SELF-CROSS-LINKING BIOPOLYMERS AS RAPIDLY GELLING, *IN SITU* FORMING BIODEGRADABLE SCAFFOLDS FOR WOUND MANAGEMENT

A Thesis Presented

by

Biji Balakrishnan

to

The Division of Polymer Chemistry in
partial fulfilment
of the requirements for the Degree of
Doctor of Philosophy
of

SREE CHITRA TIRUNAL INSTITUTE
FOR
MEDICAL SCIENCES AND TECHNOLOGY
TRIVANDRUM

June 2005

DECLARATION

I, Biji Balakrishnan hereby declare that I had personally carried out the work depicted in the thesis entitled **"SELF-CROSS-LINKING BIOPOLYMERS AS RAPIDLY GELLING, *IN SITU* FORMING BIODEGRADABLE SCAFFOLDS FOR WOUND MANAGEMENT"** except where external help sought are acknowledged.

Date: **Biji Balakrishnan**

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 Ms. Biji Balakrishnan in the Division of Polymer Chemistry 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, Trivandrum. The work relating to her thesis entitled **"SELF-CROSS-LINKING BIOPOLYMERS AS RAPIDLY GELLING, IN SITU FORMING BIODEGRADABLE SCAFFOLDS FOR WOUND MANAGEMENT"** was carried out under my direct supervision.

Dr.A. Jayakrishnan
(Guide)

The thesis

entitled

SELF-CROSS-LINKING BIOPOLYMERS AS RAPIDLY GELLING, *IN SITU* FORMING BIODEGRADABLE SCAFFOLDS FOR WOUND MANAGEMENT

Submitted

by

Biji Balakrishnan

for

Doctor of Philosophy

of

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES & TECHNOLOGY

TRIVANDRUM

evaluated and approved by

26.05.06

Dr. A. Jayakrishnan
(Guide)

(MARY RADU)
26/5/06

(Name of Thesis Examiners)

To my amma and achan.....

Acknowledgement

This thesis is the outcome of five years of work and during those days I have been accompanied and supported by many people. It is a pleasant aspect that I have now got the opportunity to express my gratitude for all of them.

The first person, I would like to thank is Dr. A. Jayakrishnan, my supervisor, whose instructions, keenness and ability to present science in a proper, practical and exciting outlook deserve special credit. His patience while writing manuscripts really astonished me. He could not even realize how much I have learned from him. The strict and extensive comments and the discussions and the interactions with him had a direct impact on the final form and quality of this thesis. Besides being an excellent supervisor, he was very understanding and affectionate to all of us. I express my sincere gratitude and obligation for his eminent guidance, constructive criticism and thought provoking questions throughout the course of the investigation.

I would like to thank the Director, SCTIMST and the Head, BMT Wing for providing me the facilities to carry out this work. Financial assistance from University Grants Commission, New Delhi, is gratefully appreciated.

I express my respect and regards to Dr. C.P. Sharma, Dr. Mira Mohanty and Dr. Krishnamanohar, members of my doctoral advisory committee. Special thanks are also due to Dr. Mira Mohanty and her division for their whole-hearted assistance during histology analysis of tissues.

In particular, I would like to thank Profs. D. Labarre and S. Lesieur of Centre d'Etudes Pharmaceutiques, University of Paris IX, Paris, France, for their help in the molecular weight measurements of biopolymers. I am extremely thankful to Dr. P.R. Umashanker, Dr. Arthur Vijayan Lal, Dr. A.C. Fernandez, Dr. P.V. Mohanan, Dr. Sachin Shenoy, Mr. Manoj Kumar, Mr. Balakrishnan Nadar, Mr. Bhuvanachandran Nair, Ms. Preetha, Ms.V.M. Remakumary, Ms. Jasmine A. Mathews and Mr. M. Prem Mohan for their help during *in vivo* studies. I note with gratitude the unreserved cooperation rendered to me by Dr. T. V. Kumary, Mr. P.R. Anilkumar and Ms. Usha Vasudev during cell culture studies. I am extremely thankful to Dr. Maya Nandakumar and Mr. S. Pradeep Kumar for their valuable suggestions and assistance during the antibacterial studies. I thank Mr. R. Sreekumar for his help with SEM analysis.

I would like to recognize the role played by Dr. S. Lakshmi, who gave me valuable instructions during the beginning of this work. Words are inadequate to express my gratitude to Dr. Nirmala Rachel James, Ms. K.K Nishi , Ms.S. Dawlee , Ms. A. Suganthi , Mr. T. Venugopal and Ms. K.G. Meenakshi whose acquaintance made my stay at SCTIMST a memorable part of my life. I was able to approach them for anything I need without any hesitation. I am thankful to all my fellow scholars especially Ms. Asha, Ms. Bernadatte, Ms. Shiny, Ms. Divya and Ms. Elizabeth for their sincere help and support. I would like to thank staffs of Library, Administration & Accounts and Stores Division for the cooperation they have extended towards me during this work. I am thankful to Ms. Beena and Mr. Varghese of Johns Studio, Plamoodu, for their assistance in setting and printing of this thesis.

I extend my deep sense of love and gratitude to my parents, sisters and brothers for their understanding and encouragement. I am very much grateful to Mr. K. R. Jayarajan and Mrs. Jayanthi Jayarajan for their support and encouragement. I would like to extend my sincere gratitude to each and every one who has rendered their help directly or indirectly during this research.

The chain of my gratitude would be definitely incomplete if I forgot to thank the first cause of this chain. Above all, I bow my head before that ultimate truth for all his blessings.

Biji Balakrishnan

Contents

List of Tables

List of Figures

Abbreviations

ACD	-	Acid Citrate Dextrose
ADA	-	Alginate Dialdehyde
AN	-	Acrylonitrile
B.P.	-	British Pharmacopeia
BCG	-	Bromocresol Green
C.D.	-	Cross-linking density
cAMP	-	Cyclic Adenosine Monophosphate
CFU	-	Colony Forming Units
DBcAMP	-	N'-2'-O-dibutyryl adenosine, 3'5'- cyclic monophosphate
EDC	-	N,N-(3-dimethylaminopropyl)-N-ethyl carbodiimide
EGTA	-	Ethylene glycol-bis(b-aminoethyl ether)-N, N, N', N'- tetraacetic acid tetrasodium salt
FITC	-	Fluorescein isothiocyanate
G	-	Guluronic acid
HBSS	-	Hanks Balanced Salt Solution
HEMA	-	2-Hydroxyethyl Methracrylate
HPLC-GEC	-	High-Performance Gel Exclusion Chromatography
I.P.	-	Indian Pharmacopeia
IL	-	Interleukin
M	-	Mannuronic acid
M_w	-	Weight average molar mass
M_c	-	Molecular Weight between Cross-links
MTT	-	3-(4,5-dimethylthiazol-2yl)-2,5, diphenyl tetrazolium bromide
OD	-	Optical Density
ORD	-	Oxidative-Reductive Depolymerization
P. aeroginosa	-	*Pseudomonas aeroginosa*
PAAc	-	Poly(acrylic acid)
PAAm	-	Poly(acrylamide)
PAN	-	Poly(acrylonitrile)
PBS	-	Phosphate Buffered Saline
PDGF	-	Platelet Derived Growth Factor
PEG	-	Poly(ethylene glycol)
PEO	-	Poly(ethylene oxide)
PHEMA	-	Poly(2-hydroxyethylmethacrylate)
PHPMA	-	Poly(N-(2-hydroxypropyl) methacrylamide)
PII	-	Primary Irritation Index
PLA	-	Poly(lactic acid)
PNIPAAm	-	Poly(N-isopropyl acrylamide)
PPO	-	Poly(propylene oxide)
PVA	-	Poly(vinyl alcohol)
S.aureus	-	*Staphylococcus aureus*
SEM	-	Scanning Electron Microscopy
TGF	-	Transforming Growth Factor
TNBS	-	Trinitrobenzene sulphonic acid
TNF	-	Tumour Necrosis Factor
UV	-	Ultraviolet
WVTR	-	Water Vapour Transmission Rate

Synopsis

SYNOPSIS

Globally, millions of people suffer from chronic non-healing skin wounds. Burn wounds have a reported incidence of 7.8 million cases per year worldwide. Taking account of the same in India, about 0.7 million burn patients get hospitalized and 0.12 million patients die every year due to burns (Health Care Management Express, 2003). A common feature in the treatment of all these wounds is that they need a covering for optimal healing. Depending upon the type of dressing material used, dressings are beneficial at different levels. Suitable dressings would help to achieve haemostasis and to control fluid loss. An effective covering would also protect the wound from the microbial contamination. Further, materials such as hydrogels have the capability to maintain the wound moist, which is beneficial for healing. Finally, some wound dressings may themselves directly promote the healing process if they contain components which directly support cell growth or migration or attract or activate cells from the immune system and secrete certain growth-promoting substances (Thomas *et al.*, 2000). Other dressings may contain anti-microbial substances, which are helpful to control infection of the wound. A wide variety of dressing materials has been developed for wound covering over the last 30 years, many of which are currently commercially available (Barlow, 1994; Quinn *et al.*, 1985; Kane *et al.*, 1996). Still there is no ideal wound dressing material which satisfies the requirements of all types of wounds.

Hydrogels are attractive materials for various biomedical applications, consisting of cross-linked networks of polymer swollen with aqueous solutions and have both liquid-like and solid-like properties. Cross-linking is usually achieved by covalent bonds, electrostatic interactions, hydrogen bonding, hydrophobic interactions, van der Waal's forces, physical entanglements and crystallite formation (Hoffman, 2002). Covalent cross-linking is the preferred method in the preparation of hydrogels. However most agents employed for cross-linking such as glutaraldehyde, diisocyanates and polyepoxy compounds are toxic to varying degrees (Kuijpers *et al.*, 2000). Hydrogels derived from naturally occurring polymers are preferred over synthetic polymers as they mimic many features of extra-cellular matrix. Hydrogels combine the features of moist wound healing with good fluid absorbance, are non-adherent to the wound bed and are transparent to allow the monitoring of healing. The simplest and the most convenient approach in wound and burns management will be to apply the material as liquid on to the wound bed so that it forms a cross-linked three-dimensional matrix in the wound bed itself. Thus, the dressing can mould into the shape of wound defect. The *in situ* application will have several advantages over the use of preformed membranes and scaffolds since it would enable conformability of the dressing on wounds without the dressing wrinkling or fluting in the area covering the wound. Most commercially available dressings in the form of membranes and sheets are problematic as far as the conformability is concerned and the *in situ* formation of the dressings will therefore be superior to such dressings.

The studies reported in this thesis are aimed at the preparation and evaluation of an *in situ*-forming wound dressing based on biopolymers such as sodium alginate and gelatin which would be beneficial at different levels of wound healing. Alginates and gelatin have a

long history of medical use as wound dressing materials (Matthew *et al.*, 1995; Choi *et al.*, 1999b), haemostatic agents (Groves & Lawrence, 1986; Cenni *et al.*, 2000), tissue engineering scaffolds (Lee & Mooney; 2001) and drug delivery matrices (Dumitriu, 2002). However, reports on the toxicity of alginate dressing prepared using calcium cross-linking method have caused concern on the use of these dressings (Matthew *et al.*, 1995). Although both alginate and gelatin have been investigated individually for many biomedical applications, published reports or patents on combining the two and thus achieving synergic beneficial effects of both for medical use have been rare. Only one report has been found on gelatin-alginate composite as a possible wound dressing material where, a mixture of gelatin and sodium alginate in water is cast as a film and cross-linked using carbodiimide in 90% acetone-water mixture (Choi *et al.*, 1999b).

The present investigation focuses on the preparation of a hydrogel matrix from gelatin and sodium alginate without the use of any such toxic compounds as cross-linking agents. The aim was to prepare an *in situ* gelling scaffold from biopolymers of well-known biocompatibility and biodegradability by the modification of the polymer itself so that they enter into cross-linking with each other without the use of any extraneous cross-linking agents. Such an *in situ* gelling system, it was envisaged, will have many biomedical applications such as *in situ*-forming wound dressings, injectable scaffolds for tissue engineering and drug delivery among other things. Here, alginate is initially oxidized using periodate to give its dialdehyde derivative. The dialdehyde derivative should cross-link proteins such as gelatin through Schiff's base formation between the amino groups in the protein and the aldehyde functions. The reaction of proteins with polyaldehdyes however, is very slow to be useful as a rapidly gelling system and different strategies were examined

to accelerate the gelling process. It was found that alginate dialdehydes having appropriate molecular weight and degree of oxidation rapidly cross-link proteins such as gelatin in the presence of small concentration of sodium tetraborate (borax) to give rapidly gelling systems. Borax has a long history of medical use and the mean lethal dose in man exceeds 700 mg/kg (www.cdc.gov/niosh/rtecs/vz26c1e0.html). Gelation time could be tailored from a few seconds to less than a minute depending on the concentrations of the reactants employed thus enabling the system to be used in a number of medical applications. The rapid gelation in the presence of borax is attributed to the slightly alkaline pH of the medium as well as the ability of borax to complex with hydroxyl groups of polysaccharides (Coviello *et al.*, 2003).

The thesis begins with an introductory chapter which provides a brief outline of wound care market, skin structure and various classifications of wounds and burns. The physiological basis of wound healing with emphasis on the effect of wound dressings on different stages of wound healing is reviewed in this chapter. The design criteria for wound coverage, literature on the preparation of different types of wound dressings and classification of hydrogels are also covered. The properties of alginate and gelatin as possible candidate materials for the present study and the aim and scope of study are also covered in this chapter.

Chapter 2 provides the details of materials and experimental methods involved in the preparation and characterization of rapidly gelling formulations from periodate oxidized alginate and gelatin. Periodate oxidation of alginate in two different media, characterization of alginate dialdehyde (ADA), molecular weight (M_w) by size exclusion chromatography, gelation reaction with gelatin under different pH conditions, physico-chemical characterization of hydrogels by swelling studies, trinitrobenzene sulphonic acid (TNBS) assay, scanning

electron microscopic (SEM) methods, estimation of the rate of water loss from gels and water vapor transmission rate are discussed. Biocompatibility evaluation of the hydrogels, *in vivo* evaluation for wound healing in a rat model and incorporation of N'-2'-O-dibutyryl adenosine, 3'5'- cyclic monophosphate (DBcAMP) in the hydrogel are also described. The methods for evaluation of hydrogels as a drug carrier and as a tissue engineering scaffold are also included in this chapter.

Results and discussion comprises the chapter 3 of this thesis. This chapter is divided into eight sections.

Section 3.1 discusses the preparation of ADA by periodate oxidation of vicinal glycols on C2 and C3 carbon atoms of the glyco-pyranose units of guluronic and mannuronic acid residues of the alginates. Reaction was conducted at 25°C in water, under dark for 6 h. Due to poor dissolution and high viscosity of the resulting aqueous solution, periodate oxidation of sodium alginate in water resulted in low yields of the oxidized product. Therefore, the oxidation of alginate as a dispersion in ethanol/water mixture was examined. It was found that the reaction in ethanol/water mixture proceeded smoothly as that in aqueous medium and the kinetics of oxidation was surprisingly similar. Periodate oxidation as a dispersion was found to have the advantage of preparing large quantities of the oxidized product in one go, a huge advantage in the preparation of large quantities of the oxidized product. Depolymerization of alginate was observed in both media, but it was extensive in ethanol/water resulting in very low molecular weight products. Low molecular weight of ADAs obtained by oxidation in ethanol/water mixture revealed that free radical mediated depolymerization is prominent in this medium. Solubility analysis showed that irrespective of the method of oxidation, the solubility of ADAs in water as well as in different buffers increases with the degree of oxidation.

Section 3.2 discusses the preparation of ADA cross-linked gelatin hydrogels. The cross-linking is due to the Schiff base formation between the aldehyde groups of ADA and ε-amino groups of lysine or hydroxylysine side groups of gelatin. The effect of medium of gelation, method of oxidation, degree of oxidation of alginate and concentration of ADA, gelatin and borax on gelling time was evaluated. The results obtained showed that medium with high pH were favorable for rapid gelation between ADA and gelatin. However, in the presence of a high pH buffers such as sodium carbonate buffer, phase separation was evident and the gels obtained were opaque in character. On the contrary, the gelation reaction in the presence of borax was rapid and transparent gels were obtained. In borax, the good solubility of ADA due to complexation lead to enhanced reaction rate as well as transparent gels. Gelation in phosphate buffered saline and water was rather slow and led to the formation of weak gels. ADAs prepared by oxidation in ethanol/water mixture gave rapid gelation as compared to those prepared in aqueous medium. This rapid gelation has been attributed to lower M.W of the product obtained by oxidation in ethanol/water in comparison to that obtained in aqueous medium. The gelling time decreased with increase in the concentration of ADA, gelatin and borax as well as with increase in the degree of oxidation of alginate. Particularly striking was the influence of borax concentration on the gelling time. These phenomena are discussed in relation to the alkaline pH of the medium as well as the ability of borax to complex with hydroxyl groups of polysaccharides (Coviello *et al.*, 2003).

Section 3.3 discusses the physico-chemical characterization of hydrogels. This comprises of the evaluation of degree of swelling, swelling ratio and cross-linking density by swelling studies, cross-linking degree by TNBS assay and degradability of the hydrogel. Generally, the cross-linking degree and cross-linking density increased with increase in

degree of oxidation. The degradation studies revealed that the hydrogels were biodegradable. This section also deals with fluid uptake ability of gels, rate of water loss from gels and water vapor transmission rate of gels which are important parameters for a wound dressing material. Fluid uptake studies revealed that the hydrogels were capable of absorbing fluid at about 90% of their weight which would prevent the wound bed from accumulation of exudates. Rate of water loss from gels revealed that the materials lose about 30-40% water content after one day when exposed to air which mimics the condition of a dry wound and secondary dressing is advocated for its use on dry wounds. Water vapor transmission studies showed that the hydrogels can maintain a moist environment over wound bed in moderate to heavily exuding wounds which would enhance epithelial cell migration during wound healing process. Porosity of the hydrogels was evaluated using SEM and image analysis.

Section 3.4 deals with the *in vitro* and *in vivo* biocompatibility evaluation of these hydrogels. Qualitative and quantitative cytotoxicity evaluation of ADA-cross-linked gelatin gel by the direct contact assay using L929 mouse fibroblast cells revealed that the gel did not induce any morphological changes to the cells confirming its non-toxic nature. Blood compatibility of the hydrogel assessed by haemolysis assay showed that the hydrogels were not haemolytic. Tests such as intracutaneous irritation on rabbits and maximization test for delayed hypersensitivity on guinea pigs were also performed.

Section 3.5 deals with the wound healing evaluation of hydrogel by applying the gel in a rat model using standard protocols. Wound size reduction was noted by measuring the wound area on 5, 10, and 15 days after the application. Gross examination revealed that subcutaneous aspects were normal for test wounds, whereas skin was rough, hard and haemorrhagic for control wounds for which scab was also present on the wound bed. Superficially, after

15 days, test wound defects filled up to 95% whereas the reduction in the defect area was only 75% for control wounds. This section also discusses the histology results of the wound specimens and percentage wound re-epithelialization obtained after definite intervals of time. After 10 days, the rate of re-epithelialization increased to 85% for test wounds; whereas for control wounds, this was 74%. The rate of re-epithelialization further increased to 90% and 82% respectively for test and control wounds after 15 days.

Section 3.6 deals with the wound healing evaluation of DBcAMP incorporated hydrogel. Cyclic AMP is a second messenger which can regulate cell proliferation (Nakamura & Nishida, 2003). Gels containing DBcAMP were evaluated using a rat model. This section deals with the histology results and percentage re-epithelialization of the wounds after 5, 10, and 15 days of observation. The results showed that after 5 days the re-epithelialization on the wounds covered with the DBcAMP incorporated gels was lower than that covered with gels without cyclic AMP. However, after 10 days, all test wounds appeared to have healed with complete epithelialization (100%).

Section 3.7 deals with the *in vitro* evaluation of hydrogel as a potential drug carrier. The gels loaded with drugs such as gentamycin and primaquine were prepared and their release profiles were evaluated. The release profile of gentamycin was studied by changing the order of mixing the drug with the biopolymer solutions. It was found that when the drug was first mixed with ADA, release was slower than that obtained when it was first mixed with gelatin solution followed by the addition of ADA. The results obtained showed that the drug was chemically conjugated to the biopolymer when it was first mixed with ADA. Finally the antibacterial property of gels incorporated with gentamycin was also evaluated.

Section 3.8 deals with the potential of hydrogel as a tissue engineering scaffold. This was evaluated by encapsulating hepatocytes inside the gel and analyzing the viability and functionality of cells within the matrix at different time intervals. Routine microscopic examination during the culture period showed that the cells were dividing inside the matrix demonstrating their live nature. The viability of hepatocytes examined by Neutral Red assay showed the presence of active cells within the matrix up to 4 weeks. Estimation of albumin secretion also showed that cells were maintaining their protein producing ability demonstrating the suitability of the scaffold for tissue engineering.

Finally, chapter 4 contains the summary, conclusion and future prospects of the investigations reported in thesis.

Chapter-1
Introduction

INTRODUCTION

1.1 Wound Care Market

Wound management is a significant clinical and economic problem. Globally, traditional dressings continue to dominate the wound care market despite the availability of advanced wound dressings that heal wounds at a quicker rate. Advanced dressings provide a moist wound-healing environment that is more conducive to healing than the traditional dry environment. However, due to the apparent high cost, care providers are sometimes reluctant to advocate their use. On the other hand, these dressings require fewer dressing changes, utilize less care provider time and offer quicker healing, thereby reducing the total resources used.

The advanced wound dressings and related technologies are now being increasingly accepted by both the hospital and alternative care markets. According to recent reports, the advanced wound care business is estimated to grow at an average annual growth rate of 7.2% and is expected to cross US $ 3 billion by 2008 (Figure 1.1) (www.bccresearch.com).

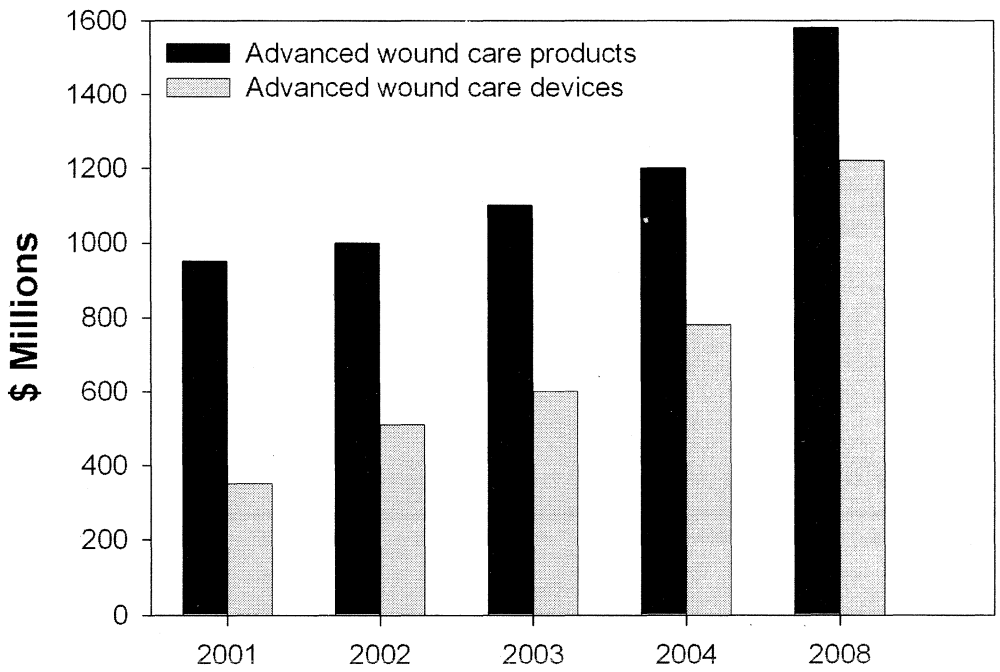
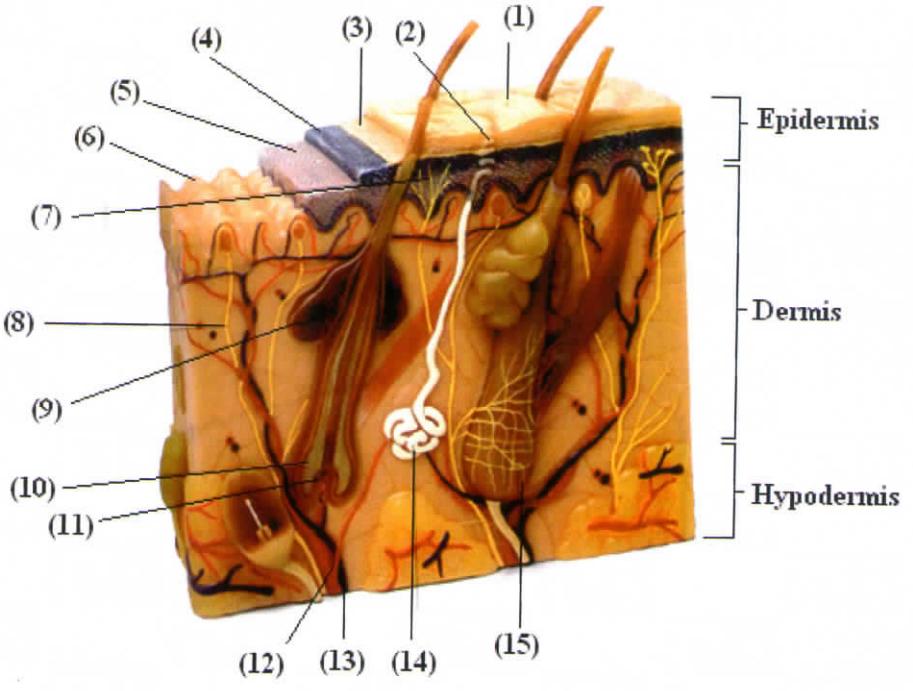


Figure 1.1: Total advanced wound care market by selected segment, 2001-2008 (www.bccresearch.com)

1.2 Skin

Skin, the largest organ of the body, also known as the cutis or integument, has surface area of 1.5 to 2 m^2. It functions as a barrier preventing the body from getting dried out and contaminated from outside with exotoxins. Skin has many other biological functions such as controlling and regulating body temperature and acts as sensory organ to external stimuli. Skin can be anatomically divided into two layers: the epidermis or cuticle and the dermis or corium. While epidermis forms the superficial protective layer, the dermis provides the firmness and elasticity of healthy skin.

The epidermis is completely cellular, typically made up of keratinized, stratified and squamous epithelium that contains five histologically distinct cell types (Figure 1.2)(Page, 2000).



gure 1. 2: Anatomical features of skin.

Stratum corneum
Stratum basale
Sebaceous gland
) vein
2) Sweat pore
6) Dermal papilla
10) Hair bulb
14) Sweat gland
3) Stratum granulosam
7) Free nerve ending
11) Papilla
15) Hair follicle
4) Stratum spinosum
8) Nerve fibre
12) Artery

ese cells are organized into layers that are arranged superficial to deep horny layer ratum corneum), clear layer (stratum lucidum), granular layer (stratum granulosum), prickle l layer (stratum spinosum) and basal layer (stratum basale). The two deepest layers are metimes grouped together as the stratum germinativum since these cells are responsible the normal physiological regeneration of the more specific cells.

The dermis is subdivided into two layers- the papillary dermis and the reticular mis. The dermal papillae and epidermal rete pegs provide a close, undulating layer in mbination between epidermis and dermis. The more superficial papillary dermis contains ch supply of blood vessels that penetrate from the deeper layers. Also contained within

this layer are numerous nerve endings, thermo-receptors and cryo-receptors. The deeper reticular dermis is mainly a layer of connective tissue in which fibroblasts are surrounded by a matrix of collagen, elastin and proteoglycans that provide the structural support for the skin. The subcutaneous tissue is deep to the dermis and contains a variety of cells - adipocytes, fibroblasts, histiocytes, plasma cells, lymphocytes and mast cells. Many of these cells are involved in the processing of foreign antigens that may be traumatically introduced into skin (Bruce, 1992).

1.3 Wounds

A wound can be defined as a disruption of the normal anatomical relationships of tissues as a result of injury. The injury may be intentional such as surgical incision, or accidental, following trauma. There is no such thing as a standard wound. Each will have different requirements and these requirements will change as healing progresses or further tissue break-down occurs. One type of dressing, therefore, is no longer sufficient to meet the needs of all wounds. Wounds can be of open and closed type. Open wounds will have an opening or break in the mucous membrane, whereas in closed wounds, skin is not broken, but the impact from the damaging object has injured or crushed tissues lying below the tissue point. These wounds are commonly known as bruises and it will be visible as discoloration resulting from the rupturing of blood capillaries in the injured area. Different types of open wounds are depicted in Figure 1.3 (Aaron *et al.*, 1979).

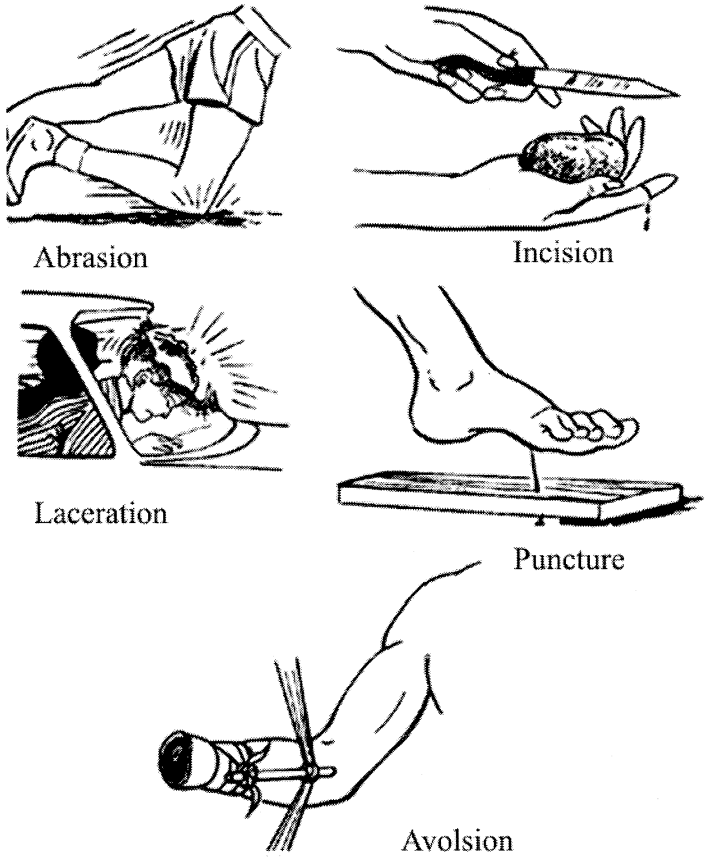


Figure 1.3: Types of open wounds

1.4 Burns

Wounds caused by exposure to heat, chemicals, radiations, electric current or to severe cold are generally known as burns. The burn wound differs from other wounds in the following ways:

a. Colonization by potentially pathogenic bacteria;

b. Presence of large amounts of nonviable tissue;

c. Exudation of large quantities of water, serum and blood.

Burns are usually classified on the basis of the extent and depth of the injury.

1.4.1 Classification Based on the Extent of Injury

The extent of a burn injury is expressed as the percentage of total body surface area affected due to burns. 'Rule of nine' (Table 1.1) is a quick way to estimate the surface area that is affected by a burn. If the entire body is burnt, then it is considered as 100% burn (www.emedicinehealth.com/articles/4871-8.asp).

Specification	Burn (%)
One hand is burnt	9
Both hands burnt	18
One leg burnt	18
Both legs burnt	36
Chest and stomach	18
Back	18
Neck and face	9
Genitals	1
Total	100

Table 1.1 : 'Rule of nine' for estimating the surface area effected by a burn

1.4.2 Classification Based on Depth of Injury

Burns are called first degree burns, if only epidermal layer is destroyed. It is second degree, if a portion of dermis is also damaged along with epidermis and in the case of third degree, there is loss of tissue through the dermis, including the hair follicles, sweat glands and extending into the hypodermis (subcutaneous) layers. Rarely, some electrical injuries may even extend downward through the subcutaneous tissue to involve tendon, bone, muscle and other deep structures. In such cases, the burn is referred to as a fourth degree.

According to new classification, first and second degree will be known under the name 'partial thickness' and the third degree as the 'full thickness' burns. Second degree burns are further classified as superficial and deep. In the superficial second degree burns, there will be enough deep epidermal or superficial dermal layers to allow spontaneous healing of the wound by re-epithelialization. In deep second degree burns, the epidermis is completely destroyed and extended further into the dermis, with large amounts of necrotic tissue together with heavy loss of fluid and chances of bacterial proliferation. These wounds, if not treated properly will result in hypertropic scarring and it should be treated as if they were third degree burns to permit better and faster healing. Third degree burn also is freely permeable to fluids, proteins and bacteria with all the epidermal cells within the wound, including those in the epidermal appendages destroyed. New epidermis seen around the edges of the wounds will not be sufficient for re-epithelialization in all, but smaller injuries. Myoblasts, a specialized fibroblast cells invade the wound and begin to pull the edges inward, resulting in contractures and restriction in movement of the surrounding skin and its structures leading to severe deformation of surrounding tissues. Prompt debridement of the wound followed by grafting or other methods is the treatment of choice in these cases in order to ensure the migration of epidermal cells. Best coverage for the wound is natural skin taken from the patient itself (autograft) to avoid specific immunological incompatibility. But for patients with more than 50% burns, obtaining autograft will be difficult and we have to depend on skin grafts from cadavers (allografts), though in some cases, it may cause immunological problems and eventual rejection. Porcine xenografts have been used after preservation, but problems regarding its storage and biocompatibility restrict their usefulness (Quinn *et al.*, 1985; Kane *et al.*, 1996).

1.5 Design Criteria for Wounds Coverage

Wound healing is a dynamic process and the performance requirements of a dressing can change as wound healing progresses. However, it is widely accepted that a warm, moist environment encourages rapid healing and most modern wound care products are designed to provide these conditions. Besides this, dressing should have certain other properties like ease of application and removal and proper adherence so that there will not be any area of non-adherence left to create fluid filled pockets for the proliferation of bacteria. Fluid balance in burn injury is very important since heavy loss of water from the body by exudation and evaporation may lead to fall in body temperature and increase in metabolic rate. Water vapour transmission rate of normal skin is approximately 8.5 ± 0.5 g m^{-2} h^{-1} while retaining proteins and electrolytes, whereas, it is at a much higher rate of 178.1 ± 5.5 g m^{-2} h^{-1} and 143 ± 4.5 g m^{-2} h^{-1} for partial and full thickness burns respectively. Ideally, the dressing should also be permeable to other gases such as oxygen and carbon dioxide. It also should provide bacterial barrier to prevent infection and mechanical characteristics to accommodate movement and durability. Materials to be used for dressings should be tested for its toxicity, sensitization and irritation and it should be sterilizable. Further, the dressing should be affordable and easily available (Quinn *et al.*, 1985; Kane *et al.*, 1996). Effective wound management requires an understanding of the process of tissue repair as well as extensive knowledge of the properties of dressings available.

1.5.1 Physiological Basis of Wound Healing

The healing of wounds is a complex dynamic process that involves many phases, each characterized by the integrated actions of different cells. It is an intricate, organized response to tissue injury that involves cellular and extracellular matrix components. Different

processes involved in wound healing include: haemostasis, inflammation, proliferation and remodeling (Figure 1.4) (Schilling, 1976).

1.5.1.1 Haemostasis

The healing starts with an inflammatory response characterized by a surge of tissue fluids into the wound site within few minutes of wounding. Following this, there will be increased blood supply followed by the aggregation and degranulation of the platelets that come into contact with damaged collagen and other tissue debris. Alpha granules released during this process contain factors that affect clotting, as well as potent polypeptide growth factors (Pessa *et al.*, 1987). The deposition and polymerization of fibrin occurs together with continued aggregation of platelets, thus forming a thrombus. The formation of the thrombus within the wound with reactive vasoconstriction of the traumatized vessels leads to haemostasis. This thrombus acts as a scaffold, providing a substrate to which inflammatory cells attach and migrate into the wound site (Weigel *et al.*, 1986). Platelets also release platelet-derived growth factor (PDGF), a chemo-attractant for smooth muscle cells (Grotendorst *et al.*, 1983) and fibroblasts (Seppa *et al.*, 1982). Transforming growth factor- β (TGF-β) released by platelets (Roberts *et al.*, 1986) has a similar chemo-attractive effect for inflammatory cells and fibroblasts.

1.5.1.2 Inflammation

Secondary vasodilation and increased capillary permeability result in the initiation of acute inflammation as neutrophils enter the wound site. By 4 hours, a layer of clear exudates will be seen above the blood clot on the wound surface. The neutrophils principally have an immunological function, controlling local bacterial contamination and aiding in the debridement

of devitalized tissue. Products which assist rapid and efficacious removal of necrotic tissue and enhance healing are an integral and essential part of the wound management.

Neutrophil infiltration peaks at about 24 hours post wounding, and slowly recedes as monocytes enter the wound. These circulating monocytes are converted into macrophages as they continue to destroy bacteria and debride the wound. Similar to platelets, macrophages also have a vital function related to subsequent healing events (Diegelmann *et al.*, 1981). Macrophages also secrete growth factors, such as TGF-β, which stimulate the proliferation of fibroblasts and thus positively affecting collagen synthesis. Additionally, other cytokines such as interleukin-1(IL-1) and tumour necrosis factor (TNF) are released from macrophages. It has been found that treatments aimed at inducing macrophages into wounds or activating them at certain stages of healing may change a non-healing wound into a healing one (Swaim *et al.*, 1993).

Other inflammatory cells involved in normal wound healing are lymphocytes, plasma cells and mast cells. The composition of wound matrix changes during the inflammatory phase. Fibrin is the initial component of the matrix, largely as a result of haemostasis. As vascular permeability increases during the onset of acute inflammation, exudation of plasma components occurs, resulting in the entry of complement, antibodies, and other plasma components into the wound. During this exudation, fibrin is replaced by glycosaminoglycans and proteoglycans. When this acute inflammatory response subsides, gelation of serous exudate layer occurs and the exposed surface will become dry due to the loss of water to the atmosphere. This dehydration results in scab formation. Once scab formation is complete, epidermal regeneration begins (Barnett & Irving, 1991). It has been found that if this scab formation is prevented, the rate of epithelialization is markedly enhanced (Winter, 1962).

1.5.1.3 Proliferative Phase

Approximately after 3 to 4 days of post wounding, fibroblast infiltration and proliferation are prominent. Endothelial cells also proliferate as neovascularization proceeds. The cellular migration that occurs is guided by the provisional matrix that exists within the wound, anatomical tissue planes and planes aligned according to the tension across the wound. The entry of fibroblasts into the wound is crucial to the healing process since these cells synthesize collagen, the primary structural component of the repaired tissue (Jackson, 1977). Collagen is the final and permanent component of the wound matrix. Smooth muscle cells, epithelial cells and endothelial cells also synthesize collagen (Stevenson & Mathes, 1988). Collagen Type- III is synthesized and deposited as the initial form of collagen in healing wounds, but it is quickly replaced by Type I, the predominant collagen of skin. As contact of fibroblasts with mature collagen fibrils leads to the reduction in collagen synthesis, collagen and pro-collagen peptide based dressings may be useful in the reduction of contractures or scarring. There has been greater interest in using biopolymers such as collagen, hyaluronate, chitosan and alginates as wound healing materials to attract cells into the wound to effect repair (Hu *et al.*, 2003; Lahiji *et al.*, 2000; Thomas *et al.*, 2000, Purna & Babu, 2000).

1.5.1.4 Remodeling

Remodeling is the last and longest phase of healing in which scar maturation occurs for months to years after the initial collagen synthesis. The major processes occurring during this phase are the dynamic remodeling of collagen and the formation of the mature scar. The net deposition of collagen in all tissues, including wounds is a balance between two opposing processes; collagenolytic activity and collagen synthesis (Peacock, 1980). However, the

wound collagen never achieves the bundled, organized pattern of normal dermal collagen. This is reflected in the fact that the strength of healed tissue never equals that of uninjured skin (Levenson *et al.*, 1965).

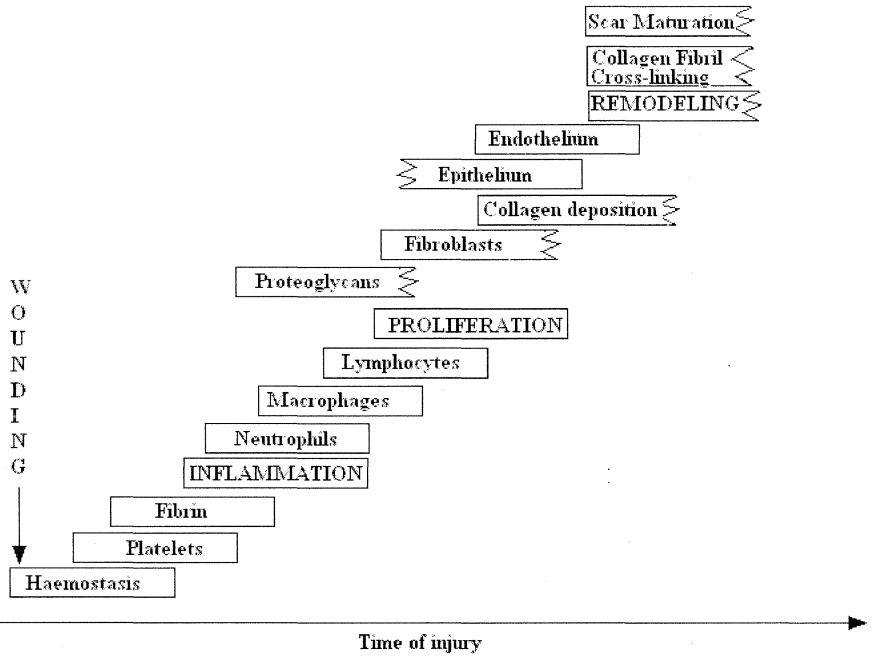


Figure 1.4 : Temporal relationship between the multiple processes occurring in dermal wound healing.

1.5.2 Types of Wound Healing

The rate of wound healing varies with the type of tissue undergoing repair. Primary healing, delayed primary healing and healing by secondary intention are the three main categories of wound healing. Wounds that have been closed shortly after injury by means of suturing or stapling, where there is no infection, heal by primary intention. Scar formation is minimal and these wounds heal rapidly by connective tissue deposition. In wounds whose edges remain open and there is severe trauma and infection, the healing is by secondary intention; the main mechanism of which is contraction, with additional contribution made by

epithelialization and connective tissue deposition. Wounds that are left open for one to two days and then surgically closed heal by delayed primary intention. Even though different categories exist, the interactions of cellular and extra-cellular constituents are similar (Lin *et al.*, 1997).

1.6 Wound Dressings-An Overview

Various substances have been used to cover wounds from the ancient times. The Egyptians (c.1550 BC) used castor oil to treat septic wounds and burns. While Gamgee used absorbent cotton for dressing in 1880, Lumiere used 'tulle grass' (paraffin impregnated gauze) in 1903 (Ulubayram & Hasirci, 1998). Various biological dressings derived from porcine skin and amniotic membrane, were also used (Park, 1978).

In the early 1960s, G.D. Winter first reported that wound healing progressed faster in a moist environment and this revolutionized the wound dressing industry (Winter, 1962). The understanding that control of the wound environment is crucial to the initiation and maintenance of repair led to the proliferation of many film dressings in the 1970s and 1980s. Synthetic polymer dressings made of thin polymer layers of silicone, polyurethane, poly(vinyl chloride) or polyethylene then arrived. However, they showed low water vapour transmission and led to pooling of exudates under the dressing. In order to provide absorbency, a number of dressings were then designed. Non-adherent and absorbent dressings are generally obtained by covering absorbent pad with non-adhering perforated films. Examples of this type of dressings are Melonin®, Telfa®, Perfron® and Lotus® (Wiseman *et al.*, 1992).

The composite foam/film dressings developed subsequently succeeded in retaining exudates away from the wound, whilst the outer film layer maintained a moist environment

at the wound surface. Yannas *et al.* was first to develop such dressings which consisted of a silicone membrane attached to an inner layer of collagen sponge (Yannas & Burke, 1980). This composite matrix having a well defined pore structure and cross-linking density optimized re-growth of the cells while minimizing scar formation. Similar bilayer wound dressings were developed by Suzuki *et al.* by modifying Yannas' technique (Suzuki *et al.*, 1990). Numerous other dressings based on these principles have been developed. Biobrane® (Woodroof, 1984) is a bilaminate membrane with an outer layer of ultra thin silicone rubber, mechanically bonded to a fine knit, flexible Nylon fabric. Epigard® (Alexander *et al.*, 1973) consists of an inner layer of reticulated polyurethane foam laminated to an outer sheet of microporous polypropylene film. Mi *et al.* (Mi *et al.*, 2002) developed a new type of bilayer chitosan wound dressing, consisting of a dense upper layer and a sponge-like lower layer, which is suitable for the topical delivery of silver sulphadiazine. A drug-impregnated polyelectrolyte complex sponge composed of chitosan and sodium alginate was prepared by Kim *et al.* (Kim *et al.*, 1999a). Dressings capable of controlled delivery of anti-bacterial agents were also developed (Lin *et al.*, 2001).

The failure of composite film/foam dressings to penetrate the market was largely due to the success of the hydrocolloids which generally contained a synthetic material such as carboxymethyl cellulose embedded in an adhesive and backed with semi-permeable films, which were introduced around the same time (e.g., Comfeel® Ulcus® and Granuflex®) (Turner, 1985; Chen, 1976).

A number of other innovative approaches were then developed. Alginate-based dressings were produced as thin non-woven films or as fibres. Upon contact with the wound fluid, they turn into a gel that has a high absorptive capacity for wound fluid. Examples include Kaltostat (Brit-Cair, UK) and Sorbsan (Steriseal, UK) (Gilchrist & Martin, 1994).

Combination dressings such as hydrogels with films and hydrocolloids with foams have also been launched in an effort to achieve a fine balance between control of exudates and retaining moist conditions at the wound surface and to get closer to the concept of the universal dressing (Barlow, 1994). Loke *et al.* (Loke *et al.*, 2000) developed a dual layer wound dressing in which upper layer is a carboxymethyl-chitin hydrogel material, while lower layer is silver sulphadiazine incorporated chitosan acetate foam which can prevent microbial invasion.

A variety of natural polymers including collagen, fibrin, fibronectin, alginate, chitosan, dextran, hyaluronic acid etc. have been studied as dressing for dermal wounds. Collagen can be formulated in many different ways as fabric, composite film, reconstituted collagen fibres, reconstituted extruded strips, sheets reconstituted on Dacron mesh, microcrystalline sheets, dermal collagen allografts and collagen sponge grafts depending on the properties desired and methods of application (Quinn *et al.*, 1985).

Sprayable wound dressings improve patient compliance and comfort, with its distinctive advantages like ease of application and localized delivery especially in the case of burns (Kane *et al.*, 1996). 'Hydron' is formed *in situ* on the burn wound by spraying the surface alternatively with poly(2-hydroxyethyl methacrylate) powder and liquid poly(ethylene glycol)-400. The membrane is easily removed from the wound bed, but their high cost limits their use over simpler dressings (Nathan *et al.*, 1975). Pluronic F-127, a block copolymer of hydroxyl terminated propylene and ethylene oxides forms a clear gel at body temperature. (Schmolka, 1972). Kirker *et al.* (Kirker *et al.*, 2002) prepared chemically cross-linked glycosaminoglycan hydrogel films by cross-linking their hydrazide derivatives with poly(ethylene glycol) propiondialdehyde.

At present, there is no one dressing satisfying all the desired requirements. Modern dressings are designed to facilitate the function of the wound rather than just to cover it. Principles of wound dressings are changing, especially in relation to debridement of wounds and control of the wound environment.

1.7 Hydrogels as Wound Dressings

Hydrogels are popular among the many categories of advanced wound dressing products available today (Eisenbud *et al.*, 2003). Most of these dressings are transparent to allow inspection of wounds. Moreover, they provide a moist wound healing environment and are impermeable to bacteria, thus preventing bacterial infection. The hydrogels adhere to the wound surface without the use of any synthetic adhesives and their absorptive capacity makes them appropriate for moderate to heavily exuding wounds (Barlow, 1994).

A number of wound dressings based on hydrogels are already in the market Examples are Geliperm® (Gelistlich Pharma, Switzerland), Vigilon® (CR Bard, USA), Koolgel® (Cambrex Hydrogels, USA) etc. Most of these dressings are preformed hydrogels in the form of sheets and membranes.

1.7.1 Hydrogels

Hydrogels are three-dimensional networks of hydrophilic polymers that can absorb large amount of water without undergoing dissolution because of their cross-linked structure. Because of this, they have physical characteristics similar to soft tissues. Over the past three decades, a number of hydrogels differing in structure, composition and properties have been developed (Graham, 1998a; Graham, 1998b; Peppas, 1987; Peppas *et al.*, 2000).

Depending on the forces responsible for this networking, they can be classified as physical gels and chemical gels. When the networks are held together by molecular entanglements or secondary forces like ionic, H-bonding or hydrophobic forces, resulting hydrogels are called reversible or physical gels (Campoccia, 1998; Prestwich, 1998). When hydrogels are covalently cross-linked networks, they are called "permanent" or "chemical" gels. The amount of water in a hydrogel will determine the absorption and diffusion of solutes through the hydrogel. This is most influenced by the composition and cross-link density of the hydrogel (Hoffman, 2002).

1.7.1.1 Physically cross-linked gels

Physical forces such as electrostatic forces, hydrophobic interactions, hydrogen bonding, van der Waal's forces or combination of these interactions can cause the association of two or more complimentary polymers, leading to the formation of hydrogels. Different methods for the preparation of physically cross-linked hydrogels are provided in Table 1.2.

1.7.1.2 Chemically cross-linked gels

Hydrogels can also be prepared by means of various chemical reactions such as radical polymerization, Schiff's base formation, addition, condensation and enzymatic reactions (Table 1.3).

Physical gels

Polymer solution forms gel on warming (e.g., PEO-PPO-PEO block copolymers in H_2O)

Polymer solution forms gel on cooling (e.g., agarose or gelatin in H_2O)

Repeated freeze-thawing of polymer in aqueous solution leads to the formation of microcrystals by cross-linking (e.g., freeze–thaw PVA in aqueous solution)

Decreasing pH of aqueous solution of two different polymers to form a H-bonded gel (e.g., PEO and PAAc)

Coacervate gel formation by simply mixing solutions of polyanion and polycation

(e.g., Sodium alginate and chitosan)

Gelation of a polyelectrolyte solution with a multivalent ion of opposite charge (e.g., Na^+ alginate + Ca^{2+} + $2Cl^-$).

Table 1.2: Methods for synthesizing physical hydrogels.

Abbreviations: PEO: poly(ethylene oxide) PAAc:poly(acrylic acid)
PVA: poly(vinyl alcohol) PPO:poly(propylene oxide) (Hoffman, 2002)

Chemical gels

Cross-link polymers in the solid state or in solution with:

Radiation (e.g., irradiate PEO in water)

Chemical cross-linkers (e.g., treat collagen with glutaraldehyde or bis-epoxide)

Multifunctional reactive compounds (e.g., PEG + diisocyanate = PU hydrogel)

Copolymerize a monomer + cross-linker in solution (e.g., HEMA + EGDMA)

Polymerize a monomer within a different solid polymer to form an IPN gel (e.g., AN + starch)

Chemically convert a hydrophobic polymer to a hydrogel (e.g., partially hydrolyze PVAc to PVA or PAN/PAAm/PAAc)

Table 1.3: Methods for synthesizing chemical hydrogels.

Abbreviations: AN:acrylonitrile, EGDMA:ethylene glycol dimethacrylate
HEMA:2-hydroxyethyl methacrylate PEO: poly(ethylene oxide)
PU: polyurethane, PAAc: poly(acrylic acid)
PVA: poly(vinyl alcohol) PPO: poly(propylene oxide)
PEG: poly(ethylene glycol) PAN: poly(acrylonitrile)
PAAm: poly(acrylamide) (Hoffman, 2002).

1.8 Alginates as Wound Dressings

Alginates have been used in a number of biomedical applications such as cell and drug delivery vehicles, dental impression materials and wound dressings (Shapiro & Cohen, 1997; Gombotz & Wee, 1998; Bouhadir *et al.*, 2000; Bouhadir *et al.*, 2001a; Cook, 1986, Schmidt *et al.*, 1993). Alginate dressings absorb moisture and maintain a moist environment in the wound bed conducive for optimal healing (Gensheimer, 1993). Some dressings have also been shown to have significant haemostatic properties (Groves & Lawrence, 1986). It has been demonstrated that alginates containing zinc ions have potential effect on pro-thrombotic coagulation and platelet activation (Segal & Hunt, 1998). Products available in market are Algosteril®, Kaltostat®, Kaltoclude®, Kaltogel®, Sorbalgon®, Sorbasan® and Tegagel®.

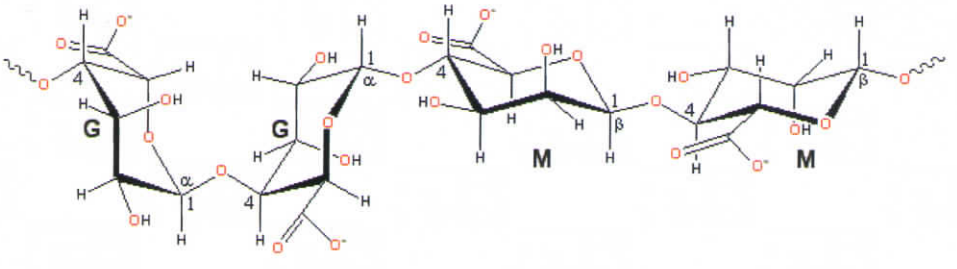
Some alginate dressings (e.g., Kaltostat) enhance wound healing through additional bioactive mechanisms. These are shown to induce the stimulation of human monocytes to produce elevated levels of TNF-α, Interleukin-6 (IL-6) and IL-1β. Production of these cytokines at the wound site would result in delivery of a pro-inflammatory stimulus which is advantageous in wound healing. It is also postulated that the high levels of bioactivity of these dressings is due to the presence of endotoxin in alginates since these are derived from natural sources (Thomas *et al.*, 2000).

Despite all these merits, studies have shown that residual alginate fibres are non-degrading and are remaining in the wound bed. In addition, cytotoxicity studies showed that these dressings are cytotoxic to fibroblasts and epidermal cells due to the diffusion of calcium ions from the gels in physiological conditions (Odell *et al.*, 1994; Barnett & Varley,

1987; Matthew *et al.*, 1995). These defects indicate that there is still room for improvement n dressing formulations based on alginates.

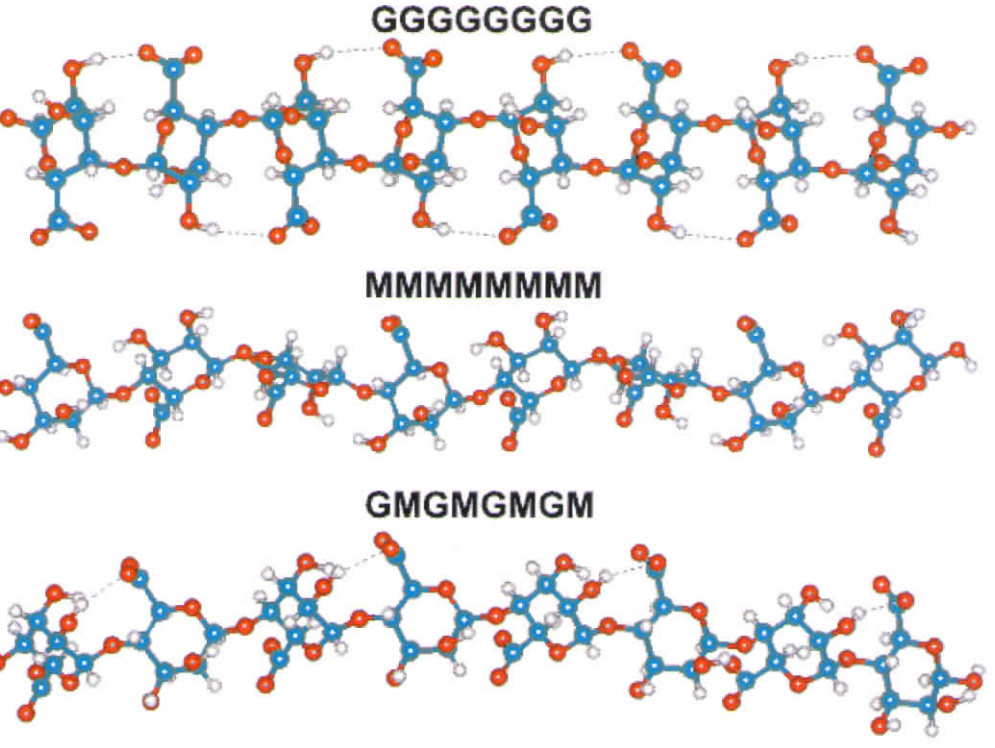
1.8.1 Structure of Alginates

Alginates are natural polysaccharides extracted from brown algae. Irrespective of he source of alginates, it is an anionic linear polysaccharide composed of 1, 4-linked β-D-mannuronate (M) residues and 1, 4-linked α-L-guluronates (G) in varying proportions Rees & Welsh, 1977). Alginates are not random copolymers but, according to the source lgae, consist of blocks of similar and strictly alternating residues (i.e. MMMMMM, GGGGGG and GMGMGMGM), each of which have different conformational preferences nd behavior. The structure of alginate is illustrated in Figure 1.5 and Figure 1.6.

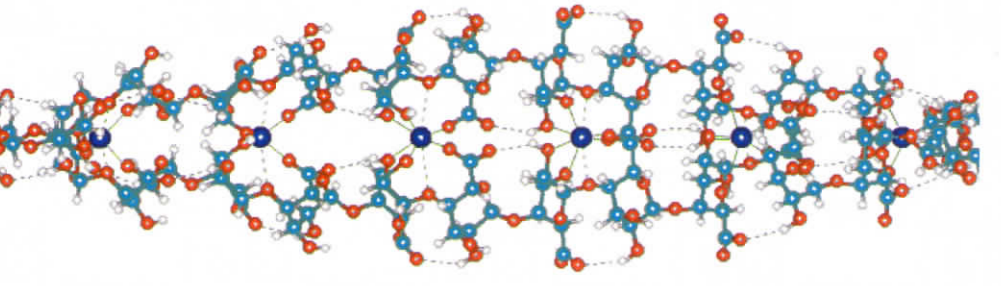


igure 1.5: Structural unit of alginate.

An important feature of alginate is its gelation in the presence of divalent cations, uch as calcium. The G-blocks are responsible for the "egg box" formation with calcium ons or other alkaline earth metal ions (Figure 1.7) (Grant *et al.*, 1973). However, alginate els dissolve in an uncontrollable manner following the loss of divalent cations releasing high nd low molecular weight alginate units (Shoichet *et al.*, 1996).



ure 1.6: Molecular structure of alginate.



ure 1.7: Hydrogen bonding and calcium binding sites of alginate.

1987; Matthew *et al.*, 1995). These defects indicate that there is still room for improvement in dressing formulations based on alginates.

1.8.1 Structure of Alginates

Alginates are natural polysaccharides extracted from brown algae. Irrespective of the source of alginates, it is an anionic linear polysaccharide composed of 1, 4-linked β-D-mannuronate (M) residues and 1, 4-linked α-L-guluronates (G) in varying proportions (Rees & Welsh, 1977). Alginates are not random copolymers but, according to the source algae, consist of blocks of similar and strictly alternating residues (i.e. MMMMMM, GGGGGG and GMGMGMGM), each of which have different conformational preferences and behavior. The structure of alginate is illustrated in Figure 1.5 and Figure 1.6.

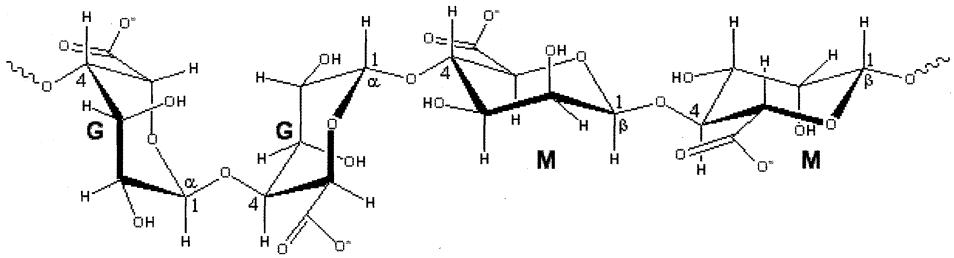


Figure 1.5: Structural unit of alginate.

An important feature of alginate is its gelation in the presence of divalent cations, such as calcium. The G-blocks are responsible for the "egg box" formation with calcium ions or other alkaline earth metal ions (Figure 1.7) (Grant *et al.*, 1973). However, alginate gels dissolve in an uncontrollable manner following the loss of divalent cations releasing high and low molecular weight alginate units (Shoichet *et al.*, 1996).

1.8.2 Degradation

1.8.2.1 Acid Hydrolysis

Generally, polysaccharides degrade via hydrolysis of glycosidic linkages by an acid catalyzed mechanism (Figure 1.8). Compared to other sugars, glycosidic linkages involving uronic acids such as M and G are quite resistant towards hydrolysis in very strong acids. However, at pH values near the pKa value of alginates (pH 1-4), protonated (-COOH) form of M and G contributes to the hydrolysis by intra molecular catalysis in addition to the free H^+ ions (Smidsrod *et al.*, 1966).

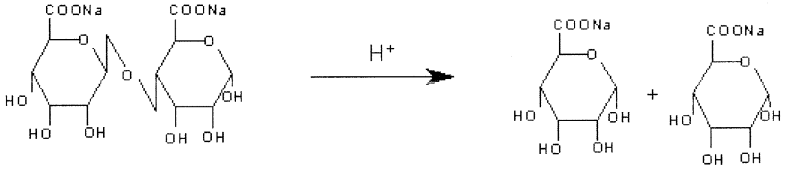


Figure 1.8: Acid hydrolysis.

1.8.2.2 Alkaline Hydrolysis

Glycosidic linkages are generally very resistant to alkaline hydrolysis. However, 4-linked uronic acids are susceptible to cleavage by an OH- catalyzed β-elimination reaction (Figure 1.9) leading to the formation of a 4, 5-unsaturated uronic acid at the non-reducing end. Therefore, the rate of this type of degradation increases rapidly at high pH-values and becomes proportional to the concentration of OH^- ions at pH–values above 10.5 (Haug *et al.*, 1967).

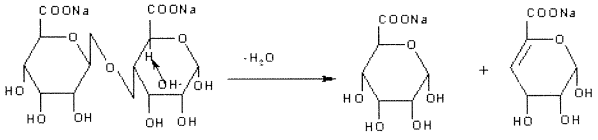


Figure 1.9: Alkaline -β–elimination in alginates.

1.8.2.3 Enzymatic Degradation

Enzymes degrading alginates have been isolated from many marine and terrestrial bacterial isolates and marine gastropods. All the enzymes characterized so far are lyases (eliminases) cleaving the polysaccharides by β–elimination. In the case of enzymatic degradation, different enzymes need different optimum pH and show different specificities (Haugen, 1990; Ostgaard & Larsen, 1993).

1.8.2.4 Oxidative–Reductive Degradation

Alkaline solutions also favour another type of degradation, called oxidative–reductive depolymerization (ORD). ORD of alginates involves a series of free radical reactions which ultimately leads to chain scission. Auto-oxidizable compounds like ascorbate, sulphites and phenols in particular will initiate ORD. In addition, molecular oxygen and transition metal ions (e.g. Fe^{2+}/Fe^{3+}) are efficient catalysts for ORD. The mechanism involves oxidation of the reducing compound yielding a peroxide, followed by decomposition of the peroxide to free radicals leading to chain reactions, some of which result in the depolymerization of the alginate.

1.8.2.5 Degradation by Temperature and Irradiation

At high temperatures, during drying or sterilization, degradation may take place. This reaction, which involves the cleavage of –C-O- linkages, is normally slow. Irradiation with γ rays may also lead to degradation. Here also ORD mechanism is involved initiated by irradiation. The absence of oxygen can decrease the rate of degradation to certain extent.

1.8.2.6 Degradation in Organisms

In organisms where there is no alginate lyase enzymes to degrade, alginates are reported to be broken down into simple glucose like residues and is absorbed (Gilchrist & Martin, 1994). This is however, controversial. Although alginates having low molecular weight (< 80 KDa), are known to be excreted from the body, recent studies (Bouhadir *et al.*, 2001b) have shown no evidence of degradation or breakdown of the alginate fibres when used as wound dressings (Matthew *et al.*, 1995). Lansdown and Payne studied the biodegradability and ability to evoke local tissue reactions of alginate swabs implanted subcutaneously in rats and found no significant evidence of biodegradation within three months (Lansdown & Payne, 1994). There are reports that alginate fibres left in an apicectomy cavity of a patient were relatively resistant to degradation and could persist for more than six months (Rosdy & Clauss, 1990).

1.8.3 Derivatives of Alginates

Modification of alginates has received very little attention so far. Modification is restricted within narrow limits due to the low reactivity of the hydroxyl group. There are two types of reported derivatives, one is alginate esters and other is a cross-linkable alginate. Of these derivatives, the only derivative having a commercial value is the propylene glycol ester of alginate that is obtained by esterification of alginate with propylene oxide under pressure (Draget *et al.*, 1997).

Cross-linkable alginate was prepared by periodate oxidation of both, guluronic acid derived from alginate having molecular weight of 6000 Da (Bouhadir *et al.*, 2000) and sodium salt of alginic acid (Bouhadir *et al.*, 1999). These were either cross-linked with

calcium ions or adipic hydrazide to prepare hydrogels suitable for tissue engineering and drug delivery. Though higher molecular weight alginates are found to be non-biodegradable, its oxidized derivative was found to be degradable. Partially oxidized alginates degraded over time to yield low molecular weight oligomers, with the rate of degradation dependent on the pH and temperature of the medium (Bouhadir *et al.*, 2001b). Alginate derived polymeric surfactants were prepared by Kang *et al.* by reducing the Schiff's base formed between oxidized alginate and long chain alkyl amines (Kang *et al.*, 2002).

1.9 Gelatin as a Wound Dressing Material

Gelatin has been known to be biodegradable, biocompatible, non-immunogenic (Lacroix *et al.*, 1995), activates macrophages (Tabata & Ikada, 1987), and has a low coagulation activity towards platelets (Tomihata *et al.*, 1994) which makes it suitable for various biomedical applications such as haemostatic sponges (Cenni *et al.*, 2000), sealant for vascular prosthesis (Guidoin *et al.*, 1987; Jonas *et al.*, 1988; Marois *et al.*, 1995) and as a drug carrier (Di Silvio *et al.*, 1994; Narayani & Rao, 1994; Lou & Groves, 1995).

Gelatin-based wound dressings have received considerable attention (Neumann *et al.*, 1981; Peterson *et al.*, 1984; Takahashi *et al.*, 1993; Yao *et al.*, 1996; Fakirov *et al.*, 1996). Gelatin can be cross-linked with various cross-linking agents (Shalaby & Park, 1994). Cross-linked gelatin fabricated into dry sponges is useful for inducing haemostasis in bleeding wounds. Commercially available sponges include Spongostan (Ferrosan, Denmark) and Gelfoam (Upjohn, USA). Gelatin-alginate sponge cross-linked using carbodiimide has been studied and evaluated as a wound dressing by Choi *et al.* (Choi *et al.*, 1999a). They also developed an artificial skin impregnated with silver sulphadiazine

using hyaluronic acid to form a cross-linked structure with gelatin in the presence of carbodiimide (Choi *et al.*, 1999b). Gelatin based spray-on foam bandage incorporated with broad spectrum antibacterial agent, 2-bromo-2-nitropropane-1,3-diol has also been evaluated (Neumann *et al.*, 1981). Genipin, a natural cross-linker has also been used to cross-link gelatin to prepare a wound dressing material (Chang *et al.*, 2003). Ulubayram *et al.* synthesized a polymeric bilayer wound dressing containing epidermal growth factor, in which a porous sponge of gelatin formed the underlying layer and a polyurethane membrane formed the external layer (Ulubayram *et al.*, 2001). Gelatin cross-linked using oxidized dextran for use as a wound dressing material has been reported (Draye *et al.*, 1998). Porous scaffolds based on gelatin and β-glucan, have been studied as bio-artificial skin (Lee *et al.*, 2003). However, the toxicity of cross-linking agent still remains the major impediment in the development of a gelatin based wound dressing. A gelatin-based wound dressing which uses a different, less toxic, cross-linking agent would be very desirable.

1.9.1 The Composition of Gelatin

Gelatin is produced by controlled acid or alkaline hydrolysis of collagen, the most abundant protein in the animal kingdom. Collagen exists in different forms, but gelatin is only derived from sources rich in Type I collagen which contains no cystine. Gelatin molecule contains repeating sequences of glycine-proline-hydroxyproline triplets, which is basically responsible for the triple helical structure of gelatin and its ability to form gel. The amino acid analysis of gelatin is variable depending on the raw material and process used, but approximate values by weight are glycine, 26.4-30.5%; proline, 14.8-18%; hydroxyproline, 13.3-14.5%; glutamic acid, 11.1-11.7% and alanine, 8.6-11.3%. Reactive groups present per 100 g of high quality gelatin are primarily, hydroxyl, carboxyl and amino at an amount of

approximately 100, 75 and 50 meq of each of these groups respectively. Modes of hydrolysis generate two different types of gelatin–Type A and Type B and reactive groups may vary a little between these two types. Acidic treatment yields Type A gelatin having an isoionic point 7 to 9 due to limited hydrolysis of the asparagine and glutamine amino acid side chains, whereas alkaline treatment hydrolyses the asparagine and glutamine relatively quickly, with the result that the Type B gelatin has an isoionic point of 4.8 to 5.2. Acid curing produces gelatin with high gel strength of 290-300 Bloom, while the same collagen source when subjected to alkaline cure yields a basic type gelatin averaging 256-260 Bloom.

1.9.2 Solubility

Gelatin is practically more convenient than commercially available collagen as it is extremely difficult to prepare collagen solution in high concentrations. Gelatin is only partially soluble in cold water; nevertheless dry gelatin swells or hydrates when stirred into water. On warming to 40 °C, it will form uniform solution when it has been allowed to hydrate for 30 min. However it forms thermally reversible gels with water and the gel melting temperature (<35 °C) is below body temperature. Gelation is markedly affected by the presence of electrolytes. Soluble sulphates, malates and citrates greatly increase the rate of gelation at any given concentration and temperature whereas chlorides, nitrates and thiocyanates retard gel formation and are called liquefiers or peptizing agents. Organic peptizing agents like urea, thiourea, ethylene chlorohydrin, sodium naphthalene sulfonate, ethyl ether and chloral hydrate also retard gelation. Even though this will render gelatin to exist in fluid form at room temperature, there is reduction in viscosity which appears to be a manifestation of unwinding of the collagen coiled fibres since there is large drop in average molecular weight associated with only a minor increase in rupture of peptide linkages (Mark *et al.*, 1967; Mac Gregor & Greenwood, 1980).

1.9.3 Cross-linking of Gelatin

The thermal and mechanical properties of the gelatin can be modified by cross-linking. Several physical and chemical cross-linking methods have been reported. Physical methods include dehydro-thermal treatment and UV-irradiation (Welz & Ofner, 1992). However, these methods cannot control the degree of cross-linking. Chemical cross-linking agents are of two types- non zero-length and zero-length. Zero-length cross-linkers are which introduce cross-links without the incorporation of foreign structures into the network e.g., by activating carboxylic acid residues to react with free amino groups in the protein molecule, resulting in the formation of an amide bond. Acyl azide (Rao, 1995) and carbodiimide cross-linking are the representatives of this class (Lee *et al.*, 1996; Kuijpers *et al.*, 2000). Non-zero length cross-linkers introduce poly- or bi- functional cross-links into the network structure by bridging free amino groups of lysine or hydroxy lysine, or free carboxylic acid residues of glutamic and aspartic acid of the protein molecule. These cross-linkers include aldehydes (formaldehyde, glutaraldehyde, glyceraldehydes) (Digenis *et al.*, 1994; Vandelli *et al.*, 2001; Miyata *et al.*, 1992; Tang & Yue, 1995; Tu *et al.*, 1994) and isocyanates (Naimark *et al.*, 1995; Petite *et al.*, 1994). However, all these agents are toxic and would be leached into the body upon biodegradation of the hydrogel.

1.10 Aim and Scope of Work

According to the latest reports (Health Care Management Express, 2003) more than 700,000 patients are hospitalized and 120,000 die annually, due to burns injury every year in India. We have only 32 burn units, and that too with inadequate facilities. Prompt replacement of the integrity of the skin is a corner stone of therapy for these patients. While

the chances of survival of severe burn patients can be enhanced by skin grafting, burn surgeons regret that cadaveric skin donation cannot be practiced due to the non-inclusion of skin in Transplantation of Human Organs Act, 1994. Live skin donation is hampered by unavailability of donors, the risks associated with viral contamination and the pain and trauma that the donor has to undergo. Under these circumstances, there is high need for the development of wound dressings which are potentially beneficial to large number of patients.

In the case of severe burns, trauma, ulcers and other conditions where there is significant damage of tissue, wounds should be covered with a dressing, which replaces the functions of the natural skin. It should reduce the evaporation of water from the wound bed and associated energy loss, prevent or minimize the microbial invasion at the wound site and stimulate the healing process by providing a support for the vascularization and tissue regeneration at the interface between the dressing and the wound surface. Also, application of dressing should be uncomplicated and cost effective.

Most commercially available dressings in the form of membranes and sheets are problematic as far as the conformability is concerned and the *in situ*-formed dressings will therefore be superior to pre-formed dressings. *In situ*-forming dressings are beneficial in the sense that it will avoid complications such as wrinkling and fluting of the dressing on the wound bed. *In situ*-forming dressings also can achieve circumferential coverage of wound with least pain, especially in the case of burns.

The present work aims at the development of an *in situ*-forming dressing, based on biopolymers which when applied onto the wound bed forms a hydrogel very rapidly. There are reports (Thomas *et al.*, 2000) suggesting that certain alginate dressings (e.g., Kaltostat) can enhance wound healing by the stimulation of human monocytes to produce elevated

levels of cytokines at the wound site resulting in a pro-inflammatory stimulus advantageous to wound healing. There are also reports proving the suitability of gelatin sponges for inducing haemostasis in bleeding wounds (Cenni *et al.*, 2000). Therefore, a composite matrix derived from both alginate and gelatin will have the synergic beneficial aspects of both polymers. Both alginate as well as gelatin has been used as a scaffold for tissue engineering and controlled drug delivery (Bauhadir *et al.*, 2001b; Kuijpers *et al.*, 2000). Therefore, sodium alginate and gelatin were selected for the present investigation in order to exploit the synergetic wound healing efficiency of the combination.

For an *in situ*-forming dressing, the gelation time should be optimal, ideally a few seconds so that once applied, the polymer construct stays at the site and does not migrate or dissolve. It has been demonstrated by Draye *et al.* (Draye *et al.*, 1998) that periodate oxidized dextran form Schiff's linkages with gelatin. However, even at 40 °C, prolonged reaction time was required for cross-linking leading to the formation of a three-dimensional gel. The present study examines the possibility of preparing an *in situ*-forming hydrogel from periodate oxidized alginate and gelatin.

Alginates form high viscous solutions in aqueous media and therefore the reaction involving alginates is usually conducted in dilute solutions. In the present study, the possibility of periodate oxidation of alginate as dispersion in ethanol/water mixture with the aim of obtaining a larger quantity of the oxidized product with minimum amount of solvent in one reaction was examined. The kinetics of oxidation reaction in both media has been compared. The properties of the oxidized product with that obtained by oxidation in aqueous media, especially the degree of oxidation, dialdehyde content and molecular weight were also examined.

This work also examines the effect of small concentrations of sodium tetraborate (borax) in facilitating rapid gelation between oxidized alginate and gelatin. Due to the slightly alkaline pH and the ability to complex with hydroxyl groups of polysaccharides, it was found that the presence of small concentrations of borax could accelerate the gelation process significantly leading to *in situ*-forming hydrogels. Borax also has a long history of medical use because of its antiseptic and antiviral activity and aqueous solutions have been used as mouth washes, eye-drops, skin lotions and cosmetics and in ointments.

The *in situ*-forming hydrogels have been characterized extensively. The gelation time has been determined with respect to the change in concentration of oxidized alginate, gelatin and borax. The cross-linking degree of the hydrogels has been determined by trinitrobenzene sulphonic acid method, cross-linking density and molecular weight between cross-links by swelling measurements, morphology by scanning electron microscopy and image analysis and rate of degradation by gravimetry.

The vital aim of the work is to evaluate the matrix as a wound dressing. With this intention, water uptake and water vapour transmission rate of hydrogels and rate of evaporation of water from hydrogels have been studied. Further, cytotoxic, irritation, sensitization and haemolytic potential of the hydrogel were evaluated as per International Standards Organization (ISO) guidelines. The wound healing efficiency of the hydrogels has been studied by forming these hydrogels *in situ* on experimental full thickness wounds in a rat model. Histological analyses at different time periods during the healing process have been carried out and the rate of wound re-epithelialization and wound size reduction have been quantified.

Cyclic adenosine monophosphate (cAMP) is one of the intracellular mediators, which function directly to regulate cell proliferation. With the aim of further improving the wound healing efficacy of these *in situ*-forming gels, N'-2'-O-dibutyryl adenosine, 3'5'-cyclic monophosphate, a water-soluble derivative of cAMP, has been incorporated in the hydrogel and the wound healing efficacy of the modified gel has been evaluated in experimental full thickness rat wound model.

The *in situ*-forming hydrogels were further evaluated as a carrier of drugs to the wound bed. Drugs containing functional groups like $-NH_2$, hydrazine etc. can enter into reaction with the aldehyde groups in periodate-oxidized alginate and will get released by the hydrolysis of the Schiff's linkages or by degradation of the matrix. To impart antibacterial property to the hydrogel, gentamycin was incorporated in the hydrogel and the antibacterial effect was examined against two strains of bacteria *S. aureus* and *P. aeroginosa*.

In order to examine the potential of the hydrogel as an injectable drug delivery system for sustained delivery, primaquine was chosen as a model drug for incorporation into the hydrogel and its release profile was examined *in vitro*.

Finally, explorative studies on the suitability of matrix as an injectable tissue engineering scaffold for cell delivery was carried out using rat hepatocytes. Hepatocytes are difficult to culture and propagate *in vitro* for extended periods. Hepatocytes were encapsulated within the hydrogel matrix and its proliferation and viability were evaluated by neutral red assay. The functionality of the encapsulated hepatocytes was also examined by estimation of albumin secreted by these cells in culture.

Chapter-2
Materials & Methods

MATERIALS AND METHODS

2.1 Materials

Sodium alginate (medium viscosity grade, viscosity of 2% solution: 3500 cps at 25 °C), gelatin (Bloom 300, Type A, M.W 100000), sodium metaperiodate, sodium tetraborate decahydrate (borax), trinitrobenzene sulphonic acid (TNBS), N'-2'-O-dibutyryl adenosine, 3'5'- cyclic monophosphate (DBcAMP), primaquine phosphate, fluorescein isothiocyanate bovine albumin (FITC albumin), phenol (99%, ultrapure), Freund's complete adjuvant, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT), Dulbecco's Minimum Essential Medium, ethylene glycol-bis(β-aminoethyl ether)-N, N, N', N'- tetraacetic acid tetrasodium salt (EGTA) and Trypan Blue were obtained from Sigma Chemicals Co., St. Louis, MO, USA. Polysaccharide standards ($M_w/M_n < 1.2$) and glucose for molecular weight determination were supplied by Polymer Laboratories, Amherst, MA, USA. Sodium thiosulphate, sodium bicarbonate, potassium iodide, starch, silver nitrate, disodium hydrogen phosphate, sodium dihydrogen phosphate and sodium chloride were procured from S.D.

Fine Chemicals, Mumbai, India. Ethanol obtained from the Hospital Wing of the Institute was distilled prior to use. Dialysis tubing (Spectra/Por®, M.W.C.O 3500) was from Spectrum Laboratories Inc., CA, USA. High density polyethylene (density, 0.934) was from Solidur Plastics Ltd, Hyderabad, India. Dynaplast, elastic adhesive bandage B.P. was obtained from Johnson & Johnson Ltd, Mumbai, India. Ketamine hydrochloride injection I.P. and pentabarbitol injection I.P were procured from Neon Laboratories Ltd, Mumbai, India. Xylaxin injection was obtained from Indian Immunologicals Ltd, Andhra Pradesh, India. Gentamycin was procured from Fluka Chemie AG, Switzerland. Mueller Hinton Agar for antibacterial activity testing and Hanks Balanced Salt solution (HBSS) for hepatocyte encapsulation studies were procured from HiMedia Laboratories, Mumbai, India. Gram-positive *Staphylococcus aureus* (ATCC 25923) and Gram-negative *Pseudomonas aeruginosa* (ATCC 27853) were obtained from American Type Culture Collection, Rockville, MD, USA. L_{929} cells for cytotoxicity evaluation were subcultured from stock culture obtained from National Centre for Cell Sciences, Pune, India. Neutral Red for measuring the viability of hepatocytes was procured from HiMedia Laboratories, Mumbai, India. Bromocresol-green (BCG) reagent kit for albumin estimation was purchased from NPIL Reagents, Mumbai, India. Multi-well tissue culture plates for cytotoxicity and hepatocytes encapsulation studies were from Nunc, Denmark. Fresh human blood anti-coagulated with acid citrate dextrose (ACD) used for haemolysis assay was procured from the Institute's blood bank. Oxidized alginate, gelatin and borax were sterilized with ethylene oxide using standard protocols. Sterile, pyrogen-free distilled water was used to prepare solutions of 0.1 M borax and of gelatin. All animal experiments were performed with the approval of the Institute's Animal Ethics Committee. Composition of buffers used for various studies is given in Appendix A.

2.2 Methods

2.2.1 Periodate Oxidation of Sodium Alginate

2.2.1.1 Oxidation in Aqueous Medium

Sodium alginate, 20 g was dissolved in 400 mL distilled water by prolonged magnetic stirring in a beaker. Different quantities of sodium metaperiodate dissolved in 100 mL distilled water were added to the solution and were stirred magnetically in the dark at 25 °C for 6 h. The solution was then dialyzed against distilled water (2.5 L) with several changes of water until it was free from periodate (48 h). Complete removal of periodate was ensured by testing the dialyzate for the absence of turbidity or precipitate with an aqueous solution of silver nitrate. The solution was then frozen at -78 °C, lyophilized and stored in a desiccator in the refrigerator at 4 °C. Yields were always within 25-35%.

2.2.1.2 Oxidation in Ethanol/Water Mixture

Sodium alginate, 20 g was dispersed in 100 mL ethanol. Different amounts of sodium metaperiodate in 100 mL distilled water were then added to the reaction mixture to get different percent oxidized alginates. This was then stirred magnetically in the dark at 25 °C for 6 h. The solution was dialyzed against distilled water (2.5 L) as before, lyophilized and stored in a desiccator at 4 °C. Yields ranged from 50 to 60%.

2.2.2 Characterization of Alginate Dialdehyde (ADA)

2.2.2.1 Kinetics of Periodate Oxidation

The extent of periodate oxidation in water and in ethanol/water mixture was followed by iodometric titration of the residual periodate present in the reaction mixture using the modified Muller-Friedberger method (Guthrie, 1953). Briefly, 5 mL aliquot of the reaction

mixture was neutralized with 10 mL of 10% solution of sodium bicarbonate and iodine was liberated by the addition of 20% potassium iodide solution (2 mL). This was kept under dark for 15 min. The amount of excess periodate in the reaction mixture was estimated by titrating liberated iodine against standard sodium thiosulphate solution using starch as indicator. All experiments were done in triplicate.

$$IO_4^- + 2I^- + H_2O \rightarrow IO_3^- + I_2 + 2OH^-$$

2.2.2.2 Determination of Dialdehyde Content

Approximately 50 mg of lyophilized oxidized alginate was dissolved in 10 mL of 0.25 N hydroxylamine hydrochloride-methyl orange solution. The solution was allowed to stand at room temperature for 2 h and was then titrated against standard sodium hydroxide solution until the red-to-yellow end point was achieved by matching the colour of the sample solution with that of a blank one (Zhao & Heindel, 1991). Values reported are average of a minimum three estimations.

2.2.2.3 Molecular Weight Measurements

The weight average molar mass (M_w) of the polymers was determined by using HPLC-GEC (Lesieur *et al.*, 1993). A 30 cm x 0.75 cm TSK-G4000 PW column (Toyo Soda, Tokyo, Japan) preceded by a 2-mm filter (Rheodyne, CA) was equipped with a Hitachi pump (Model L-6000), a precision injection valve (Rheodyne, 50 mL sample loading) and a differential refractometer (R401 Waters Associates, France) connected to a computer for sample detection. Polymer standards and glucose were injected at a concentration of 1 mg/mL to establish the selectivity curve of the column. Injection volume was 50 µL for all analyses. The mobile phase was 100 mM $NaNO_3$ aqueous solution (pH 7) at a flow rate of

1 mL/min. A plot of log M_w versus K_d was used for the determination of the molar mass of the alginate derivatives.

The elution parameter K_d was calculated according to the following equation:

$$K_d = \frac{(V_e - V_0)}{(V_t - V_0)}$$

Where, V_e,V_0 and V_t are the sample elution, void and total volume. The void and total volumes were determined experimentally from the intercept of the baseline with the half-height tangent to the left side of the elution peak given by the 1,660,000-standard and from the maximum of the elution peak given by a 100 mM $NaNO_3$ aqueous solution, respectively. In these conditions, the selectivity curve of the TSK-G4000 PW column was fitted (0.997 correlation coefficient) by the following polynomial:

$$\log (M_w) = 6.69136 - 12.25285\ K_d + 35.07149\ K_d^2 - 47.62062\ K_d^3 + 20.01476\ K_d^4$$

2.2.3 Characterization of ADA Cross-linked Gelatin Hydrogel

2.2.3.1 Gelling Time Determination

One mL of ADA at different concentrations was reacted with 1mL aqueous solution of gelatin in glass vials of 15 mL capacity (diameter 20 mm) under magnetic stirring using a Teflon-coated stir bar (diameter 5 mm, length 10 mm) at 37 °C. Gelling time was noted as the time required for the stir bar to stop according to Mo *et al.* (Mo *et al.*, 2000). The effect of medium on gelation was studied by noting the gelling time in various media like borax (0.1 M, pH 9.4), carbonate buffer (0.1M, pH 9.4), Tris buffer (0.1 M, pH 8.5), PBS (0.1 M, pH 7.4) and sodium hydroxide (0.1 M, pH 10). Values reported are average of 4 to 5 determinations ± S.D.

Viscosity change during gelation reaction between ADA and gelatin was also determined by using a programmable viscometer (Brookfield, Model DV-II+, Brookfield Laboratories, MA, USA) at 37 °C using spindle SLV-31 and small sample adapter (10 mL) at 200 rev/min. Gelling time was noted as the time at which the viscosity of mixture rose to infinity.

2.2.3.2 Degree of Cross-linking

Degree of cross-linking was evaluated by modifying the method of Bubnis & ofner (Bubnis & Ofner, 1992; Lee *et al.*, 2000). Gels were frozen within a minute after solutions of ADA and gelatin were mixed and lyophilized. About 5 mg of lyophilized gel was treated with a mixture of 1 mL of 0.5% solution of TNBS and 1 mL of 4% sodium bicarbonate at 60 °C for 4 h. The unreacted gelatin in the hydrogel reacts with TNBS and forms a soluble complex. One mL of this solution was further treated with 3 mL of 6 N HCl at 40 °C for 1.5 h and its absorbance was spectrophotometrically (Spectronic, Genesys 2, NY, USA) determined at 334 nm after dilution. A standard curve was plotted for non-cross-linked gelatin by treating various concentrations of gelatin by TNBS in a similar manner.

$$\text{Degree of cross-linking (\%)} = \left\{1 - \frac{\text{Absorbance of cross-linked gel}}{\text{Absorbance of non-cross-linked gel}}\right\} x100$$

2.2.3.3 Swelling Measurements

Hydrogels were prepared using different percent oxidized ADAs in 0.1 M borax or in 0.1 M PBS with different concentrations of gelatin. Concisely, about 0.5 mL each of 20% solution of 87and 57 oxidized ADA in 0.1 M borax and PBS and different concentrations (w/v, 10%, 15%, 20%) of gelatin were injected into 10 mL screw capped vials to prepare different gels using a dual syringe fibrin glue applicator. The solutions get

mixed in the mixing chamber by pushing the common plunger and the gel is extruded out of the hypodermic needle facilitating the placement of the insoluble hydrogel. The fibrin glue applicator used is shown in Figure 2.1.

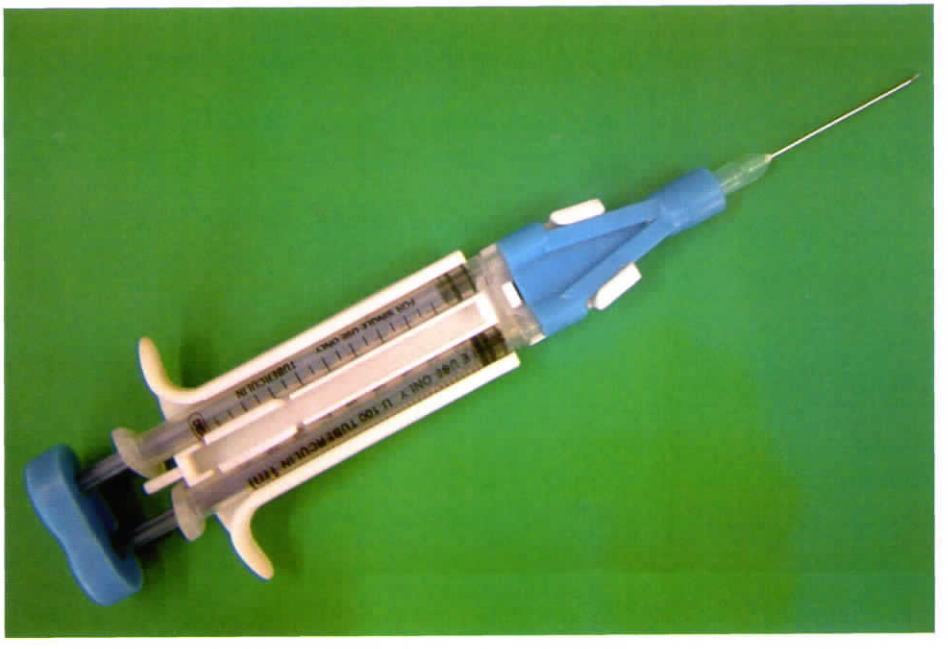


Figure 2.1: Fibrin glue applicator.

After 10 min, hydrogels were allowed to swell in 5 mL of PBS (0.1 M, pH 7.4) for 24 h at 37 °C and excess PBS on the hydrogel was removed and the gels were weighed. Degree of swelling (Q) was defined as the reciprocal of the volume fraction of the polymer in the hydrogel (v_2) and calculated using the equation,

$$Q = v_2^{-1} = [(1/\rho_p)][(Q_m/\rho_s) + (1/\rho_p)]^{-1}]^{-1}$$

where ρ_p is the polymer density (0.825 g/cm^3), ρ_s is the density of water (0.9971 g/cm^3 at 25 °C) and Q_m is the swelling ratio, defined as the mass ratio of absorbed water and the dried gel.

2.2.3.4 Internal Structure of Hydrogels

Internal structure of ADA cross-linked gelatin gels was examined by scanning electron microscopy (SEM). Lyophilized gels were cut using as razor blade, placed on double-sided tape, sputter coated with gold and examined in the microscope (Hitachi, Model S-2400, Japan). SEM images were analysed for pore size determination using the image analysis software (Optimas™ 6.1, West Ford, MA, USA).

2.2.3.5 *In Vitro* Degradation of Hydrogel

Gels were prepared using 0.5 mL of a 20% solution of ADA having degree of oxidation 57% or 87% in 0.1 M borax and an equal volume of 15 % solution of gelatin in distilled water and incubated with 5 mL PBS at 37 °C (n=3). Degradation of gels was followed every week by aspirating the medium followed by freeze-drying the gel to dryness and normalizing the weight obtained to their initial values.

2.2.3.6 *In Vivo* Degradation of Hydrogel

Male, Wistar rats (n=2) weighing approximately 250 g were anesthetized by intramuscular injection of ketamine and xylaxin, at a dose of 40 mg and 5 mg respectively per kg of the body weight of rats. Gels were injected intramuscularly to the gluteal muscle on the dorsal side using a double syringe fibrin glue applicator, in which one syringe was filled with the solution of ADA in 0.1 M borax and other with equal volume of gelatin in water. The applicator was fitted with a 20 G needle. The mixing of the polymer solutions inside the hypodermic needle on pushing the common plunger in the applicator led to gelation and cross-linking in a few seconds leading to the formation of the hydrogel. After one month, the rats were anesthetized with sodium pentabarbitol and sacrificed by its excess dose and histology of the sections were analysed.

2.2.4 Characterization of Hydrogel as a Wound Dressing Material

2.2.4.1 Fluid Uptake Ability of Gels

The fluid absorbing capacity of the hydrogel is one of the important criteria for maintaining a moist environment over wound bed. One half mL of a 20% solution of ADA having degree of oxidation 87% and 57% in 0.1 M borax and equal volume of 15% solution of gelatin in water were introduced into screw-capped test tubes of 10 mL capacity using the fibrin glue applicator to form different gels (26.4 mm diameter and 2 mm thickness). Gelation occurred within seconds after the mixture was extruded out of the needle. After 10 min, 5 mL PBS was introduced and the tubes were incubated at 37 °C. At regular intervals of time, the weight of the gel was noted after removing the PBS and gently blotting the gels with a filter paper. Weights of gels were noted until equilibrium swelling was reached

$$\text{Equilibrium fluid content (\%)} = \frac{W_s - W_d}{W_s} x100$$

where, W_s and W_d represent the weight of swollen and dry sample, respectively. All experiments were done at least in triplicate.

2.2.4.2 Water Vapour Transmission Rate

The moisture permeability of the hydrogel was determined by measuring the water vapour transmission rate (WVTR) across the material as stipulated by ASTM standard (ASTM E96-00, 2000; Queen *et al.*, 1987). Gels having a diameter of 35 mm and thickness 3 mm were prepared by mixing 2 mL of 20% solution of 57% oxidized alginate in 0.1 M borax and equal volume of 15% gelatin solution in tissue culture wells. The hydrogels were mounted on the mouth of cylindrical plastic cups (34 mm diameter) containing 10 mL water

with negligible water vapour transmittance. The material was fastened using Teflon tape across the edges to prevent any water vapour loss through the boundary and kept at 37 °C and 35% relative humidity in an incubator. The assembly was weighed at regular intervals of time and weight loss versus time plot was constructed. From the slope of the plot, WVTR was calculated by the following formula,

$$\text{WVTR} = \frac{\text{Slope x } 24}{\text{A}} g / m^2 / day$$

where, A is the test area of the sample in m^2. Experiments were done in triplicate.

2.2.4.3 Rate of Evaporation of Water from Gel

Hydrogels were prepared in a similar manner as that used for WVTR measurements. Gels were kept at 37 °C and at regular intervals of time, change in weight was noted. Percentage weight loss was found out by the equation

$$\text{Weight loss \%} = \frac{W_t - W_0}{W_0}$$

W_t = Weight at time 't'

W_0 = Weight at time 0

2.2.4.4 Biocompatibility studies

2.2.4.4.1 Cytotoxicity studies

Qualitative cytotoxicity evaluation of ADA-cross-linked gelatin gel was carried out by the direct contact assay with a monolayer of L_{929} mouse fibroblast cells according to ISO standards (ISO10993-5, 1992). Gels were prepared from a 15% solution of gelatin and 20% solution of 57% oxidized ADA in 0.1 M borax. Briefly L_{929} cells were subcultured from stock culture by trypsinization and seeded onto multi-well tissue culture plates. Cells

were fed with Dulbecco's minimum essential medium supplemented with bovine serum and incubated at 37 °C in 5% carbon dioxide atmosphere (Nuiare CO_2 water-jacketed incubator, Minnesota, USA). When the cells attained a monolayer, material (0.75 cm^2) was kept in contact with the cells in triplicate for direct contact assay. After incubation at 37 ± 1°C for 24 h, cell culture was examined microscopically using a phase contrast inverted microscope (Leica, WILD MPS32, Germany) for cellular response. The morphology of the cells was assessed in comparison with negative (high density polyethylene) and positive control (copper wire). Cellular responses were scored as 0, 1, 2 and 3 according to non-cytotoxic, slightly cytotoxic, moderately cytotoxic and severely cytotoxic.

Cytotoxicity of gels was quantitatively assessed by MTT staining assay (Ciapetti *et al.*, 1993) which measures the metabolic reduction of 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide to a coloured formazan by viable cells. Toxicity was evaluated after preparing extract of the material by incubating the gel with media containing serum at an extraction ratio of 0.75 cm^2/mL for 24 h at 37 ± 1°C. MTT dissolved at a concentration of 5 mg/mL in sterile PBS, filtered through a 0.22 μm filter to remove any formazan crystals and stored at -20 °C was used as working solution. Cells were cultured as before in multi-well tissue culture plates and when monolayer was attained, culture medium was removed, rinsed with PBS and 100 μL each of extract of gel, and negative control (high-density polyethylene) and 100 μL of positive control (diluted phenol) were added to different pre-labelled wells containing cells. Cells with medium alone served as control. Culture medium (100 μL) was used as reagent blank. Plates were incubated for 24 h at 37 ± 1°C, in 5% carbon dioxide atmosphere. After 24 h, the extracts/media were removed and 200 μL of MTT working solution was then added using a multi-channel pipette into each well. Plates

were wrapped with aluminium foil and incubated at 95% humidified atmosphere at 37 °C for 8 h. After removing the reagent solution and rinsing with PBS, 200 µL of isopropanol was added to each well and incubated for 20 min at 37 °C in a shaker incubator (Labline Instruments, Melrose Park, USA). The absorbance of the resulting solution was recorded immediately at 570 nm using automated micro plate reader (Bio-Tek Instruments, Vermont, USA). Results were expressed as optical density (OD) after blank (i.e.; medium only) subtraction. Reported values are mean of three replicates.

2.2.4.4.2 Intracutaneous reactivity test

To assess the potential of the hydrogel to produce irritation following intradermal injection of its extract, intracutaneous reactivity test was performed. The test was performed on New Zealand white adult rabbit weighing not less than 2000 g as per ISO standards (ISO 10993-10, 2002). Healthy, smooth skinned animals (n=2) were selected and the fur was closely clipped, swabbed the skin with alcohol and ensured to be free of mechanical trauma or signs of irritation. Sterile hydrogel was prepared by introducing 2 mL each of 20% solution of ADA having degree of oxidation 57% and 15% solution of gelatin into sterile petri dishes using a fibrin glue applicator. The hydrogel was extracted in physiological saline (4g/20 mL; in duplicate) at 70 ± 2 °C for 24 h under stirring at a speed of 50 rev/min in a reciprocating bath-shaker (Model SW-23, Julabo Labortechnik, Seelbach, Germany). At the end of extraction period, the test and control (physiological saline) extract solutions were decanted into sterile beakers and was used for intracutaneous injection into 5 sites (0.2 mL/site), each on the upper portion of 2 rabbits using 22 G needle. The control extract was injected into 5 sites each on the lower portion of same rabbits (Figure 2.2).

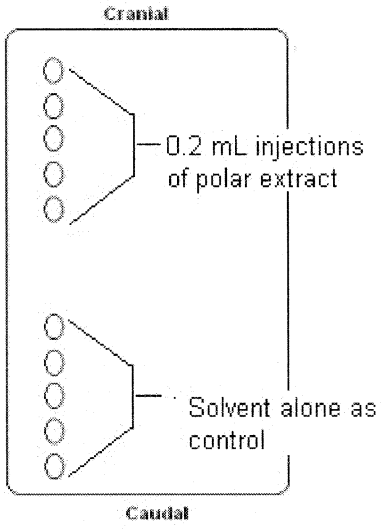


Figure 2.2: Arrangement of injection sites.

All the injected sites were observed for the evidence of any tissue reaction immediately and at 24 h, 48 h and 72 h after injection and scored.

2.2.4.4.3 Maximization test for delayed hypersensitivity

In order to evaluate the skin sensitization potential of the hydrogel, the maximization test for delayed hypersensitivity was performed. This was done on young adult albino guinea-pigs of either sex weighing from 300 g to 500 g as per ISO guidelines (ISO 10993-10, 2002). Test was conducted on the extract (4 g/20 mL) of the material in physiological saline. Hydrogel prepared by using 20% solution of ADA having degree of oxidation 57% in 0.1 M borax and 15% solution of gelatin was used for preparing the extract. Ten animals were treated with the extract of the test material and 5 animals served as control. The fur of selected animals was closely clipped and the skin was swabbed with alcohol. There are three phases of testing which include intradermal induction phase, topical induction phase and challenging phase.

During the intradermal induction phase, a pair of 0.1 mL of each of the following was injected at three different sites as shown in Figure 2.3.

a) A 50: 50 (v/v) mixture of Freund's complete adjuvant mixed with the physiological saline in both test and control animals.

b) The extract of the gel in the test animals and solvent alone in the control animals.

c) The extract of the gel emulsified in a 50:50 (v/v) mixture of Freund's complete adjuvant and the solvent in test animals and solvent mixed with adjuvant in the control animals.

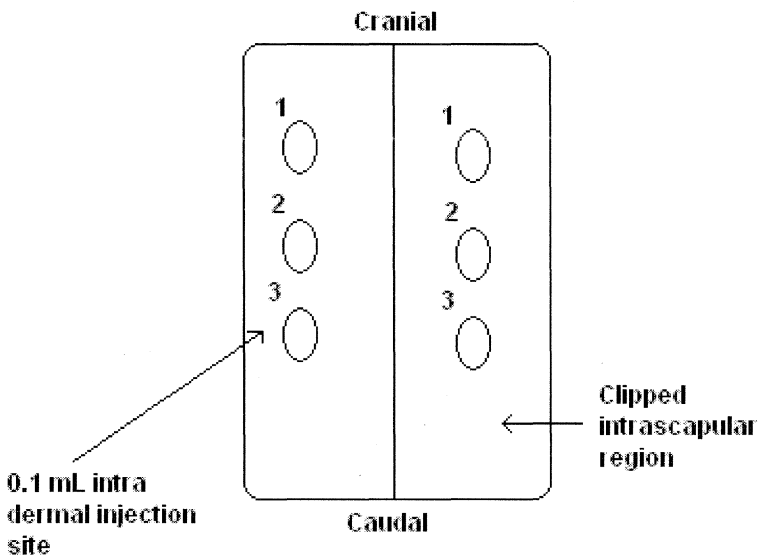


Figure 2.3: Location of intradermal injection sites.

In the topical induction phase (seven days after completion of the intradermal induction phase), the extract of the gel was topically applied to the intrascapular region of each animal using 20 mm x 40 mm absorbent gauze pad, so as to cover the intradermal injection sites. Sites were then covered with an elastic adhesive bandage. Control animals were treated similarly using the solvent alone. After 48 h, the dressings and patches were removed.

Fourteen days after the topical induction phase, i.e., in the challenge phase, again extract of the gel was topically applied to one flank of all test and control animals by soaking the patches in the extract. The sites were then covered with elastic adhesive bandage and the patches and dressings were removed after 24 h. The appearance of the challenged skin sites of the test and control animals were observed 24 h, 48 h and 72 h after the removal of the dressings and skin responses was scored (erythema, oedema formation) and graded for sensitization.

2.2.4.4.4 Haemolysis assay

Haemolytic potential of the hydrogels was determined according to O'Leary & Guess method (O'Leary & Guess, 1969). About 0.2 mL of human blood anti-coagulated with ACD was added to 12.5 mL of PBS containing hydrogels. Hydrogels were prepared by mixing 0.3 mL of 20% solution of ADA having degree of oxidation 57% in 0.1 M borax and an equal volume of 15% solution of gelatin. A separate positive (100% haemolysis induced by replacing the PBS with 12.5 mL of 0.1% sodium carbonate solution) and a negative (PBS with no material added having 0% haemolysis) control was set up. Each set of experiments were done in triplicate. All the test tubes containing samples and control were incubated for 60 min at 37 °C. After incubation, the tubes were centrifuged at 3000 rev/min for 5 min. The percentage haemolysis was calculated by measuring the OD of the supernatant solution at 545 nm in a UV-Visible spectrophotometer (Spectronic, Genesys 2, NY, USA) as per the following formula

$$\text{Haemolysis (\%)} = \frac{OD\ of\ the\ test\ sample - OD\ of\ negative\ control}{OD\ of\ positive\ control} X100$$

2.2.5 Evaluation of Wound Healing Efficacy of the Hydrogel

2.2.5.1 Wound Healing

The wound healing characteristics of the *in situ* formed hydrogel were evaluated using a rat model. Male, Wistar rats weighing approximately 250 g were anesthetized by intramuscular injection of ketamine and xylaxin, at a dose of 40 mg and 5 mg per kg body weight respectively. The skin of the animal was shaved and disinfected using 70% ethanol. Two full thickness skin wounds of 1 cm^2 area were prepared by excising the dorsum of the animals (Figure 2.4). Photograph of wound, by placing a sterile ruler along its side, was taken to measure the wound area.

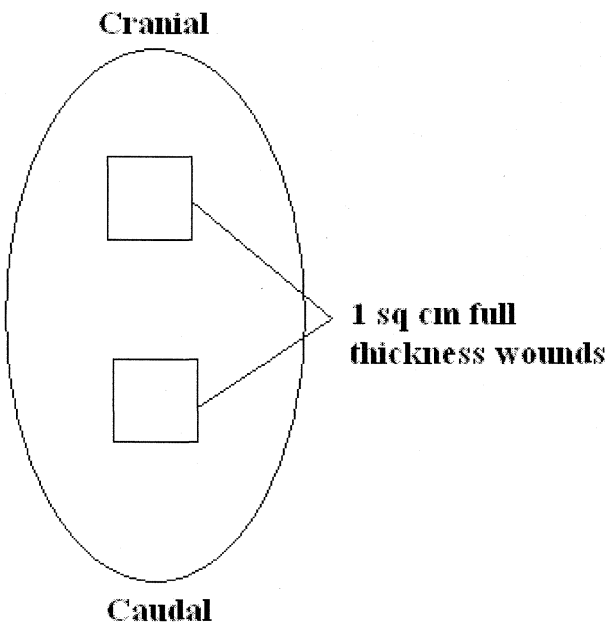


Figure 2.4: Location of full thickness wounds excised on the dorsum of rat.

About 0.2 mL of 20% solution of ADA having degree of oxidation 57% in 0.1 M borax and an equal volume of 15% solution of gelatin were introduced onto the wound bed

using the double syringe fibrin glue applicator. Spreading of the gel evenly on the wound bed was done immediately on application with the aid of fire-polished glass rod tip. The test wounds (n = 6) were then covered with sterile gauze, which was then fixed with elastic adhesive bandage (Dynaplast®). Similarly, control wounds (n = 6) also were covered with sterile gauze and elastic adhesive bandage without the test material. After experiment, animals were kept in separate cages and fed with commercial rat feed and water *ad libitum* until they were sacrificed.

The rats were sacrificed by excess dose of sodium pentabarbitol on day 5, 10 and 15 after surgery. The wounds were grossly examined and photographed for measurement of wound size reduction. For histology, the skin including the entire wound with adjacent normal skin was excised and fixed in 10% buffered formalin. The specimen included the dermis and the subcutaneous tissue. The wound size measurements taken at the time of surgery and at the time of biopsy were used to calculate the percent reduction in wound size using equation

$$\text{Wound size reduction (\%)} = \frac{[A_0 - A_t]}{[A_0]} \times 100$$

where, A_0 and A_t are initial wound area and wound area after a time interval 't'. Area was measured from the photographs of the wounds using the image analysis software (NIH Image tool III, Maryland, USA).

2.2.5.2 Histology

Excised wound sites fixed in formalin were processed and embedded in paraffin, and sections of 3-5 μm were stained with hematoxylin and eosin. Percentage of wound re-epithelialization was determined by using image analysis (Optimas™ 6.1, West Ford, MA,

USA). The distance from right wound margin to left wound margin was measured. The length of newly generated epithelium across the surface of the wound was determined as the sum of the new epidermis growing from right and left margins of the wound. This length was expressed as a percentage of entire wound length.

2.2.6 Evaluation of Wound Healing Efficacy of DBcAMP incorporated Hydrogel

In order to improve the wound healing efficiency of the hydrogel, the growth promoter, DBcAMP was incorporated into the system while mixing two components. DBcAMP was mixed with ADA solution in 0.1 M borax, such that each wound (n= 6 in 3 animals) was covered with a gel containing about 1.5 mg (in 0.2 mL of ADA) of DBcAMP. This solution was sterilized using 0.22 μm filter (Millex® GV Filter unit, Malsheim, France) prior to use. Control wounds (n = 6 in 3 animals) received no hydrogel. The procedure for applying the dressing was as described before and histology of the sections and reduction in wound area were analyzed in a similar way.

2.2.7 Evaluation of Hydrogel as a Drug Carrier

2.2.7.1 *In Vitro* Release Of Gentamycin

Gels were incorporated with gentamycin, a commonly used drug to prevent wound infection. Gentamycin, a hydrophilic drug with three primary amino groups, can enter into Schiff's reaction with ADA. One half mL of a 20% solution of ADA having a degree of oxidation of 87 and 57% in 0.1 M borax containing gentamycin to give a drug payload of 2% or 5% or 10% in the final gel and an equal volume of a 15% solution of gelatin in water were introduced into screw-capped test tubes of 10 mL capacity using a double syringe

fibrin glue applicator fitted with a 20 G needle. Gelation occurred within seconds after the mixture was extruded out of the needle. After 10 min, PBS, 5 mL was introduced and the tubes were incubated at 37 °C. At regular intervals, 1 mL aliquots were withdrawn and replenished with 1 mL fresh PBS. Withdrawn aliquot was then treated with 1 mL of O-phthaldialdehyde reagent and concentration of gentamycin released was noted spectrophotometrically (Spectronic, Genesys 2, NY, USA) at 332 nm.

In order to study the effect of process of gel preparation on drug release, gels were also prepared by inversing the component in which gentamycin was mixed and followed the release profile. Instead of mixing gentamycin with ADA solution, it was first mixed with gelatin solution in such a way that final gel will have 5% drug payload. The gel was prepared and release was noted in a similar manner as described above.

2.2.7.1.1 Preparation of O-phthaldialdehyde reagent

The O-phthaldialdehyde reagent was prepared following a procedure given by Zhang *et al.* (Zhang *et al.*, 1994). It was formulated by adding 2.5 g of O-phthaldialdehyde, 62.5 mL methanol and 3 mL of 2-mercaptoethanol to 560 mL of 0.04 M sodium borate in deionized water solution. The reagent was stored in a brown bottle in dark and kept to settle for at least 24 h prior to use.

2.2.7.2 *In Vitro* Release Of Primaquine From Hydrogels

In vitro release experiments were performed in a similar manner as reported by Jeong *et al.* with minor modifications (Jeong *et al.*, 1997). Primaquine has two amino groups, one primary and another secondary, which also can enter into Schiff's reaction with ADA. One half mL of a 20% solution of ADA having a degree of oxidation of 87 and 57%

in 0.1 M borax and an equal volume of a 15% solution of gelatin in water containing primaquine to give a drug payload of 5% were introduced into screw-capped test tubes of 10 mL capacity using a double syringe fibrin glue applicator fitted with a 20 G needle. After 10 min, PBS, 5 mL was introduced and the tubes were incubated at 37 °C. At regular intervals, 1 mL aliquots were withdrawn and replenished with 1 mL fresh PBS. Absorbance of the released primaquine was read at 355 nm in a spectrophotometer (Spectronic, Genesys 2, NY, USA). All experiments were done in triplicate.

2.2.7.3 Evaluation Of Antibacterial Activity Of Hydrogels Incorporated With Gentamycin

Antibacterial activity of gentamycin impregnated hydrogel was evaluated on two strains of bacteria: *Pseudomonas aeruginosa ATCC 27853* and *Staphylococcus aureus ATCC 25923* by using the procedure reported by Kim *et al.* (Kim *et al.*, 2000). The test microorganisms were inoculated into the Mueller Hinton agar plate at a density of 1×10^5 CFU/mL by pour plate method. The gels (1.5 cm^2) were placed on the agar plate and incubated at 37 °C for 48 h. The drug payload used was 2.5% (9.97 mg/gel). After 2 days, the gel was removed and then 1 cm^2 of the agar beneath the gel was cut out and homogenized in 5 mL of sterile PBS. The PBS was then subjected to serial dilution. The dilutions were subcultured on nutrient agar plates and incubated for 2 days at 37 °C. The number of colonies grown in all was counted and total count was thus calculated. All the experiments were done in triplicates.

2.2.8 Evaluation of Hydrogel as an Injectable Tissue Engineering Scaffold

2.2.8.1 Hepatocyte Isolation

Hepatocytes were isolated from adult rat liver (Wistar, female, 2-3 weeks old, 200-300 g). A two step perfusion method was employed as outlined by Seglen with slight modification (Seglen, 1976). Rat was anaesthetized with xylaxine and ketamine (10 mg and 90 mg per kg body weight respectively) followed by midline laparotomy and cannulation of portal vein. Liver was first perfused with buffered HBSS containing 0.5 mM EGTA for 15 min and then with same buffer without EGTA. Next, 0.05 % collagenase in buffered HBSS containing 4 mM $CaCl_2.2H_2O$ was circulated for 10 min. Glisson's capsule was teased apart to disrupt liver and collected cells in serum free medium. Tissue pieces were removed by passing cell suspension through sterile cheese cloth and then centrifuging at 40 g for 2 min. Hepatocytes were further purified by repeating the centrifugation of cells resuspended in serum free medium. Isolated hepatocytes were cultured in Iscove's Modified Dulbecco's Medium supplemented with 10% foetal calf serum and antibiotics. Cells were stained with Trypan Blue and counted in hemocytometer to check the viability. Trypan Blue reagent solution was prepared in PBS at a concentration of 3μg/ mL. About 8000 cells were seeded per well.

2.2.8.2 Encapsulation Of Hepatocytes

About 50 μL of 20% (w/v) solution of ADA having degree of oxidation 57% in 0.1M borax was introduced into 32 wells of 96 well plate, followed by 30 μL of cell suspension (containing 2.65 x 10^5 cells/mL) and 50 μL of gelatin solution to form gels. Gels, without cell were prepared in another 32 wells in the similar way. Cell suspension

alone was kept in 16 wells. Another 16 wells were coated with gelatin and cell suspension was added to it. About 150 µL of culture medium was added to all wells then and incubated at 37 °C. All the samples were sterilized prior to use.

2.2.8.3 Neutral Red Assay

The viability and proliferation of encapsulated hepatocytes were studied by Neutral Red assay (Lowik *et al.*, 1993). A stock solution of Neutral Red (1 mg/mL) in de-ionised water was prepared and diluted (1:1) with 1.8% NaCl prior to use. Culture medium from the wells was removed and collected for albumin estimation. About 100 µL of PBS was added to each well and centrifuged at 1500 rev/min for 3 min. PBS was then removed without disturbing the cell pellet. About 100 µL of working solution of Neutral Red was then added to each well and kept for 6 h at 37 °C. After the removal of the dye solution by inverting the plates, the gels were rapidly washed twice with PBS. The dye was then extracted from the cells by the addition of 100 µL of 0.05 M NaH_2PO_4 in 50% isopropyl alcohol. OD was read at 630 nm using a microplate reader (Bio-Tek Instruments, Vermont, USA). This was studied after 24 h, 1 week, 2 week, 3 week and 4 weeks.

2.2.8.4 *In Vitro* Release of FITC Albumin from the Hydrogel

In order to examine whether the albumin secreted by the hepatocyte will get released into the medium, FITC albumin was incorporated within the gel and release profile was studied. Hydrogel was prepared using 20% solution of ADA having degree of oxidation 57% in 0.1 M borax and 15% solution of gelatin in a similar manner as used for drug release experiments. FITC albumin was mixed with gelatin in such a way that albumin payload was 1%. After 10 min, PBS (pH 7.4), 5 mL was introduced and the tubes were

incubated at 37 °C. At regular intervals, 1 mL aliquots were withdrawn and replenished with 1 mL fresh PBS. Absorbance of the released FITC albumin was read at 496 nm in a spectrophotometer. All experiments were done in triplicate.

2.2.8.5 Albumin Estimation

Culture medium removed from the tissue culture plate after regular intervals of time was used for albumin estimation. Albumin synthesized by the cells was estimated by BCG method. Albumin will form complex with BCG at pH 4.2. About 3 mL of the reagent (as provided with kit) was added to the test tubes containing 10 µL of the culture medium. To another test tube was added, 10 µL of standard solution of albumin (2.8 g/ dL) and made to react with 3 mL of the BCG reagent. This was then kept for 5 minutes at room temperature. Concentration of albumin was then estimated by measuring the absorbance of test sample and standard against reagent blank at 578 nm. The concentration of albumin of the sample is obtained in g/ dL using the equation,

$$\text{Concentration} = \text{Concentration of standard} \times \frac{\text{Absorbance (sample)}}{\text{Absorbance (standard)}}$$

2.2.9 Statistical Analysis

Statistical analysis of data was performed by one way analysis of variance (ANOVA), assuming confidence level of 95% ($p < 0.05$) for statistical significance. All the data were expressed as mean ± standard deviation (SD).

Chapter-3
Results & Discussion

RESULTS & DISCUSSION

3.1 Periodate Oxidation of Sodium Alginate

3.1.1 Background

The cleavage of the carbon-carbon bond employing periodates, in compounds in which the two carbon atoms, each bearing an oxygen atom either as a hydroxyl or carbonyl group (Figure 3.1.1), was first observed by Malaprade (Malaprade, 1928). This was extensively used in elucidating the ring structure of sugars later by Jackson and Hudson (Jackson & Hudson, 1937). Although periodate can completely degrade a free sugar, the oxide ring in glycosides is sufficiently stable to resist opening by this reagent. Periodate can also oxidize certain α-amino ethanol derivatives such as hydroxylysine residues in collagen. These reactions, however usually occur at a slower rate than oxidation of vicinal diols. It is reported that cis-glycols are more rapidly oxidized than trans-isomers (Price & Knell, 1942). The reaction has been also used to introduce functional groups into the biomacromolecules

like cellulose (Singh *et al.*, 1982), dextran (Draye *et al.*, 1998), hyaluronic acid (Lovekamp & Vyavahare, 2001) and alginates (Lee *et al.*, 2000; Bouhadir *et al.*, 2001a) thus providing unique sites for their chemical modification. This is a convenient method for activation and attachment of polysaccharide chains to proteins.

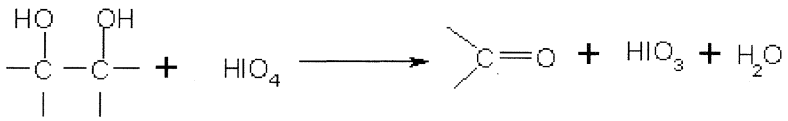


Figure 3.1.1: Schematic representation of periodate oxidation of vicinal glycols.

3.1.2 Oxidation of Alginate

Alginates, derived from brown sea weed are anionic linear polysaccharides composed of 1,4-linked β-D-mannuronate and 1,4-linked α-L-guluronate residues in varying proportions. The alginate used for present study contained 61% of residues of D-mannuronic acid and 39% of residues of L-guluronic acid and its 2% solution had a viscosity of 3500 cps and a weight average molar mass (M_w) of 4, 89, 000 ± 50, 000 g/mol.

Periodate oxidation of sodium alginate was performed initially by treating an aqueous solution of alginate (4% solution) with different quantities of sodium periodate in order to produce alginate dialdehyde (ADA) of different degrees of oxidation. Modulating the concentration of sodium periodate can direct this oxidation to convert all available diols to aldehydes. It has been reported that guluronic acid residues are more selectively oxidized by periodate (Scott & Harbinson, 1969). The reaction was conducted under dark to avoid the decomposition of sodium periodate. Reaction temperature was slightly lower than the room temperature (25 °C) so as to minimize the non-specific oxidation reaction.

Temperatures lower than room temperatures are necessary to prevent hydrolysis of acetal linkages in acid solution. It is reported that oxidation of carbohydrate derivatives is usually fastest at pH 3-5 (Guthrie, 1953). Sodium metaperiodate is most soluble in neutral and weekly acidic solutions. As the pH of un-buffered aqueous solution of sodium periodate is 4, aqueous solution of the sodium periodate was sufficient enough to get a fast rate of oxidation.

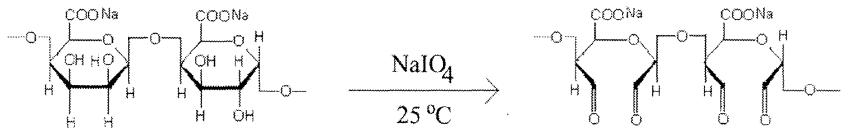
Alginate molecule is highly extended in solution even at infinite ionic strength or low pH, where the mutual repulsion of the charged carboxylic groups does not contribute to the extension of the chain (Smidsrod & Painter, 1973). When periodate was added to sodium alginate aqueous solution, it was found that there was abrupt decrease in its viscosity. The rapid, initial decrease in viscosity of alginate solutions containing periodate has been shown by light scattering and ultracentrifugation to be due to scission of the alginate molecule. Three possibilities have been discussed for this dramatic decrease in viscosity of alginate solutions. One postulates that splitting of the C-2 and C-3 glycol groups converts a rigid pyranose monomer into a flexible link between unaltered segments of the polymer chain, consequently decreasing total stiffness (Scott & Tigwell, 1976). Glycol cleavage in polysaccharides has also been reported to increase polymer flexibility, decreasing hydrodynamic interactions and thereby lowering viscosity in solution. This decrease in viscosity is observed only on periodate oxidations of polyethers and polyacetals (Scott & Tigwell, 1973). The other mechanism invokes the cleavage of polymer chain by extensive depolymerization and extent of depolymerization varied considerably from sample to sample (Painter & Larsen, 1970). Periodate-induced degradation of alginate ensues by two mechanisms; one fast, not mediated by hydroxyl free-radical, and the other slow, possibly

due to low concentrations of hydroxyl radicals. These free radicals are generated during oxidation due to the presence of phenolic impurities present along with alginate. The free radical independent fast degradation implies that there are infrequent and unusual monomers in the alginate molecule which are introduced during biosynthesis or by subsequent *in vivo* or *in vitro* processes which can be cleaved by periodate resulting in the breakage of the chains (Scott & Tigwell, 1973).

The free radical-mediated depolymerization reaction of alginates has been shown to be in inhibited by certain buffer salts and aliphatic alcohols, by acting as free radical scavengers. The presence of propanol has been shown to reduce depolymerization of sodium alginate (Painter & Larsen, 1970). Kang *et al.* also oxidized sodium alginate in a mixture (4:1) of water and *n*-propanol in order to prevent this free radical mediated depolymerization (Kang *et al.*, 2002).

Though the periodate oxidation was proceeding well in aqueous medium, the reaction was very difficult in this medium as alginates form very viscous solutions even at low concentrations and takes more time to dissolve. In aqueous medium, a maximum of only 4% solution could be prepared from medium viscosity alginate derived from *Macrocystis pyrifera*. Bouhadir *et al.* (Lee *et al.*, 2000; Bouhadir *et al.*, 2001a) conducted periodate oxidation of both hydrolyzed alginate with high guluronic acid content (M.W. 6000 Da) and sodium alginate (M.W. 358 kDa) in aqueous medium. These authors used 1% solution of alginate for oxidation in aqueous medium. To make the reaction much handier, the reaction was conducted in 1:1 ethanol/ water medium as a dispersion. Twenty grams of sodium alginate was dispersed in 100 mL of ethanol and periodate dissolved in 100 mL of distilled water was then added to it and kept magnetically stirred under dark at 25 °C for 6 h. By

doing the reaction in ethanol/water medium, solvent quantity needed was small even for preparing larger quantity of the oxidized product, which made the reaction handier. Figure 3.1.2 gives the schematic representation of periodate oxidation of sodium alginate.



10% sodium alginate in ethanol/water mixture as dispersion

Oxidized alginate

Figure 3.1.2: Schematic representation of periodate oxidation of sodium alginate.

3.1.2.1 Degree of Oxidation

The degree of oxidation reaction (%) was studied indirectly by measuring the periodate consumption during the oxidation after regular intervals of time. The periodate consumption was determined by iodometry (Guthrie, 1953). The aliquot was neutralized first with sodium hydrogen carbonate solution and potassium iodide was added to liberate iodine from periodate only.

The degree of oxidation obtained in ethanol/water mixture was compared to that obtained in aqueous medium (Figure 3.1.3). For this, the reaction was carried out in such a way that sodium alginate was treated with stoichiometric amounts of sodium periodate so that the C-2 and C-3 bonds of all units (both guluronic acid and mannuronic acid) of sodium alginate will undergo periodate oxidation. It was observed that the reaction in ethanol/ water mixture proceeded smoothly as that in aqueous medium (Figure 3.1.3).

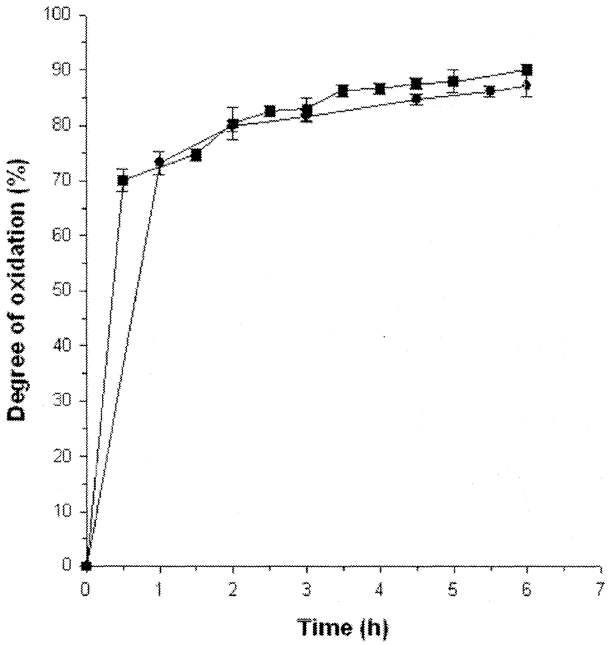


Figure 3.1.3: Variation of degree of oxidation of alginate in aqueous (■) and in ethanol/water (●) media with time.

On further analysis, it was found that in both media the reaction was very fast initially with an abrupt increase in the degree of oxidation to about 70% within 1 h. After that, the reaction was rather slow and attained no complete periodate consumption and therefore, no complete oxidation. It has been reported that periodate oxidation of alginates in aqueous medium initially proceeds in a random manner, that only one monomeric unit in a given chain is oxidized at a time, and the protection of either one or both neighbouring units ensues immediately by intra-molecular, inter-residue hemi-acetal formation (Figure 3.1.4), before the next oxidative attack on the chain occurs. Hemi-acetal formation between oxidized and un-oxidized alginate residue prevents the complete consumption and therefore complete oxidation of the chains (Painter and Larsan; 1970).

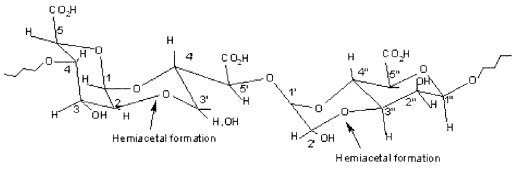


Figure 3.1.4: An oxidized site at the centre of a long chain of un-oxidized residues in an alginate chain.

It has been reported that the oxidation limit of higher molecular weight alginates except at conditions at which alginate is insoluble or glycol cleavage is impaired, vary between 0.49 and 0.55 mol of periodate per uronic acid unit. This was reported for periodate oxidation under highly dilute conditions where alginate is completely dissolved (Painter and Larsan, 1970). In the present study, the oxidation limit obtained in both reaction conditions is between 0.87 and 0.89 moles. This can be attributed to incomplete dissolution of alginate in aqueous medium or minimal dissolution of alginate in ethanol/water medium.

The effect of the concentration of periodate on the degree of oxidation in both media was examined by varying the molar concentration of periodate by keeping the concentration of sodium alginate constant. The degree of oxidation was found to increase with increase in the concentration of periodate and here again the kinetics were very much similar (Table 3.1.1).

Sodium alginate (mmol)	Sodium metaperiodate (mmol)	Periodate equivalents (%)	Degree of oxidation (%)
101.00	29.59	29.3	27.4 ± 0.4 (27.4 ± 0.2)
101.00	50.51	50.0	48.0 ± 0.4 (49.0 ± 0.5)
101.00	65.66	65.0	57.5 ± 0.2 (61.8 ± 0.2)
101.00	95.96	95.0	87.0 ± 0.3 (87.1 ± 1.0)

Table 3.1.1: Experimental degree of oxidation of ADAs obtained by periodate oxidation in ethanol/water mixture and in aqueous medium (values in parenthesis)

3.1.2.2 Dialdehyde Content

Dialdehyde content in oxidized alginate was measured by the hydroxylamine hydrochloride method. When hydroxylamine hydrochloride in methyl orange indicator is reacted with aldehydes in ADAs at pH 4, alginate polyoxime is produced, releasing HCl equivalent for each aldehyde group. By titrating the solution against standard sodium hydroxide, the dialdehyde content (%) was estimated. The dialdehyde content was found to increase with increase in the amount of periodate employed in both media. There was no significant difference between the dialdehyde content of oxidized products obtained in aqueous and ethanol/water mixture (Table 3.1.2).

Periodate equivalents (%)	Dialdehyde content (%) (Ethanol/water)	Dialdehyde content (%) (Aqueous)
29.3	20.4 ± 2	26.2 ± 1
50.0	43.0 ± 2	47.3 ± 1
65.0	55.0 ± 1	58.0 ± 1
94.5	83.7 ± 2	84.2 ± 4

Table 3.1.2: Amount of dialdehyde produced by periodate oxidation of sodium alginate at various periodate concentrations in ethanol/water mixtures and in aqueous solution for 6 h at 25 °C

3.1.2.3 Molecular Weight Determination

Molecular weights of ADA prepared by two methods were determined by HPLC-GEC. There is a gradual reduction in the molecular weight with increase in the amount of periodate employed in aqueous medium due to increase in the extent of oxidation and associated depolymerization (Table 3.1.3). There was no such regular reduction in molecular weights of ADAs obtained by periodate oxidation in ethanol/water mixture except for very high periodate equivalent.

Periodate equivalent (%)	M_w (g/mol) (Ethanol/water)	M_w (g/mol) (Aqueous)
29.3	28,790 ± 1440	69,620 ± 3480
50.0	31,240 ± 1560	56430 ± 2820
65.0	30,245 ± 1510	43,060 ± 2150
94.5	11,340 ± 570	22,930 ± 1150

Table 3.1.3: Molecular weight of ADAs prepared by periodate oxidation of sodium alginate at various periodate concentrations in ethanol/water mixtures and in aqueous solution for 6 h at 25 °C

It is already mentioned that depolymerization of alginates is a free radical-mediated reaction. Figure 3.1.5 shows the different stages of depolymerization reaction involving the free radicals. There are reports about certain buffer salts and aliphatic alcohols such as propanol acting as hydroxyl radical scavengers to prevent depolymerization giving rise to higher M_w alginates (Painter and Larsan, 1970). Therefore, it was expected that the presence of ethanol would prevent free radical-mediated depolymerization and give rise to oxidized alginate of high M_w. On the contrary, the molecular weights of ADAs obtained in ethanol/ water mixture were considerably lower than those obtained for corresponding ADAs prepared by oxidation in aqueous medium. This extensive degradation is believed to be due to the generation of reactive 1-hydroxyethyl radicals during oxidation (as opposed to the more stable and less reactive 2-hydroxypropyl radicals) along with hydroxyl radicals cleaving the glycosidic bonds in alginate in the presence of ethanol. Except for very high periodate equivalents, the M_w of the product was found to remain the same due to the presence of excess ethanol in the reaction medium predominantly influencing the depolymerization of the alginate. At very high periodate equivalent, the effect is believed to be synergic, both free radical-mediated and periodate-mediated depolymerization influencing the cleavage of the alginate chains (Table 3.1.3).

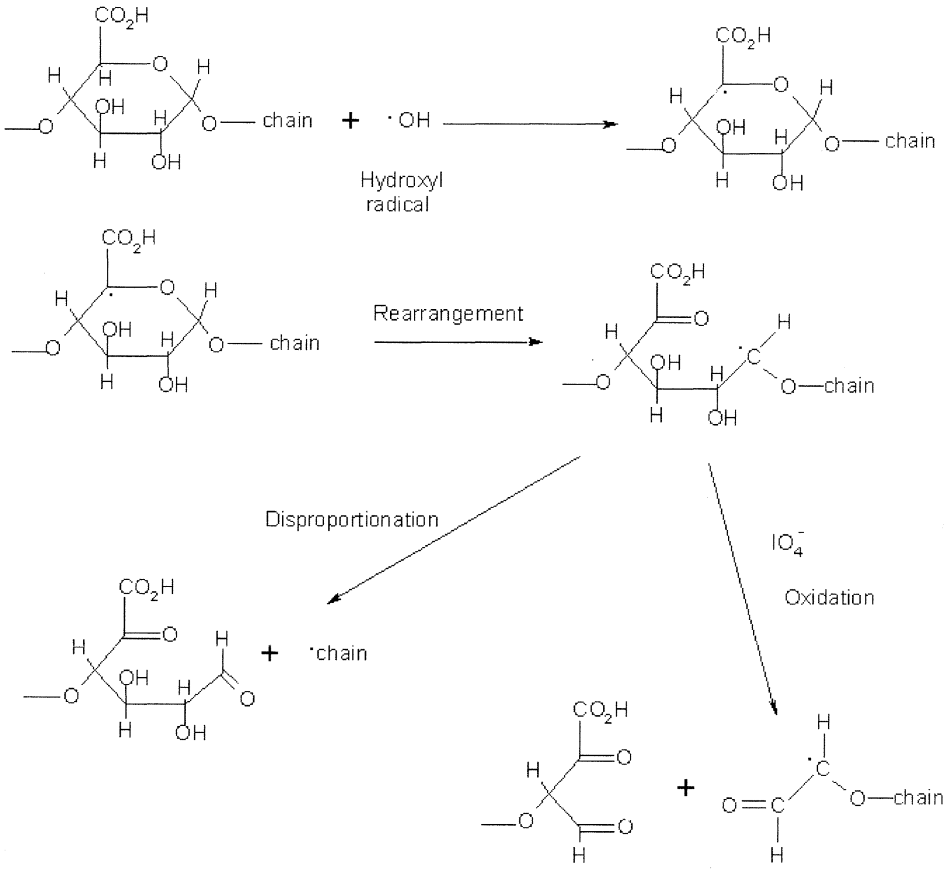


Figure 3.1.5: Mechanism of depolymerization of alginates in the presence of hydroxyl radicals (Scott & Tigwell, 1973).

3.1.2.4 Solubility

ADAs obtained were found to be readily soluble in both 0.1 M borax and 0.1M PBS and solubility was found to increase with increase in the degree of oxidation. Maximum concentration obtained in 0.1 M borax and in 0.1 M PBS was 20% for ADAs having degree of oxidation 57% and 87% (Table 3.1.1) and dissolution was fast in the presence of borax. For ADA having degree of oxidation 27%, only a 10% solution could be prepared in 0.1 M PBS as well as in 0.1 M borax. The fast dissolution in borax is attributed to the ability of borax to complex with the hydroxyl groups (Figure 3.1.6) of the polysaccharides (Coviello *et al.*, 2003).

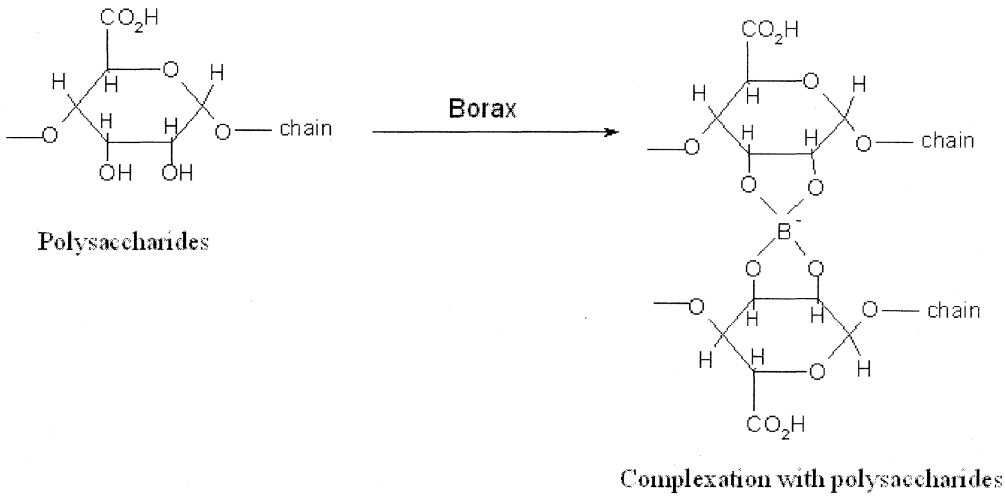


Figure 3.1.6: Schematic representation of borax complexation with polysaccharides.

3.1.3 Conclusion

The study reported in this section has shown that periodate oxidation of alginate in 1:1 ethanol/water medium as a dispersion is a facile method for periodate oxidation of sodium alginate to obtain larger quantities of the oxidized product in one reaction. Both the degree of oxidation and dialdehyde content measurements showed that periodate oxidation is proceeding with same speed in both media. Just as in aqueous medium, periodate consumption was never complete in ethanol/water mixture. This pointed to the possibility of intra-molecular, inter-residue hemi-acetal formation in ethanol/water mixture also. Molecular weight determination showed that there was extensive depolymerization in ethanol/water mixture in comparison with reaction in water alone due to the presence of more reactive hydroxyethyl radicals. Solubility of oxidized alginates was found to be better than alginate. They were easily soluble in borax due to the complexing ability of borax.

3.2 Preparation of Alginate Dialdehyde Cross-linked Gelatin Hydrogel

3.2.1 Background

Hydrogels derived from naturally occurring polymers mimic many features of extra-cellular matrix and thus have the potential to direct the migration, growth and organization of cells during tissue regeneration and wound healing and for stabilization of encapsulated and transplanted cells. Many of them also demonstrate adequate biocompatibility and biodegradability. *In situ*-gelling formulations from biopolymers are achieved by photo-polymerization of their custom-made monomers (Kim *et al.*, 1999), enzymatic cross-linking (Crescenzi *et al.*, 2002; Chen *et al.*, 2003), chemical cross-linking with metal ions (Martinsen *et al.*, 1989), or by cross-linking agents such as glutaraldehyde, carbodiimide, adipic dihydrazide etc.(Shalaby & Park, 1994). However, photo-polymerization often requires a photo-sensitizer and prolonged irradiation limiting their use. Cross-linking with metal ions is often reversible in the body and exerts cytotoxic effects (Lee & Mooney, 2001). Agents that are incorporated into the polymer matrix such as glutaraldehyde, polyepoxides and isocyanates are highly toxic and are prone to leach out into the body on matrix biodegradation (Kuijpers *et al.*, 2000). Agents that cross-link without incorporation by activating the carboxylic acid residues in biopolymers such as acyl azides and carbodiimides are considered less toxic. The toxicity of cross-linking agents is the major obstacle in the use of these polymers as *in situ*-forming polymer scaffolds, since their seepage into body fluids even at low concentrations can be catastrophic (Speer *et al.*, 1980). Potentially less toxic reagents such as adipic acid dihydrazide (Lee *et al.*, 2000; Bouhadir *et al.*, 2001a) and oxidized mono-, di- and polysaccharides have been investigated as cross-linking agents (Cortesi *et al.*, 1998; Draye *et al.*, 1998). The gelation reaction

leading to the three dimensional network is rather slow with many of these reagents to be of practical use as injectable systems and modification of the biopolymer has been attempted to reduce the gelling time (Mo *et al.*, 2000). This section outlines the way in which an injectable system was prepared by the cross-linking of ADA and gelatin without employing any extraneous cross-linking agents.

3.2.2 Preparation of Hydrogels by Chemical Cross-Linking of Gelatin using ADA

Carbonyl groups such as aldehydes, ketones and glyoxals can react with amines to form Schiff's base intermediates that are in equilibrium with their free forms (Figure 3.2.1).

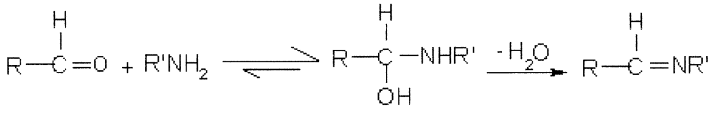


Figure 3.2.1: Scheme representing the mechanism of Schiff's base formation.

It has been reported that the reducing sugars particularly those which are α-hydroxy aldehydes when continually exposed to proteins in blood, first form reversible Schiff's base linkage with the α-amino or ε-amino groups of the proteins. This reaction is also called Maillard reaction (Horvat & Jakas, 2004). This bond then undergoes an Amadori rearrangement to form a stable ketamine derivative. The measurement of glycated hemoglobin is a clinically important parameter in the management of diabetes mellitus. Similar approach was adopted for the preparation of hydrogels based on alginate and gelatin. Cross-linking leading to gelation is predominantly due to Schiff's base formation between the ε-amino groups of lysine or hydroxylysine side groups of gelatin and the available aldehyde of ADA (Figure 3.2.2). The rather labile Schiff's base interaction can also be chemically stabilized via reduction by the addition of sodium borohydride or sodium cyanoborohydride, creating

a secondary amine linkage between the two molecules (Hermanson, 1996). The present study does not employ these reducing agents as these will exert cytotoxic effects when used as *in situ*-formulations.

Both gelatin-Type A and Type B were employed for cross-linking with ADA. It was found that gelatin–Type A which bears more number of amino groups, gave fast gelation reaction with ADA. Therefore, it was chosen for all future studies. During the Schiff's base formation, ADA and gelatin may be cross-linked to several distinguishable levels. Initially, branched polymers are formed which remain soluble which is known as sol stage. As cross-links increase, clusters form and size of clusters increases and eventually the structure becomes infinite in size. Continued cross-linking produces compositions where ultimately all the polymer chains are linked to other chains at multiple points resulting in one giant covalently bonded molecule. This reaction stage referred to as the sol-gel transition is called the gel point. At the gel point, the viscosity of the system becomes infinite, and the equilibrium modulus climbs from zero to finite values. In simple terms, the polymer goes from being a liquid to being a solid.

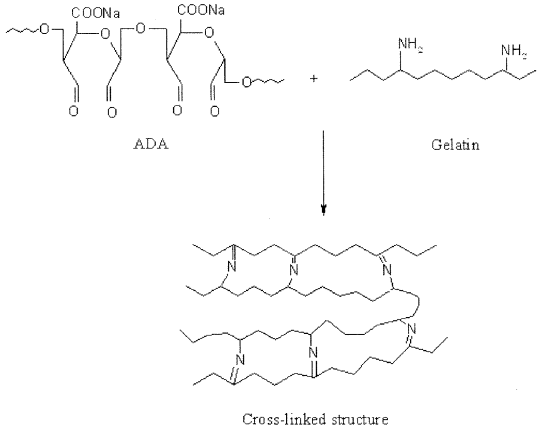


Figure 3.2.2: Scheme of cross-linking reaction between gelatin and ADA.

3.2.2.1 Effect of Medium of Oxidation on Gelation Reaction

As already discussed in section 3.1, alginates were oxidized in aqueous medium and as dispersion in ethanol/water mixture. ADAs obtained by both methods have almost similar degree of oxidation and dialdehyde content. However, the molecular weight of the product obtained by oxidation in ethanol/water mixture was lower than that obtained by oxidation in aqueous medium. In order to find out whether the ADA obtained by periodate oxidation in ethanol/water mixture could function as an efficient cross-linker for gelatin, the gelation reaction between ADAs prepared by two different methods with gelatin using dynamic viscosity measurements was examined. Two media selected for this study were 0.1 M PBS and 0.1 M borax. Five mL each of 10% solution of ADA prepared by both methods in 0.1 M borax or in 0.1 M PBS and 10% aqueous solution of gelatin were mixed in the small sample adapter of programmable viscometer at 37 °C. Gelation reaction was monitored by following the change in viscosity in the viscometer using the Wingather 32® software (Brookfield Engineering Laboratories Inc. MA, USA).

3.2.2.1.1 Gelation in the presence of 0.1 M PBS

Figure 3.2.3 shows the viscosity change during gelation reaction between ADAs obtained by periodate oxidation in both media using 65% periodate equivalents and gelatin in the presence of 0.1 M PBS. It was found that the oxidized product obtained by reaction in ethanol/water mixture gave a fast gelling formulation as compared to that obtained by oxidation in aqueous medium. While ADA obtained by oxidation in ethanol/water mixture gave gelation in about 2 min, gelation of the same obtained by oxidation in aqueous medium occurred only after 10 min.

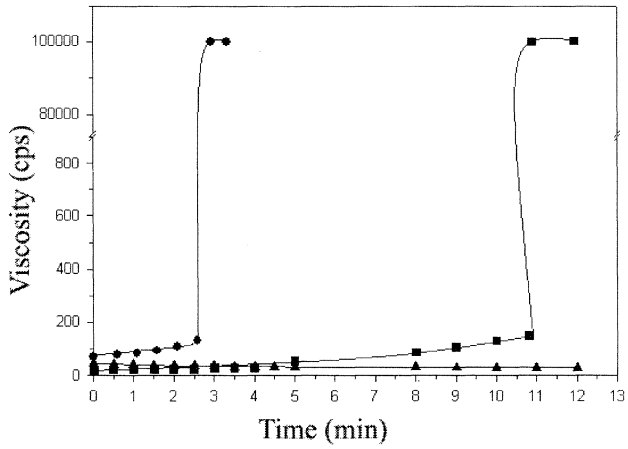


Figure 3.2.3: Viscosity change at 37 °C during gel formation between 10% solution of oxidized alginate obtained by periodate oxidation in aqueous (■) and in 1:1 ethanol/water mixture (●) using 65% periodate equivalents and 10% solution of gelatin in the presence of 0.1 M PBS. Control 10% gelatin solution (▲). Degrees of oxidation of ADA by using 65% periodate equivalents in aqueous and ethanol/water mixture are 61 and 57% respectively.

3.2.2.1.2 Gelation in the presence of 0.1 M borax

The effect of medium of oxidation on gelling time in the presence of 0.1 M borax was also evaluated using ADAs prepared by employing 30, 65 and 95% periodate equivalents. Figure 3.2.4 (a) gives the viscosity change observed during gelation reaction between ADAs obtained by oxidation using 65% periodate equivalents by two methods in the presence of 0.1 M borax. It was found that ADA prepared by oxidation in ethanol/ water mixture gave fast gelation (~ 50 s) as compared to that prepared in aqueous medium (~110 s) as observed in PBS. Further, the gelation reaction between ADA prepared by employing 95% and 29.3% periodate equivalents and gelatin was examined. Figure 3.2.4 (b,c) revealed that for ADAs having higher and lower oxidations also, gelation reaction was fast for those obtained by oxidation in ethanol/water mixture. Therefore, irrespective of the degrees of oxidation, gelation reaction was fast for all ADAs obtained by oxidation in ethanol/water mixture. This fast gelation can be attributed to the lower M_w of the product

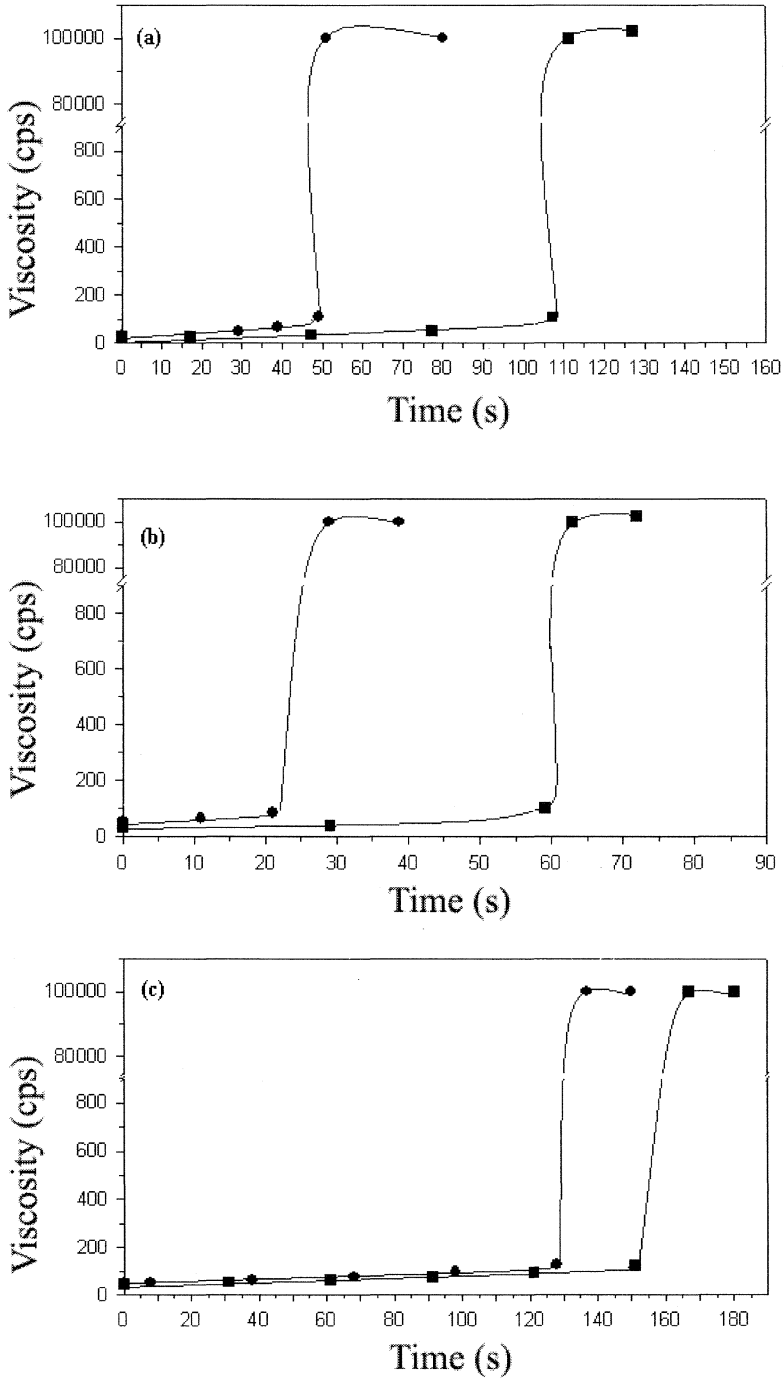


Figure 3.2.4: Viscosity change during gel formation between 10% solution of oxidized alginate obtained by periodate oxidation in ethanol/water (●) and aqueous (■) media using (a) 95, (b) 65 and (c) 29.3 % periodate equivalents and 10% solution of gelatin in the presence of 0.1 M borax.

obtained by oxidation in ethanol/water mixture in comparison with the product obtained by oxidation in aqueous medium. A higher molecular weight or higher viscosity likely resulted in increased hindrance and friction among polymer chains leading to slower rearrangement of the polymer chain conformations to form effective cross-links. This shows that the conformational rearrangement of the polymer chains also is important for cross-link formation. This study showed that periodate oxidation of alginate in ethanol/water mixture yielding a low molecular weight product having sufficient aldehyde content, is more favourable for a fast gelation reaction with gelatin. Therefore, all further studies reported in this thesis are confined to oxidized alginate prepared by periodate oxidation in 1:1 ethanol/water mixture.

3.2.2.2 Effect of Medium on Gelling Time

Gelation reaction was followed in various buffers and solutions. Only solutions having neutral or alkaline pH were chosen for this study as an slightly alkaline pH favours Schiff's reaction between amino groups and aldehyde groups. Gelation studies by dynamic viscosity measurements were hampered for certain compositions where the gelling was too rapid to be followed by viscometry. Hence, the method reported by Mo *et al.* (Mo *et al.*, 2000, See section 2.2.3.1) was adopted for determining the gelling time. The method has a certain amount of subjectivity, but the advantage was that it offered a simple method to compare the gelation behaviour of different systems in a reproducible manner.

One mL of 20% solution of ADA obtained by periodate oxidation in ethanol/water mixture having degree of oxidation 57% was dissolved in different media and mixed with equal volume of 20% solution of gelatin and stirred magnetically at 37 °C. Gelling time was noted as time required for stir bar to stop. Media chosen for the study and gelling time obtained are depicted in Table 3.2.1.

It has already been reported that the optimum pH for the Schiff's base formation is alkaline and good yield can be realized from pH 7-10. Further at pH 9-10, the formation of the Schiff's bases is more efficient and highly favourable (Hornsey *et al.*, 1986). Gelation was fast in all media except in Tris buffer even though the pH is 8. This is attributed to the presence of competing amines in the Tris buffer, which also can undergo reaction with aldehydes present in ADAs thus preventing its further reaction with amino groups of gelatin.

Medium of gelation	pH	Gelling time (s)
Phosphate buffered saline, 0.1 M	7.4	121 ± 15
Tris buffer, 0.1 M	8.0	No gelation
Carbonate buffer, 0.1 M	9.4	35 ± 3
Borax, 0.1 M	9.4	21 ± 2
NaOH, 0.1 M	11.0	105 ± 2

Table 3.2.1: Gelling time obtained for 20% solution of ADA having degree of oxidation 57% in different media and 20% solution of gelatin.

Although gelation was rapid in carbonate buffer, the gels formed were opaque. This is attributed to phase separation between solution of ADA and gelatin in carbonate buffer. Though the gels formed in PBS and NaOH were transparent, gelation was not rapid, whereas, the gelation in 0.1 M borax was very rapid and the gels formed were transparent. Borax has been used as a cross-linker for polymers containing hydroxyl groups (Deuel, 1948; Davis & Mott, 1980). Due to complexation in borax, the solubility of ADA would be high leading to enhanced reaction rate (Strauss & Kral, 1982; Coviello *et al.*, 2003, Ricardo *et al.*, 2004). The non-reversibility and stability of these gels also can be attributed to this complexation with borax giving rise to a cyclic structure (Figure 3.2.5).

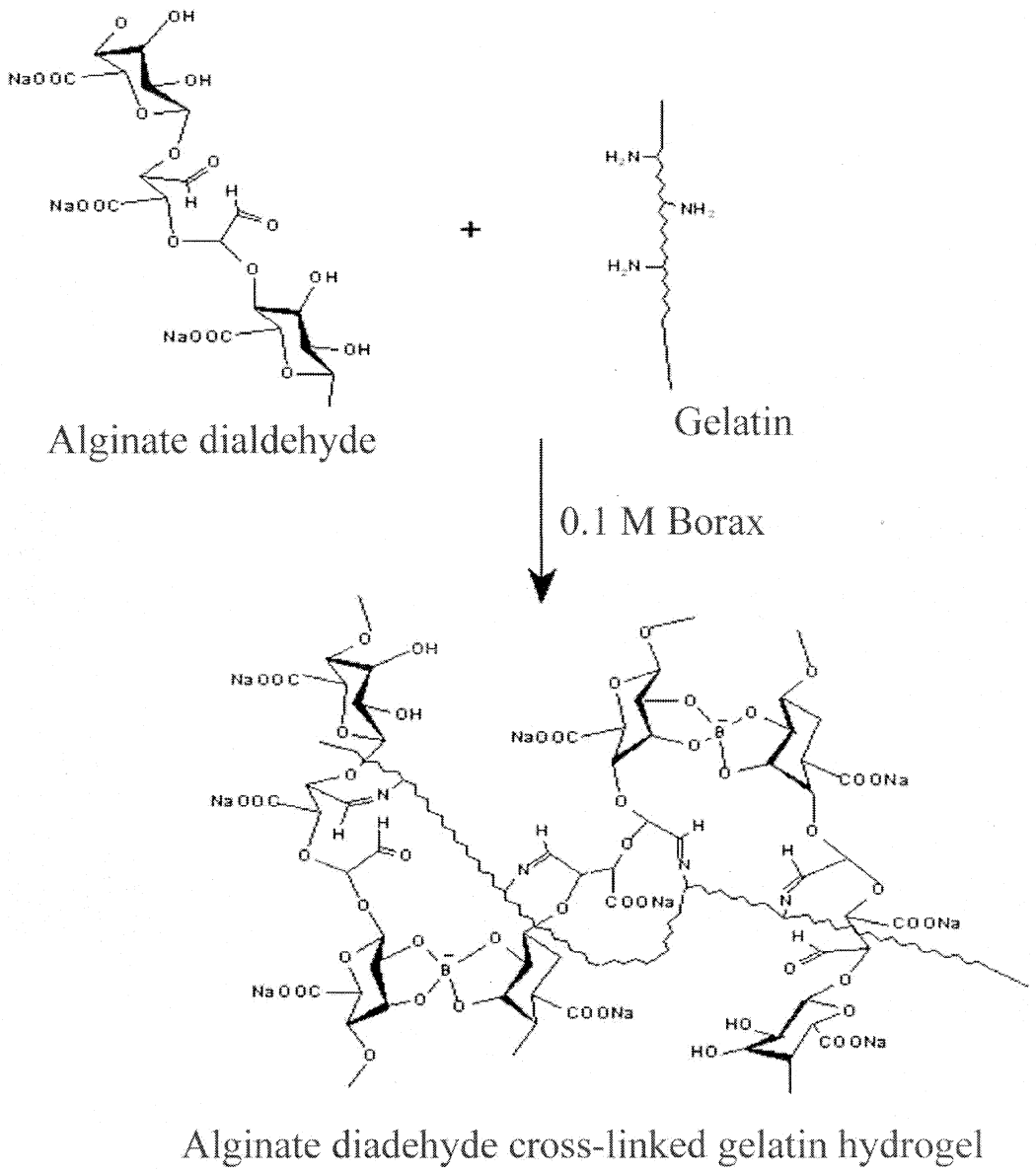


Figure 3.2.5: Schematic representation of ADA cross-linking gelatin in the presence of 0.1 M borax.

Borax has a long history of medical use and the mean lethal dose in man exceeds 700 mg/kg (www.cdc.gov/niosh/rtecs/vz26c1e0.html). Toxicity or discomfort has not been reported in humans receiving equivalent to 100 mg of boron intravenously (Jansen *et al.*, 1984). Boron is reported to prevent osteoporosis at a dose between 3-6 mg/day and in

persons with arthritis a dose of 3 mg of boron per day for 2-4 months is indicated (Kelly, 1997). Therefore the use of borax for the preparation of *in situ*-formulations from ADA and gelatin is acceptable for various biomedical applications.

3.2.2.3 Effect of Degree of Oxidation on Gelling Time

One mL of 20% solutions of ADAs having degree of oxidation 48, 57 and 87% or 10% solution of ADA having degree of oxidation 27% (only 10% solution could be prepared from ADA having degree of oxidation 27%; See section 3.1.2.4) in 0.1 M borax was mixed with equal volume of 20% solution of gelatin and stirred magnetically. The gelling time was found to decrease with increase in the degree of oxidation (Table 3.2.2). This can be attributed to the increase in the reactivity due to increase in the number of available aldehyde groups to react with amino groups of gelatin.

Periodate equivalents (%)	Degree of oxidation (%)	Gelling time (s)
30.0	27.4 ± 0.4	25 ± 2*
50.0	48.0 ± 0.4	22 ± 2†
65.0	57.5 ± 0.2	21 ± 2†
95.0	87.0 ± 0.3	13 ± 1†

Table 3.2.2. Gelling times for 10% (*) and 20% (†) solutions of ADA with 20% solution of gelatin in 0.1 M borax.

Further, 10% solution of ADA in 0.1 M PBS or in 0.1 M borax was mixed with gelatin (10%) and change in viscosity was monitored using dynamic viscosity measurements to examine the effect of degree of oxidation more accurately. It was found that, the more oxidized the alginate is, the faster the gelation reaction. This was evident from the gelling

time obtained by varying the degree of oxidation of sodium alginate (Figure 3.2.6 (a, b)). In the presence of PBS, this difference was not prominent since gelation is slow and chains get enough time to rearrange, leading to the formation of maximum possible cross-links in all formulations. However, in the presence of borax, this difference can be seen more clearly (Figure 3.2.6 (a)). While 10% solution of ADA having degree of oxidation 87% gave a gelling time of 29 s, ADA having degree of oxidation 57% and 27% gave a gelling time of 51 and 102 s respectively.

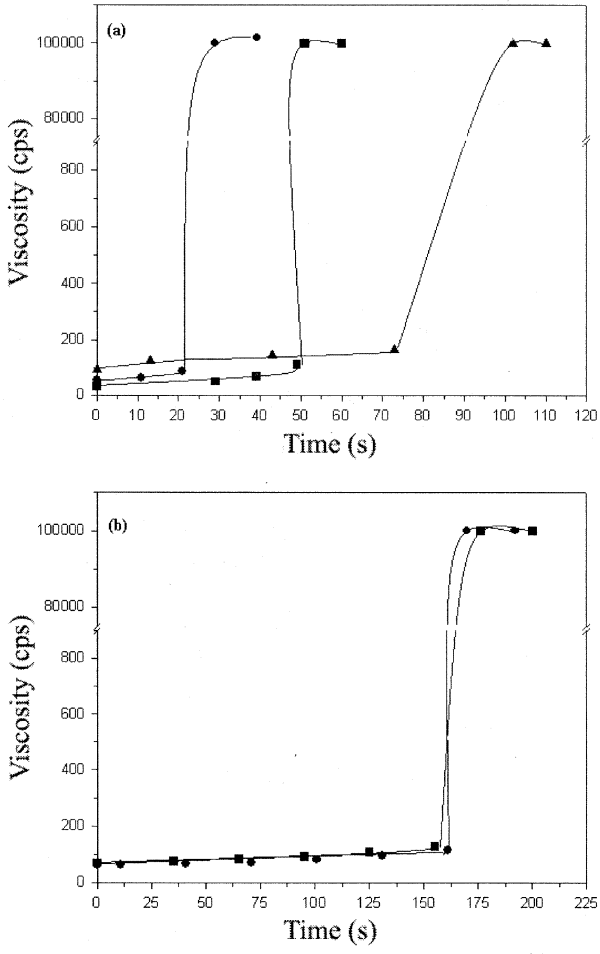


Figure 3.2.6: Viscosity change during gel formation between 10% solution of ADA having degree of oxidation 87 (●), 57 (■) and 27%(▲) and 10% solution of gelatin in (a) 0.1 M borax and (b) in 0.1 M PBS.

3.2.2.4 Effect of Concentration of ADA on Gelling Time

The effect of concentration of ADA on the gelling time was evaluated by varying the concentration of ADA (5%, 10%, 15% and 20%) in 0.1 M borax while keeping the concentration of gelatin constant as 20%. On increasing the concentration of ADA, decrease in gelling time was found as more aldehyde groups will be available for Schiff's base formation. Figure 3.2.7 gives the variation of gelling time with change in concentration of ADA having two different degrees of oxidations.

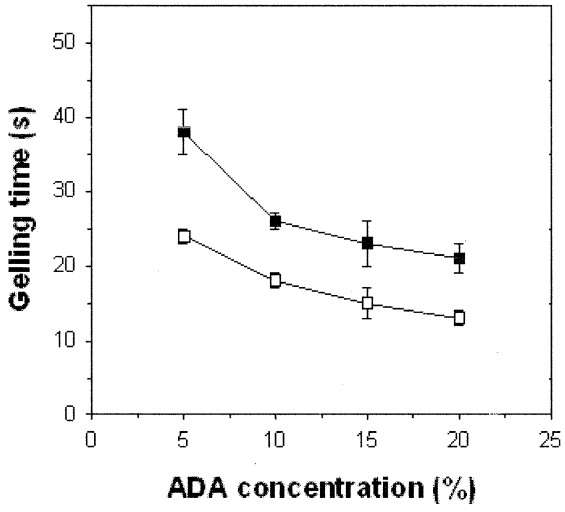


Figure 3.2.7: Variation of the concentration of ADA having degree of oxidation 87% (□) and 57% (■) in 0.1 M borax on the gelling time of 20% solution of gelatin.

3.2.2.5 Effect of Concentration of Gelatin on Gelling Time

The effect of gelatin concentration on gelling time was also evaluated. The concentration of gelatin was varied and gelling time was noted keeping the concentration of ADA as 20% in 0.1 M borax. The gelling time was found to decrease with increase in the concentration of gelatin (Figure 3.2.8). The availability of amino groups to enter into Schiff's

reaction with aldehyde groups increases with increase in the concentration of gelatin resulting in more rapid gelation.

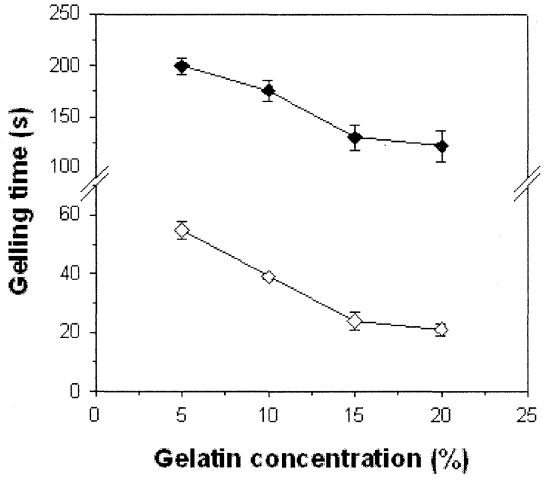


Figure 3.2.8: Variation of the concentration of gelatin on the gelling time of 20% solution of ADA having degree of oxidation 57% in 0.1 M borax (◇) and 0.1 M PBS (◆).

3.2.2.6 Effect of Concentration of Borax on Gelling Time

The effect of concentration of borax on gelling time was also evaluated by varying the concentration keeping the concentration of ADA and gelatin as 20%. Particularly striking was the decrease in gelling time (Figure 3.2.9) with increase in borax concentration. This is believed to be due to the alkaline pH of the medium which facilitates the formation of the Schiff's base as well as the ability of borax to complex with hydroxyl groups of polysaccharides. Since the gelling time decreased rapidly with increase in the concentration of borax, it supports the fact that not only the alkaline pH of the medium, but the ability of borax to complex with hydroxyl groups is also responsible for the rapid gelation.

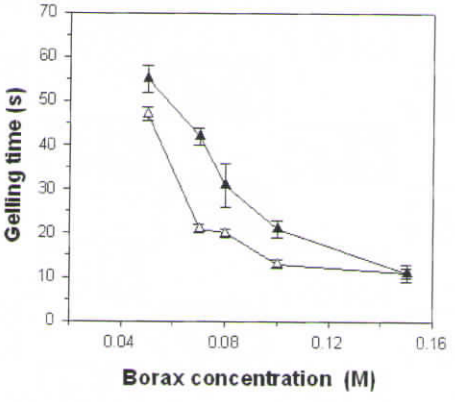


Figure 3.2.9: Variation of the concentration of borax on the gelling time of 20% solution of ADA having degree of oxidation 87% (△) and 57% (▲) and 20% solution of gelatin.

3.2.3 Stereo Microscopic View of Hydrogel

For an *in situ*-gelling system, the gelation time should be optimal (ideally a few seconds), so that once injected, the polymer construct stays at the site of injection and does not migrate or dissolve. The gelation between ADA and gelatin was examined in real life situations by using a double syringe fibrin glue applicator fitted with a 20 G needle. Figure 3.2.10 (a) shows the formation of the gel after the mixture was ejected out of the needle.

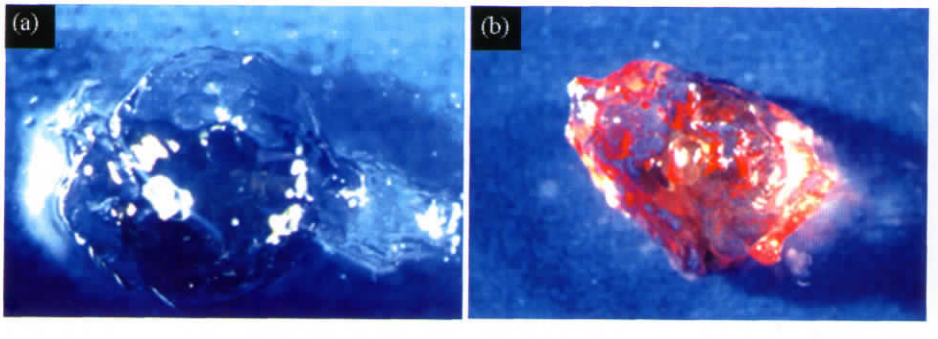


Figure 3.2.10: Magnified stereomicroscopic views (30x) of gel formed from 15% solution of gelatin and 20% solution of 57% oxidised ADA in 0.1 M borax soon after emerging out of the hypodermic needle (a) and gel explanted from the gluteal muscle of rat 1 h after injection (b).

Figure 3.2.10(b) gives the stereomicroscopic view of gel explanted from the rat gluteal muscle 1 h after injection. The gel was maintaining its shape demonstrating the potential of the system as an injectable *in situ*- forming scaffold.

3.2.4 Conclusion

Fast gelling formulations based on alginate and gelatin, were prepared. The mode of oxidation of alginate, medium of gelation, concentration of ADA, gelatin and borax were varied and gelling time was examined to optimize the system. It was found that ADAs obtained by oxidation in ethanol/water mixture gave fast gelation compared to that obtained by oxidation in aqueous medium. Degree of oxidation and medium of gelation also are important parameters in reducing the gelling time. It was found that the more the degree of oxidation of alginate, faster the gelation. Also, gelation was more favourable in the presence of borax due to its alkaline pH and ability to complex with hydroxyl groups of ADA. Gelling time can also be varied by varying the concentration of ADA, gelatin and borax. As the concentration of ADA, gelatin and borax increased, gelling time was found to decrease. Therefore by varying several factors such as mode of oxidation of alginate, media of gelation, degree of oxidation and the concentrations of the reactants, the gelation time can be varied from a few seconds to few minutes allowing the system to be used in a number of applications.

3.3 Physico-chemical Characterization of ADA Cross-linked Gelatin Hydrogels

3.3.1 Background

Hydrogels are three dimensional, water swollen structures composed of mainly hydrophilic homopolymers or copolymers and they resemble natural living tissue more than any other class of synthetic biomaterials (Ratner & Hoffman 1976; Peppas, 1986; Peppas, 1994).

Different models to predict the network structure of hydrogels in order to understand their structure at the molecular level have been developed. There are presently three distinct models used for examining network structure formation: kinetic models, statistical models, and Monte Carlo simulations. Due to the non-ideal thermodynamic behavior of polymer networks in electrolyte solutions, no theory can predict exact behavior. However, the Flory-Rehner analysis, and its various modifications are used with reasonable success. This theoretical framework describes gels as neutral, tetrafunctionally-cross-linked networks with polymer chains exhibiting a Gaussian distribution (Peppas, 1994).

The most important parameters used to characterize the network structure of hydrogels are the polymer volume fraction in the swollen state (v_2), cross-linking density (v_e) and molecular weight of the polymer chain between two neighbouring cross-linking points (M_c) (Figure 3.3.1). The polymer volume fraction in the swollen state is a measure of the amount of fluid absorbed and retained by the hydrogel. It also gives an idea about the interaction between polymer chains. The molecular weight between two consecutive cross-links is a measure of the extent of cross-linking of the polymer. Due to random nature of the polymerization process itself, only average values of v_e and M_c can be calculated (Peppas

et al., 2000). The knowledge of ν_e and M_c is of great importance because of its effect on the mechanical and physical properties of the hydrogels.

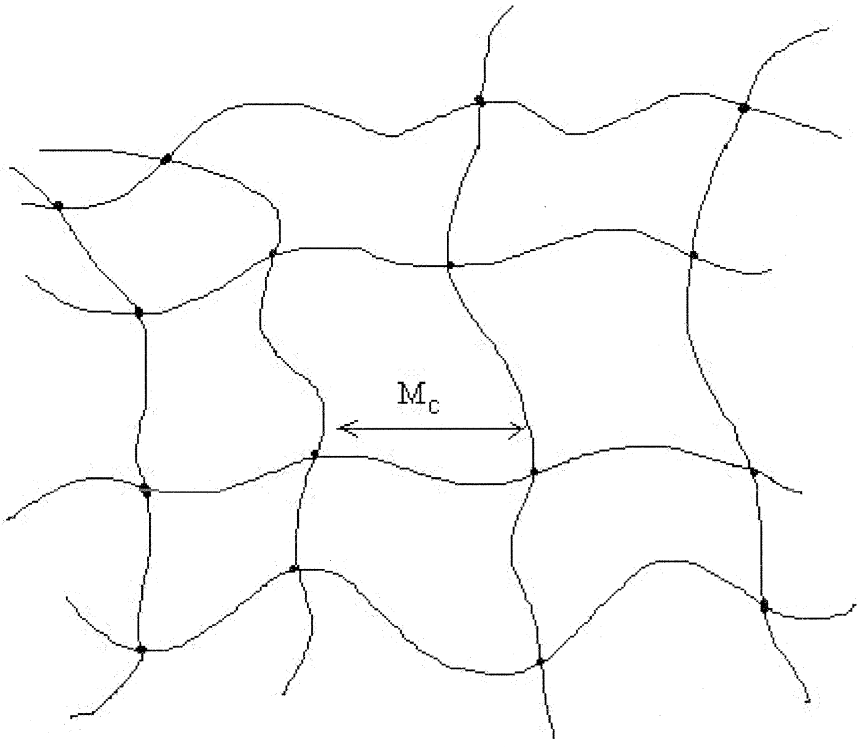


Figure 3.3.1: Schematic representation of the cross-linked structure of the hydrogel. M_c is the molecular weight of the polymer chains between cross-links.

In order to understand more about the nature of cross-linking, the degree of cross-linking of hydrogels was evaluated by TNBS assay. TNBS assay quantitatively analyzes the amount of gelatin undergoing cross-linking. Evaluation of equilibrium fluid content, water vapour transmission rate (WVTR) and rate of evaporation of hydrogels will enable us to understand moisture retaining ability of these hydrogels. Degradability and porous structure of the hydrogels were also examined.

Since ADA having a degree of oxidation of 27% showed only limited solubility, and the gels produced were also mechanically very weak, further characterizations were confined to ADAs having a degree of oxidation of 57% and 87% in order to arrive at the formulations having more desirable features for forming *in situ*-forming hydrogels.

3.3.2 Swelling Studies

Equilibrium swelling theory developed by Flory and Rehner was employed for the study of ADA cross-linked gelatin hydrogels (Flory & Rehner, 1943). Peppas and Merril modified the original Flory-Rehner theory for hydrogels prepared in the presence of water (Peppas & Merril, 1976; Peppas & Merril, 1977). The theory describes equilibrium volume-swelling ratio of cross-linked polymers based on the postulate that the elastic retractive forces of the polymer chains and the thermodynamic compatibility of the polymer with the solvent molecules balance each other during swelling.

Hydrogels were allowed to swell in PBS for 24 h at 37 °C and weight of the swollen gels was noted. Swelling ratio (Q_m) was calculated from the ratio of weight of PBS uptake to weight of dried gel. Degree of swelling (Q) is the reciprocal of volume fraction of the polymer (v_2) in the hydrogel, which is a measure of interaction between polymer chains. Typical values of Q for highly swollen gels vary from 5 to 100 and sometimes even 1000. Typical values of moderately swollen gels vary from 1.5 to 5. Hydrogels prepared by the cross-linking of ADA and gelatin gave a value of Q in the range 5-10, showing that these gels swell moderately.

As the network is swollen by the absorption of solvent, the chains between the cross-links assume elongated configuration. As swelling proceeds, thermodynamically driven swelling force is counter balanced by elastic retractive force of the cross-linked structure. Finally, a state of equilibrium is reached at which both forces are equal. By considering the

expressions of the ordinary free energy of mixing and elastic free energy consequential to the expansion of the network structure, Flory and Rehner derived an equation for calculating the cross-linking density (ν_e, mol/cm^3) of a swollen network which is written as,

$$\nu_e = -[\ln(1-\nu_2) + \nu_2 + \chi_1 \nu_2^2][V_1 (\nu_2^{1/3} - 2\nu_2/f)]^{-1}$$

where, χ_1 is the Flory-Huggins interaction parameter, f is the cross-linking functionality, V_1 is the molar volume of water (18.062 cm^3/mol) and ν_2 is the volume fraction of polymer in the hydrogel when its reaches the equilibrium swelling state.

The polymer-water interaction parameter, χ_1 was introduced independently by Flory and Huggins (Peppas & Barr-Howell, 1986; Lowman & Peppas, 2000). In general, for a polymer to be soluble in water at a particular temperature, χ_1 must be below 0.5. The value of χ_1 was assumed to be 0.35 for ADA as it has been previously reported for similar interaction (Lee *et al.*, 2000). It has been reported that the reactive functional groups present per 100 g of high quality gelatin are primarily, hydroxyl, carboxyl and amino at an amount of approximately 100, 75 and 50 meq of each of these groups respectively (Mark *et al.*, 1967). On this basis, the functionality of gelatin in terms of reactive amino groups was assumed to be 50. Assumptions made on these calculations are that the hydrogels are neutral, swelling is isotropic and polymer chains are having Gaussian distribution.

Hydrogels were prepared by using 20% solution of ADAs having different degree of oxidation 87 and 57% in 0.1 M borax or in 0.1 M PBS at different concentrations of gelatin such as 10%, 15% and 20%.

3.3.2.1 Hydrogels Prepared by Using ADA having Degree of Oxidation 87%

Table 3.3.1 gives the swelling characteristics of hydrogels prepared by cross-linking of ADA having degree of oxidation 87% with different concentrations of gelatin in the presence of 0.1 M borax. Gels obtained using 10% solution of gelatin, were found to be rather weak. This was reflected in the swelling characteristics also. These gels gave high values for Q_m and Q which shows that network structure is loose enough to accommodate more water. The cross-linking was found to be less efficient for these hydrogels, even though in all formulations there are sufficient aldehyde and amino functionalities. Further, upon increasing the concentration of gelatin to 15%, a reduction was observed in the values of Q_m and Q, which can be attributed to more efficient cross-linking between ADA and gelatin. Polymer volume fraction also increased with increase in gelatin concentration showing that there is increase in polymer chain interactions. M_c measurements supported these results. It was found that the value obtained was twice that obtained for the gels with 15% gelatin solution demonstrating better cross-linking. However, this difference was not statistically significant ($p > 0.05$) when gelatin concentration was further increased to 20%.

Concentration of gelatin (%)	Swelling ratio (Q_m)	Degree of swelling (Q)	Polymer volume fraction (x 10^2) (ν_2)	Mol.Wt between cross -links (M_c)
10	10.65 ± 0.07	10.37 ± 0.06	9.65 ± 0.06	3923 ± 61
15	5.82 ± 0.52	6.18 ± 0.31	16.18 ± 0.10	1453 ± 138
20	6.65 ± 0.39	6.61 ± 0.35	15.10 ± 0.80	1484 ± 238

Table 3.3.1: Characteristics of hydrogel prepared by using 20% solution of ADA having degree of oxidation 87% in 0.1 M borax.

Cross-linking of ADA having degree of oxidation 87% and gelatin were performed in the presence of PBS also. It has already been discussed in section 3.2 that gelation reaction is rather sluggish in the presence of PBS. Here the effect of concentration of gelatin on cross-linking was not profound. Swelling studies demonstrated no significant difference ($p > 0.05$) in the swelling parameters (Table 3.3.2) as the concentration of gelatin increased from 10% to 20%. Gelation is slow as chains get enough time to rearrange, leading to the formation of maximum possible cross-links in all formulations in the presence of PBS. M_c of the gels prepared in the presence of PBS was found to be larger than that obtained for the same composition in the presence of 0.1 M borax except for those gels with 10% concentration of gelatin, showing that in the presence of PBS, cross-linking is less in comparison with borax. The alkaline pH and complexation property of borax favours fast gelation as well as efficient cross-linking resulting in lower values of M_c.

Concentration of gelatin (%)	Swelling ratio (Q_m)	Degree of swelling (Q)	Polymer volume fraction (x 10^2) (ν_2)	Mol.Wt between cross -links (M_c)
10	7.92 ± 1.47	7.97 ± 1.29	12.70 ± 2.00	2420 ± 749
15	7.90 ± 0.60	7.95 ± 0.53	12.60 ± 0.80	2391 ± 307
20	7.19 ± 0.04	7.33 ± 0.35	13.63 ± 0.09	2035 ± 28

Table 3.3.2: Characteristics of hydrogel prepared by using 20% solution of ADA having degree of oxidation 87% in 0.1 M PBS.

Cross-linking density measurements also showed similar behaviour (Figure 3.3.2). In the presence of borax, the difference in the value of ν_e was profound when gelatin concentration was increased from 10% to 15%. However, there was no statistically significant difference ($p > 0.05$) in the cross-linking density values on further increasing the concentration

of gelatin to 20%. The gels prepared in the presence of PBS showed constant value of ν_e, irrespective of the gelatin concentration.

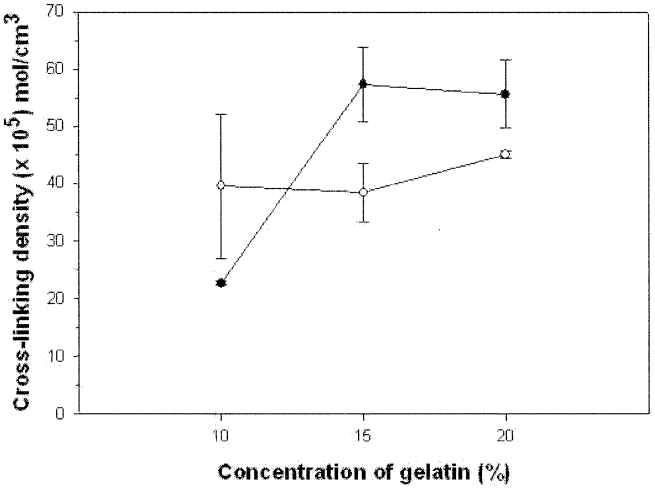


Figure 3.3.2: Variation of cross-linking density of gels prepared by using ADA having degree of oxidation 87% with concentration of gelatin in 0.1 M borax (●) and in 0.1 M PBS (○)

3.3.2.2 Hydrogels Prepared by Using ADA having Degree of Oxidation 57%

Swelling characteristics of gels prepared by employing 20% solution of ADA having degree of oxidation 57% and different concentrations of gelatin in the presence of 0.1 M borax or 0.1 M PBS were also studied. The values of Q_m and Q, obtained were slightly higher in comparison with values obtained with 87% oxidized ADA. This is because these gels are slightly less cross-linked. The value of ν_2 was increased with increase in concentration of gelatin showing that polymer chain interactions was more for gels prepared using 15% and 20% solution of gelatin. This was supported by the reduction seen in the values of M_c with increase in the concentration of gelatin. As the molecular weight of ADAs having degree of oxidation 57% and 87% is different, the comparison of values of M_c will not give a clear picture about the relative efficiency of cross-linking in these gels. However, it can

safely be concluded that gels prepared using ADA having degree of oxidation 57% with 15% and 20% gelatin are most cross-linked (Table 3.3.3).

Concentration of gelatin (%)	Swelling ratio (Q_m)	Degree of swelling (Q)	Polymer volume fraction (x 10^2) (ν_2)	Mol.Wt between cross -links (M_c)
10	12.74 ± 0.87	11.75 ± 0.134	8.2 ± 0.5	5368 ± 624
15	7.12 ± 0.63	7.53 ± 0.31	12.4 ± 0.5	2137 ± 68
20	7.15 ± 0.41	7.29 ± 0.36	13.7 ± 0.6	2019 ± 193

Table 3.3.3: Characteristics of hydrogel prepared by using 20% solution of ADA having degree of oxidation 57% in 0.1 M borax.

Studies were extended to the gels prepared in the presence of PBS. The trend observed for gels prepared using ADA having degree of oxidation 87% was seen here also. No statistically significant ($p > 0.05$) difference was observed in the values of Q_m, Q, ν_2 and M_c upon increasing the concentration of gelatin (Table 3.3.4).

Concentration of gelatin (%)	Swelling ratio (Q_m)	Degree of swelling (Q)	Polymer volume fraction (x 10^2) (ν_2)	Mol.Wt between cross -links (M_c)
10	7.72 ± 0.68	7.79 ± 0.35	14.26 ± 2.00	1918 ± 533
15	8.20 ± 0.53	8.22 ± 0.52	12.17 ± 0.12	2543 ± 43
20	6.91 ± 0.61	7.08 ± 0.041	14.15 ± 0.90	1904 ± 64

Table 3.3.4: Characteristics of hydrogel prepared by using 20% solution of ADA having degree of oxidation 57% in 0.1 M PBS.

Cross-linking density (Figure 3.3.3) also increased with the increase in the concentration of gelatin for the gels prepared in the presence of 0.1 M borax as observed in the case of gels prepared using ADA having degree of oxidation 87%. Similarly, the gels showed no significant ($p > 0.05$) difference in the cross-linking densities in PBS.

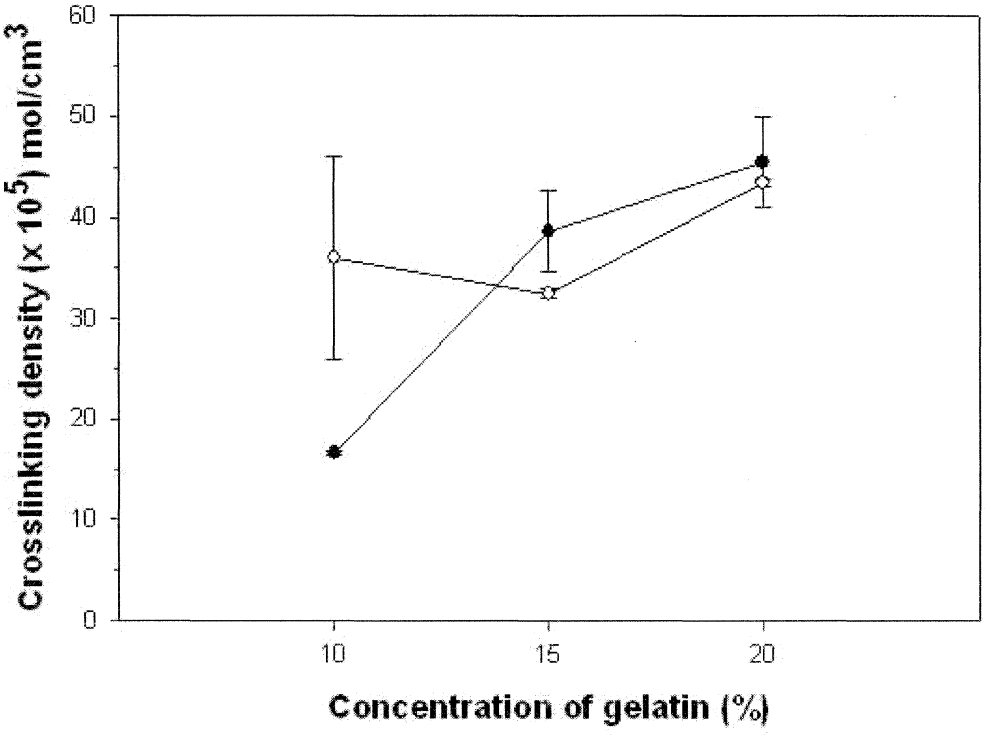


Figure 3.3.3: Variation of cross-linking density of gels prepared by using ADA having degree of oxidation 57% with concentration of gelatin in 0.1 M borax (●) and in 0.1 M PBS (○).

3.3.3 Degree of Cross-linking by TNBS Assay

Swelling studies revealed that cross-linking in the presence of borax was more efficient. Also it was found that 20% solution of ADAs having degree of oxidation 87 and 57% and 15% or 20% solution of gelatin were optimal for efficient cross-linking. However, 20% solution of gelatin is difficult to handle due to fast physical gelation and high viscosity. Therefore, cross-linking degree was examined only for gels prepared by using 20% solution of ADA having degree of oxidation 57 and 87% in 0.1 M borax and 15% solution of gelatin. Table 3.3.5 gives the degree of cross-linking calculated by TNBS assay.

Degree of oxidation (%)	Degree of cross-linking (%)
57	50.6 ± 2
87	77.6 ± 2

Table 3.3.5: Degree of cross-linking of hydrogels

It was found that the higher the degree of oxidation of the alginate, the higher the degree of cross-linking as the presence of a large number of aldehyde groups facilitates the gel formation.

3.3.4 Fluid Uptake Ability of Gels

It has already been mentioned in the introductory chapter that an ideal dressing should prevent accumulation of exudates on the wound bed. At the same time, it should provide a moist environment over the wound. In order to examine whether the hydrogel under investigation has the ability to absorb exudates, its fluid uptake ability was evaluated by incubating in PBS at 37 °C. Hydrogels were prepared using 0.5 mL each of ADA having degree of oxidation 87% and 57% in 0.1 M borax and 15% solution of gelatin. Studies were performed on gels prepared at two different concentrations of ADA (10 & 20%) to know the effect of concentration of ADA on fluid uptake ability. Figure 3.3.4 shows the kinetics of swelling of hydrogel. Initial fluid content of the gel is about 82-85%. This is in agreement with the concentrations of the reacting solutions employed. Noteworthy here is the fact that on cross-linking, the gel does not exude the fluids and retain the initial amount present. On equilibration, the swelling increased to only about 90%. This was interesting from the point of gel strength; a significant swelling following equilibration would lead to poor mechanical properties. Statistical analysis revealed that there is no statistically significant difference ($p > 0.05$) in the equilibrium fluid content of gels using ADA having different degree of oxidation at different concentrations.

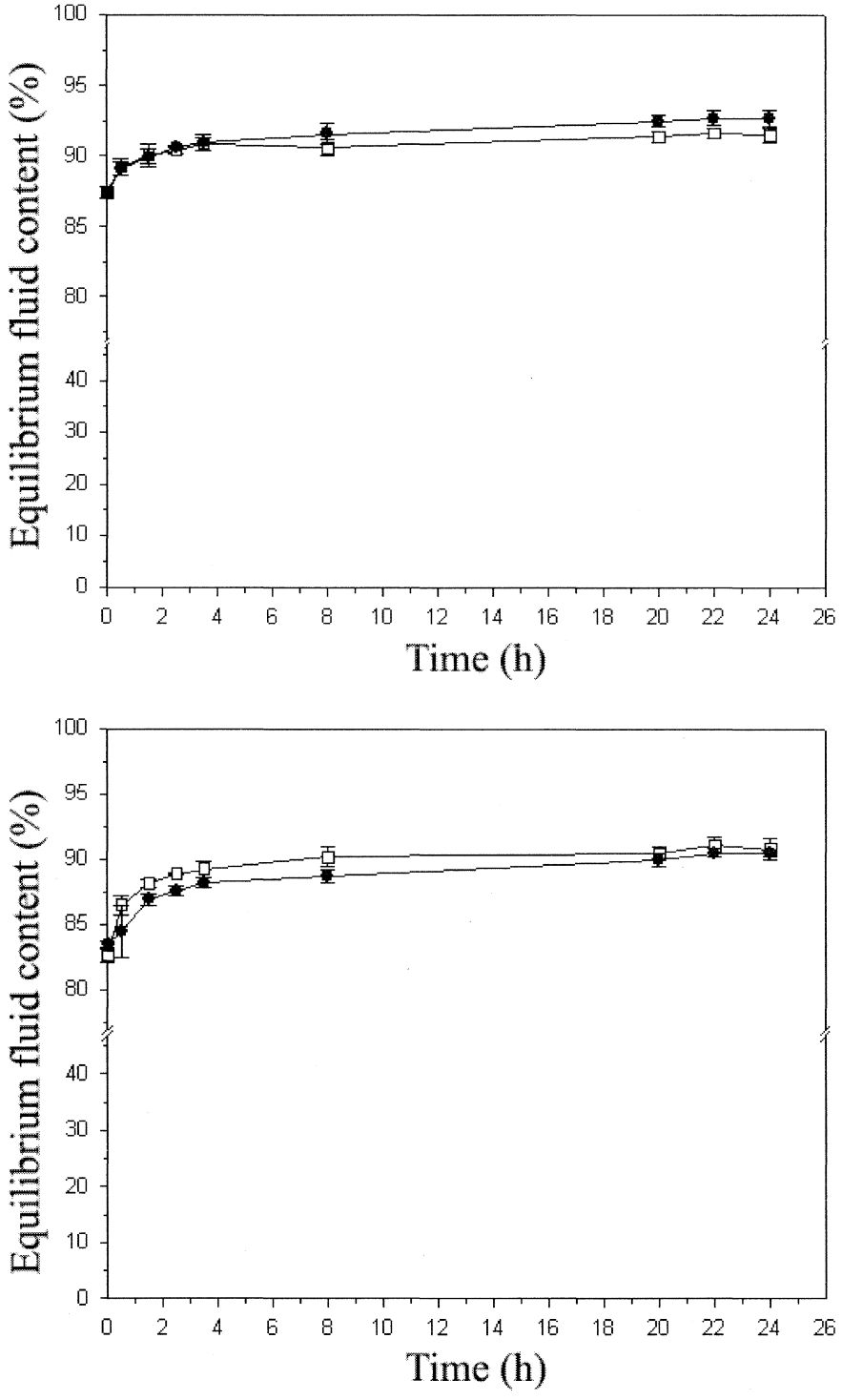


Figure 3.3.4: Equilibrium PBS content of hydrogel prepared by using 10% solution of ADA having degree of oxidation 87% (□) and 57% (●) (a) ; 20% solution of ADA having degree of oxidation of 87% (□), 57% (●) in 0.1 M borax (b). Gelatin concentration is 15%.

3.3.5 Water Vapour Transmission Rate (WVTR) of Gels

An ideal wound dressing must control the water loss from a wound at an optimal rate. Lamke *et al.* (Lamke *et al.*, 1977) reported the evaporative water loss for normal skin as 204 ± 12 g/m^2/day and that for injured skin can range from 279 ± 26 g/m^2 /day for a first degree burn to 5138 ± 202 g/m^2/day for a granulating wound. The water vapour permeability of a wound dressing should prevent excessive dehydration as well as build up of exudates. It has been recommended that a rate of 2000-2500 g/m^2/day would provide adequate level of moisture without risking wound dehydration (Queen *et al.*, 1987). WVTR of the hydrogel was calculated as the gradient of the weight loss versus time plot. Figure 3.3.5 shows the loss of water vapour with time through the hydrogel when placed in a moisture-rich environment. Wound dressings available in market such as Geliperm® (Geistlich Ltd, Switzerland) and Vigilon® (Bard Ltd., Crawley, UK) were found to have a WVTR of 9009 ± 319 g/ m^2/day and 9360 ± 34 g/m^2/day respectively and thus acts as water-free surface (Wu *et al.*, 1995). Such high WVTR would lead to total dehydration of the wound surface enabling the dressing adhere to the wound.

The hydrogels were prepared by cross-linking of 20% solution of ADAs having degree of oxidation 57and 15% solution of gelatin. As the gelation was too rapid for ADA having 87% oxidation, it was difficult to prepare a uniform film for WVTR measurements. Therefore, this study was confined to hydrogels prepared using ADAs having degree of oxidation 57 %.

Hydrogels showed a WVTR of 2686 ± 124 g/m^2/day close to the range appropriate to maintain a proper fluid balance on the wound bed, which can facilitate cellular migration and enhance re-epithelialization.

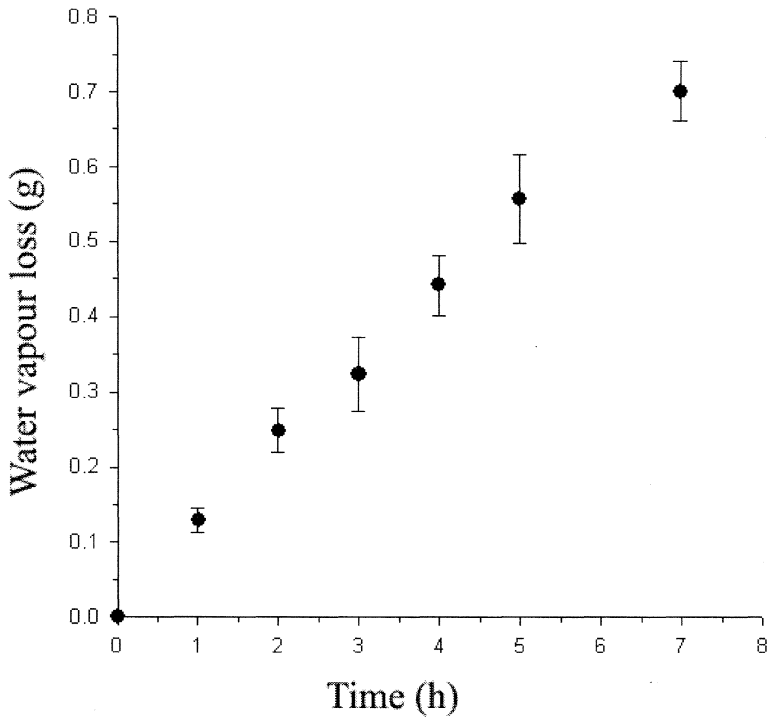


Figure 3.3.5: Water vapour transmission loss from hydrogel prepared by cross-linking 15% solution of gelatin using 20% solution of ADA having degree of oxidation 57% in 0.1 M borax.

3.3.6 Rate of Evaporation of Water from Gels

The extent of water loss from the hydrogel on exposure to the air was evaluated to examine its behaviour when used as a dressing over a dry wound. Hydrogels were prepared using ADAs having different degrees of oxidation (87 and 57) in 0.1 M borax and 15% solution of gelatin. Two ADA concentrations, 10 % and 20% were employed to study the effect of concentration of ADA on rate of evaporation.

3.3.6.1 Hydrogel Prepared using ADA having Degree of Oxidation 87%

Figure 3.3.6 shows that for hydrogels prepared by using ADA having degree of oxidation 87%, evaporative loss was more for the gels prepared from 10% solution of

ADA. This is expected as water loss will be more from the gels having more water content and less cross-links. After the first day, it was found that evaporative water loss was 32 and 42% respectively for gels prepared from 20 and 10% solution of ADA. After 4 days, the gels lost about 84-87% water and subsequently there was no further loss. The hydrogel showed different rates of evaporation at different intervals of time. It was found that up to 5 h, the gels prepared by employing 10% solution of ADA, lost water at a rate of 24 $g/m^2/h$. On the next two days, a rate of 17 $g/m^2/h$ was observed. After that, the rate decreased to 3 $g/m^2/h$ and there was no further water loss. Similarly gels prepared by employing 20% solution of ADA lost water at a rate of 18 $g/m^2/h$ up to 5 h and on next two days a rate of 13 $g/m^2/h$ was observed. After that, the rate decreased to 5%.

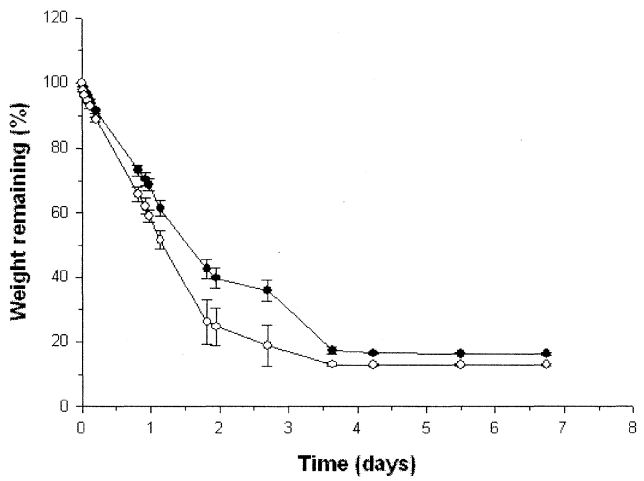


Figure 3.3.6: Evaporative loss of water from hydrogels prepared by using (●) 20% and (○) 10% solution of ADA having degree of oxidation 87% in 0.1 M borax and 15% solution of gelatin.

3.3.6.2 Hydrogel Prepared using ADA having Degree of Oxidation 57%

Irrespective of concentration of ADA, it was found that the loss of water increased linearly with time for the first two days. After one day, the loss was approximately 30-40%

and this increased slowly to about 80% over 4 days (Figure 3.3.7). Subsequently, there was no water loss from the gel. The hydrogel showed different rates of evaporation at different intervals of time. It was found that up to 5 h, the gel lost water at a rate of 20 g/m^2/h. On the next two days, a rate of 15 g/m^2/h was observed. After that, the rate decreased to 7 g/m^2/h.

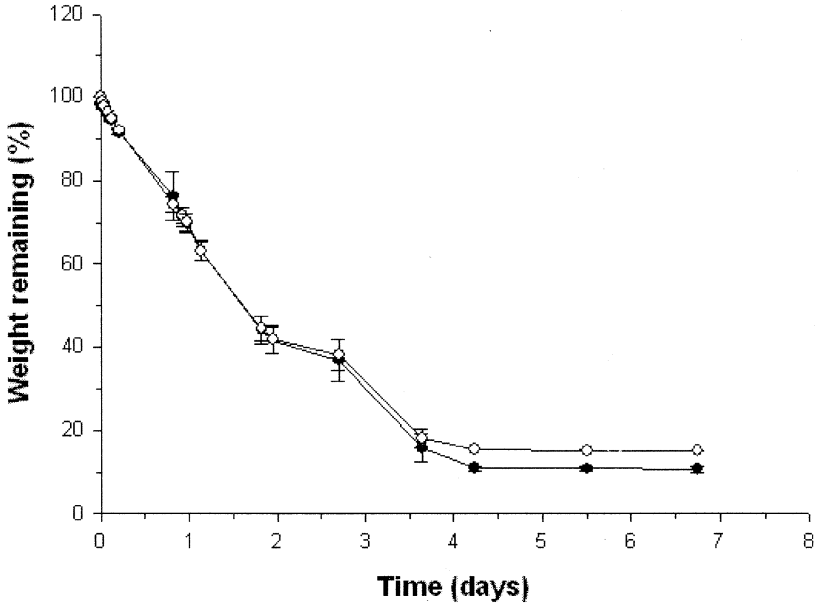


Figure 3.3.7: Evaporative loss of water from hydrogels prepared by using (●) 20% and (○) 10% solution of ADA having degree of oxidation 57% in 0.1 M borax and 15% solution of gelatin.

It is clear from these studies that the hydrogels will lose its water content when exposed to air under dry conditions over long periods. Thus, these dressings will be more beneficial to wounds with moderate exudates rather than for dry wounds. It may be pointed out that the dressings can be kept moist if desired by spraying saline or water since these hydrogels rapidly imbibe water. Commercially available dressing like Geliperm® (Geistlich Ltd., Switzerland) has also been reported to show similar behaviour loosing about 50% of its bound water after 12 h, and retaining about 30% water after 24 h. It has been reported

that this water loss enables the gel to take up exudates and oedema fluid from the wound into the dressing by an active upward-directed process when used in exudating wounds (KichÖfen *et al.*, 1986).

3.3.7 Degradation Studies

3.3.7.1 *In Vitro* Degradation

The degradation of hydrogels was examined under *in vitro* conditions in PBS at 37 °C. Reduction in weight was noted after lyophilizing the gels incubated in PBS at regular intervals of time. As gels prepared using ADAs having degree of oxidation 57 and 87% showed faster gelation, high cross-linking density and degree of cross-linking, they were more suitable for application as *in situ*-gelling systems. Therefore, the degradation profile of hydrogels based on these ADAs was studied.

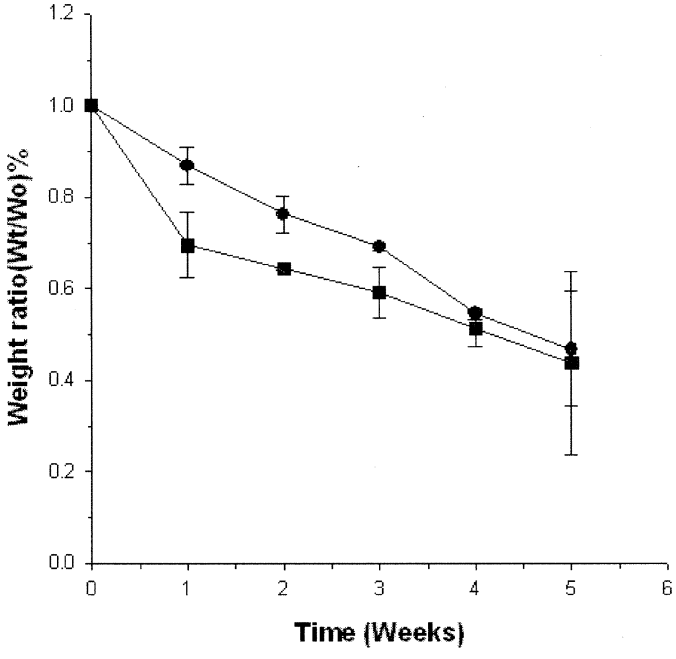


Figure 3.3.8: Degradation profile of gel prepared from 20% solution of (■) 87% and (●) 57% oxidized ADA and 15% solution of gelatin.

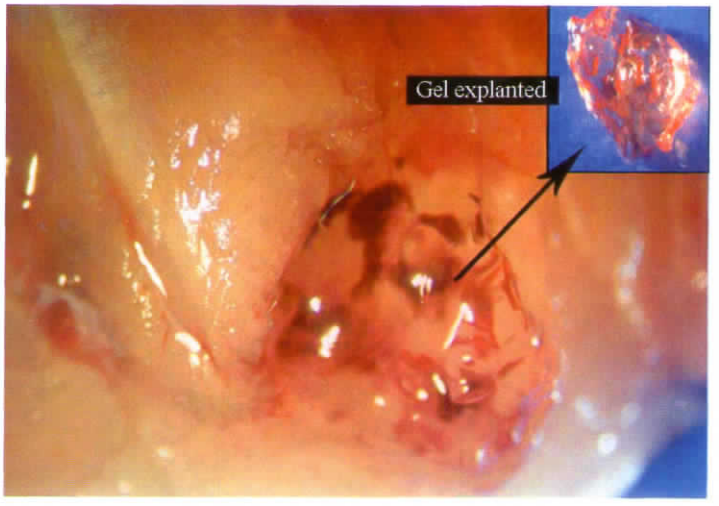
For both gels, a linear decrease in weight with time was seen up to 4 weeks with complete dissolution of the gel at the end of 5 weeks demonstrating the degradability of the matrix (Figure 3.3.8). However, weight loss was slightly more rapid for gels prepared using 87% oxidized ADA which can be attributed to lower molecular weight of ADA due to severe depolymerization.

3.3.7.2 *In Vivo* Degradation

Gels were injected intra muscularly on the dorsal side of Wistar rats (n=2) using a double syringe fibrin glue applicator. After 1 h, one of these animals was sacrificed by excess dose of sodium pentabarbitol and explanted the gel from the muscular region.

Figure 3.3.9 shows that the gel retains its three dimensional shape at the injection site, rather than forming a sheet like structure thereby demonstrating the potential of the system as an injectable *in situ*-forming scaffold that can fill any shape of a defect. After one month, the other animal was sacrificed and histology of sections was analyzed. There was no evidence of implant remnants in any section.

It has been recently reported that oxidized alginates are rapidly degraded at physiological pH unlike alginates (Bouhadir *et al.*, 2001b), which are not broken down in mammals and have a very slow clearance from the body (Al-Shamkhani & Duncan, 1995). The periodate oxidation alter the chair conformation of uronate residues of alginate to an open-chain adduct. This may allow free rotation on the β-glycosidic linkage of alginate, which could behave like an acetal group, susceptible to hydrolysis. Also it is reported that rate of degradation increases with increase in pH and temperature (Bouhadir *et al.*, 2001b). Therefore, the hydrolytic susceptibility of Schiff's linkage between gelatin and oxidized alginate and the degradability of both gelatin and ADA would make the scaffold fully biodegradable with time in the body.



ure 3.3.9: Magnified stereomicroscopic views of gel formed from 15% solution of gelatin and 20% solution of 57% oxidised ADA in 0.1 M borax within the gluteal muscle (x 150) and gel explanted from the gluteal muscle of rat 1 h after injection (x 30)(inset).

.8 SEM Analysis

Hydrogels were lyophilized and their internal structure was examined by slicing the and examining them in SEM. Figure 3.3.10 shows the interconnecting pores in different tin-ADA gel matrices prepared by varying the degree of oxidation of ADA. Pore size lysis gave an average pore diameter of 80 -100 μm (Figure 3.3.11) and pore size ibution was found to vary with change in composition of the gel. Numerous applications uire a highly interconnected, porous structure within a scaffold system to encourage al and fibrovascular ingrowth, promote uniformity *in vitro* cell seeding, and facilitate ration of both seeded cells and cell migrating from a neighboring *in vivo* site. rconnecting porous structure of a matrix also promotes cell attachment and exchange of ients and waste materials (Mikos *et al.*, 1993).

The porosity and pore dimensions of these hydrogels obtained in the present study on lyophilized gels which do not necessarily reflect their true nature in the hydrated

state. However, as a close approximation, it is possible to conclude that the gels are macroporous with interconnecting pores pointing to their suitability as injectable scaffolds for tissue engineering and drug delivery.

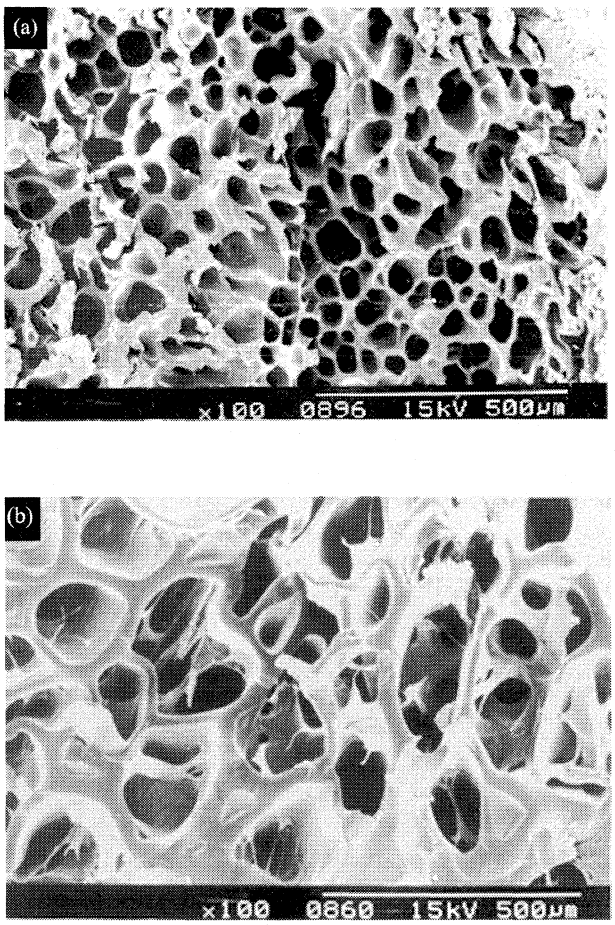


Figure 3.3.10: SEM image of internal structure of lyophilized hydrogels prepared from 20% solution of 57% (a) and 87% oxidized ADA (b) with 15% solution of gelatin.

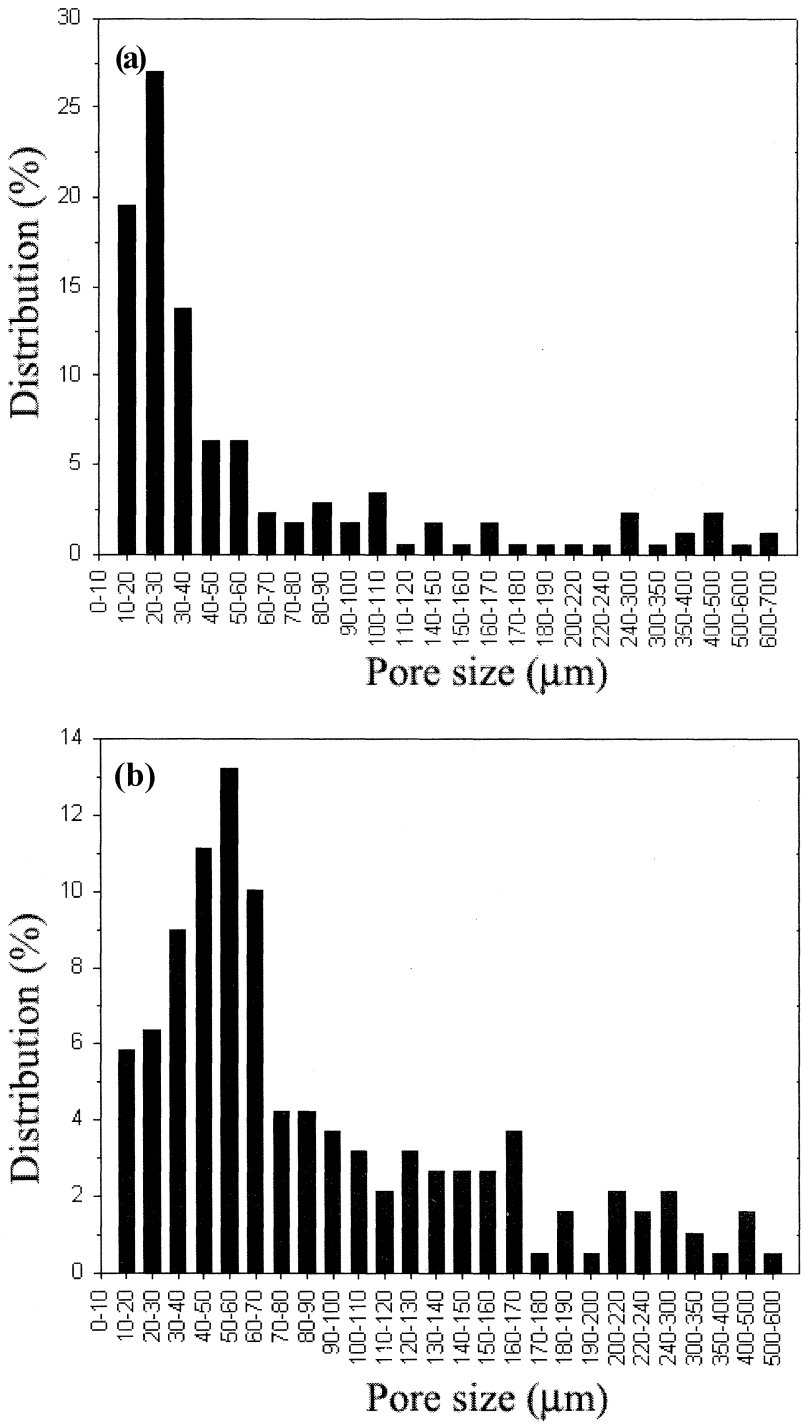


Figure 3.3.11: Pore size distribution of hydrogels prepared from 20% solution of 57% (a) and 87% (b) oxidized ADA in 0.1 M borax and 15% solution of gelatin.

3.3.9 Conclusion

The physico-chemical characterization of the hydrogels under investigation has been carried out. By swelling measurements, the cross-linking density and molecular weight between cross-links were determined. Further, the degree of cross-linking was analyzed by TNBS assay. The studies showed that hydrogel prepared using 20% solution of ADA having degree of oxidation 87% and 15% solution has the highest cross-linking density and degree of cross-linking as compared to other gels. However, a 20% solution of ADA having degree of oxidation 57% and a 15% solution of gelatin were optimal with respect to dissolution, ease of handling and gelation time in the preparation of hydrogels for many applications. The fluid uptake ability and WVTR of these hydrogels were found to be highly optimal for maintaining a moist environment conducive for wound healing. *In vitro* studies showed that the hydrogels are completely degradable under physiological conditions. SEM analysis of internal structure of lyophilized gels showed that they possess a macroporous structure with interconnecting pores.

3.4 Biocompatibility Evaluation of ADA Cross- Linked Gelatin Hydrogel

3.4.1 Background

With the increasing number of synthetic materials being introduced into the biomedical field, there is an increasing demand for more discriminating tools to evaluate their safety and efficacy. Any severe reactions by the host toward the biomaterial will probably result in failure. Consequently, the need for standardized methods and protocols for assessing the biological response of materials has never been greater. International standard - ISO 10993 provides guidelines concerning the safety-in-use of medical devices and materials. It is intended to assess the biological response of medical devices and materials as part of the overall evaluation and development of devices and materials.

As per ISO guidelines, for materials that contact breached or otherwise compromised body surfaces, different biological tests such as cytotoxicity, sensitization and irritation or intracutaneous reactivity are mandatory (ISO 10993-1, 1997). Therefore, the hydrogel based on ADA and gelatin was evaluated for its cytotoxicity, intracutaneous reactivity and delayed hypersensitivity. In addition to the above tests, the hydrogel was also screened for its haemolytic potential. This section discusses the biocompatibility evaluation of the hydrogel under investigation.

3.4.2 Cytotoxicity Evaluation

Cytotoxicity tests enable us to separate reactive from non-reactive materials, providing predictive evidence of material biocompatibility. A negative result indicates that a material is free of harmful extractables or has an insufficient quantity of them to cause acute effects under exaggerated conditions with isolated cells. The test is conducted either on an extract of the material or the material itself. The extracting conditions are selected in such a

way as to exaggerate the clinical use conditions so as to define the potential toxicological hazard without causing significant changes such as fusion or melting of the material pieces or alter the chemical structure. The test was performed on a sub-confluent monolayer of L_{929} mouse fibroblast cells.

Cytotoxicity can be determined by either qualitative or by quantitative means. In qualitative evaluation, cells are microscopically examined to assess for changes in, for example, general morphology, vacuolization, detachment, cell and membrane lysis. In quantitative evaluation, cell death, inhibition of cell growth, cell proliferation or colony formation is measured. In general, in these tests, approximately one-half million to one million cells are present in each culture dish, and toxicity is verified after a period of exposure (typically 24–72 hours) of the cells to the extract or device. Positive control materials (e.g., copper wire for direct contact assay and diluted phenol for test on extract) and negative control materials (e.g., USP-grade high-density polyethylene) are similarly tested alongside to validate the test results (ISO 10993-5, 1992). *In vitro* methods using tissue cells have advantages like sensitivity, reproducibility, economy and speed compared to *in vivo* method. It can be some times be faulted in the actual biological conditions, but still it can very well be used as a screening test for selecting materials for biomedical applications.

3.4.2.1 Qualitative Evaluation

Qualitative cytotoxicity evaluation of the hydrogel under investigation was carried out by direct contact assay using L_{929} mouse fibroblast cell line. Studies were performed on hydrogel prepared from 20% solution of ADA having degree of oxidation 57% in 0.1M borax and 15% solution of gelatin. In direct contact assay, hydrogel (0.75 cm^2) was placed directly on a sub-confluent monolayer of cells.

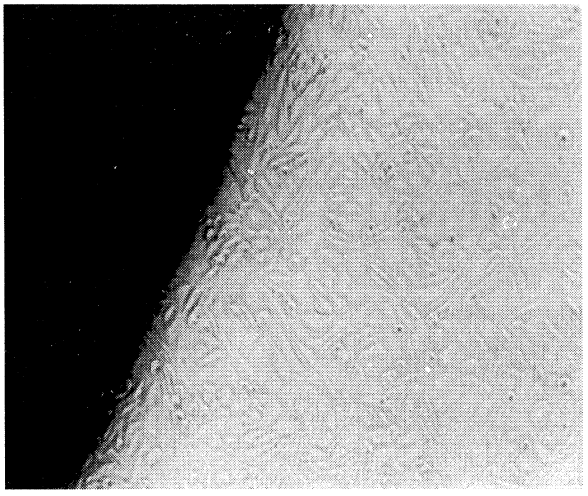


Figure 3.4.1: Optical photomicrograph (x 200) of monolayer of L_{929} mouse fibroblast cells on contact with negative control (high density polyethylene).

The morphology of fibroblasts was examined under microscope and cellular responses were scored. Fibroblast cells are spindle shaped as shown in Figure 3.4.1 on contact with high density polyethylene (negative control) and any change in its morphology on contact with material denotes its cytotoxic nature. Figure 3.4.2 shows the photomicrograph of the monolayer on contact with copper wire (positive control). The cells lost their characteristic spindle shape and become rounded showing cell death.

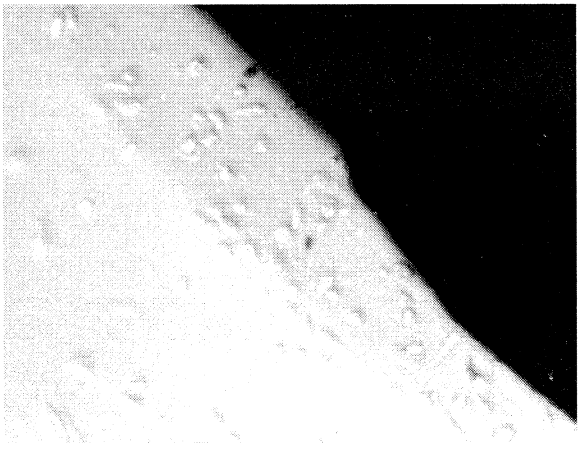


Figure 3.4.2: Optical photomicrograph (x 200) of monolayer of L_{929} mouse fibroblast cells on contact with positive control (copper wire).

Figure 3.4.3 shows the photomicrograph of ADA cross-linked gelatin hydrogel placed in contact with monolayer of fibroblast cells. The cells showed the same morphology and same characteristics, which were shown by negative controls. The cellular response was scored as zero for ADA cross-linked gelatin hydrogel.

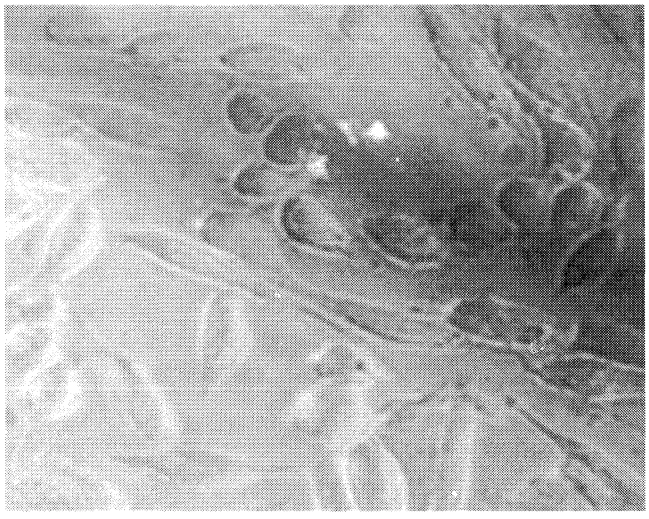


Figure 3.4.3: Optical photomicrograph (x 320) of monolayer of L_{929} mouse fibroblast cells on contact with ADA cross-linked gelatin hydrogel.

3.4.2.2 Quantitative Evaluation

Quantitative assessment of cytotoxicity was done by MTT assay of cells after contact with material extract. MTT assay is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form dark blue formazan crystals which is largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. The number of surviving cells is directly proportional to the level of the formazan product created (Mosmann, 1983). The color can then be quantified using a simple colorimetric assay. The results can be read on a multiwell scanning spectrophotometer.

From the absorbance noted, it was found that cells after contact with the material extract showed 93% metabolically active cells compared to cells without the material extract for 24 h of contact. Statistical analysis of absorbance values obtained for control and gel samples showed that there was no statistically significant difference ($p > 0.07$). The MTT reduction for 24 h contact for the gel is shown in Figure 3.4.4 along with the reduction observed for positive and negative controls.

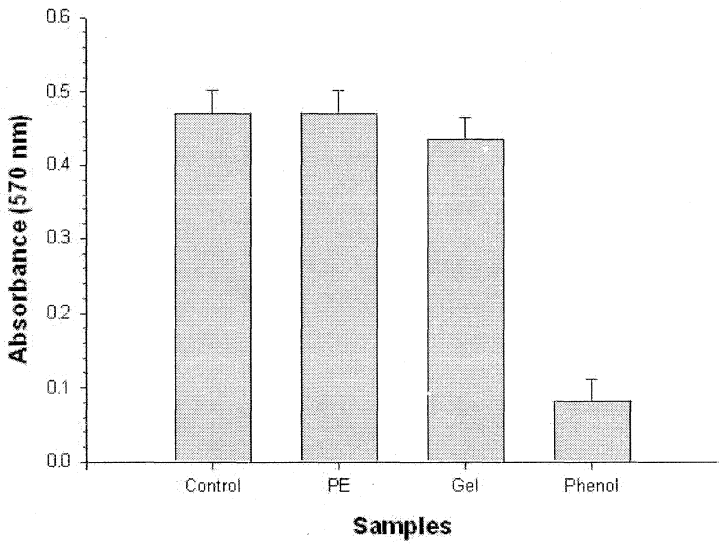


Figure 3.4.4: MTT reduction by L_{929} mouse fibroblast cells challenged with gel extract in comparison with control (cells with medium alone), and negative (high density polyethylene-PE) and positive control (dilute phenol) for 24 h.

3.4.3 Intracutaneous (Intradermal) Irritation Test

Irritation is a localized inflammatory response to single, repeated or continuous application of a material, without the involvement of an immunological mechanism. A primary irritant is a substance, which produces inflammatory changes in the skin as a result of a direct damaging effect characterized by the presence of inflammation, or in the case of

severe irritant, vesiculation and/or necrosis. The irritation potential of a material or its leachables when administered to human patients can be extrapolated from the response obtained by injecting the extract of the test material intracutaneously to test animals. Dermal irritation in small animals is performed to identify substances, which may be potential human skin and/or mucosal tissue irritants. Intracutaneous (Intradermal) reactivity test is one of tests to assess the potential of the material under test to produce irritation following intradermal injection of extracts. In this test, the extract of the material will be injected at different sites on rabbits and observed for erythema and oedema. Erythema is the reddening of the skin or mucous membrane and oedema is swelling due to abnormal infiltration of fluid into the tissues. The rabbit is the preferred test animal due to large amount of dermal irritation formation in this animal. The minimum number of animals required for this test is two (Upman *et al.*, 2003).

As body tissues differ in vascularization, composition and response, irritation potential needs to be tested using the same contact conditions as those encountered during actual usage (e.g., eye irritation tests for eye products). As the hydrogel under investigation is meant for wound management, intradermal irritation test was adopted. Hydrogel prepared by using 20% solution of ADA having degree of oxidation 57% in 0.1 M borax and 15% solution of gelatin were employed for this test as this composition was most suited for *in situ*-forming dressing with respect of dissolution of ADA, ease of handling and gelation time.

Two rabbits received a series of five skin injections (0.2 mL) of the extract. Any resulting erythema (redness of the skin) and oedema (excessive fluid build-up in a localized area) were scored daily for three consecutive days according to the classification system given in Table 3.4.1. These scores were evaluated and compared to the control sites at the

end of this time period. This comparison was made to determine if any substances that may have been leached or extracted from the test material are capable of producing irritation following intradermal injections. For each animal, the primary irritation scores for both erythema and oedema were added together separately at each time specified and divided by the total number of observations. A similar assessment was made of the sites injected with the reagent control. The primary irritation index (PII) was determined by dividing the sum total of irritation scores of all animals by the number of animals.

Skin responses	Score
Erythema formation	
No Erythema	0
Very slight erythema (barely perceptible)	1
Well defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet-redness) to slight eschar formation (injuries in depth)	4
Oedema formation	
No oedema	0
Very slight oedema (barely perceptible)	1
Slight oedema (edges of area well-defined by definite raising	2
Moderate oedema (raised approximately 1.0 mm)	3
Severe oedema (raised more than 1.0 mm and extending beyond exposure area)	4
Total possible score for irritation	8

Table 3.4.1: Classification system for intracutaneous (intradermal) injections.

The requirements of the test will be met only if the difference between the test sample score and the control mean score is 1.0 or less. All rabbits gave a score of zero for erythema and oedema at all time intervals and hence got a PII value of zero (Table 3.4.2) for the hydrogel material. This indicated that the hydrogel does not produce any irritation following intra-dermal injection of physiological saline extract. Hence the hydrogel meets the requirements of the test as per ISO 10993-10:2002 (E) –Biological evaluation of medical devices: Part 10. These results suggest that ADA cross-linked gelatin hydrogel is non-irritant.

Animal No:	Extract	Irritation score	
		Erythema	Odema
1. 304	Control Test	0 0	0 0
2. 315	Control Test	0 0	0 0
Average irritation score		0	

Table 3.4.2: Average irritation score obtained for the hydrogel

3.4.4 Maximization Test for Delayed Hypersensitivity

Sensitization is an allergic response involving immunological systems that have been activated by prior exposure of a material. The material must be able to penetrate the skin and react with skin protein to become antigenic. Langerhans cells at the epidermal/dermal border present the antigen to specific lymphocytes, which are then activated to initiate the immune response. A small percentage of these lymphocytes are long-lived memory cells and these serve as the primary activators during the challenge phase. Thus, subsequent re-exposures can result in adverse reactions that are mediated by lymphokines released by

the activated lymphocytes and other inflammatory cells that are attracted to the area of the lesion. There are several methods for determining skin sensitization in guinea pigs. The two most commonly used methods are the maximization (Magnusson & Kligman) and closed patch (Buehler) methods. It is only necessary to evaluate the material by one of these methods. With regard to the evaluation of extracts of the material, Magnusson & Kligman maximization test is regarded as the most sensitive and preferred method (Magnusson & Kligman, 1969).

In this test method, fluid extracts of the test material were prepared in saline and guinea pigs were exposed repeatedly to the extracts. The guinea pigs were first injected with an extract along with Freund's complete adjuvant intended to enhance an immune response and then received a topical application. Following a two-week rest or recovery, the animals were covered with a topical patch containing the extract. The use of a saline extract simulates extraction by body fluids that first contact the dressing. The appearance of challenged skin sites of the test and control animals were examined for reactions (redness and swelling) 24 h, 48 h and 72 h after the removal of dressings. Skin reactions for erythema and oedema were described and graded according to the grading given in the Table 3.4.3 for each challenged site and at each time interval. Maximization test was carried out on hydrogels prepared by using 20% solution of ADA having degree of oxidation 57% in 0.1 M borax and 15% solution of gelatin. Grades of 1 or greater in the test group generally indicate sensitization, provided grades of less than 1 are seen on control animals.

Reactions	Numerical grading
Erythema formation	
No Erythema	0
Very slight erythema	1
Well defined erythema	2
Moderate erythema	3
Severe erythema to slight eschar formation	4
Oedema formation	
No oedema	0
Very slight oedema	1
Slight oedema	2
Moderate oedema	3
Severe oedema	4

Table 3.4.3: Classification system for skin sensitization reactions

Skin reactions graded as per Table 3.4.3 for the hydrogel and control at 24 h, 48 h and 72 h after the removal of dressings in the challenge phase are given in Table 3.4.4.

The results indicated that none of the animals in the test and control group showed any adverse skin reaction during the induction or challenge period. The extract of the test material induced a numerical grading of '0' for erythema and oedema. Therefore, the physiological saline extract of hydrogel meets the requirements of the test as per ISO 10993-10, 2002 (E) clause 7.4, Maximization test for delayed hypersensitivity. The hydrogels are shown to be not producing any sensitization reaction on skin.

Animal no:	Group	Skin reaction					
		Erythema			Oedema		
		24 h	48 h	72 h	24 h	48 h	72 h
1	Hydrogel	0	0	0	0	0	0
2	Hydrogel	0	0	0	0	0	0
3	Hydrogel	0	0	0	0	0	0
4	Hydrogel	0	0	0	0	0	0
5	Hydrogel	0	0	0	0	0	0
6	Hydrogel	0	0	0	0	0	0
7	Hydrogel	0	0	0	0	0	0
8	Hydrogel	0	0	0	0	0	0
9	Hydrogel	0	0	0	0	0	0
10	Hydrogel	0	0	0	0	0	0
11	Control	0	0	0	0	0	0
12	Control	0	0	0	0	0	0
13	Control	0	0	0	0	0	0
14	Control	0	0	0	0	0	0
15	Control	0	0	0	0	0	0

Table 3.4.4: Grading obtained for hydrogel and control (physiological saline)

3.4.5 Haemolysis

Hydrogels were also evaluated for their haemolytic potential. The objective of this study was to evaluate the presence of any leachable chemicals from the hydrogel that would cause *in vitro* red blood cell haemolysis. Haemolysis testing of medical device materials has historically been used to measure blood compatibility *in vitro*.

Blood is composed of about 90–95% water and the various cellular elements including the red blood cells (RBCs), white blood cells, platelets, etc. RBCs are the cells that harbor haemoglobin, the protein responsible for oxygen transport and cellular respiration. Lysis of RBCs would result in leakage of free haemoglobin into the plasma, potentially leading to severe hepatic and renal injury among other effects. In the haemolytic assay, the potential of material to destruct the RBCs is evaluated by exposing the material to human blood anti-coagulated with ACD.

Hydrogels were prepared by using 20% solution of ADA having degree of oxidation 57% in 0.1 M borax and 15% solution of gelatin. The haemolytic potential of the hydrogel was found to be 0.04 ± 0.02. Permissible level of the extent of haemolysis is $< 5\%$ (O'Leary & Guess, 1969). The haemolytic potential of the hydrogel was very negligible.

3.4.6 Conclusion

Hydrogels formed from ADA and gelatin was evaluated for its biocompatibility as per ISO guidelines. Qualitative and quantitative cytotoxicity, intradermal irritation potential, delayed hypersensitivity and haemolytic potential of the system were evaluated. Studies have shown that the hydrogel is non-cytotoxic, non-irritant, non-sensitive and non-haemolytic.

3.5 Evaluation of ADA Cross-linked Gelatin Hydrogel in Wound Healing

3.5.1 Background

In the case of severe loss of skin or difficult and non-healing wounds, immediate coverage of the wound surface with a dressing is needed. It is also well recognized that the right choice of dressing for a particular wound at a particular healing stage is crucial as the control of the micro-environment of the healing wound is very important. The healing of a wound proceeds in three overlapping phases, namely inflammation, granulation tissue formation and matrix formation and remodeling (See section 1.5.1). This sequential process requires the interaction of cells in the dermis and epidermis, as well as the activity of chemical mediators released from inflammatory cells, fibroblasts and keratinocytes (Clark, 1985). Modern dressings are designed to nurture the cellular environment of the wound.

Early studies of healing tissues by wound dressings were carried out using experimental animals, particularly small mammals and the results were extrapolated to the human condition. Preparation of model wound bed by full thickness excision with a scalpel appears to simulate very closely one which is generated by a burn. Experimentally, it is very difficult to produce a standard lesion and consequently the speed of healing is variable. It has been suggested that more knowledge can be gained from a qualitative assessment of the stage of healing reached by full thickness wounds at any given time rather than attempting to produce quantitative data for statistical analysis (Barnett & Irving, 1991).

Wounds exposed to the air form a scab and healing takes place beneath this dry crust. Cotton gauze has been used as a control because it is a widely used, long established standard hospital dressing. It can be used as a pad held in place with an adhesive plaster.

The histology of the specimens from a given number of comparable wounds has to be evaluated at regular intervals of time until epidermal repair is complete. Also, autopsy specimens should be cut with dressings in place on the wound surface to avoid damage to the wound during removal of the dressing and to allow the precise relationship between the dressing and the wound to be examined in the microscope.

3.5.2 Evaluation of Wound Healing

The wound healing efficacy of the hydrogels was evaluated in 1cm x 1cm full thickness wounds on Wistar rats (Figure 3.5.1). Only male rats were selected in order to avoid the effect of hormonal variations.

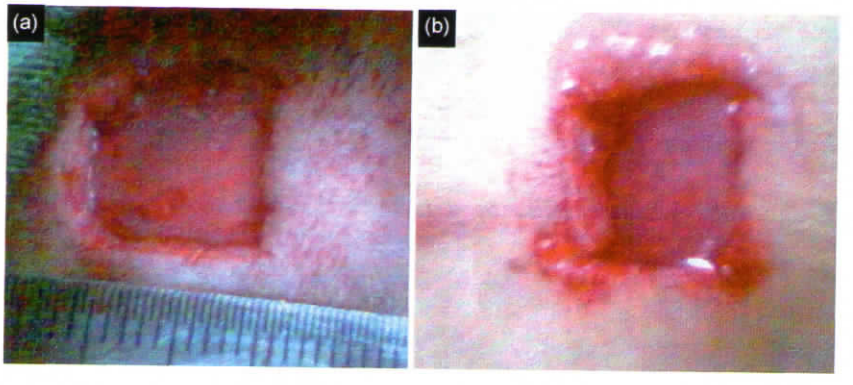


Figure 3.5.1: Photograph showing excised 1cm x 1cm wound (a) and the wound after application of the hydrogel (b).

All wound healing studies were carried out using a 20% solution of ADA having a degree of oxidation 57% in 0.1 M borax and 15% solution of gelatin. Gels were applied on each wound using the dual syringe fibrin glue applicator in which one syringe was filled with ADA solution in borax and other with 15% aqueous solution of gelatin. About 0.2 mL each of both solutions was injected to the wound site (Figure 3.5.1(b)). Immediately on application, the gel was spread evenly on the wound bed using a sterile fire-polished glass rod tip. The wound was then covered with gauze followed by an elastic adhesive bandage.

Wounds in control animals did not receive any hydrogels and were covered using cotton gauze followed by adhesive bandage.

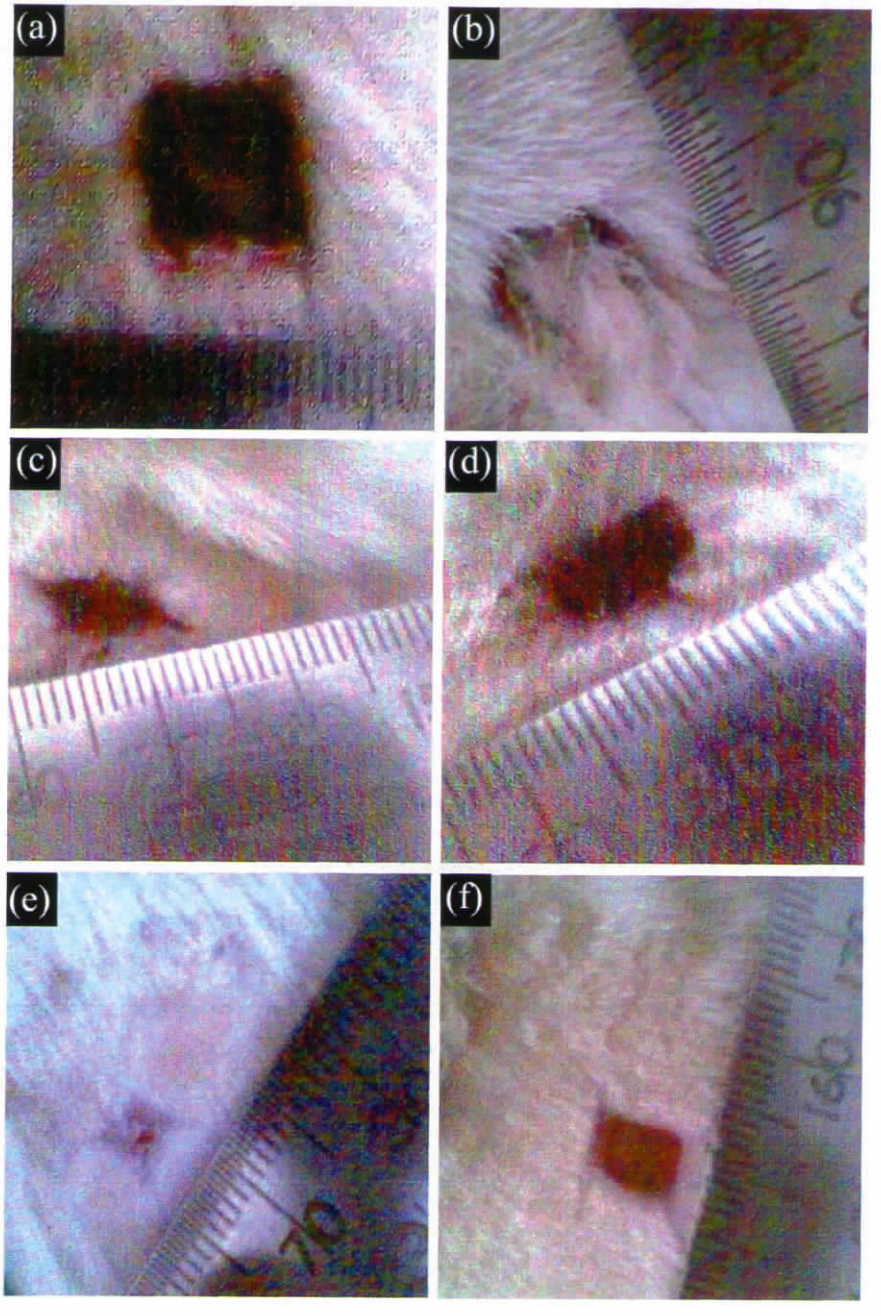
3.5.2.1 Gross Examination

Grossly, each wound (both test and control) was observed for a period of 5, 10 and 15 days post treatment (Figure 3.5.2). At 5 days, subcutaneous aspect appeared grossly normal for the test samples and there was no evidence of infection or contraction of the wound. The skin was haemorrhagic for some control samples and scab was present on the wound bed. It has been reported that epithelialization is retarded by the dry scab. Winter showed that epithelialization can be accelerated if the wound is kept moist (Winter, 1962). Exact mechanism for this accelerated epithelialization under moist conditions of the wound is still not exactly known. One probable explanation is that keratinocytes migrate more easily over a moist wound surface than underneath a scab (Winter & Scales, 1963). Epidermal cells can migrate at a speed of about 0.5 mm per day over a moist wound surface which is twice as fast as under a scab in dry wounds (Winter, 1972). Subcutaneous aspects appeared grossly normal for test and control wounds at 10 days of post wounding. At 15 days, majority of the test wounds appeared to be healed.

3.5.2.2 Wound Size Reduction

By measuring the wound area before and after definite intervals of time, reduction in wound defect area was calculated. At 5 days, there was no reduction in wound defect area for both test and control. At 10 days, healing started leading to about 72.5% fill in wound defect for control wounds whereas for test wounds this was only 68.9% (Table 3.5.1). Statistical analysis revealed that this difference was not significant ($p>0.05$). However,

5 days, wound defect filled up to 95.3% in the case of test wounds whereas for control unds this was about 75% which was statistically significant ($p< 0.05$).



ure 3.5.2: Representative photographs of macroscopic appearance of 1 x 1 cm test wounds at 5 (a), 10 (c) and 15 days (e) and control wounds at 5 (b), 10 (d) and 15 days (f).

Days of observation	Wound size reduction (%)		Wound re-epithelialization (%)	
	Test	Control	Test	Control
5	-	-	42.2 ± 9	11.0± 6
10	68.9 ± 2	72.5 ± 6	85.2 ± 11	74.6± 27
15	95.3 ± 5	75.5 ± 5	90.4 ± 9	81.7± 9

Table 3.5.1: Percent wound size reduction and wound re-epithelialization on 5, 10 and 15 days post wounding

3.5.2.3 Histological analysis

Healing pattern of wounds was studied by examining the histology of the test and control samples at 5, 10 and 15 days of post wounding.

3.5.2.3.1 Fifth day of observation

In the superficial layers, moderate necrosis with severe inflammation was observed in both test and control wounds during this period (Figure 3.5.3 (a & b)). Inflammatory granulation tissue was seen in dermis. Inflammatory phase is a normal and necessary prerequisite to healing (Kirsner & Eagstein, 1993). This can be initiated by numerous causes, one of which is injury. Therefore, during early stage of wound healing, it is difficult to assess whether the inflammatory response is a part of normal healing or due to the effect of material. During this stage, macrophages are seen at the wound site. In test wounds during this period, foreign body type giant cell response was observed in the dermis. It is reported that as a consequence of macrophage/biomaterial interactions, there is fusion of adherent macrophages leading to the formation of multinucleated foreign body giant cells (FBGCs) on biomaterial surfaces (Murch *et al.*, 1982; Anderson, 1988). This phenomenon is accompanied by FBGC-mediated biomaterial degradation (Zhao *et al.*, 1991; Wiggins

et al., 2001). This is believed to result from the action of reactive oxygen species within an acidified closed compartment between FBGCs and their biomaterial substrate (Heiple *et al.*, 1990).

Focal irregular areas of new epithelium on edge were also noted for some of the test wounds during this period (Figure 3.5.3 (a)), whereas it was found only in one of the control wounds. In test samples, collagen appeared mature in lower dermis. Bacterial colonies were found in control wounds (Figure 3.5.3 (b)) whereas no bacterial colony was found in any of the test wounds. The absence of bacterial colonies leading to infection in test wounds is noteworthy and is attributed to the mild antiseptic properties of borax present in the matrix. The presence of borax also therefore serves the important function of preventing bacterial infection of the wound bed.

3.5.2.3.2 Tenth day of observation

At 10 days, test wounds appeared reduced in size with new epithelium noted at both the edges of the defect with the proliferation of basal layer and formation of the rete pegs (Figure 3.5.3 (c)). New collagen formed in the dermis appeared to be matured. Granulation tissue was seen in dermis. Granulation tissue formation is essential for permanent wound closure, since it fills the defects and prepares the way for epithelialization. These findings support that ADA/gelatin hydrogel is able to provide suitable condition for granulation tissue formation.

3.5.2.3.3 Fifteenth day of observation

At 15 days, in test wounds, the defect area became small and filled with fibro-proliferative tissue (Figure 3.5.3 (d)). Inflammatory cells were absent. The entire surface of

the defect was covered with new epithelium. Mature collagen was present in dermis. Figure 3.5.3 (e) shows the presence of mature collagen under polarized light. However, for some control wounds, though the entire surface of the defect was covered with new epithelium, moderate number of inflammatory cells, predominantly lymphocytes and macrophages were still present in the upper dermis. Immature collagen fibres filled the dermis (Figure 3.5.3 (f)).

There are reports of foreign body giant cell reactions seven months after the use of calcium sodium alginate dressing (Kaltostat) (Matthew *et al.*, 1995). However, in the present study, foreign body reaction subsided within 15 days demonstrating that the material was undergoing degradation on the wound bed and the degradation products were not inducing any adverse reaction. We have shown earlier (section 3.3.7) that the ADA-cross-linked gelatin network is completely degradable under physiological environment. Therefore, unlike calcium cross-linked alginates which are less susceptible to biodegradation and have a long residence time in the body, the ADA cross-linked gelatin gels appear to be fully degradable with time on the wound bed.

3.5.2.4 Wound Re-epithelialization

The length of newly generated epithelium across the surface of the wound was determined as the sum of the new epidermis growing from right and left margins of the wound and was expressed as a percentage of entire wound length. Though superficially neither control nor test wounds showed any reduction in defect area at 5 days, on measuring the wound re-epithelialization it was found that both wounds have started healing. However, due to scab formation over control wounds, the wound epithelialization was lower in control wounds than in test wounds (Table 3.5.1). At 10 days, the rate of re-epithelialization increased

to 85.2 ± 11 % for test wounds; whereas for control wounds, this was 74.6 ± 27%. The wound re-epithelialization further increased to 90.4 ± 9 % and 81.7 ± 9% respectively for test and control wounds at 15 days. Statistical analysis however revealed that though there was significant difference between control and test wounds in wound re-epithelialization at 5 days, the difference was not significant at 10 and 15 days due to the large standard deviation observed.

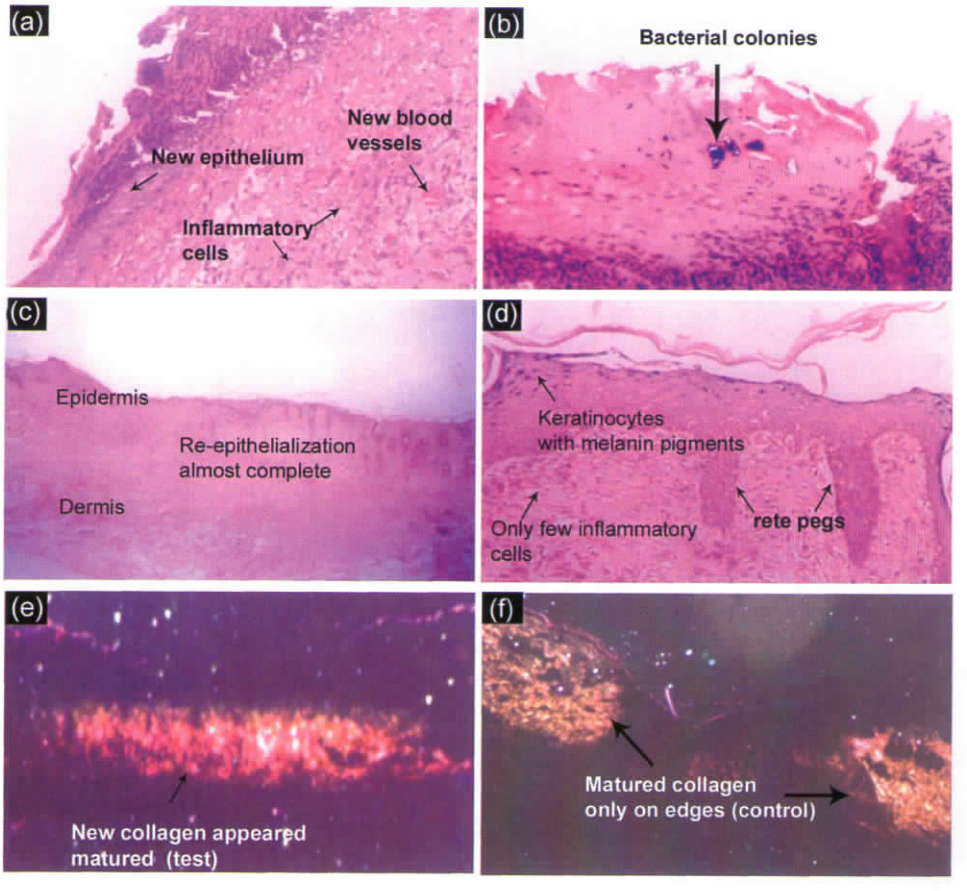


Figure 3.5.3: Histology of wound sections stained with haematoxylin and eosin. Epithelialization at test wound edges at 5 days (a, 150 x); bacterial colonies present in control wounds (b, 300 x); neat test wound section at 10 days (c, 60x); test wound with rete pegs at 10 days (d, 150 x); test wound (e, 15 x) and control wound (f, 15 x) under polarised light at 15 days.

3.5.3 Conclusion

The *in situ*-forming hydrogel wound dressing based on ADA cross-linked gelatin combines the beneficial properties of both alginate and gelatin and eliminates the use of extraneous cross-linking agents such as calcium, glutaraldehyde, carbodiimide or di-isocyanate in the formation of the hydrogel. The presence of borax, apart from accelerating the Schiff's reaction between the aldehyde groups of oxidized alginate and the amino groups in the gelatin leading to the swift formation of the three dimensional network, also exerts an antiseptic effect to prevent bacterial colonization of the wound. The hydrogel thus serves an important function by preventing bacterial infection on the wound bed. The experiments showed that in the early stages of wound healing, the wound re-epithelialization was very high in wounds covered with the hydrogel dressing as compared to control wounds. This is very important for the initiation of the healing process. The experiments also demonstrated that at 15 days, wound defect filled up to 95.3 % in the case of test wounds whereas for control wounds this was only about 75.5 %. While inflammatory cells were completely absent in wounds covered with the hydrogel and the entire surface of the defect was covered with new epithelium at 15 days, moderate number of inflammatory cells was still present in the control wounds. The wound healing efficacy of these *in situ*-forming hydrogels can be further improved by incorporating drugs or growth factors which can be done by simply mixing the desired drug or growth factor with one of the components and forming the hydrogel *in situ* in the wound bed and is dealt with the ensuing section.

3.6 Evaluation of ADA Cross-linked Gelatin Hydrogel Containing DBcAMP in Wound Healing

3.6.1 Background

Collagen deposition and re-epithelialization are major requirements for normal wound healing. Re-epithelialization is for the re-establishment of integrity of skin and collagen deposition is necessary for the restoration of strength. Re-epithelialization occurs by migration and proliferation of keratinocytes from the wound edges and by the differentiation of stem cells from remaining hair follicle bulbs. Collagen deposition occurs by influx of growth factors secreted by macrophages, platelets and fibroblasts, by the proliferation of fibroblasts and subsequent synthesis and remodeling of collagenous dermal matrix (Clark, 1996). In the case of full-thickness acute burn injuries and chronic wounds such as pressure ulcers, venous ulcers and diabetic foot ulcers, these processes are defective (Adair, 1977; Herrick *et al.*, 1992). New approaches are being devised to improve the healing under such conditions. Since 1960s, various wound management strategies have been developed, for the treatment of different types of wounds. These are generally classified as passive, interactive and bioactive methods based on its nature of action. Growth factors and other bioactive molecules have been used as wound healing agents and formulated into dressings and ointments (Ono *et al.*, 1999; Kiyohara *et al.*, 1993).

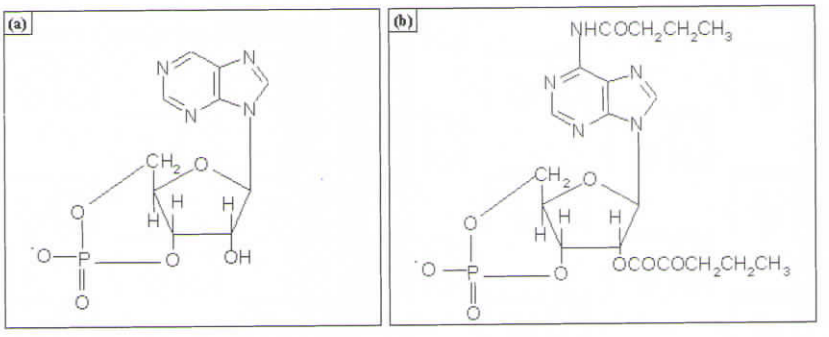
A crucial element in controlling the metabolism of cells is the chemical messages sent from one cell to another. These are being sensed by the receptors on the cell membrane or in the cytoplasm. The receptors are often integral membrane proteins on cell surfaces. The activated receptor interacts with enzymes in the cytoplasm or on the membrane facing the cytoplasm and generates a second messenger. The second messenger is a substance that can diffuse throughout the cell and alter metabolism by exerting allosteric effects on

various enzymes. One of the best known second messengers is cyclic adenosine 3', 5'-monophosphate (cAMP). Its chemical structure is depicted in Figure 3.6.1(a). It has been found that cAMP can regulate the human keratinocyte proliferation and migration in a dose dependent manner. It can either inhibit or promote cell proliferation and migration depending on dose (Iwasaki *et al.*, 1994; Dunlap & Donaldson, 1980). It has also been reported that cAMP potentiates the stimulation of corneal epithelial migration by EGF *in vitro* (Nakamura & Nishida, 2003).

The use of intercellular mediators as wound healing agents which function directly to regulate cell proliferation has been attempted by using N'-2'-O-dibutyryl adenosine cyclic AMP (DBcAMP) (Figure 3.6.1(b)), a lipophilic analog of cAMP which possesses considerable membrane permeability (Braumann & Jastroff, 1986). Clinical studies have shown that ointment containing DBcAMP promotes wound healing by stimulating cytokine secretions and facilitates cell proliferation (Zhou & Ono, 2000). Ointment therapy requires dressing changes every day. This could be considerably reduced by incorporating the DBcAMP into a dressing. Shibata *et al.*, have prepared DBcAMP incorporated spongy collagen sheet and its wound healing efficiency was evaluated. They found that dressing containing DBcAMP was effective in promoting the granulation tissue formation and epithelialization (Shibata *et al.*, 1997).

With the aim of improving the wound healing efficiency of the ADA cross-linked gelatin hydrogels, DBcAMP was incorporated into the hydrogel. It has been reported that films or hydrocolloid type dressings were more capable of retaining cytokines than ointments, particularly intrinsic growth factors secreted at the wound site (Ono *et al.*, 1995). The incorporation of DBcAMP into a hydrogel will further stimulate cytokine secretion, which is conducive for fast wound healing. The wound healing efficiency of this DBcAMP

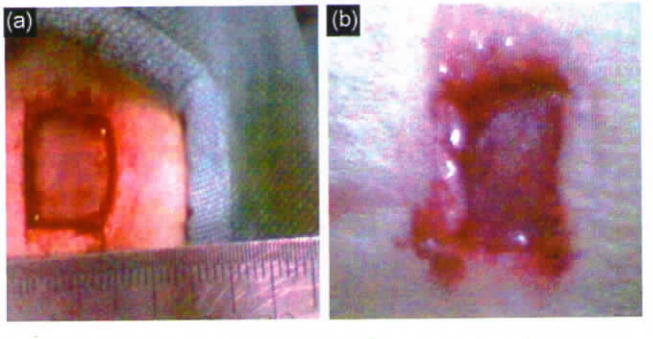
corporated hydrogel was examined by applying on experimental full thickness wounds cised in a rat model.



gure 3.6.1: Chemical structure of (a) cAMP and (b) DBcAMP.

6.2 Evaluation of Wound Healing

Hydrogels were applied on wounds created on rats as before using the fibrin glue plicator (Figure 3.6.2). One syringe was filled with 20% solution of ADA in 0.1 M borax d DBcAMP and the other with 15% solution of gelatin. DBcAMP was added to ADA lution and mixed before syringing, such that 0.2 mL of the solution contains 1.5 mg of BcAMP. As mentioned in Chapter 2 (section 2.2.6), ADA solution containing DBcAMP as filter-sterilized prior to use. Wounds (n = 6) treated with hydrogel alone and those vered with gauze were served as control (see section 3.5.2).



igure 3.6.2: Representative photographs of macroscopic appearance of 1 x 1 cm wound excised on rat (a), gel applied on wound (b).

3.6.2.1 Gross Examination

Grossly, each wound (n = 6) was observed for a period of 5, 10 and 15 days post treatment. At 5 days, subcutaneous aspect appeared grossly normal for the test samples (Figure 3.6.3 (a)) and there was no evidence of infection or contraction of the wound similar to wounds treated with hydrogel without DBcAMP (See section 3.5.2.1). It may be recalled that that wounds covered with gauze was haemorrhagic and scab was present on the wound bed (section 3.5.2.1). Subcutaneous aspects appeared grossly normal for test and control wounds at 10 days of post wounding also. All the test wounds appeared to be healed by 10 days (Figure 3.6.3 (b)); whereas the complete healing of wounds treated with hydrogel without DBcAMP occurred only by 15 days (See section 3.5.2.1; Figure 3.5.2 (e)). During this period, wounds treated with gauze also were not healed properly (see section 3.5.2.1; Figure 3.5.2 (d)).

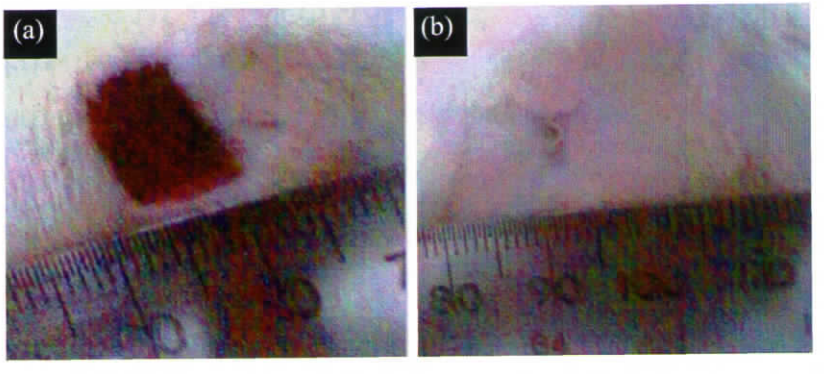


Figure 3.6.3: Representative photographs of macroscopic appearance of the test wounds at 5 (a) and 10 (b) days.

3.6.2.2 Wound Size Reduction

By measuring the wound area at different intervals of time, reduction in wound defect area was calculated. At 5 days, there was reduction in wound defect area for test

(Table 3.6.1 & Figure 3.6.3 (a)); whereas no reduction was observed for wounds treated with hydrogel without DBcAMP or with gauze at 5 days (see section 3.5.2.2, Table 3.6.1). At 10 days, healing was complete for wounds that received hydrogel containing DBcAMP, leading to about 100% fill in wound defect (Figure 3.6.3 (b)). This was only about 68.9% in the set of experiments where wounds covered with hydrogel alone were compared with control wounds. The enhanced wound size reduction observed for wounds treated with hydrogel containing DBcAMP at 10 days showed that there is release of DBcAMP from the gels to the wound bed in its active form, which stimulates epithelial migration and proliferation. The effect of DBcAMP in enhancing the rate of wound size reduction is particularly striking as the wounds treated with hydrogels without DBcAMP took 15 days for 95 ± 3 % wound size reduction. Statistical analysis showed that there was significant ($p < 0.05$) difference in the wound size reduction between wounds treated with DBcAMP incorporated hydrogel and wounds treated with hydrogel alone and those treated with gauze at 10 days.

Days of observation	Wound size reduction (%)			Wound re-epithelialization (%)		
	Test	Control (Hydrogel)	Control (Gauze)	Test	Control (Hydrogel)	Control (Gauze)
5	16.2 ± 7	-	-	29.8 ± 11	42.2 ± 9	11.0 ± 6
10	100	68.9 ± 2	72.5 ± 6	100	85.2 ± 11	74.6 ± 27
15	100	95.3 ± 5	75.5 ± 5	100	90.4 ± 9	81.7 ± 9

Table 3.6.1: Percent wound size reduction and wound re-epithelialization on 5, 10 and 15 days of post wounding

3.6.2.1 Gross Examination

Grossly, each wound (n = 6) was observed for a period of 5, 10 and 15 days post treatment. At 5 days, subcutaneous aspect appeared grossly normal for the test samples (Figure 3.6.3 (a)) and there was no evidence of infection or contraction of the wound similar to wounds treated with hydrogel without DBcAMP (See section 3.5.2.1). It may be recalled that that wounds covered with gauze was haemorrhagic and scab was present on the wound bed (section 3.5.2.1). Subcutaneous aspects appeared grossly normal for test and control wounds at 10 days of post wounding also. All the test wounds appeared to be healed by 10 days (Figure 3.6.3 (b)); whereas the complete healing of wounds treated with hydrogel without DBcAMP occurred only by 15 days (See section 3.5.2.1; Figure 3.5.2 (e)). During this period, wounds treated with gauze also were not healed properly (see section 3.5.2.1; Figure 3.5.2 (d)).

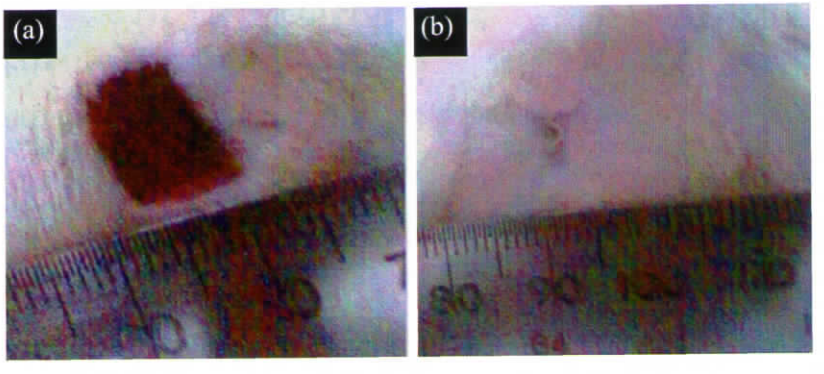


Figure 3.6.3: Representative photographs of macroscopic appearance of the test wounds at 5 (a) and 10 (b) days.

3.6.2.2 Wound Size Reduction

By measuring the wound area at different intervals of time, reduction in wound defect area was calculated. At 5 days, there was reduction in wound defect area for test

3.6.2.3 Histological Examination

Healing pattern of wounds was studied by examining the histology of the test and control samples at days 5, 10 and 15 of post wounding.

3.6.2.3.1 Fifth day of observation

On the 5th day of post wounding, histology analysis revealed that test wounds have severe inflammation (Figure 3.6.4 (a)) as seen previously with wounds covered with hydrogel alone or wounds covered with gauze alone (See section 3.5.2.3.1; Figure 3.5.3 (a & b). Fragments of dressing were seen with foreign body response. Inflammatory granulation tissue was seen in dermis. Inflammatory phase can be initiated by numerous causes, one of which is injury. Therefore, it was difficult to assess whether the inflammatory response was part of normal healing process or due to the effect of material, during this early stage of wound healing as described earlier. Granulation tissue formation is essential for permanent wound closure, since it fills the defects and prepares the way for epithelialization. These findings support that DBcAMP incorporated ADA/gelatin hydrogel also provides suitable conditions for granulation tissue formation.

Macrophages are the notable feature at the wound site during this period. In test wounds during this period, foreign body type giant cell response was observed in the dermis, which can be attributed to macrophage/biomaterial interactions, where there is fusion of adherent macrophages leading to the formation of multinucleated foreign body giant cells (FBGC) on biomaterial surfaces (Murch *et al.*, 1982; Anderson, 1988). This phenomenon is accompanied by FBGC-mediated biomaterial degradation (Zhao *et al.*, 1991; Wiggins *et al.*, 2001). Mild re-epithelialization was also noted at one end of the test wounds during

this period. These observations closely resembled those seen in the case of hydrogel alone (section 3.5.2.3.1).

3.6.2.3.2 Tenth day of observation

At 10 days, test wounds completely healed with new epithelium noted at both the edges of the defect. This was associated with mild contracture of some of the wounds (Figure 3.6.4 (b)). Wound contraction is mediated by specialized phenotypically altered fibroblasts (myofibroblasts) found within the granulation tissue (Kato *et al.*, 1988). These cells are reported to contract collagen gel, which were newly synthesized in the site of the healing wound (Moulin *et al.*, 1998; Gomathi *et al.*, 2003). Wound contraction seen in wounds treated with DBcAMP can be due to the enhanced activity of these fibroblasts. It has been found that the wounds treated with hydrogel without DBcAMP healed without any contracture (section 3.5.2).Therefore, it should be possible to control the contraction by varying the dose of the DBcAMP. Even though the test wounds were completely healed, foreign body reactions were still found in the dermis, which can be attributed to the degradation of the hydrogel matrix. It has already been shown that these hydrogels are biodegradable and foreign body reaction would be subsided after 15 days due to complete degradation of the matrix. This was corroborated with the absence of foreign body giant cells on histology analysis of test wounds after 15 days (Figure 3.6.4 (c)).

Wounds treated with hydrogel alone appeared reduced in size with new epithelium noted at both the edges of the defect with the proliferation of basal layer and formation of the rete pegs (see section 3.5.2.3.2; Figure 3.5.3 (c). Bacterial colonies were found in control wounds (see section 3.5.2.3.1; Figure 3.5.3 (b)) covered with gauze alone whereas

no bacterial colony was found in any of the test wounds as observed in the case of hydrogel alone which was attributed to the mild antiseptic properties of borax present in the matrix.

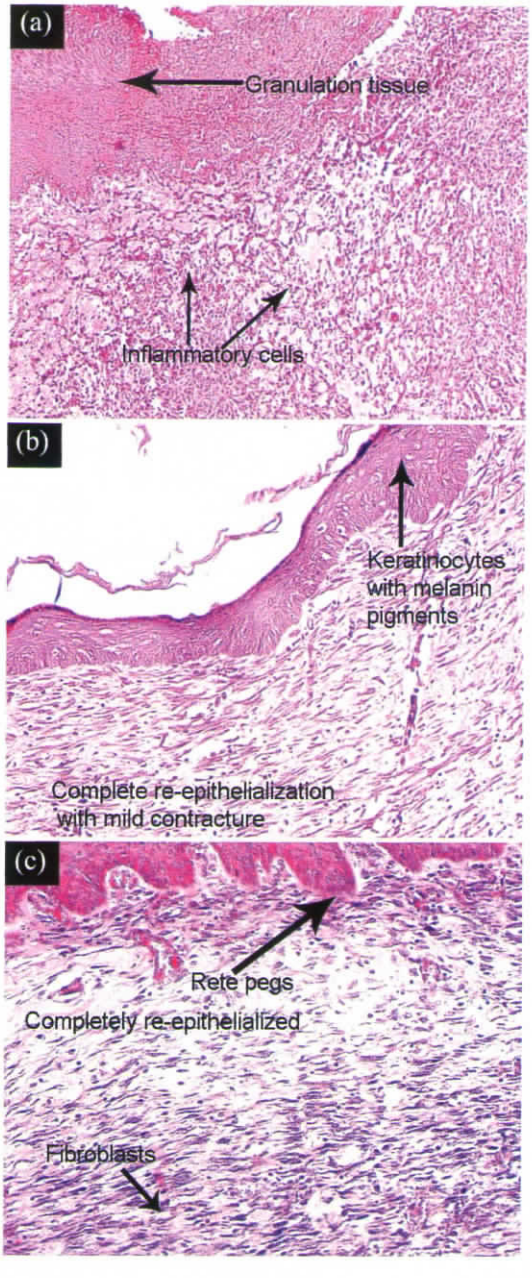
3.6.2.3.3 Fifteenth day of observation

At 15 days also, test wounds were completely healed (Figure 3.6.4 (c)) with complete re-epithelialization across the wounds and foreign body reactions due to the material was completely absent unlike the situation observed at the 10 day period. For wounds covered with hydrogel alone also, entire surface of the defect was covered with new epithelium (section 3.5.2.3.3). However, for wounds covered with gauze alone, moderate number of inflammatory cells, predominantly lymphocytes and macrophages were still present in the upper dermis (section 3.5.2.3.3).

3.6.2.4 Wound Re-epithelialization

At 5 days, wounds treated with hydrogel alone showed a wound re-epithelialization of 42 ± 9% (Table 3.6.1) whereas, those which received hydrogels with DBcAMP showed a wound re-epithelialization of 29.8 ± 11%. However, this difference was not statistically ($p < 0.05$) significant due to large standard deviation observed. For wounds covered with gauze alone, wound re-epithelialization was negligible which can be attributed to the scab formation by which the rate of re-epithelialization was retarded (see section 3.5.2.4, Table 3.6.1). At 10 days, the rate of re-epithelialization increased to 100% for test wounds, whereas this was 85.2% in the case of hydrogel alone. For wounds covered with gauze, this was only 74.6%. Statistical analysis revealed that there was significant difference between control and test wounds in the rate of re-epithelialization at 10 days. Earlier, it was found that ADA cross-linked hydrogels heal wounds by 15 days (see section 3.5.2). The wounds

l much more efficiently in the presence of DBcAMP and all the wounds undergo complete epithelialization within 10 days.



gure 3.6.4: Histology of wound sections stained with haematoxylin and eosin. Inflammatory granulation tissue formation at test wound edges at 5 days (a, 150x) and test wounds healed with complete re-epithelialization at 10 days (b, 150 x) and 15 (c, 300x) days.

3.6.3 Conclusion

It was shown earlier that ADA cross-linked gelatin hydrogel promotes granulation tissue formation and epithelialization when applied over wounds excised on rats. In order to improve its wound healing efficacy, a second messenger, DBcAMP was incorporated within the hydrogel and its wound healing efficacy was analyzed both by gross macroscopic examination and histology evaluation. It was found that DBcAMP enhances the rate of epithelialization resulting in the complete healing by 10 days. However, mild contracture was observed, which could be reduced by careful selection of the dose of DBcAMP incorporated within the hydrogel.

3.7 ADA Cross-linked Gelatin Hydrogel for Controlled Drug Delivery

3.7.1 Background

Colonization of bacteria leading to infection is one of the major problems in wound management, especially burns. Within 12-24 h, unprotected burn wounds are colonized by bacteria and within 48 h the level of microbes increase to 100 million per gram of tissue (Monafo & West, 1992). Many dressings cannot effectively prevent subsequent microbial invasion of the burn wounds. In those cases, the bacteria preferentially target wounds beneath the dressing materials, leading to serious infections necessitating frequent removal of the dressing and excision of cutaneous wounds (Kuroyanagi *et al.*, 1987; Fox *et al.*, 1969; Sayman *et al.*, 1973; Robb & Nathan., 1981). Naturally, by phagocytosis or other immune processes, proliferation of bacteria in wounds can be reduced. However, in the case of immuno-compromised patients, topical antimicrobial treatment of infected chronic wounds may be necessary (Price *et al.*, 1990; Leaper, 1994). Traditionally, the antibacterial cream will be applied on the injured skin once or twice a day. An important issue for treatment of an infected wound is to sustain sufficient drug concentration at the site of infection. Wound dressings containing antibiotics with sustained release are now being developed for the inhibition of wound infection (Mi *et al.*, 2002; Kim *et al.*, 1999a; Choi *et al.*, 2001; Kuroyanagi *et al.*, 1992; Grybowski *et al.*, 1997; Loke, 2000). Such dressings can decrease the wound infection and avoid the laborious replacement of wound dressings.

Spray-on films have been in existence since 1950's. These *in situ*-forming dressings could mould into the shape of wound defect. But the major difficulty concerning these dressings was the problem of bacterial spread when used over open third degree wounds

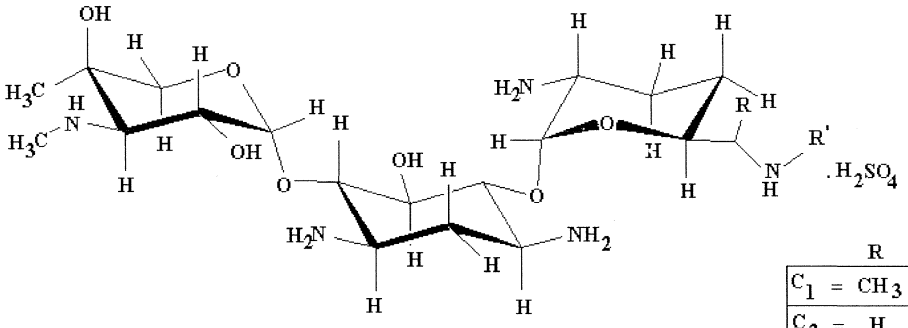
(Kane *et al.*, 1996). Gelatin-based spray on foam bandage incorporated with antibiotics has been found to possess antimicrobial activity against Gram-positive, Gram-negative and fungal contaminants (Neumann *et al.,* 1981).

In order to examine the potential of ADA cross-linked gelatin a matrix for controlled delivery of antibiotics to prevent wound infection, gentamycin was incorporated into the hydrogel and its release profile was studied *in vitro*. The release profile was also examined by varying the mode of incorporation of gentamycin, its payload and also the degree of oxidation of ADA. The antibacterial property of the drug loaded hydrogel was evaluated using two bacterial strains *P. aeroginosa* and *S. aureus.*

The study was also extended to primaquine, a potential anti-malarial drug. Although not directly related to wound management, this study was undertaken to examine the potential of the system further as an injectable delivery vehicle.

3.7.2 Gentamycin-Loaded Hydrogels

Gentamycin, a hydrophilic antibiotic belongs to the group of drugs called aminoglycosides, and is commonly used to prevent wound infection. Aminoglycosides are usually derived from *Streptomyces* or *Micromonospora*. Gentamycin appears to prevent bacteria from making their cell walls by irreversibly binding with 30S ribosomal unit, causing cell death. It is used to treat many sensitive Gram-negative and some Gram-positive bacteria. Gentamycin used for the present study is derived from *Micromonospora Purpurea*.



	R	R'
C_1	CH_3	CH_3
C_2	H	CH_3
C_{2A}	H	H

Figure 3.7.1: Structure of gentamycin sulphates.

Gentamycin, available in three different forms (C1, C2 and C2A), has three free primary amino groups (Figure 3.7.1), which can enter into reaction with aldehyde groups of ADA. Therefore, the mode of drug incorporation into the hydrogel will have influence on the release of drug from the matrix. The drug-containing hydrogels were prepared by two different methods. In one, gentamycin was first mixed with ADA solution (in 0.1 M borax) and in the other, it was first mixed with gelatin. Hydrogels were prepared in the usual way. The reaction of three amino groups of gentamycin with aldehyde groups of ADA is more facile when the drug is first mixed with ADA solution and the resulting solution is reacted with gelatin. In comparison, mixing the drug with gelatin first does not result in any chemical reaction. On subsequent treatment of the drug-containing gelatin solution with ADA, the amino groups present in the drug will have to compete with the amino groups of gelatin to enter into reaction with aldehyde groups in ADA. Thus, the probability of free drug in the latter preparation is expected to be more. The release profile of gentamycin was studied in PBS.

3.7.2.1 *In Vitro* Release of Gentamycin from Hydrogels: Gentamycin First Mixed with Gelatin Solution

Hydrogels were prepared from 0.5 mL of 20% solution of ADA having degree of oxidation, 57% and 87% in 0.1 M borax and 0.5 mL of gelatin solution mixed with gentamycin to give a payload of 5% by weight. Continuous release of drug from the hydrogels was observed (Figure 3.7.2). Release pattern was same for both hydrogels irrespective of the degree of oxidation of ADA used for hydrogel preparation. However, release appeared to be slightly higher for gels prepared using ADA having degree of oxidation 57%. The difference observed in the cumulative release as well as the rate of drug release from these hydrogels was however, not statistically significant ($p > 0.05$). Therefore we could infer that mechanism of release is similar in both gels. It may be recalled that the swelling ratio and the M_c of hydrogels prepared from 57 and 87% oxidized alginate and 15% solution of gelation were not very different (Section 3.3). Therefore, a dramatic difference in the release profile was also not expected. The release seems to be mainly diffusion controlled in the present case.

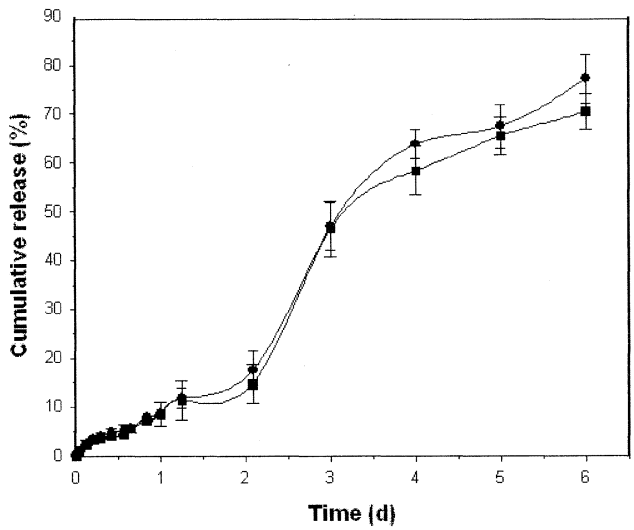


Figure 3.7.2: Cumulative release of gentamycin from hydrogels prepared from 20% solution of ADA having degree of oxidation (●) 57% and (■) 87% in 0.1 M borax and 15% solution of gelatin. (Gentamycin mixed with gelatin solution; drug payload 5%).

3.7.2.2 *In Vitro* Release of Gentamycin from Hydrogels: Gentamycin First Mixed with ADA Solution

Gentamycin was first mixed with ADA solution in 0.1 M borax and hydrogels were prepared with 15% solution of gelatin in the usual manner. Drug payload was kept at 5% by weight. Figure 3.7.3 shows the cumulative release of gentamycin from these gels. There was no significant difference observed in cumulative release with change in the degree of oxidation of ADA as in the previous case (Section 3.7.2.1). Only about 25% of the incorporated drug was released into the PBS in 8 days. The release seems to reach a plateau after this time period. On the other hand, when gentamycin was first mixed with gelatin, about 70% of the drug is released in 6 days. Looking at the extent of release in 5 days, it is seen that while 10-15% of the drug is released from the system where the drug is first mixed with ADA, around 60%, amounting to four times is released from the system where the drug is first mixed with gelatin. One should expect further release of gentamycin from the former system concurrent with hydrolysis of the Schiff's linkages as well as the degradation of the hydrogel matrix. The release profile observed from the system where drug is mixed with ADA clearly demonstrates conjugation of the drug to the matrix through Schiff's linkages. The matrix thus offers the opportunity for designing a delivery system with different delivery profiles for drugs possessing free amino groups.

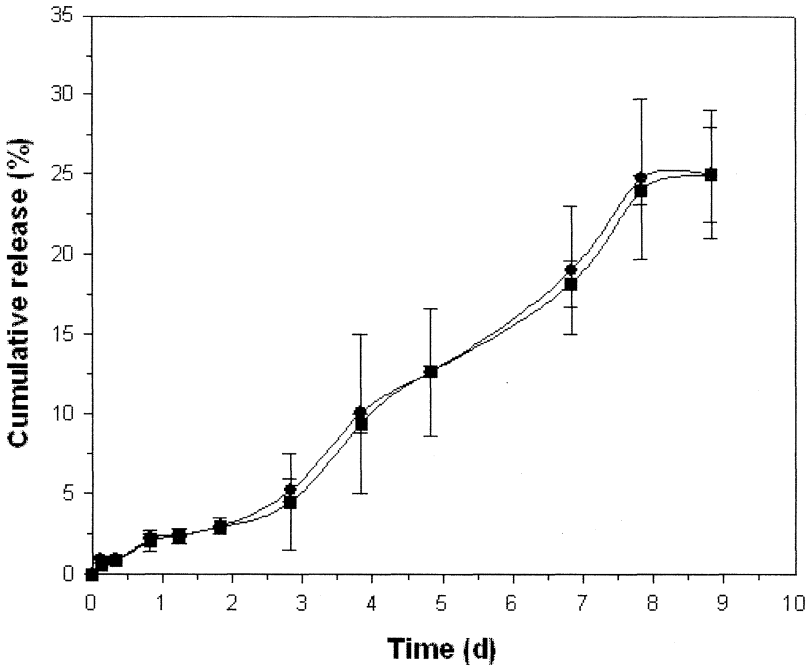


Figure 3.7.3: Cumulative release of gentamycin from hydrogels prepared from 20% solution of ADA having degree of oxidation (●) 57% and (■) 87% and 15% solution of gelatin.(Gentamycin mixed with ADA solution; drug payload 5%).

3.7.2.3 *In Vitro* Release of Gentamycin from Hydrogels: Effect of Drug Payload

Release of gentamycin from hydrogels in which the drug was first mixed with ADA was also examined by varying the drug payload. While about 55% of drug incorporated was released from hydrogels having a drug payload of 10%, only 25% and 15% of the incorporated drug was released respectively from same hydrogels with 5% and 2% drug payload during the same period (Figure 3.7.4). Thus, the release was also dependent on the drug payloads. Figure 3.7.4 shows that the cumulative release increases with the increase in the drug payload. The release of gentamycin from ADA cross-linked gelatin hydrogels are thus guided by two factors; the drug payload and the mode of mixing of gentamycin during hydrogel preparation.

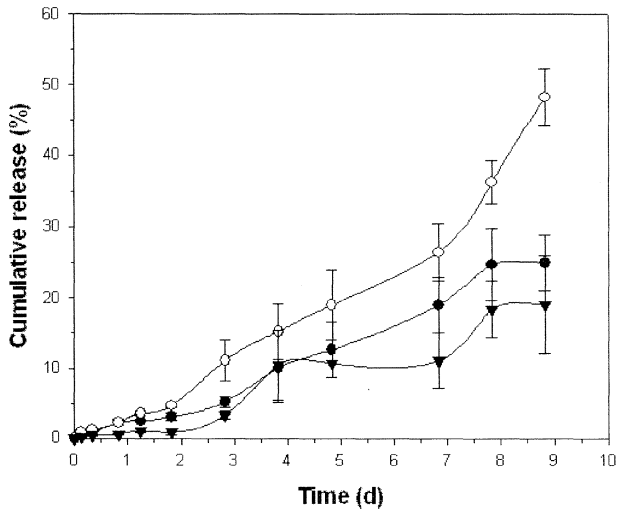


Figure 3.7.4: Variation on cumulative release of gentamycin from the hydrogels prepared from 20% solution of ADA having degree of oxidation 57% in 0.1 M borax and 15% solution of gelatin with change in drug payload: (▼) 2%, (●) 5% and (○) 10% wherein gentamycin is mixed with ADA solution.

3.7.3 *In Vitro* Release of Primaquine

The potential of the ADA/gelatin as an injectable drug delivery vehicle was examined. Primaquine was chosen as the model drug as its amino functions can enter into Schiff's reaction with the aldehyde groups in ADA and give rise to both diffusion and degradation controlled release (Figure 3.7.5). Primaquine was first mixed with gelatin and then reacted with ADA to form the hydrogel.

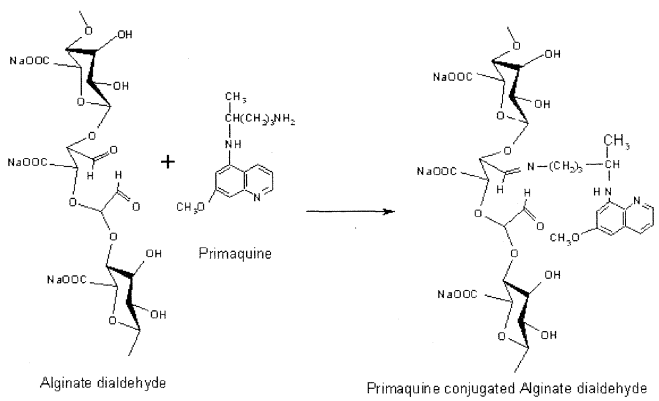


Figure 3.7.5: Scheme representing the conjugation of primaquine with ADA.

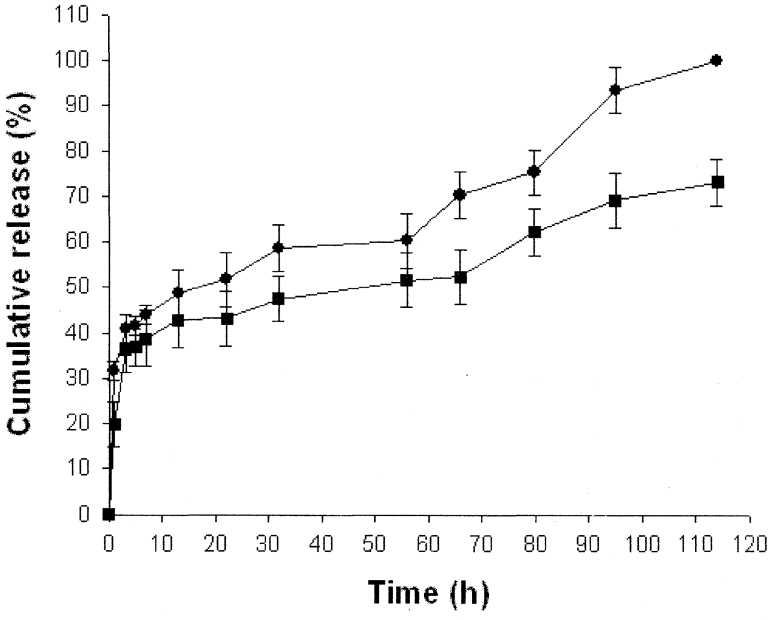


Figure 3.7.6: Cumulative release of primaquine from hydrogels having payload 5% prepared from 20% solution of ADA having degree of oxidation (■) 87% and (●) 57% in 0.1 M borax and 15% solution of gelatin.

As opposed to gentamycin, a burst release was observed from hydrogels prepared from both 57 and 87% oxidized ADA and gelatin (Figure 3.7.6). About 20-30% of the incorporated drug was found to be released during this burst. It was also seen that drug release from hydrogels prepared from 57% oxidized alginate was more compared to release from matrix composed of 87% oxidized alginate as opposed to the profile seen in the case of gentamycin. This is a pointer to better drug conjugation with the more oxidized alginate unlike the case of gentamycin. Cumulative release seems to taper off from this system with time; the release is about 60% of the incorporated drug as opposed to 90% seen from the system composed of 57% oxidized alginate. Primaquine is also a small molecule unlike gentamycin and the diffusional release of the drug is more facile in the case of primaquine which could explain the burst effect seen with primaquine and not observed with gentamycin.

If primaquine is first mixed with ADA, better conjugation of the drug with the matrix would be possible leading to both diffusion and degradation controlled release. However, this was not attempted in the present study.

The data obtained suggest that the *in situ*-forming hydrogels from oxidized alginate and gelatin has the potential to be used as an injectable drug delivery vehicle. More studies of course would be needed to optimize the system for prolonged delivery of drugs *in vivo*. The limited *in vitro* experiments can not directly be translated into *in vivo* situations as the drug release profile *in vivo* could be quite different from what is seen *in vitro*. Nevertheless, the observations made in the study are significant enough to be a pointer to the possibility of constructing an injectable drug delivery system with these hydrogels.

The system may also be useful for the controlled release of therapeutic peptides and proteins, since at lower aldehyde contents, these molecules will be less chemically conjugated to the matrix enabling release in their most native form (Draye *et al.*, 1998).

3.7.4 Antibacterial Effect of Gentamycin-incorporated Hydrogel

Bactericidal efficacy of gentamycin incorporated ADA cross-linked gelatin hydrogel was examined by using the procedure reported by Kim *et al.* (Kim *et al.*, 2000). Gentamycin was mixed with 15% solution of gelatin and was cross-linked with solution of ADA in 0.1 M borax. Drug payload used was 2.5%. Two strains used for the study were *P. aeruginosa* and *S. aureus*. Initial seeding density used for both strains was 1×10^5 CFU/mL. Gel having the same composition, but without the drug was used as the control. Figure 3.7.7 shows the photographs of culture plates on which the gel containing gentamycin was placed and incubated with two different strains of bacteria for 48 h at 37 °C. A region (~ 31 mm) of inhibition was found around the gels containing the drug for *P. aeruginosa*. Similarly a

region of inhibition of 30 mm diameter was found for the same gels placed on culture plates inoculated with *S. aureus.* This clearly shows that gels were releasing gentamycin to the agar, in active form to inhibit the growth of bacteria.

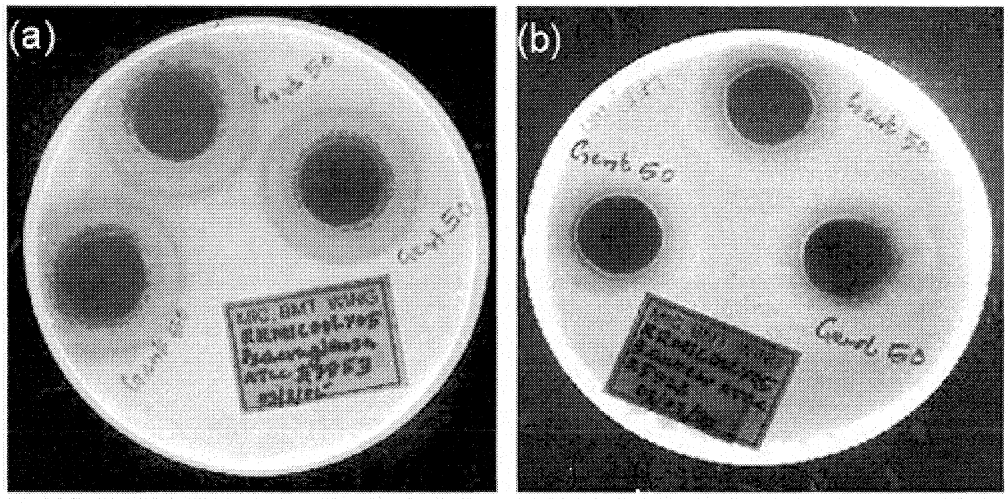


Figure 3.7.7: Photographs of culture plates: Gentamycin impregnated hydrogel on (a) *P. aeroginosa* (ATCC 27853) and (b) *S. aureus* (ATCC 25923).

In order to evaluate whether the gels could function as an antibacterial dressing which could prevent the bacterial growth beneath the dressings, agar beneath the gels (1 cm^2) was removed after 48 h and homogenized using sterile PBS. The PBS suspension was then cultured for measuring the viable count by optical microscopy. It was found that while control samples gave a count of 5×10^3 CFU and 1.7×10^6 CFU respectively for *S. aureus and P. aeroginosa,* test samples completely inhibited the survival and proliferation of both bacterial strains (Figure 3.7.8).

The absence of bacteria beneath the gentamycin-impregnated gels clearly indicated that gentamycin released from the hydrogel was still in its active form and was able to prevent their survival and proliferation. Also it was found that results were similar for both strains.

In preparing the hydrogels, gentamycin was first mixed with gelatin and then with ADA in borax. This was done in order to make the drug continuously available in culture. From the *in vitro* release study using this system, continuous release of gentamycin was seen as opposed to the system in which gentamycin was first mixed with ADA. It is noteworthy that although borax has mild antibacterial properties, the control gel does not have a bactericidal effect. It may be recalled that in the *in vivo* wound healing studies, no bacterial colonies were present on the wounds covered with the hydrogel dressing. But, in situations where there are large colonies of bacteria already present, it should be inferred that borax does not exert a significant bactericidal effect.

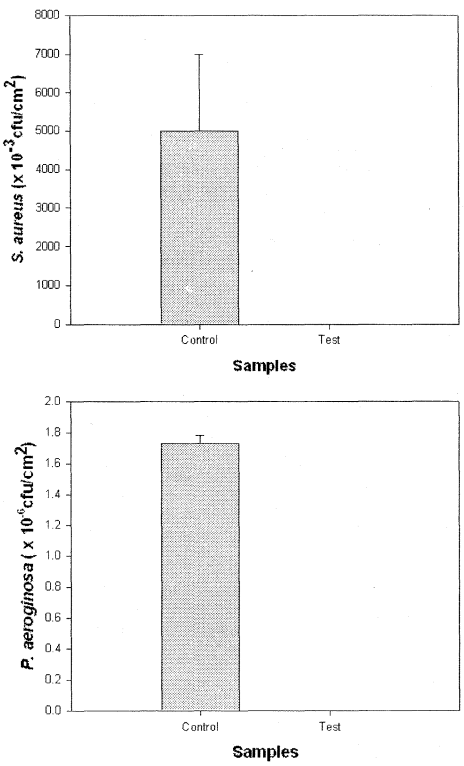


Figure 3.7.8: Number of residual bacteria in the agar plate covered with gentamycin impregnated wound dressing.

3.7.5 Conclusion

ADA cross-linked gelatin hydrogel could be used as a vehicle for the controlled delivery of drugs. In the case of drugs such as gentamycin and primaquine which contain primary amino groups, the possibility of drug conjugation to the hydrogel through Schiff's base is a distinct possibility. The order of mixing the drug plays a significant role in determining the type of release. By first mixing the drug with gelatin, a more facile and more rapid diffusional release is possible for both drugs as opposed to mixing the drug first with oxidized alginate. Such manipulations provide considerable opportunity in modulating the drug release from the hydrogel matrix. In the case of gentamycin, the release was studied by varying the order of mixing the drug and found that release was fast when drug was mixed with gelatin solution. While gentamycin did not show a burst effect, primaquine showed a significant burst which could be attributed to the small size of primaquine molecule. Another factor that determined the release was the drug payload and this was demonstrated in the case of gentamycin. The antibacterial property of gels impregnated with gentamycin using two bacterial strains, *S. aureus and P. aeroginosa,* showed that gels were able to prevent the survival and proliferation of bacteria completely on contact.

3.8 ADA Cross-linked Gelatin Hydrogel as a Tissue Engineering Scaffold

3.8.1 Background

An exciting and revolutionary strategy to treat patients who need a new organ or tissue is the engineering of man made organs or tissues. Tissue engineering is "an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain and improve the function of the damaged tissues and organs" (Skalak & Fox, 1988; Langer & Vacanti, 1993). Of the different strategies for engineering of tissues or organs, an appealing approach will be the combination of patient's own cells with polymer scaffolds. Scaffolds in tissue engineering, provide cultured cells that grow on them, the physical and chemical cues to guide their differentiation and assembly into three dimensional tissues. Critical element in this approach is the polymer scaffold. These scaffolds should be biocompatible, bio-resorbable, easily reproducible and able to provide mechanical support to maintain a space for tissue to form (Mooney & Langer, 1995). Various types of polymers have been studied and utilized to date in tissue engineering. Aliphatic polyesters including poly(glycolic acid), poly(lactic acid) and copolymers of these materials are the most widely used synthetic polymers (Cima *et al.*, 1991a; Cima *et al.*, 1991b). Though these polymers have a long history of use in medical applications and considered safe in many situations by the US Food and Drug Administration, the use of these scaffolds requires the surgeon to make incisions sufficiently large to enable placement of the polymer/cell constructs.

An exciting alternative approach to cell delivery for tissue engineering will be the use of polymeric scaffolds that can be injected into the body. Injectable systems offer specific advantages over preformed scaffolds, which include ease of application, confined

delivery for a site-specific action and improved patient compliance and comfort (Mallapragada & Narasimhan, 2002). Various methods have been employed for the preparation of injectable hydrogel systems. Water-soluble, thermosensitive and pH sensitive polymers exhibiting reversible sol-gel transition and photo-polymerizable hydrogels have been tailor-made as injectables (Jeong *et al.*, 1997; Vernon *et al.*, 1996; Skjak-Braek *et al.*, 1992; Nguyen & West, 2002). However, photo-polymerization often requires a photo-sensitizer and prolonged irradiation limiting their use and thermosensitive polymers form just physical gels and not cross-linked structures.

The gel forming property of alginates in the presence of divalent ions like Ca^{2+}, has been much exploited for tissue engineering applications. Cells entrapped within the gel beads maintain viability and metabolic function and are capable of secreting a variety of protein products into external medium. Alginates have been extensively researched for the encapsulation of pancreatic islet cells for controlled release of insulin to treat Type I diabetes. This application of alginates has been comprehensively reviewed (Clayton *et al.*, 1993; Draget & Skjak-Braek, 1990). Despite many advantages, the use of calcium alginates as tissue engineering scaffolds is limited due to their poor stability due to loss of divalent ions into surrounding medium and subsequent dissolution of the scaffold, under physiological conditions. More over, the molecular weights of many alginates are typically above the renal clearance threshold of the kidney (Lee & Mooney, 2001). Recently, biodegradable, covalently cross-linked hydrogels derived from alginate were reported. In this approach, the authors have employed adipic di-hydrazide to covalently cross-link alginate as well as slightly oxidized alginate (Lee *et al.*, 2000; Bouhadir *et al.*, 2001a).

It was therefore very interesting to examine the potential of ADA cross-linked gelatin hydrogels as an injectable tissue engineering scaffold. Since the gelation reaction leading to the three-dimensional hydrogel is very fast with the systems based on ADA, borax and gelatin, it would be possible to inject the cell/scaffold construct directly into the body. In order to demonstrate this approach, hepatocytes, one of most difficult cells to culture, were chosen and encapsulated within the gel matrix with the anticipation that other cells also could be cultured on the matrix. This section deals with the encapsulation of hepatocytes inside the hydrogel and their viability, functionality and proliferation within the matrix.

3.8.2 Encapsulation of Hepatocytes

Hydrogels prepared by employing 20% solution of ADA having degree of oxidation 57% in 0.1 M borax and 15% solution of gelatin were used for this study. Hepatocytes can be readily isolated from livers taken from a wide range of species. For this study, hepatocytes were isolated from Wistar rats by a modification of Seglen's two step collagenase perfusion procedure as described in Chapter 2, section 2.2.8.1. The viability of cells measured by Trypan Blue staining was found to be more than 90%.

The isolated hepatocytes suspension (containing 2.65 x 10^5 cells/mL) was added to ADA solution followed by addition of gelation solution to form a hydrogel. Since 50 mL each of gelatin and ADA was mixed with 30 mL of cell suspension (see section 2.2.8.2), \ this particular gel was characterized for its physical properties. The degree of cross-linking of the gel was found to be 54.48 ± 3 %. The swelling ratio (Q_m) and the degree of swelling (Q) were found to be 9.68 ± 0.5 and 9.52 ± 0.5 respectively. The cross-linking density of the gel, ν_e (x 10^5) was determined to be 23.5 ± 7 mol/cm^3.

3.8.2.1 Evaluation of the Viability of Hepatocytes

Hepatocytes are anchorage-dependent cells with the matrix playing an important role in cell shape, division, differentiation and function and are difficult to culture and propagate *in vitro* for extended periods (Atala & Mooney, 1997). In 2-D cultures, cell matrix interaction is minimal resulting in limited cell survival and proliferation. By encapsulating hepatocytes within a matrix, the cells are being protected from the immune response of the host. Hydrogels act as extracellular matrix and thus assist the external cell metabolism and prolong survival time by mimicking *in vivo* hepatocytes environment. It also helps to maintain a three dimensional structure for hepatocytes.

Successful hepatocyte transplantation requires the maintenance of morphology of the cells within the matrix. It has been reported that hepatocytes were well maintained by sand-witching the cells between two hydrated collagen layers (Langer & Vacanti, 1993). As the hydrogel under investigation is based on biopolymers, similar results were expected. Routine microscopic examination of hepatocytes encapsulated within the hydrogel during the culture period showed that the cells were maintaining their morphology and were dividing inside the matrix demonstrating their live nature. Figure 3.8.1 shows the morphology of hepatocytes encapsulated within the gel.

The proliferation of hepatocytes within the gel was indirectly measured by estimating the number of viable cells using Neutral Red assay. As the hydrogel also was taking stain along with the cells, commonly used MTT assay was not useful for the quantitative estimation of viable cells encapsulated within the gel. Neutral Red assay is very simple assay for the determination of number of viable cells cultured in tissue culture plates. It has been reported that optical density of Neutral Red, after treating the cells with the stain and extracting the

stain from the cells by the addition of 0.05 M NaH_2PO_4 in 50% isopropanol, is directly proportional to the number of cells (Lowik *et al.*, 1993). Neutral Red will only be taken up by actively dividing viable cells. Figure 3.8.2 gives the viability of hepatocytes indirectly estimated by measuring the optical density of the Neutral Red uptake by the cells after regular intervals of time. Absorbance increase up to 4 weeks can be attributed to the presence of more active cells within the matrix.

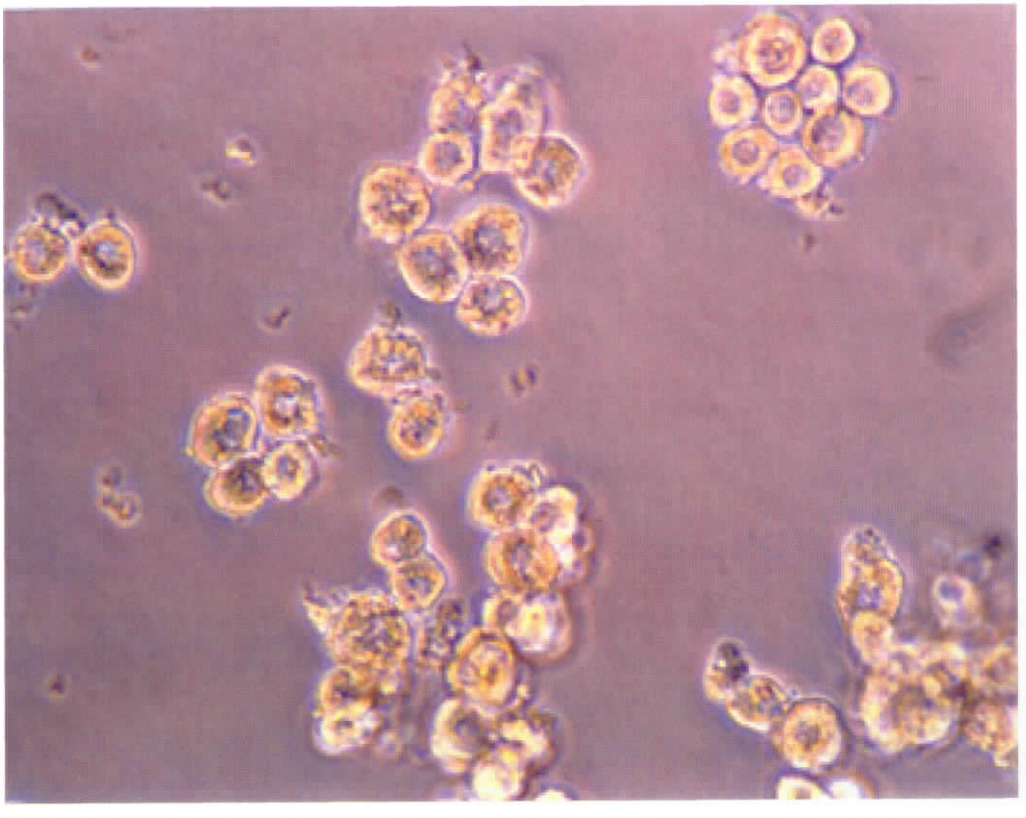


Figure 3.8.1: Optical photomicrographs (x 320) of hepatocytes encapsulated within the gel derived from 15% solution of gelatin and 20% solution of 57% oxidized ADA in 0.1 M borax 7 days after encapsulation. Note that the cells are inside the matrix although they might appear to be on the surface.

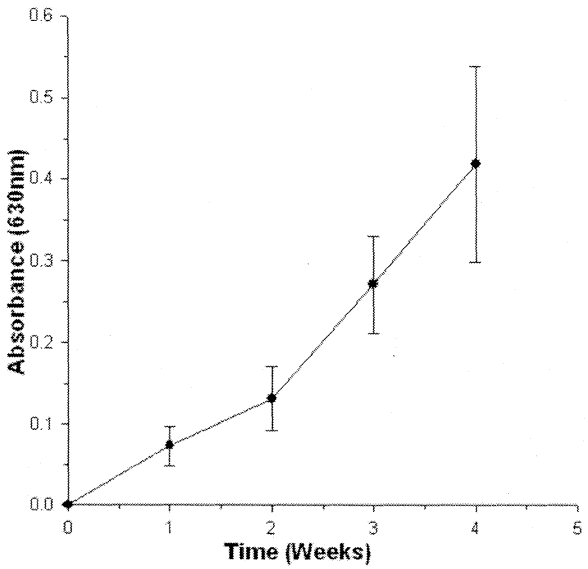


Figure 3.8.2: Viability of hepatocytes encapsulated in the gel with respect to time as shown by increase in absorbance.

3.8.2.2 Evaluation of the Functionality of Hepatocytes

Due to dedifferentiation, the isolated hepatocytes have been observed to lose a number of liver specific functions with time. A part of this functionality loss can be prevented by encapsulating the cells within a matrix which can act as an extra-cellular matrix. Hepatocytes must be anchored to a substrate in order to function properly. One of the liver specific functions, albumin secretion was examined in order to evaluate the potential of the hydrogel to maintain the functionality of cells encapsulated within.

In order to examine whether the albumin secreted by the hepatocytes within the matrix will get released into the medium, *in vitro* release of FITC albumin incorporated to the gel at a payload of 1% was examined. It was found that albumin does not enter into Schiff's reaction with ADA and complete release occurred from the matrix after a period of 60 h (Figure 3.8.3).

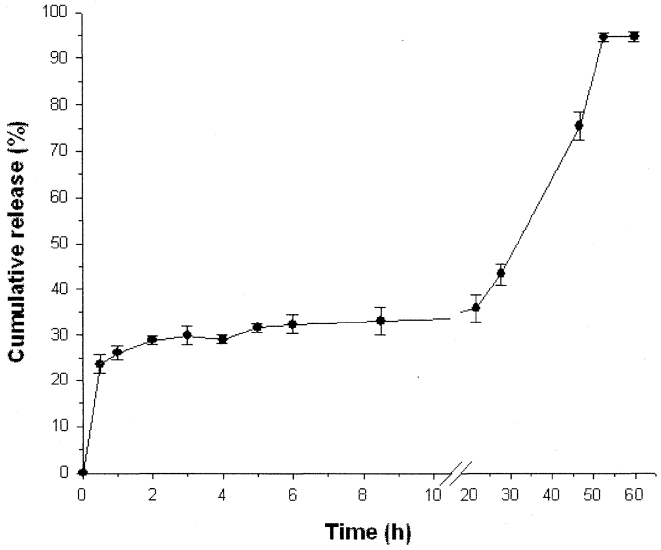


Figure 3.8.3: *In vitro* release of FITC albumin from the gel.

Further, the albumin secreted by the hepatocyte and then released into the culture medium was analyzed after regular intervals of time as outlined in section 2.2.8.5. Estimation of albumin secretion also showed that cells were maintaining their protein producing ability (one of the most liver-specific functions) (Figure 3.8.4) demonstrating the suitability of the scaffold for tissue engineering.

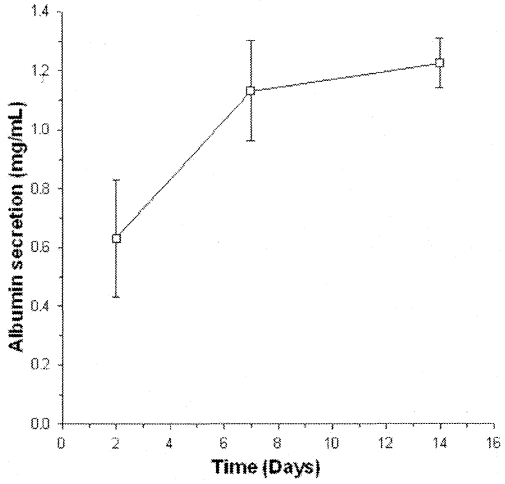


Figure 3.8.4: Albumin secretion of encapsulated hepatocytes with respect to time.

3.8.3 Conclusion

The potential of ADA cross-linked gelatin hydrogel as a tissue engineering scaffold has been examined by encapsulating hepatocytes within the matrix. It was found that hepatocytes maintain normal morphology within the matrix. The cells were found to be viable and proliferating. Albumin secretion analysis revealed that the encapsulated hepatocytes maintain its one of the most specific functions over a period of 4 weeks. It should therefore be possible to use the hydrogel as an injectable scaffold for tissue engineering applications.

Chapter-4
Summary, Conclusions & Future Prospects

SUMMARY, CONCLUSIONS AND FUTURE PROSPECTS

4.1 Summary and Conclusions

Hydrogels are an important class of biomaterials and hydrogels derived from many biopolymers such as proteins and polysaccharides possess structural similarity to extracellular matrices in the body. The development of an *in situ*-forming hydrogel system based on biopolymers without using any extraneous cross-linking agents has been a major challenge in the field of biomaterials.

The major aim of the present work was the development of a rapidly forming hydrogel from biopolymers such as sodium alginate and gelatin to be used as *in situ*-forming wound dressing. Both biopolymers are known to be non-toxic, biodegradable and possess wound healing efficacy. Periodate oxidized alginate having appropriate molecular weight and degree of oxidation was shown to react rapidly with gelatin in the presence of borax to give *in situ*-forming hydrogels within a few seconds.

Alginates form high viscous solutions even at low concentrations and the reaction is usually conducted in dilute solutions. Therefore, the possibility of oxidizing alginate as a

dispersion in ethanol/water mixture with the aim of obtaining larger quantity of the oxidized product with minimum amount of the solvent in one go was examined. The degree of oxidation, dialdehyde content and the M_w of the alginate dialdehyde (ADA) prepared by the two methods were compared. It was found that the reaction in ethanol/water mixture proceeded smoothly as that in aqueous medium and the kinetics of oxidation in both media were surprisingly similar. The ethanol/water medium also gave rise to better yield of the oxidized product (50-60%) as compared to the yield from aqueous medium which was always within 25-35%. The M_w of oxidized alginates obtained demonstrated extensive depolymerization in both media; the degradation was drastic in ethanol/water resulting in very low molecular weight products. This new approach to periodate oxidation has many advantages such as increased yield, use of less quantity of the solvent and the ability to prepare large quantities of the oxidized product in one reaction.

The gelation reaction between gelatin and oxidized alginate prepared by the two different methods was examined. The gelation of gelatin using the ADAs obtained from aqueous medium was rather sluggish. However, when oxidized product obtained from ethanol/water mixture was used, the gelation occurred rapidly. Degree of oxidation, molecular weight of the ADA, concentration of reactants and medium of gelation were important parameters in determining the gelling time. It was found that in the presence of borax, the Schiff's reaction between ADA and gelatin leading to gelation was most rapid as opposed to many other buffers and media with alkaline pH. This was attributed to the unique ability of borax to complex with the hydroxyl groups of polysaccharides as well as the slightly alkaline pH of borax solutions. It was demonstrated that the system can be manipulated by varying different factors that influence the gelation reaction to obtain gelling time from a few

seconds to a minute or more thereby allowing the system to be used in a variety of applications.

A thorough physico-chemical characterization of the hydrogel was carried out and the swelling parameters, cross-linking density, degree of cross-linking and molecular weight between cross-links of the hydrogels were evaluated systematically. It was shown that the higher the degree of oxidation of alginate, higher the cross-linking degree. The fluid uptake ability, rate of evaporation of water and WVTR of these hydrogels were found to be optimal for a wound dressing for maintaining a moist environment conducive for wound healing. Studies have also shown that the hydrogels were completely degradable under physiological conditions and are porous in structure.

It was found that a 20% solution of ADA having degree of oxidation 57% and a 15% solution of gelatin were optimal with respect to dissolution, ease of handling and gelation time for the preparation of hydrogels for many applications. Therefore, this composition was chosen for further studies. The biocompatibility of the hydrogel was evaluated as per ISO guidelines. Qualitative and quantitative cytotoxicity, intradermal irritation potential, delayed hypersensitivity and haemolytic potential of the hydrogel were evaluated. It was shown that the hydrogel was non-cytotoxic, non-irritant, non-sensitive and non-haemolytic.

Wound healing experiments using the *in situ*-forming hydrogel on full thickness rat wound model showed that wound defect filled up to 95% and the defect was covered with new epithelium in two weeks. The presence of borax was found to exert an antiseptic effect to prevent bacterial colonization of the wound. In order to improve the wound healing efficacy, a second messenger, DBcAMP was incorporated within the hydrogel. The presence

of DBcAMP enhanced the rate of wound re-epithelialization resulting in complete healing by 10 days.

The hydrogel was further evaluated as a vehicle for the controlled delivery of drugs like gentamycin and primaquine. In the case of gentamycin, the release was studied by changing the order of mixing of the drug with the gelling biopolymers. While the release was faster when the drug was first mixed with gelatin solution, the release was considerably retarded when the drug was first mixed with oxidized alginate pointing to drug conjugation with the biopolymer. Antibacterial testing using two bacterial strains *S. aureus and P. aeroginosa,* showed that gels were able to prevent bacterial colonization completely. With primaquine as the drug, the limited study undertaken showed that the release profile of the incorporated drug could be manipulated to provide sustained release. These studies demonstrated that the system could be used as an injectable drug delivery vehicle for controlled drug delivery.

The potential of the *in situ*-forming hydrogel as a tissue engineering scaffold was examined by encapsulating hepatocytes within the matrix. It was found that hepatocytes maintained normal morphology within the matrix and the cells were viable and proliferating. This study showed that the matrix has the potential as an injectable tissue engineering scaffold.

The results obtained in the present study showed that the *in situ* forming hydrogels from oxidized alginate and gelatin have significant potential to be used in number of medical applications such as wound dressings and injectable drug and cell delivery systems.

4.2 Future Prospects

These rapidly gelling systems also have the potential to be used for embolization, a procedure in interventional radiology for obliteration of blood vessels supplying a pathological area. Embolization is used in the treatment of haemorrahges, haemoptysis, arterio-venous malformations and tumours. Using double lumen catheters and state-of-the-art imaging and road mapping technology available at the disposal of interventional radiologists, it should be possible to introduce this two-component gelling system into the blood vessel that is to be occluded for such treatments. Such approach will be especially valid for chemoembolization for treatment of tumours by incorporating appropriate cytotoxic agents in the hydrogel for prolonged release to the tumour site.

Another area that could be explored in the biomedical field with these rapidly gelling systems is tissue sealing. Tissue adhesives are receiving increasing importance and these gels could form soft, compliant, biodegradable sealants in surgery.

Although hydrogels obtained were found to be sufficiently strong for applications such as wound dressing and drug delivery, their mechanical properties were not examined in a quantitative manner in the present study. The shear modulus of these hydrogels could be examined further in order to get a quantitative picture about their mechanical strength. Also the change in shear modulus during degradation could be followed as this study is very crucial for tissue engineering applications.

It was demonstrated that wound healing efficacy of these hydrogels was further enhanced by incorporating DBcAMP within the gel. By varying the concentration of DBcAMP, wound healing pattern could be evaluated further, so that the appropriate dose for enhanced wound healing with minimal contraction could be found out.

The results obtained by encapsulation of hepatocytes within the gels were particularly striking. Further studies could be performed in that direction to develop this as an injectable system for cell delivery in real life situations. The hydrogel could also find application as an injectable a matrix for sustained delivery of many growth factors.

Bibliography

BIBLIOGRAPHY

Aaron JE, Bridges AF, Ritzel DO, Lindauer LB. First Aid and Emergency Care Prevention and Protection of Injuries. second edition, USA: Macmillan, 1979, pp.71-5.

Adair HM. Epidermal repair in chronic venous ulcers. Br J Surg 1977;**64**:800-04.

Alexander JW, Wheeler LM, Rooney RC, Macdonald J, MacMillan BG. Clinical evaluation of Epigard, a new synthetic substitute for homograft and heterograft skin. J Trauma 1973;**13**:373-83.

Al-Shamkhani A, Duncan R. Radioiodination of alginate via covalently bound tyrosinamide allows monitoring of its fate in vivo. J Bioact Compat Polym 1995;**10**:4-13

Anderson JM. Inflammatory response to implants. Am Soc Artif Intern Organs 1988;**11**:101-7.

ASTM Standard E96-00. Standard test methods for water vapour transmission of materials, Annual book of ASTM standards, Vol. 4.06, Philadelphia: ASTM, 2000.

Atala A, Mooney DJ. Editors. Synthetic Biodegradable Polymer Scaffolds. Boston: Birkhauser: 1997.

Barlow YM. Developments in Wound Care: International trends and opportunities. Clinica Reports, UK: PJB Publications, 1994.

Barnett SE, Irving SJ. Studies of wound healing and the effect of dressings. In: Szycher M, Editor. High Performance Biomaterials. Lancaster: Technomic, 1991, pp.583-620.

Barnett SE, Varley SJ. The effects of calcium alginate on wound healing. Ann R Coll Surg Engl 1987;**69**:53-155.

Bouhadir KH, Alsberg E, Mooney DJ. Hydrogels for combination delivery of antineoplastic agents. Biomaterials 2001a;**22**:2625-33.

Bouhadir KH, Hausmana DS, Mooney DJ. Synthesis of cross-linked poly(aldehyde guluronate) hydrogels. Polymer 1999;**40**:3575-84.

Bouhadir KH, Kruger GM, Lee KY, Mooney DJ. Sustained and controlled release of daunomycin from cross-linked poly(aldehyde guluronate) hydrogels. J Pharm Sci 2000;**89**:910-9.

Bouhadir KH, Lee KY, Alsberg E, Damm KL, Anderson KW, Mooney DJ. Degradation of partially oxidised alginate and its potential application for tissue engineering. Biotechol Prog 2001b;**17**:945-50.

Braumann T, Jastroff B. Physical chemical characterization of cyclic nucleotides by reversed phase high performance liquid chromatography. II. Quantitative determination of hydrophobicity. J Chromatogr 1986;**350**:105-18.

Bruce AM. The Skin. In: Cohen IK, Diegelmann RF, Lindblad WJ, Editors, Wound Healing Biochemical and Clinical aspects. USA: WB Saunders, 1992, pp.344-55.

Bubnis WA, Ofner CM. The determination of ε-amino groups in soluble and poorly soluble proteinaceous materials by a spectrophotometric method using trinitrobenzenesulfonic acid. Anal Chem 1992;**207**:129-33.

Campoccia D, Doherty M, Radice P, Brun P, Abatangelo G, Williams DF. Semisynthetic resorbable materials from hyaluronan esterifications. Biomaterials 1998;**19**:2101-27.

Cenni E, Ciapetti G, Stea S, Corradini A, Carozzi F. Biocompatibility and performance *in vitro* of a hemostatic gelatin sponge. J Biomater Sci Polym Edn 2000;**11**:685-99.

Chang W-H, Chang Y, Lai P-H, Sung H-W. A genipin-cross-linked gelatin membrane as wound-dressing material: *in vitro* and *in vivo* studies. J Biomater Sci Polym Edn 2003;**14**:481-95.

Chen JL Surgical bandage. US Pat 3972328, 1976.

Chen T, Embree HD, Brown EM, Taylor MM, Payne GF. Enzyme-catalyzed gel formation of gelatin and chitosan: potential for *in situ* applications. Biomaterials 2003;**24**:2831-41.

Choi YS, Hong SR, Lee YM, Song KW, Park MH, Nam YS. Studies on gelatin-containing artificial skin: II. Preparation and characterization of cross-linked gelatin-hyaluronate sponge. J Biomed Mater Res Appl Biomater 1999b;**48**:631-9.

Choi YS, Hong SR, Lee YM, Song KW, Park MH, Nam YS. Study on gelatin-containing artificial skin: I. Preparation and characteristics of novel gelatin-alginate sponge. Biomaterials 1999a;**20**:407-9.

Choi YS, Lee SB, Hong SR, Lee YM, Song KW, Park MH. Studies on gelatin-based sponges. Part III: a comparative study of cross-linked gelatin/alginate, gelatin/hyaluronate and chitosan/hyaluronate sponges and their application as a wound dressing in full-thickness skin defect of rat. J Mater Sci Mater Med 2001;**12**:67-73.

Ciapetti G, Cenni E, Pratelli L, Pizzoferrato A. *In vitro* evaluation of cell/biomaterial interaction by MTT assay. Biomaterials 1993;**14**:359-64.

Cima LG, Langer R, Vacanti C, Ingber D, Mooney D, Langer R. Tissue engineering by cell transplantation using degradable polymer substrates. J Biomech Eng 1991a;**113**:143-51.

Cima LG, Langer R, Vacanti JP. Polymers for tissue and organ culture. J Bioactive Compat Polymers 1991b;**6**:232-40.

Clark RA. Cutaneous tissue repair: basic biologic considerations. J Am Acad Dermatol 1985;**13:**701-2

Clark RAF. Wound repair: overview and general considerations. In: The Molecular and Cellular Biology of Wound Repair. Clark RAF, Editor. London: Plenum Press, 1996; pp.3-50.

Clayton HA, James RF, London NJ. Islet microencapsulation: a review. Acta Diabetol 1993;**30**:181-9.

Cook W. Alginate dental impression materials-chemistry, structure and properties. J Biomed Mater Res 1986;**20**:1-24.

Cortesi R, Nastruzzi C, Davis SS. Sugar cross-linked gelatin for controlled release: microspheres and disks. Biomaterials 1998;**19**:1641-9.

Coviello T, Grassi M, Lapasin R, Marino A, Alhaique F. Scleroglucan/borax: characterization of a novel hydrogel system suitable for drug delivery. Biomaterials. 2003;**24**:2789-98.

Crescenzi V, Francescangeli A, Taglienti A. New gelatin based hydrogels via enzyme networking. Biomacromolecules 2002;**3**:1384-91.

Davis HB, Mott CJB. Interaction of boric acid and borates with carbohydrates and related substances. J Chem Soc Faraday I 1980;**76**:1991-2002.

Deuel H, Neukom H, Weber F. Reaction of boric acid with polysaccharides. Nature 1948;**161**:96-7.

Di Silvio L, Gurav N, Kayser MV, Braden M, Downes S. Biodegradable microspheres: a new delivery system for growth hormone, Biomaterials 1994;**15**:931-6.

Diegelmann RF, Cohen IK, Kaplan AM. The role of macrophages in wound repair: A review. Plast Reconstr Surg 1981;**68**:107-13.

Digenis GA, Gold TB, Shah VP. Cross-linking of gelatin capsules and its relevance to their *in vitro-in vivo* performance. J Pharm Sci 1994;**83**:915-20.

Draget KI, Skjak- Brek G, Smidsrod O. Alginate based new materials. Int J Biological Macromolecule 1997;**21**:47-55.

Draget KI, Skjak-Braek G. Alginate as immobilization matrix for cells. Trends Biotechnol 1990;**8**:71-8.

Draye J-P, Delaey B, Van de Voorde A, Van Den Bulke A, Bogdanov B, Schacht E. *In vitro* release characteristics of bioactive molecules from dextran dialdehyde cross-linked gelatin hydrogel films. Biomaterials 1998;**19**:99-107.

Dumitriu S. Polymeric Biomaterials. second edition, New York: Marcel Dekker, 2002.

Dunlap MK, Donaldson DJ. Effect of cAMP and related compounds on new epidermal cell migration both *in vivo* and *in vitro*. J Exp Zool 1980;**212**:13-9.

Eisenbud D, Hunter H, Kessler L, Zulkowski K. Hydrogel wound dressings: where do we stand in 2003? Ostomy Wound Manage 2003;**49**:52-7.

Fakirov S, Sarac Z, Anbar T, Boz B, Bahar I, Evstatiev M, Apostolov AA, Mark JE, Kloczkowski A. Mechanical properties and transition temperatures of cross-linked oriented gelatin. Collod Polym Sci 1996;**274**:334-41.

Flory PJ, Rehner J. Statistical mechanics of cross-linked polymer networks. II. Swelling. J Chem Phys 1943;**11**:521-6.

Fox CL, Rappole BW, Stanford W. Control of Pseudomonas infection in burns by silver sulfadiazine. Surg Gynecol Obstet 1969;**128**:1021-6.

Gensheimer D. A review of calcium alginates. Ostotomy Wound Manage 1993;**39**:34-8.

Gilchrist T, Martin AM. Wound treatment with Sorbsan^{R}- an alginate wound dressing. Biomaterials 1994;**15**:317-20.

Gomathi K, Gopinath D, Ahmed MR, Jayakumar R, Quercetin incorporated collagen matrices for dermal wound healing processes in rat. Biomaterial 2003;**24**:2767-72.

Gombotz WR, Wee SF. Protein release from alginate matrices. Adv Drug Deliv Rev 1998;**31**:267-85.

Graham NB. Hydrogels: their future, Part 1. Med Device Technol 1998a;**9**:18-22.

Graham NB. Hydrogels: their future, Part 11. Med Device Technol 1998b;9:22 -5.

Grant GT, Morris ER, Rees DA, Smith PJC, Thom D. Biological interactions between polysaccharides and divalent cations: the egg box model. FEBS lett 1973;**32**:195-8.

Grotendorst CR, Chang T, Seppa HEJ, Kleinman HK, Martin GR. Platelet-derived growth factor is a chemoattractant for vascular smooth muscle cells. J Cell Physiol 1982;**113**:261-6.

Groves AR, Lawrence JC. Alginate dressing as a donor site haemostat. Ann R Coll Surg 1986;**68**:27-8.

Grybowski J, Kolodziej W, Trafny EA, Struzyna J. A new anti-infective collagen dressing containing antibiotics. J Biomed Mater Res 1997;**36**:163-6.

Guidoin R, Marceau D, Rao TJ, King M, Merhi Y, Roy PE, Martin L, Duval M. *In vitro* and *in vivo* characterization of an impervious polyester arterial prosthesis: the Gelseal Triaxial graft. Biomaterials 1987;**8**:433-41.

Guthrie RD. Periodate Oxidation. In: Whistler RL, Wolfram ML, Editors. Methods in carbohydrate chemistry. New York: Academic Press, 1953. p. 432-47.

Haug A, Larsen B, Smidsrod O. Alkaline degradation of alginate. Acta Chem Scand 1967;**21**:2859-70.

Haugen F, Kortner F, Larsen B. Kinetics and specificity of alginate lyases: Part I, a case study. Carbohydr Res 1990;**198**,101-9.

Heiple JM, Wright SD, Allen NS, Silverstein SC: Macrophages form circular zones of close apposition to IgG-coated surfaces. Cell Motil Cytoskel 1990;**15**:260-70.

Hermanson GT. Editor, Bioconjugate techniques. San Diego: Academic press, 1996, pp 32-8.

Herrick SE, Sloan P, McGurk M, Freak L, McCollum CN, Ferguson MW. Sequential changes in histologic pattern and extracellular matrix deposition during the healing of chronic venous ulcers. Am J Pathol 1992;**141**:1085-95.

Hoffman AS. Hydrogels for biomedical applications. Adv Drug Delivery Rev 2002;**43**:3-12.

Hornsey VS, Prowse CV, Pepper DS. Reductive amination for solid phase coupling of protein. A practical alternative to cyanogens bromide. J Immunol Methods 1986;**93**:83-8.

Horvat S, Jakas A. Peptide and amino acid glycation: new insights into the Maillard reaction. J Pept Sci 2004;**10**:119-37.

Hu M, Sabelman EE, Cao Y, Chang J, Hentz VR. Three- dimensional hyaluronic acid grafts promote healing and reduce scar formation in skin incision wounds. J Biomed Mater Res B Appl Biomater 2003;**67**:586-92.

International Standardization Organization, "Biological evaluation of medical devices. Part 1. Evaluation and testing," ISO10993-1, 1997.

International Standardization Organization, "Biological evaluation of medical devices. Part 5. Tests for Cytotoxicity: *In vitro* methods," ISO10993-5, 1992.

International Standardization Organization, "Biological evaluation of medical devices. Part 10. Tests for irritation and delayed type hypersensitivity," ISO10993-10, 2002.

Iwasaki T, Chen JD, Kim JP, Wynn KC, Woodley DT. Dibutyryl cyclic AMP modulates keratinocyte migration without alteration of integrin expression. J Invest Dermatol 1994;**102**:891-7.

Jackson DS. Development of fibrosis. Cell proliferation and collagen biosynthesis. Ann Rheum Dis (Suppl) 1977;**36**:2-4.

Jackson EL, Hudson CS. Application of the cleavage type of oxidation by periodic acid to starch and cellulose. J Am Chem Soc 1937;**59**:994-1003.

Jansen JA, Anderson J, Schou JS. Boric acid single dose pharmokinetics after intravenous administration to man. Arch Toxicol 1984;**55**:64-7.

Jeong B, Bae YH, Lee DS, Kim SW. Biodegradable block copolymers as injectable drug -delivery systems. Nature 1997;**388**:860-2.

Jonas RA, Ziemer G, Schoen FJ, Britton L, Castaneda AR. A new sealant for knitted Dacron prostheses: minimally cross-linked gelatin. J Vasc Surg 1988;**7**:414-9.

Kane JB, Tomkins RG, Yarmush ML, Burke JF. Burn dressings. In: Ratner BD, Hoffman AS, Schoen FJ, Lemons JE, Editors, An Introduction to Materials in Medicine, Biomaterials Science. USA: Academic Press, 1996, pp.360-70.

Kang H-A, Jeon G-J, Lee M-Y, Yang J-W. Effectiveness test of alginate derived polymeric surfactants. J Chem Technol Biotechnol 2002;**77**:205-10.

Kato K, Terao S, Shimamoto N, Hirata M. Studies on scavengers of active oxygen species. 1. Synthesis and biological activity of 2-O-alkyl ascorbic acids. J Med Chem 1988;**31**:793-8.

Kelly GS. Boron: A Review of its Nutritional Interactions and Therapeutic Uses. Alt Med Rev 1997;**2**:48-56.

Kichöfen B, Wokalek H, Scheel D, Ruh H. Chemical and physical properties of a hydrogel wound dressing. Biomaterials 1986;**7**:67-72.

Kim H-J, Choi E-Y, Oh J-S, Lee H-C, Park S-S, Cho C-S. Possibility of wound dressing using poly(L-leucine)/poly(ethylene glycol)/poly(L-leucine) triblock copolymer. Biomaterials 2000;**21**:131-41.

Kim H-J, Lee H-C, Oh J-S, Shin B-A, Oh C-S, Park R-D, Yang K-S, Cho C-S. Polyelectrolyte complex of chitosan and sodium alginate for wound dressing application. J Biomater Sci Polymer Edn 1999a;**10**:543-56.

Kim SH, Won CY, Chu CC. Synthesis and characterisation of dextran-maleic acid based hydrogel. J Biomed Mater Res 1999b;**46**:160-70.

Kirker KR, Luo Y, Nielson JH, Shelby J, Prestwich GD. Glycosaminoglycan hydrogel films as biointeractive dressings for wound healing. Biomaterials 2002;**23**:3661-71.

Kirsner RS, Eaglstein WH. The wound healing process. Dermatol Clin 1993;**11**:629-40.

Kiyohara Y, Nishiguchi K, Komada F, Iwakawa S, Hirai M, Okumura K. Cytoprotective effects of epidermal growth factor (EGF) ointment containing nafamostat, a protease inhibitor, on tissue damage at burn sites in rats. Biol Pharm Bull 1993;**16**:1146-9.

Kuijpers AJ, Engbers GHM, Krijgsveld J, Zaat SA, Dankert J, Feijen J Crosslinking and characterisation of gelatin matrices for biomedical applications. J Biomater Sci Polymer Ed 2000;**11**:225-43.

Kuroyanagi Y, Kim E, Kenmochi M, Ui K, Kageyama H, Nakamura M, Takeda A, Shioya N. A silver sulphadiazine-impregnated synthetic wound dressing composed of poly-L-leucine sponge matrix: An evaluation of clinical cases. J Appl Biomater 1992;**3**:153-61.

Kuroyanagi Y, Koganei Y, Shioya N. Supression of bacterial growth by a new temporary wound dressing containing antibacterial antibacterial drug. Jpn J Burn Inj 1987;**13**:206-12.

Lacroix H, Boel K, Nevelsteen A, Suy R. Early inflammatory response to gelatin- and collagen-sealed Dacron prostheses. Ann Vasc Surg 1995;**9**:152-4.

Lahiji A, Sohrabi A, Hungerford DS, Frondoza CG. Chitosan supports the expression of extracellular matrix proteins in human osteoblasts and chondrocytes. J Biomed Mater Res 2000;**51**:586-95.

Lamke LO, Nilsson GE, Reithner HL. The evaporative water loss from burns and the water permeability of grafts and artificial membranes used in the treatment of burns. Burns 1977;**3**:159-65.

Langer R, Vacanti JP. Tissue engineering. Science 1993;**260**:920-6.

Lansdown ABG, Payne MJ. An evaluation of the local reaction and biodegradation of calcium sodium alginate (Kaltostat) following subcutaneous implantation in rat. J R Coll Surg Edinb 1994;**39**:284-8.

Leaper DJ. Prophylactic and therapeutic role of antibiotics in wound care. Am J Surg 1994;**167**:15S-20S.

Lee JM, Edwards HL, Pereira CA, Samii SI. Cross-linking of tissue derived biomaterials in 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC). J Mater Sci Mater Med 1996;**7**:531-5.

Lee KY, Bouhadir KH, Mooney DJ. Degradation behaviour of covalently cross-linked poly (aldehyde guluronate) hydrogels. Macromolecules 2000;**33**:97-101.

Lee KY, Mooney DJ. Hydrogels for tissue engineering. Chem Rev 2001;**101**:1869-79.

Lee SB, Jeon HW, Lee YW, Lee YM, Song KW, Park MH, Nam YS, Ahn HC. Bio-artificial skin composed of gelatin and (1—>3), (1—>6)-beta-glucan. Biomaterials 2003;**24**:2503-11.

Lesieur S, Grabielle-Madelmont C, Paternostre M, Ollivon M. Study of size distribution and stability of liposomes by high performance gel exclusion chromatography. Chem Phys Lipids 1993;**64**:57-82.

Levenson SM, Geever EF, Crowley LV, Oates JF 3rd, Berard CW, Rosen H. The healing of rat skin wounds. Ann Surg 1965;**161**:293-308.

Lin PH, Hirko MK, von Fraunhofer JA, Griesler HP. In: Chu CC, von Fraunhofer JA, Greisler HP, Editors, Wound Healing and Inflammatory Response to Biomaterials. Boca Raton: CRC Press, 1997, pp.7-19.

Lin S-Y, Chen K-S, Liang R-C. Design and evaluation of drug-loaded wound dressing having thermoresponsive, adhesive, absorptive and easy peeling properties. Biomaterials 2001;**22**:2999-3004.

Loke W-K, Lau S-K, Yong LL, Knor E, Sum CK. Wound dressing with sustained antimicrobial capability. J Biomed Mater Res Appl Biomater 2000;**53**:8-17.

Lou Y, Groves MJ. The use of gelatin microparticles to delay the release of readily water-soluble materials. J Pharm Pharmacol 1995;**47**:97-9.

Lovekamp J, Vyavahare N. Periodate-mediated glycosaminoglycan stabilization in bioprosthetic heart valves. J Biomed Mater Res 2001;**56**:478-86.

Lowik CWGM, Alblas MJ, van de Ruit M, Papapoulos SE, van der Pluijm G. Quantification of adherent and nonadherent cells cultured in 96-well plates using the supra-vital stain neutral red. Anal Biochem 1993;**213**:426-33.

Lowman AM, Peppas NA. Hydrogels. In: Mathiowitz E, Editor, Encyclopedia of Controlled Drug Delivery. New York: Wiley, 2000, pp.397-417.

Mac Gregor EA, Greenwood CT. Polymers in Nature. New York: John Wiley & sons, 1980, pp. 142-3.

Magnusson B, Kligman AM. The identification of contact allergens by animal assay. The guinea pig maximization test. Invest Dermatol 1969;**52**:268-76.

Malaprade L. Oxidation of polyalcohols by periodic acid: application. Compt Rend 1928;**186**:382-4.

Mallapragada KS, Narasimhan B, Guest Editors. Special issue. Injectable Polymeric Biomaterials. Biomaterials 2002;**23**:4305-33.

Mark HF, Norman G, Gaylord NG, editors. Encylopedia of Polymer Science and Technology. Vol 7, New York: Interscience, 1967, pp.446-60.

Marois Y, Chakfe N, Deng X, Marois M, How T, King MW, Guidoin R. Carbodiimide cross-linked gelatin: a new coating for porous polyester arterial prostheses. Biomaterials 1995;**16**:1131-9.

Martinsen A, Skjak-Braek G, Smidsrod O. Alginate as immobilization material: 1. Correlation between chemical and physical properties of alginate gel beads. Biotechnol Bioeng 1989;**33**:79-87.

Matthew IR Brownie RM, Frame JW, Millar BM. Subperiosteal behaviour of alginate and cellulose wound dressing materials. Biomaterials 1995;**16**:265-74.

Mi FL, Wu YB, Shyu SS, Schoung JY, Huang YB, Tsai YH, Hao JY. Control of wound infections using a bilayer chitosan wound dressing with sustainable antibiotic delivery. J Biomed Mater Res 2002;**59**:438-49.

Mikos AG, Bao Y, Cima LG, Ingber DE, Vacanti JP, Langer R. Preparation of Poly(glycolic acid) bonded fiber structures for cell attachment and transplantation. J Biomed Mater Res 1993;**27**:183-9.

Miyata T, Taira T, Noishiki Y. Collagen engineering for biomaterial use. Clin Mater 1992;**9**:139-42.

Mo X, Iwata H, Matsuda S, Ikada Y. Soft tissue adhesive composed of modified gelatin and polysaccharides. J Biomater Sci Polymer Ed 2000;**11**:341-51.

Monafo WW, West MA. Current treatment recommendations for topical burn therapy. Drugs 1992;**40**:364-73.

Mooney DJ, Langer RS. Engineering biomaterials for tissue engineering: the 10-100 micron size scale. In: Bronzino JD, Editor, The Biomedical Engineering Handbook. Boca Raton: CRC press, 1995,1608-15.

Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;**65**:55-63.

Moulin V, Castilloux G, Auger FA, Garrel D, O'Connor-McCourt MD, Germain L. Modulated response to cytokines of human wound healing myofibroblasts compared to dermal fibroblasts. Exp Cell Res 1998;**238**:283-93.

Murch AR, Grounds MD, Marshall CA, Papadimitriou JM. Direct evidence that inflammatory multinucleate cells form by fusion. J Pathol 1982;**137**:177-80.

Naimark WA, Pereira CA, Tsang K, Lee JM. HMDC cross-linking of bovine pericardial tissue: a potential of the solvent environment in the design of bioprosthetic materials. J Mater Sci Mater Med 1995;**6**:235-41.

Nakamura M, Nishida T. Potentiation by cyclic AMP of the stimulatory effect of epidermal growth factor on corneal epithelial migration. Cornea 2003;**22**:355-8.

Narayani R, Rao PKJ. Controlled release of anticancer drug methotrexate from biodegradable gelatin microspheres. Microencapsulation 1994;**11**:69-77.

Nathan P, MacMillan BG, Holder IA. In situ production of a synthetic barrier dressing for burn wounds in rats. Infect and Immun 1975;**12**:257-60.

Neumann PM, Zur B, Ehrenreich Y. Gelatin-based sprayable foam as a skin substitute. J Biomed Mater Res 1981;**15**:9-18.

Nguyen KT, West JL. Photopolymerizable hydrogels for tissue engineering applications. Biomaterials 2002;**23**:4307-14.

O'Leary RK, Guess WL. Toxicological studies on certain medical grade plastics sterilized by ethylene oxide. J Pharm Sci 1969;**58**:1007.

Odell Ew, Oades P, Lombardi T. Symptomatic foreign body reaction to haemostatic alginate. Br J Oral Maxillofac Surg 1994;**32**:178-9.

Ono I, Gunji H, Zhang JZ, Maruyama K, Kaneko F. Studies on cytokines related to wound healing in donor site wound fluid. J Dermatol Sci 1995;**10**:241-5.

Ono I, Tateshita T, Inoue M. Effects of a collagen matrix containing basic fibroblast growth factor on wound contraction. J Biomed Mater Res 1999;**48**:621-30.

Ostgaard K, Larsen B. Substrate conversion and product inhibition of mannuronate lyase from Haliotis1. Carbohydr Res 1993;**246**:229-41.

Page M. Editor. Ultimate visual dictionary 2000. London: Dorling Kindersley, 2000, p. 235.

Painter T, Larsen B. Formation of hemiacetals between neighbouring hexuronic acid residues during the periodate oxidation of alginate. Acta Chem Scand 1970;**24**:813-33.

Park GB. Burn wound dressings-a review. Biomat Med Dev Art Org 1978;**6**:1-35.

Peacock EE Jr. Collagenolysis: The other side of the equation. World J Surg 1980;**4**:297-302.

Peppas NA, Barr-Howell BD. Characterization of cross-linked structures of hydrogels. In: Peppas NA, Editor, Hydrogels in Medicine and Pharmacy, Vol 1, Fundamentals. Boca Raton: CRC Press, 1986, pp. 27-57.

Peppas NA, Bues P, Leobandung W, Ichikawa H. Hydrogels in pharmaceutical formulations. Eur J Pharm Biopharm 2000;**50**:27-46.

Peppas NA, Langer R. New challenges in biomaterials. Science 1994;**263**:1715-20.

Peppas NA, Merril EW. Cross-linked poly(vinyl alcohol) hydrogels as swollen elastic networks. J Appl Polym Sci 1977;**21**:1763-70.

Peppas NA, Merril EW. Poly(vinyl alcohol) hydrogels-Reinforcement of radiation cross-linking networks by crystallization. J Polym Sci Part A-Polym Chem 1976;**14**:441-57.

Peppas NA. Hydrogels in Medicine and Pharmacy. Vols II-III, Boca Raton: CRC Press, 1987.

Pessa ME, Kirby KI, Copeland EM. Growth factors and determinants of wound repair, J Surg Res 1987;**42**:207-17.

Peterson JK, Kroggsgaard J, Nielson KM, Norgaadr EB. A comparison between 2 absorbable hemostatic agents: gelatin sponge (Spongostan) and oxidized regenerated cellulose. Int J Oral Surg 1984;**13**:406-10.

Petite H, Frei U, Huc A, Herbage D. Use of diphenyl phosphorylazide for collagen based biomaterials. J Biomed Mater Res 1994;**28**:159-65.

Prestwich GD, Marecak DM, Marecak JF, Vercruysse KP, Ziebell MR. Controlled chemical modification of hyaluronic acid. J Controlled Release 1998;**53**:93-103.

Price CC, Knell M. The kinetics of the periodate oxidation of 1,2-glycols II. ethylene glycols, pinacol and cis-and trans-cyclohexene glycols. J Am Chem Soc 1942;**64**:552-4.

Price CI, Horton JW, Baxter CR. Topical liposomal delivery of antibiotics in soft tissue infection. J Surg Res 1990;**49**:174-8.

Purna SK, Babu M. Collagen based dressings-a review. Burns 2000;**26**:54-62.

Queen D, Gaylor JDS, Evans JH, Courtney JM, Reid WH. The preclinical evaluation of the water vapour transmission rate through burn wound dressings. Biomaterials 1987;**8**:367-71.**(a)**

Quinn KJ, Courtney JM, Evans JH, Gaylor JDS. Principles of burn dressings. Biomaterials 1985;**6**:369-77.

Rao PKJ. Recent developments of collagen-based materials for medical applications and drug delivery systems. J Biomater Sci Polym Edn 1995;**7**:623-7.

Ratner BD, Hoffman AS. Synthetic hydrogels for biomedical applications, In: Andrade JD. Editors. Hydrogels for Medical and Related Applications. ACS Symp Series, No. 31, Washington Dc: ACS, 1976, pp.1-36.

Rees DA, Welsh EJ. Secondary and tertiary structure of polysaccharides in solution and gells. Agnew Chem Int Ed Engl 1977;**16**:214-24.

Ricardo A, Carrigan MA, Olacott AN, Benner SA. Borate minerals stabilize ribose. Science 2004;**303**:196.

Robb EC, Nathan P. Control of experimental burn wound infections: comparative delivery of the antimicrobial agent (silver sulphadiazine) either from a cream base or from a solid synthetic dressing. J Trauma 1981;**21**:889-93.

Roberts AB, Sporn MB, Assoian RK, Smith JM, Roche NS, Wakefield LM. Transforming growth factor type: Rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation *in vitro*. Proc Natl Acad Sci USA 1986;**83**:4167-71.

Rosdy M, Clauss LC. Cytotoxicity testing of wound dressings using normal human keratinocytes in culture. J Biomed Mater Res 1990;**24**;363-77.

Sayman DG, Nathan P, Holder IA. Control of experimental burn wound infection: Skin versus synthetic grafts. Appl Microbiol 1973;**25**:921-34.

Schilling JA. Wound healing. Surg Clin North Am 1976;**56**:859-74.

Schmidt RJ, Chung LY, Andrews AM, Spyratou O, Turner TD. Biocompatibility of wound management products- a study of the effects of various polysaccharides on murine L929 fibroblast proliferation and macrophage respiratory burst. J Pharm Sci 1993;**45**:508-13.

Schmolka IR. Artificial skin. 1. Preparation and properties of Pluronic F-127 gels for the treatment of burns. J Biomed Mater Res 1972;**6**:571-82.

Scott JE, Harbinson RJ. Periodate oxidation of acid polysaccharides 11. Rates of oxidation of uronic acids in polyuronides and acid mucopolysaccharides. Histochemie 1969;**19**:155-61.

Scott JE, Tigwell MJ. On the mechanism of scission of alginate chains by periodate. Carbohydr Res 1976;**47**:105-17.

Scott JE, Tigwell MJ. Periodate-induced viscosity decreases in aqueous solutions of acetal-and ether-linked polymers. Carbohydr Res 1973;**28**:53-9.

Segal HC, Hunt BJ. The effects of alginate and non-alginate wound dressings on blood coagulation and platelet activation. J Biomater Appl 1998;**12**:249-57.

Seglen PO. Preparation of isolated rat liver cells. Methods Cell Biol 1976;**13**:29-83.

Seppa HEJ, Grotendorst Gr, Seppa S I, Schiffman, E, Martin GR. The platelet-derived growth factor is a chemoattractant for vascular smooth muscle cells. J Cell Biol 1982;**92**:584-8.

Shalaby WSW, Park K. Chemical modification of proteins and polysaccharides and its effect on enzyme-catalyzed degradation. In: Shalaby SW, Editor. Biomedical Polymers: Designed to Degrade Systems. Munich: Hanser Publishers, 1994, pp.213-58.

Shapiro L, Cohen S. Novel alginate sponges for cell culture and transplantation. Biomaterials 1997;**18**:583-90.

Shibata H, Shioya N, Kuroyanagi Y. Development of new wound dressing composed of spongy collagen sheet containing dibutyryl cyclic AMP. J Biomater Sci Polym Ed 1997;**8**:601-21.

Shoichet MS, Li RH, White ML, Winn SR. Stability of hydrogels used in cell encapsulation: An in vitro comparison of alginate and agarose. Biotechnol Bioeng 1996;**50**:374-81.

Singh M, Ray AR, Vasudevan P. Biodegradation studies on periodate oxidized cellulose. Biomaterials 1982;**3**:16-20.

Skalak R, Fox CF. Editors, Tissue engineering. New York: Alan R. Liss, Inc., 1988.

Skjak-Braek G, Anthonsen T, Sandford P, Editors. Chitin and Chitosan: Sources, Chemistry, Biochemistry, Physical Properties and Applications. London: Elsevier Applied Science, 1992.

Smidsrod O, Haug A, Larsen B. The influence of pH on the rate of hydrolysis of acidic polysaccharide. Acta Chem Scand 1966;**20**:1026-34.

Smidsrod O, Painter T. Effect of periodate oxidation upon stiffness of the alginate molecule in solution. Carbohydr Res 1973;**26**:125-32.

Speer DP, Chavapil M, Eskelson CD, Ulreich J. Biological effects of residual glutaraldehyde in glutaraldehyde-tanned collagen biomaterials. J Biomed Mater Res 1980;**14**:753-64.

Stevenson TR, Mathes SJ. Wound healing. In: Miller TA, Rowlands BJ, Editors. Physiologic Basis of Modern Surgical Care. St. Louis, CV Mosby, 1988, pp. 1010-3.

Strauss G, Kral H. Borate complexes of amphotericin -B: polymeric species and aggregates in aqueous solutions. Biopolymers 1982;**21**:459-70

Suzuki S, Matsuda K, Isshiki N, Tamada Y, Ikada Y. Experimental study of a newly developed bilayer artificial skin. Biomaterials 1990;**11**:356-60.

Swaim SF, Riddell K, McGuire JA. Joint meeting of the wound healing society and the european tissue repair society. Amsterdam, The Netherlands, 1993.

Tabata Y, Ikada Y. Synthesis of gelatin microspheres containing interferon. Pharm Res 1989;**6**:422-7.

Takahashi H, Miyoshi T, Boki K. Study on hydrophilic properties of gelatin as a clinical wound dressing. II. Water absorbing property and haemostatic effect of gelatine. Tokushima J Exp Med 1993;**40**:169-75.

Tang Z, Yue Y. Crosslinkage of collagen by polyglycidyl ethers. ASAIO J 1995;**41**:71-6.

Thomas A, Harding KG, Moore K. Alginates from wound dressings activate human macrophages to secrete tumour necrosis factor-α. Biomaterials 2000;**21**:1797-802.

Tomihata K, Burczat K, Shiraki K, Ikada Y. Crosslinking and biodegradation of native and denatured collagen. In: Shalaby SW, Ikada Y, Langer RS, Williams J, Editors, Polymers of Biological and Biomedical Importance. ACS Symp Series, Vol.540, Washington DC: ACS, 1994.

Tu R, Shen S-H, Lin D, Hata C, Thyagarajan K, Noishiki Y, Quijano RC. Fixation of bioprosthetic tissues with monofunctional and multifunctional polyepoxy compounds. J Biomed Mater Res 1994;**28**:677-81.

Turner TD. Semiocclusive and Occlusive dressings. In: The Role of Occlusion, International Congress and Symposium Series. 88, London: Royal Society of Medicine, 1985.

Ulubayram K, Hasirci. N. Polymeric materials in wound healing. In: Hincal, Kas. Editor, Biomedical Science and Technology. New York: Plenum, 1998.

Ulubayram K, Nur Cakar A, Korkusuz P, Ertan C, Hasirci N. EGF containing gelatin-based wound dressings. Biomaterials 2001;**22**:1345-56.

Upman PJ, Anderson J, Tasse L. An analysis of ISO intracutaneous reactivity test results to justify a reduction in animal requirements. Lab Anim (NY). 2003;**32**:26-7.

Vandelli MA, Rivasi F, Guerra P, Forni F, Arletti R. Gelatin microspheres cross-linked with D, L-glyceraldehyde as a potential drug delivery system : preparation, characterisation, *in vitro* and *in vivo* studies. Int J Pharm 2001;**215**:175-80.

Vernon B, Gutowska A, Bae YH, Kim SW. Thermally reversible polymer gel for biohybrid artificial pancreas. J Macromol Chem Phys 1996;**109**:155- 67.

Weigel PH, Fuller GM, LeBoeuf RD. A model for the role of hyaluronic acid and fibrin in the early events during the inflammatory response and wound healing. J Theor Biol 1986;**119**:219-34.

Welz MM, Ofner CM. Examination of self-cross-linked gelatin as a hydrogel for controlled release systems. J Pharm Sci 1992;**81**:85-9.

Wiggins MJ, Wilkoff B, Anderson JM, Hiltner A. Biodegradation of polyether polyurethane inner insulation in bipolar pacemaker leads. J Biomed Mater Res 2001;**58**:302-7.

Winter GD, Formation of the scab and the rate of epithelialization of the superficial wounds in the skin of the young domestic pig. Nature 1962;**193**:293-4.

Winter GD, Scales JT. The effect of air drying and dressings on the surface of a wound. Nature 1963;**197**:91.

Winter GD. In: Maibach HI, Rovee DT, Editors. Epidermal Wound Healing. Year Book Medical Publishers: Chicago, 1972, p71.

Wiseman DM, Rovee DT and Alvarez OM, Wound dressings: design and use, In: Cohen IK, Diegelmann RF, Lindblad WJ, Editors, Wound Healing: Biochemical and Clinical aspects. Philadelphia: WB Saunders, 1992, pp.560-80.

Woodroof EA. Biobrane. A biosynthetic skin prosthesis. In: Wise DL, Editor, Burn Wound Coverings. Vol II, FL: CRC Press, 1984, pp.1-20.

Wu P, Fisher AC, Foo PP, Queen D, Gaylor JD. In vitro assessment of water vapour transmission of synthetic wound dressings. Biomaterials 1995;**16**:171-5.

Yannas IV, Burke JF. Design of an artificial skin 1. Basic design principles. J Biomed Mater Res 1980;**14**:65-81.

Yao CH, Sun JS, Lin FH, Liao CJ, Huang CW. Biological effects and cytotoxicity of tricalcium phosphate and formaldehyde cross-linked gelatin composite. Mater Chem Phys 1996;**45**:6-14.

Zhang X, Wyss UP, Pichora D, Goosen MFA. Biodegradable controlled antibiotic release devices for osteomyelitis: optimization of release properties. J Pharm Pharmacol 1994;**46**:718-24.

Zhao H, Heindel ND. Determination of degree of substitution of formyl groups in polyaldehyde dextran by the hydroxylamine hydrochloride method. Pharm Res 1991;**8**:400-2.

Zhao Q, Topham NS, Anderson JM, Hiltner A, Lodoen G, Payet R. Foreign body giant cells and polyurethane biostability: *in vivo* correlation of cell adhesion and surface cracking. J Biomed Mater Res 1991;**25**:177-183.

Zhou L-J, Ono I. Stimulatory effects of dibutyryl cyclic adenosine monophosphate on cytokine production by keratinocytes and fibroblasts. British Journal of Dermatology 2000;**143**:506-12.

WEB PAGES

Elder MA. C-077U. Market for advanced wound care technologies, Business Communications Company, Inc. Connecticut, USA, 2004. Available at URL: http:// www.bccresearch.com/biotech/C077U. html. Last accessed on 13.05.2005.

Health care management express, Indian Express Newspapers, Bombay, 2003. Available at URL: http://www.expresshealthcaremgmt.com/20030215/burns1.shtml. Last accessed on 13.06.2005

The Registry of Toxic Effects of Chemical Substances, National Institute for Occupational Safety and Health. Available at URL:http://www.cdc.gov/niosh/rtecs/vz26c1e0.html. Last accessed on 13.05.2005.

Thermal (heat or fire) burns. Available at URL: http://www.emedicinehealth.com/articles/4871-8.asp. Last accessed on 16.05.2005.

Appendix A

Composition of Buffers

Carbonate buffer (0.1 M, pH 9.4)

A.	Sodium carbonate	: 1.06 g
	Distilled water	: 100 mL
B.	Sodium bicarbonate	: 0.84 g
	Distilled water	: 100 mL

Add 4.4 mL of solution A to solution B. Check pH and adjust if necessary.

Tris buffer (0.1 M, pH 8.0)

Tris base	: 12 g
Sodium chloride	: 32 g
Potassium chloride	: 0.8 g
Distilled water	: 100 mL

Adjust pH with 1 M HCl.

Phosphate Buffered Saline (0.1 M, pH 7.4)

Disodium hydrogen phosphate	: 17.927 g
Monosodium hydrogen phosphate	: 5.73 g
Sodium chloride	: 9 g
Distilled water	: 1000 mL

Acid Citrate Dextrose (ACD)

Trisodium citrate	: 22g
Citric acid	: 8 g
Dextrose	: 25 g
Distilled water	: 1000 mL

Appendix B

List of Publications

Publications from the thesis work

Balakrishnan B, Lesieur S, Labarre D, Jayakrishnan A. Periodate Oxidation of Sodium Alginate in Water and in Ethanol/Water Mixture: A Comparative Study. Carbohydr Res 2005;**340**:1425-29.

Balakrishnan B, Jayakrishnan A. Self cross-linking biopolymers as injectable *in situ* forming biodegradable scaffolds. Biomaterials 2005;**26**:3940-51.

Balakrishnan B, Mohanty M, Umashanker PR, Jayakrishnan A. Evaluation of an *in situ* forming hydrogel wound dressing based on oxidized alginate and gelatin. Biomaterials (In press).

Balakrishnan B, Mohanty M, Fernandez AC, Mohanan PV, Jayakrishnan A Evaluation of the effect of incorporation of dibutyryl cyclic adenosine monophosphate in an in situ forming hydrogel wound dressing based on oxidized alginate and gelatin. Biomaterials (Communicated).

Author's other publications

Balakrishnan B, Kumar DS, Yoshida Y, Jayakrishnan A. Chemical modification of poly(vinyl chloride) resin using poly(ethylene glycol) to improve blood compatibility. Biomaterials 2005;**26**:3495-3502.

Balakrishnan B, James NR, Jayakrishnan A. Tween 20-modified poly(vinyl chloride) exhibits enhanced blood-compatibility. Polym Int (Published online, 19 May, 2005).

S. Dawlee, A. Sugandhi, Biji Balakrishnan, D. Labarre, and A. Jayakrishnan. Oxidized chondroitin sulfate-cross-linked gelatin matrixes: A new class of hydrogels. Biomacromolecules (Published online, April 14, 2005).

Patent

Jayakrishnan A, Balakrishnan B. Alginate Dialdehyde Crosslinked Gelatin As A Wound Dressing Material (Indian Patent pending).

Presentations

"Self cross-linking biopolymers as injectable *in situ* forming biodegradable scaffolds" at Macro 2004 organized by SPSI held at Mascot Hotel, 14-17 December, 2004.

"Self cross-linking biopolymers as injectable *in situ* forming biodegradable scaffolds" at MRSI meeting at RRL, Trivandrum on January 8, 2005.