Surface functionalization of porous chitosan microsphere with silver nanoparticle and carbon dot

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Abstract
Porous microspheres have enormous specific surface area due to the presence of micropores. This makes them suitable for all applications that involves surface adsorption e.g. chromatographic separation of biomolecules, catalytic reactions and drug delivery. The surface property may further be tuned up by functionalization of microsphere surface with different nanoparticles like silver nanoparticle and carbon dot (CD). In this study porous chitosan microsphere (PCM) was synthesized by ‘phase inversion of emulsion’ technique. Silver nanoparticle (AgNP) was synthesized, in-situ during the process of surface modification, using silver nitrate solution. CD was synthesized by solvothermal method using urea and EDTA. PCM, AgNP and CD were of ~9 μm, ~27 nm and ~14 nm diameter, respectively. From FTIR study it was confirmed that the amino group of chitosan backbone was responsible for reduction of Ag⁺ ion to Ag° species which were clustered as AgNP and attached to the surface of PCM. The same amino group of chitosan molecule was also responsible for conjugation of CD to the microparticle surface. The optimized AgNP functionalized PCM had 5.36 × 10¹¹ AgNP per mg dried mass. The release of AgNP was triggered at pH ≤ 4.5. The CD functionalized PCM had 56.82 ± 2.8 % conjugation efficiency and 7.83 ± 1.7 % quantum yield with respect to quinine sulphate.

Abbreviations
AgNP Silver Nanoparticle.
PCM Porous Chitosan Microparticle.
EDTA Ethylenediamine-tetra acetic acid.
CD Carbon dot.
SFPCM Silver Nanoparticle functionalized Porous Chitosan Microparticle.
CFPCM Carbon dot functionalized Porous Chitosan Microparticle.

1. Introduction
Porous microsphere was discovered in 1990 as potential material for the development of drug delivery system and biomedical device [1]. Two types of pores are present. External pores are present on the surface and internal pores are situated within the core. These pores are usually interconnected [2]. High porosity, low density and large specific surface area are their unique properties over traditional microspheres. Their applications are mainly based on their porosity, pore size and surface area. Compared to nonporous microspheres, they are unique regarding drug entrapment and release characteristics. The active ingredients are either absorbed at the surface or dispersed into the matrix [3]. Again, due to enormous specific surface area, porous microsphere may be an ideal stationary phase for separation of complex mixture of molecules. The micropores present on the...
particle surface provide the enormous surface area required for adsorption of the analyte molecules, whereas the macropores provide the easy passage for the mobile phase to pass through. Many studies have shown that porous microspheres can be used for high-performance column chromatography with high column efficiency, high dynamic capacity, and low flow resistance [4–6].

Chitosan is biodegradable, biocompatible and non-toxic polymer. Porous chitosan microsphere has widely been studied as drug carrier for pulmonary drug delivery [7, 8], scaffold for tissue engineering [9] and micro-carrier for 3D cell culture [10]. Porous polyelectrolyte complex of poly(L-glutamic acid) (PLGA) and chitosan was found useful for cartilage regeneration [11]. Chitosan-modified porous silicon micro particles enhanced the permeability of insulin across intestinal cell monolayers [12].

Surface modification of porous particle opened a new horizon of applications. Venkatesan et al reported the antimicrobial and anticancer activities of porous chitosan-alginate biosynthesized silver nanoparticles [13]. The internal pore surfaces of open porous poly (methyl methacrylate) (PMMA) microspheres were coated with polydopamine for spontaneous reduction of silver nitrates into solid silver nanoparticles (AgNPs) within the pores of the prepared microspheres [14]. Recently carbon-dot wrapped ZnO nanoparticle-based photo-electrochemical sensor for selective monitoring of H$_2$O$_2$ released from cancer cells [15]. Surface functionalization of porous ZnO microparticle with multi-band light active carbon dots was reported [16]. It had superior photo-catalytic activity due to the superior photoelectrical properties of carbon dot.

In this study we have developed a simple method for synthesis of porous chitosan microsphere (PCM) and functionalized the surface of synthesised microsphere with silver nanoparticle and carbon dot.

2. Experimental section

2.1. Materials

Chitosan (nominal molecular weight: $\sim$ 19 kDa, degree of deacetylation 95 %), urea and di-sodium ethylenediamine-tetra acetic acid (EDTA, di sodium salt) were purchased from Sisco Research Laboratories Pvt. Ltd, Mumbai, India. Acetic acid was purchased from Thermo Fisher Scientific India Pvt. Ltd, Mumbai, India. Tween® 20 was purchased from Merck Specialities Private Limited (Merck), Mumbai, India. Span®80 was procured from Central Drug House (CDH), Chennai, India. Sodium hydroxide (NaOH) was bought from SD fine-Chem limited, Mumbai, India. Propylene glycol was purchased from Nice Chemicals Pvt. Ltd Kerala, India. Silver nitrate (AgNO$_3$) was purchased from M P Biomedicals, Solon, India. Other solvents were purchased from local vendors and used as such. Milli-Q water was used.

2.2. Methods

2.2.1. Preparation of porous chitosan microspheres

100 mg chitosan was dissolved in 1% (v/v) acetic acid solution to prepare the aqueous phase [17]. Span®80, Tween® 20 and Petroleum ether (60 °C–80 °C) were mixed together using a magnetic stirrer to prepare the oil phase. After 0.5 h the aqueous solution was added drop wise into the oil phase. A white emulsion was formed. The flask was closed tightly and stirring was continued for 4 h followed by freezing at $\sim$20 °C. After 2 h the flask was taken back at room temperature to allow the ice to start melting. Now, 30 ml ethanol was added drop wise under continues stirring (2000 rpm) using a magnetic stirrer (Remi Laboratories Instrumentations, Mumbai, India). 220 ml 1% (w/v) NaOH in 9% (v/v) ethanol in water solution was added drop wise under continues stirring. Stirring was continued under air tight condition overnight.

The content was kept in a separating funnel to allow the phases to get separated out. After 24 h, three layers were found; the top layer was of petroleum ether, whereas aqueous layer remains at the bottom due to its high density. The porous particles formed a layer at the junction between the aqueous and oil phase. The aqueous layer together with the layer of porous particles were taken out and the upper oil phase was discarded. Thus, an aqueous dispersion of porous particle was obtained. It was kept in a tightly closed container at room temperature till used further.

2.2.2. Surface modification of PCM with AgNP

The dispersion of porous particle was centrifuge at 5000 rpm (Remi Laboratories Instrumentations, Mumbai, India) for 5 min to separate out the particles at the top. The aqueous phase from the bottom was discarded using a syringe to reduce the volume to 50 ml. 1 ml, 2 ml, 3 ml, 4 ml and 5 ml (F1 to F5) of this product was taken in 5 different test tube and 10 ml 1 mM silver nitrate solution was added to each of them. They were kept under mild shaking for 2 h. Deep brown particles were obtained at the top of aqueous phase (light phase). The particles were separated by centrifuging at 5000 RPM for 5 min. The AgNP conjugated porous Chitosan particle were obtained as the bottom product.
2.2.3. Synthesis of carbon dot (CD)

The fluorescent CD was synthesized by one-step direct solvo-thermal reaction. EDTA (0.2 mM) and urea (2 mM) were added in propylene glycol and heated at 150 °C for 4 h with vigorous stirring in an oil bath. The colour of solution changed from colourless changed to yellow and finally to dark brown. The reaction mixture was taken out of oil bath and cooled down at room temperature. It was filtered through sintered glass filter having a pore size of 15–40 μm. The filtrate was centrifuged at 10,000 rpm for 15 min (Remi- R8C). The supernatant was filtered through syringe filter of pore size 0.2–0.45 μm to get the final product.

2.2.4. Surface functionalization of PCM with CD

After centrifugation of dispersion of PCM at 5000 r.p.m for 25 min, the separated layer of PCM was divided into five equal parts (2 ml each) in test tubes. 50 μl, 100 μl, 200 μl, 300 μl and 400 μl of CD was added in each test tube. After incubation for overnight yellowish-brown particles were obtained at the top of aqueous phase. Centrifugation was done at 5000 r.p.m for 45 min to separate out the particles, the aqueous bottom phase was discarded with a syringe to collect the CD coated PCM at the top. This was followed by dialysis for 1 h using cellulose membrane with molecular weight cut off 12–14 kD (LA- 387–5 MT, Himedia®). Milli-Q water was used dialysis medium.

2.2.5. Quantitative estimation of incorporation of AgNP in PCM

40 μl sample was dried at 50 °C. The mass was dissolved in 2 ml acetate buffer of pH 4.0. After 10 times dilution with Milli-Q water, the samples were scanned within the range of 300–700 nm using UV-vis spectrophotometer (Jasco, V-730). Number of AgNP was calculated from the OD (Optical Density) value at 405.6 nm using molar extinction co-efficient (1.45 × 10^10) [18].

Figure 1. Synthesis and characterization of porous chitosan microspheres. (A): Schematic representation of fabrication of porous chitosan microspheres by phase inversion regeneration method. (B): Observation after separation of phases in separating funnel. Due to low density porous chitosan microspheres were floating at the junction of oil and aqueous phases. (C): Image of microsphere under scanning electron microscope at a magnification of 7.5 K.
2.2.6. Quantitative estimation of conjugation of CD to the surface of PCM

The prepared CD and CD functionalized PCM (at the same degree of dilution) were scanned within the range of 200–700 nm using UV-vis spectrophotometer (Jasco, V-730). The absorbance at 280 nm was used to calculate the conjugation efficiency using the following formula:

\[
\text{Conjugation Efficiency} = \frac{\text{OD}_{\text{CD}} - \text{OD}_{\text{CFPCM}}}{\text{OD}_{\text{CD}}} \times 100\% \tag{1}
\]

- \(\text{OD}_{\text{CD}}\) - Optical density of Carbon Dot
- \(\text{OD}_{\text{CFPCM}}\) - Optical density of Carbon dot functionalized PCM

2.2.7. Estimation of quantum yield

The fluorescence quantum yield (QY) of CD and CFPCM was calculated by comparing the gradient from the plot of integrated fluorescence intensity and the absorbance values of test samples (CD and CFPCM) and standard sample using the following equation. Quinine sulphate was used as the standard [19]. 280 nm was used as excitation wave length. In order to avoid the inner-filter effect and re-absorption effects, the optical density of all samples was kept below 0.1. Spectro-fluorometer (JASCO FP-8500, North America) was used for all fluorescence measurements.

\[
\text{QY} = \phi_T \left( \frac{\text{Grad}_T}{\text{Grad}_S} \right) \times \left( \eta^2 T / \eta^2 S \right) \tag{2}
\]

where, subscripts T and S denotes Test sample and Standard, ‘Grad’ means the gradient from the plot of integrated fluorescence intensity versus absorbance, and \(\eta\) is the refractive index of the solvent.
2.2.8. **Fourier-transform infrared spectroscopy (FTIR) study**

FTIR spectra of Chitosan, blank porous chitosan microspheres, AgNP functionalized PCM and CD functionalized PCM were recorded using (Parkin Elmer, 1000 spectrum, USA) in the range of 4000–400 cm$^{-1}$.

2.2.9. **Scanning electron microscopy**

Morphology of the PCM, SFPCM & CFPCM were examined using scanning electron microscope (Hitachi S-3400, Japan) at an acceleration voltage of 15kv. Liquid samples were taken on cover slips and was dried at 50°C for 2 h. After gold sputtering, samples were scanned under the electron microscope.

2.2.10. **Particle size distribution and zeta potential analysis**

Measurement of particle size distribution and zeta potential were done with Zetasizer Nano ZS (Malvern Instruments Limited, U K) at 25°C and a laser (633 nm) scattering angle of 90°. Diameter corresponding to the maximum % of particle was considered as mean.

3. Results and discussion

3.1. **Synthesis of porous chitosan microspheres**

The strategy to synthesize porous Chitosan microsphere is shown in figure 1(A). The solution of Chitosan in 1% (v/v) acetic acid solution was emulsified in oil phase. The resulting bluish white emulsion was freezed at $-20$ °C for 2 h. At this low temperature, the dispersed water droplets containing dissolved chitosan were converted into ice particles. Thus, the polymeric chains of chitosan were quenched [20]. At room temperature when the ice starting melting, ethanol was added to make the melting process faster than the rate of relaxation of quenched polymeric chains of chitosan. Being a dehydrating agent, ethanol takes out the water molecules from polymeric network. Thus, the pores were generated. Ethanol also acts as cosurfactant. So, drop wise addition of 220 ml 1% (w/v) NaOH in 9% (v/v) ethanol in water solution helps in phase inversion of emulsion from W/O to O/W. The role of NaOH was to shift the pH of aqueous phase from acidic to basic. In basic pH deprotonation of amino and hydroxyl groups of chitosan molecules makes it insoluble. Thus, addition of NaOH hardens the porous polymeric microparticles of chitosan. Finally, the reaction mixture was allowed to stand undisturbed for
24 h in a separating funnel. The dispersed oil phase got separated out on the top of aqueous phase (figure 1(B)). The light porous microspheres were floating at the junction of aqueous and oil phase (figure 1(B)). After discarding the aqueous phase, the microspheres were collected from the bottom leaving the oil phase in the separating funnel. The porous nature of chitosan microspheres was confirmed from the electron microscopic image (figure 1(C)). The particles were spherical in shape and had a diameter of \( \sim 9 \mu m \). The particle surface was irregular and full of pores.

3.2. Synthesis of AgNP functionalized porous chitosan microspheres (SFPCM)

Simply addition of silver nitrate solution into the separated porous chitosan microspheres (PCM) resulted in the conjugation of silver nanoparticle on the surface of microsphere (figure 2). The volume of 1 mM AgNO\(_3\) solution was fixed to 10 ml whereas the amount of PCM was varied from 1 ml to 5 ml (F1–F5). Deep brown particles were obtained at the top of aqueous phase (figure 2(A)).

Their surface morphology (figures 2(B), (C)) was quite different from that of uncoated particles (figure 1(C)). At higher magnification (figure 2(C)), clusters of silver nanoparticles were found attached on the surface of PCM. Thus, the average particle size was increased to \( \sim 12 \mu m \). The release of AgNP was triggered at pH \( \leq 4.5 \) [21].

The surface conjugation of silver nanoparticle was further confirmed with spectroscopic analysis (figure 3(A)) and dynamic light scattering technique (figure 3(B)). Dried particles were dissolved in acetate buffer (pH 4.0) and diluted with water before collecting absorbance spectra. The spectra were typically of silver nanoparticle, observed due to the surface plasmon resonance [22]. Spectrum of F3, F4 and F5 were sharp and had low FWHM (97.9 nm, 71.7 nm and 98.3 nm respectively). The \( \lambda_{max} \) value was 405.6 nm, corresponding to a particle size of 27 nm (figure 3). Spectrum of F1 and F2 were broad and had higher FWHM values (165.6 nm and 132.5 nm respectively). The \( \lambda_{max} \) values were also shifted to higher values (red shift), indicating the presence of
large aggregated silver nanoparticles conjugated to surface of PCM. Among F3, F4 and F5 if we consider the number of silver nanoparticles conjugated per mg dried product, F4 had the highest value of $5.36 \times 10^{11}$.

The mechanism behind conversion of $\text{Ag}^+ \text{ion}$ into silver nanoparticle was further investigated with FTIR analysis (figure 4(A)). In case of PCM, the peaks at 2923 cm$^{-1}$ and 2853 cm$^{-1}$ were due to the stretching vibration of C–H bond present in Chitosan backbone [23]. The characteristic peaks of amino group were found at 1561 and 699 cm$^{-1}$ due to the stretching vibration of $\text{N}–\text{H}$ bond. In chitosan molecule amino group is attached to the carbon at C2 position. The peaks due to vibration of C–N linkage were found at 1151 and 1078 cm$^{-1}$ wave number [24]. In case of Silver nitrate, the broad peak with strong intensity at 1384 cm$^{-1}$ was due to the N=O stretching vibration of $\text{NO}_3^-$ ion [25]. In case of SFPCM, the peaks at 2922 cm$^{-1}$ and 2852 cm$^{-1}$ were characteristic peaks of chitosan (stretching vibration of C–H bond). The intensity of peak at 1564 cm$^{-1}$ (due to amino group) was decreased in comparison to that of the uncoated particle (PCM). It indicated the involvement of amino group to convert $\text{Ag}^+$ ion to silver nanoparticle [23, 26]. Again, there was a new absorption peak found at 849 cm$^{-1}$. It is a characteristic peak of out-of-plane wagging of $\text{N}–\text{H}$ bond of amino group. Thus, the amino groups of chitosan backbone were responsible for reduction of $\text{Ag}^+$ ion to $\text{Ag}^0$ species which ultimately led to the formation of cluster of silver nanoparticle attached on the surface of PCM.

3.3. Synthesis and characterization of CD

CD were obtained as dark brown dispersion in propylene glycol. It was 500 times diluted with water before characterization. The average particle size and zeta potential of this diluted sample was estimated to be 14 nm and $-3.12 \text{ eV}$ respectively using (figures 5(A) and (B)). The UV-VIS absorbance spectrum is shown in figure 5(C). Optical density at 280 nm was below 0.1. After excitation at a wavelength of 280 nm, emission spectrum is shown in figure 5(D). It was within the range of 530 nm to 555 nm with emission maximum at 543 nm.

3.3.1. Conjugation of CD on the surface of PCM

Simply mixing of CD and PCM resulted in conjugation of CD on the surface of PCM. The yellowish-brown particles were seen floating on the aqueous reaction medium (figure 6(A)). The change of colour of PCM from white to yellowish brown is an indication of surface conjugation. The floating tendency was an indicative of
porousness of the surface functionalized PCM. As per the electron microscopic images (figure 6(B)) these particles were spherical in shape. At higher magnification, their surface texture (figure 6(C)) could easily be differentiated from that of uncoated PCM (figure 1(C)). The CFPCM had cluster of fine particles attached to the surface. Thus, the average size of CFPCM was significantly increased to $\sim 36 \mu m$. The surface conjugation of CD of PCM surface can further be explained with the FTIR study (figure 4(B)). In case of blank PCM the peaks at 2923 & 2853 cm$^{-1}$ were due to stretching vibration of C–H present in the backbone [23]. At 1561 and 699 cm$^{-1}$, the characteristic peaks were found due to stretching vibration of N–H linkage of amine group (–NH$_2$). In chitosan molecule amino group is attached to the carbon at C2 position. The peaks due to vibration of C–N linkage were found at 1151 and 1078 cm$^{-1}$ [24]. In case of C-dot, peak due to bending vibration of O–H was found at 3369 cm$^{-1}$ [27]. At 2974 cm$^{-1}$, the characteristic peak was found due to C–H stretching vibration [27]. The characteristic peaks for N–H stretching vibration of amines groups (–NH$_2$) were found at 1044 cm$^{-1}$ and 772 cm$^{-1}$. The peak at 1654 cm$^{-1}$ was due to stretching vibration of C=C linkage of alkene. This peak has frequently been reported as characteristic peak of C-dot [28]. In case of C-dot coated PCM, peak due to C–H stretching vibration was found at 2923 cm$^{-1}$. Peaks at 1258 cm$^{-1}$ and 1081 cm$^{-1}$ were for the C–O–C ether linkage present in the chitosan backbone [29]. The characteristic peak of C-dot was found at 1654 cm$^{-1}$. New characteristic peaks for alkene group (C=C) were found at 991 cm$^{-1}$ and 838 cm$^{-1}$. They also represent the presence of C-dot. If we compare the spectrum of PCM and CFPCM, the % transmittance of signature peak for amino group (N–H stretching vibration) in chitosan moiety was significantly decreased in C-dot coated PCM. This disappearance of amino group may be related to the interaction of CD with the amino group of chitosan molecule.

The relative amount of CD and PCM was varied to find out the optimum ratio in terms of conjugation efficiency and quantum yield of product (figure 7). In 2 ml dispersion of PCM, 50 µl (P1), 100 µl (P2), 200 µl (P3), 300 µl (P4) and 400 µl (P5) of CD was added. Product P3 had highest % quantum yield (7.83 ± 1.7) with maximum % conjugation efficiency (56.82 ± 2.8).
4. Conclusion

A new method was developed to synthesize porous chitosan microsphere. Its surface was successfully functionalized with silver nanoparticle and carbon dot. The optimized AgNP functionalized PCM had \(5.36 \times 10^{11}\) AgNP per mg dried mass, whereas CD functionalized PCM had 56.82 ± 2.8 % conjugation efficiency and 7.83 ± 1.7 % quantum yield with respect to quinine sulphate.

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Conflict of Interest

The authors have NO conflict of interest.

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Figure 7. Optimization of CD functionalized PCM. The relative amount of CD and PCM was varied to find out product having highest conjugation efficiency and quantum yield. In 2 ml dispersion of PCM, 50 μl (P1), 100 μl (P2), 200 μl (P3), 300 μl (P4) and 400 μl (P5) of CD was added.
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