Organized Shells on Clay Nanotubes for Controlled Release of Macromolecules

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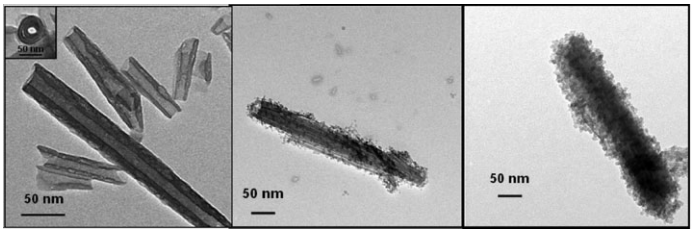
The use of tubular halloysite clay as a nanotemplate for layer-by-layer (LbL) shell assembly and its utilization for controlled release of drug macromolecules are studied. The LbL nanoshell allowed additional control for the sustained release of drug loaded halloysite tubes. The number of polymeric layers in the shell and molecular weight of the assembled polymers influences the drug release rate. Three bilayer shells of chitosan and gelatin of 15 nm thicknesses gave the best encapsulation and retardation in the release rate of dexamethasone. An encapsulation of the macromolecules inside the lumen of the biocompatible clay nanotubes coupled with the polyelectrolyte shell formation provides a novel formulation for the controlled release of bioactive agents.



Introduction

In recent years nanoscale formulations allowed for essential progress in pharmaceuticals. Nanoparticle drug formulation combined with the design of nano-containers for controlled release, targeting, and making availability of low soluble drugs are in development.[1–3] Bio-responsive or biomimetic materials used to create drug delivery systems typically include synthetic polymers and natural materials such as lipids, polysaccharides and proteins. One such nano/sized delivery system is naturally available clay nanotubes called halloysite. Halloysite is tubular aluminosilicate clay of 15 nm inner lumen diameter and 0.5–1 μm in length (Figure 1). Tubes are formed by the rolling of two layered aluminosilicate kaolin due to the strain caused by lattice mismatch between the adjacent sheets of silicon dioxide and aluminum oxide.[4,5] Halloysite is a cheap natural material excavated from deposits in hundred tons.[6] Halloysite nanotubes are biocompatible as shown in cell growth experiments.[7] Halloysite is a promising nanomaterial due to its tubule structure and capability for macromolecule sustained release from its lumen. The modification of electrical and physicochemical properties of halloysite particles was also achieved by surface coating with metals, for example, using the electroless plating technique to deposit a thin nickel film.[6] Due to their narrow lumen and chemically active external and internal surfaces, halloysite particles can be used as a cheap nano-container for the controlled release of active agents. Halloysite tubes have silicon dioxide external and aluminum dioxide internal surfaces. This chemical difference results in a negatively charged outer surface and positively charged inner lumen surface at pH

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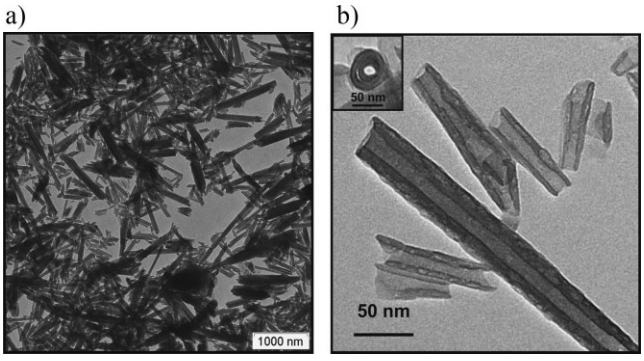

Figure 1. TEM images of halloysite nanotubes: (a) and with cross-section inserted (b).

range 2–8.[8] The negative charge on the outer surface was used in the production of halloysite multilayer films by layer-by-layer (LbL) nanoassembly.[9] The positively charged inner surface was used to fill the lumen with negative macromolecules including biocides, pharmaceuticals, enzymes, and other chemically active agents, e.g., anticorrosion for protective coating, for processes which benefit from their sustained release.[10–12] We earlier reported the use of halloysite nanotubes for entrapment and subsequent slow release of three drugs: nifedipine (anti-anginal), furosemide (antihypertension), and dexamethasone (corticosteroid).[8] The charge difference in outer and inner surfaces of halloysite was recently exploited for loading benzotriazole (corrosion inhibitor) and incorporation of loaded halloysite tubes into the sol–gel coating.[13] Usually, materials were loaded into halloysite from water, alcohol, or acetone solutions, or a molten state by using vacuum pulling. Typical macromolecule release time from halloysite nanotubes was 5–10 h. In attempts to reduce the release rate, the solvent viscosity in the lumen was increased by the addition of glycerol or polyvinylpyrrolidine.[5] Presently there is no general method for control and adjustment of release from halloysite nanotubes.

In this paper we elaborated an LbL assembly of polyelectrolyte multilayer shells on clay nanotubes and thus decreased the release rate of dexamethasone by four times: from 7 h release for bare clay tubes to 30 h for polyelectrolyte coated tubes. LbL assembly of the shell on the tubes allows thickness tunability in the nanometer range, control over permeability, modification of the surface properties of similar bulk materials, and is a simple water-based process, which makes this process inexpensive and effective.[14,15] Biocompatible chitosan and gelatin multilayers were used for the encapsulation of halloysite tubes loaded with dexamethasone. For the first time, we utilized 50 nm tubes as a template for polyelectrolyte shell assembly. The current majority of LbL encapsulation works was performed on isometric nanocores, such as latex spheres or drug microparticles.[16–19] An earlier paper[20] described LbL coating on lipid microtubules which were much larger than halloysite nanotubes.

Experimental Part

Processed halloysite was obtained from Atlas Mining Corporation (USA) and used without further treatment. Polyelectrolytes of different molecular weight: poly(allylamine) hydrochloride (PAH), $\overline{M}_w$ 70 000 and 15 000, sodium poly(styrene sulfonate) (PSS), $\overline{M}_w$ 70 000 and 32 000; poly(ethyleneimine) (PEI), $\overline{M}_w$ 1 100, and poly(acrylic acid) (PAA), $\overline{M}_w$ 5 200; were purchased from Sigma–Aldrich (USA). Natural polyelectrolytes: chitosan ($\overline{M}_w$ 1 MDa gelatin from bovine skin (gelatin-B, $\overline{M}_w$ 70 000), and silica nanoparticles (7 nm diameter) were also purchased from Sigma–Aldrich. The drug dexamethasone was purchased from Spectrum Chemicals and Laboratory Products, CA (USA). Dexamethasone has a molecular weight of 341 Da and is negatively charged at pH 6.5.[8]

LbL assembly on halloysite was performed following a traditional procedure as described elsewhere.[16–19] Negatively charged halloysite tubes were used as a template for the first step adsorption of cationic PAH ($\overline{M}_w$ 70 000 and 15 000), PEI ($\overline{M}_w$ 2 000), and chitosan ($\overline{M}_w$ 1 MDa). At the following step, an anionic PSS ($\overline{M}_w$ 70 000 and 32 000), PAA ($\overline{M}_w$ 5 100), gelatin-B ($\overline{M}_w$ 70 000), or silica nanoparticles were deposited. The assembly was performed at pH 6.5 in order to achieve strongly charged species for all the polycations and polyanions used. Typically, polycations (0.75 mL of 3 mg $\cdot$ mL^{-1} and polyanions 0.75 mL of 3 mg $\cdot$ mL^{-1} in DI water) were alternatively added into 0.75 mL of halloysite suspension in water containing 10 mg of nanotubes. Two intermediate water washes were performed between the adsorption cycles with centrifugation clay sedimentation at a speed of 5 000 rpm for 5 min (Eppendorf 5804R Centrifuge). For LbL encapsulation of the drug loaded halloysite the same procedure was used, and total shell assembly time did not exceed 1 h. In this work, for the first time, we used clay nanotubes of 50 nm diameter as a template for polyelectrolyte assembly. The majority of earlier studies of LbL encapsulation works were performed on isometric nanocores.[19]

The assembly of polyelectrolyte layers on halloysite tubes was monitored by controlling the surface ξ-potential alternation after deposition of each layer using a ZetaPlus Potential Analyzer (Brookhaven Instruments Corp). The coating was confirmed by TEM images of the shells with terminated layers of silica nanoparticles (Zeiss-Libra TEM, 120 kV).

Loading of halloysite with dexamethasone was performed from saturated drug solution as described earlier.[5,8] In the first step, 100 mg of halloysite was mixed with 5 mg of dexamethasone and made into paste by adding 50 μL of DI water. This slurry was sonicated for 30 min and vacuumed. Pumping out was performed three times until the fizzing stopped due to complete air replacing in the lumen. Finally, the tubes were washed twice with water (with centrifugation separation at 5 000 rpm for 1 min at room temperature) and vacuum dried. The loading of the drug into tubes was confirmed by porosity measurements using mercury porosimeter (micromeretics). 100 mg of dry halloysite powder (loaded or unloaded) was taken, and mercury was forced through the pores of the sample by slowly increasing its pressure up to

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30 000 psi (≈2 000 atm). At each stage of the pressure increase, the amount of mercury intruded into the pores gave the pore volume.

In vitro cytotoxicity testing is one of the important biological test methods for checking the biocompatibility of a material as per ISO-10993 standards. For cytotoxicity testing two types of cells were used, one is 3T3 fibroblast cells and the other is human breast cancer MCF-7 cells. Each type of cell was grown separately at 37 °C in a humidified atmosphere (95% air and 5% CO_2), Dulbecco's Modified Eagle Medium (DMEM) supplemented with phenol red, 3% L-glutamine, penicillin (100 $U \cdot mL^{-1}$), gentamycin (100 $\mu g \cdot mL^{-1}$), and 10% fetal calf serum. Briefly, each type of cell was separately seeded approximately 10 000 cells/well into a flat bottomed 96-well plate. Halloysite (both coated and uncoated) was added separately in three different rows around 100 $\mu g \cdot mL^{-1}$ of the medium. For positive control some rows were left untreated and for negative control some rows were treated with cytotoxic levels of NaCl. After certain time of incubation, the plate was washed and reagent (CellTiter 96 from promega) was added. The resulting change in color was read on a plate reader at 490 nm. The percentage of live cells was calculated based upon initial number of cells seeded.

The in vitro dexamethasone release studies were performed using 2 mL centrifuge tube. 10 mg of drug loaded halloysite were suspended in 1.5 mL of DI water at pH 6.5 with continuous stirring. Samples (750 μL) were collected from the supernatant after centrifuging every 10 min at the beginning of the release and every half hour later. We collected a series of ten process samples total, for each release study. Concentration of the dexamethasone in the collected samples was determined by absorption values at 240 nm with UV–Vis spectrophotometer (Agilent-8453). Every release curve was drawn based on the average results of three experiments. Finally, the total amount of drug loaded was calculated based on complete dexamethasone release after 1 h sonication of halloysite.

Results and Discussion

First, we established halloysite as a nanotubular template for LbL assembly by sequentially adsorbing layers of polyelectrolytes of different molecular weights (small: $\overline{M}_w$ 2–5 kDa, medium: $\overline{M}_w$ 15–32 kDa, high: $\overline{M}_w$ 70 kDa, and very high: $\overline{M}_w$ 1 MDa), with a final layer of silica nanoparticles. We then applied LbL assembly to the nanotubes loaded with dexamethasone to study the influence of shell composition on the rate of drug release.

The thickness of polymer/nanoparticle bilayers on solid surface was monitored in separate experiments with Quartz Crystal Microbalance (QCM, USI-System, Japan, 9 MHz silver plated electrodes) using experimental scaling: ΔL (nm) $= -0.016\ \Delta F$ (Hz).[16b] The bilayer thickness of hydrated PEI/PAA as well as PAH/PSS was approximately 2.5 ± 0.5 nm. An average thickness of 7 nm diameter silica layer was deposited sequentially with polycations, gave 6 ± 1 nm thickness increment. Typically, shells of four polycation/polyanion bilayers correspond to ca. 10 nm coating thickness in a hydrated state.

The ξ-potential of alternating halloysite surfaces for each sequential layer adsorbed is shown in Figure 2. Beginning with the third step, one can see a symmetric alternation of the surface potential with positive values approximately +60 mV and negative values approximately −30 mV corresponding to sequential polycation/polyanion deposition steps. No discernible difference in the process was found for differing molecular weights or polyelectrolytes. An additional silica nanoparticle coating on the outermost positive layer reduced ξ-potential to −25 mV. Polycation PEI provided higher positive ξ-potential as compared with PAH. It is interesting to compare the fully extended length of these polycations: for linear PEI 2 000 $\overline{M}_w$ corresponds to polymerization degree 50 and extended length of ca. 20 nm, PAA length is of ca. 32 nm, for PAH of 70 000 $\overline{M}_w$ polymerization is 800 and the length is ca. 240 nm, and for PSS of $\overline{M}_w$ 70 000 the length is ca. 120 nm. Therefore, in the case of PEI and PAA, polyelectrolytes adsorbed evenly on the tube surface which are much larger (tube diameter 50 nm, and circle length is $\pi D = 157$ nm). In the case of higher molecular weight PAH and PSS, polymer length is larger than tube diameter, and one may expect one of the following scenarios: (i) the polymer is winding around each tube, or (ii) a polymer is not closely bound to the surface and produces loops encompassing several tubes, leading to aggregation. An analysis of the shell TEM images provides evidence for the first scenario, but there is also evidence for the second scenario that some tubes are aggregated. The TEM images of the PAH/PAA shells with silica finishing are shown in Figure 3. A uniform coating of silica nanoparticle adsorbed to the outer cationic LbL layer is observed both for the first and second layer of nanoparticles/polycation

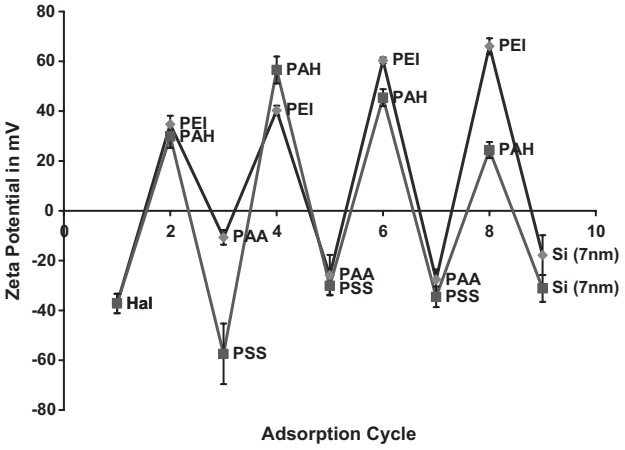

Figure 2. ξ-Potential readings for LbL coating halloysite through sequential polycation/polyanion adsorption process (PEI $\overline{M}_w$ 2 kDa, PAA $\overline{M}_w$ 5 kDa, and PAH $\overline{M}_w$ 70 kDa, PSS $\overline{M}_w$ 70 kDa).

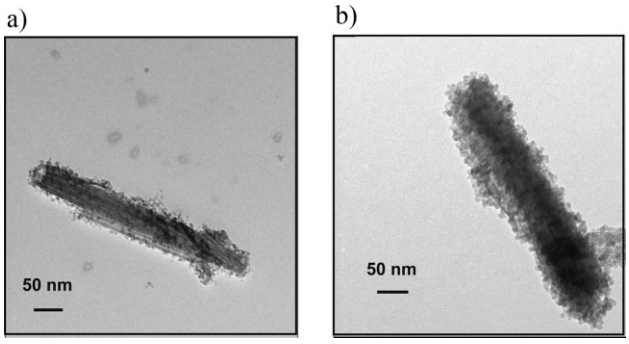

Figure 3. Transmission electron microscopy image of (PEI/PAA) 2.5 shell assembly with 7 nm silica outermost (a,one silica layer; b, two silica layers, PEI of 2 000 $\overline{M}_w$, and PAA of 5 100 $\overline{M}_w$).

coatings demonstrating an even encapsulation of the tubes and sealing off their openings (Figure 3b). Total thickness of the coated tubes in the Figure 3b is ca. 90 nm which corresponds to a 50 nm tube diameter and a 20 nm composite layer on every side consisting of a 10 nm polymer plus a 12 nm double silica layer. These are estimated values with an error of ca. 10%.

In order to confirm the loading of dexamethasone by the procedure described above, the porosity of the dexamethasone loaded halloysite nanotubes was compared with the porosity of the unloaded tubes. Figure 4 shows reduction in the pore volume for the dexamethasone loaded nanotubes. Quantitatively, the porosity of the halloysite was reduced from 68 ± 1% in the unloaded state to 50 ± 1% after loading dexamethasone. The measured porosity was a summation of lumen volume porosity and porosity due to the stacking of halloysite in the form of a tablet. However, one can see that the characteristic maximum at 50 nm relates to the lumen volume, which almost disappeared after tube loading. From UV experiments, the dexamethasone loading was estimated at 7 vol.-% which is approximately the lumen volume (it is ca. 10% of the total tube volume as follows from simple geometric estimations taking into account that D ext = 50 nm and D int = 15 nm).

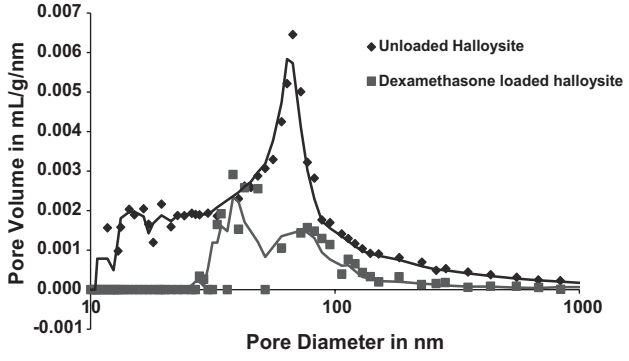

Figure 4. Porosity test by mercury porosimeter of halloysite (loaded and unloaded).

In the next step, encapsulation of the dexamethasone loaded halloysite was studied with the optimization of the shell composition: (i) using 1, 2, 6, and 9 polycation/polyanion bilayers in the coating; (ii) using multilayers of polyelectrolytes consisting of small, medium, and large molecular weights; and (iii) using natural biocompatible polyelectrolytes. First, the effect of the number of adsorbed layers on the release rate of dexamethasone was studied using several bilayers of low molecular weight PEI/PAA. The release profiles of dexamethasone from the 1, 3, 6, and 9 LbL bilayers shells were compared with uncoated drug loaded halloysite. A marginal retardation in the release rate was observed in 1–3 bilayer coated samples as compared to uncoated halloysite. In the case of 6 and 9 bilayer coated samples the release rates of dexamethasone increased from 2 to 5 h. For multiple step coatings we observed a large loss of dexamethasone during the shell assembly. One may conclude that an optimal coating thickness is three bilayers, and then test the influence of polyelectrolyte molecular weight on the shell permeability. Sufficient retardation achieved for three bilayers might not be due to the high molecular weight of polymers used for encapsulation, and thus may be connected with the possible penetration of these polyelectrolytes into the tube lumen.

To confirm this hypothesis we studied the effect of molecular weight of the adsorbed polymers on the rate of drug release. Three variations of the molecular weight range have been studied: low molecular weight polymers PEI ($\overline{M}_w$ 2 000)/PAA ($\overline{M}_w$ 5 100), medium molecular weight polymers PAH ($\overline{M}_w$ 15 000)/PSS ($\overline{M}_w$ 32 000), and high molecular weight polymers PAH ($\overline{M}_w$ 70 000)/PSS ($\overline{M}_w$ 70 000). The release profiles of dexamethasone from the three polyelectrolyte bilayer coated tubes with different

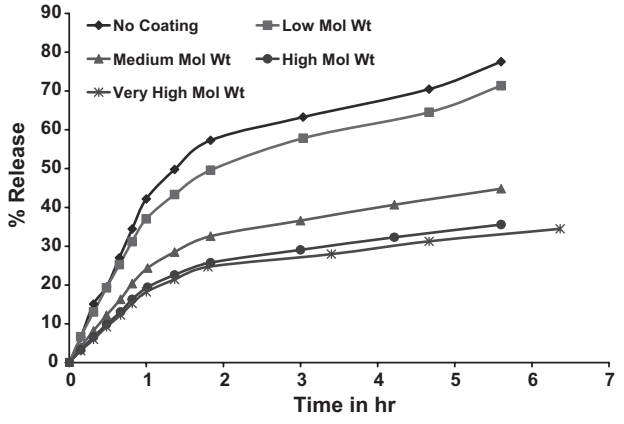

Figure 5. Release profiles of dexamethasone from the three bilayers LbL shelled halloysite depending on the polyelectrolyte molecular weight. The upper line is halloysite without coating, below (PEI 2 kDa/PAA 5 kDa)$_3$, next with large interval (PAH 15 kDa/PSS 30 kDa)$_3$, following (PAH 70 kDa/PSS 70 kDa)$_3$ and the bottom line is (chitosan 1 MDa/gelatin 70 kDa)$_3$.

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molecular weights show an increase in the retardation of the drug for the same number of layers (Figure 5). This increase can be attributed to more dense packing and higher stability of the formed shell. Therefore, higher molecular weight polyelectrolytes provided both better encapsulations combined with a lower release rate.

As the next optimization step, we used natural biodegradable polymers for the shell formation to study its influence on release rate. We have chosen very high molecular weight natural polymers chitosan ($\overline{M}_w$ 1 MDa) and gelatin B ($\overline{M}_w$ 70 000). ξ-Potential alternation for the subsequent layer adsorption of cationic chitosan ($\overline{M}_w$ 1 MDa) and anionic gelatin B ($\overline{M}_w$ 70 000) on dexamethasone loaded halloysite tubes gave up/down steps similar to those shown in Figure 2. One can see a release curve from halloysite with biocompatible and biodegradable (chitosan/gelatin)$_3$ shells in Figure 5. This shell gives slower release rate as compared with release rates with any shell composition used earlier. This again confirms that higher molecular weight polymers provide better LbL encapsulation, and retardation. Natural chitosan and gelatin have biocompatibility and biodegradability advantages over synthetic polymers in making novel clay polyelectrolyte composites for drug formulations and sustained release.

The cytotoxicity of the halloysite was tested after 24 and 48 h incubation time for two types of cells (fibroblast and human breast cancer). The percentage of live cells as determined by celltiter-96 reagent was measured for various concentrations of testing agents (raw halloysite, gelatin/chitosan coated halloysite, and sodium chloride (negative control). It was observed that bare halloysite as well as natural polymer coated halloysite are nontoxic to the cells. Similar experiments conducted for dexamethasone loaded halloysite also yielded $98 \pm 1\%$ of live cells. Therefore, results indicated that shelled halloysite is a noncytotoxic material and can be utilized as a component in drug delivery systems.

Conclusion

Halloysite tubule clay was used as a nanotemplate for a LbL assembly of polyelectrolyte shells. The LbL assembly of polymers was confirmed by ξ-potential monitoring and TEM images. Multilayer of (PEI/PAA) $2.5 + 7$ nm silica nanoparticles finishing gave uniform coating over the clay tube and its openings with a thickness of ca. 20 nm. LbL shell assembly was applied to dexamethasone loaded halloysite to retard the drug release from the nanotubes. The loading of the drug was found to be ca. 7 vol.-%, confirmed by reduction in lumen porosity combined with UV monitoring. Three high molecular weight bilayers in the shell were optimal for retarding the rate of drug release. Encapsulation of dexamethasone in the lumen of the halloysite, accomplished with the assembly of natural polymer shells, provides a novel nanotube formulation for controlled release of macromolecules, including drugs, biocides, and anticorrosion agents.

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