Morphology Controlled Growth of Chitosan-Bound Microtubes and a Study of their Biocompatibility and Antibacterial Activity

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Self-assembled peptide microtubes are fabricated with the biopolymer chitosan. The microtubes are covalently attached to chitosan and the morphology of the chitosan assembled on the surface of the microtubes can be tuned by altering the pH of the growth solution. Cytotoxicity studies in the presence of mouse embryonic fibroblasts indicate that the chitosan-bound microtubes are highly biocompatible and the cells are able to survive and proliferate at a similar rate to the control. Antibacterial studies in the presence of E. coli prove that the chitosan-bound microtubes are bactericidal. This simple method for the development of biocompatible microstructures will facilitate cell targeting, fabrication of efficient carrier devices, and the preparation of highly efficient antibacterial materials.

Introduction

Micrometer- and nanometer-scale materials have been gaining significant attention because of their potential applications in the development of drug delivery systems, biosensors, optoelectronics, sensors, antimicrobial agents, enzyme substrates, medical implants, and ceramics. Such materials are advantageous because of their unique sizes and ability to be modified in order to facilitate attachments to biomolecules such as DNA, proteins, and lipids. Self-assembly, bio mineralization, and functionalization are some of the common methods utilized in order to improve biocompatibility, biomolecular recognition, and fabrication of many composite materials that mimic nature. In addition to proteins, DNA and bio-inorganic composites, natural materials that contain complex oligosaccharides, glycoproteins and carbohydrate moieties such as alginates, mucin, cellulose, dextran, and chitosan are being increasingly used for the development of new biomaterials because of their vital role in cellular recognition, biocompatibility, and gelation properties in order to advance the development of new therapeutics,
diagnostics, biochips, and biosensors. Amongst the various natural carbohydrate polymers, chitosan is the second most abundant polysaccharide and is derived from crustacean shells. Its biocompatibility, biodegradability, non-immunogenicity, and biological reactivity make it a highly desirable material for the development of wound healing materials, transdermal drug delivery, potential mucosal delivery, gene delivery devices, biodegradable packaging, tissue engineering, and antibacterial applications. In order to further enhance the properties and applications of chitosan, many derivatives of chitosan have been synthesized. For example, recently, a chitosan derivative, succinyl chitosan, was synthesized and found to exhibit pH dependent swelling behavior, which allowed for protection of encapsulated drugs from the harsh environment. Multilayer films of chitosan and alginate have been fabricated for loading and immobilizing specific antibodies on those films. Chitosan has also been blended with polymers such as poly(vinyl acetate) (PVA), poly(ethylene glycol) (PEG), poly(butylene succinate), polyalanine, and polycaprolactone to develop highly porous materials, films with high electrical conductivity, to enhance cell attachment, and for the preparation of antibacterial materials, respectively. Nanoparticles of N-trimethyl chitosan and N-diethyl chitosan as well as chitosan-integrated poly(ethylene oxide) (PEO) and hydroxy propyl cellulose (HPC) films have been shown to have significant antimicrobial effects. Nanocomposites of chitosan–metal nanoparticles as well as chitosan–gold hybrid nanospheres have been prepared for potential use in sensing and imaging techniques. Protein–hydrogel networks that integrate chitosan and ovalbumin have recently been prepared by self-assembly for efficient drug delivery.

Although there has been significant progress in the investigation of properties and applications of chitosan, few studies have attempted to study the properties of chitosan on surfaces of nanotubes and microtubes. Recently, some researchers have bound carbon nanotubes with chitosan, however, to our knowledge interactions of chitosan with self-assembled peptide nanotubes and microtubes are yet to be explored. Peptide and lipid-based nanotubes and microtubes display several properties that make them promising biomaterial candidates, including facile self-assembly in aqueous solutions and adaptability to functionalization for enhanced biocompatibility.

Designing a system that involves functionalizing peptide-based microtubes and nanotubes with chitosan is likely to impart interesting properties to the tubes and augment their applications as novel biomaterials. In this work, we have functionalized self-assembled peptide microtubes with chitosan and investigated the effect of pH on the growth and morphology of chitosan attached to the microtubes. The integration of chitosan on the microtubes was confirmed using scanning electron microscopy (SEM), FT-IR spectroscopy, and zeta potential analysis. Furthermore, we examined the cytotoxicity and cell proliferation of mouse embryonic fibroblast cells in the presence of the chitosan-bound microtubes. We also investigated the potential of the chitosan-bound microtubes as bactericidal materials. The development of such microstructures could improve or potentially help in cell changed to targeting, fabrication of efficient carrier devices, and the preparation of highly efficient antibacterial materials at the micro and nanometer scale.

Experimental Part

Materials

Medium molecular weight chitosan (75–85% deacetylated), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDAC), and N-hydroxysuccinimide (NHS) were obtained from Sigma Aldrich. Buffer solutions of various pH were purchased from Fisher Scientific. In order to prepare template microtubes for the incorporation of chitosan, the bolaamphiphile bis(N-α-amidometyr-tyr-tyrosine)-1,5-pentane dicarboxylate (8 × 10⁻³ M) was synthesized and self-assembled into microtubes at pH 5 over a period of two weeks. The bolaamphiphile peptide monomer was synthesized according to previously established methods and the details of the synthesis and the self-assembly process are described elsewhere. The tubes were sonicated, washed with nanopure water, and centrifuged thrice before reaction with chitosan.

Methods

Preparation of Chitosan-Bound Microtubes

Samples of self-assembled peptide microtubes (500 μL) were added to 200 μL buffer solutions of various pH values in the range of pH 3–9. NHS (0.25M, 3 M) was added to 200 μL buffer solutions of various pH values in the range of pH 3–9. NHS (0.25 M, 50 μL) and EDAC (0.50 M, 100 μL) were then added to each of the solutions in order to activate the carboxylic groups of the microtubes. After 60 min, chitosan (300 μL, 1 mg·mL⁻¹) in 0.1% acetic acid was added to the solutions and agitated with shaking for 48 h at 4 °C. The samples were then centrifuged and washed three times to remove any unbound chitosan.

Characterization

Scanning Electron Microscopy (SEM)

The incorporation of chitosan onto the microtubes was analyzed by SEM (Hitachi S-2600N) operated at 25 kV. The washed samples were dried and carbon coated before analysis.

FT-IR Spectroscopy

In order to confirm the incorporation of chitosan onto the peptide microtubes, FT-IR analyses were performed (IR DIGILAB, Excalibur FT-IR Series FTS 3100). The samples were dried at room temperature and mixed with KBr to make pellets and then analyzed. All spectra were taken at 4 cm⁻¹ resolution with 100 scans taken for
averaging. Sample measurements were carried out between 400–4000 cm⁻¹.

Zeta Potential Analysis

The zeta potentials were measured using a PSS-NICOMP 380 ZLS, Zeta Potential/Particle Sizer system (Santa Barbara, California, USA). Measurements of the ζ-potential of the samples were carried out at 25 °C after equilibrating with 0.1 × 10⁻³ M KCl in order to maintain constant ionic strength. The samples were measured at varying pH values (3.0–9.0). All analyses were performed in triplicate.

Cell Viability Assays

Mouse embryonic fibroblasts (MEF) (a generous gift from Dr. Zahra Zakeri, Queens College) were cultured in Dulbecco’s Modified Eagle’s Medium (GIBCO) supplemented with 100 units · mL⁻¹ penicillin, 100 μg · mL⁻¹ streptomycin, and 10% fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO₂. For analysis, cells were collected using 0.25% trypsin, 0.1% EDTA in Hank’s Balanced Salt Solution, and plated in 2 mL cell culture medium in 12-well Costar plates at a density of 5 × 10⁵ cells per well (1.3 × 10⁶ cells · cm⁻²). After allowing the cells to attach and spread for five hours, we then added 50, 100, 200, or 300 μL of 50 mg · mL⁻¹ of chitosan-bound microtubes (prepared at pH 5) to each well and incubated for up to 7 d. To the control wells, which contained the same number of cells, we added either 50 or 300 μL of water, the microtube solvent. To determine cell viability, the adherent and any unattached cells were collected from each well by trypsinization and the number of live and dead cells was determined by trypan blue exclusion. For cell-proliferation studies, the samples were examined at different periods of time and counted using a hemocytometer.

To record the cell morphology, photographs were taken using an Olympus C-50 zoom digital camera directly through the eyepiece of an inverted Olympus CK 30 phase contrast microscope.

Antibacterial Studies

A single colony of *Escherichia coli* (E. coli; XL-1 blue strain) was grown overnight in Luria–Bertani broth (LB) at 37 °C and agitated with shaking (240 rpm) until late log phase. A 100 μL aliquot was then transferred into 28 mL of LB. Two milliliters of the diluted bacteria was placed into separate sterile tubes with either 100, 200, or 300 μL of 50 mg · mL⁻¹ of chitosan-bound microtubes (prepared at pH 5) that were 50 mg · mL⁻¹ in ultrapure water (UP water; distilled and then deionized with a Barnstead EASYpure UV/UF system). As diluent controls, we added 100, 200, or 300 μL of UP water that did not contain microtubes to 2 mL of diluted bacteria. As a positive control for an antibacterial compound, we added 50 μg · mL⁻¹ of the antibiotic ampicillin to 2 mL of diluted bacteria. All samples were then agitated with shaking (240 rpm) at 37 °C for approximately 16 h. We assayed the bacteriostatic properties of the microtubes by determining the relative number of bacteria in the cultures by measuring the turbidity of three separate 100 μL aliquots in 96-well plates using 630-nm light in a BioTek ELx 800 microplate reader. The optical density of 100 μL of LB with no additions was subtracted from the values obtained from the other wells and served to ‘blank’ the spectrophotometer.

To assay for the bacteriocidal properties of the chitosan-bound microtubes, we grew overnight cultures of *E. coli* and then 100 μL was pelleted (2 000 × g for 5 min) and resuspended in 28 mL of UP water. To sterile test tubes that contained 2 mL of this diluted bacteria in UP water, we added 0 or 300 μL of chitosan-bound microtubes that were 50 mg · mL⁻¹ in UP water and then incubated at 37 °C with shaking (240 rpm) for 16 h. To assay the bacteriocidal activity of the microtubes, 50 μL of the treated bacteria (or 50 μL of the control bacteria) was spread onto the surface of 10 cm LB agar plates and incubated at 23 °C for 3 d. Single live bacteria were identified by the formation of macroscopic bacterial colonies. Images of the plates were captured using a NucleoVision Gel Documentation System (NucleoTech Inc., CA).

**Results and Discussion**

The bis(N-α-amido-tyr-tyr-tyrosine)-1,5-pentane dicarboxylate bolaamphiphile was synthesized according to previously established methods and the details of the synthesis and self-assembly of the microtubes are provided elsewhere.[33a] In general, the microtubes formed by self-assembly at room temperature within a period of two weeks at pH 5. The bolaamphiphiles are pH sensitive because of the presence of the carboxyl groups and hydroxyl groups of tyrosine. In general, both hydrogen bonding interactions between the amide and carboxyl groups as well as the hydrophobic interactions between the tyrosine moieties allowed for the facile self-assembly of the microtubes. It has been observed that such assemblies are promoted by protonation and/or hydrophobic interactions between the peptide moieties. The three-dimensional chemical structure of the bolaamphiphile is shown in Figure 1a, while a plausible three-dimensional assembly mechanism for tubular formations from the peptide bolaamphiphile is shown in Figure 1b. The self-assembled microtubes were sonicated, centrifuged, and washed thoroughly with nanopure water before conducting reactions with chitosan. The scheme for the fabrication of chitosan-bound microtubes is shown in Figure 2. Chitosan has reactive amino groups that can react with carboxylic acid groups under mild conditions.[35] The peptide microtubes possess hydrogen bonded as well

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**Figure 1.** a) The three-dimensional structure of the bis(N-α-amido-tyr-tyr-tyrosine)-1,5-pentane dicarboxylate. b) Proposed self-assembly mechanism leading to the formation of the microtubes.
as free amide and carboxylic acid groups.\textsuperscript{[32,36]} Therefore, the carboxylic acid groups present in the peptide microtubes can be covalently bound to the amine groups of chitosan by amide bond formation. In lieu of this, the carboxylic acid groups of the microtubes were activated using EDAC and NHS and allowed to react with chitosan. After reaction between the chitosan and the microtubes, the samples were washed and centrifuged to remove any unbound chitosan and the morphologies of the microtubes after incorporation of chitosan were examined by SEM. In general, the tubes used were 500 nm in diameter on average. Figure 3 shows SEM images that indicate the morphologies of the microtubes under various conditions. Figure 3a shows an SEM image of a microtube before reaction with chitosan. As seen from Figure 3a, the tube has a smooth surface. Figure 3b shows the SEM image of chitosan grown on microtubes at pH 3 and Figure 3c and d show the SEM images of chitosan grown on microtubes at pH 5 and pH 7, respectively. Upon comparison of the peptide microtubes before reaction with chitosan (Figure 3a) and after growth of chitosan on the tube surfaces (Figure 3b–d), there is a significant difference observed on the surface of the tubes. This indicates that chitosan was bound to the surface of the tubes.

It appears that under highly acidic conditions, pH < 5, microparticulate structures of chitosan formed on the surface of the microtubes, while at higher pH, uniform films of chitosan formed. It has been reported that, in general, coupling reactions that involve NHS–EDAC are pH dependent and the reaction is relatively slower at lower pH.\textsuperscript{[37]} In addition, the electrostatic repulsion of the positively charged EDAC molecules and the NH\textsubscript{3}\textsuperscript{+} groups of the chitosan at highly acidic pH may result in less deposition and binding of chitosan on the microtubes. Hence the growth of chitosan on the microtubes under highly acidic pH conditions is not uniform and in some
cases aggregation/clumping is also observed, as seen in Figure 3b. In Figure 3c and d, uniform films of chitosan are observed on the entire surface of the microtubes. In some cases, the openings of the microtubes are also wrapped with the chitosan films, as seen in the inset in Figure 3c. Such sealed microtubular structures could be very useful in the packaging industry, in particular for the packaging of antibacterial materials. In general, uniform coatings of chitosan films were observed between pH 5 and 7. It is well known that the \( pK_a \) of chitosan is 6.3. \[38,39\] Thus at pH 5, the amine groups would be relatively more deprotonated compared to highly acidic conditions (pH 3) and at pH 7, all the \( \text{–NH}_3^+ \) groups from chitosan would be present in the \( \text{–NH}_2 \) form, which leads to more favorable electrostatic interactions and efficient binding between the carboxylic groups of the tubes and the amine groups of the chitosan. Hence high yields of uniform films of chitosan were observed in a pH range of 5–7. Furthermore, the coupling reaction in the presence of NHS–EDAC would be more favored under those conditions as well. Under basic conditions (pH > 8), chitosan self-precipitates, \[40\] and flocculation is observed, hence the conditions are not appropriate for the preparation of uniformly chitosan-bound microtubes. Thus, the pH-dependent behavior, hydrogen bonding, and electrostatic interactions, allows for morphology controlled growth of chitosan on the microtubes. It is to be noted that the microtubes also possess –OH groups capable of hydrogen bonding because of the presence of the tyrosine moieties, and its presence helps to interact with the –OH groups of chitosan as well, thus aiding the incorporation of chitosan onto the microtubes. Overall, this method could lead to the development of smart chitosan scaffolds for the fabrication of new devices for various bioapplications.

### FT-IR Spectroscopy

The incorporation of chitosan on to the peptide microtubes was confirmed by FT-IR analysis. Figure 4 shows the FT-IR spectra of the neat microtubes (Figure 4a), neat chitosan (Figure 4b), and chitosan bound microtubes at pH 5 (Figure 4c) in the region between 3 800 and 1 000 cm\(^{-1}\). For the neat microtube (Figure 4a), the IR spectrum shows the characteristic amide I peaks at 1 639 and 1 654 cm\(^{-1}\), which are assigned to hydrogen bonded as well as free amide peaks of the peptide microtubes. \[38a\] Peaks at 1 725 and 1 562 cm\(^{-1}\) are also observed and are assigned to the free carboxyl peak and amide II peak of the peptide microtubes. The region between 3 100 and 3 500 cm\(^{-1}\) indicates the peaks arising from the –OH of the carboxyl group, aromatic C–H stretches, and hydroxyl peaks of the tyrosine moiety. The peak around 2 900 cm\(^{-1}\) is assigned to asymmetric C–H stretching vibrations. For neat chitosan (Figure 4b), researchers have established that the FT-IR
spectra includes a NH$_3^+$ peak and a peak attributable to the NH$_2$ (or CO–NH$_2$) groups.[41] We observed peaks at 1 653 and 1 572 cm$^{-1}$, which were attributed to the CO–NH$_2$ and free –NH$_3^+$ groups of neat chitosan, respectively. The broad peak in the region at 3 500–3 000 cm$^{-1}$ was attributed to N–H and –OH stretching vibrations, as well as intermolecular hydrogen bonding of chitosan molecules.[42–45] A weak peak at around 2 900 cm$^{-1}$ is attributed to the C–H stretch. Figure 4c indicates the IR spectra of chitosan-bound microtubes at pH 5. There is a stark difference observed, compared to the microtubes alone (Figure 4a), particularly in the 1 700 cm$^{-1}$ region as well as in the 2 500 to 3 500 cm$^{-1}$ region. The peak at 1 725 cm$^{-1}$ attributable to the free carboxyl groups is absent, which indicates that those carboxyl groups are involved in formation of amide bonds with the amine groups of chitosan. In addition, the amide I peak of the microtubes is red shifted to 1 646 cm$^{-1}$. The peak at 1 571 cm$^{-1}$ (as seen for the –NH$_3^+$ of chitosan) is diminished and a red-shifted amide II peak is observed at 1 538 cm$^{-1}$. This suggests that the –NH$_3^+$ groups of chitosan are involved in amide bond formation with the microtubes. A strong peak at 1 272 cm$^{-1}$ is also observed as a result of C–O stretching. The region between 2 500 and 3 500 cm$^{-1}$ appears similar to that observed for chitosan. It is to be noted that similar results were observed at pH 6–7 (data not shown) as well. These results confirm the integration of chitosan uniformly on the peptide microtubes.

Zeta Potential Analysis

The incorporation of chitosan onto the microtubes was also confirmed using zeta potential analysis. In order to maintain constant ionic strength, 0.1 M KCl was added to all the reaction mixtures before carrying out zeta potential analyses. Figure 5 shows the variation of zeta potential with pH. In comparison with chitosan, both the microtubes as well as the chitosan-bound microtubes have lower zeta potentials. However, the zeta potential of the microtubes increased significantly upon binding with chitosan. The positive zeta potential values in the case of the lower pH range are mainly a result of the positively charged amino groups. As the pH is increased, consequently lesser free amine groups are present on the surface of the chitosan-bound microtubes because of formation of amide bonds with the carboxyl groups of the microtubes, which leads to a gradual decrease in zeta potential with pH. The point of zero-potential is observed to be at pH 6.8, which is close to the pK$_a$ of chitosan that is known to be around 6.3.[46,47] The zeta potentials of chitosan-bound microtubes appear to be dominated by the effect of chitosan although the incorporation on the surface of the microtubes with chitosan does lower the magnitude of the zeta potentials of the chitosan-bound microtubes. At basic pH, the carboxylate groups of the microtubes become increasingly deprotonated, which gives rise to a negative zeta potential for the microtubes, and the chitosan itself self-precipitates at basic pH, consequently not much binding is observed with the microtubes and thus the zeta potentials of those blends are relatively negative as well.

Cell Viability Studies

We next determined whether the chitosan-bound microtubes were biocompatible with mammalian cells. We used well-characterized mouse embryonic fibroblasts (MEF) in culture in cytotoxicity and cell proliferation studies. Varying concentrations of chitosan-bound microtubes were added to recently plated MEFs in complete media and the morphology, cell number, and cell viability were followed for 7 d. Using a phase contrast microscope we observed no cell lysis or changes in cell morphology of MEFs incubated with chitosan-bound tubes for periods of 1, 2, or 7 d (Figure 6).

In addition to normal morphology, the chitosan-associated microtubes did not kill the MEFs nor stop their movement through the cell cycle. Using trypan exclusion assays we calculated that approximately 95% of the cells in the wells containing 1.2–6.5 mg·mL$^{-1}$ of microtubes for 7 d were alive (Figure 6b). This percentage of live cells is the same as for the control wells to which we added water and no microtubes. We also determined the number of cells in the wells at 2 and 7 d and found that the cells had divided on average between 1.5 to 2.5 times during this period. These results indicate that chitosan-bound microtubes are non-toxic to mammalian cells and are consistent with studies that show that synthesized UV-curable chitosans implanted into murine subcutaneous tissues were enclosed by vascularized fibrous connective tissue that contained no immunoresponsive cells.[12a]

Figure 5. Comparison of zeta-potential analyses of chitosan-bound microtubes with chitosan and the microtubes alone.
Antibacterial Studies

Previous work from other laboratories has shown that various forms of chitosan (microparticles, nanoparticles, films) have important antimicrobial properties.[48–50] We wished to determine whether the chitosan-bound microtubes prepared here shared these properties. Because the most uniform coating was observed for chitosan that was grown on the microtubes at pH 5, in all cases those microtubes were used in the antimicrobial studies. We found that at all concentrations of chitosan-bound microtubes assayed (1.2–6.5 mg·mL⁻¹) that there was no detectable E. coli growth after overnight incubation at 37 °C. We detected no increase in turbidity of the cultures that contained microtubes, while the same number of bacteria in the diluent control samples that did not contain microtubes increased in turbidity to an O.D. value of 0.7. Samples that contained microtubes and bacteria; samples that contained the antibiotic ampicillin and bacteria, and samples that contained only LB with no added bacteria all had no increase in turbidity following incubation. These results indicate that the chitosan-bound microtubes are highly bacteriostatic. To determine whether the chitosan-microtubes were also bactericidal, a 50 μL aliquot of an overnight incubation of E. coli with microtubes or E. coli alone was spread onto the surface of LB agar plates and allowed to grow so that single bacteria formed macroscopic colonies. We found that bacteria incubated in the absence of microtubes formed thousands of colonies on the plate, while plates containing microtube-treated bacteria contained only several colonies (Figure 7). These results in conjunction with the turbidity measurements confirm that chitosan-conjugated microtubes are potent bacteriostats and bacteriocides.

Conclusion

Self-assembled peptide microtubes were functionalized with chitosan. The pH effect on the morphology of the chitosan attached to the microtubes was examined. We observed that at low pH, aggregates of chitosan microparticles were formed on the surface of the self-assembled microtubes, while at pH 5–7, uniform films of chitosan formed. Such engineered self-assembling systems could help to control the morphology of the chitosan grown on tubular surfaces. The effects of pH on the charge density of the chitosan-bound microtubes were also studied by zeta potential analysis and the results showed that there was an increase in zeta
potential of the microtubes upon incorporation of chitosan. FT-IR analysis confirmed the incorporation of chitosan onto the surface of the microtubes. Cytotoxicity studies in the presence of fibroblasts indicated that the chitosan-bound microtubes were highly biocompatible with mammalian cells, and the cells were able to proliferate in the presence of the tubes as well. Antibacterial studies in the presence of *E. coli* proved that the microtubes had bactericidal effects as well. Such materials could help develop new biocompatible, biodegradable devices for applications in tissue engineering, environmental remediation, and drug delivery.

Acknowledgements: The authors thank Dr. Patrick Brock at the Department of Earth and Environmental Sciences, Queens College for use of the scanning electron microscope. I.B. thanks the Fordham University faculty Research Grant and K.F. thanks the Professional Staff Congress of The City University of New York for financial support.

Received: August 1, 2008; Revised: September 21, 2008; Accepted: September 26, 2008; DOI: 10.1002/mabi.200800220

Keywords: antibacterial; biocompatibility; chitosan; microtubes