Hydrophobic quercetin encapsulated hemoglobin nanoparticles: formulation and spectroscopic characterization

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Communicated by Ramaswamy H. Sarma

\textbf{ABSTRACT}

Various natural proteins are finding application in drug delivery for their high biodegradability and biocompatibility. Albumins are well explored and now focus is shifting to other proteins like hemoglobin (Hb) with unique structural properties. In the present study Hb is allowed to denature at pH 5.0 and model hydrophobic drug quercetin (Q) is encapsulated via self-assembly and hydrophobic interactions. Fluorimetric titrations record highest binding between Hb and Q at pH 5.0, rendering significant structural changes in Hb as captured in CD spectra. A decrease in fluorescence life time of tryptophan residues from 3.31 ns in Hb to 2.89 ns in presence of Q at pH 5.0; surmises efficient binding of Q at the hydrophobic core housing tryptophan. Peak shifts in Fourier transform infrared spectroscopy spectra of Hb-Q compared to Hb evidence significant interactions between them at pH 5.0. Significant spectral changes in soret band region of Hb on addition of Q at pH 5.0 envisages unfolding of porphyrin ring and binding influence of Q. Efficient formation of Hb-Q nanoparticles (NPs) at pH 5.0 is established by DLS, SEM and TEM.

\textbf{1. Introduction}

Nanotechnology has developed a myriad of functional nano structures to facilitate the delivery of therapeutic and imaging agents. The advantages of such delivery vehicles are their ability to improve solubility of hydrophobic anti-cancer drugs. Polymer nano particles have good loading efficiency if modified on the surface (Pal & Saha, 2017; Saha et al., 2016). Liposomes are limited by complicated steps associated with preparation and purification, low loading efficiency for hydrophobic drugs, burst release kinetics of encapsulated drugs and instability during storage leading to short life (Das et al., 2019). Our studies show that proteins like albumins, enzymes and histones bind to flavonoids as well as anti-cancer drugs efficiently and can be used as carriers (Majumdar et al., 2017; Pal et al., 2012, 2014; Saha et al., 2016). Proteins as natural macromolecules can emerge effective as they get degraded naturally in a controlled manner and are biocompatible compared to synthetic drug-carriers (Bennet & Kim, 2014). Proteins as drug carriers are advantageous because of their amphipathic nature with hydrophilic outer surface and a hydrophobic core facilitating solubility in aqueous medium and encapsulation of hydrophobic drugs in core. Among the proteins plasma albumin is the most abundant with inherent half-life of 19 days rendering it potential drug carrier. Albumin based nanoparticles (NPs) can accumulate in tumor by both the enhanced permeability and retention (EPR) effect and specific interaction of albumin with the gp60 receptors expressed on tumor cell surface (Desai, 2007; Kratz, 2008; Sage et al., 1984).

Hemoglobin (Hb) with roughly spherical shape is also a promising drug delivery material but less investigated than albumin (Wang et al., 2015, 2017). Targeted delivery of drugs in Hb NPs is possible due to erythropoietin receptors (EPORS) expressed on cancerous tissues (Rozsas et al., 2013). The structure of Hb is pH dependent and this attributes is used in NPs synthesis and drug encapsulation by pH variation (Wu et al., 2010). The spherical structure of Hb maintained by two alpha and two beta globin chains and is followed in the present study by circular dichroism at varying pH and ligand concentrations. Heme as a prosthetic group in Hb has a Fe\(^{2+}\) at the centre and is a favored binding site for ligands (Wu et al., 2010) and in the present study, the influence of drug binding on porphyrin structure is investigated. The fluorescence from six tryptophan (Trp) chromophores in Hb tetramer is exploited to trace conformational changes in the protein on pH variation and ligand binding. The ligand a cancer hydrophobic drug model used in our study is quercetin (Q), a flavonol found ubiquitously in fruits and vegetables bestowed with anti-oxidant, anti-cancer, anti-viral as well as free radical scavenging activity (Cos et al., 1998; Kumar & Pandey, 2013; Procházková et al., 2011). Due to poor water solubility, Q like most anti-cancer drugs needs to be delivered via hydrophobic carrier. This aspect is explored...
in the present by encapsulating Q in Hb through molecular self-assembly where molecules organize themselves in defined patterns or structures without any influence of outside sources. Molecular self-assembly can be a simple and cost effective way to encapsulate a hydrophobic drug into a hydrophobic core (Dawei et al., 2014; Lunelli et al., 1993; Wang et al., 2015). The formulation and characterization of Hb-Q NPs using spectroscopic techniques, SEM and TEM is reported here.

2. Materials and methods

2.1 Chemicals

Human hemoglobin, quercetin, dimethyl sulfoxide (DMSO) and methanol were obtained from Sigma-Aldrich, St. Louis, MO, USA. Hemoglobin from human blood was used without further purification. Hemoglobin was kept soluble in phosphate buffer 50 mM (pH 7.0).

2.2 Synthesis of Hb-Q nanoparticles

For synthesis of Hb-Q NPs, 5 mg of Hb was dissolved in 5 mL buffer of pH 5.0 at room temperature. The solution was allowed to stand for 10 minutes and to it 0.5 mg Q dissolved in 1 mL ethanol was added drop wise to get the desired nanoparticles. This was allowed to stand for 1 hr and latter ultra-centrifuged to remove free Q. A final 2 mL concentrated solution of Hb-Q NPs was stored for later use.

2.3 Fluorescence spectroscopy

Fluorescence spectra were recorded on Perkin Elmer fluorescence spectrophotometer (LS-55) equipped with 150 W Xenon flash lamp. In all experiments a fluorescence-free quartz cuvette of 1 cm path length was used. Fluorimetric titration was performed with 760 μL of 2 μM Hb in a quartz cuvette at pH 4.0, 5.0, 6.0 and 7.0 with successive addition of 2 μL Q (4 × 10⁻⁵ M) up to 40 μL until saturation in fluorescence emission is observed. The fluorescence emission spectra of Hb when excited at 280 nm were followed between 300 nm and 450 nm. The changes in the spectra on successive addition of Q were recorded as illustrated in Figure 1. Slit widths (5.0 nm) and scan speed (100 nm/sec) were kept constant for each data set. From the double log plots of log \( F_0 - F/F \) and log [Q], binding constants were calculated using the Stern-Volmer equation

\[
\log (F_0 - F/F) = \log K + n \log [Q]
\]

where \( F_0 \) is the fluorescence intensity of Hb at 350 nm and \( F \) is the consecutive fluorescence intensity on addition of Q, \( K \) is the binding constant and \( n \) is the number of binding sites. Plot of log \( F_0 - F/F \) against log [Q] was used to determine the values of \( K \) and \( n \) from the intercept and the slope respectively.

2.4 Circular dichroism (CD) spectroscopy

Circular dichroism (CD) spectra were acquired on a Jasco-815 (JB15 model) automatic spectropolarimeter (Jasco International Co. Ltd. Hachioji, Japan) equipped with Peltier temperature controller (PFD 425 L/15). A cuvette having path length 1 mm and loading volume 400 μL was used in all experiments. The spectra of Hb (1 × 10⁻⁶ M) in absence and presence of Q in ratio of 1:5, 1:10 and 1:20 at pH 4.0, 5.0, 6.0 and 7.0 were recorded at scanning speed of 100 nm/min and band width of 2.0 nm at 20°C. Three scans were averaged and smoothed to improve signal to noise ratio. The molar ellipticity values are expressed in terms of mean residue molar ellipticity, in units of deg cm² dmol⁻¹.

2.5 Time resolved fluorescence spectroscopy

The changes in the fluorescence life time of tryptophan residues in Hb (2 × 10⁻⁶ M) were monitored with Q in 1:12 ratio by time-correlated single-photon-counting spectrophotometer (Horiba Jobin Yvon). Nanosecond LED of 295 nm was used as excitation source to follow the decay kinetics at 350 nm.

2.6 Fourier transform infrared spectroscopy (FTIR)

The Fourier Transform Infrared Spectroscopy (FTIR) spectra were recorded with Perkin-Elmer, Spectrum GX equipment using ZnSe window. FTIR spectra of free Q (1.47 × 10⁻³ M), Hb (1 × 10⁻⁵ M) and Hb–Q complex (1:5) ratio were recorded to identify changes in secondary structure of Hb. A volume of 200 μL of each sample was spread on thick FTIR glass slide and left to dry completely in a desiccator under vacuum. Background spectra were corrected before scanning each sample and spectra recorded between 400–4000 cm⁻¹ at room temperature.

2.7 Dynamic light scattering (DLS) measurements

Hb (1 × 10⁻⁶ M) solutions at pH 4.0, 5.0 7.0, and 8.0 were conditioned to self-assemble with and without Q (1 × 10⁻⁵ M) as explained in synthesis section. The size distribution of self-assembled NPs was recorded on Zetasizer Nano ZS (Malvern instrument), the scattered intensity was recorded at 173° and the laser beam was set at 632.8 nm wavelength. Quartz cuvette which were washed minimum 10 times by doubles distilled water in a dust free atmosphere are used for all measurements. Same solutions of a duplicate set of pH 5.0 and pH 7.0 were kept for 48 h in room temperature and measured for DLS to follow any aggregation of NPs.

2.8 Zeta potential measurements

The Zetasizer Nano ZS (Malvern Instrument, UK) was used to determine zeta potential (mV) by Laser Doppler Anemometry. The zeta cells treated with gold plated electrodes (Malvern Instrument, UK) and the caps were thoroughly cleaned by
water, ethanol and finally by water again and dried before putting the solution. Test solutions of Hb and Hb-Q NPs were loaded by disposable syringe while avoiding air bubble formation into cells and their Zeta potential was measured.

### 2.9 Soret band displacement assay

The Soret band of Hb was traced in a quartz cuvette of path length 1.0 cm on UV visible spectrophotometer (CARY 100 Bios). An absorption maximum was observed between 300–450 nm. Any shift in Soret band peaks on addition of Q was followed in $1 \times 10^{-6}$ M Hb solutions of pH 4.0, 5.0, 6.0 and 7.0 when titrated with $1 \times 10^{-5}$ M solution of Q (up to 40 µL).

### 2.10 Scanning electron microscopy (SEM)

Surface morphology of Hb and Hb-Q NPs was imaged by scanning electron microscope (ZEISS EV018). A10 µL aliquots of Hb and Hb-Q NPs formulated at pH 5.0 as described earlier were drop casted on carbon coated copper TEM grid (150 mesh, Ted Pella Inc. Redding, CA), and allowed to vacuum dry in a desiccator. The images were recorded at 15.00 KV voltages and 50 K magnification.

### 2.11 Transmission electron microscopy (TEM)

JEOL JEM-2100 HR Transmission Electron Microscope EELS was used to capture the image of Hb and Hb-Q NPs. A 10 µL aliquots of Hb and Hb-Q NPs formulated at pH 5.0 as described earlier were drop casted on carbon coated copper TEM grid (150 mesh, Ted Pella Inc. Redding, CA), and allowed to vacuum dry in a desiccator. The images were visualized at an accelerating voltage of 200 KV under the transmission electron microscope.

### 3. Results

#### 3.1 Fluorescence spectroscopy

Fluorescence spectroscopy is used to get primary information about the conformational and dynamic changes in protein structures. From the fluorescence emission of tryptophan in hydrophobic core of hemoglobin, changes in conformation can be monitored at varying pH (4.0–7.0). Fluorescence emission spectra of Hb ($2 \times 10^{-6}$ M) and changes in the spectra on consecutive addition of Q ($4 \times 10^{-5}$ M) were recorded and are represented in Figure 1 (A1, B1, C1 and D1) for pH (4.0, 5.0, 6.0 and 7.0), respectively. The double logarithmic plot of log $F$ against log ($F_0 - F/ F$) are shown in Figure 1 (A2, B2, C2 and D2) for pH (4.0, 5.0, 6.0 and 7.0) respectively. On consecutive addition of Q there is a progressive hypochromic shift in the emission spectra of Hb. Isosbestic point observed in the spectra signifies equilibrium between the bound and unbound ligand (Berlett et al., 2000). The calculated binding constants of Hb and Q at various pHs and the number of binding sites are summarized in Table 1. From the table it is observed that Q binds to Hb with higher affinity at lower pH 5.0.

### 3.2 Circular dichroism (CD) spectroscopy

CD spectroscopy is one of the commonly used methods to study protein conformation in solution. In Figure 2(A–D), conformational changes taking place in secondary structure of Hb on interaction with hydrophobic drug Q at varying pH (4.0 to 7.0) are represented. The CD spectra of Hb at different pH exhibited two negative minima in the ultraviolet region at 208 nm and 222 nm, which are signatures of $\alpha$-helical structure of protein (Tschesche & Gruyter, 2012). From Figure 2B it is evident that peaks at 208 nm and 222 nm show significant decrease in intensity at pH 5.0 in presence of Q (1:20), suggesting loss in $\alpha$-helicity due to conformational changes. At pH 4.0 and pH 6.0 less significant changes are noticed with no spectral changes at pH 7.0, evidencing higher unfolding of secondary structure of Hb at pH 5.0.

### 3.3 Time resolved fluorescence spectroscopy

The fluorescence observed in protein is mainly due tryptophan (Trp) and tyrosine (Tyr) residues. Hb has six tryptophan residues whose microenvironment can change on binding with Q and influence their fluorescence lifetimes (Tang et al., 2012). When excited between 290–295 nm, the emission of proteins is generally dominated by the Trp fluorescence because Tyr fluorescence is often quenched efficiently by carbonyl groups of the peptide backbone or neighboring residues. Time resolved fluorescence decay of Hb and Hb-Q NPs formulated at pH 5.0 and 25 °C ($\lambda_{ex} =295$ nm) are shown in Figure 3. The decay profile reveals decrease in fluorescence life time of Trp from 3.31 ns in Hb to 2.89 ns in Hb–Q NPs. From this decrease in fluorescence life time it is surmised that Q binds close to Trp residues in the hydrophobic core of Hb.

### 3.4 Fourier transform infrared spectroscopy (FTIR)

FTIR is another relevant spectroscopic technique to study changes in protein secondary structure on ligand/drug binding. Specific stretching and bending vibration of peptide backbone in amide I, II, and III bands provide information about $\alpha$-helix, $\beta$-sheet, turns and random coil. Among all the amide bands of the peptide group, amide I, which gives rise to infrared bands in region between approximately 1600 and 1700 cm$^{-1}$ is most sensitive marker of protein secondary structure. The approximate FTIR bands referred to in most protein studies are; 1651–1658 cm$^{-1}$ ($\alpha$-helix), 1618–1642 cm$^{-1}$ ($\beta$-sheet) and 1666–1688 cm$^{-1}$ (turns) (Kong & Yu, 2007).

The FTIR spectra of free Hb and Hb-Q NPs formulated at pH 5.0 along with Q were recorded and are shown in Figure 4. On comparing the spectral characteristics of Hb with Hb-Q NPs, changes in both shape and peak position are observed, demonstrating changes in the secondary structure in Hb-Q NPs. A predominant band centered at 1651.50 cm$^{-1}$ in amide I region of Hb spectrum evidences predominant $\alpha$-helical secondary structure at pH 5.0. The band centered at 1651.50 cm$^{-1}$ in Hb is shifted to 1652.19 cm$^{-1}$ with significant decrease in intensity in the Hb-Q NPs spectrum,
confirming loss in α-helical secondary structure and encapsulation of Q. The spectral region from 3500 to 3000 cm\(^{-1}\) is assigned to the amide A band which is contributed by the N-H stretching vibration and is primarily governed by the stretching vibrations of the C=O and to some extent C-N groups. A peak at 3382.19 cm\(^{-1}\) is recorded and displayed in Figure 4 and is attributed to this amide A band and shift in this peak along with decrease in intensity is observed in the Hb-Q NPs evidencing structural changes along amide bond on Q encapsulation (Kong & Yu, 2007).

### 3.5 Size determination by DLS

DLS is used for hydrodynamic size determination (Bhattacharjee, 2016; Kam Zvi et al., 1986). The DLS

#### Table 1. Binding constants and the number of binding sites of Hb-Q at different pH.

| pH     | Binding constant (M\(^{-1}\)) | Number of binding sites |
|--------|-------------------------------|-------------------------|
| pH 4.0 | \(1.2 \times 10^5\)           | 1                       |
| pH 5.0 | \(3.6 \times 10^7\)           | 1                       |
| pH 6.0 | \(1.5 \times 10^7\)           | 1                       |
| pH 7.0 | \(1.1 \times 10^3\)           | 1                       |
measurements of Hb and Hb-Q NPs ascertain that the size of NPs varies with pH. At pH 5.0 the size is around 7.0 nm (Figure 5A) and is reduced to around 6.0 to 5.5 nm at lower and higher pH respectively. In acidic pH, Hb encapsulates Q to record significant increase in diameter in DLS measurements with maximum of 11.2 nm at pH 5.0 as displayed in Figure 5B and tabulated in Table 2. After 48 h of incubation at room temperature, an increase in size of Hb-Q NPs is observed of in the range of 30–35 d.nm and it is attributed to aggregation on standing and a less percentage of distribution is recorded for aggregates with diameter up to 150 d.nm demonstrated as in Table 2 and Figure 5C.

### 3.6 Zeta potential measurement

Zeta potential can be considered as one of the best method to characterize the dispersive nature and stability of colloidal suspension (Bhattacharjee, 2016; Clogston & Patri, 2011; Dukhin & Parlia, 2014). Zeta potential value recorded for Hb-Q NPs at pH 4.0 is +18.0 mV, pH 5.0 is +15.0 mV, pH 7.0 is −10.0 mV and pH 8.0 is −14.0 mV as plotted in Figure 6.

![Figure 2](image1.png)

Figure 2. Changes in the CD spectra of Hb (1 × 10⁻⁶ M) and Hb on addition of Q at 25°C in three consecutive ratio 1:5, 1:10 and 1:20 at four different pH of 4.0, 5.0, 6.0 and 7.0.

![Figure 3](image2.png)

Figure 3. Time resolved fluorescence decay of tryptophan residue in Hb and Q encapsulated Hb in 1:12 ratio at 25°C ($\lambda_{ex} = 295$ nm).

### 3.7 Soret band displacement assay

The Soret band spectral peak shift is the measure to ascertain the binding of drug in the vicinity of metal porphyrin
Absorption spectra of Hb-Q NPs in the Soret band 390–420 nm region is shown in Figure 7 at different pH. The spectra at pH 4.0, 7.0 and 8.0 shows a hyperchromic shift which is attributed to successive addition of Q in with an absorption peak at 390 nm and is not assigned to changes in the porphyrin ring (400–435 nm; B-band). At pH 4.0 a blue

![Figure 4](image1.png)

**Figure 4.** Fourier transformed infrared spectra in the region of 4000–1000 cm⁻¹ for Hb, Q, and Hb-Q at pH 5.0.

![Figure 5](image2.png)

**Figure 5.** Size distribution of Hb and Hb-Q NPs formulated at pH 5.0 (A) Hb, (B) Hb-Q after 1 hour, (C) Hb-Q after 48 h.

![Figure 6](image3.png)

**Figure 6.** Zeta potentials of Hb-Q NPs at different pH.

**Table 2.** Size of Hb and Hb-Q NPs at pH 5.0 as measured by DLS.

| Nano formulations | Size, d nm at pH 5.0, presented in highest percentage | Time after formulation |
|-------------------|----------------------------------------------------|------------------------|
| Hb NPs            | 7.0                                                | 1 h                    |
| Hb-Q NPS          | 11.0                                               | 1 h                    |
| Hb-Q NPS          | 32.7                                               | 48 h                   |

**Table 3.** Size of Hb and Hb-Q NPs at different pH as measured by DLS.

| pH    | Size of Hb NPs in d nm, presented in highest percentage | Size of Hb-Q NPs in d nm, presented in highest percentage |
|-------|--------------------------------------------------------|----------------------------------------------------------|
| 4.0   | 6.0                                                    | 8.1                                                       |
| 5.0   | 7.0                                                    | 11.2                                                      |
| 7.0   | 6.5                                                    | 7.6                                                       |
| 8.0   | 5.5                                                    | 7.0                                                       |
shift in the Soret band is observed surmising delocalization of metal ion at the porphyrin ring and other structural changes. At pH 5.0, quenching is observed on addition of Q in the Soret band region, evidencing structural changes in the tetrameric protein, like opening of the core to accommodate Q near porphyrin ring (Giovannetti, 2012). It is concluded here that hypochromicity and saturation at the Soret band region with no bathochromic or hypsochromic shift suggests that Q does not directly bind to the metallo porphyrin ring but near to it (Ke-wei Ding et al., 2018; Satake & Kobuke, 2005).

3.8 Electron microscopy (SEM and TEM)

SEM and TEM are techniques used for getting insight into surface morphology, shape and size of NPs (Akhtar et al., 2019). SEM and TEM images recorded for Hb-Q NPs at pH 5.0 are presented in the Figure 8A and 8B respectively. Images evidence unfolding and refolding by self-assembly driven forces to encapsulate hydrophobic drug. The structures of Hb-Q NPs are spherical in shape with smooth surface morphology as compared to the smaller void Hb nano particles. The size of Hb-Q NPs as observed in SEM micrographs corroborates with size determined by DLS after 48 hrs; we reason here that the drop casted on carbon–copper grid takes time to dry and the self-assembly goes on to reach optimum size which is larger than that recorded after 1 hr on DLS. The results from two methods namely DLS and SEM used for determination of size distribution of NPs, are comparable here, though the diameter shown in SEM is somewhat higher, as SEM micrographs are more comprehensible beyond 50 nm whereas DLS can measure up to few nms. Here it is brought to light that the purpose and sample preparation methodology are independent in DLS and SEM and that brings the inevitable divergent results as also reported by others (Bootz et al., 2004). It can be concluded here that the size distribution varies between 30 nm and 150 nm for Hb-Q NPs with major population in the 30–35 nm region as demonstrated in DLS after 48 hrs. The TEM image in Figure 8B resonates with SEM findings.

Discussion

High affinity of small-molecule antioxidants for Hb is reported (Puscas et al., 2018) and here we have chosen Q as the antioxidant with anti-cancer and MDR reversing properties. Fluorescence studies reported here evidence high affinity between Hb and Q at pH 5.0 (Figure 1 and Table 1) and
demonstrate loss in secondary structure in the recorded CD spectra shown in Figure 2B. The strong interaction of Q and Hb is also substantiated by FTIR spectral peak shifts in Figure 4 which demonstrates change in secondary structure of Hb to accommodate Q. Maximum decrease in the fluorescence intensity and lifetime of Trp residues is recorded at pH 5.0 envisaging high interaction between Hb and Q at pH 5.0 compared to other pH conditions. The decrease in Soret band intensity on addition of Q only at pH 5.0 reinforces Hb unfolding, exposure of porphyrin ring and binding of Q close to it. From these results pH 5.0 was chosen to produce Hb-Q NPs and the formation of NPs is demonstrated by SEM and TEM images as represented in Figure 8A and 8B. The size recorded of NPs in these images is between 35-60 nm and spherical in shape. DLS measurements observed increase in hydrodynamic size of Hb NPs from 7.0 to 11.2 nm on addition of Q and this size further increased to 32.7 nm after 48 hrs which is attributed to aggregation on standing. Similar size is also recorded in SEM and TEM images as the samples are left to dry after casting for few days. The positive charge of the nanoparticles as measured by

Figure 8. (A) SEM images of Hb and Hb-Q NPs formulated at pH 5.0. a.1, a.2 and a.3 are of void Hb NPs; a.4, a.5 and a.6 are of Hb-Q NPs in different scale of size distribution. (B) TEM image of Hb-Q NPs formulated at pH 5.0.
zeta potential studies is favorable for drug delivery (Honary & Zahir, 2013) as the cancerous cells are negatively charged due to excess glycol proteins expressed. The Hb-Q NPs formation is favored by unfolding of Hb at pH 5.0 and exposure of hydrophobic core to encapsulate Q by self-assembly. These NPs are expected to be stabilized through hydrophobic interactions as reported earlier (Huang et al., 2013; Wang et al., 2017).

Conclusion

Hemoglobin as carrier for drug delivery needs to be explored further with other potential hydrophobic drugs. The shape of Hb is roughly spherical and has high surface area to encapsulate drugs. Positive charge of Hb-Q NPs at low pH favours binding to cancerous cells and increases their potential as drug delivery systems of future. The size of Hb NPs is 30–50 nm, and can reach 150 nm on standing due to aggregation. The size is small enough for efficient delivery by EPR effect, compared to polymer NPs and liposomes tested by us for drug delivery.

Acknowledgements

The authors are thankful to UGC-DAE Consortium for Scientific Research, Kolkata for the financial research grant and for providing us instrumental facilities like DLS, Zeta potential, ITC, Fluorimeter and UV-Vis Spectrophotometer. Thanks are also extended to Centre for Research in Nano Science and Nano Technology, Kolkata for providing us with SEM and TEM facility and Bose Institute for CD facility. The Vice-Chancellor of MAKAUT, Prof. Saikat Maitra is acknowledged for his constant support and encouragement.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

The authors are thankful to UGC-DAE Consortium for Scientific Research, Kolkata for the financial research grant and for providing us instrumental facilities like DLS, Zeta potential, ITC, Fluorimeter and UV-Vis Spectrophotometer.

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