Synthesis and characterization of thiol functionalized cholic acid based poly(ε-caprolactone) biodegradable polymer and encapsulation of noble metal nanoparticles for biomedical applications

1. Introduction

In recent years, considerable research efforts have been devoted for the preparation biocompatible polymeric materials and co-polymers using both synthetic and biologically derived (or natural) building block such as starting materials, due to their potential applications in various fields like drug delivery and tissue engineering, DNA sensors, tumor therapy etc., [1-3] The surface properties of these polymeric materials are determined by their hydrophobicity, charge, and morphology, which are particularly important to the potential regulation of biological phenomena such as protein adsorption and cell adhesion [4,5]. During the last two decades, significant advances have been made in the preparation of poly(ester) based on polylactides (PLA), polyglycolides (PGA), poly(ε-caprolactone) (PCL) as biodegradable polymeric biomaterials through ring opening polymerization [6,7]. However, owing to their copolymer crystallization, hydrophobic properties, lack of functional groups, low biodegradation rate the biological application is reasonably limited.

To meet the increasing demands for better performances and satisfy the requirements of some specific applications, the properties of these materials can further be tailored and improved by modifications and functionalization. Moreover, these synthetic polymers have great design flexibility in which the composition and structure can be tailored to the specific needs. For instance, Hwang et al. reported various cholesteryl oligo(L-lactic acid) materials to promote cell proliferation [8]. In their study, cholesteryl oligo(L-lactic acid) was synthesized by the ring-opening polymerization of L-lactide initiated by aluminum alkoxide. Cheng and co-workers has reported a series of cholic acid contained star oligo/polymers by incorporation of cholic acid
moiety into poly(DL-lactide), poly(ε-caprolactone) and poly(2,2-dimethyltrimethylene carbonate) for the drug delivery applications [9]. Since, bile acid are naturally occurring steroidal detergents in mammals, enantiomeric purity, rigid structure, and acid-base properties, which make them a useful building blocks for the synthesis of biocompatible polymers [10,11]. Moreover, these derivatives possess some facial amphiphilicity, owing to the presence of hydroxyl groups located on one side of the molecule and self-assemble to form primary and secondary structures that play an important role in the solubilization of cholesterol and lipids for example. Incorporation of bile acid derivatives within a polymer backbone should therefore allow easy tuning of the hydrophobicity, as well as the rigidity of the polymer chains. This, in turn, will have important consequences on the mechanical, degradation and drug-delivery properties of the materials.

Preparation of polymer-coated noble metal nanoparticles in particularly silver and gold nanoparticles (Ag and Au NPs) great interest in the fields of biomedical and biotechnological applications [12,13]. These materials have been shown to combine both the intrinsic features of noble metal nanostructures and the biological features presented by renewable source polymers. Also, these NPs were exhibit vibrant optical absorbance, high dispersibility in aqueous medium, chemical inertness, and biocompatibility. A small change in the NP size, shape, surface nature, and the distance between particles leads to tunable changes in their optical properties [14]. These features have been employed for a number of applications including high resolution biomedical imaging, catalysis, sensing, drug delivery, as therapeutic agents, and treatment of various diseases [15,16]. Even though a large number of polymer-coated noble metal nanoparticles have been fabricated over the past decade, most of these materials still present some challenges emanating from their synthesis. Hence in the present investigation we synthesized thiol functionalized cholic
acid based biodegradable polymers and encapsulation of noble metal nanoparticles for biological applications.

2. Object of the proposal

The aim of the proposal is to synthesis and characterization of thiol functionalized cholic acid based poly(ε-caprolactone) biodegradable polymer through ring opening polymerization and encapsulation of Ag and Au NPs for biological applications.

3. Experimental Section

(a) Materials and Methods

Silver nitrate (AgNO₃), tetrachloroaurate trihydrate (HAuCl₄·3H₂O), cholic acid was purchased from SRL chemicals Pvt. Ltd. (India) and were used without additional purification. ε-caprolactone, cysteamine, and diethylenetriaminophosphonate (DEPC) were obtained from MERK chemicals. Triple distilled water was used for preparation of all solutions. HR-TEM images were obtained using a FEI-TECHNAI, G2 MODEL (T-30 S-TWIN) at an acceleration voltage of 250 kV. The samples were drop casted onto a carbon-coated copper grid and were allowed to dry at room temperature before analysis. Dynamic light scattering (DLS) measurements were performed for colloidal solutions using Nanotrac Ultra NPA 253 from Microtrac, U.S.A. UV-visible spectra of Ag and Au NPs formation were recorded on Techcomp UV-2301 spectrophotometer operated at a resolution of 1 nm. The FT-IR spectra was recorded using PerkinElmer FT-IR spectrometer (Spectrum one) with 1 cm⁻¹ resolution. The polymer was grinded with KBr to obtain pellet for recording FT-IR analysis. The morphology of the samples was analyzed by FE-SEM using a HITACHI SU6600 field emission-scanning electron microscopy. The number average and weight average molecular weight of the polymer was obtained with a Shimadzu instrument using THF as an eluent at a flow rate of 0.3 mL/min. A styragel column of pore size 103-106 Å was used. ¹H NMR spectra were recorded in CDCl₃ using TMS as an internal standard on JEOL 400 MHz NMR
spectrometer at room temperature. The TGA (Waters TA Instrument, model SDT Q600) of thiol functionalized CA-(PCL) templated Au and Ag NPs were carried out in the temperature range from 50 to 800°C under nitrogen atmosphere, with heating rate of about 20°C/min.

(b) Synthesis of thiol functionalized cholic acid

Thiol functionalized cholic acid was synthesized by adopting the following procedure. About 1.0 mmol of cysteamine was added into the magnetically stirred solution of 0.5 mmol of cholic acid (1) in dry DMF (2 mL), Et₃N (0.8 mL), and DEPC (0.6 mmol), the resulting suspension was stirred at RT for an hour. After completion of reaction the product was extracted with EtOAc, and the combined extracts were washed with double distilled water and evaporated to dryness. The residue was then refluxed for 30 min in 10 mL of 5% methanolic NaOH. The solvent was evaporated under reduced pressure, and the hydrolyzed product was dissolved in 10 mL of water and acidified with 5% H₂SO₄ with constant stirring. The precipitated solid was filtered, washed with water and dried to afford thiol functionalized cholic acid (2) with 80% of yield (Scheme-1).

(c) Synthesis and characterizations of thiol functionalized cholic acid based poly (ε-caprolactone)
The ring-opening polymerization is an efficient method for producing aliphatic polyesters with high molecular weights where the ring-opening polymerization can be initiated by active hydrogen of present in the amine and alcohol ring-opening polymerization is accelerated in presence of carboxyl groups. Here, we used OH groups of cholic acid as an initiator for the ring-opening polymerization of ε-caprolactone. The thiol functionalized cholic acid based poly(ε-caprolactone) (3), can be obtained as shown in Scheme-2.

![Scheme-2](image_url)

Scheme-2 Synthesis of thiol functionalized cholic acid based poly (caprolactone).

In this work, the polymerization was carried out under anhydrous conditions. The monomer ε-caprolactone and the initiator cholic acid were carefully dried to avoid the initiation by water molecules, which results in a mixture of poly(ε-caprolactone) and are characterized by FTIR, $^1$H NMR and GPC techniques. In FT-IR spectra, the presence of OH in carboxyl group of cholic acid moiety and OH in the terminals of the branches is supported by the band appeared around 3412 cm$^{-1}$ (Figure 1). Besides, the peaks appeared at 1519 cm$^{-1}$, 1640 cm$^{-1}$ and 1752 cm$^{-1}$.
corresponds to the stretching mode of C=O in caprolactone repeating units and CO-NH group respectively.

Figure 1. FT-IR spectrum of thiol functionalized cholic acid based poly (ε-caprolactone).

In $^1$H NMR spectra, for thiol functionalized cholic acid based poly(ε-caprolactone), the typical signals from cholic acid moiety and ε-caprolactone repeating units can be observed. $^1$H NMR: a: 0.7 ppm, b: 0.9 ppm, c: 1.2 ppm (cholic acid moiety: CH$_3$), d: 1.4-2.3 ppm (cholic acid moiety: CH$_2$, CH), e: 3.71, 3.86, 4.52 ppm (cholic acid moiety connected to ε-caprolactone repeating units: CHOOCO), and B terminal: 4.4 ppm (terminal caprolactone repeating unit: CHO). The synthesized polymer was well dissolved in THF, indicating no crosslinked microgel formed during the polymerization. These evidences confirm that the star polymer with a well-defined three-branched structure was obtained. The molecular weight of the polymer was determined by the GPC technique. The GPC trace of polymer is presented in the Figure 2. The number and weight
average molecular weight of the CA-(PCL) were 25490 g/mol and 37579 g/mol respectively, with the polydispersity value of 1.47.

Figure 2. GPC trace of thiol functionalized cholic acid based star poly (ε-caprolactone).

4. Result and discussion

(a) Synthesis of thiol functionalized cholic acid based poly(ε-caprolactone) encapsulated Au and Ag NPs

UV-visible absorption spectroscopy is one of the main techniques to examine the formation of nanoparticles in aqueous suspensions. The effect of polymer concentration on the AuNPs formation was analyzed by adding different quantities of CA-(PCL) stock solution to 50 µL of HAuCl₄ (1x10⁻³ M) in THF under UV light irradiation. The change of color gradually changed from pink to red and then purple, depending upon the amount of CA-(PCL) in the reaction mixture, which could be attributed to the changes in the size and shape of the AuNPs. The corresponding UV-visible spectra shown in Figure 3 clearly indicates that the position of the surface plasmon resonance (SPR) exhibited by AuNPs is influenced by the amount of CA-(PCL) used in the reaction mixture. At lower concentration of polymer the SPR band appeared at 556 nm becomes weak and broad, upon increasing the amount of polymer to 60 µL the intensity of SPR band
gradually increases along with small shift towards lower wavelength. Furthermore, the intensity of SPR significantly increases upon increase in the amount of polymer and it attained maximum intensity when the amount of polymer was reached to 120 µL. However, further increase in the polymer amount results to decrease in the intensity of the SPR band, and the peak becomes broader.

Figure 3 (a) UV-visible spectra of thiol functionalized CA-(PCL)-AuNPs obtained after mixing 50 µL of HAuCl₄ (1x10⁻³M) and different amount of polymer (i) 10 µL, (ii) 20 µL, (iii) 40 µL, (iv) 60 µL, (v) 80 µL, (vi) 100 µL, (vii) 120 µL, (viii) 140 µL, (ix) 160 and (x) 180 µL.

Hence, we choose 120 µL is the optimized amount for the preparation of AuNPs, because in this polymer concentration the formed particles were smaller in size and highly stable and were further supported by SEM, TEM and DLS analysis. Also, the functional groups present in the polymer acts as both reducing and stabilizing agent for the formation of AuNPs. The effect of
polymer concentration on AgNPs formation was analyzed by adding different quantities of CA-(PCL) stock solution to 50 µL of AgNO₃ (1x10⁻³ M) in THF under UV light irradiation. The change in the color of the solution from light yellow to brown, depending upon the amount of polymer, could be attributed to the formation of different size and shaped AgNPs. The corresponding UV-visible spectra shown in Figure 4 clearly indicates that the position of the surface plasmon resonance (SPR) at 420 nm clearly indicates the formation of AgNPs which was influenced by the concentration of CA-(PCL) used in the reaction mixture.

Figure 4 (a) UV-visible spectra of thiol functionalized CA-(PCL)-AgNPs obtained after mixing 50 µL of AgNO₃ (1x10⁻³M) and different amount of polymer: (i) 10 µL, (ii) 20 µL, (iii) 40 µL, (iv) 60 µL, (v) 80 µL, (vi) 100 µL, (vii) 120 µL, (xii) 140 µL, (ix) 160 and (x) 180 µL.

At lower amount of polymer the SPR band appeared at 420 nm with broad absorption, while increasing the amount of polymer the intensity of SPR band gradually increases without any shift in the λmax. However, no change in the intensity of SPR band was noted while increasing the
concentration of polymer (120 µL), hence we choose 120 µL is the optimized concentration for the preparation of AgNPs, because in this concentration the formed particles were smaller in size and highly stable. Here, also the AgNPs were formed without any extra reducing agent could be due to the functional groups present in the polymer acts as both reducing and stabilizing agent for the formation NPs.

(b) Morphological studies of thiol functionalized cholic acid based poly (ε-caprolactone) templated Au and Ag NPs.

FE-SEM analysis was used to study the morphology of polymer and polymer-nano hybrid materials. FE-SEM images of pure CA-(PCL) shows popcorn like structure (Figure 5a). Images of polymer capped AgNPs and AuNPs hybrids show that the monodispersed Ag or Au NPs were embedded homogeneously throughout the polymer matrix as shown in Fig. 5b & 5c.

Figure 5 FE-SEM images of thiol functionalized CA-(PCL) (a), CA-(PCL) encapsulated AgNPs (b) and AuNPs (c).
Transmission electron microscopy and dynamic light scattering studies provide further insight into the morphology and size details of the Ag and Au NPs. To observe the influence of the stabilizing agent in the size and shape of the AgNPs, TEM and DLS were measured at fixed amount of polymer stabilized NPs under optimized conditions. Figure 6 show the TEM images of fixed amount of polymer (120 µL) stabilized Ag and Au NPs and the results revealed that the obtained particles were almost spherical in shape.

Figure 6. TEM images and DLS histogram shows optimized amount of polymer stabilized AuNPs (a) and AgNPs (b). The inset of TEM shows corresponding SAED pattern.
The average size of the particles 23 and 19 nm for Au and Ag NPs respectively. The inset of Fig. 6 show the corresponding selected area electron diffraction (SAED) pattern obtained for the Au and Ag NPs. The Scherrer ring pattern characteristic of the face centered cubic (fcc) Au and Ag were clearly observed, showing that the structures noted in the TEM images were nanocrystalline in nature. The DLS study provides detailed knowledge about the particle dispersion, i.e. monodispersed, or polydispersed. In this study, the average hydrodynamic radii of polymer stabilized Au and Ag NPs was found to be 28.3 and 22.4 respectively. The difference in the TEM and DLS data are due to the fact that DLS measurements, records higher values, since the light scattered from both core particles as well as a layer on the surface of the NPs. Whereas in TEM measurement only the metallic particle core is measured. The formed NPs were stable for more than six months without any sign of aggregation therefore which could be used as probe for biological applications.

(c) TGA analysis of polymer capped AgNPs and AuNPs

Thermogravimetric analysis (TGA) was used to study the thermal-stability of the polymer and polymer-nano hybrid materials. The TGA curve of polymer capped AgNPs and AuNPs were measured and compared with pure polymer as shown in Figure 7. The TGA curve for pure polymer Fig. 7(curve a) shows the first drop at 84.2°C asserting the water molecules removal from the pure polymer. After reaching at 182°C showed a weight loss about 18% indicates the decomposition of thiol functionalized molecules and weakening of secondary force between the polymer chains. Upon further increase in temperature there is a gradual weight occurred could be due to the onset of degradation polymer molecules. The TGA curve for AgNPs stabilized polymer molecules in Fig. 7(curve b) shows major weight loss at 270°C could be due to the decomposition
of thiol molecules bounded on the surface of the NPs and also showed second stage at 450°C where weight loss occurred due to decomposition of polymer molecules.

**Figure 7**  TGA of the pure polymer (curve a) and polymer capped AgNPs (curve b) and AuNPs (c) obtained under nitrogen atmosphere and at a heating rate of 20°C min⁻¹.

In the case AuNPs stabilized polymer a gradual weight loss occur up to temperature 310°C could be due to the elimination of water molecules and decomposition of thiol molecules anchored on the NPs surfaces. However, above this temperature there is a gradual weight loss occur could be due to the degradation of polymer molecules. The higher degradation temperature of polymer nano hybrids compared to pure polymer indicates that the improvement of thermal stability this may be due to interaction of polymer on the NPs surface.

(d) **Antibacterial activity of thiol functionalized cholic acid based poly(ε-caprolactone) templated Au and Ag NPs.**

Antibacterial activity of various AAs stabilized AgNPs was tested against various bacterial isolates such as *Acinetobacter Sp.*, *E. coli*, *Klebsiella Sp.*, *Pseudomonas aeroginosa* and *Proteus*
using Kirby-Bauer disc diffusion method. About 30 μL of NPs was added to sterile discs (6 mm) to obtain the desired concentration of 0.02 mg/disc. The disc was allowed to dry for 5 minutes under sterile condition. Overnight grown cultures of about 1mL were centrifuged at 6,000 rpm for 10 minutes and the supernatant was discarded. To the pellet about 2 mL of sterile physiological saline was added and mixed well, adjusted to get the 0.5 OD at 580 nm. Cultures were then swapped on sterile Muller-Hinton agar surface using sterile cotton swaps. AgNPs impregnated discs were placed on the swapped surface. The zone of inhibition (mm) was measured after 24 h incubation at 37 °C. Cefazolin (CZ 30 μg) and Carbenicillin (CB 100 μg) were used as the positive standards and 30 μL of sterile water was used as the negative standard. The polymer stabilized Ag and Au NPs show very low solubility (20 μL) in aqueous medium at basic pH. Therefore we tested the antibacterial of Ag and Au NPs against all five bacteria and compared with the control.

<table>
<thead>
<tr>
<th>S.No</th>
<th>Compound</th>
<th>Acinetobacter sp</th>
<th>E. coli</th>
<th>Klebsiella sp</th>
<th>P. aeruginosa</th>
<th>P. mirabilis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AuNPs</td>
<td>16</td>
<td>18</td>
<td>15</td>
<td>19</td>
<td>14</td>
</tr>
<tr>
<td>2</td>
<td>AgNPs</td>
<td>18</td>
<td>20</td>
<td>19</td>
<td>20</td>
<td>19</td>
</tr>
<tr>
<td>3</td>
<td>+ve control (mm)</td>
<td>-</td>
<td>15</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>CZ 30 μg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CB 100 μg</td>
<td>14</td>
<td>21</td>
<td>18</td>
<td>35</td>
<td>16</td>
</tr>
<tr>
<td>4</td>
<td>-ve control (mm)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

In the present study five gram negative bacterial strains were used for antibacterial activity with equal concentration of NPs. The diameters of zone of inhibition (ZOI in mm) varied for all the test bacteria for polymer stabilized Ag and Au NPs and the results were displaced in Table-1. The results revealed that, AgNPs shows moderate to better antibacterial activity compared to AuNPs.
Conclusions

In conclusion, thiol functionalized cholic acid based poly (caprolactone) with feed ratio 1/120 was prepared through the ring-opening polymerization of ε-caprolactone initiated by hydroxyl groups present in the cholic acid. The prepared polymer was thoroughly characterized by FT-IR and NMR spectroscopy. The synthesized polymer was used as a template to prepare nearly monodispersed, spherical shaped Ag and Au NPs under UV light irradiation without any external reducing agent. The SEM studies revealed that the synthesized NPs were homogeneously embedded through the polymeric matrix. The formation of NPs was monitored by UV-visible spectroscopy and the average size of the NPs was confirmed by TEM technique which are found to be ~19 nm and ~23 nm for Ag and Au NPs respectively. The size of the NPs was further confirmed DLS technique. Thermal stability of the polymer and polymer-nano hybrid materials was studied by TGA analysis. The antibacterial studies reveals that AgNPs show better antibacterial activity compared to AuNPs. Hence, the polymer capped Ag and Au NPs could be used as potential probe for biological applications.

Acknowledgement:

The authors thank the Tamil Nadu State Council for Higher Education (TANSCHE), Government of Tamil Nadu, for their financial support. I also thank National Centre for Nanoscience and Nanotechnology (NCNSNT), University of Madras, for the HRTEM measurements.
Output of the project work:


References


