Polyelectrolyte Multilayer Film Coated Silver Nanorods: An Effective Carrier System for Externally Activated Drug Delivery

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Abstract: Nanoparticle anisotropy offers unique functions and features in comparison with spherical nanoparticles (NPs) and makes anisotropic nanoparticles (ANPs) promising candidates in applications like drug delivery, imaging, biosensing and theranostics. Presence of surface active groups (e.g. amine, and carboxylate groups) on their surface provides binding sites for ligands or other biomolecules, and hence, this could be targeted for specific part or cells in our body. In the quest of such surface modification, functionalization of ANPs along Layer-by-Layer (LbL) coating of oppositely charged polyelectrolytes (PE) reduces cellular toxicity and promotes easy encapsulation of drugs. In this work, we report the silver nanorods (AgNRs) synthesis by adsorbate directed synthetic approach using cetyltrimethyl ammonium bromide (CTAB). The formed ANPs is investigated by scanning electron microscopy (SEM) and UV-Visible (UV-Vis) spectroscopy revealing the shaping of AgNRs of 3-16 nm aspect ratio with some presence of triangles. These NRs were further coated with bio polymers of chitosan (CH) and dextran sulphate (DS) through LbL approach and used for encapsulation of water soluble anti-bacterial drugs like ciprofloxacin hydrochloride (CFH). The encapsulation of drugs and profiles of drug release were investigated and compared to that of spherical silver nanoparticles (AgNPs). The added advantages of the proposed drug delivery system (DDS) can be externally activated to release the loaded drug and used as contrast agents for biological imaging under exposure to NIR light. Such system shows unique and attractive characteristics required for drug delivery and bioimaging thus offering the scope for further development as theranostic material.

Keywords: Anisotropy, drug delivery, polyelectrolytes, silver nanorods.

![Scheme 1: Schematic illustration of PE coating on AgNRs, drug encapsulation into assembled film and release of loaded drug from film during application.](image-url)
1. INTRODUCTION

Past two decades in the design of drug delivery system (DDS) with novel functionalities is of tremendous interest for scientific communities because of their potential applications like biosensing, bioimaging, theranostics, sustained and targeted drug delivery [1]. Drug delivery carrier aims to achieving large drug encapsulation, controlled release, retained circulation in blood and target specific area at cells, so that they greatly improve the drug efficacy and reduces the drug toxicity and other side effects during application. Various nanocarriers such as liposomes, polymer capsules, micelles, emulsions and nanoparticles (NPs), have been extensively investigated and proved to be efficient DDS. In the recent times, noble metal anisotropic nanoparticles (ANPs), especially made of gold (Au) and silver (Ag), have attracted significant attention because of their unique chemical and physical properties. The unique properties such as enhanced optical near-infrared (NIR) absorption, large extinction cross sections, enhanced photothermal heating capacity, chemical stability, and biocompatibility, have made them promising therapeutic paradigm especially for imaging and cancer therapy. The most interesting feature of ANPs is the surface plasmon resonance (SPR) bands in NIR region, which induces photothermal conversion by converting the light absorption to heat when it matches with the wavelength of irradiated light. A range of 1D, 2D, 3D ANPs such as nanotubes [2], nanorods (NRs) [3], nanowires [4], triangles [5], [6], sheets and plates [7], [8], pyramids [9], [10], stars [11], [12], nanocages [13], multipods [14], [15] and flowers [16], [17], have been reported for applications ranging from electronics and sensing to bio- imaging and therapeutics.

The chemical and physical properties of NPs are greatly defined by their shape as the movement of electrons occurs in that confined space and the type of electron motion is accompanied by well defined energies. Larger space gives weaker confinement and thereby less energy separation between various types of motion [18]. The same is the case with metals which determine its insulating and conducting properties. As ANPs have large surface to volume ratios, the coherent oscillation of free conduction band electrons or SPR results in intense absorption at NIR range. Plasmon absorption maxima and bandwidth of ANPs is greatly influenced by size, shape and composition [19]. Among the reported ANPs, NRs were extensively used for biological applications as it has absorption peaks at the biological window (650 - 900 nm) where tissues and blood components absorb light very low.

The synthesis of NRs have prepared by different methods namely electrochemical [20], photochemical [21] or by seed mediated synthesis [22], [23]. The parameters like nature of seed and surfactant, concentration of seed, and presence of charged species like ions play a key role in the formation of NRs and its aspect ratio [24], [25]. The presence of surfactant layer on NR surfaces not only useful in stabilizing NP suspension but is also useful for the biomolecules entrapment and chemotherapeutic agents used in the targeted cancer therapy. Cetyltrimethyl ammonium bromide (CTAB) is most widely used surfactant for NR synthesis and provides hydrophobic characteristics to the resulted NRs. As a result, hydrophobic model drug 1-naphthol was successfully loaded over NRs surfaces (14.6 ± 2.2 x 10^3 molecules per AuNR).
Despite of all the advantages, the issues such as surfactant cytotoxicity and burst release of loaded therapeutics limit in the drug delivery applications using NRs [27], [28]. To circumvent these limitations, the possibility of using NRs coated polyelectrolytes (PEs) via layer by layer (LbL) assembly. PE coating improves solubility in water and forms stable aqueous suspension could be directly used without any prior modifications. Furthermore, it is helpful in the encapsulation of drugs and targeting by providing binding site for biomolecules through covalent and non-covalent interactions. The release of loaded molecules is induced by either concentration gradient based diffusion process or rupture of PE film due to heat generated under the exposure to NIR light [29]. The other advantage of NRs based DDS applied NIR lights useful for both drug delivery and imaging in in-vivo applications. For instance, AuNRs were coated with poly (diallydimethylammonium chloride) (PDDAC) and polyethyleneimine (PEI) were used for for HIV treatment by encapsulation of adjuvant-DNA vaccine [30]. NR based formulations have also been reported for respiratory syncytial virus (RSV) and HIV treatment [31]. Wang et al., demonstrated successful release of loaded doxorubicin (Dox) from polyethylene glycol (PEG) and polyacrylic acid (PAA) coated AuNRs in prostate cancer cells [32]. In-vitro cytotoxicity evaluation with breast cancer cells (MCF-7) showed enhanced therapeutic efficiency for AuNR-PSS-Dox when compared to Dox free complex[33].

The driving force for drug encapsulation is the electrostatic interaction between the drug molecules and polyelectrolytes adsorbed on NRs surfaces. Encapsulation ranging from 10 to 50% has been achieved [32]-[34], which is influenced by charge density of drugs and number of deposited layers [34]. Upon irradiation of light, controlled release of 80% Dox was observed in 30 min [34]. Though there have been reports on silver nanorods (AgNRs) as imaging tools using NIR absorption and ligand functionalization but only handful of reports are present for NRs being efficient DDS. In this paper, we report seed mediated synthesis of AgNRs using CTAB as capping agent. The NR formation was investigated by UV-Vis spectroscopy and SEM. NRs were obtained and coated with bio polymers like dextran sulphate (DS), chitosan (CH) and used for encapsulation, release of water soluble anti-bacterial drug, ciprofloxacin hydrochloride (CFH). The profiles of encapsulation and release were investigated by UV-Visible spectroscopy and the results obtained were compared to that of spherical silver nanoparticles (AgNPs). As PE coating reduces cytotoxicity issues, the use of such drug delivery systems can be extended to in-vivo applications. This work will not only expand the knowledge on the preparation of NR based DDS, but also would benefit the application of externally activated drug delivery.

2. EXPERIMENTAL SECTION

2.1 Materials

Dextran sulphate (Mw > 500,000 Da), chitosan (Mw – 50,000-190,000 Da), CTAB, L-ascorbic acid were purchased from Sigma Aldrich, India and CFH, silver nitrate (AgNO3), sodium borohydride (NaBH4), sodium citrate tribasic, sodium hydroxide (NaOH), sodium chloride
(NaCl), acetic acid were obtained from SRL Pvt. Ltd., India. All chemicals were used as received. Milli-Q water was used for all the experiments. Stocks of all the chemicals were freshly prepared.

2.2 Synthesis of spherical AgNPs

Spherical AgNPs of 20 nm size were prepared by a co-reduction approach as reported elsewhere [35]. In brief, a mixture of 24 mL of 1 mM NaBH₄ and 24 mL of 3.5 mM sodium citrate tribasic was taken and heated at 60°C for 30 min in dark with vigorous stirring. Thereafter, 2 mL of 1 mM AgNO₃ was added drop wise and temperature was increased to 90°C. After reaching 90°C, the pH of the solution was adjusted to 10.5 using 0.1 M NaOH and heating was continued till there was a change in color (maximum of 20 min). The co-reduction occurs in two steps: first, the reduction of silver nitrate at 60°C by NaBH₄ which resulted in new silver nuclei, and second, where Ag ion reduction occurred at 90°C by sodium citrate tribasic. The continuous addition of silver atoms formed at the second step on the surface of nuclei, resulted in growth of nuclei into larger AgNPs. The prepared AgNPs were washed three times with water by centrifugation at 12000 rpm for 15 min, and purified spherical AgNPs were redispersed in DI water and stored at 4°C.

2.3 Synthesis of PE coated spherical AgNPs

Polymer solution of DS and CH (1 mg/mL) was used for LbL assembly. First, the synthesized AgNPs were incubated in 2 mL of CH solution and incubated for 15 min at pH 5. After CH adsorption, the NPs were separated out by centrifugation and washed three times with water to remove unbound excess polymer. Then, the NPs were dispersed in DS solution for coating of second layer of DS. The centrifugation and washing procedure was repeated again. In this way, NPs with two polymer layers (NP-CH/DS) were prepared and used for encapsulation and release experiments. Since the synthesized NPs have negative zeta potential values due to adsorbed citrate molecules, the LbL assembly was started with CH. Two kinds of samples (as prepared NPs, NP₁; and NPs with three polymer layers, NP₂) were prepared and used.

2.4 Synthesis of AgNRs

AgNRs were prepared by seed mediated approach using CTAB as reported elsewhere [23]. For preparation of seeds, 20 mL solution of 0.25 mM AgNO₃ and 0.25 mM sodium citrate tribasic in water was prepared. While stirring the mixture vigorously, 0.6 mL of 10 mM NaBH₄ was quickly added and stirring was then stopped. The prepared seed (3-4 nm) were used after 2 h for synthesis of AgNRs. For preparation of AgNR growth solution, 10 mL of 80 mM CTAB, 0.25 mL of 10 mM AgNO₃ and 0.5 mL of 100 mM L-ascorbic acid was prepared and mixed with varying volume of seed solution (2, 1, 0.5, 0.25, 0.125 and 0.06 mL) to investigate the
formation of NR as a function of seed solution. Lastly, 0.1 mL of 1M NaOH was added that leads to the formation of AgNR. The particle formation is visualized by color change from orange to red to grey which is depending on the size of NRs. As the synthesis also resulted in formation of spherical NPs, the suspension was centrifuged at 2000 rpm for 6 min to separate NRs from the suspension. The synthesized AgNRs were washed and redispersed in DI water for further use. For all the encapsulation and release experiments, least seed concentration (0.06 mL) was used.

2.5 Synthesis of PE coated AgNRs

Polymer solution of DS and CH (1 mg/mL) was used for LbL assembly. First, the synthesized AgNRs were incubated in 2 mL of DS solution and incubated for 15 min at pH 5. After DS adsorption, the NPs were separated out by centrifugation and washed three times with water to remove unbound excess polymer. Then, the NRs were dispersed in CH solution for coating of second layer of CH. The centrifugation and washing procedure was repeated again. In this way, NRs with one polymer layer (NR-DS) and NRs with three polymer layers (NR-DS/CH/DS) were prepared and used for encapsulation and release experiments. Since the synthesized NRs have positive zeta potential values due to adsorbed CTAB molecules, the LbL assembly was started with DS. Two kinds of samples (NRs with one polymer layer, NR$_1$; and NRs with three polymer layers, NR$_2$) were prepared and used.

2.6 Encapsulation of ciprofloxacin hydrochloride

Encapsulation was performed by incubating PE coated AgNRs and AgNPs with 2 mL of CFH solution (3 mg/mL) at pH 5 for 1 h at room temperature. After incubation, the suspension was centrifuged at 8000 rpm for 20 min. The amount of drug present in the supernatant (un-encapsulated drug) was estimated by measuring the absorbance at 276 nm. The amount of encapsulated drug (actual drug loading) was directly calculated by subtracting free drug present in the supernatant from the original amount used for drug encapsulation. Encapsulation efficiency (EE%) was calculated by following equation:

$$ EE\% = \frac{ActualDrugLoading}{TheoreticalDrugLoading} \times 100 $$

2.7 Release of ciprofloxacin hydrochloride

The *in-vitro* drug release study from PE coated AgNRs and AgNPs was performed by incubating drug loaded NPs in 2 mL of Milli-Q water. The release was performed as a function of time. By measuring the absorbance value at 276 nm, the drug release as a function of time was estimated. At every 1 h, 1.4 mL of supernatant was withdrawn and replaced with fresh
water to favor the release by maintaining the concentration gradient.

2.8 UV-Visible Spectroscopy

The drug encapsulation and release experiments were investigated by UV-Visible spectrophotometer (Nanodrop 2000c Spectrophotometer, Nanodrop Technologies, U.S.A.). Milli-Q water was used as blank. Nanoparticle suspensions filled in quartz cuvette were directly used to acquire UV-Visible spectra.

2.9 Size and Zeta Potential measurements

The zeta potential of AgNRs and AgNPs were measured by Zetasizer (Malvern Nano ZS90, Malvern Instruments, UK) based on dynamic light scattering principle. Diluted particle suspensions were directly used for measurement.

2.10 Scanning Electron Microscopy (SEM)

SEM images were obtained using Zeiss scanning electron microscope, Germany, operating at 10 kV. Samples were prepared by overnight drying of a drop of sample suspension on silicon substrate followed by gold sputtering.

3. RESULTS AND DISCUSSION

3.1 Synthesis of AgNRs

The AgNRs synthesis in suspension has always been a challenging task and several methods have been developed to synthesize NRs with good control over the NR size and aspect ratio. In this work, AgNRs were prepared through seed mediated synthesis using CTAB as shape directing agent. The synthesis is a two step process, in which seed crystals that are obtained by reduction of AgNO$_3$ with the use of NaBH$_4$ (strong reducing agent), undergo regrowth process in the presence of CTAB (structure-directive additives) and result in AgNRs. In the first step, NaBH$_4$ was used as reducing agent for the formation of seeds whereas ascorbic acid (weak reducing agent) was used as reducing agent in the second step to grow the seeds into rods. Figure 1 shows the SEM images of synthesized seed crystals and NRs. Notably, the seed crystals are about 4 nm in size, which resulted in NRs of length 400 nm in growth solution. It can be seen that the NRs are uniformly distributed in the sample and have the aspect ratio of about 16 ± 2 nm. It is noteworthy that some fraction of other shapes like spheres and nanotriangles were also observed along with the NRs, which could be neglected as their number was very less. To separate the NRs from the mixture, the suspension was centrifuged at 2000
rpm for 6 min. The aspect ratio as well as the size of NRs can be easily changed by varying the volumetric composition of the reaction mixture. To gain more detailed information about the role of seed volume on NR size and aspect ratio, the seed volume was varied from 0.06 - 2 mL. When the seed volume was increased, the NRs became shorter with aspect ratio varying from 16 ± 2 nm (figure 1d) to 9 ± 2 nm (figure 1c) and 3 ± 1 nm (figure 1b) as shown in SEM images. As a result, the diameter of NRs started to increase continuously as a function of seed volume. In other words, the aspect ratio of the NRs is inversely proportional to the seed volume. Figure 2 shows the UV-Visible spectra of seed crystals and NRs formed. Notably, the spectrum of seed shows one characteristic absorption peak at 390 nm. On the other hand, UV-Visible spectra of AgNRs showed both the conventional transverse band of spherical NPs at 400 - 450 nm and longitudinal plasmon band of AgNRs at 600-700 nm [19]. As the seed concentration was decreased, the aspect ratio of formed NRs increased, which could be seen by visible color changes of NR suspension (figure 3). The absorption spectra of AgNR obtained from different growth solutions reveals the shift of second peak to higher wavelength (red shift) with the increase in aspect ratio of NR as shown in figure 2c. The zeta potential of the NRs was estimated about 20 ± 3 mV, which is due to the presence of CTAB on the surface of the NRs [33].

Figure 1: SEM images of (a) Ag Seeds, (b) AgNRs (2 mL seed volume), (c) AgNRs (0.5 mL seed volume) and (d) AgNRs (0.06 mL seed volume).
Figure 2: UV-Visible spectra of synthesized seeds, NRs and spherical NPs. (a) Silver seed, (b) Silver nanorod aspect ratio (16 ± 2) used for PE coating and drug release study, (c) red shift of spectrum as a function of seed concentrations, and (d) Spherical silver nanoparticle.

Figure 3: Seed solution and aqueous solutions of AgNRs with visible colour changes depending on the aspect ratio.
3.2 Synthesis of spherical AgNPs

The spherical AgNPs were prepared by two step reduction method, wherein small nuclei formed in the first step undergo growth process and resulted in spherical AgNPs. The UV-Visible spectrum of spherical AgNPs shows a sharp extinction peak at 394 nm wavelength indicating the formation of spherical AgNPs (figure 2d) [35]. Figure 4 shows the TEM image of synthesized AgNPs. It can be seen that NPs of 20 nm in size were uniformly distributed. Zeta potential measurement shows that the synthesized NPs have the zeta potential of $-25 \pm 2$ mV. As synthesis involves the utilization of citrates for NP synthesis, the resulted NPs have the negative zeta potential [35].

3.3 Preparation of PE coated AgNPs and AgNRs

Biopolymer were chosen for LbL assembly as it improves the biocompatibility of NRs and NPs which is beneficial for drug targeting and cell internalization processes in future studies. The polyelectrolyte coating not only improves aqueous stability and biocompatibility of synthesized NPs and NRs, but also provides binding site for attaching drug and other biomolecules. As previously explained, AgNPs were negatively charged whereas AgNRs were positively charged due to the adsorbed monolayers of citrate and CTAB on its surface respectively [33], [35]. Hence, the LbL assembly was started with CH and DS coating as first layer for NPs and NRs, respectively. Assembly was performed at pH 5 which maintained both the polyelectrolytes at the fully charged state (p$K_a$ value of DS, $< 2.5$; p$K_a$ value of CH, 6.5) [36]. Alternative deposition of CH and DS on the surface of NRs and NPs resulted in polyelectrolyte coated NRs and NPs. Scheme 1 shows the schematic illustration of polyelectrolyte coating on synthesized NRs, drug encapsulation into the assembled film and release of loaded drug from the film during the application. Zeta potential measurements were performed after each layer of deposition to investigate the layer growth on the NP surfaces. Figure 5 shows the zeta potential as function of number of layers. The change of zeta potential values from positive to negative or vice versa after each layer of polymer coating confirms the successful polymer deposition on the surfaces of NRs and NPs. The zeta potential values of $> 25$ mV shows that the polymer coated NRs or NPs are stable in the suspension.
Encapsulation studies

Drug encapsulation was performed by incubating PE multilayer coated NRs and NPs in 3 mg/mL of CFH solution for 1 h. The antibiotic drug, CFH is a small water soluble drug ($M_W$ 385.8 g mol$^{-1}$), which undergoes protonation at lower pH values ($pK_a$ 1 = 6.09 for carboxylic acid group; $pK_a$ 2 = 8.74 for nitrogen on piperazinyl ring), and thus, is easily encapsulated into the polyelectrolyte multilayer film that rely on electrostatic interactions. The advantage of using
Ciprofloxacin for drug delivery is that it has a lower inhibitory concentration, and hence, minimum amount of released drug from the film is enough to induce desired therapeutic effect. The amount of loading was estimated using UV–Visible spectroscopy, by measuring the absorbance difference in the supernatant prior to and after loading. The encapsulation was performed at pH 5.0, at which, both the film and CFH are highly charged. The fact that the loading conditions lay in between the pKa values of DS and CFH, it facilitates higher encapsulation via electrostatic interactions [37]. Previous studies showed that the encapsulation performed under such conditions improved the amount of encapsulation efficiency significantly [37]-[39]. As electrostatic interactions were involved in the drug encapsulation process, particles with negative surface potentials (NP₁, NP₂, NR₁ and NR₂) were used for drug encapsulation process. Notably, the zeta potential values of both NRs and NPs changed to positive values after drug encapsulation (figure 5), which corroborate well with literature data that the encapsulation is driven by electrostatic interactions. The PE coated NRs and NPs showed excellent loading of CFH. The average total loading of NR suspension was estimated to be around 1500–1600 µg for 1 mL of suspension. The estimated encapsulation efficiency was 56.2% for 1 layer coated NR₁ and 54.1% for 3 layer coated NR₂. For spherical NPs, the average total loading was estimated to be around 1200–1300 µg for 1 mL of suspension. The estimated encapsulation efficiency was 43.3% for NP₁ and 40.43% for 2 layer coated NP₂. A high encapsulation efficiency of AgNR can be attributed to their larger surface area and higher surface energy. Notably, the layer thickness of 1 bilayer does not influence the encapsulation efficiency. Thus, it can be concluded that PE coated NPs and NRs can be used as drug reservoir for encapsulation of water soluble drugs.

3.5 In-vitro drug release studies

The release kinetics of CFH from AgNRs and AgNPs was studied as a function of time by dispersing them in water at pH 7.4. The amount released was estimated by measuring the absorbance increase in supernatant using UV–Visible spectrophotometry. The drug release as a function of time is shown in figure 6. In all the cases, the release was found to occur in two phases; initial burst release followed by a sustained release over a period of 12 h. The burst release in the first 2 h is due to faster diffusion of surface bound drug molecules from higher to lower concentration in the bulk solution. The total release at the end of 12 h was estimated to be about 78%, 83%, 89% and 90% for NR₁, NR₂, NP₁ and NP₂, respectively.
Figure 6: a) Cumulative release of drug as a function of time and b) decrease in supernatant concentration as a function of time.

4. CONCLUSION

We have demonstrated a sustained drug delivery system based on polyelectrolyte multilayer coated AgNRs for encapsulation and release of water soluble drugs. Spherical AgNPs and AgNRs were prepared through co-reduction and seed mediated approaches, respectively. The decrease of seed volume resulted in longer NRs. AgNRs of aspect ratio ranging from 3 to 16 were synthesized by varying the seed volumetric composition. Polyelectrolyte multilayer coated NRs were used as carriers for encapsulation of antibacterial drug, ciprofloxacin hydrochloride. The average total loading was estimated to be around 1500–1600 µg for AgNRs and 1200–1300 µg for spherical AgNPs. Two phase release was observed, initial burst release followed by a sustained release up to 12 h. As NRs are emerging as contrast agents for bioimaging as well as carriers for drug delivery, drug delivery system demonstrated here has great potential for the use in targeted and external activated drug delivery, which we will further exploit in the near future. The detailed information obtained from the current study will be highly valuable for the design of NR based drug delivery system for cancer therapy.

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