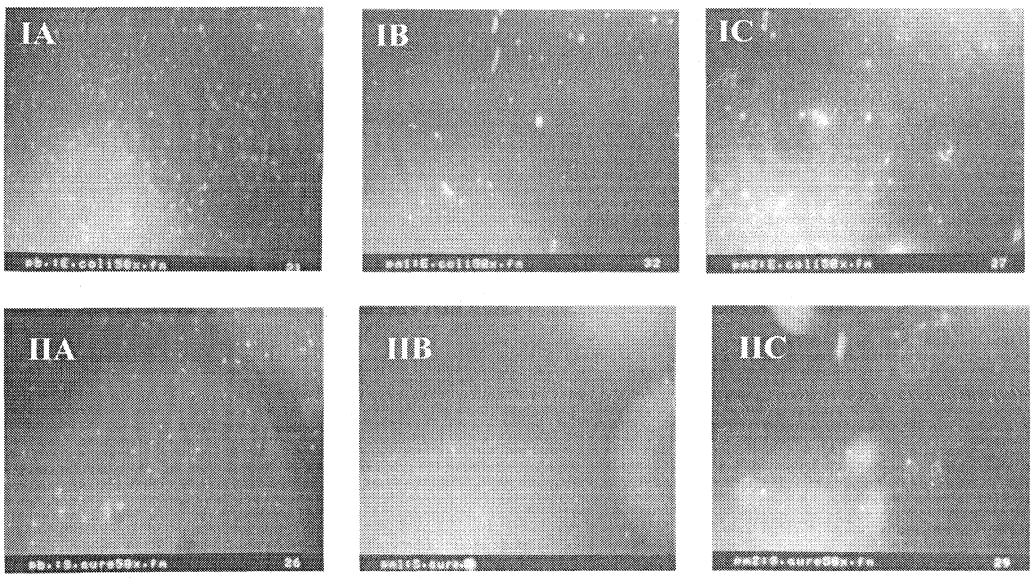


Figure-3.14.1: Bacterial adhesion studies on supported lipid surfaces. Escherichia coli (I), Staphylococcus aureus (II) (Inoculum density of about 10^6 (fu/ ml) cells). Bare PolyC (A). PolyC modified with OCMC (B), PCMCc(C), supported lipid surface

The adhesion of the E. coli is more than Staph. aureus on all the surfaces. Another interesting observation is that on to PE incorporated surface (Figure 3.14.1(II C), (Table- 3.14.1) the E. coli adhesion appeared higher than the OCMC surface.

Table-3.14.1 Colony forming assay

Sample No	Adhered bacteria adhered per cm^2	
	Eschericia coli - G-ve (ATCC 25922)	Staphylococcus aureus - G+ve (ATCC 25923)
Polycarbonate bare	1.5×10^4	2.5×10^6
PC modified with OCMC	4.4×10^3	5.8×10^4
PC modified with PCMCc	4×10^2	2.1×10^5

The reduction in protein adsorption on supported lipid surfaces might be the reason for the reduction in bacterial adhesion. The difference in bacterial adhesion between the OCMC and PCMCc modified lipid surface may be due to specific interaction between the lipid surface and the bacteria.

3.15 Drug release across the monolayers

Thin film strategy could very well explored for the surface modification of devices for the systemic drug delivery of various therapeutic agents for degenerative diseases. Here understanding about the effect of surface modification on controlled drug delivery is important. For that we have attempted to understand the small molecule and peptide delivery across these monolayers, by two different strategies.

3.15.1 Small molecules loaded into nanoparticles

For that nanoparticle of PVADICb have been developed (as per the procedure 2.1.3.1.3). Here the drug DIC with high partition coefficient is covalently immobilized onto the large molecular weight PVA, and the nanoparticles are prepared out of PVA-DIC pendant polymer, by simple solution precipitation. During the synthesis of the pendant polymer PVADICb, at a particular feed ratio of the drug/ polymer, the polymer recovery is almost impossible with any of the solvents. Salting out at 60°C recovered the amphiphilic

polymer. However at other drug/ polymer ratios, simple solvent precipitation could be able to recover the polymers.

3.15.1.1 Sedimentation-Dispersibility studies

The sedimentation-dispersibility studies has demonstrated that the nanoparticles are getting redispersed, (Figure-3.15.1.1.1A), (Figure-3.15.1.1.1B), (Figure-3.15.1.1.1C) at ambient temperature conditions (25°C), and are aggregated (Figure-3.15.1.1.1A (C&D) at elevated temperature. Further to verify the mechanism of the aggregation behavior we have studied the temperature dependence of the nanoparticles by DLS. The Figure- 3.15.1.1.1B shows that, the size of the nanoparticles is increasing with increase in temperature. This is due to swelling of the nanoparticle by the reduction in hydrophobic interactions between the pendant groups of the polymeric chain. It also reduces the refractive index as well as the count rate of the nanoparticles (Figure-3.15.1.1.1C). Further, the reduction in count rate (Figure-3.15.1.1.1C 3&4(B)) of the nanoparticle suspension after sedimentation-dispersibility studies demonstrated the association behavior.

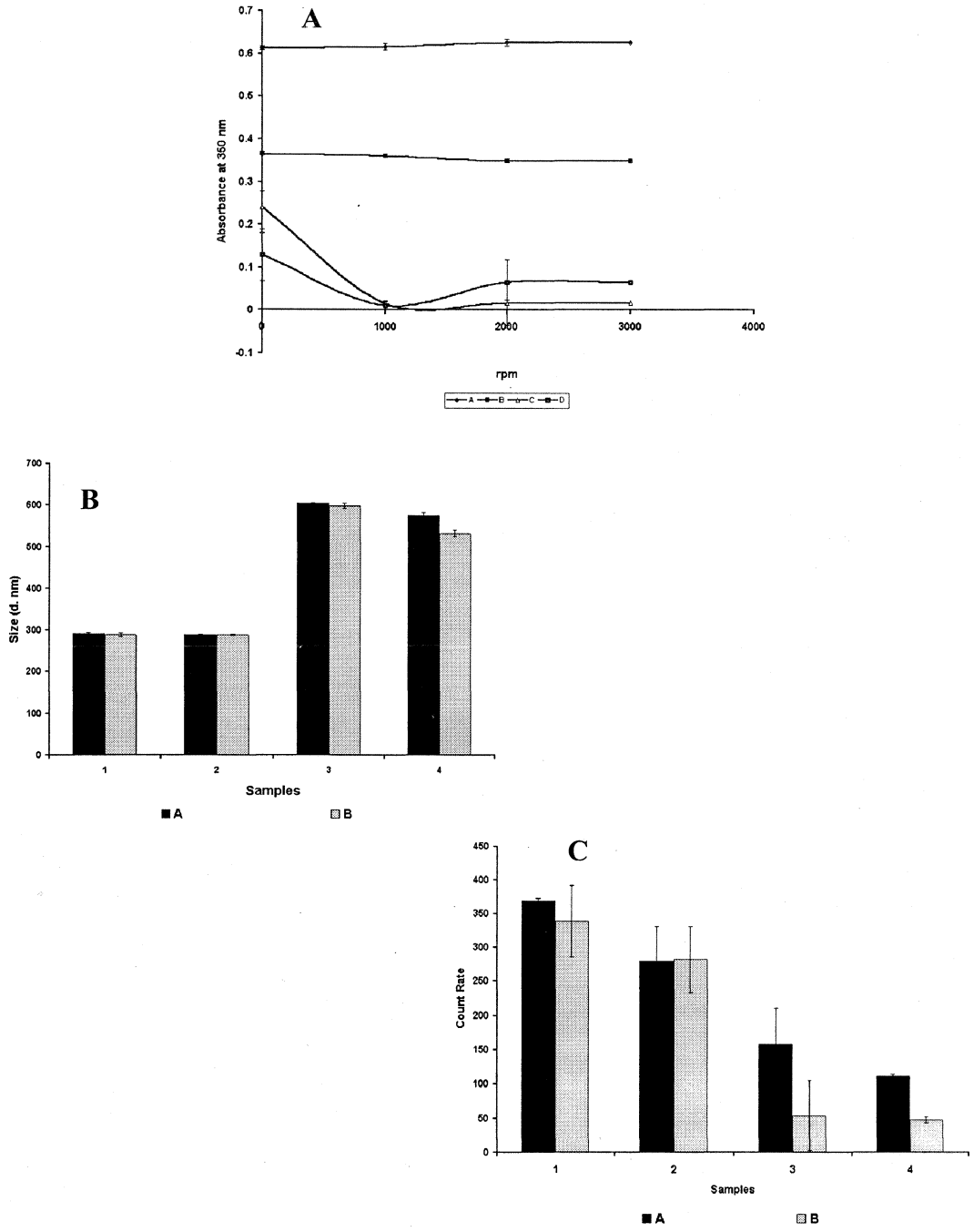


Figure-3.15.1.1.1: Dispersibility of the nanoparticles
Absorption of the nanoparticles at 350nm by UV/Vis Spectroscopy (A); Nano particle suspension: water dilution ratio; (1:2) A, (1:4) B at 25°C, and (1:2) C, (1:4) D at 70°C. DLS Experiments, Hydrodynamic radius of the nanoparticles (B), Count rate of the nanoparticles (C), The samples 1&2 are native size or count rate at 25°C and 3&4 at 70°C as well as before (A), after (B) centrifugation at 3000rpm.

Earlier, change in refractive index as well as count rate, has been observed in the case of proteins, polymer molecules and poly N- isopropylacrylamide (PNIPAM). In the case of PNIPAM the increase in count rate has been associated with conformational change from random coil to condensed globular state, while in the case of proteins and polymers swelling and association has been proposed as the mechanism for the decrease in count rate [285]. Here the association of nanoparticles at elevated temperature (70°C) may be due to the interaction of hydrophobic groups of the pendant polymer, like in the case of soluble globular proteins.

Earlier the surface morphology of the nanoparticles processed at different temperature conditions have been studied by scanning electron microscopy (SEM) (Figure-3.5.2.3.5.1). The Figure-3.5.2.3.5.1A, shows unidisperse globular nanoparticles formed at ambient (25°C) conditions. However at 70°C the nanoparticles (Figure-3.5.2.3.5.1B) are deformed, adhered to each other and grown to become micro fibrils. These micro fibrils are further aligned (Figure-3.5.2.3.5.1C) to form the microfilaments. These microfilaments have a rod or star shaped (Figure-3.5.2.3.5.1D) morphology. This is due to local control of growth of the microfilaments by self-assembly by the nanoparticles still in solution.

Further DLS studies (Figure-3.5.2.3.3.1A) at different temperature conditions have demonstrated that the nanoparticle has a denaturation temperature (DT) at 59°C, above the temperature the nanoparticles are irreversibly aggregated. Further, the temperature cycling studies below the DT has demonstrated that the nanoparticles has shape memory effect, in solution (Figure- 3.5.2.3.3.1C). Which is lost gradually on multiple cycling (hysterisis). This is one of the typical characters of soluble globular proteins [286]. However different from globular proteins, these nanoparticles are forming fibrils at higher temperatures as evident from the SEM.

Apart from that, earlier it has been observed that some protein architectures with a strong hydrophobic core, holds light harvesting capabilities[287]. Further we have evaluated the photochemical behavior of these nanoparticles by UV/Vis and Fluorescence spectroscopy (Figure-3.15.1.2.1). UV irradiation of these nanoparticles (Figure-3.15.1.2.1A) shows green fluorescence, which indicates that the nanoparticles are monodisperse and hold a specific structural conformation. Further UV/ Vis spectroscopy (Figure-3.15.1.2.1.B) has demonstrated that, depending upon the drug to polymer ratio there has been a hypsochromic shift in the absorption spectra. This property is lost at other drug/polymer ratios Figure-3.15.1.2.1.A. The figure-3.15.1.2.1.B shows a strong absorption at 244nm and 310nm. At higher drug concentration the peak are at 266nm. Pure drug has given a peak at 274 nm but did not show any fluorescence. The absorption peak at 310nm is assigned to aryl π- π^* transition due to the π- π stacking of the neighboring hydrophobic side chains.

This indicates that the π- π stacking (due to pendant groups) balanced by the hydrogen bonding interactions (hydrophilic PVA chain) regulate the self-assembly process in the formation of nanoparticles. In fluorescent spectroscopy (as per the procedure 2.2.13), (figure-3.15.1.2.1.C) we have found interestingly, at excitation wavelength of 266 and 274 as well as 310 nm the immobilized samples are not emitting any light. But when excited at 244nm only these nanoparticles (Figure-3.15.1.2.1.C) are showing emission peaks at 497nm.

3.15.1.2 Photoresponsiveness of protien like nanoparticles

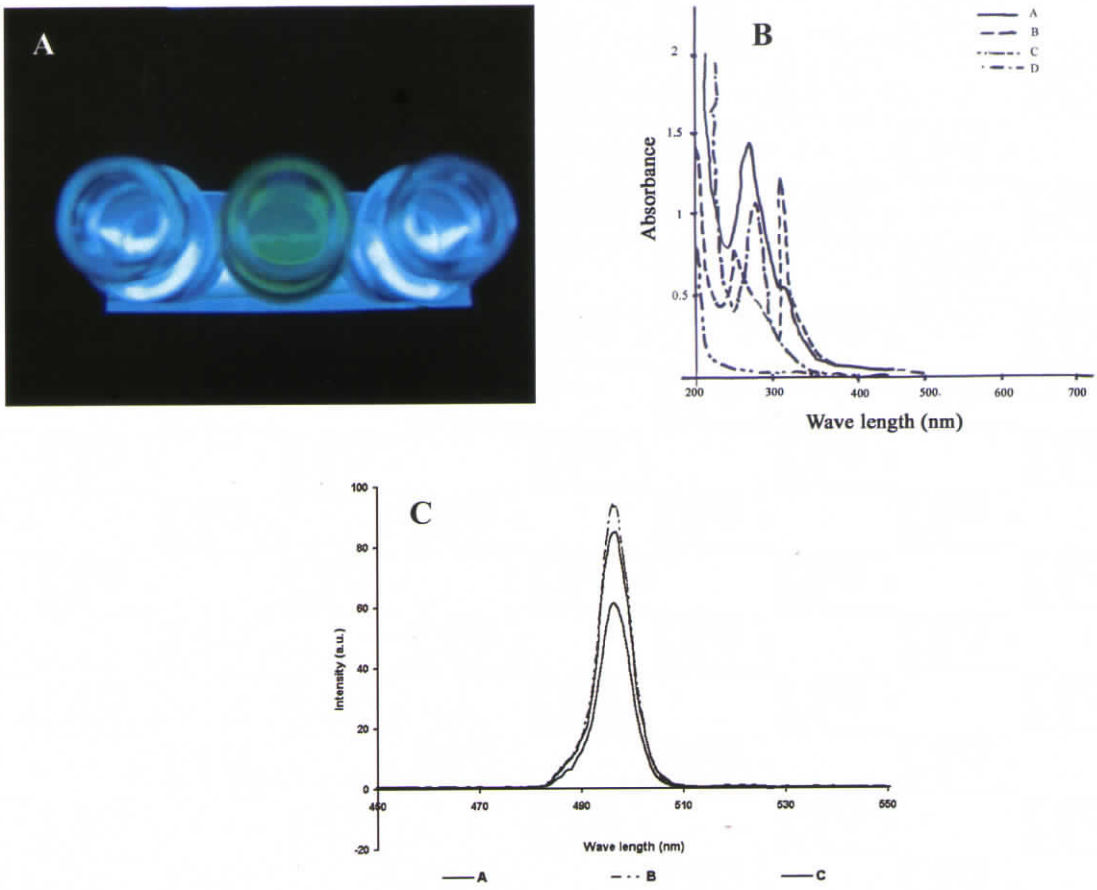


Figure-3.15.1.2.1:Photochemical evaluation of the nanoparticles. UV irradiation of the samples (A), Drug to Polymer Concentration (0.025:1) (PVADICa) (A), (PVADICb) (0.125:1)(B), (PVADICc)(0.25:1)(C). UV/Vis Absorbance spectra of the nanoparticles (B), Drug (A) Pendant polymer PVADICa (B), PVADICb (C), PVADICc (D), Fluorescence spectra of the nanoparticles (C), Three different concentration of the nanoparticles of PVADICb, 0.1(A), 0.05(B), 0.025(C) gm%.

Earlier soluble Phtoactive Yellow Protien (PYP) have shown such fluorescent phenomena [288]. The energy harvesting has been expected to be due to π- π interactions of the hydrophobic core of the proteins. The co-factor binding modifies the π- π stacking of the hydrophobic core and adjust the light harvesting ability. Such proteins have Per-Arnt-Sim (PAS) like sequence similarities. It is

common in Drosophila Period clock protein, (PER) Drosophila Single-minded protein (SIM)[289], the vertebrate Aryl hydrocarbon Receptor Nuclear Translocator (ARNT) [290] etc. In all these proteins signal detection is enhanced with an associating co-factor[291]. Such strong regulation of hydrophobic interactions due to π-π stacking has also been observed in the formation of amyloid bodies[292]. All these things indicate that the presence of a closely packed hydrophobic core seems to play an important role in the signal harvestment in the case of proteins[293].

Here we have demonstrated the development of nanoparticles, mimicking globular proteins. The hydrophobic pendant groups through π-π stacking balanced by the hydrogen bonding regulated self-assembly, controls the formation of globular nanoparticles from solution. These nanoparticles found to have strong light harvesting ability like PYP and other signal harvesting proteins. These nanoparticles have a DT at 59°C like other globular proteins. We have also demonstrated that, like other globular proteins, above the DT the nanoparticles tend to self-aggregate, due to the reduction in hydrogen bonding interactions and eventually the π-π stacking has become the principal force that regulate the self-assembly and formation of fibrils. The fibril formation may be due to linear self-assembly of the adjacent pendant groups of the neighbouring polymeric chains. Below the denaturation temperature the nanoparticles have shape memory effect which shows hysteresis on multiple thermal cycling. It clearly indicates that these nanoparticles are supramolecular architecture formed due to helical arrangement of polymeric chains to form globular architectures. These helical architectures show a protein like conformation typically demonstrated with a hydrophobic core.

We have attempted to check whether this core can identify like molecules, so that they can induce the condensation of soluble molecules with the pendant molecular imprints. Further diclofenac as a model drug is loaded into ceramic nanoparticles synthesized into the nanodomains of PVADICb nanoparticles and it is coated with the OCMC and PCMCc monolayers. The release kinetics of these nanoparticles have been studied.

3.15.1.3 Drug Condensation

The drug condensation has been done inside the nanoparticles as per the procedure 2.1.3.1.3.1. It indicates that heavy drug loading can be done inside these nanoparticles by inducing co-operative activity inside the nanodomains. This is due to structure aided condensation of the drug molecules. The strong co-operative activity at the hydrophobic domains aided by the molecular imprints (pendant groups) under less solvation helps the drug molecule to condense. Since the drug condensation is aided by salting out process due to calcium followed by phosphate ionic interactions, large amount of drug could be able to load into these nanoparticles.

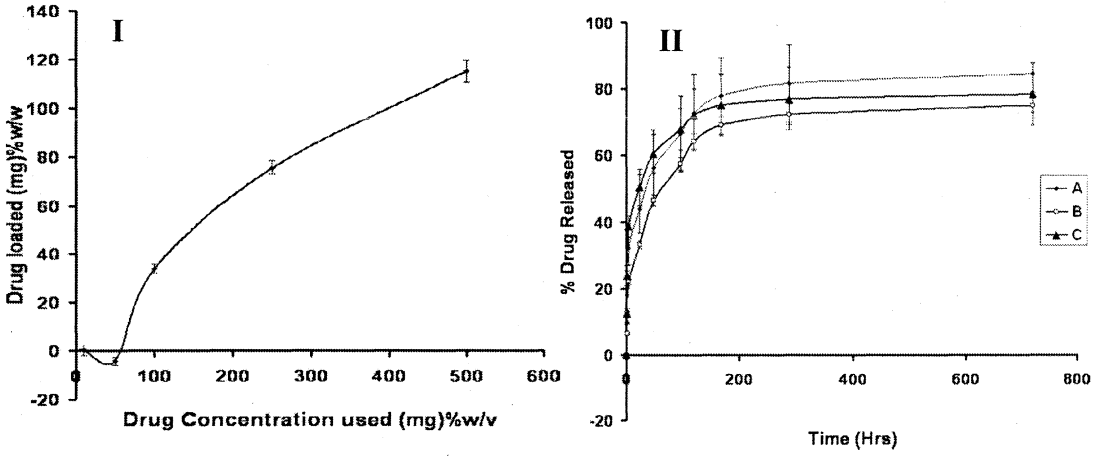


Figure-3. 15.1.3.1: Drug condensation to the nanodomains of the protein like nanoparticles. Drug loading (1). Release studies (2), Drug loaded nanoparticles (A), Modified with OCMC (B), PCMCc

The release kinetics of the nanoparticles is principally guided by the solubility of the drug calcium phosphate complex and is prolonged beyond 800hrs. The surface modification does not significantly influence the release kinetics of such small molecules (Figure 3.15.1.3.1). Further we have attempted to study the macromolecular (protein) release across the monolayers.

3.15.2 macromolecular release across the monolayers

For that we have developed porous microspheres of CHIT as per the procedure 2.1.2.1. The Table-3.15.2.1 shows the physical properties of the microsphers.

Table-3.15.2.1 Structure of lipomat

Structure of lipomat	
Size	3.18+/-0.12mm
Weight	0.2914+/-0.016mg
Pore volume (as per the procedure 2.2.6)	3.89+/-0.018ml/gm
Internal Architecture	
Larger Pore Dia	188-200μm
Small Pore Dia	80-120 μm
Inter Connected Channels	40-55 μm
Bridges	2.5 μm
Outer Film Thickness	2-5μm

It indicates that the microspheres have an internal porous structure, which can hold large amount of liquid medium. The figure-3.15.2.1 below shows the structure of the microspheres. They form uniform spherical microspheres, with porous cavities, thin walls and a continuous outer membrane. The large pores are connected by small pores and they are interconnected. The interconnected pores form continuous porous architecture through out the microspheres. The outer thin membrane is continuous through out the microspheres. This helps in the controlled drug delivery using these microspheres. The porous architecture can act as a scaffold for the cells to attach and proliferate. They can be further modified with cell mimetic lipid monolayers for controlling the ligand supplementation from the surface. Further we have studied the release kinetics of proteins using insulin as a model drug out of the microspheres after modifying the surface with lipid layers.

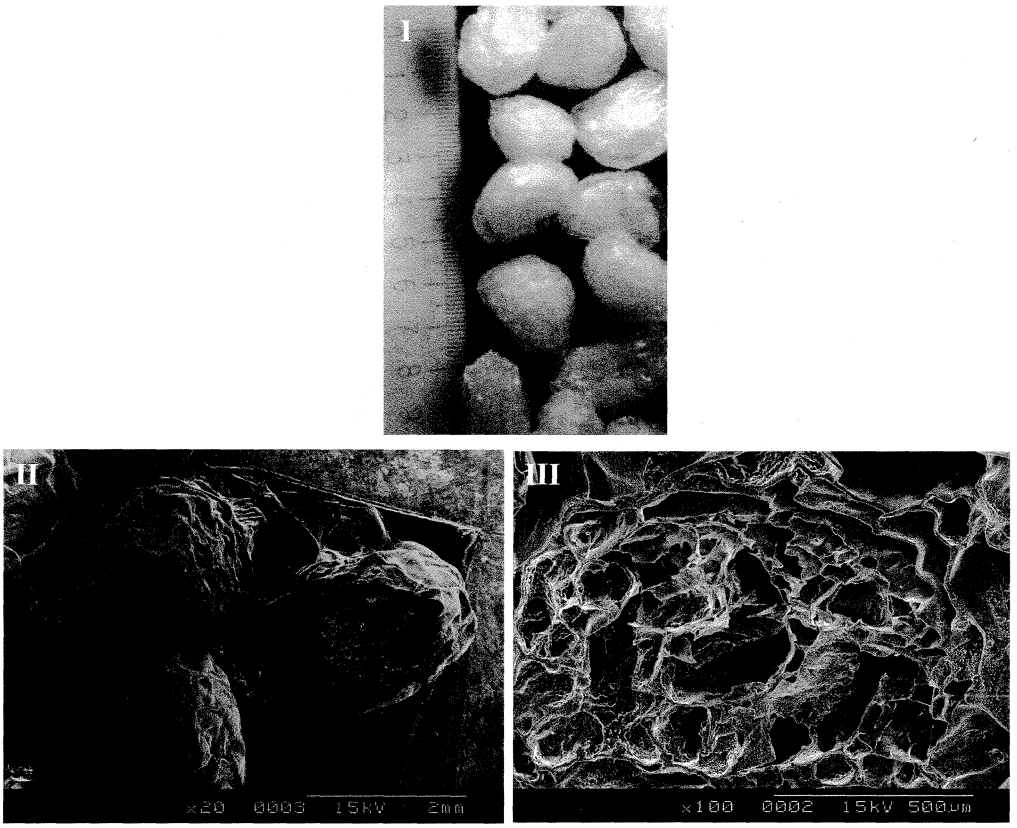


Figure-3.15.2.1 : Blend of microspheres which can deliver mixture of stimuli both soluble and insoluble stimuli for tissue engineering applications. Structure of microsphere (I) Surface Topography (II), Internal architecture (III)

For that the microspheres have been loaded with insulin (as per the procedure 2.1.5) and the surface is modified with PC, OCMC and PCMC monolayers (as per the procedure 2.2.4.2.2). The drug release studies have been done as per the procedure 2.2.8 in PB 7.4 at ambient conditions. The figure 3.15.2.2 shows the drug release across the lipid monolayers.

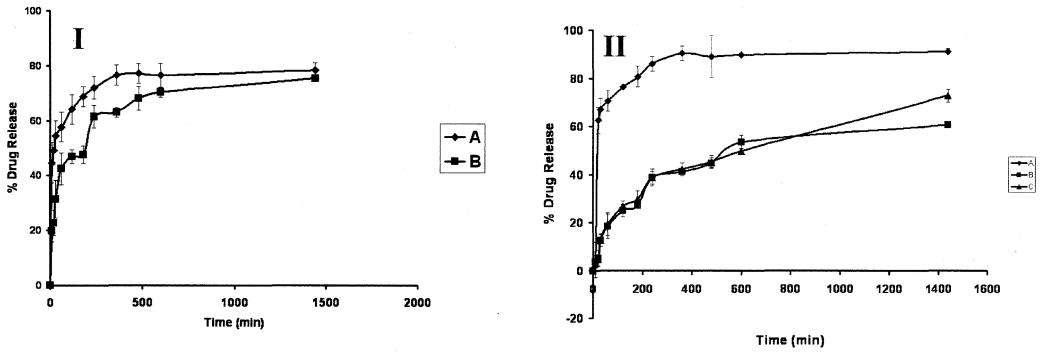


Figure-3.15.2.2: Insulin release profile out of the liposome delivering matrix. Cell mimetic lipid modified microspheres (II) Bare microsphere (A) modified with OCMC (B) modified with PCMCc (C)

The lipid coating over the microspheres has reduced the initial burst release and sustained drug delivery have been observed for days. In the case of PC modified surfaces (figure- 3.15.2.2 I) sustained release was observed upto 500hrs. While in the case of OCMC and PCMC modified surfaces (figure- 3.15.2.2 II) it has prolonged beyond 1400hrs. This clearly indicates that lipid composition plays an important role in regulating the release profile of macromolecules across the monolayers. Further to check the mode of release profile we have done the dye test after loading the different micropheres with Brilliant blue as a model dye. The figure - 3.15.2.3 shows that liposomes are forming out of this lipid modified microspheres.

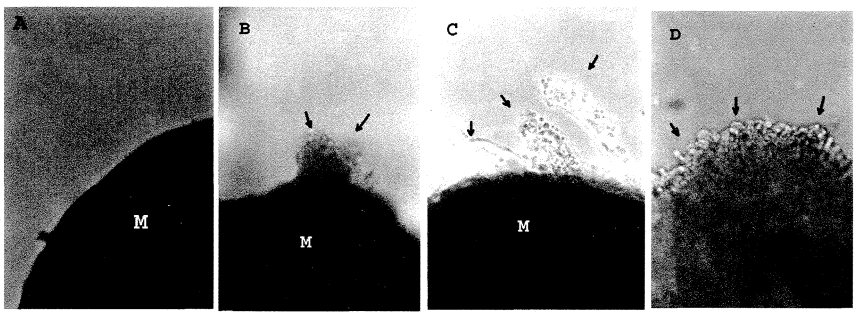
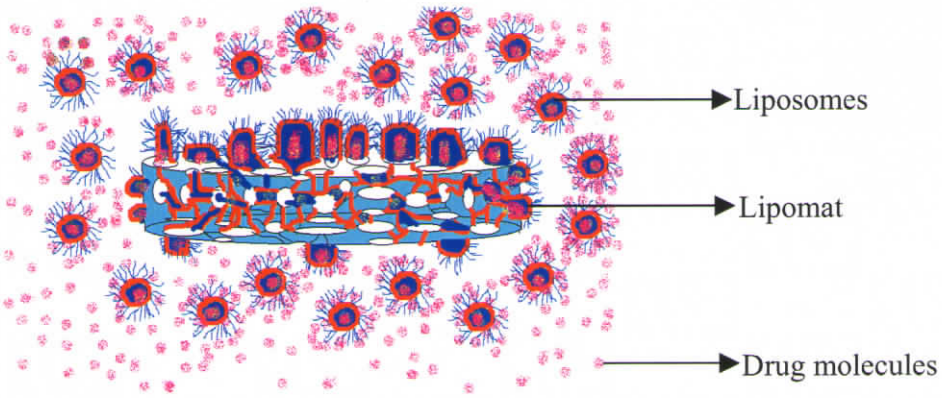


Figure-3.15.2.3: The hydration of microspheres modified with lipid layers (dye test). 0hr (A), 10min(B), 20min(C), 30min (D). The liposomes and tube like architectures are found to be large multi lamellar vesicles (200- 500nm)

The OCMC and PCMCc monolayers form long liposomes, may be due to the lateral stability of the lipid films. While with PC alone, liposomes are delivered out of the matrix.



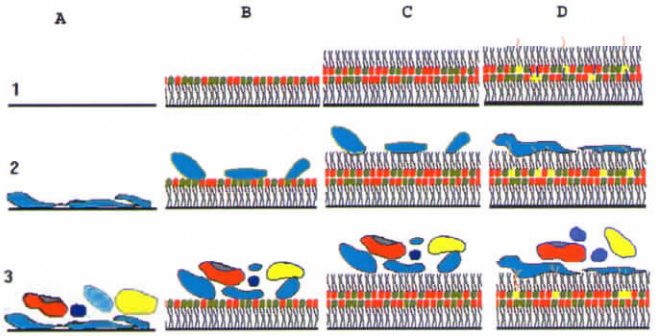
Scheme-3.15.2.3.1 Macromolecular release by the microspheres

Liposome delivering matrices (Lipomat) has far reaching implications in systemic local drug delivery for tissue engineering applications. The formed liposomes can deliver the drug to a distant site (Scheme-3.15.2.3.1). These micropheres either alone or as a blend with various drugs and mitogens could be explored for multiple drug delivery.

Further we have checked the Biofriction of the lipid modified surfaces. Here we have used PC coatings for the polymeric gel and PC as well as OCMC in the case of microspheres. As we know from the air/ water interfacial studies the film of PC is existing in the fluid state through out the compression. The OCMC and PCMC also remain in the fluid state. Therefore the results with PC give information with other lipid layers also.

Surface active phospholipids have boundary lubrication properties at synovial joints[294] and lung[295]. Satisfying the boundary lubrication of injectable medical devices has therapeutic significance[296]. Therefore we have studied the

supported monolayer surfaces prepared (3.3.1.3) accordingly are (1) Closely packed ordered hydrophobic surface - PC modified with the combination (PTC: Chol: Gal) (1: 0.35: 0.125), (2) Loosely packed ordered hydrophobic surface - PC modified with the combination (PTC: Chol) (1: 0.35), (3) Closely packed ordered hydrophilic surface - PC modified with the combination (PTC: Chol)(1: 0.7). The lipid modified substrates are highly hydrophilic, irrespective of orientation and packing density (3.4.2). Their interaction with ions (3.7.1) , proteins (3.8.1) and cells (3.9.1) have been studied. Based on the experiments we hypothesise that the net cellular interactions to lipid modified surface remain same irrespective of orientation and packing density of the compressed monolayers, eventhough the interaction with ions and proteins are different. It is schematically represented in scheme-4.1.1.1.



Scheme- 4.1.1.1**. Schematic representation of the proposed orientation of the deposited mono and bilayers on hydrophobic PolyC polymer surface and their protein as well as blood cell interaction. Horizontally, PolyC bare (A), PC modified with (PC: Chol) (1: 0.7) (B), PC modified with (PC: Chol) (1: 0.35) (C), PolyC modified with (PC: Chol: GalC) (1: 0.35: 0.125) (D). Vertically, The unmodified and modified substrates (1) Protein adsorption to the substrates (2) Cell adhesion to the substrates (3). PC ( ), Chol ( ), GalC ( ), protein ( ), erythrocyte ( ), leukocyte ( ), Platelets ( )**

This is mainly attributed by the hydrophilicity of the modified substrates, fluidity and the reduction in free energy parameter. Here we have optimized a heterogeneous ternary lipid composition based on the air/water (3.2.2) and air/solid

Biofriction at lipid layers after immobilizing them onto microspheres and polymeric gels.

3.16 Biofriction at the lipid interface

For that we have immobilized PC by layer by layer deposition (as mentioned in the procedure 2.2.4.4) and coated over microspheres (as mentioned in the procedure (2.2.4.2.2). Biofriction studies have been done by protrusion method (as per the procedure 2.2.4.4.1) in the case of polymeric gels and sliding method (as per the procedure 2.2.4.2.2.1) in the case of microspheres.

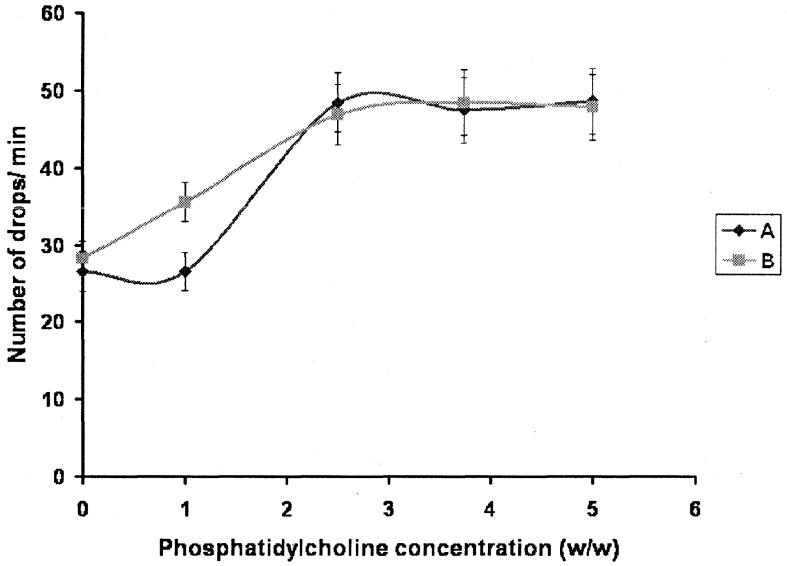


Figure-3.16.1: Extrudability of polymeric gel: PVA (A), PVA-DICa (B)

The figure 3.16.1 demonstrates that biofriction has uniformly decreased in both the kind of polymeric gels with respect to the phospholipid concentration. The reduction is independent of polymeric gel. The maximum reduction has been observed at 2.5% w/w PC concentration and after that it is going through a plateau state. This clearly indicates that the reduction in boundary friction is a function of lipid monolayer saturation.

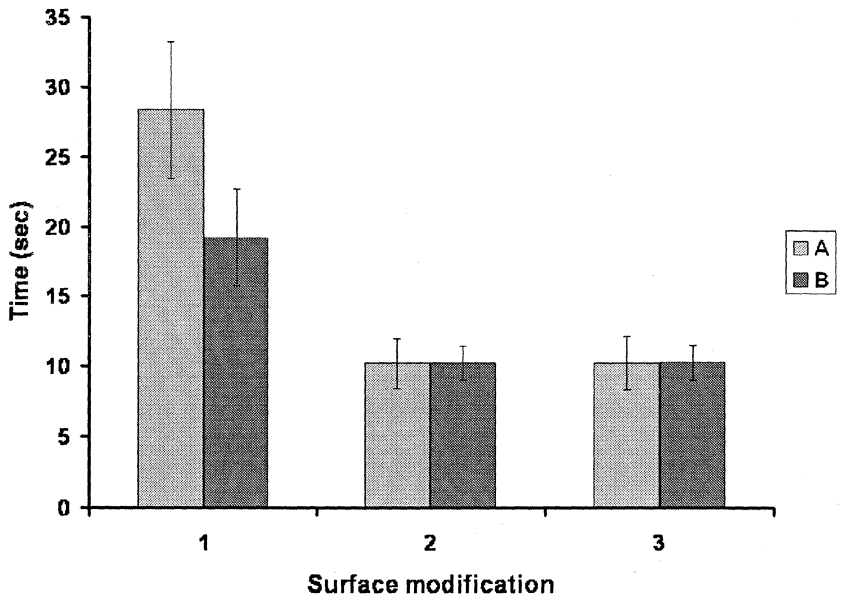
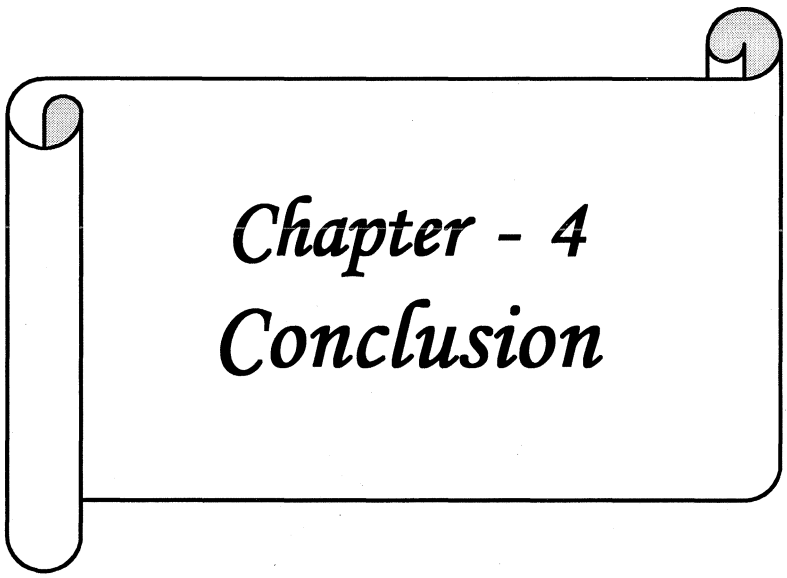


Figure-3.16.2: Movement of microsphere on wet glass surface: Glass surface uncoated (A), Surface coated with PC (B), Microsphere uncoated (1), PC coated (2), Coated with OCMC (3)

Another study of biofriction between rigid surfaces (using microsphere on glass surface under wetting) (Figure-3.16.1&2) modified with lipid layers demonstrated that surfaces modified with lipid layers on both the sides has significantly reduced the biofriction, uniformly by the PC and OCMC monolayers. It also revealed that the moving surface need to be surface modified than the stable surface.

During the implantation of injectable drug delivery and tissue engineering devices, one of the major problems is biofriction during implantation. The biofriction alone can alter the surface properties of these devices. Here we have demonstrated that the lipid modified surfaces can significantly reduce the biofriction. It can also functionally satisfy the boundary lubrication needed for synovial fluid or during the administration of stents or Intra Ocular Lens etc.



Chapter - 4
Conclusion

CONCLUSION

Over the last 30 years various post synthetic surface modification strategies has been proposed for biomaterials. From the experience it is evident that the redundancy of stable surface (by covalent surface modification) in a biological environment changes the course of the device patency with time. The demanding need of miniaturization of biomedical devices explores enormous opportunity in the field of post synthetic surface modification. Here the challenge of dealing a dynamic environment for an optimum performance is best achieved through a dynamic interface. The interface has to be less rigid, flexible, do controlled ligand supplementation, antifouling and renewable.

Thin films formed by self-assembly are a promising alternative. Here close control of organization of components into a supramolecular architecture is to be acquired for attaining optimum efficiency. Biomimicry, specifically cell membrane mimetics, helps as a limelight in this scenario.

4.1 Development of thin film of cell membrane components

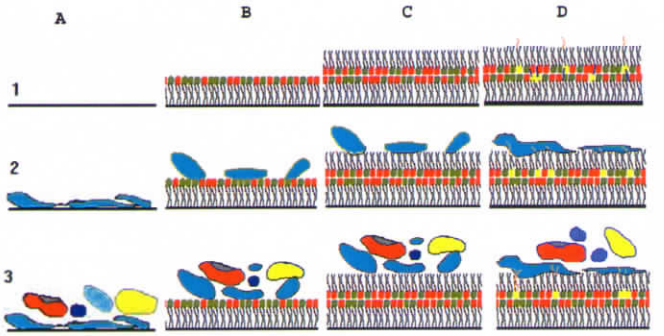
In this present investigation we have attempted to develop thin cell membrane mimetic lipid films for the post synthetic surface modification of materials. Further we have compared effect of nonspecific protein adsorption and preferential albumin adsorption with respect to these lipid modified substrates. All the experiments related to understanding about the material-biology interactions have been done on polymeric films or flat surfaces. This information is explored for post synthetic surface modification of miniaturized devices.

To develop antifouling lipid thin films we have mimicked the outer cell membrane lipid components (1.3.2.1). For that we have developed co-solvent system for solubilising heterogeneous phospholipid, glycolipid and chol, together. Further a co-solvent system has been optimized for incorporating other macromolecules like proteins, polysaccharides and polymers. It has been optimized in such a way that it does not affect the properties of base polymeric substrate(3.1). We have used phosphatidylcholine (PC) for phospholipid, galactocerebroside (GalC) for glycolipid and cholesterol (Chol) based on the head group structure to represent the major lipid components of the endothelial luminal cell membrane. The interfacial behavior of various combinations of PC, GalC and Chol monolayers have been studied at the air/ water interface and deposited on hydrophobic polycarbonate (PolyC) polymer substrates with the help of the langmuir blodgett (LB) trough (1.3.2.3) for the modeling studies and different deposition parameters were optimized. The intermolecular self-assembly between the components of lipid film (3.2) and intramolecular self-assembly between the monolayer and substrates (3.3) have been studied

4.1.1 Effect of orientation and packing density of the lipid monolayer

The packing and orientation of the supported monolayers have been varied by means of changing the lipid composition rather than the deposition parameters. This approach seems to be more similar to the In vivo conditions. The different

supported monolayer surfaces prepared (3.3.1.3) accordingly are (1) Closely packed ordered hydrophobic surface - PC modified with the combination (PTC: Chol: Gal) (1: 0.35: 0.125), (2) Loosely packed ordered hydrophobic surface - PC modified with the combination (PTC: Chol) (1: 0.35), (3) Closely packed ordered hydrophilic surface - PC modified with the combination (PTC: Chol)(1: 0.7). The lipid modified substrates are highly hydrophilic, irrespective of orientation and packing density (3.4.2). Their interaction with ions (3.7.1) , proteins (3.8.1) and cells (3.9.1) have been studied. Based on the experiments we hypothesise that the net cellular interactions to lipid modified surface remain same irrespective of orientation and packing density of the compressed monolayers, eventhough the interaction with ions and proteins are different. It is schematically represented in scheme-4.1.1.1.



Scheme- 4.1.1.1**. Schematic representation of the proposed orientation of the deposited mono and bilayers on hydrophobic PolyC polymer surface and their protein as well as blood cell interaction. Horizontally, PolyC bare (A), PC modified with (PC: Chol) (1: 0.7) (B), PC modified with (PC: Chol) (1: 0.35) (C), PolyC modified with (PC: Chol: GalC) (1: 0.35: 0.125) (D). Vertically, The unmodified and modified substrates (1) Protein adsorption to the substrates (2) Cell adhesion to the substrates (3). PC ( ), Chol ( ), GalC ( ), protein ( ), erythrocyte ( ), leukocyte ( ), Platelets ( )**

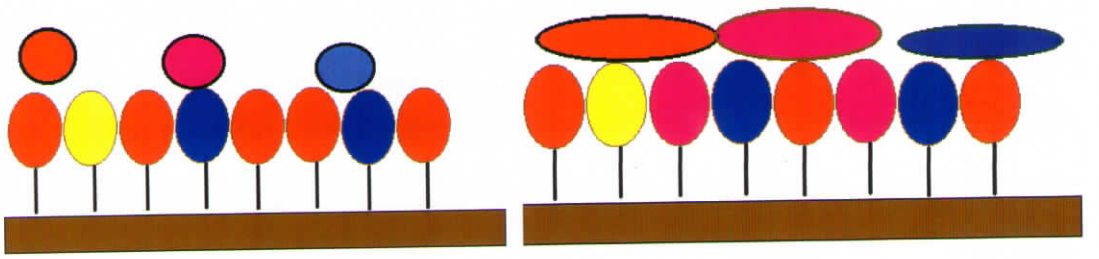
This is mainly attributed by the hydrophilicity of the modified substrates, fluidity and the reduction in free energy parameter. Here we have optimized a heterogeneous ternary lipid composition based on the air/water (3.2.2) and air/solid

transfer studies (3.3.1.2). This outer cell membrane mimetic lipid composition (OCMC) is stable on hydrophobic surfaces (3.5) under static conditions. Surface topography of this OCMC modified surface shows cell membrane like topography (3.6.1). The most interesting observation is that this surface significantly reduces platelet (3.10), macrophage (3.11.1.2) and polymorphonuclear cell activation (3.12.1). It does not change the secretory profile of the macrophages (3.11.2) or PMN cells (3.12.2) under normal conditions. Here the most interesting observation is that the liposomes or nanoparticles of these thin films do not alter the normal phenotype of the cells or during activation, (3.11.1.2) and (3.12.2). It does not influence the proliferation of PMN cells (3.12.3), but significantly influences the proliferation of fibroblasts (3.13) and bacteria (3.14). This discrepancy may be due to that the PMN cells require least adhesion sequence to proliferate as compared to fibroblasts or bacteria.

4.1.2 Effect of head group structure

Among the various lipids outer cell membrane lipids are less thrombogenic or reduce cell activation and inner cell membrane components are thrombogenic and induces cellular activation (1.3.2). However the role of PE which reside in both the side of cell membrane are least studied. Here we have incorporated PE into these lipid monolayers and supported over hydrophobic polymer substrates. The air/water interfacial studies indicate that the packing density is getting reduced after the incorporation of PE in a concentration dependent manner. Further it is reflected in the reduction of the size of the domains in surface morphometric studies. Further the interaction of ions, proteins and cells have been done onto these substrates. Calcification has been reduced on this PCMCc surface like the OCMC surface. However protein adsorption has been further reduced on PCMCc surface. The most interesting observation is that the PCMCc surface slightly enhances platelet (3.10), macrophage (3.11.1.2) and polymorphonuclear cell activation (3.12.1). It does not change the secretory profile of the macrophages (3.11.2) or PMN cells (3.12.2). The proinflammatory cytokine profile of the

macrophages under depleted serum concentrations has reduced (3.11.2.1.1 .1) due to lipid surface modification. Interestingly PE incorporated surfaces enhances cell-cell interaction without altering the secretory profile (3.11.1), and elicit an organotypic multi-cellular spheroid formation. However in the case of PMN cells, stabilized lipid monolayers with pendant polymer elicits a Th1 response (3.12.2) which may be due to the intercalation of the stimulant to the lipid layer. The stabilized layer has more rigidity as compared to the unstabilized monolayer. This enhanced stability can elicit the minimum traction force required for the cell activation. However such effect is not distinguishable in the case of macrophage cells (3.11.2), may be due to its high sensitivity even to soluble stimulants. Here the most interesting observation is that the liposomes of these thin films does not alter the normal phenotype of the cells or during activation, (3.11.1.2) and(3.12.2). It does not influence the proliferation of PMN cells (3.12.3), but slightly enhances the proliferation of fibroblasts (3.13) and bacteria (3.14). Under reduced protein adsorption to these surfaces the cell activation process may be regulated through specific activation of the cells. From our studies we hypothesize that PE enhances cellular activation process through specific interaction with the cells. This effect is uniformly visible on both exogeneous and endogeneous pathological cells. The concept is schematically represented in the scheme-4.1.2.1.



Scheme-4.1.2.1Cell interaction to the phospholipid monolayer supported polymer substrates., Polycarbonate supported OCMC(A)., PCMCc (B).(More cell adhesion and spreading in B). Polymer substrate ( )., PC ( )., PE ( )., Chol ( )., GalC ( ). Platelets non spread ( ) more adhesion and activation ( ),. Endogeneous pathological cells, non spread ( ), more adhesion and proliferation ( ). Exogeneous pathological cells, less adhesion ( ), more adhesion ( ).

This information is helpful for doing controlled ligand supplementation from the material surface.

4.1.3 Effect of lateral stabilization

Further we have attempted to stabilize these monolayers laterally by covalent immobilization of the intercalated protein molecules (3.5.1) and by physically using pendant polymers (3.5.2).

For covalent lateral stabilization, we have mimicked the lateral stabilization of plasma membrane by transmembrane proteins (1.3.2.3). For that soluble globular protein (albumin) is conformationally changed to expose its hydrophobic core and intercalated into the monolayer. Further hydrophilic ionic macromolecule heparin and DAPEG have been introduced into the monolayer and compressed to expel to the hydrophobic side of the monolayer, to form a three dimensional network around the monolayer (3.2.4). A bilayer of this lipid film from the interface has been transferred to functionalized PMMA films and laterally stabilized by carbodiimide chemistry (3.5.1). The interaction of ions (in terms of calcification (3.7.3.)) protein (in terms of adsorption (3.8.3)) and cells (in terms of adhesion (3.9.3)) is minimal on this laterally stabilized surfaces.

However this approach has its own limitation because it can be applied only on functionalized substrates. For versatility we have attempted to develop physical strategies for the lateral stabilization using supramolecular assemblies (1.3.2.4), so that it can be applied on any kind of substrates. For that we have developed nanoparticles of pendant polymers (3.5.2) and immobilized onto different kind of substrates (3.5.2.2.5) and further the lipid monolayers are stabilized (3.5.2.2.8). Here careful selection of the polymers (structural advantage (3.5.2.1)) and pendant groups (based on partition coefficient (3.5.2.2)). For the value addition, pendant groups have been selected from a class of drugs with high safety and albumin binding avidity. The screening of the pendant group has been done based on *in silico* docking studies with albumin (3.5.2.2.1). Then they were

ionically immobilized in CHIT and studied the preferential adsorption of albumin (3.5.2.2). The Diclofenac has been found to be the best candidate and prepared PVADIC pendant polymers. Wet lab studies indicated that surfaces modified with the pendant polymers (3.5.2.3.4,5&6) of the nanoparticles retain the albumin binding avidity (3.5.2.3.8). This nanoparticle modified surfaces hold thermoresponsiveness like PNIPAAM due to their protein like structure (3.5.2.3.7). This is an important advancement where a lipid layer can be laterally stabilized, when it is removed it can preferentially adsorb albumin, both the events favours inhibition of cellular activation processes. This is confirmed with macrophages and PMN cells (3.11.1.2) and (3.12.2), where albumin preferentially adsorbing surface and the lipid modified surface behave similarly. The nanoparticles of the pendant polymer or which is coated with the thin films does not alter the phenotype of the cells under normal or activated conditions (3.11.1.2) and (3.12.2).

4.1.4 Drug release across the monolayers

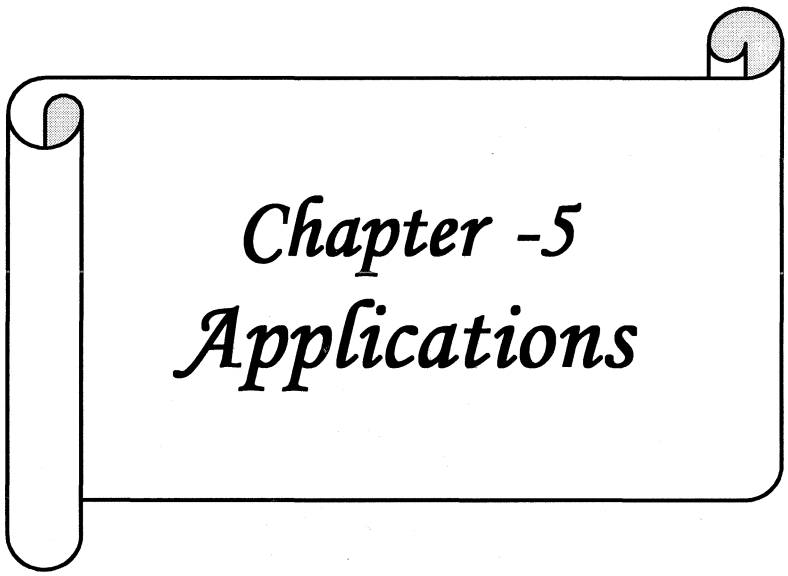
We have attempted to study the drug release across the monolayers. For that high load of drug DIC has been condensed into the nanoparticles of PVADIC and which is modified with the OCMC and PCMCc monolayers (3.15.1). The release studies demonstrates that the surface modification does not alter the release kinetics of the nanopartiocles.

Further we have attempted to check the release kinetics of the protein insulin across the monolayers (3.15.2). In this case the osmotic pressure induced by the insulin found to induce liposome formation. This clearly indicates that size of the drug play an important role in maintaining the integrity of the monolayers when released out of the matrix.

4.1.5 Biofriction at the lipid interface

Our studies indicate that biofriction is significantly reduced after the surface modification using lipids (3.16.1& 2). Biofriction is an important events while implanting the medical devices.

In conclusion renewable self-assembled thin films with high flexibility generate a dynamic interface which can interact with the dynamic biological environment. Here self- assembled lipid systems offer possibility of controlled ligand supplementation from the surface. They can be laterally stabilized using nanoparticles of high functional aspect ratio. These systems do not change the intrinsic property of the thin films. A renewable albumin adsorbing surface also works equally well. A combination of it may have long survival time in the biological environment. Here by delineating the force component exerted by the cellular actin filaments through ECM to the substrate, these renewable surfaces inhibit all the actin mediated pathological events. When controlled stimuli has been supplemented from the surface, cells form multicellular spheroids, may be due to enhanced cell-cell adhesion. This organotypic changes may be explored for tissue engineering applications. The antifouling properties of the OCMC could be explored for surface modification of various kinds of devices for drug delivery, biosensors stents etc. Its lateral stabilization with the pendant polymer with thermoresponsive properties could be explored for cell layer tissue engineering applications. Protien intercalated lipid layer could be explored as a cheap substitute as a lung surfactant. Reduced biofriction by these monolayers could explore towards synovial fluid substitution. These studies unveil few of the tremendous opportunity of these renewable systems.

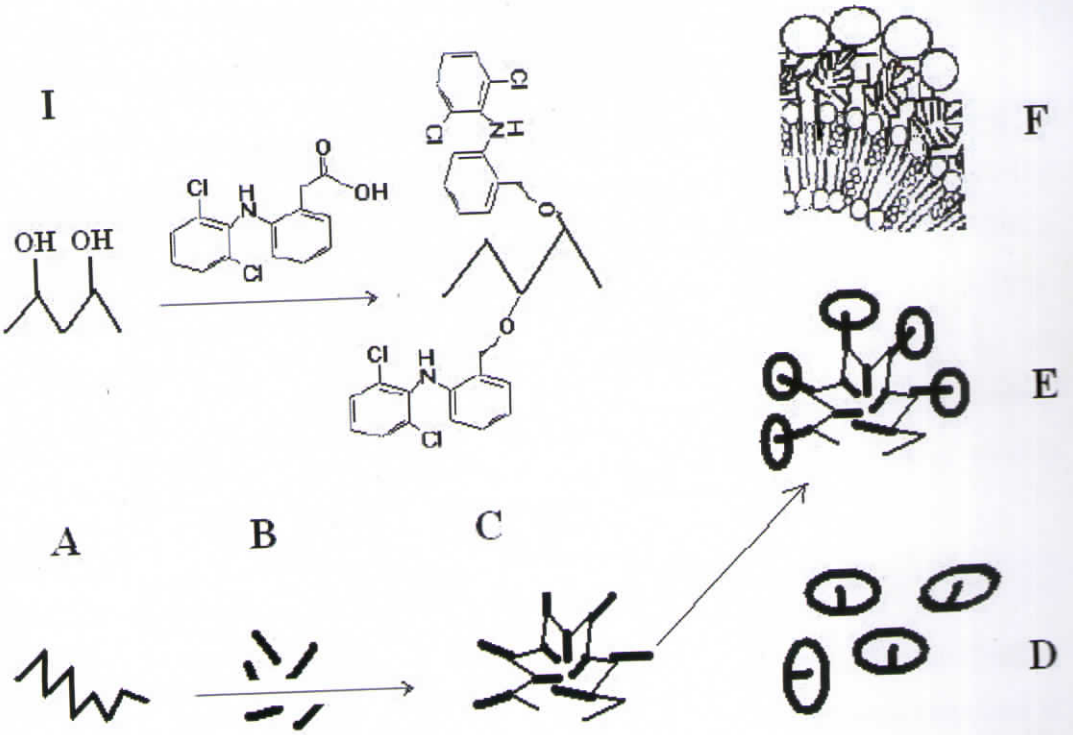


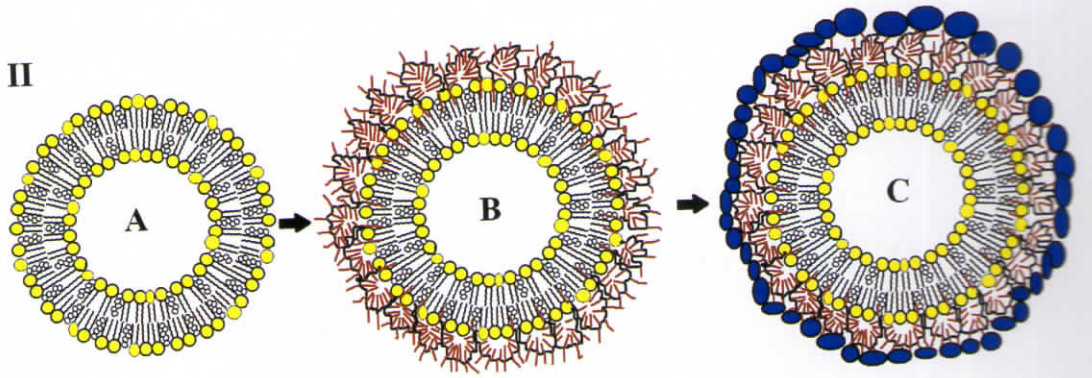
Chapter -5
Applications

APPLICATIONS

5.1 Albumin self assembled liposomes

Albumin-adsorbing surfaces are famously, haemocompatible. However guided self-assembly rather than non-specific adsorption improves the functional proficiency of the protein layer. Here we are proposing a novel ligand- receptor mediated self-assembly of albumin for the surface modification of nano-systems. For that PVADICa was coated over liposomes. This was further coated with albumin molecule. This system demonstrates the albumin self-assembly, over the modified liposomes, in presence of other proteins and it could be explored for enhancing the circulation time of the liposomes and other nanosystems.





Scheme-5.1.1 Schematic representation of the albumin self assembled liposomes. PVA (A), DIC (B), PVADICa (C), Albumin (D), Albumin self assembled PVADICa (E), Albumin self- assembled liposomes (F). Scheme-II, Liposomes (A), Polymer coated liposomes (B), Albumin self- assembled liposomes (C)

5.1.1 Drug immobilization into the polymer

The immobilization of the drug to the polymer has been evaluated by quantifying the drug content of the supernatant solution. Here we have chosen the particular composition (with drug to polymer ratio of 0.025:1w/w) PVA-DICa pendant polymer, where the drug pay load has been evaluated to be 6.58+/-0.12%w/w, and is water soluble. The other drug compositions PVA-DICb&c are water insoluble and are not useful for the said purpose. The liposome binding affinity (3.5.2.3.2.1.1) of the pendant polymer and its albumin binding avidity (3.5.2.3.2.2, 3.5.2.3.8 & 3.5.2.2.1) has already discussed.

5.1.2 Gross structure of the albumin coated liposomes

Further the gross structure of the albumin molecule has been studied by TEM (as per the procedure 2.2.18.1). The Figure-5.1.1 shows the gross structure of the albumin self-assembled liposomes. All the samples has been negatively stained. To get the surface morphology of the coated liposomes we have dried the liposomes for 24 h, till the liposomes spread over the surface. The schematic representation of the albumin self-assembled liposomes and its preparation is shown in Scheme-5.1.1. This illustrates the overall form of the liposomes.

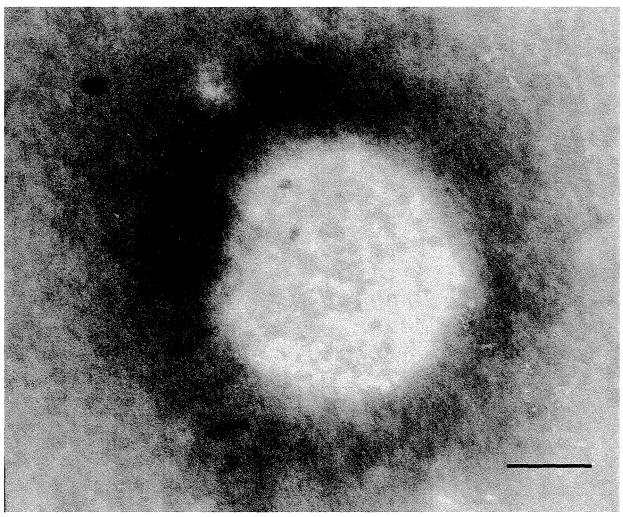


Figure-5.1.1: TEM of the albumin coated liposomes. (Mag = X 50, 000), (bar =15nm)

The understanding of the supramolecular chemistry which operates between the pendant polymer and the liposome as well as the self-assembling of albumin over the liposomes, is an important development, with implications for both pendant polymer and liposomes. This research outcome has far reaching implications for self-assembling of proteins, using designed chemical moieties. Apart from that it sends light to a new method of surface modification of nanosystems for various applications like implantable electrodes, drug delivery systems, tissue engineering devices etc. With this development it may be possible to increase the circulation time of the liposomes. The recent progress in the development of targeted drug delivery systems has specificity under in vitro conditions, but loses its specificity in vivo. The self-assembly of albumin around the liposomes gives another opportunity in this direction to hide the target molecules into the supramolecular assembly.

Application-5.2: Thermoresponsiveness of the amphiphilic polymer

Lipid monolayers are self-assembled systems, formed through intermolecular interaction of the hydrophobic straight chains of the lipid amphiphiles. The self-assembly is temperature dependent. Even though they didn't show change in polarity with respect to temperature, they can create a dynamic interface, which can disturb the ECM protein layer at different temperature condition. Further the thin film of nanoparticles formed out of the PVADICc pendant polymer has shown temperature dependent polarity differences like PNIPAAM. We have attempted to check the thermoresponsiveness of these modified surfaces towards cell removal. Macrophage adhesion studies were done with RAW 264.7 macrophage cell line (as per the procedure 2.3.3.5). For that the PS tissue culture plates were modified as per the procedure 2.2.4.1.2.

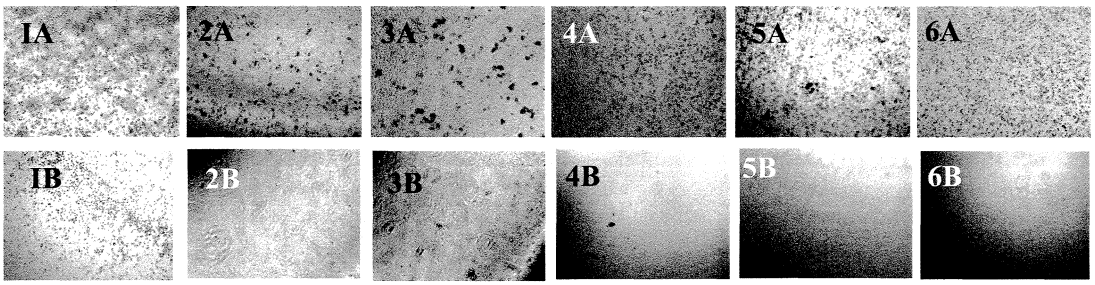


Figure5.2.1: Macrophage cell removal after changing the temperature from 25 to 10°C for 10min and washing with sterile PBS .Before washing (A), After Washing (B), Bare polymer (1), Modified with OCMC (2), PCMCc (3), PVADICc(TRP) (4), TRP+OCMC(5), TRP+PCMCc(6)

The figure-5.2.1 demonstrates that all the modified surfaces are thermo-responsive. When the temperature is reduced from 25 to 10°C the adhered cells are removed. Almost all the cells adhered on TRP modified surface has been removed. Therefore cell layer removal studies has been done with this particular surface after 5days (The medium has been changed on every alternate day) culture.

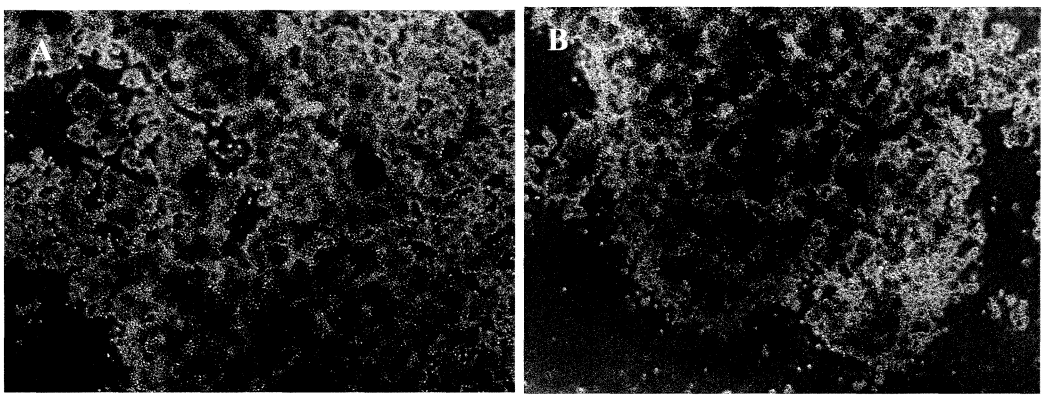
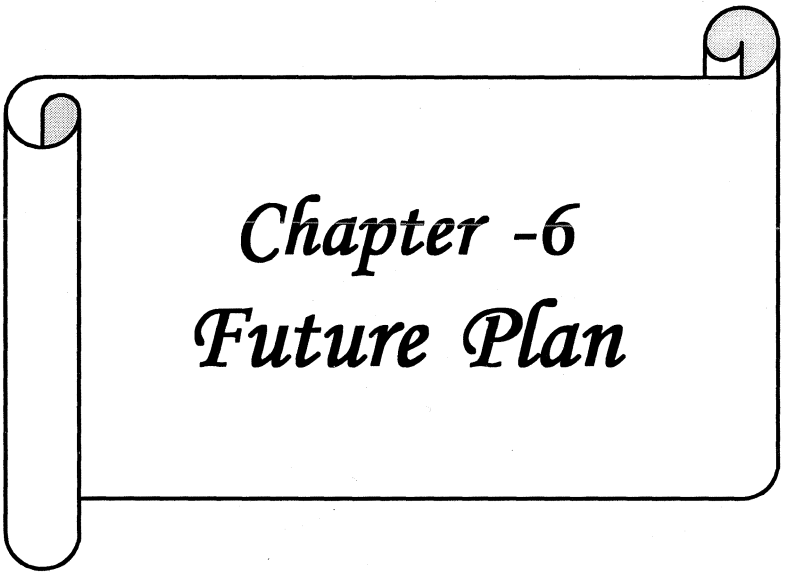


Figure-5.2.2: Macrophage Cell layer pealing off, after changing the temperature from 25 to 10°C for 5min. Before (A), After (B)

The figure-5.2.2 demonstrates that when the temperature is changed from 25 to 10°C the cell layer is getting pealed off without any assistance. The mechanism of change of polarity with respect to the temperature is already discussed (3.5.2.3.7). This is an important development, as it can be explored for cell layer tissue engineering.



Chapter -6
Future Plan

FUTURE PLAN

6.1 Modification of substrates for blood compatible applications

The thin films developed here by biomimetic strategies have tremendous opportunity for the surface modification of blood contacting devices. Since they are dynamic and renewable, it can elicit a sustained bioactive response for long time. In the case of devices with larger surface area, lipids can be entrapped into pits and which can form continuous renewable monolayer. The pendant polymer can further invite albumin from the blood and prolong the bioactive response in absence of the lipid layer. This strategy can be explored for the surface modification of stents, small diameter vascular grafts etc.

In the case of miniaturized devices these thin films can be self-assembled over the surface by physical means. The core of these self-assembled architectures could be explored as a compartment for the synthesis of nanoparticles, which can be applied for drug delivery imaging or tissue regenerative applications.

6.2 Modification of substrates for tissue compatible applications

These thin films could also be explored for the post synthetic surface modification of tissue engineering devices. Where it can do controlled ligand supplementation for regeneration of the tissue. On the other hand they can act as thermoresponsive surface for cell layer tissue engineering. They can also reduce biofriction and can be explored towards boundary lubrication for rheumatoid arthritis or as a lung surfactant. Here incorporation of proteins into the monolayer can be explored as a cheap substitute for the surfactant proteins.

6.3 Modification of substrates for drug delivery applications

These thin films have tremendous opportunity in the surface modification of drug delivery systems. Particularly for drug targeting, the compartmentalization efficiency added with controlled ligand supplementation and the antifouling properties could be explored for the targeting applications. It can also be explored for local systemic drug delivery as a liposome delivering matrix. Here a combination of drugs can be delivered at different rates to the inflammatory site.

These systems can be developed in 2D or 3D form for the various therapeutic applications. These thin films are versatile in their functional aspect ratio and could be explored for bottom up or post synthetic surface modification of miniaturized devices. It has numerous therapeutic potential as demonstrated in the thesis and further studies are planned in these directions.

REFERENCES

1. Ratner BD, Bryant SJ. Biomaterials: where we have been and where we are going. Annu Rev Biomed Eng. 2004; 6:41-75.

2. Von Recum, A. F., Handbook of Biomaterials Evaluation, Taylor & Francis, 1999.

3. Kakisis JD, Liapis CD, Breuer C, Sumpio BE. Artificial blood vessel: the Holy Grail of peripheral vascular surgery. J Vasc Surg. 2005; 41(2):b 349-54.

4. Daly CD, Campbell GR, Walker PJ, Campbell JH. In vivo engineering of blood vessels., Front Biosci. 2004; 1;9: 1915-24.

5. Hopkins RA. Tissue engineering of heart valves: decellularized valve scaffolds. Circulation. 2005; 31, 111(21): 2712-4.

6. Vacanti, JP, Langer R. Tissue engineering: the design and fabrication of living replacement devices for surgical reconstruction and transplantation. Lancet. 1999; 354, 1:SI32-4.

7. Chan C, Berthiaume F, Nath BD, Tilles AW, Toner M, Yarmush ML, Hepatic tissue engineering for adjunct and temporary liver support: critical technologies. Liver Transpl. 2004; 10(11): 1331-42.

8. Mooney DJ, Kim BS, Vacanty JP, Langer R, Atala A. tissue engineering of genitourinary system, In: Principles of Tissue engineering, Edited by R. P. Lanza, R. Langar, W. L. Chick, Academic Press, NY, 1997, 591- 600.

9. Tiranathanagul K, Eiam-Ong S, Humes HD. The future of renal support: high-flux dialysis to bioartificial kidneys. Crit. Care Clin. 2005, 21(2): 379-94.

10. Lanza RP, Chick WT. Endocrinology: Pancreas., In: Principles of Tissue engineering., Edited by R. P. Lanza, R. Langer, W. L. Chick, Academic Press, New York, 1997, 405- 425.

11. Miyamoto M. Current progress and perspectives in cell therapy for diabetes mellitus. Hum Cell. 2001, 14(4): 293-300.

12. Nerem RM, Ensley AE. The tissue engineering of blood vessels and the heart.Am J Transplant.4 Suppl 2004, 6:36-42.

13. Langer R, Vacanti JP. Tissue engineering. Science. 1993, 14; 260(5110): 920-6.

14. van der Voort van Zyp J, Conway WC, Thamilselvan V, Polin L, Basson MD. Divalent cations influence colon cancer cell adhesion in a murine transplantable tumor model. Am J Surg. 2005, 190(5): 701-7.

15. Dvorak MM, Riccardi D. Ca2+ as an extracellular signal in bone Cell, Calcium. 2004, 35(3): 249-55.

16. Mehrotra, R., Disordered mineral metabolism and vascular calcification in nondialyzed chronic kidney disease patients. J Ren Nutr. 2006, 16(2): 100-18.

17. Kaladhar. K.; Sharma, CP, "Surface passivation and controlled ligand supplementation of cellular activation processes- Strategies for bottom up synthesis of bioactive surfaces", Trends Biomater. Artif. Organs, 2007, Vol 21(1), 29-62.

18. Ertel SI, Chilkoti A, Horbett TA, Ratner BD. Endothelial cell growth on oxygen-containing films deposited by radio-frequency plasmas: the role of surface carbonyl groups. J Biomater Sci Polym Ed. 1991; 3(2): 163-83.

19. Steele JG, Johnson G, McFarland C, Dalton BA, Gengenbach TR, Chatelier RC,. Underwood PA, Griesser HJ. Roles of serum vitronectin and fibronectin in initial attachment of human vein endothelial cells and dermal

fibroblasts on oxygen- and nitrogen-containing surfa ces made by radiofrequency plasmas. J Biomater Sci Polym Ed. 1994, 6(6): 511-32.

20. Skarja GA, Brash JL. Physicochemical properties and platelet interactions of segmented polyurethanes containing sulfonate groups in the hard segment. J Biomed Mater Res. 1997; 15; 34(4): 439-55.

21. Barbosa JN, Barbosa MA, Aguas AP.Inflammatory responses and cell adhesion to self-assembled monolayers of alkanethiolates on gold. Biomaterials. 2004; 25(13):2557-63.

22. Dahners LE, Mullis BH. Effects of nonsteroidal anti-inflammatory drugs on bone formation and soft-tissue healing. J Am Acad Orthop Surg. 2004, 12(3): 139-43.

23. Sharma CP, Hari PR. The effect of anasthetic and analgesics on protein adsorption, platelet adhesion, and plasma recalcification time at blood polymer interface, Artif. Organs, 1991, 15,498.

24. Kim YH, Han DK, Park KD, Kim SH. Enhanced blood compatibility of polymers grafted by sulfonated PEO via a negative cilia concept. Biomaterials. 2003, 24(13):2213-23.

25. Di Martino A, Sittinger M, Risbud MV. Chitosan: a versatile biopolymer for orthopaedic tissue-engineering. Biomaterials. 2005, 26(30):5983-90.

26. Goda T, Ishihara K. Soft contact lens biomaterials from bioinspired phospholipid polymers. Expert Rev Med Devices. 2006, 3(2):167-74.

27. Leung MY, Liu C, Koon JC, Fung KP. Polysaccharide biological response modifiers. Immunol Lett. 2006, 15; 105(2): 101-14.

28. Hatakeyama H, Kikuchi A, Yamato M, Okano T. Influence of insulin immobilization to thermoresponsive culture surfaces on cell proliferation and thermally induced cell detachment. Biomaterials. 2005, 26(25): 5167-76.

29. Cobb BA, Kasper DL, Zwitterionic capsular polysaccharides: the new MHCII-dependent antigens.Cell Microbiol. 2005, 7(10):1398-403.

30. Bain CD, Manning-Benson S, Darton RC. Rates of Mass Transfer and Adsorption of Hexadecyltrimethylammonium Bromide at an Expanding Air-Water Interface. J Colloid Interface Sci, 2000; 1; 229(1): 247-256.

31. Leonard EF, Vroman L. Is the Vroman effect of importance in the interaction of blood with artificial materials? J Biomater Sci Polym Ed. 1991, 3(1):95-107.

32. Serrano AG, Perez-Gil J. Protein-lipid interactions and surface activity in the pulmonary surfactant system.Chem Phys Lipids. 2006, 141(1-2):105-18.

33. Bruck SD. Current activities and future directions inbiomaterials research, Ann. N.Y. Acad. Sci., 1977, 283, 332- 355.

34. Ito A, Ino K, Hayashida M, Kobayashi T, Matsunuma H, Kagami H, Ueda M, Honda H. Novel methodology for fabrication of tissue-engineered tubular constructs using magnetite nanoparticles and magnetic force. Tissue Eng. 2005, 11(9-10):1553-61.

35. Joung IS, Iwamoto MN, Shiu YT, Quam CT. Cyclic strain modulates tubulogenesis of endothelial cells in a 3D tissue culture model. Microvasc Res. 2006, 71(1): 1-11.

36. Dorsey WC, Ford BD, Roane L, Haynie DT, Tchounwou PB. Induced mitogenic activity in AML-12 mouse hepatocytes exposed to low-dose ultra-wideband electromagnetic radiation. Int J Environ Res Public Health. 2005, 2(1): 24-30.

37. Silver FH, Siperko LM. Mechanosensing and mechanochemical transduction: how is mechanical energy sensed and converted into chemical energy in an extracellular matrix. Crit Rev Biomed Eng. 2003, 31(4): 255-331.

38. Dmitrieva NI, Michea LF, Rocha GM, Burg MB. Cell cycle delay and apoptosis in response to osmotic stress. Comp Biochem Physiol A Mol Integr Physiol. 2001, 130(3): 411-20.

39. Szabo I, Kappelmayer J, Alekseev SI, Ziskin MC. Millimeter wave induced reversible externalization of phosphatidylserine molecules in cells exposed in vitro. Bioelectromagnetics. 2006, 27(3): 233-44.

40. Kikuchi A, Okano T. Nanostructured designs of biomedical materials: applications of cell sheet engineering to functional regenerative tissues and organs. J Control Release. 2005, 3; 101(1-3):69-84.

41. Ingber DE. The mechanochemical basis of cell and tissue regulation. Mech Chem Biosyst. 2004, 1(1):53-68.

42. Lyman DJ, Muir WM, Lee IJ. The effect of chemical structure and surface properties of polymers on the coagulation of blood. i. surface free energy effects. Trans Am Soc Artif Intern Organs. 1965; 11:301-6.

43. Baier RE. The role of surface energy in thrombogenesis. Bull N Y Acad Med. 1972; 48(2):257-72.

44. Andrade JD, Lee HB, Jhon MS, Kim SW, Hibbs JB Jr. Water as a biomaterial. Trans Am Soc Artif Intern Organs. 1973; 19: 1-7.

45. Hoffman AS. Principles governing biomolecule interactions at foreign interfaces. J Biomed Mater Res. 1974; 8(3):77-83.

46. Sharma CP. Surface--interface energy contributions to blood compatibility. Biomater Med Devices Artif Organs. 1984-1985, 12(3-4): 197-213.

47. Ikada Y, Iwata H, Horii F, Matsunaga T, Taniguchi M, Suzuki M, Taki W, Yamagata S, Yonekawa Y, Handa H. Blood compatibility of hydrophilic polymers.J Biomed Mater Res. 1981, 15(5):697-718.

48. Curtis A. Wilkinson C. New depths in cell behaviour: reactions of cells to nanotopography. Biochem. Soc. Symp. 1999; 65:15-26.

49. Gao L, McCarthy TJ. The "lotus effect" explained: two reasons why two length scales of topography are important. Langmuir. 2006 Mar 28;22(7):2966-7.

50. Graziano A, d'Aquino R, Cusella-De Angelis MG, Laino G, Piattelli A, Pacifici M, De Rosa A, Papaccio G. Concave pit-containing scaffold surfaces improve stem cell-derived osteoblast performance and lead to significant bone tissue formation. PLoS ONE. 2007, 6;2:496-501.

51. Arciola CR, Cenni E, Tarabusi C, Caramazza R, Pizzoferrato A. Corneal endothelium cell adhesion on intraocular lenses in vitro. J Appl Biomater. 1993; 4(3):249-52.

52. Noskov BA, Loglio G, Lin SY, Miller R. Dynamic surface elasticity of polyelectrolyte/surfactant adsorption films at the air/water interface: dodecyltrimethylammonium bromide and copolymer of sodium 2-acrylamido-2-methyl-1-propansulfonate with N- isopropylacrylamide. J Colloid Interface Sci. 2006; 15;301(2):386-94.

53. Svitova TF, Wetherbee MJ, Radke CJ. Dynamics of surfactant sorption at the air/water interface: continuous-flow tensiometry. J Colloid Interface Sci. 2003, 1;261(1):170-9.

54. Pedersen JA, Swartz MA. Mechanobiology in the third dimension. Ann. Biomed. Eng.; 2005, 33(11): 1469-90.

55. Roeder BA, Kokini K, Robinson JP, Voytik-Harbin SL. Local, three-dimensional strain measurements within largely deformed extracellular matrix constructs. J Biomech Eng. 2004, 126(6):699-708.

56. Aez A, Buguin A, Silberzan P, Ladoux B. Is the mechanical activity of epithelial cells controlled by deformations or forces Biophys J. 2005; 89(6):L52-4.

57. Du Roure O, Saez A, Buguin A, Austin RH , Chavrier P, Silberzan P, Ladoux B. Force mapping in epithelial cell migration Proc. Natl. Acad. Sci. 2005, 15, 102 7, 2390-2395.

58. Ingber DE. Mechnical signaling and cellular response to Extra cellular matrix in angiogenesis and cardiovascular physiology, Circ. Res. 2002, 91, 877- 887.

59. Rizzo V, Sung A, Oh P, Schnitzer JE, Rapid mechanotransduction in situ at the luminal cell surface of vascular endothelium and its caveolae. J. Biol. Chem. 1998, 273, 26323–26329.

60. Ingber DE, Cellular mechanotransduction: putting all the pieces together again. FASEB J. 2006, 20, 811-827.

61. Clark ER, Clark EL. Microscopic observations on the growth of blood capillaries in the living mammal. Am J Anat. 1938, 64: 251–301.

62. Takagi J. Structural basis for ligand recognition by RGD (Arg-Gly-Asp)-dependent integrins. Biochem Soc Trans. 2004, 32(Pt3): 403-6.

63. Itoh S, Matsuda A, Kobayashi H, Ichinose S, Shinomiya K, Tanaka J. Effects of a laminin peptide (YIGSR) immobilized on crab-tendon chitosan tubes on nerve regeneration. J Biomed Mater Res B Appl Biomater. 2005; 73(2): 375-82.

64. Matsuda A, Kobayashi H, Itoh S, Kataoka K, Tanaka J. Immobilization of laminin peptide in molecularly aligned chitosan by covalent bonding. Biomaterials. 2005; 26(15):2273-9.

65. Hunter DD, Cashman N, Morris-Valero R, Bulock JW, Adams SP, Sanes JR. An LRE (leucine-arginine-glutamate)-dependent mechanism for adhesion of neurons to S-laminin. J Neurosci. 1991, 11(12): 3960-71.

66. Girotti A, Reguera J, Rodriguez-Cabello JC, Arias FJ, Alonso M, Matestera A. Design and bioproduction of a recombinant multi(bio)functional elastin-like protein polymer containing cell adhesion sequences for tissue engineering purposes. J Mater Sci Mater Med. 2004; 15(4): 479-84.

67. Mizuno M, Fujisawa R, Kuboki Y, Type I collagen-induced osteoblastic differentiation of bone-marrow cells mediated by collagen-alpha2beta1 integrin interaction. J Cell Physiol. 2000; 184(2): 207-13.

68. Maeda T, Oyama R, Titani K, Sekiguchi K. Engineering of artificial cell-adhesive proteins by grafting EILDVPST sequence derived from fibronectin. Biochem (Tokyo). 1993; 113(1):29-35.

69. Floquet N, Hery-Huynh S, Dauchez M, Derreumaux P, Tamburro AM, Alix AJ. Structural characterization of VGVAPG, an elastin-derived peptide. Biopolymers, 2004, 76(3): 266-80.

70. Jenney CR, Anderson JM. Adsorbed serum proteins responsible for surface dependent human macrophage behavior. J Biomed Mater Res. 2000;15; 49(4): 435-47.

71. Unsworth LD, Sheardown H, Brash JL. Polyethylene oxide surfaces of variable chain density by chemisorption of PEO-thiol on gold: adsorption of proteins from plasma studied by radiolabelling and immunoblotting. Biomaterials. 2005; 26(30): 5927-33.

72. Krishnan A, Siedlecki CA, Vogler EA. Mixology of protein solutions and the Vroman effect. Langmuir. 2004; 8; 20(12): 5071-8.

73. Noh, H., E. A. Vogler. Volumetric interpretation of protein adsorption: Competition from mixtures and the Vroman effect. Biomaterials. 2007 Jan;28(3):405-22.

74. Kaladhar, K; Sharma, CP; Modification of chitosan using N- aryl anthranilic acid derivative for blood compatible applications, International

Symposium on Advanced Materials and Processing; Dec 6-8, IIT Kharagpur., India, 2004.

75. Chun MH. Serum signaling factors and spheroids Crit Rev Oncol Hematol., 2000; 36, 2-3: 89-98.

76. Hunt AN, Skippen AJ, Koster G, Postle AD, Cockcroft S. Acyl chain-based molecular selectivity for HL60 cellular phosphatidylinositol and of phosphatidylcholine by phosphatidylinositol transfer protein α. Biochim Biophys Acta - Mol. Cell Biol. of Lipids 2004; 1686: 50-60.

77. Yamaji-Hasegawa A, Tsujimoto M. Asymmetric distribution of phospholipids in biomembranes. Biol Pharm Bull. 2006; 29(8):1547-53.

78. Stepanenko Iiu, Strakhovskaia MG, Belenikina NS, IuA. Nikolaev AL, Miliukin AN, Kozlova A. Revina A, El'-Registan GI, Protection of Saccharomyces cerevisiae against oxidative and radiation-caused damage by alkyl hydroxybenzenes.; Mikrobiologiia. 2004; 73(2):204-10.

79. Zhu Y, Granick S. Viscosity of interfacial water. Phys Rev Lett., 2001, 27; 87(9): 096104-8.

80. Alonso C, Zasadzinski JA Linear dependence of surface drag on surface viscosity. Phys Rev E Stat Nonlin Soft Matter Phys. 2004; 69(2 Pt 1): 021602.

81. Greenfield ML, Ohtani H. Packing of simulated friction modifier additives under confinement. Langmuir. 2005; 2;21(16):7568-78.

82. Starov VM. Surfactant solutions and porous substrates: spreading and imbibition. Adv Colloid Interface Sci. 2004; 29;111(1-2):3-27.

83. Vilmin T, Raphael E. Dynamic instability of thin viscoelastic films under lateral stress. Phys Rev Lett. 2006; 21;97(3):036105.

84. Serrano AG, Perez-Gil J. Protein-lipid interactions and surface activity in the pulmonary surfactant system. Chem. Phys. Lipids. 2006; 141(1-2):105-18.

85. Serro AP, Bastos M, Pessoa JC, Saramago B. Bovine serum albumin conformational changes upon adsorption on titania and on hydroxyapatite and their relation with biomineralization. J Biomed Mater Res A. 2004; 1;70(3):420-7.

86. Krishnan A, Cha P, Liu YH, Allara D, Vogler EA. Hematology at Biomaterial Interfaces Research Group Interfacial energetics of blood plasma and serum adsorption to a hydrophobic self-assembled monolayer surface. Biomaterials. 2006; 27(17):3187-94.

87. Sethuraman A, Han M, Kane RS, Belfort G. Effect of surface wettability on the adhesion of proteins Langmuir. 2004; 31; 20(18): 7779-88.

88. Eberhart RC, Munro MS, Williams GB, Kulkarni PV, Shannon Jr WA, Brink BE, Fry JW. Albumin adsorption and retention on C18-alkyl-derivatized polyurethane vascular grafts. Artif Organs. 1987; 11(5):375-82.

89. Zwaal RFA, Comfurius P Van deenen IIM. Membrane asymmetry and blood coagulation, *Nature* 1977; 268, 358 – 360.

90. Baier RE."An overview of biomaterials", Edited by Sharma CP, Szycher M, Blood Compatible Materials and Devices, CRC Press, New York, 1991; 1-3.

91. Xu Y, Lorf T, Sablinski T, Gianello P, Bailin M, Cooper RDKC, Monroy DH, Kozlowski T, Removal of anti-porcine natural antibodies from human and nonhuman primate plasma in vitro and in vivo by a Gal1–3Gal1–4Glc–X immunoaffinity column.Transplantation, 1998; 65: 172-179.

92. Brash JL. "Role of plasma protein adsorption in the response of blood to foreign surfaces- An overview of biomaterials", Edited by Sharma CP, Szycher M. Blood Compatible Materials and Devices, CRC Press, 1991; 3-25.

93. Anderson JM. et.al., "Host reaction to biomaterials and their evaluation", In: 2nd edition, Biomaterial Science, An introduction to materials in medicine, Edited by Ratner, BD, Hoffman AS, Schoen FJ, Lemons JE, Elsevier Academic Press pp. 2004; 237- 291.

94. Miyamoto S, Teramoto H, Coso OA, Gutkind JS, Burbelo PD, Akiyama SK, Yamada KM, Integrin function: molecular hierarchies of cytoskeletal and signaling molecules. J. Cell Biol. 1995, 131 791–805.

95. Galbraith CG, Yamada KM, Sheetz MP. The relationship between force and focal complex development. J. Cell Biol. 2002;159 pp. 695–705.

96. Balaban NQ, Schwarz US, Riveline D, Goichberg P, Tzur G, Sabanay I, Mahalu D, Safran S, Bershadsky AAddadi, L, Geiger B. Force and focal adhesion assembly: a close relationship studied using elastic micropatterned substrates Nat. Cell Biol. 2001; 3: 466–472.

97. Ingber DE. Cancer as a disease of epithelial mesenchymal interaction and extracellular matrix regulation, Differentiation, 2002; 70, 547- 560.

98. Parent CA, Devreotes PN. A cell's sense of direction, science, 1999; 284, 765-769.

99. Ridley AJ, Paterson HF, Johnston CL, Diekmann D, Hall A. The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. Cell, 1992, 70, 401–410.

100. Pelham RJ Jr, Wang YL. Cell locomotion and focal adhesions are regulated by substrate flexibility. Proc. Natl.Acad. Sci. USA 1997, 94, 13661–13665.

101. Dedhar SB, Hannigan WG. Integrin-linked kinase (ILK): a regulator of integrin and growth-factor signalling. Trends Cell Biol. 1999; 9(8): 319-23.

102. Takahashi MO, Takahashi Y, Iida K, Okimura Y, Kaji H, Abe H, Chihara K. Growth hormone stimulates tyrosine phosphorylation of focal adhesion kinase (p125(FAK)) and actin stress fiber formation in human osteoblast-like cells, Saos2. Biochem. Biophys. Res. Commun. 1999; 16; 263(1): 100-6.

103. Kunitake T. Synthetic bilayer membranes: molecular design, self-organization, and application. Angew Chem Int Ed Engl, 1992; 31: 709-726.

104. Vauthey S, Santoso S, Gong H, Watson N, Zhang S. Molecular self assembly of surfactant-like peptides to form nanotubes and nanovesicles. Proc Natl Acad Sci USA,; 2002; 99: 5355-5360.

105. Velonia K, Rowan AE, Nolte RJM. Lipase polystyrene giant amphiphiles. J Am Chem Soc, 2002; 124: 4224-4225.

106. Kimizuka N, Kawasaki T, Hirata K, Kunitake T. Supramolecular membranes. Spontaneous assembly of aqueous bilayer membrane via formation of hydrogen bonded pairs of melamine and cyanuric acid derivatives. J Am Chem Soc, 1998; 120: 4094-4104.

107. Kaler EW, Murthy AK, Rodriguez B, Zasadzinski JA. Spontaneous vesicle formation in aqueous mixtures of single-tailed surfactants. Science, 1989; 245:1371-1374.

108. Hayward JA, Chapman D. Biomembrane surfaces as models for polymer design: the potential for haemocompatibility.Biomaterials 1984; 5, 135.

109. Svedhem S, Dahlborg D, Ekeroth J, Kelly J, Ho¨o¨k F, Gold J. In Situ Peptide-Modified Supported Lipid Bilayers for Controlled Cell Attachment. Langmuir, 2003; 19, 6730.

110. Ishihra K, Aragaki R, Ueda T, Watanabe A, Nakabayashi N. Reduced thrombogenicity of polymers having phospholipid polar groups J. Biomed. Mater. Res., 1990; 24, 1069.

111. Ishihara K, Fujita H, Yoneyama T, Iwasaki Y. Antithrombogenic polymer alloy composed of 2-methacryloyloxyethyl phosphorylcholine polymer and segmented polyurethane. J Biomater Sci Polym Ed. 2000; 11(11):1183-95.

112. Ishihara K, Fukumoto K, Iwasaki Y, Nakabayashi N. Modification of polysulfone with phospholipid polymer for improvement of the blood compatibility. Part 2. Protein adsorption and platelet adhesion. Biomaterials, 1999; 20, 1553.

113. Virtanen A, Cheng KH, Somerharjus P. Phospholipid composition of the mammalian red cell membrane can be rationalized by a superlattice model. Proc. Natl. Acad. Sci. U.S.A., 1998; 95, 4964.

114. Keller SL, Pitcher III WH, Huestis WH, McConnell HM. Red Blood Cell Lipids Form Immiscible Liquids Phys. Rev. Lett. 1998; 81, 5019.

115. Demel RA, Geurts Van Kessel WSM, Zwaal RFA, Roelofsen B, Van Deenen LLM. Relation between various phospholipase actions on human red cell membranes and the interfacial phosholipid pressure in monolayers, Biochim. Biophys. Acta, 1975; 406, 97.

116. Dupree JL, Suzuki KPB. Galactolipids in the formation and function of the myelin sheath Microsc. Res. Tech., 1998; 41 (5), 431.

117. Verkleij AJ, Zwaal RFA, Roelofsen B, Comfurius P, Kastelijn D, van Deenen LL. Phospholipid composition of the mammalian red cell membrane can be rationalized by a superlattice model, Biochim Biophys Acta. 1973; 11, 323, 2, 178.

118. Bevers EM, Comfurius P, Dekkers DW, Zwaal RFA. Lipid translocation across the plasma membrane of mammalian cells. Biochim Biophys Acta. 1999; 18, 1439, 3, 317.

119. Bevers EM, Comfurius P, Dekkers DW, Harmsma M, Zwaal RFA. Regulatory mechanisms of transmembrane phospholipid distributions and

pathophysiological implications of transbilayer lipid scrambling Lupus. 1998; 7, 2, S126.

120. Frasch SC, Henson PM, Kailey JM, Richter DA, Janes MS, Fadok VA, Bratton DL. Regulation of phospholipid scramblase activity during apoptosis and cell activation by protein kinase Cdelta. J Biol Chem., 2000; 28, 275, 30, 23065.

121. Pereira J, Palomo I, Ocqueteau M, Soto M, Aranda E, Mezzano D. Platelet aging in vivo is associated with loss of membrane phospholipid asymmetry,Thromb Haemost., 1999; 82, 4, 1318-21.

122. Schwartz RS, Tanaka Y, Fidler IJ, Chiu DT, Lubin B, Schroit AJ. Increased adherence of sickled and phosphatidylserine-enriched human erythrocytes to cultured human peripheral blood monocytes. J Clin Invest. 1965, 1985, 75, 6.

123. Fadok VA, de Cathelineau A, Daleke DL, Henson PM, Bratton DL. Loss of Phospholipid Asymmetry and Surface Exposure of Phosphatidylserine Is Required for Phagocytosis of Apoptotic Cells by Macrophages and Fibroblasts J Biol Chem. 2001; 12, 276, 2, 1071.

124. Robert G, Ed. Langmuir- Blodgett Films, Plenum Press, New York 1990.

125. Petty MC. Possible applications for. Langmuir–Blodgett films, Thin solid films, 1992; 417, 210-211.

126. McKiernan AE, Ratto TV, Longo ML. Domain growth, shapes, and topology in cationic lipid bilayers on mica by fluorescence and atomic force microscopy. Biophys J. 2000; 79(5): 2605–2615.

127. J-M Lehn, Supramolecular chemistry, Science, 1993, 260, 1762.

128. Fuhrhop JH, Koning J. Membranes and Molecular Assembles: The Synkinetic Approach , The Royal Society of Chemistry, Cambridge, 1994.

129. Gattuso G, Nepogodiev SA, Stoddart JF. Synthetic Cyclic Oligosaccharides. Chem Rev. 1998 Jul 30;98(5):1919-1958.

130. Tanford C. Formation of micelles & Biological membranes In: The hydrophobic effect, Wiley Interscience, New York, 1973

131. Kauzmann W. Some factors in the interpretation of protein denaturation. Adv. Protein chem. 1959; 14, 1-5.

132. Bglockzijl W, Eugberts JBFN. Hydrophobic effects. Opinions. and facts. Angew. Chem. Int. Edn. Engl. 1993; 32, 1545-79.

133. Kunitake T, Synthetic bilayer-membranes—molecular design, self-organization, and application. Angew. Chem. Int. Edn. Engl. 1992; 31, 709-26.

134. Elemans JAAW, Rde Gelder AE, Rowman AE, Nolte RJM. Bipyridine functionalized molecular clips. Self-assembly of their ruthenium complexes in water, Chem. Commun. 1998; 1553.

135. Sommerdijk NAJM, Buynsters PJJA, Pistourius AMA, Wang M, Feiters MC, Notte RJM, Zwanenburg B. Tuning the Supramolecular Expression of Chirality: Phospholipid Analogues containing Amide linkages J. Chem. Soc. Chem. Commun. 1994; 1941-42.

136. Hafkamp RJH, Feiters MC, Nolte RJM. Tunable supramolecular structures from a gluconamide containing imidazole, Angew. Chem. Int. Ed. Eng. Engl. 1994; 33, 986-7.

137. Schnur JM, Lipid tubules: a paradigm for molecularly engineered structures. Science, 1993; 262, 1669- 1676.

138. Nakashima N, Sakuma S, Kunitake T. Optical microscopic study of helical superstructures on chiral bilayer membranes J. Am. Chem. Soc. 1989; 111, 4567- 4570.

139. Frankel DA, O'Brian DF. Supramolecular assemblies of diacetylenic aldonamides J. Am. Chem. Soc. 1994; 116, 22, 10057- 10069.

140. Fuhrhop JH, Helfrich W. Fluid and Solid Fibers Made of Lipid Molecular Bilayers Chem Rev. 1993; 1565- 1582.

141. Israelachivilli JN, Mitchel DJ, Ninham BW. Theory of Self-Assembly of Hydrocarbon Amphiphiles into Micelles and Bilayers. J. Chem. Soc. Faraday Trans., 1976; 2. 72, 1525.

142. Langmuir I. The Constitution and Fundamental Properties of. Solids and Liquids, J. Am. Chem. Soc., 1917; 39, 1848.

143. Blodgett KB. Films built by depositing successive unimolecular. layers on a solid surface J. Am. Chem. Soc. 1935; 57, 1007.

144. Harkins WD, The physical Chemistry of surface films, Reinhold, New York, 1952.

145. Kuhner M, Tampe R, Sackmann E. Lipid mono- and bilayer supported on polymer films: composite polymer-lipid films on solid substrates. Biophys J. 1994; 67(1): 217-26.

146. Boxer SG. Molecular transport and organization in supported lipid membranes, Curr. Opin. in Chem. Biol. 2000; 4: 704–709.

147. Nissen J, Gritsch S, Wiegand G, Rädler JO. Wetting of phospholipid membranes on hydrophilic surfaces — concepts towards self-healing membranes. Eur Phys J B, 1999; 10: 335-344.

148. Cremer PS, Boxer SG. Formation and spreading of lipid bilayers on planar glass supports. J Phys Chem, 1999; 103: 2554-2559.

149. Kloboucek A, Behrisch A, Faix J, Sackmann E. Adhesion-induced receptor segregation and adhesion plaque formation: A model membrane study. Biophys J. 1999; 77(4): 2311–2328.

150. Nelson KD, Eisenbaumer R, Pomerantz M, Eberhart RC. High affinity polyethylene oxide for improved biocompatibility. ASAIO J.; 1996; 42(5): M884-9.

151. Denizli A, Salih B, Kozluca A, Piskin E. Comparison of albumin binding capacities of three different reactive dye-derivatized poly(ethylene glycol dimethacrylate-hydroxyethyl methacrylate) microbeads. J Biomater Sci Polym Ed.; 1997; 8(6): 411-20.

152. Amiji M, Park H, Park K. Study on the prevention of surface-induced platelet activation by albumin coating. J Biomater Sci Polym Ed. 1992; 3(5): 375-88.

153. Kaladhar, K; Sharma CP;"Modification of chitosan using n-aryl anthranilic acid derivative for insulin delivery" Macro2004, "International Conference on Polymers for Advanced Technologies",) Dec 15-17, Thiruvananthapuram, India, 2004.

154. Tanford C, Reynolds J. Nature's robots: A history of proteins" In Roskoski Biochemistry and Molecular Biology Education. 2002; 30, 343-345.

155. du Roure O, Saez A, Buguin A, Austin RH, Chavrier P, Silberzan P, Ladoux B, Force mapping in epithelial cell migration. Proc Natl Acad Sci U S A. 2005; 15; 102(7): 2390-5.

156. Ghuman J, Zunszain PA, Pettipas I, Bhattacharya AA, Curry S. Human serum albumin complexed with myristate and azapropazone, PDB, PDB ID No: 2BXI, www.rcsb.org.

157. Singh N, Jabeen T, Sharma S, Bhushan A, Singh TP. Crystal structure of a complex formed between group II phospholipase A2 and aspirin at 1.86 A resolution, PDB, PDB ID No: 1TGM, www.rcsb.org.

158. Ghuman J, Zunszain PA, Pettipas I, Bhattacharya AA, Curry S. Human serum albumin complexed with myristate, azapropazone, and indomethacin,PDB, PDB ID No: 2BXK, www.rcsb.org.

159. Klabunde T, Petrassi HM, Oza VB, Kelly JW, Sacchettini JC. Crystal structure of human transthyretinin complex with diclofenac, PDB, PDB ID No: 1DVX, www.rcsb.org.

160. Thompson MA, ArgusLab 4.0.1, Planaria Software LLC, Seattle, WA, http://www.arguslab.com.

161. Takeuchi H, Kojima H, Yamamoto H, Kawashima Y. Polymer coating of liposomes with a modified polyvinyl alcohol and their systemic circulation and RES uptake in rats J. Cont. Rel, 2000, 68, 195.

162. Takeuchi H, Kojima H, Yamamoto H, Kawashima Y. Evaluation of circulation profiles of liposomes coated with hydrophilic polymers having different molecular weights in rats, J Control Release. 2001 Jul 10;75(1-2): 83-91.

163. KSV Instruments Application notes 2005.

164. S. Choudhury, R. Chitra, and J. V. Yakhmi, "Studies on the formation of Langmuir monolayer and Langmuir-Blodgett films of octadecyl amine-bromocresol purple dye complex," Thin Solid films, 2003; 44, 240- 246.

165. Nakajima N, Ikada Y. Mechanism of amide formation by carbodiimide for bioconjugation in aqueous media. Bioconjug. Chem. 1995; 6, 1:123-30.

166. Young JJ, Cheng KM, Tsou TL, Liu HW, Wang HJ. Preparation of cross-linked hyaluronic acid film using 2-chloro-1-methylpyridinium iodide or

water-soluble 1-ethyl-(3,3-dimethylaminopropyl)carbodiimide. J Biomater Sci Polym Ed. 2004; 15, 6: 767-80.

167. Takeuchi, H.; Kojima, H.; Yamamoto, H.; Kawashima, Y., *J. Cont. Rel*, 2000, 68, 195.

168. Kim K, Kim C, Byun Y. Preparation of a PEG-grafted phospholipid Langmuir-Blodgett monolayer for blood-compatible material. J. Biomed. Mater. Res. 2000; 15, 52, 4: 836- 40

169. Lowry O, Rosenberg N, Farr A, Randall R. Protein. measurement with the Folin phenol reagent. J. Biol. Chem, 1951, 193, 265.

170. Higuchi A, Sugiyama K, Yoon BO, Sakuri M, Hara M, Sumita M, Shuichi S, Shirai T. Serum protein adsorption and platelet adhesion on pluronic-adsorbed polysulfone membranes, Biomaterials 2003, 24, 3235.

171. Golomb G, Wagner D. Development of a new in vitro model for studying implantable polyurethane calcification, Biomaterials 1991, 12, 397.

172. Brown WE, Lehr JR, Smith JP, Frazier AW. Crystallography of octacalcium phosphate J. Amer. Chem. Soc. 1957, 79, 5318.

173. Heughebaert JC, Zawacki SJ, Nancollas GH. The growth of octacalcium phosphate on beta tricalcium phosphate J. Crystal growth, 1983.; 63, 83.

174. Chiu TC, Metcalf LC, Lyman DJ. Electrophoretic analysis of protein adsorbed on polymer surfaces. J. Biomed. Mater. Res. 1981, 15, 781.

175. Serro AP, Bastos M, Costa Pessoa J, Saramago B. Bovine serum albumin conformational changes upon adsorption on titania and on hydroxyapatite and their relation with biomineralisation. J Biomed Mater Res. 2004; 70A: 420- 427.

176. Alder AJ, Greenfield NJ, Fasman GD, Circular Dichroism and Optical Rotary Dispersion of Proteins and Polypeptides Meth. Enzymology 1973; 27, 675.

177. Andrade MA, Chacon P, Merelo JJ, Moran F. Evaluation of secondary structure of proteins from UV Circular Dichroism using an unsupervised learning neural network. Protein Eng. 1993; 6: 383- 390.

178. Merelo JJ, Andrade MA, Prieto A, Moran F. Proteoinitropic future maps. Neurocomputing, 1994; 6: 443- 454.

179. Serro AP, Bastos M, Costa Pessoa J, Saramago B. Bovine serum albumin conformational changes upon adsorption on titania and on hydroxyapatite and their relation with biomineralisation. J Biomed Mater Res. 2004; 70A: 420- 427.

180. Chandy T, Sharma CP. Changes in albumin/platelet interaction with an artificial surface--due to a antibiotics, pyridoxal phosphate, and lymphocytes Artif. Org. 1988, 12 (2), 143.

181. Faucheux N, Schweiss R, Lutzow K, Werner C, Groth T. Self-assembled monolayers with different terminating groups as model substrates for cell adhesion studies Biomaterials. 2004 25, 14, 2721.

182. Bruinsma GM, van der Mei HC, Busscher HJ. Bacterial adhesion to surface hydrophilic and hydrophobic contact lenses. Biomaterials. 2001. 22, 24, 3217.

183. Zhang WN, He HB, Feng YQ, Da SL. Separation of phosphatidylcholine and phosphatidylethanolamine by using high-performance displacement chromatography J Chrom A 2004, 1036, 2, 21 145-154.

184. Dong A, Meyer JD, Kendrick BS, Manning MC, Carpenter JF. Effect of secondary structure on the activity of enzymes suspended in organic solvents. Arch Biochem Biophys.1996; 15, 334, 2: 406-14.

185. Khmelnitsky YL, Mozhaev VV, Belova AB, Sergeeva MV, Martinek K. Denaturation capacity: a new quantitative criterion for selection of organic solvents as reaction media in biocatalysis. Eur. J. Biochem. 1991; 23, 198, 1: 31-41.

186. Kim K, Kim C, Byun Y. Preparation of a PEG-grafted phospholipid Langmuir-Blodgett monolayer for blood-compatible material. J. Biomed. Mater. Res., 2000; 15, 52, 4, 836- 40.

187. Larvor MP, Cerdan R, Gumila C, Maurin L, Seta P, Roustan C, Vial H. Characterization of the lipid-binding domain of the Plasmodium falciparum CTP:phosphocholine cytidylyltransferase through synthetic-peptide studies Biochem. J. 2003, 375, 653-58.

188. Mattjus P, Bittman R, Slotte JP. Molecular interactions and lateral domain formation in monolayers containing cholesterol and phosphatidylcholines with acyl- or alkyl-linked C16 chains, Langmuir 1996, 12, 1284-89.

189. Shah DO, Schulman JH. The ionic structure of lecithin monolayers J. Lipid Res. 1967, 8, 227-302.

190. Phillips MC, Chapman D. Monolayer characteristics of saturated 1,2,-diacyl phosphatidylcholines (lecithins) and phosphatidylethanolamines at the air-water interface, Biochim. Biophys. Acta. 1968, 163, 301-306.

191. Johnston DS, Chapman D. The properties of brain galactocerebroside monolayers, Biochim. Biophys. Acta- Biomembranes. 1988, 937, 10-22.

192. McConnell HM. Equilibration rates in lipid monolayers , PNAS, 24, 1996; 93(26): 15001 – 15003.

193. Frazier ML, Wright JR, Pokorny A, Almeida, PFF. Investigation of domain formation in sphingomyelin/cholesterol/POPC mixtures by fluorescence resonance energy transfer and Monte Carlo simulations. Biophys J. 2007, 1;92(7):2422-33.

194. Demel RA, Geurts Van Kessel WSM, Zwaal RFA, Roelofsen B, Van Deenen LLM. Relation between various phospholipase actions on human red cell membranes and the interfacial phospholipid pressure in monolayers. Biochim. Biophys. Acta, 1975, 406, 97.

195. Virtanen A, Cheng KH, Somerharjus P. Phospholipid composition of the mammalian red cell membrane can be rationalized by a superlattice model. Proc. Natl. Acad. Sci. USA., 1998, 95, 4964-9.

196. Keller SL, Pitcher WH III., Huestis WH, McConnell HM. Red Blood Cell Lipids Form Immiscible Liquids, Phys. Rev. Let., 1998, 81, 5019- 22.

197. Moon HT, Lee YK, Han JK, Byun Y. Improved blood compatibility by sustained release of heparin-deoxycholic acid conjugates in a PCL-PEG multiblock copolymer matrix. J. Biomater. Sci. Polym. Ed. 2002; 13, 7: 817-28.

198. Hattori T, Kimura K, Seyrek E, Dubin PL. Binding of bovine serum albumin to heparin determined by turbidimetric titration and frontal analysis continuous capillary electrophoresis. Anal Biochem. 2001; 15, 295, 2: 158-67.

199. Marsh D. Elastic constants of polymer-grafted lipid membranes. Biophys J, 2001; 81: 42154-2162.

200. Srisailam S, Kumar TK, Srimathi T, Yu C. Influence of backbone conformation on protein aggregation, J Am Chem Soc. 2002; 6, 124, 9: 1884-8.

201. Andrade MA, Chacon P, Merelo JJ, Moran F. Evaluation of secondary structure of proteins from UV Circular Dichroism using an unsupervised learning neural network. Protein Eng. 1993; 6: 383- 390.

202. Merelo JJ, Andrade MA, Prieto A, Moran F. Proteoinitropic future maps. Neurocomputing, 1994; 6: 443- 454.

203. Lenaz G. Lipid fluidity and membrane protein dynamics Biosci. Rep., 1987;7, 11: 823-37.

204. Osborn TD, Yager Y. Modeling success and failure of Langmuir-Blodgett transfer of phospholipid bilayers to silicon dioxide, Biophysical J. 1995, 68, 1364.

205. Briscoe WH, Titmuss S, Tiberg F, Thomas RK, McGillivray DJ, Klein J. Boundary lubrication under water Nature, 2006; 444, 191–194.

206. Sanii B, Parikh AN. Surface-energy dependent spreading of lipid monolayers and bilayers, Soft Matter, 2007, 3, 974-977.

207. Kim K, Kim C, Byun Y. Preparation of a stable phospholipid monolayer grafted onto a methacryloyl-terminated substrate as blood compatible materialsm. J. Biomater. Sci. Polym. Ed. 2003, 14, 9: 887-902.

208. Sheetz MP. Microtubule motor complexes moving membranous organelles. Cell. Struct. Funct. 1996; 21, 5: 369-73.

209. Tanford C. How protein chemists learned about the hydrophobic factor. Protein Sci. 1997; 6, 6: 1358-66.

210. Tanford C. The hydrophobic effect and the organization of living matter. Science 1978; 2, 200, 4345: 1012-8.

211. Lenaz G. Lipid fluidity and membrane protein dynamics Biosci. Rep., 1987;7, 11: 823-37.

212. Curry H, Mandelkow P, Franks BN. Crystal structure of human serum albumin complexed with fatty acid reveals an asymmetric distribution of binding sites. Nat. Struct. Biol. 1998; 5: 827.

213. Sharma CP, Sunny MC. Albumin adsorption on to aluminium oxide and polyurethane surfaces. Biomaterials. 1990; 11, 4: 255-7.

214. Nozaki Y, Reynolds J. A, Tanford C. The interaction of a cationic detergent with bovine serum albumin and other proteins. J. Biol. Chem., 1974; 25, 249, 14: 4452-9.

215. Shosheva A, Miteva M, Christova P, Atanasov B. pH-dependent stability of sperm whale myoglobin in water-guanidine hydrochloride solutions. Eur. Biophys. J. 2003; 31, 8: 617-25.

216. Cooper A. Heat capacity of hydrogen-bonded networks: An alternative view of protein folding thermodynamics. Biophys. Chem. 2000; 31, 85, 1: 25-39.

217. Fernandez A, Kardos J, Goto Y, Protein folding: could hydrophobic collapse be coupled with hydrogen-bond formation? FEBS Lett. 2003; 11, 536, 1, 3: 187-92

218. McColl MD, Greer IA. Low-molecular-weight heparin for the prevention and treatment of venous thromboembolism in pregnancy. Curr. Opin. Pulm. Med. 2004; 10, 5: 371-5.

219. Siguret V, Pautas E, Gouin I, Safety Profile of Different Low-Molecular Weight Heparins Used at Therapeutic Dose. Curr. Opin. Pulm. Med. 2004; 10, 5: 366-70.

220. Needham D, Kim DH, PEG-Covered Lipid Surfaces: Bilayers and Monolayers. In Protein and Cell Repellent Surfaces, Col. Surf B Biointer. 2001; 18, 3, 4:183-195.

221. Kim K, Kim C, Byun Y, Preparation of a PEG-grafted phospholipid Langmuir-Blodgett monolayer for blood-compatible material. J. Biomed Mater Res. 2000; 15, 52, 4: 836-40.

222. Sackmann E. Supported membranes: scientific and practical applications. Science 1996; 5, 271, 5245: 43-8.

223. Goennenwein S, Tanaka M, Hu B, Moroder L, Sackmann E. Functional incorporation of integrins into solid supported membranes on ultrathin films of cellulose: impact on adhesion. Biophys J. 2003, 85, 1: 646-55.

224. Borga O, Borga B. Serum protein binding of nonsteroidal antiinflammatory drugs: a comparative study, J Pharmacokinet Biopharm. 1997, 25, 1, 63.

225. Aspirin, Indomethacin, Diclofenac Monograph.; In The merck index 13th edition an encyclopedia of chemicals, drugs and biologicals, Merck research laboratories, Divn of Merck Co Inc. 2001.

226. Wallace JL. Distribution and expression of cyclooxygenase (COX) isoenzymes, their physiological roles, and the categorization of nonsteroidal anti-inflammatory drugs (NSAIDs). *Am J Med.* 1999,13, 107, 6A, 11S.

227. Russeva V, Mihailova D. Modelling of indometacin and diclofenac sodium binding to the molecule of human serum albumin, Arzneimittelforschung. 1996, 46, 3, 288.

228. Menasse R, Hedwall PR, Kraetz J, Pericin C, Riesterer L, Sallmann A, Ziel R, Jaques R. Pharmacological properties of diclofenac sodium and its metabolites, Scand. J. Rheumatol. 1978, 22, 5.

229. Deschamps Labat L, Pehourcq F, Jagou M, Bannwarth B. Relationship between lipophilicity and binding to human serum albumin of arylpropionic acid non-steroidal anti-inflammatory drugs, J Pharm. Biomed. Anal. 1997, 16, 2, 223.

230. El-Kamel A, Sokar M, Naggar V, Al Gamal S. Chitosan and sodium alginate-based bioadhesive vaginal tablets, AAPS Pharm. Sci. 2002, 4, 4, E44.

231. Wang J, El Sayed MA Temperature Jump-Induced Secondary Structural Change of the Membrane Protein Bacteriorhodopsin in the Premelting Temperature Region: A Nanosecond Time-Resolved Fourier Transform Infrared Study Biophys J. 1999, 76, 5, 2777.

232. Grobe GL, Gardella JA, Chin RL, Salvati L. Characterization of Solution-Cast Extracts from Cardiothane-51® by FT-IR and ESCA Applied Spectroscopy, 1988, 42, 6, 989.

233. Coughlan DC, Quilty FP, Corrigan OI. Effect of drug physicochemical properties on swelling/deswelling kinetics and pulsatile drug release from thermoresponsive poly(N-isopropylacrylamide) hydrogels. J Control Rel. 2004 Jul 23;98(1):97-114.

234. Kragh-Hansen U. Molecular aspects of ligand binding to serum albumin. Pharmacol. Rev. 1981, 33, 17-53.

235. Celej MS, Montich GG, Fidelio GD. Protein stability induced by ligand binding correlates with changes in protein flexibility, Protein Science, 2003,12, 1496-1506.

236. Dzwolak W, Ravindra R, Lendermann J, Winter R. Aggregation of bovine insulin probed by DSC/PPC calorimetry and FTIR spectroscopy, Biochemistry. 2003, 30, 11347.

237. Rosso S, González M, Bagatolli L, Duffard R, Fidelio G, Evidence of a strong interaction of 2,4-dichlorophenoxyacetic acid herbicide with human serum albumin Life Sci. 1998, 63, 2343.

238. Yamasaki K, Rahman MH, Tsutsumi Y, Maruyama T, Ahmed S, Kragh-Hansen U, Otagiri M, Circular Dichroism Simulation Shows a Site-II-to-Site-I Displacement of Human Serum Albumin-Bound Diclofenac by Ibuprofen AAPS Pharm Sci Tech. 2000, 1, 12.

239. Rahman MH, Yamasaki K, Shin YH, Lin CC, Otagiri M. Characterization of high affinity binding sites of non-steroidal anti-inflammatory drugs with respect to site-specific probes on human serum albumin. Biol Pharm Bull. 1993, 16, 1169.

240. Curry S, Brick P, Franks NP. Fatty acid binding to human serum albumin: new insights from crystallographic studies. Biochim. Biophys. Acta. 1999, 1441, 131.

241. Li H, Frieden C. NMR studies of 4-19F-phenylalanine-labeled intestinal fatty acid binding protein: evidence for conformational heterogeneity in the native state Biochemistry. 2005, 22, 44, 7, 2369.

242. Janzen J, Le Y, Kizhakkedathu JN, Brooks DE. Plasma protein adsorption to surfaces grafted with dense homopolymer and copolymer brushes containing poly(N-isopropylacrylamide. J. Biomater. Sci. Polym. Ed. 2004, 15, 9, 1121.

243. Eberhart RC, Munro MS, Williams GB, Kulkarni PV, Shannon WA, Jr, Brink BE, Fry WJ. Albumin adsorption and retention on C18-alkyl-derivatized polyurethane vascular grafts, Artif Organs, 1987, 11, 5, 375.

244. Krishna OD, Kim K, Byun Y. Covalently grafted phospholipid monolayer on silicone catheter surface for reduction in platelet adhesion. Biomaterials, 2005, 26, 34, 7115-23.

245. Tanaka M, Sackmann E. Polymer-supported membranes as models of the cell surface. Nature. 2005 Sep 29;437(7059):656-63.

246. Milhiet PEC, Domec MC, Giocondi N, Van Mau F, Heitz C, Le Grimellec. Domain formation in models of the renal brush border membrane outer leafletBiophys. J. 2001, 81, 547.

247. Yuan C, Johnston LJ. Atomic force microscopy studies of ganglioside GM1 domains in phosphatidylcholine and phosphatidylcholine/cholesterol bilayers, Biophys. J. 2001; 81, 1059.

248. Kaasgaard T, Leidy C, Crowe JH, Mouritsen OG, Jorgensen K. Temperature-controlled structure and kinetics of ripple phases in one- and two-component supported lipid bilayers, Biophys J. 2003, 85, 1, 350.

249. Gupta RK, Suresh KA. AFM studies on Langmuir-Blodgett films of cholesterol, Eur Phys J E Soft Matter. 2004, 14, 1, 35.

250. Tagawa A, Mezzacasa A, Hayer A, Longatti A, Pelkmans L, Helenius A. Assembly and trafficking of caveolar domains in the cell: caveolae as stable, cargo-triggered, vesicular transporters. J Cell Biol. 2005; 29;170(5):769-79.

251. Mayor S, Rao M. Rafts: scale-dependent, active lipid organization at the cell surface. Traffic. 2004; 5, 4: 231-40.

252. Silvius JR. Role of cholesterol in lipid raft formation: lessons from lipid model systems. Biochim. Biophys. Acta., 2003;10, 1610, 2:174-83.

253. Giachelli CM. Vascular calcification: in vitro evidence for the role of inorganic phosphate, J Am Soc Nephrol. 2003, 14, 9, 4, 300.

254. Bailey MT, Pillarisetti S, Xiao H, Vyavahare NR. Role of elastin in pathologic calcification of xenograft heart valves, J Biomed Mater Res A. 2003, 1, 66, 1, 93.

255. Goldberg M, Septier D. Phospholipids in amelogenesis and dentinogenesis Crit. Rev. Oral. Biol. Med. 2002, 13, 3, 276.

256. Mann S, Douglas D, Archibald JMD, Douglas T, Heywood BR, Fiona C, Nicholas MFC, Reeves J. Crystallization at Inorganic-Organic Interfaces - Biominerals and Biomimetic Syntheses. Science. 1993; 261:1286- 92.

257. Brown WE, Lehr JR, Smith JP Frazier AW, Crystallography of octacalcium Phosphate. J. Amer. Chem. Soc. 1957; 79: 5318.

258. Heughebaert JC, Zawacki SJ, Nancollas GH. The growth of octacalcium phosphate on beta tricalcium phosphate. J. Crystal growth. 1983; 63: 83.

259. Arakawa T, Timasheff SN. Mechanism of poly(ethylene glycol) interaction with proteins. Biochemistry 1985;19, 24, 24: 6756-62.

260. Mann S, Douglas D, Archibald JMD, Douglas T, Heywood BR, Fiona C, Nicholas MFC, Reeves J. Crystallization at Inorganic-Organic Interfaces - Biominerals and Biomimetic Syntheses. Science. 1993; 261:1286- 92.

261. Goldberg M Septier. "Phospholipids in amelogenesis and dentinogenesis," Crit. Rev. Oral. Biol. Med. 2002; 13, 3: 276- 90.

262. Sharma CP, Chandy T. Influence of steroid hormones on protein-platelet interaction at the blood-polymer interface, Biomaterials. 1989, 10, 609-16.

263. Malmsten M, Lassen B. Competitive adsorption at phospholipid surfaces. Col. and surf. B Biointer. 1995, 4, 173.

264. Ishihara K, Fukumoto K, Iwasaki Y, Nakabayashi N. Modification of polysulfone with phospholipid polymer for improvement of the blood compatibility. Part 2. Protein adsorption and platelet adhesion, Biomaterials 1999, 20,1553-9.

265. Bertucci C, Domenici. E. Reversible and covalent binding of drugs to human serum albumin: methodological approaches and physiological relevance Curr. Med. Chem. 2002, 9(15), 1463.

266. Halperin A. Polymer Brushes that Resist Adsorption of Model Proteins: Design parameters Langmuir 1999, 15, 2525.

267. Sackmann E. Supported Membranes: Scientific and Practical Applications,Science 1996, 271, 43.

268. Oliver JA, Albrecht RM. Colloidal gold labeling of fibrinogen receptors in epinephrine and ADP-activated platelet suspensions. Scanning Microsc. 1986, 1(2), 745.

269. Sharma CP, Chandy T. Influence of steroid hormones on protein-platelet interaction at the blood-polymer interface. Biomaterials. 1989; 10:609- 16.

270. Baier RE, De Palma VA, Goupil DW, Cohen E. Human platelet spreading on substrata of known surface chemistry. J. Biomed. Mater. Res. 1985; 19:1157- 67.

271. Broberg M, Eriksson C, Nygren H, GpIIb/IIIa is the main receptor for initial platelet adhesion to glass and titanium surfaces in contact with whole blood. J. Lab. Clin. Med. 2002; 139, 3: 163-72.

272. Broberg M, Nygren H. Exposure of fibrinogen-adherent platelets to plasma proteins: a new method for studying protein interactions with platelets. J Biomed Mater Res. 2003; 1, 66A, 2: 403-10.

273. Ye SH, Watanabe J, Iwasaki Y, Ishihara K. Antifouling blood purification membrane composed of cellulose acetate and phospholipid polymer. Biomaterials. 2003; 24, 23, 41: 43-52.

274. Chung CW, Kim HW, Kim YB, Rhee YH. Poly(ethylene glycol)-grafted poly(3-hydroxyundecenoate) networks for enhanced blood compatibility. Int J Biol Macromol. 2003; 32, 1-2: 17-22.

275. Baier RE, De Palma VA, Goupil DW, Cohen E. Human platelet spreading on substrata of known surface chemistry, J. Biomed. Mater. Res. 1985, 19, 1157.

276. Goodman SL, Grasel TG, Cooper SL, Albrecht RM. Platelet shape change and cytoskeletal reorganization on polyurethaneureas J. Biomed. Mater. Res. 1989, 23, 105.

277. Salthouse TN. Some aspects of macrophage behavior at the implant interface. J Biomed Mater Res 1984;18:395–401.

278. Rabinovitch M, DeStefano MJ. Macrophage spreading *in vitro*. I. Inducers of spreading. Exp Cell Res 1973;77:323–334.

279. Webb DS, Shimizu Y, Van Seventer GA, Shaw S, Gerrard TL. LFA-3, CD44, and CD45: Physiologic triggers of human monocyte TNF and IL-1 release. Science 1990;249:1295–1297.

280. Harada A, Sekido N, Akahoshi T, Wada T, Mukaida N, Matsushima K. Essential involvement of interleukin-8 (IL-8) in acute inflammation. J Leukoc Biol 1994;56:559–564.

281. McNally AK, Anderson JM. Complement C3 participation in monocyte adhesion to different surfaces. Proc Natl Acad Sci USA 1994;91:10119–10123.

282. Mohanty M. Cellular basis for failure of joint prosthesis. Biomed Mater Eng. 1996;6(3):165-72.

283. Shanbhag AS, Jacobs JJ, Black J, Galante JO, Glant TT. Cellular mediators secreted by interfacial membranes obtained at revision total hip arthroplasty. J Arthroplasty. 1995 ;10(4):498-506.

284. Chandy T, Mooradian DL, Rao GH. Platelet adhesion and spreading on protein-coated surfaces: variations in behavior in washed cells, PRP, and whole blood. J Biomater Appl. 1998, 13, 1, 46.

285. http://www.azonano.com/details.asp?ArticleID=1232

286. Nozaki Y, Reynolds JA, Tanford C. The interaction of a cationic detergent with bovine serum albumin and other proteins J. Biol. Chem., 1974; 25, 249, 14: 4452.

287. Wang Y, Hu X. A quantum chemistry study of binding carotenoids in the bacterial light-harvesting complexes J Am Chem Soc. 17;124(28): 2002, 8445.

288. Kyndt JA, Savvides SN, Memmi S, Koh M, Fitch JC, Meyer TE, Heyn MP, Beeumen JJ, Cusanovich MA. Structural role of tyrosine 98 in photoactive yellow protein: effects on fluorescence, gateway, and photocycle recovery. Biochemistry. 2007 Jan 9;46(1):95-105.

289. Crews ST, Thomas JB, Goodman CS. The Drosophila single-minded gene encodes a nuclear protein with sequence similarity to the per gene product. Cell, 52, 1988, 143

290. Hoffman EC, et. al. Cloning of a factor required for activity of the Ah (dioxin) receptor. Science. 252, 1991, 954.

291. Getzoff ED, Tainer JA, Stempien MM, Bell GI, Hallewell RA. Proteins Struct. Funct. Genet. 5, 1989, 322.

292. Zanuy D, Nussinov R, Aleman C. From peptide-based material science to protein fibrils: discipline convergence in nanobiology. Phys Biol. 2006, 31;3(1):S80-90.

293. Genick UK, Borgstahl GE, Ng K, Ren Z, Pradervand C, Burke PM, Srajer V, Teng TY, Schildkamp W, McRee DE, Moffat K, Getzoff ED. Structure of a protein photocycle intermediate by millisecond time-resolved crystallography. Science. 1997, 7;275(5305):1471-5.

294. Blewis ME, Nugent-Derfus GE, Schmidt TA, Schumacher BL, Sah RL. A model of synovial fluid lubricant composition in normal and injured joints. Eur Cell Mater. 2007 Mar 6;13:26-39.

295. Serrano AG, Perez-Gil J.Protein-lipid interactions and surface activity in the pulmonary surfactant system. Chem Phys Lipids. 2006; 141(1-2):105-18.

296. Kawano T, Miura H, Mawatari T, Moro-Oka T, Nakanishi Y, Higaki H, Iwamoto Y.Mechanical effects of the intraarticular administration of high molecular weight hyaluronic acid plus phospholipid on synovial joint lubrication and prevention of articular cartilage degeneration in experimental osteoarthritis. Arthritis Rheum. 2003; 48(7):1923-9.

LIST OF PUBLICATIONS OUT OF THE THESIS

1. **Kaladhar K**, Sharma CP, Supported cell mimetic monolayers and their interaction with blood. *Langmuir.* **(2004)** Dec 7; 20(25): 11115-22.
2. **Kaladhar K**, Sharma, CP, Cell Mimetic Lateral Stabilization of Outer Cell Mimetic Bilayer on Polymer Surfaces by Peptide Bonding and Their Blood Compatibility"., *J Biomed Mater Res A.* **(2006)** Oct;79(1):23-35.
3. Smitha M, **Kaladhar K,** Sharma CP, Cell Mimetic Monolayer Supported Chitosan- Haemocompatibility Studies, *J Biomed Mater Res A.* **(2006)** Oct; 79(1): 147-52.
4. **Kaladhar K,** Sharma CP, Novel Albumin Self-Assembled Liposomes, *Langmuir* **(2006)** (Communicated).
5. **Kaladhar K,** Sharma CP, A new strategy to improve the Haemocompatibility of materials using monomolecular layer immobilization of albumin adsorbing drugs, *Langmuir*, **(2006)** (Communicated).
6. **Kaladhar K,** Sharma CP, Supported outer and pathological cell mimetic model lipid compositions and their blood compatibility, *Biomacromolecules*, **(2006)** (Communicated).
7. **Kaladhar K,** Sharma CP, Protein like polymeric nanoparticles prepared from hydrophobic ally modified PVA. *Biomacromolecules* **(2006)**, (Communicated).

International Conferences

1. **Kaladhar. K**; Sharma CP; "Blood Compatibility of Cell Mimetic Monolayers" 7th World Biomaterial Conference **(2004)** Sydney, Australia.
2. **Kaladhar, K**; Sharma, CP; "Modification of chitosan using N- aryl anthranilic acid derivative for blood compatible applications" International Symposium on Advanced Materials and Processing; Dec 6-8, **(2004)**., IIT Kharagpur., India.
3. **Kaladhar, K**; Sharma CP;"Modification of chitosan using n-aryl anthranilic acid derivative for insulin delivery" Macro2004, "International Conference on Polymers for Advanced Technologies",) Dec 15-17, **(2004)**, Thiruvananthapuram, India.
4. **Kaladhar, K.**; Sharma CP.; "Niomat- A Novel Solid Drug Delivery System to Deliver the Niosomes or Proniosomes with Drugs", Indo-Australian Conference on Biomaterials, Implantable Devices and Tissue Engineering", Jan 2-5; **(2005)**., Thiruvananthapuram, India.

5. **Kaladhar, K.**; Sharma, CP.; "A novel Solid Liposome Based Drug Delivery System- Concept Proving Approach", Society for Biomaterials., 30th Anniversary meeting &Exposition; New Applications and Technologies; April 27- 30; **(2005).**, Memphis Cook Convension center; Memphis; USA.
6. **Kaladhar, K.**; Sharma, CP.; "Novel Albumin Self-Assembled Liposomes for Drug Delivery Applications", Society for Biomaterials., Annual meeting: Biomaterials- The enabling Technology. April 26- 29; **(2006)** Pittsburgh, Pennsylvania, USA.
7. **Kaladhar. K.;** Sharma, CP.; "Effect of phosphatidylethanolamine in fibroblast cell adhesion and proliferation.", Tissue Engineering and Regenerative Medicine International Society., Regenerate- World Congress on Tissue Engineering and Regenerative Medicine. April 24- 27.; **(2006)**, Westin Convention Center, Pittsburgh, Pennsylvania, USA.
8. **Kaladhar.K.**; Asha Rani. V.; Sharma CP. Screening of ligands for blood compatible applications by *In silico* methods: NSAID's like Aspirin, Indomethacin & Diclofenac affinity for albumin. BITE & RM (**2007**). Thiruvananthapuram, Kerala, India.
9. **Kaladhar**.K.; Sharma CP. Development of thin solid films of thermoresponsive nanoparticles and their characterisation. BITE & RM, Indo- Australian Conference, **(2007)**. Thiruvananthapuram, Kerala, India.
10. **Kaladhar. K.**; Sharma, CP.; "Calcification at micro domains: Effect of insulin and ethanol", Society for Biomaterials, 31st Annual meeting: A multi dimensional: multi disciplinary approach to biomaterials, April 18-21, (**2007**), Illinois, Chicago, USA.
11. **Kaladhar. K.**; Sharma, CP.; "Thermoresponsive protein like nanoparticles" Society for Biomaterials, 31st Annual meeting: A multi dimensional: multi disciplinary approach to biomaterials, April 18-21, (**2007**), Illinois, Chicago, USA.

Book Chapter

1. **Kaladhar. K.**; Sharma, CP.; "Possibilities of using cord blood for improving the biocompatibility of implants", IN Frontiers of Cord Blood Science.; E by Dr. Niranjan Bhattacharya & Prof. Phillip Stubblefield, Radcliffe Publishing House, Oxford, UK (**2006**) (IN Press).

2. **Kaladhar, K.**; Sharma, CP.; "Supported cell mimetic monolayers and their blood compatibility. IN "Advanced Biomaterials", E by Bikramjith Basu, JOHN WILEY & SONS, INC. (**2007**) (Under Review).

Patents in process

1. **Kaladhar, K.**; Sharma, CP.; Novel Albumin Self-Assembled Liposomal drug delivery systems: preparation and characterization, **(2006),** Indian Patent (Applied).

2. **Kaladhar, K.**; Sharma, CP.; Novel Amphiphilic Nanoparticle Based Thermo-Responsive Coatings on Surfaces for Tissue Engineering Applications, **(2007),** Indian patent (Applied).

Reviews

1. **Kaladhar. K,** Sharma, CP, "Surface passivation and controlled ligand supplementation of cellular activation processes- Strategies for bottom up synthesis of bioactive surfaces", Trends Biomater. Artif. Organs, (**2007**), Vol 21(1), 29-62.