# GOLD NANORODS FOR TARGETED PHOTODYNAMIC THERAPY AND FLUORESCENCE IMAGING: FINAL REPORT

1. Title of the Project: **Gold nanorods for targeted photodynamic therapy and fluorescence imaging**

2. Unique ID of the Project (provided by ICMR): **NO.5/13/128/2011/NCD-III**

3. Principal Investigator and Co-Investigators: **Dr.R.S.Jayasree , Scientist E**
**Dr.Sreenivasan. K, Scientist-G (Superannuated recently)**
**Dr. Kumary TV, Scientist-G**

4. Implementing Institution and other collaborating Institutions: **Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala-695 012**

5. Date of commencement: :**1/12/2013**

6. Duration :**3 years**

7. Date of completion :**31/05/2017**

8. Objectives as approved:
   - **Development of Gold nanorod photosensitiser conjugates and characterization.**
   - ***In vitro* PDT and imaging studies in cancerous and normal cell line.**
   - **Animal model development for subcutaneously implanted tumour**
   - ***In vivo* therapeutic and follow up imaging studies using the gold nanorods**

9. Deviation made from original objectives if any, while implementing the project and reasons thereof:
**No Deviation**

10. Field/ Experimental work giving full details of summary of methods adopted:
**Methods:**

**Preparation of gold naorod (GNR & GSH@GNR):**

Gold nanorods were synthesised using seed mediated procedure. Seed solution was prepared by treating 0.2mM $HAuCl_4$ with $NaBH_4$ as reducing agent and CTAB as stabilizing agent. Keep the solution in $20^0$c for 3hrs. The growth solution was prepared by treating 0.2mM $HAuCl_4$ with CTAB, AgNO3, ascorbic acid followed by 200ul of seed solution keep the solution for growth overnight at room temperature. After the completion of growth keep the solution in $4^0$c for 1hr, and then keep it in room temperature to settle down the excess CTAB. After removing the settled particle then centrifuged in 1200rpm for 20minutes and repeated for three times.

This rod was again subjected to ligand exchange reaction in ice cold condition. The centrifuged GNR was treated with 0.25M reduced glutathione by simple stirring in 500 rpm for 3hrs. Purification steps are same as in GNR. The so formed product is abbreviated as GSH@GNR.

**Preparation of photosensitizer/ Cell targeting system (P@GNR/F@PGNR complex):**

Protoporphyrin IX was treated with double the concentration of GSH@ GNR through EDC/NHS coupling. First 3mg/5ul of EDC was treated with protoprphyrin IX at pH 4 for 3hrs. After that 3mg/500µl of NHS was added stirred for 15 minutes then added the GSH@GNR and make the pH to above 9. Keep on stirring for 12hrs and then centrifuged with 14000 rpm speed. The obtained product was abbreviated as P@GNR.

P@GNR was again functionalised with folic acid through EDC/NHS coupling (Refer above).

**Characterisation:**

The GNR, GSH@GNR, P@GNR and F@GNR were characterized by Fourier Transform infrared spectrometer to confirm the functionalisation and chemical changes after functionalisation of GNR. Spectra were recorded in transmission mode using KBr method ranging from 400-4000 $cm^{-1}$over 32 scans. Zeta potential of each step was investigated using Malvern Zetasizer NanoZS 90 to investigate the change in charge with functionalisation. Surface morphology was confired using TEM analysis. UV-Visible study was done using Shimadzu UV spectrometer- UV 2600 to study the absorbance and from which to confirm the functionalisation of GNR. The emission property was studied using in Varian Carl Eclipse fluorescence spectrophotometer at different excitation wavelength. The fluorescence of synthesised material was checked using IVIS instrument. Also the fluorescence emission was confirmed using UV light irradiation. Fluorescence life time measurements were carried out using time-correlated single-photon counting (TCSPC) method with excitation wavelength of 455 nm using Fluoro Max-4C Spectrofluorometer (Horiba Instruments, USA) instrument.

***In Vitro* Cytotoxicity evaluation:**

L929 mouse fibroblast cells are used for the cytotoxic studies.  L929 cells were seeded in each well of 96 well plates at a density of 9000 cells per well and incubated for 12h. Thereafter the GNR, GSH@GNR, P@GNR and F@GNR were diluted using cell cultured medium to obtain 1 mg/ml, 0.5 mg/ml, 0.1 mg/ml, 0.05 mg/ml, 0.01 mg/ml, 0.005 mg/ml and 0.001 mg/ml concentrations. The existing medium was replaced with 100ul of fresh medium containing the materials of different concentration and incubated for 3 h. For the control group same amount of fresh medium without material. After the medium was replaced with fresh medium and then incubated for 46h. Typical MTT assay were carried out to obtain the cytotoxicity.

**Cellular Uptake of P@GNR and F@PGNR Complex Confocal Analysis**:

To examine the cellular internalisation of P@GNR and F@PGNR was performed with confocal investigation of cancer cells (MCF7). The cells were seeded on a cover glass with a density of 9000cells. After 24h incubation cells were washed with PBS and were Dappy stained and mounted on a confocal glass plate.

***In Vitro* Photothermal Treatment:**

MCF7 cells were used for the photothermal studies.  MCF7 cells were seeded in each well of 96 well plates at a density of 9000 cells per well and incubated for 12h. Thereafter the GNR,

GSH@GNR, P@GNR and F@GNR were diluted using cell cultured medium to obtain 1 mg/ml, 0.5 mg/ml, 0.1 mg/ml, 0.05 mg/ml, 0.01 mg/ml, 0.005 mg/ml and 0.001 mg/ml concentrations. The existing medium was replaced with 100ul of fresh medium containing the materials of different concentration and incubated 3 h. For the control group same amount of fresh medium without material. Then 808nm laser was irradiated for 50s with a laser power of 0.5W. Then MTT assay were carried out.

Cells treated with 1mg/ml solution after laser irradiation live dead assay was done Ethedium bromide and acridine orange. The procedure followed was 100 µL of both the dyes were mixed and added 200 µl for each well kept it for 2 minutes. Then Microscopic image were taken.

***In vivo* Photodynamic Treatment:**

MCF7 cells were used for the photothermal studies. MCF7 cells were seeded in each well of 96 well plates at a density of 9000 cells per well and incubated for 12h. Thereafter the GNR, GSH@GNR, P@GNR and F@GNR were diluted using cell cultured medium to obtain 1 mg/ml, 0.5 mg/ml, 0.1 mg/ml, 0.05 mg/ml, 0.01 mg/ml, 0.005 mg/ml and 0.001 mg/ml concentrations. The existing medium was replaced with 100ul of fresh medium containing the materials of different concentration and incubated 3 h. For the control group same amount of fresh medium without material. Then 530nm laser was irradiated for 40s with a laser power of 0.05W. Then MTT assay were carried out.

Cells treated with 1mg/ml solution after laser irradiation live dead assay was done Ethelium bromide and Acryline red. Microscopic image were taken.

***In vivo* Studies:**

All animal studies were approved by Institutional Animal Care and Use Committee. Balb/c 20g was used for the *in vivo* experiments. MCF7 cells were injected with subcutaneously into the hind flank of each mouse. After 2 week tumour growth animals were used for experiments.

For *in vivo* NIR fluorescence imaging, PDT and PTT the animals were divided into 3 (1 control and 2 test) different group having 6 animals each. The first group animals were injected with PBS were used as control. Second group were injected with 0.5 mg/ml of P@GNR were given and third group were injected with 0.5 mg/ml of F@PGNR in 500 µl PBS. After half an hour then the animals were imaged using IVIS Animal imaging system. Among them 1 from each group were sacralised and images were collected from excised organs. Rest of the animal 3 from each group were undergone treatment.

For PDT studies each animals (both control and test) were exposed to laser 530 nm for about 15 minutes and recorded the fluorescence spectrum. Repeated till tumor was disappears.

In case of PTT animals both test and control were exposed with laser 808 nm wavelength for 15 minute. Recorded the fluorescence spectrum and repeated till the tumor volume decreases.

11. Supported by necessary tables, charts, diagrams and photographs.

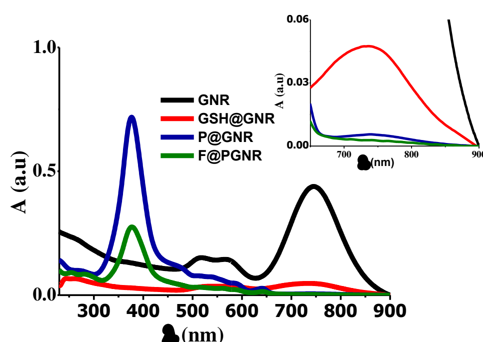


**Figure 1. Absorbance spectra of GNR, GSH@GNR, P@GNR and F@PGNR. Inset shows the absorbance of GSH@GNR, P@GNR and F@PGNR in the region 600-900 nm.**

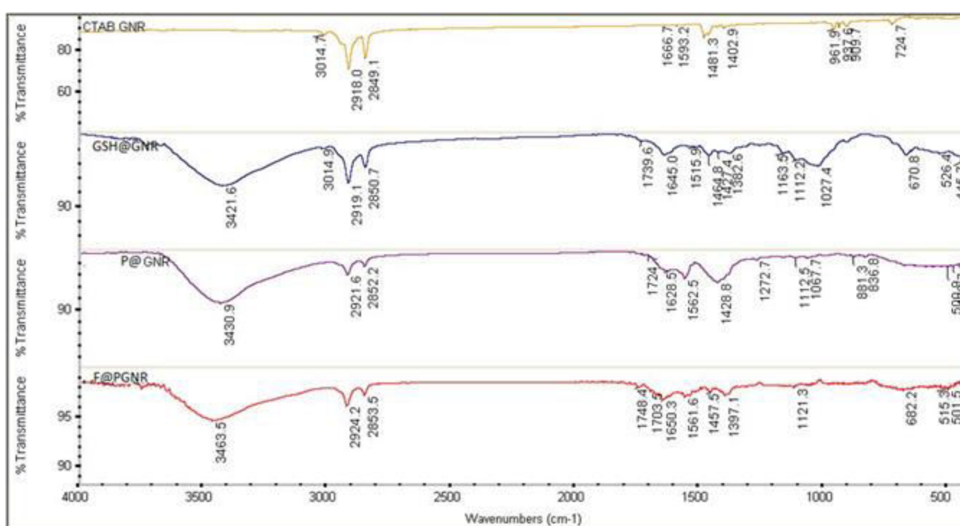


**Figure 2: FTIR Spectrum of GNR,GSH@GNR, P@GNR and F@PGNR.**

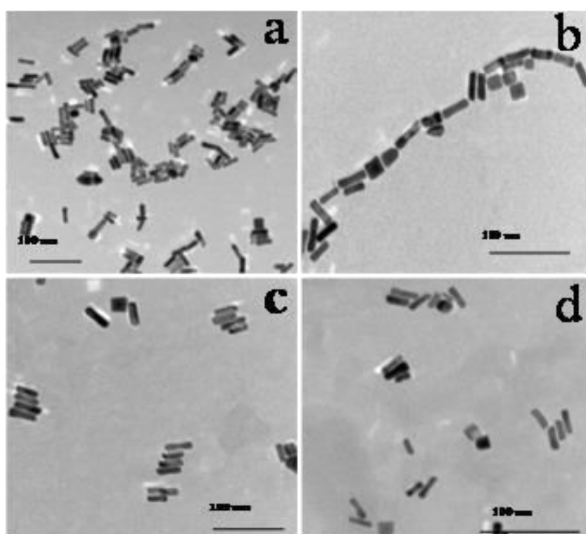


**Figure 3. TEM images of a) GNR b) GSH@GNR c) P@GNR and d) F@GNR.**

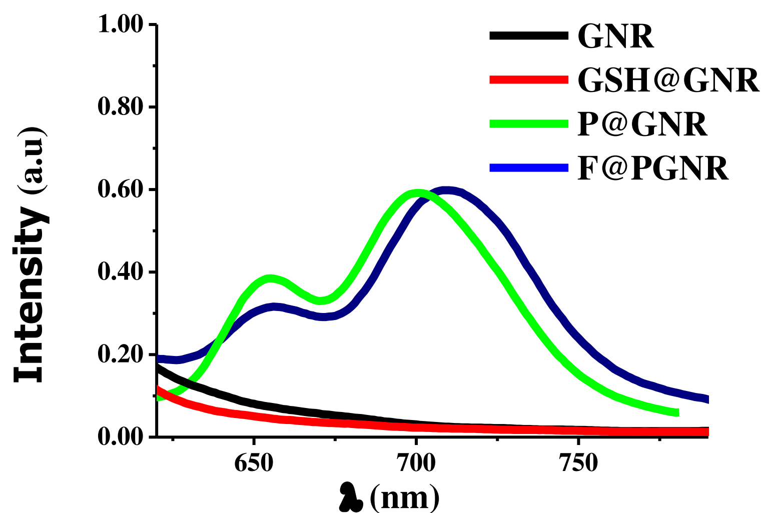


**Figure 4. Fluorescence spectra of GNR, GSH@GNR, P@GNR and F@PGNR.**

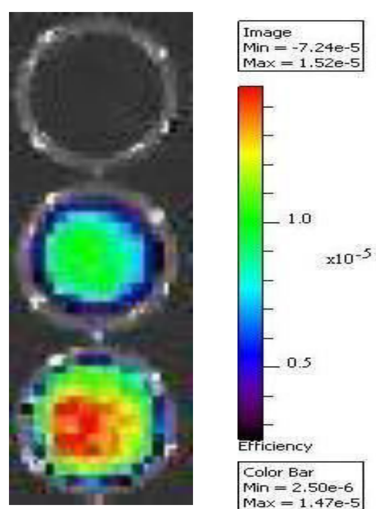


**Figure 5: Imaging property of water, P@GNR and F@PGNR.**

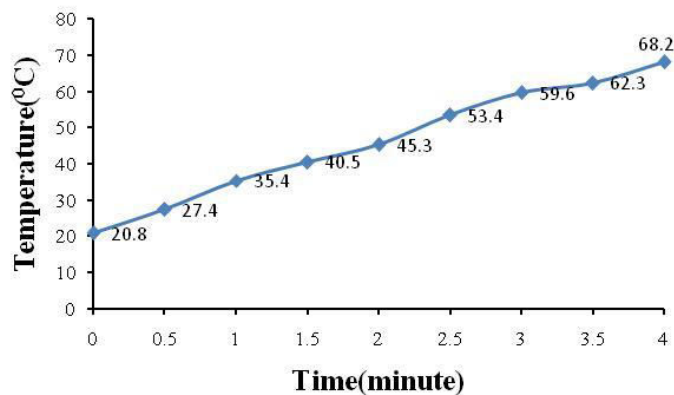


**Figure 6: Time dependant temperature increase of F@PGNR on exposure with 808 nm laser.**

**Figure 7: PDT, evaluation of singlet oxygen capacity of P@GNR, F@PGNR using PGNR as standard.**

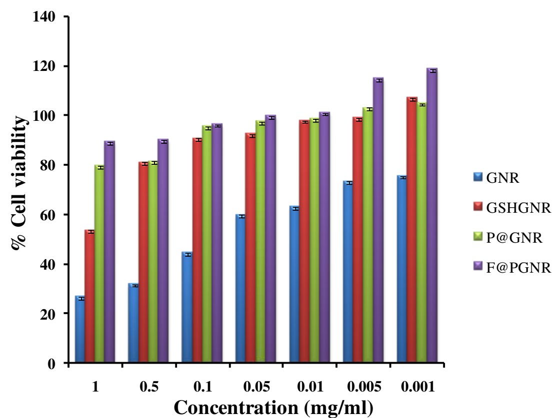


**Figure 8: Cytotoxicity study (MTT assay) of GNR, GSH@GNR, P@GNR and F@PGNR using L929 cell.**

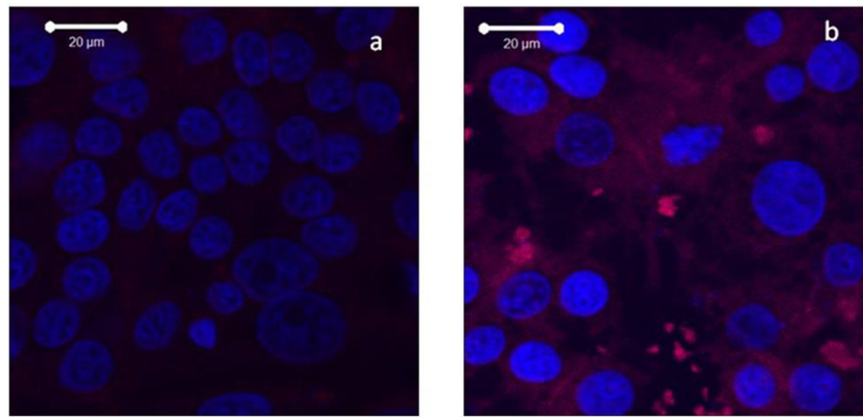


**Figure 9: Uptake of P@GNR and F@PGNR using MCF7**

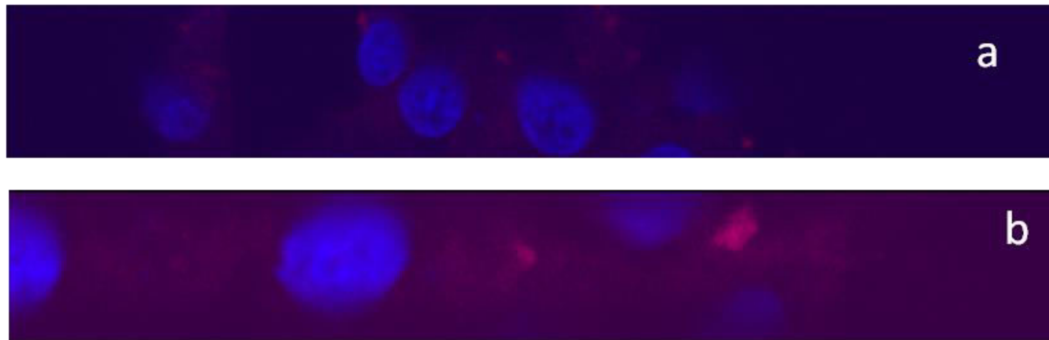


**Figure 10: Z-Stack image of cellular uptake of a) P@GNR and b)F@PGNR**

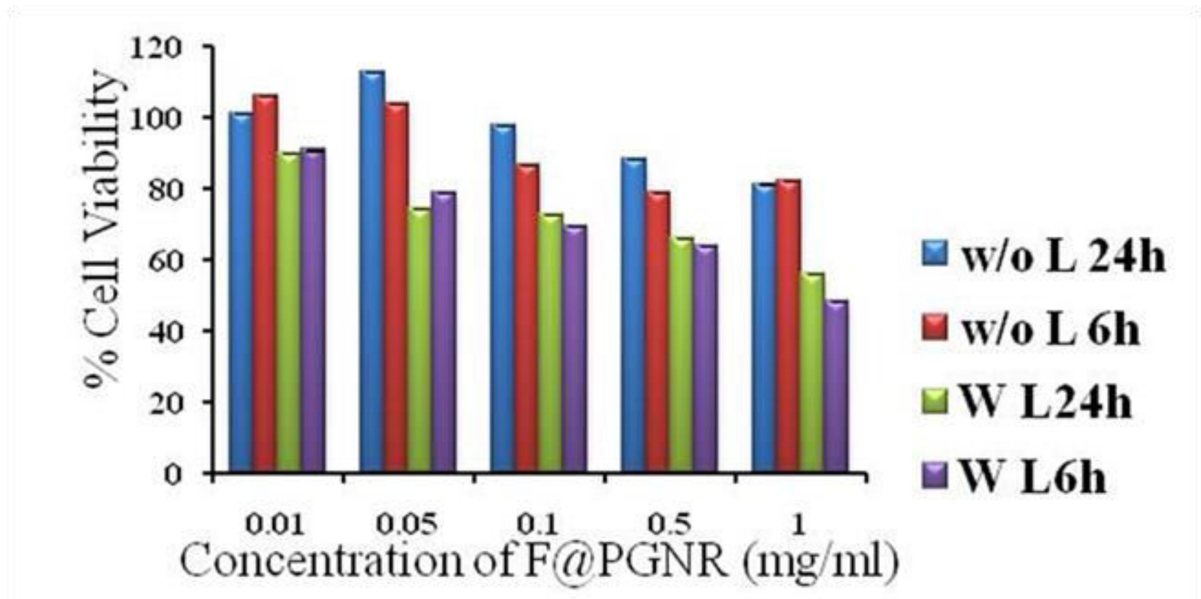


**Figure 11: Cellular viability different concentration of F@GNR With and without 808nm laser (PTT) using MCF7 cells at two different time period incubation**

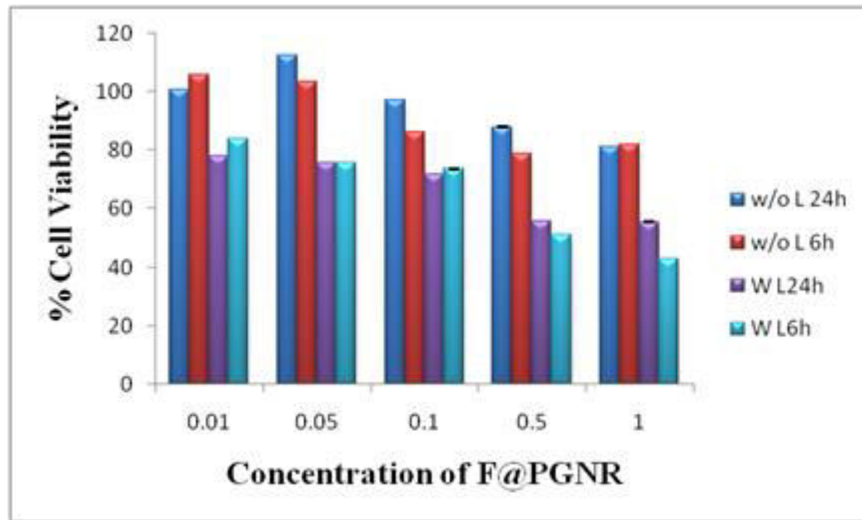


**Figure 12: Cellular viability different concentration of F@GNR With and without 530 nm laser (PDT) using MCF7 cells at two different time period incubation**

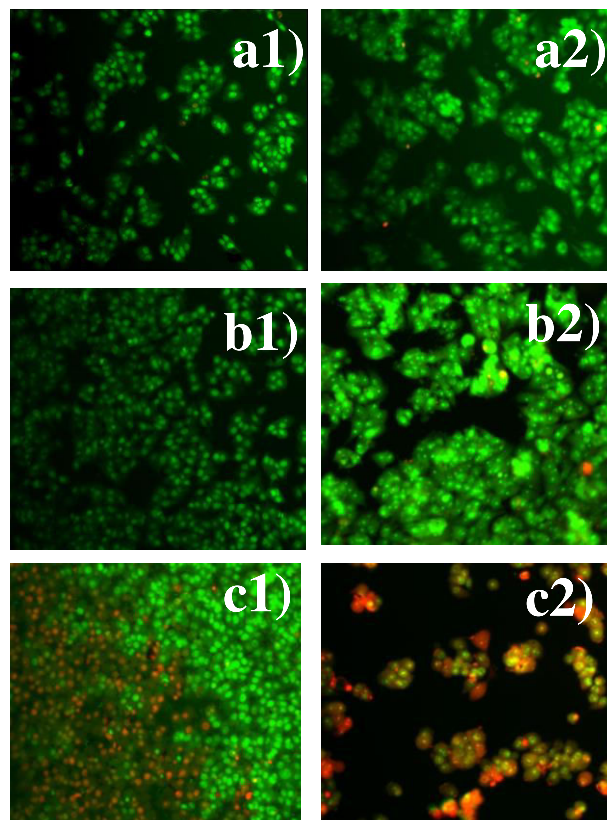


**Figure13: a1, b1,c1 PTT control, P@GNR, and F@PGNR , a2, b2, c2, PDT control, P@GNR, F@PGNR.**

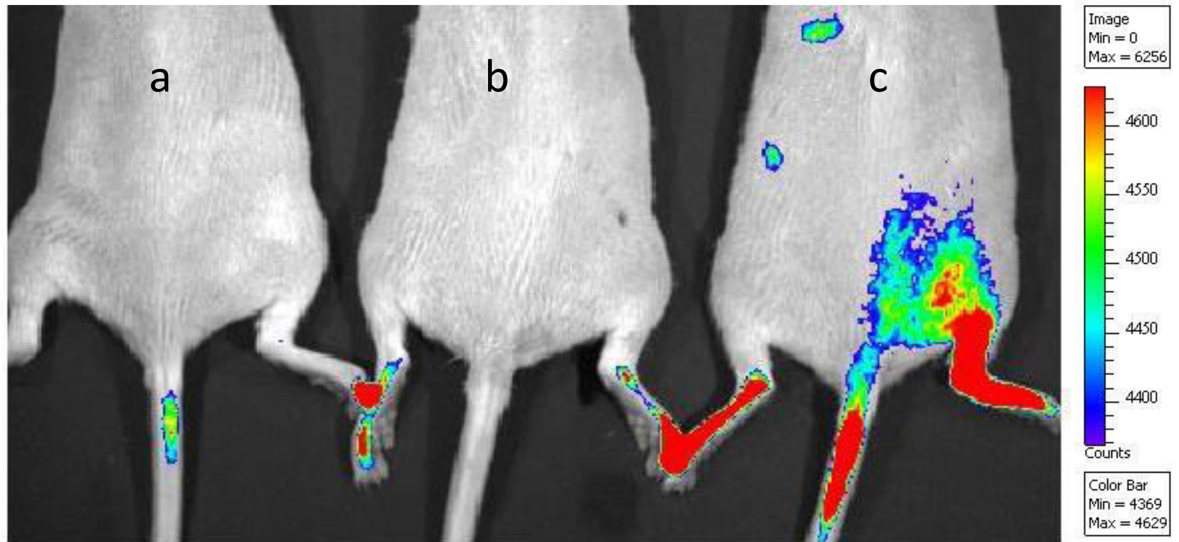


**Figure 14: In vivo imaging of a) control b) P@GNR and c) F@PGNR**

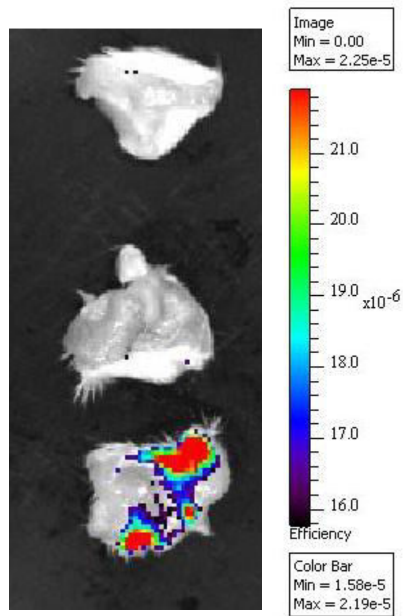


**Figure 15 : *Ex vivo* Image of Tumor**

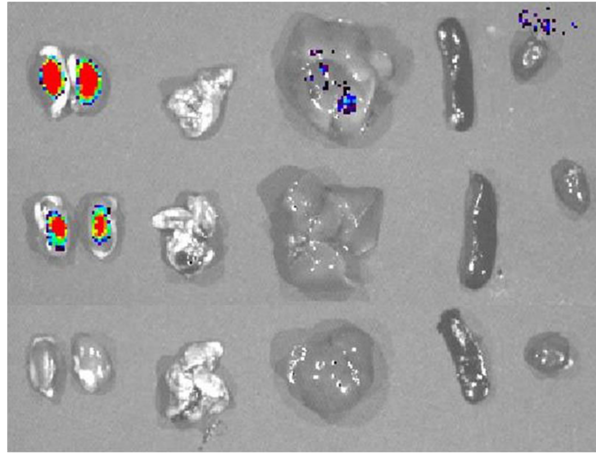

**Figure 16: Bio distribution of P@GNR and F@PGNR comparing with control important organs other than tumor.**

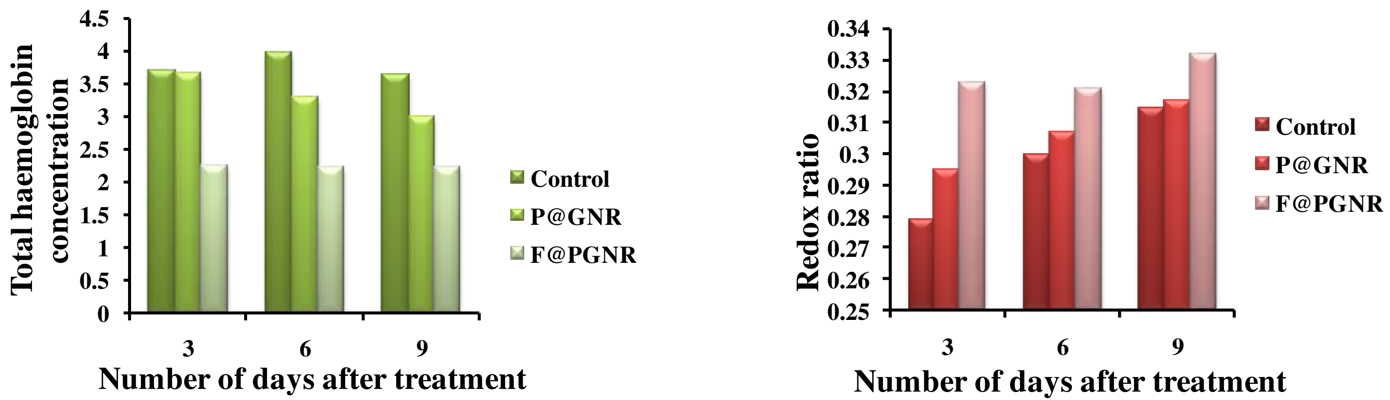


**Figure 17:** ***In vivo*** **photo thermal therapy efficiency by spectral analysis**

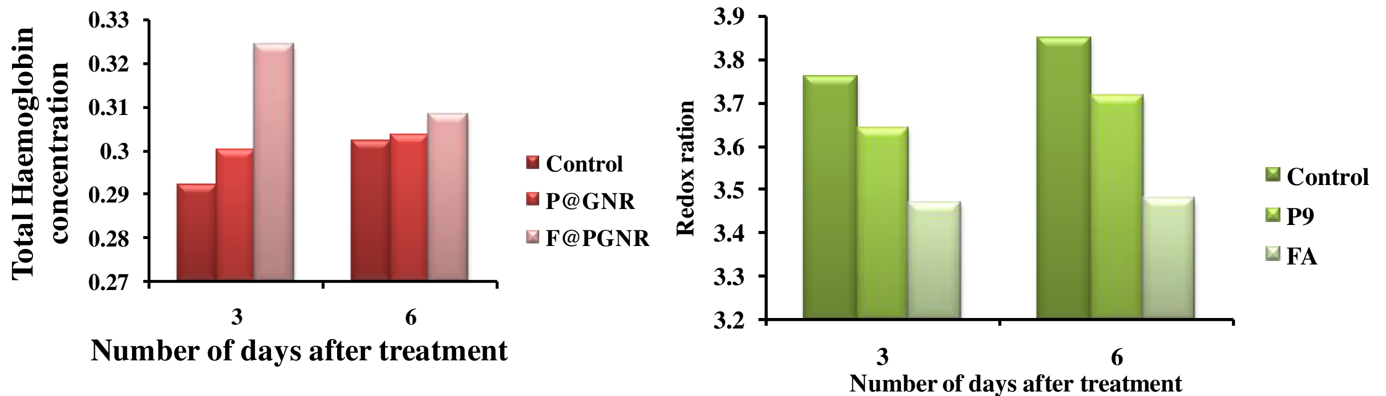


**Figure 18: In vivo photo dynamic therapy efficiency by spectral analysis**

**Table 1: Zeta Potentials of GNR, GSH@GNR, P@GNR and F@PGNR**

| Particle | Zeta potential |
|---|---|
| GNR | +16.2mV |
| GSH@GNR | +4.02mV |
| P@GNR | -35.5mV |
| F@PGNR | -43.4mV |

12. Detailed analysis of results:

GNR was synthesized with CTAB as capping agent using seed mediated method. For further studies and to increase biocompatibility, CTAB was replaced with reduced glutathione (GSH) to get GSH@GNR. GSH@GNR was further modified for additional properties like PDT and fluorescence emission using photosensitizer (PS), protoporphyrin IX. Porphyrins, due to its large conjugated aromatic system are reported to have excellent properties for surface functionalization. They also exhibit unique optical properties at the same time provide strong π–π interactions resulting controlled molecular self-assemblies. PS was covalently functionalized to GSH@GNR to get P@GNR. Finally the developed system was conjugated with a cancer targeting moiety, folic acid for cancer specificity. Here also the -$NH_2$ group of folic acid covalently binds to the -COOH groups of P@GNR.

The synthesized GNR has two characteristic plasmon absorptions (523&743 nm) due to transverse and longitudinal surface plasmon resonance (Figure 1). GSH@GNR retains both the plasmon peaks. After PPIX conjugation, P@GNR shows prominent bands characteristic of the same (376 nm) and with Q- bands along with characteristics peaks of GSH@GNR. Additional absorbance peaks c.a. 284 & 254 nm is observed on folic acid conjugation in F@PGNR.

The functionalization at different stages of preparation was confirmed by using suitable analytical techniques. The initial system, GNR showed the characteristic vibrational peaks of trimethyl ammonium ion in the range 961, 936, 1484 $cm^{-1}$ in the FTIR spectrum confirming the stabilization by CTAB (Figure 2). Absence of these peaks is indicative of the complete replacement of CTAB by

reduced glutathione in GSH@GNR. The absence of thiol peak at 2524 $cm^{-1}$ indicates the formation of gold-thiol bond. Vibrations of the amine group of glutathione are observed as a strong broad peak around 3421 $cm^{-1}$. On functionalization with PPIX, the peak corresponding to the amine group of glutathione is retained in the range 3400 $cm^{-1}$. Formation of amide bond is clear from the 1272 $cm^{-1}$ peak. Presence of excess of carboxylic acid in the system gives rise to the 1724 $cm^{-1}$ peak. Similar observations are seen in the case of F@PGNR.

GNR morphology is clear from the TEM images (Figure 3a) whose aspect ratio was found as 3.3±0.3. Formation of GSH@GNR witnessed a linear assembly (Figure 3b) which is attributed to the formation of intermolecular hydrogen bonding between glutathione molecules at the long ends of the rods. The low molecular weight of reduced glutathione is expected to trigger end to end assembly of GNR. The terminal glutathione molecules are flexible and free to make hydrogen bond with another glutathione molecule of a second GNR. On conjugating with protoporphyrin IX, the linear assembly is broken (Figure 3c) and a peculiar lateral assembly is formed. This indicates the formation of oligomeric aggregates due to the covalent binding of activated COOH with the two NH2 moieties of GSH@GNR. PPIX cannot form stable bond at the short end of GNR due to the large size and hence it forms side by side assembly by breaking the weak bonds which was responsible for linear assembly in GSH@GNR. The higher molecular weight of PPIX also favors the lateral assembly of GNR. Finally, on folic acid conjugation, the system retains the same orientation with a partial breakage of lateral assembly. It may be due to the formation of amide bond between self assembled protoporphyrin IX and folic acid. Since the concentration of folic acid used was very less, breakage of the bonds is not complete and it follows the same lateral assembly with lesser number.

Fluorescence emission spectra of synthesized products were recorded (Figure 4). GNR and GSH@GNR did not show any characteristics emission in the NIR region. While on conjugation with PPIX it (P@GNR) shows emission at 703 and 660 nm. On further surface functionalization of P@GNR with folic acid, the emission of F@PGNR red shifts to 730 nm, which can be explained based on the change in the functionality over PPIX ring. The shift in the spectrum indicates the decrease in aggregation of PPIX in solution. The NIR emission exhibited by the final system makes it suitable for *in vivo* imaging application.

Considering the importance of zeta potential during different functionalization over GNR, zeta potential at each stages of functionalization was measured (Table 1). While GNR had a zeta potential of +16.2 mV, it has decreased to +4.02 mV for GSH@GNR, which could be due to the $NH_2$ and COOH groups of GSH. Further, conjugation with PPIX increased the zeta potential to -35.5 mV, due

to the presence of more –COOH groups. F@PGNR showed a zeta potential value of -43.4 mV attributing high stability to the final system.

The imaging capability of P@GNR and F@PGNR was evaluated using IVIS imaging system. In comparison with PBS, both P@GNR and F@PGNR showed high fluorescence signal with F@GNR showing maximum signal (Figure 5). Enhanced signal in the case of F@GNR may be due to the formation of covalent coupling of the folic acid to PPIX which result in breakage of lateral assembly. Further to exploit the therapeutic potential of the developed system, photothermal efficacy of F@PGNR was evaluated using 808 nm laser irradiation. The strong absorption of GNR in the NIR region could will facilitate thermal therapy applications. Heat generation and increase in temperature to the order of $60^0$C was achieved within 4 minutes of irradiation, at which temperature cancer cells are prone to cell death while undergoing photothermaltherapy (PTT) (Figure 6).

The singlet oxygen generation capacity of the system was also evaluated for PDT, to impart dual therapeutic approach to increase the efficiency of treatment. Compared to PPIX alone which is an FDA approved PS for PDT, P@GNR shows a singlet oxygen yield of 44.7% (Figure 7). The final system, F@PGNR showed an increase in the singlet oxygen generation to 67.56%. This may be due to the covalent coupling of folic acid over P@GNR which resulted in less aggregation of PPIX, which is evident from the red shift in emission spectrum of F@PGNR. The final system, F@PGNR possess all the desired properties like NIR emission, high thermal efficiency and singlet oxygen yield which is required for multifunctional applications like *in vivo* imaging and dual mode therapeutic applications.

Biocompatibility of the developed materials was checked in normal mouse fibroblast cell line (L929) using MTT assay (Figure 8). GNR is found to be less cytocompatible due to the presence of highly toxic CTAB over it. The toxicity is reduced substantially in GSH@GNR due to the replacement of CTAB with GSH. The final multifunctional system (F@PGNR) shows ~100% cell viability even at 1 mg/ml concentration for 48h incubation.

To prove the cellular uptake and targeted PTT and PDT at cellular level, breast cancer cell line, MCF7 was used. F@PGNR shows more uptakes (Figure 9A) after 3h of particle treatment in comparison with P@GNR (Figure 9B). This is due to the folate receptor mediated internalization of F@PGNR in comparison with P@GNR. From Z stacking images, it is evident that most of the particles are localized inside the cytoplasm with little uptake in the nucleus due to the non specific uptake of the nanoparticles by the nucleus (Figure 10).

Targeted PDT and PTT of P@GNR and F@GNR were also checked in MCF 7 cells by irradiating it with 530 nm and 808 nm lasers respectively. Concentration dependent toxicity of cancer cells was quantified by measuring the activity of live cells using mitochondrial reductase enzyme assay (Figure 11& 12). MCF7 cells treated with P@GNR and F@PGNR without laser irradiation showed no cell death. IC 50 values for PTT and PDT are found to be 1 mg/ml and 0.5 mg/ml respectively. The higher IC 50 value for PTT compared to that of PDT may be because PTT depends on the concentration of GNR present in the system. But in the case of PDT, it is the singlet oxygen generated by the system which determines the cell death. Even a lower concentration of PPIX produces a long chain of singlet oxygen generation.

PDT was confirmed using live-dead assay in MCF7 cells. After treating the cells with the particles for 3h, the washed cells were irradiated with 530 nm laser for 40 s with a power of 0.1 W. The Acridine orange & propedium iodide stained F@PGNR treated cells showed considerable cell death compared to P@GNR treated group (Figure 13-a2-c2). Live dead assay performed for the cells that has undergone PTT for 50 s after incubating the material F@PGNR also showed considerable cell death (Figure 13-a1-c1).

Followed by the proof of successful PDT and PTT efficiency *in vitro,* synthesised materials were evaluated *in vivo* in mouse tumor models. Initially the targeted imaging capacity of the material was evaluated by intravenously injecting 500 µl of 1mg/ml P@GNR and F@PGNR in two groups of animals and a third group of tumor bearing animals without any particle injection served as control. The imaging potential of the developed system for live animal imaging was facilitated using Xenogen IVIS animal imaging system. It is clear that fluorescence in the NIR region of the developed system is capable of imaging the tumor site using optical technique. It is also clear that the uptake of folic acid conjugated particle by the tumour cells is much higher compared to the animals injected with P@GNR (Figure 14). After 3h, animals were sacrificed and organs were imaged to see the bio-distribution which shows that the tumour mass shows maximum fluorescence (Fig 15 & Fig 16).

Subsequent to the imaging experiments, two separate groups of animals were subjected to *in vivo* PDT and PTT. Animals under the PDT groups were irradiated with 530 nm laser, where two separate groups which received P@GNR and F@PGNR injection were used. Tumour bearing animals without particle injection, but with 530 nm laser irradiation served as control. After successive evaluation using fluorescence spectral analysis, it was observed that F@PGNR treated animal showed a

considerable reduction in the tumor and showed properties similar to that of the normal animals within a short duration of 3 days (Figure 17), confirming the PDT efficacy.

Again, another set of animals in three groups were subjected to PTT with 808 nm laser irradiation with P@GNR and F@PGNR injected animals, separately. Tumour without particle treatment and with laser irradiation served as control. Tumour bearing mice without treatment served as normal. Here also, after successive evaluation using spectral analysis it was observed that F@PGNR treated animal showed a considerable reduction in tumor within 6 days (Figure 18).

13. Contributions made towards increasing the state of knowledge in the subject.

A nanosystem which can perform both diagnosis and therapeutic functions like PDT, PTT and imaging simultaneously have not been reported so far. We are hopeful that this system with some minor modifications could be tested for clinical trials after necessary regulatory clearance in future. In such case many of the limitations of chemotherapy like severe side effects could be avoided. The currently developed system has tumor targeting property and hence it will not influence normal cells adversely. The work also has significant contribution towards basic science research. In GNR based systems, the fluorophores loses its emission property when in the proximity of GNR and hence quenching takes place. However, in the present study, there is no fluorescence quenching; instead enhanced fluorescence is observed which is made use for the imaging application. Hence the completed project has contributed significantly to the basic science and applied science fields.

14. Conclusions summarizing the achievements and indication of scope for future work:

In conclusion, GNR-PS based system which retains its fluorescence, the inherent thermal property of GNR and singlet oxygen yield of PS was developed. For retaining the fluorescence emission of the photosensitizer, GNR was considered as the donor in the developed system by making use of its weak fluorescence emission property. The energy transfer from GNR to PS makes it suitable for imaging applications. Using this system *in vitro* and *in vivo* tumor imaging and therapy were successfully performed. In future such a system will be very useful for the evaluating of bio-distribution of drugs, imaging and therapy.

15. Science and Technology benefits accrued:

I. List of research publications with complete details:

1. Resmi V Nair, Lakshmi V Nair, Ramapurath S Jayasree, Ayyappanpillai Ajayaghosh, Luminescent Gold Nanorod based multifunctional FRET system for Bioimaging and Targeted cancer therapy *(Communicated).*
2. Lakshmi V Nair, Resmi. V Nair, Jayasree R.S. An insight into the optical properties of a sub nanosize glutathione stabilized gold cluster; Dalton Trans., , 45, 11286-11291,2016.
3. Lakshmi V. Nair, Shaiju S. Nazeer, R.S. Jayasree and A. Ajayaghosh. Fluorescence Imaging Assisted Photodynamic Therapy Using Photosensitizer-Linked Gold Quantum Clusters; ***ACS Nano*,** 9 (6), 5825–5832, 2015.
4. Resmi.V.Nair, R. S. Jayasree. Gold nanorod based Multifunctional system for *in vivo* cancer therapy, Indo-Australian Conference held at Chennai from 5th to 7th February, 2015. ( **Best Poster Award**)
5. Ms Resmi V Nair, R.S. Jayasree. A gold nanorod based imaging and photothermal therapy System, National Seminar on Photonics and its Applications, held at Department of Optoelectronics, University of Kerala during 9 to 11 December, 2015. (**Best Poster Award**).

II Manpower trained in the project:

| | | |
|---|---|---|
| a. | Research Scientists or Research Fellows | :1 |
| b. | No. of Ph.Ds produced | :1 |
| c. | Other Technical Personnel trained | :Nil |

III. Patents taken, if any : Nil

IV Products developed, if any. : Arrived at the proof of concept for further development

16. Abstract (300 words for possible publication in *ICMR Bulletin*).

Multifunctional nanostructures are emerging nanomaterials which have got wide interest in biomedical applications due to their potential use in multiple parallel applications especially in disease diagnosis and its treatment. Cancer is the second leading cause of death in most of the developed countries. The main imaging techniques like MRI, CT etc and treatment modalities like chemotherapy, radiation therapy etc suffers from a lot of limitations. Major challenge in the field of

disease diagnosis is the difficulty to identify the tumors at its early stage which results in a treatment delay. Non specific cancer therapy faces damage to normal cells. To address some of these problems, we aim to develop a multifunctional nanocarrier for early stage tumor diagnosis and its therapy. In the present study, we were developed a multifunctional gold nanorod (GNR) based system for targeted tumor imaging and multimodal therapy. And evaluated the efficacy of the probe *in vitro* and *in vivo*. A GNR based multifunctional nanocarrier containing a fluorescent molecule and a tumor targeting agent was developed (Fa@PGNR). Various physico- chemical and biological characterizations were carried out for *in vivo* imaging and therapy. Fa@PGNR having emission at 730 nm is found to have tumor targeting, imaging and photothermal properties. Due to the presence of photosensitizer protoporphyrin IX the system showed a significant photodynamic therapy also. Tumor specific uptake of the material helped in the targeted diagnosis and multimodal therapy. The *in vivo* potential of the developed system was also demonstrated and found to be effective for targeted imaging and multimodal therapy when evaluated in tumor bearing mice.

17. Procurement/usage of Equipment

a.

| S.No. | Name of Equipment | Make/ Model | Cost FE/₹ | Date of Installation | Utilisation rate % | Remarks regarding maintenance/breakdown |
|---|---|---|---|---|---|---|
| 1 | FTIR-Spectrometer | CARY 660 | 2,055,283.62 | 29.09.2016 | 75 | The instrument is under warranty and is working perfectly |

b. Suggestions for disposal of equipment: On completion of the project the instrument becomes the part of the institute and is regularly used by the PhD students and other scientists of the institute and neighbouring universities and colleges

Name and
signature with date

1.______________________________
(Principal
Investigator)

2. ______________________________

(Co-Investigator)

3. ______________________________

(Co-Investigator)