Scientific and Technical Completion Report (STPR)[1st year]

(R&D projects)

Section-A : Project Details

A1. **Project Title:** Blood brain barrier targeted Nanoconstructs for the diagnosis of brain diseases and the delivery of therapeutics into the brain.

A2. **DBT Sanction Order No. & Date:** BT/PR14763/NNT/28/961/2015
27/07/2016

A3. **Name of Principal Investigator:** Dr. Jayasree R S

Name of Co-PI/Co-Investigator: Nil

A4. **Institute:** Sree Chitra Thirunal Institute of Medical Sciences and Technology

A5. **Address with Contact Nos. (Landline & Mobile) & Email :**

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A6. **Total Cost: 1145200/-**

A7. **Duration:** 1 year

A8. **Approved Objectives of the Project:**

1. Synthesis of NIR emitting gold nanocluster (AuC) for brain imaging and its conjugation with BBB targeting molecule.
2. Tagging of therapeutic agents/drug molecule to these nanoclusters for therapeutic application.
3. *In vitro* BBB targeting and delivery of therapeutics into brain.
4. *In vivo* efficacy of the developed probe for brain imaging and therapy.

A9. **Specific Recommendations made by the Task Force (if any):** Nil

Section-B : Scientific and Technical Progress

B1. Progress made against the Approved Objectives, Targets & Timelines during the Reporting Period (1500-2500 words; 2500-3500 words for final report; data must be included in the form of 2-7 figures and/or tables).

Project was sanctioned to SCTIMST on 25/07/2016 wide sanction No. SAN No.102/IFD/SAN/1706/2016-2017 and the same was communicated to the PI by the order BT/PR14763/NNT/28/961/2015 for a period of 1 Year 0 Month at a total cost of Rs.1145200.00. A total amount of Rs. 1145200.00 out of which Rs. 345000.00 was released as non recurring on 26/09/16 and Rs. 803200.00 was released as recurring on 19.10.16. The SRF interview was held on 25/01/2017 by the panel of 3 scientists in the relevant field after advertising the post and the candidate selected was joined on 20/02/2017 at SCTIMST.

Technical Report

Introduction

Brain disorders are major concern of modern world in terms of economic liability and human suffering, with the increased number of aged population as a result of the long life expectancy. The main challenge in the treatment of many of the neurodegenerative diseases is the presence of a polarized layer of endothelial cells that comprises the blood–brain barrier (BBB) which precludes access of systemically administered diagnostic or therapeutic agents to the Central Nervous system (CNS). Currently more than 98% of all small molecules and 100% of large-molecule pharmaceutics do not cross the BBB unless it is disrupted or loosened.[1] Overcoming the BBB is therefore an important field of current research that seeks better treatment option for diseases of the central nervous system. Currently, targeted disruption of BBB is often employed using techniques like ultrasound for effective delivery of therapeutics.[2] But these techniques have serious implications because alterations in membrane integrity of BBB could lead to neuroinflammation and neurodegeneration.[3] BBB also restricts entry of imaging contrast agents to the brain which could enable early diagnosis of diseases and monitoring of the drug delivery to the brain. [4,5] So the delivery of neurotherapeutics and/or diagnostics to CNS is possible only at a later stage of the disease when the integrity of the barrier is compromised due to degeneration.

However, this tight junction allows selective transport of nutrients and oxygen essential for the brain through specific membrane transport proteins. These transport receptors also allow the transport of macromolecules into the brain [6-11] and may be explored for an effective delivery of imaging moiety or drug to the brain facilitating early diagnosis and effective therapy.

With this background, we aim to design a self fluorescing multifunctional probe for brain imaging and drug delivery. Gold quantum clusters, due to its inherent fluorescence emission and non toxicity is one of the promising candidates to quantify the NP-BBB interaction.

Methodology

Synthesis of GSH Stabilized Gold Quantum Clusters (GQC)

Gold Quantum Clusters (GQC) were synthesised in two steps method. Initially, gold nanoparticles were synthesised using mercaptosuccinic acid and chloroauric acid. Then GQC was synthesized by etching of gold nanoparticles with reduced glutathione (GSH). For tuning the emission property to NIR region, the reaction conditions were optimized and ultimately mercaptosuccinic acid conjugated gold nanoparticles (GMSA) were treated with GSH at 0^{o}C, for this. In a typical experiment, 2.8 mg GMSA at pH 1.5 and 12 mg GSH (at 0^{o}C) were dissolved in 30 ml MQ water. After 15 min this system was allowed to react at 70^{o}C at 500 rpm for 24 hrs. As the time progressed, the color of the solution changed from brown to yellow. The gold cluster (GQC) was collected by centrifugation at 3000 rpm for 10 minutes, to remove any GMSA. Supernatant was collected and precipitated in 1:1 methanol isopropanol mixture to remove the unreacted GSH from the cluster.

Conjugation of L-Dopa to GQC (Dopa@GQC)

For BBB targeting GQC was conjugated with L-dopa (L-3,4-dihydroxyphenylalanine). For this, 5 mg of synthesized GQC was dissolved in 5 ml DI water. 0.05 M 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) was added and pH adjusted to 4 and stirred for 2 h. N-Hydroxysuccinimide (NHS) (0.05 M) was added, followed by the addition of L-dopa in basic pH. The final pH of the solution was set at 9 and the reaction was continued for another 12 h. Dopa@GQC was centrifuged at 15000 rpm for 15 min in 1:1 methanol isopropanol mixture. The product was washed twice with DI water to remove excess of unreacted ligands.

Development i*n Vitro* BBB Model.

For the construction of BBB models, transwell inserts (millipore, 0.4 micron) were first placed onto a culture plate well, and DMEM growth medium was added to the basolateral side of each well until the membrane in each insert was completely moistened with the growth medium. Then bEnd.3 cells were seeded onto the inside of the insert above the membrane at a seeding density of 1×10^4 cells/well and cultured in DMEM growth medium with 10% fetal bovine serum. Cells were maintained at 37 °C and 5% CO_2 in a humidified incubator. In all the further study GQC and Dopa@GQC were used for the BBB experiments, both *in vitro* and *in vivo*

***In Vitro* Cytotoxicity Study**

In vitro cytotoxicity of GQC and Dopa@GQC was measured by MTT assay which measures the metabolic reduction of MTT reagent to colored formazan by viable cells. For determining the cell viability, normal cell line L929 mouse fibroblast cells were seeded in a 96-well tissue culture plate at 5 x103 cells/well in 100 µL MEM containing 10 % FBS. After achieving 40 - 50 % confluence, cell lines were exposed to various concentrations of nanoclusters (1 mg, 0.5 mg, 0.1 mg, 0.05 mg, 0.01 mg, 0.005 mg, 0.001 mg). After 72 h, the derivatives in the medium were removed and washed with phosphate buffered saline. To that 50 µL of MTT (2 mg/ml in MEM) and 200µL of media was added and incubated for 4h under 37oC and 5% CO2. Four hours later, MTT was removed and 100 µ L of isopropanol was added into each well. The absorbance of the resulting solution was recorded immediately at 570 nm using automated micro plate reader (BioTek Instruments, Vermont, USA). Results were expressed as absorbance after blank (i.e., cells without material) subtraction. Values reported are the mean of experiments done in triplicate.

% Cell Viability = ([Abs]sample/[Abs]Control) x 100.

Barrier Potential Measurement in b-End3 Cell

Brain barrier is reported to exhibit inherent potential which gets disturbed on loss of barrier integrity. Hence barrier potential measurement will give an idea on the integrity of the formed BBB. Barrier potential of the cells was measured using Milli Cell ERS (Millipore). Cells were cultured on a transwell membrane having 0.4 micron pore size (Millipore) by keeping it on a 12 well cell culture plate. Barrier potential was monitored daily on subsequent days. After 6th day the potential of the cells reached around 1548 ± 35 Ω. Cell material interaction was studied by seeding the probes on the 6th day. 100 µL

of the 1 mg/ ml of GQC and Dopa@GQC was added to each well and monitored the change in potential for 30 S, 1 h, 2 h, 3 h, 6 h and 24 h. Barrier potential was measured in triplicate for both the probes and control. Cell without material served as a control.

Barrier Permeability Measurement In b-End3 Cell

After addition of 100 µl of 1 mg of GQC and Dopa@GQC, media in the 12 well plates were collected after 1 h, 2 h, 3 h, 6 h and 24 h for assessing the barrier permeability of the probes. Amount of nanoparticles present in the collected media were estimated spectrometrically by drawing the calibration plot of known concentration of GQC and Dopa@GQC. Cells without material and the millicell insert without bEnd.3 cells served as a control.

***In Vitro* Celluar Uptake**

Cellular uptake of GQC and Dopa@GQC were monitored using fluorescence (Leica), and TEM (Jeol) microscopes. For this, cells were grown on a cover slip with a seeding density of 1x105 cells/ well. When the cells formed a complete monolayer, 1 mg/ mL of GQC and Dopa@GQC were added, in separate wells. After 3hrs, cells were washed with PBS.

For fluorescence microscopic study nucleus was stained with hoechst as per the manufactures instruction. Washed with PBS and fixed with 1% paraformaldehyde. Again washed with PBS and viewed under fluorescence microscope (Leica DMI 3000 B, Germany).

Blood Compatibility Study

Hemolysis

Blood samples were collected in anticoagulant [trisodium citrate (Sigma)] containing vials from healthy individuals. Blood was centrifuged at 3000 rpm for 3 min and washed four times in saline. 100 µl of RBC was collected and diluted to 1 ml and used for the studies. For hemolysis, different concentrations of GQC and Dopa@GQC (1 mg, 0.5 mg, o.1 mg, 0.05 mg, and 0.01 mg/ ml) were added. As control, same amount of water and saline were added to RBC. Percentage hemolysis was measured using the formula

$$\text{Percentage Hemolysis} = \frac{(\text{Absorbance of sample}) - \text{Absorbance of blank})}{\text{Absorbance of positive control}} \text{x } 100$$

RBC, WBC and Platelet Aggregation

To check the aggregation of RBC with respect to saline, 1 mg of GQC and Dopa@GQC were added to RBC in 6 well plate. The materials were incubated for 3 h. Aggregation was monitored using phase contrast microscope.

For the separation of WBC and platelet, histopaque (Sigma) was layered onto the anticoagulated blood and kept for 30 min for the formation of 3 layers. Top 2 layer contain WBC and Platelet. Both these layers were collected carefully and centrifuged at 1000 rpm for 10 min. 1 mg of the samples was added to WBC and platelet and aggregation was monitored under phase contrast microscope.

Results and discussion

Synthesised GQC and Dopa@GQC were characterised using different techniques. Figure 1a shows absorption spectra of GQC and Dopa@GQC. It shows distinct absorption features indicating quantum confinement in both cases. Figure 1b indicates emission spectra of GQC, L-Dopa and Dopa@GQC. L-Dopa conjugated GQC retains its NIR emission with an additional peak at 534 nm, due to the emission of L-dopa. Quantum yield of GQC and Dopa@GQC for the NIR emission are 0.24 and 0.013 respectively with respect to nileblue. The zeta potential at different pH indicates the stability of both GQC and Dopa@GQC (Figure 1e and f). Functionalization of L-Dopa over GQC is evident from DLS study and FT- IR spectroscopy (Figure 1g). Isoelectric point of 6.62 and 6.52 for L-Dopa and Dopa@GQC respectively are consistent with the amino acid composition of the later material. The occurrence of amide band at 1588 cm^{-1} and a shift in the C=O peak is also an indirect evidence for the functionalization of L-dopa onto the surface of GQC through an amide bond between GSH and L-dopa.

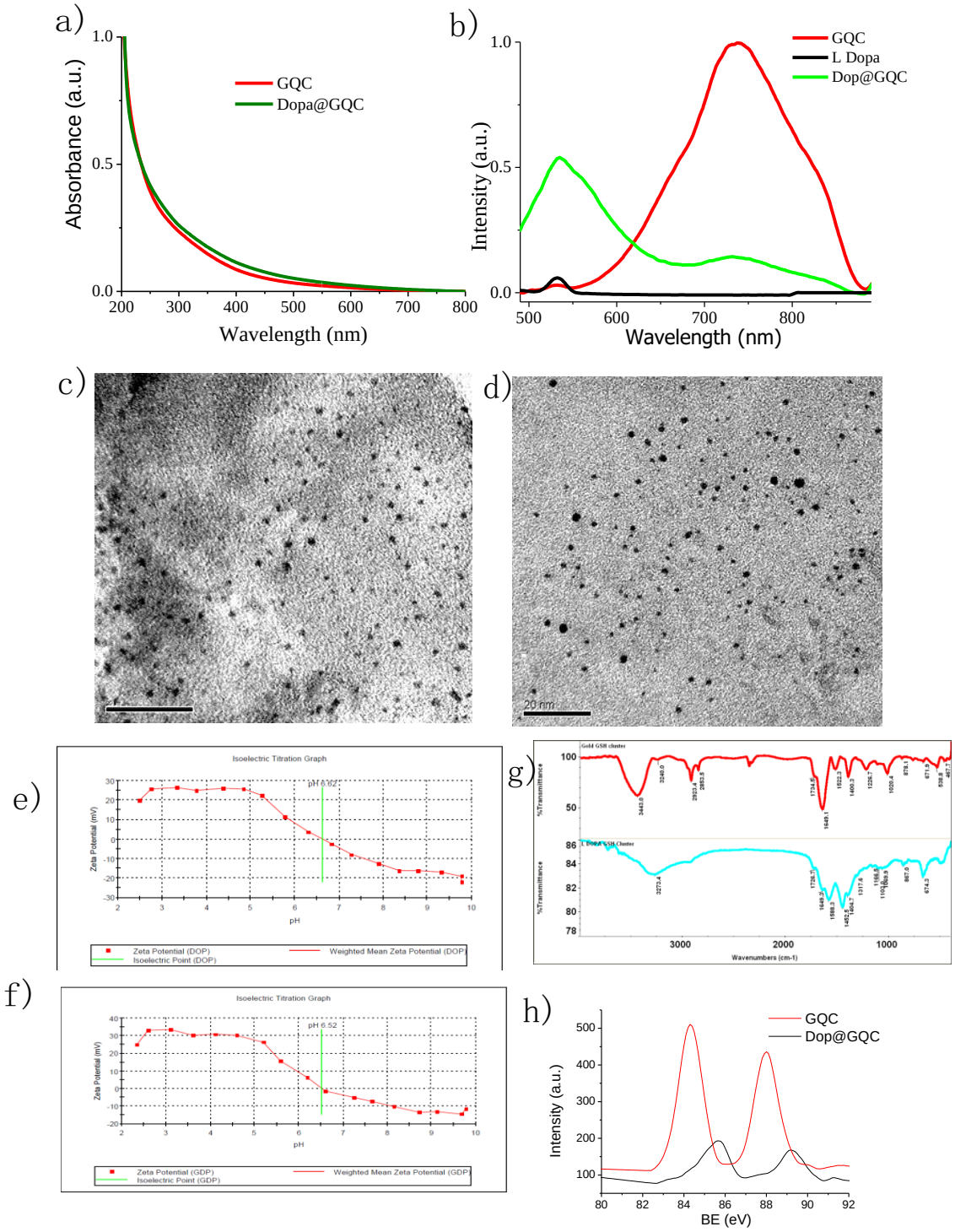

Figure 1. UV/ Vis absorbance spectra (a) and Photoluminescence spectra (b) of GQC and Dopa@GQC. TEM of (c) GQC and (d) Dopa@GQC. Scale bar in both cases are 20 nm. Isoelectric point measurement using zeta sizer (e) L- Dopa and (f) Dop@GQC. (g) FT-IR spectra of GQC(red) and Dopa@GQC (cyan). (h) XPS spectra of GQC and Dopa@GQC

Particle size of GQC and Dopa@GQC ranged from 0.7 to 1.4 nm respectively (Figure 1c and 1d). Binding energy evaluated using XPS study shows a difference of 1.61 eV and

1.41 eV respectively for the Au 4f7/2 and Au 4f5/2 states of GQC and Dopa@GQC (Figure 1h). This difference in the binding energy of Dopa@GQC is either due to the minor increase in the average particle size or due to the increase in the distance of Au core on binding with L-dopa. Increase in the particle size is due to the binding of the amine moiety of L-Dopa to the carboxylic acid present in GSH. This particular size will meet the particular study.

Cell Compatibility and *In vitro* BBB studies.

After successful characterisation before going to the in vivo studies in vitro studies are essential to prove the efficacy of particular system. Initially, cell compatibility of different concentrations of the two probes considered was proven by standard MTT assay using L929 mouse fibroblast cell lines (Figure 2a).

In order to prove the *in vitro* BBB permeability of the developed materials, brain endothelial cells of mouse origin (bEnd.3 from ATCC) were grown on milli cell insert with a pore size of 0.4 μm. Barrier potential of the cells was regularly monitored to ascertain the the monolayer formation and integrity so that it exactly mimics the BBB. Completely confluent monolayer with barrier potential of the order of 1400 Ω was considered as *in vitro* model for BBB. To this BBB model, 1 mg/ mL of GQC and Dopa@GQC was added, separately. On addition of GQC and Dopa@GQC, the original barrier potential dropped to 812 Ω and 982 Ω respectively within 30 min. Later, the potential of the cells regained almost completely within 2-3 h (Figure 2b). The concentration of the materials that crossed the barrier cells and passed out through the insert at different time interval was quantified using UV/ Vis absorption spectroscopy. At 3h, 44% of Dopa@GQC crossed the *in vitro* barrier and cleared out from the cells (Figure 2c) where as only 14% of GQC only has crossed and cleared out during this time. It took nearly 24 h for 56% of the material to cross and clear out in the case of GQC. More than 90% of Dopa@GQC crossed the *in vitro* barrier and cleared from the cell in 5h. From this result, it is inferred that the entry of Dopa@GQC through the barrier cells and its clearance is faster than that of GQC.

The cellular uptake efficacy of GQC and Dopa@GQC (1 mg/ mL) was again checked using fluorescence microscope at 3, 6, 12 and 24 h of incubation period (Figure 2d).The

NIR emission of gold cluster helps to visualize the particles under microscopy and gives an indication about the uptake at different time points. It is clear that more particles of GQC remain inside the cell compared to Dopa@GQC at 3h. At different time intervals, the uptake efficacies of both the materials are in good agreement with the permeability study. At 6 h, cells with GQC show intense emission of the gold cluster where as it is comparatively very less in the cells with Dopa@GQC confirming the finding of barrier permeability study, that Dopa@GQC clears from the cells quickly.

Based on these observations, we hypothesize that Dopa@GQC enters through a large amino acid transport pathway while GQC enters through a normal carrier mediated pathway by the activation of γ-glutamyl transpeptidase [12-15]. This hypothesis is based on the fact that presence of transporters on both luminal and abluminal side of the cells facilitate the quick intake and clearance of Dopa@GQC by the microvascular brain endothelial cells. In the case of GQC, non enzymatic degradation of GSH caused the delay in the intake and clearance. It can be concluded that the rates of transport of GQC and Dopa@GQC across the cells are different. The barrier integrity of the cells was checked and found that it was intact in the case of Dop@GQC treated cells whereas it was found to be disturbed and damaged in the case of GQC treated cells (Figure2e &f). This is an indication of the fact that the developed probe Dop@GQC can cross the barrier at very early stages of diseses when it is not damaged and could be used for the early diagnosis and treatment.

Percentage hemolysis, and RBC, WBC and platelet aggregation studies of the nanoprobes were evaluated and are found to be suitable for further *in vivo* evaluation (Figures 2g& h).

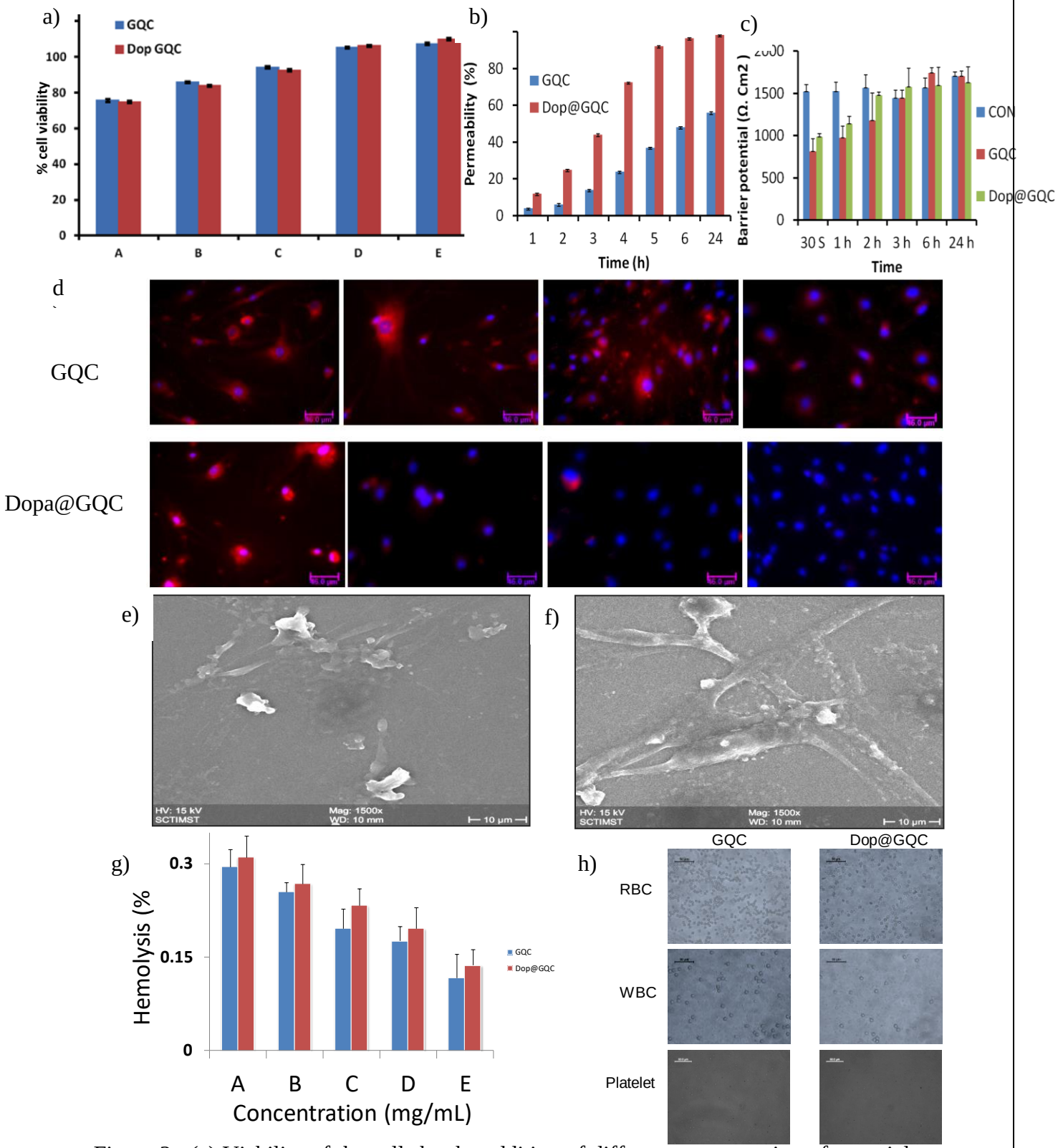

Figure 2. (a) Viability of the cells by the addition of different concentration of materials using MTT assay. A, B, C, D, and E represents 1, 0.5, 0.1, 0.05 and 0.01 mg/ mL of material respectively. (b) Barrier permeability and (c) barrier potential measurement in bEnd.3 cells on addition of 1 mg/ mL of GQC and Dopa@GQC at different time period. (d) Cellular uptake of the material for 3, 6, 12 and 24 h using fluorescence microscope. Bend3 cells with GQC for 3 h, 6 h, 12 h and 24h represents the same with Dop@GQC. SEM images of cells treated with (e) GQC and (h) Dop@GQC. (g) Percentage hemolysis of GQC and Dop@GQC. A, B, C, D, and E represents 1 mg, 0.5 mg, o.1 mg, 0.05 mg, and 0.01 mg/ mL of material respectively. (h) Response of blood cells towards the nanoparticles.

Cellular uptake Using TEM Ananlysis and animal study

The cellular uptake of cluster and L-dopa conjugated system were proved by using transmission electron studies (Figure 3). Further the systems were extended *to in vivo* imaging. Swiss Albino mice were used to demonstrate the *in vivo* imaging efficacy of gold clusters. The materials were administered in normal mice through tail vein and after 1h the animals were imaged using IVIS animal imaging system (optical imaging system). The NIR emission of the gold cluster facilitated the brain imaging by avoiding the auto fluorescence signal from the animal body. Image acquisition was done by using Living Image software (Figure 4a). The enhanced signal intensity due to the entry of the L-dopa@GQC was observed implying the brain targeting potential and the barrier permeability of the system. In the case of GQC administered animals the signal from the brain was less. Moreover, the signals were seen from different organs other than brain due to the nonspecificity of GQC to target brain. This is in good agreement with the permeability study and the *in vitro* barrier and permeability study, where the faster uptake and clearance of Dopa@GQC was observed compared to those of GQC (Figure 5). The *ex-vivo* image (Figure 4b) of brain also confirms the more and targeted uptake of L-Dopa conjugated system.

After 2h the animals were sacrificed and brain sections were used for further microscopic analysis. Scattered and enhanced fluorescence was observed from different areas of the brain in the case of Dopa@GQC, whereas GQC mostly concentrated in blood vessels. (Figure 6). This is also in agreement with other findings of *in vitro* and *in vivo* studies. Fluorescence signal pattern of Dopa conjuaged system injected brain sections indicates that the material reached upto the hippocampal area of brain, where the blood vessels are comparatively less. These results were further confirmed by repeating the *in vivo* experiment using dopa conjugated system, injected after saline perfusion. The presence of gold in the brain was quantified using ICP-OES after 1 h of injection. The amount of gold estimated was 3.3 ppm in dopa conjugated system. To check the drug delivery efficiency of the developed probe, Dopa@GQC was physically conjugated with a model drug Pilocarpine, a fast acting seizure inducing drug with neurological symptoms like Temporal Lobe Epilepsy (TLE). The *in vitro* drug release was studied in different physiological conditions (figure 7). Which indicate the percentage of drug released increases with time till 360 min after which it slowly saturates, implying that the drug release is slow and sustained. Again the drug release

was confirmed *in vivo* by comparative behavioral studies of material (Dopa@GQC, pilocarpine-Dopa@GQC, pilocarpine) injected mice (table 1). The results also confirm the slow and sustained release of pilocarpine into the brain. Accordingly the pilocarpine injected animals showed characteristic behaviour of seizure and attained TLE within 20 minutes of injection whereas, though the pilocarpine conjugated Dopa@GQC showed the characteristic behaviour of seizure very slowly, it never attained TLE, because of the slow release of drug to the brian (Table 1). The results favour the use of the developed gold cluster based dopamine conjugated probe for drug delivery to brain.

Table 1: Behavioral changes of material injected mice**.**

	Activity	Salivation	Tail stiffness	Seizure
1.Pilocarpine	+++	+++	++	+++
2.Pilocarpine-Dopa@GQC	++	+	-	-
3.Dopa@GQC	++	-	-	-

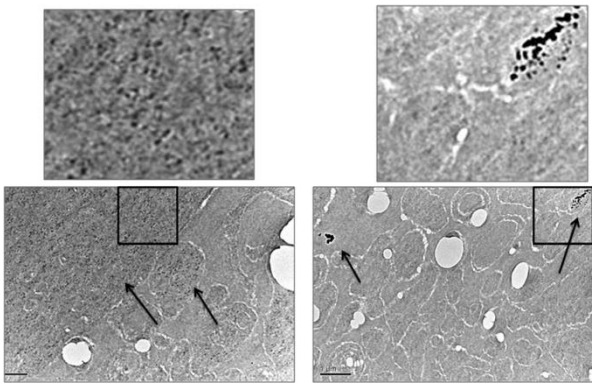

Figure 3: TEM images of bEnd.3 cells incubated with GQC (left) and Dopa@GQC (right) for 3 h. Magnified images of the marked areas are shown above. Well dispersed particles are seen in case of Dopa@GQC where as clumps are seen in case of GQC.

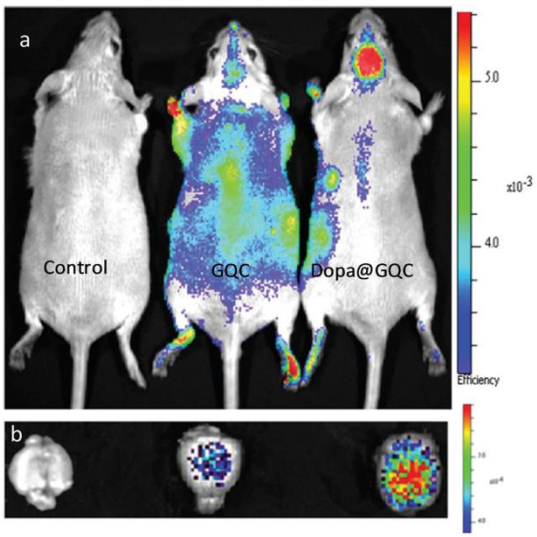

Figure 4: Brain imaging efficacy of GQC and Dopa@GQC *in vivo* (a) and *ex vivo* (b) showing the signal in the mice brain. Highly intense and concentrated signals are obtained from Dopa@GQC injected animals.

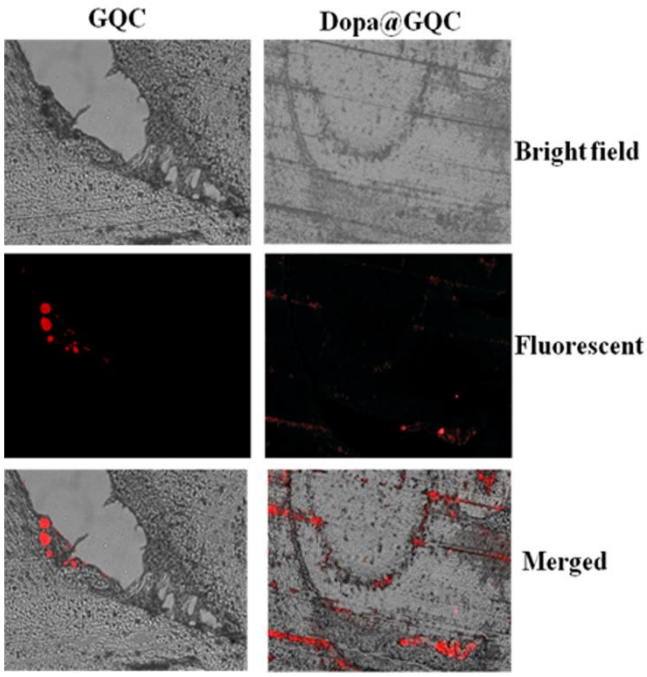

Figure 6: Images of brain sections showing the fluorescence in the case of Dopa@GQC and GQC. The confinement of GQC inside the blood vessel is clear in the case of GQC. In the case of Dopa@GQC, bright fluorescence from areas of hippocampus is very clear.

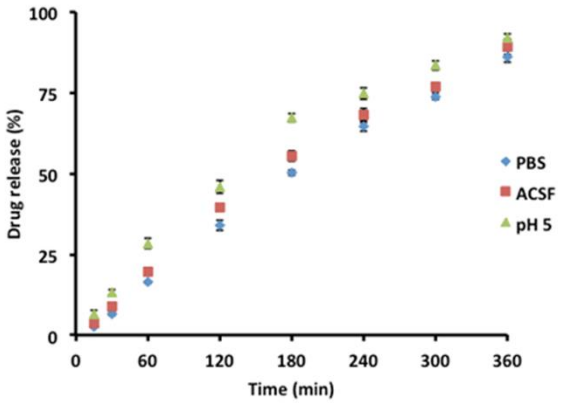

Figure 7: Cumulative pilocarpine release profile of pilocarpine-Dopa@GQC in PBS (pH 7.3), ACSF and phosphate buffer (pH5). Slow and sustained release is observed.

To summarise, a near infrared emitting glutathione gold cluster was developed and modified it with a brain targeting molecule L-dopa. The combined system has proven to have all properties to use it as an imaging and drug delivery agent and at the same time possess the BBB crossing potential. The brain targeting and BBB crossing were demonstrated *in vitro* in BBB model and *in vivo* in normal mice models. Further, the sustained drug delivery potential of the system was also monitored and proven by incorporating a model drug-pilocarpin. To conclude, dop@GQC is a promising candidate for brain imaging and drug delivery without further damage to the brain cells.

References:

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B2. Summary and Conclusions of the Progress made so far (minimum 100 words, maximum 200 words)

NIR emitting gold nanocluster were synthesized and the particular system was functionalized with L-Dopa for brain targeting. An *in vitro* BBB model was developed using bEnd.3cells. It was proven that the synthesized particles have good cell uptake property and also could cross *in vitro* BBB model. On comparison with bare cluster, L-dopa functionalized system showed quick BBB entry and quick clearance as well indicating that the functionalisation facilitate crossing of BBB. The efficacy of the system was checked in the *in vivo* normal mice models and it was proven to be work as well. A model drug pilocarpine was incorporated into the system and checked the *in vitro* and *in vivo* drug release of the same and showed a sustained and slow drug relase pattern, in both systems.It is concluded that that Dop@GQC is a good carrier for drugs that can cross BBB without disturbing it. The process and product was unique and innovative so that the patent application has been filed.

B3. Details of New Leads Obtained, if any:

Brain diseases are one of the major concerns in the society. In majority of the cases the treatment is difficult because delivery of the medicines is restricted to the brain by the presence of a tight junction across the brain called blood brain barrier. Current practices of drug delivery include forceful destruction of the BBB which in future can be harmful in many ways. In order to overcome this limitation, we have attempted a nanotechnological approach for drug delivery and imaging of brain without disturbing

the natural integrity of the brain, at the same time it can cross a tight junction. For this, we developed a very small gold based nanocluster which exhibits size dependent fluorescence due to quantum confinement. The emission has been tuned to be in the NIR region to facilitate brain imaging without the hindrance form autofluorescence from the body which is in the visible region. The concept of BBB crossing and drug delivary has been proven using brain endothelial cells which is considered as an efficient experiment model for BBB. Later, the concept has also been proved successfully in normal animals for imaging and drug delivery efficacy. The process and product has identified to have potential for patenting and a patent has been filed by the institute.

B4. Details of Publications & Patents, if any:

1. **Publication :** L. V. Nair, R. V. Nair, S. J. Shenoy, A. Thekkuveettil and R. S. Jayasree; Blood brain barrier permeable gold nanocluster for targeted brain imaging and therapy: an *in vitro* and *in vivo* study, *J. Mater. Chem. B*, **2017**,5, 8314-8321.
2. L.V.Nair, R.V.Nair, S.J. Shenoy, R.S.Jayasree, Pre clinical evaluation of the L-dopa functionalized fluorescent gold nanocluster for targeted brain imaging (Communicated)
3. **A patent** application entitled "Gold quantum cluster for targeted brain imaging and delivery of foreign molecules (drugs, nutrients, therapeutics etc) through blood brain barrier" is under consideration (application No: 201641019235).

B5. Details of the lead of the project to pursue further (in two to three lines) **in case project will be completing its tenure soon**.

The project has already been completed

B6. List of publications in the project (enclosed reprints on the papers published and manuscripts communicated).

1. L. V. Nair, R. V. Nair, S. J. Shenoy, A. Thekkuveettil and R. S. Jayasree; Blood brain barrier permeable gold nanocluster for targeted brain imaging and therapy: an *in vitro* and *in vivo* study, *J. Mater. Chem. B,* **2017**,5, 8314-8321.
2. L.V.Nair, R.V.Nair, S.J. Shenoy, R.S.Jayasree, Pre clinical evaluation of the L-dopa functionalized fluorescent gold nanocluster for targeted brain imaging (Communicated)