Published in final edited form as:

Biomacromolecules. 2010 September 13; 11(9): 2352–2358. doi:10.1021/bm100481r.

Zwitterionic chitosan derivatives for pH-sensitive stealth coating

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Abstract

Zwitterionic chitosan, a chitosan derivative with a unique pH-dependent charge profile, was employed to create a stealth coating on the cationic surface of drug carriers. Zwitterionic chitosans were synthesized by amidation of chitosan with succinic anhydride. The succinic anhydride-conjugated chitosan had an isoelectric point, which could be easily tuned from pH 4.9 to 7.1, and showed opposite charges below and above the isoelectric point. The succinic anhydride-conjugated chitosan was able to inhibit the protein adsorption to the cationic surface at physiological pH, compatible with blood components, and well tolerated upon intraperitoneal injection. The succinic anhydride-conjugated chitosan has the potential to serve as a coating material to prevent protein adsorption to cationic surfaces, which can be removed in a pH-responsive manner.

Keywords

Zwitterionic chitosan; pH-sensitive; drug delivery; stealth coating; biocompatibility

Introduction

Nanocarriers with cationic surface charge are known to enter cells relatively easily, due to adsorptive interactions with the cell membrane.^{1,2} Therefore, cationic nanocarriers are often employed to enhance cellular delivery of payloads that are unable to enter the cells (e.g., nucleic acids³) or poorly retained in the cells (e.g., antineoplastic drugs in multidrug-resistant cells⁴). However, cationic nanocarriers are easily cleared by the reticuloendothelial system, severely compromising drug delivery to the target tissues.⁵ To circumvent this problem, polyethylene glycol (PEG), so called ‘stealth coating’, is often employed to mask the cationic surface and reduce opsonization.⁶

On the other hand, recent studies suggest that the very presence of PEG can also limit the interaction between carriers and the target cells^{7–11} and inhibit effective cellular uptake of

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the loaded drug.¹² Recognition of this limitation has prompted recent efforts to develop removable stealth coatings.^{9,12} For example, tumoral extracellular pH is known to be more acidic in general (pH 6.5 to 7.2),^{13,14} due to the increased glycolysis and the plasma membrane proton-pump activity of tumor cells.^{15,16} Accordingly, PEG is modified with pH-sensitive moieties so that the PEG lose its stealth function in the weakly acidic extracellular environment.^{4,17-19} For example, pH-responsive micelle systems were developed using variants of poly(lactic acid)-*b*-PEG-*b*-poly(L-histidine) (PLA-*b*-PEG-*b*-polyHis) triblock copolymer^{4,19}. The imidazole ring of polyHis endows the polymer with pH-sensitivity and allows the PEG layer to transform to expose cell-interactive ligands on the particle surface at weakly acidic pH. Alternatively, a polymeric micelle was prepared with a diblock copolymer conjugated to a cationic ligand, which was masked by another diblock copolymers of PEG and pH-sensitive polymer.^{18,20} The surface copolymers were designed to remain associated with the cationic surface of the micelle via electrostatic interactions only at neutral pH but get removed from the micelle at lower pH. The same pH-sensitive diblock copolymer has also been used to coat a cationic gene-polymer complex to allow gene transfection to occur in a pH-sensitive manner.²¹ The difficulty in these approaches is, however, that it is quite challenging to design a specific polymer that responds sensitively to a desired range of pH while maintaining the stealth function during circulation.²¹⁻²⁵ The challenge is even more aggravated when the choice of materials has to be limited to biocompatible and biodegradable ones for *in vivo* applications.²¹

In an attempt to address these challenges, we develop an alternative strategy to modify the surface of nanocarriers using succinic acid-conjugated chitosans, which show pH-dependent charge profiles similar to those of proteins. Chitosan has been widely used for a variety of biomedical applications for the relatively good biocompatibility and biodegradability.²⁶ Succinic acid-conjugated chitosan, created by amidation of primary amines of chitosan with succinic anhydride, has been previously reported as a chitosan derivative soluble at neutral pH²⁷⁻³¹ and used as a macromolecular drug carrier.²⁷⁻²⁹ In this study, we find that the chitosan derivative shows a unique pH-dependent charge profile, which could be conveniently exploited to create pH-sensitive surface coating on the cationic surface of drug carriers, and the chitosan modification method has a great deal of flexibility in tuning the pH-sensitivity. The chitosan derivative shows opposite charges and is soluble in water below and above the isoelectric points (IEP). The IEP can be tuned anywhere between 4 and 7 simply by controlling the feed ratio of reactants. To signify the unique zwitterionic properties, the chitosan derivatives are named as 'zwitterionic chitosans.' Here, we report synthesis and characterization of the zwitterionic chitosan and demonstrate its pH sensitivity and ability to serve as a protective coating for *in vivo* applications. To investigate the safety of systemic application of the zwitterionic chitosan, hemocompatibility and tissue compatibility are evaluated using *in vitro* assays and a mouse intraperitoneal injection model, respectively.

Experimental Section

Materials

Low molecular weight chitosan (MW: 15,000 Da) was purchased from Polysciences. Chitosan glutamate was purchased from Novamatrix (FMC BioPolymer AS, Norway), and branched polyethylenimine (MW: 25,000 Da) was purchased from Aldrich. Fluorescent dyes Flamma FPG-456, FPR-553, and FPR-648 were a gift of BioActs (Incheon, Korea). Eudragit E100, butyl methacrylate-(2-dimethylaminoethyl)methacrylate-methyl methacrylate copolymer (1:2:1), was a gift of Degussa (Germany). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and used as received unless specified otherwise.

Synthesis of succinic anhydride-conjugated low molecular weight chitosan (SALM-CS)

Chitosan was first dissolved in 1% acetic acid and centrifuged at 4000 rcf for 20 min, and the clear supernatant was collected and freeze-dried to obtain an acetate salt form of chitosan. Chitosan acetate 200 mg was dissolved in 30 mL deionized (DI) water. Succinic anhydride was added as solid to the chitosan solution in varying quantities according to the desired molar feed ratio of anhydride to amine (An/Am ratio), over 5 min under vigorous stirring. For example, for An/Am ratio of 0.7, 140 mg of succinic anhydride was added to 400 mg chitosan. After 1 hour of reaction, the pH of the reaction mixture was adjusted to 8-9 with 0.2 N NaHCO₃. After overnight reaction at room temperature under stirring, the reaction mixture was dialyzed against water (molecular weight cut-off: 3500), whose pH was adjusted to 10-11 with 1 N NaOH. The purified succinic anhydride-conjugated low molecular weight chitosan (SALM-CS) was freeze-dried and stored at -20°C. ¹H NMR spectra of SALM-CS dissolved in 2% CD₃COOD/D₂O were obtained on a Bruker ARX (300 MHz). The proton signals were recorded at 70°C. δ (ppm): 5.41 (m), 5.13 (m), 4.41-4.20 (br), 3.71 (t), 3.16 (t), 2.58 (s).

Measurement of zeta potential and transmittance of SALM-CS solution

To determine the isoelectric point of the SALM-CS, zeta potential of the SALM-CS solution was measured at different pHs. SALM-CS was prepared as 0.5 mg/mL solution in 10 mM NaCl. Zeta potential of the solution was monitored with a zetasizer (Nano ZS-90, Malvern) at various pHs ranging from 3 to 9. The solution pH was adjusted using 0.1 N HCl. To investigate the pH dependence of aqueous solubility of the SALM-CS, transmittance of the SALM-CS solution was monitored at various pHs with a UV-VIS spectrophotometer at 500 nm.

Acid-base titration

Acid-base titration was performed to evaluate the buffering capacity of SALM-CS. The SALM-CS was prepared as 0.5 mg/mL solution in 10 mM NaCl. The initial pH of SALM-CS solution was set to 9-10 with 0.1 N NaOH as needed. Subsequently, pH of the solution was measured after each addition of 0.1 N HCl.

Fluorescence labeling

Branched polyethyleneimine (bPEI, MW: 25,000) and SALM-CS were labeled with FPG-456 ($\lambda_{\text{abs}}/\lambda_{\text{em}} = 495/526$ nm) and FPR-553 ($\lambda_{\text{abs}}/\lambda_{\text{em}} = 553/587$ nm), respectively, according to the manufacturer's protocol. Bovine serum albumin (BSA) was labeled with FPG-456, FPR-553 or FPR-648 ($\lambda_{\text{abs}}/\lambda_{\text{em}} = 647/672$ nm). Fetal bovine serum (FBS) protein was labeled with FPG-456. Unreacted free dye was removed by dialysis against water (molecular weight cut-off: 10,000) for 2 days. The fluorescently labeled materials were named according to the maximum absorption wavelength: e.g., SALM-CS-553 or BSA-648.

Protein adsorption to a cationic film

A cationic polymer film was prepared by casting 180 μ L of 300 mg/mL Eudragit E100 solution in acetone on a 18 $\times$ 18 mm glass coverslip and drying in air for 4 hours followed by vacuum-drying for 6 hours. The E100 film on the coverslip was equilibrated overnight in phosphate buffered saline (PBS, 10 mM phosphate, 150 mM NaCl, pH 7.4). Subsequently, the E100 film was incubated in PBS or SALM-CS solution in PBS (0.2 to 5 mg/mL) for 2 hours at room temperature, rinsed twice with fresh PBS, and then incubated in 0.2 mg/ml BSA-456 or FBS-456 for 2 hours. After removing the loosely bound protein by rinsing the films with fresh PBS twice, the films were incubated in 1% sodium dodecyl sulfate (SDS) solution at 37°C for 3 hours to recover the adsorbed protein. The protein in 1% SDS solution

was quantified by measuring the solution fluorescence intensity using a Tecan Spectrafluor Plus microplate reader (Ex/Em = 485/525 nm).

Protein adsorption to DP-complex and FRET

Plasmid DNA (pEGFP) was prepared as previously reported.³ A DNA-polymer (DP-456) complex was prepared with the pEGFP and bPEI-456 at the N/P ratio of 10, as previously described.³ One milliliter of the DP-456 complex solution was placed in a 35-mm dish with a glass window (MatTek). One milliliter of PBS buffer containing SALM-CS-553 or BSA-553 (0.1 mg/mL) was added to the DP-456 complex and incubated for 30 min, followed by addition of BSA-648 (0.1 mg/mL) and further incubation for 30 min. The emission spectra of the DP-456, (DP-456 + SALM-CS-553), (DP-456 + BSA-553), (DP-456 + SALM-CS-553 + BSA-648), and (DP-456 + BSA-553 + BSA-648) were acquired with a confocal laser scanning microscope (Olympus X81) using the lambda function of the Fluoview software (Olympus, Japan). The samples were excited with a 488-nm laser, and the emission signals were collected stepwise from 490 nm to 750 nm at the step length of 5 nm and at the bandwidth of 10 nm.

Hemolytic activity assay

Blood was obtained from Sprague-Dawley rats by cardiac puncture, according to the protocol approved by the Purdue Animal Care and Usage Committee. The hemolytic activity assay was performed as described in the literature.³² Briefly, red blood cells (RBC) were washed with 210 mM NaCl solution until no red color was visible in the supernatant. The washed RBC were incubated with SALM-CS and unmodified chitosan solution in PBS (pH 7.4) in various concentrations (10-100 μ g/mL) at 37°C for 1 hour. The unmodified chitosan solution was first prepared as 1 mg/mL chitosan acetate solution and then diluted with PBS, in which the unmodified chitosan precipitated out over time. PBS (pH 7.4) and deionized water were used as a negative- and a positive control. After incubation with the chitosan solutions, the RBC suspension was divided into two parts. One part was imaged with a Nikon eclipse TE200 microscope to count the intact RBC. The other part was centrifuged at 1000 rcf for 5 min to take a picture of the tube.

Complement activation assay

The pooled human plasma (Innovative Research, Inc) was incubated with SALM-CS in various concentrations (10-1000 μ g/mL), as described in the NCL protocol.³³ Briefly, 10 μ L of the SALM-CS (An/Am = 0.7) solution was incubated with 10 μ L of the plasma at 37°C for 1 hour. Cobra venom factor (Quidel Corp.) and Ca^{2+} / Mg^{2+} -free Dulbecco's PBS (DPBS) were used as a positive- and a negative control, respectively. The resulting mixture were separated on 10% Tris-glycine gel in a minigel apparatus and transferred to a nitrocellulose membrane (0.2 μ m). The membrane was blocked in 5% nonfat milk in Tris-buffered saline containing 0.1% Tween20, and then incubated with goat polyclonal anti-C3 antibody (1:2000, EMD Biosciences Inc), followed by peroxidase-linked anti-goat IgG (1:50000 dilution). The immunoblot bands were detected by the luminescence method (ECL Western blotting detection kit, Pierce). The intensity of the bands was recorded with Gel documentation system (BioRad, Gel DOC XR) and quantified with BioRad Quantity One software (version 4.6.1).

IP injection of chitosan

ICR mice (25g) were purchased from Harlan (Indianapolis, IN) and cared for in compliance with protocols approved by the Purdue Animal Care and Usage Committee, in conformity with the NIH guidelines for the care and use of laboratory animals. Two kinds of SALM-CS (An/Am ratio of 0.7 and 0.3) and chitosan glutamate were prepared as 20 mg/mL solution in

PBS (pH 7.4) and water, respectively. The chitosan solutions and buffer controls (PBS, pH 7.4 or glutamate buffer, pH 5) were sterilized by filtration through 0.22 μ m membrane filters. The animals were anesthetized with ketamine 50 mg/kg SC and xylazine 10 mg/kg SC. A 0.5 cm skin incision was made in the skin 0.5 cm above the costal margin, revealing the translucent abdominal wall. The peritoneum was nicked with a 24-gauge catheter (<1 mm incision), and the polyurethane tube was then advanced 1 cm, enough to insert into the peritoneal cavity. Air was insufflated to confirm the positioning and 0.5 ml of the chitosan solutions (i.e., 400 mg/kg chitosan) or buffer controls was instilled into the peritoneal cavity. The skin was closed with 1 or 2 size 4-0 sutures, and 0.02 mg/kg buprenorphine was given SC immediately after the procedure. The mice were sacrificed after 7 days, and the presence of residue, adhesions, and visible signs of inflammation (nodules, increased vascularization) were evaluated. Tissues were sampled, fixed in 10% formalin, and processed for histology at the Indiana Animal Disease Diagnostic Laboratory, West Lafayette.

Statistical analysis

All data were expressed as averages with standard deviations. Mean comparisons were made by the Student t-test. The difference was considered significant when the p-value was less than 0.05. For the western immunoblotting assay, ANOVA was used to determine the statistical difference among the groups, and then pair-wise comparison was made using the paired t-test. A p-value of <0.05 on a 2-tailed test was considered statistically significant.

Results and Discussion

Synthesis and characterization of SALM-CS

Zwitterionic chitosans were created by amidating a fraction of chitosan amine groups with carboxylic acid anhydrides. For example, succinic anhydride-conjugated low molecular weight chitosans (SALM-CS), one of the zwitterionic chitosan series, was created by conjugating succinic anhydride to chitosan. The appearance of new peaks between 3.0 and 3.3 ppm confirmed amidation of chitosan amine groups (Fig. 1). The degree of amidation, defined as the ratio of amidated amines (m) to total amines (1+m), was proportional to the molar feed ratio of anhydride to amine (An/Am ratio) (Supporting Table and Supporting Fig. 1), similar to a previous study.³⁴ The acid-base titration experiment was performed to evaluate the buffering capacity of SALM-CS. The buffering pH region shifted from pH 6-7 to pH 3.5-4.5 with the increase in the degree of amidation, due to the increase of carboxylic groups relative to amine groups (Supporting Fig. 2).

The aqueous solubility of SALM-CS with different An/Am ratio was monitored by observing the transmittance of the solution increasing the pH (Fig. 2A). Unlike the unmodified chitosan, which started to precipitate and became turbid at pH 6.5, SALM-CS were soluble at both basic and acidic pHs except at the intermediate pH. For example, the SALM-CS prepared with the An/Am ratio of 0.3 was soluble at above pH 7.38 and below pH 6.7. The zeta potential of the SALM-CS was measured at different pH's to correlate with its pH-dependent solubility profile. The SALM-CS showed positive charges at acidic pH and negative charges at basic pH (Fig. 2B). The pH ranges at which SALM-CS were charged corresponded to those SALM-CS showed good aqueous solubility. The IEP point of SALM-CS decreased from 7.1 to 4.9 with the increase of the An/Am ratio from 0.3 to 0.7. On the other hand, the unmodified chitosan was completely deprotonated at pH 8 (Fig. 2B), which corresponds to the pH value that its turbidity started to reach the plateau (Fig. 2A).

Inhibition of protein adsorption to cationic surfaces by SALM-CS

For a polymer to serve as a coating material that ensures the long-term circulation of a nanocarrier in the blood stream, it should effectively prevent the nanocarrier from

interacting with blood components, in particular, the opsonin proteins that mark it for phagocytic destruction.⁶ To test the potential of SALM-CS as a stealth polymer, its ability to prevent protein adsorption to a cationic surface was evaluated using a polymer film. A polymer film carrying positive charges at physiological pH was prepared with Eudragit E100, a cationic polymethacrylate with dimethylaminoethyl groups.³⁵ Eudragit E100 is water-soluble at <pH 5, where the tertiary amines are mostly protonated, but not water-soluble at higher pH, where the amines are only partly protonated. At pH 7.4, Eudragit E100 served as a water-insoluble platform with cationic charges that would attract anionic proteins. BSA or FBS was incubated with the E100 film or the film coated with SALM-CS at pH 7.4, and the amount of protein adsorbed to each film was quantified. BSA was chosen as a representative protein, since it accounts for ~60% of plasma protein.³⁶ Fig. 3A and Supporting Fig. 3 show that the SALM-CS coating reduced the protein adsorption to the cationic polymer film in a dose-dependent manner. When pre-coated with 5 mg/mL SALM-CS (An/Am=0.7, IEP value of 4.9), BSA adsorption to the E100 film decreased by 75%. A similar effect was observed with FBS (Supporting Fig. 3). Here, FBS adsorptions to E-100 film decreased by 56% and 43% when the film was pre-coated with 5 mg/mL SALM-CS (An/Am=0.63, IEP value of 6) and SALM-CS (An/Am=0.7), respectively. The fact that SALM-CS with IEP of 6 was able to reduce protein adsorption to the cationic surface at pH 7.4 is significant, because it is more likely to be relevant to targeting the tumoral pH.

Next, the stealth function of SALM-CS was examined on the nanocarrier level using the Förster resonance energy transfer (FRET) spectroscopy. A cationic nanocarrier was formed by complexing plasmid DNA and branched polyethyleneimine (bPEI, MW: 25,000), a polycation routinely used for non-viral gene delivery.³⁷ The DNA-polymer (DP) complex was either directly incubated with BSA or pre-incubated with SALM-CS prior to addition of BSA. To determine the interactions among DP complex, SALM-CS, and BSA using FRET, they were labeled with fluorescence dyes, FPG-456 ($\lambda_{\text{abs}}/\lambda_{\text{em}} = 495/526$ nm), FPR-553 ($\lambda_{\text{abs}}/\lambda_{\text{em}} = 553/587$ nm), and FPR-648 ($\lambda_{\text{abs}}/\lambda_{\text{em}} = 647/672$ nm), to form DP-456, SALM-CS-553, BSA-553, and BSA-648. The FRET phenomenon occurs only when the distance between donor and receptor fluorophores is less than 10 nm.³⁸ Here, the FRET pairs are FPG-456 and FPR-553, and FPR-553 and FPR-648.

When the positively charged DP-456 was incubated with BSA-553, a FRET emission at ~565 nm appeared (Fig. 3B, DP456 + BSA-553) upon excitation with 488-nm laser, in addition to its original emission at 520 nm, showing that the BSA-553 adsorbed to the DP-456. When BSA-648 was added to the mixture of DP-456 and BSA-553, additional FRET signal appeared at ~660 nm (Fig. 3B, DP456 + BSA-553 + BSA-648). This indicates that BSA-648 and BSA-553 were close to each other, as both adsorbed to the DP-456. No fluorescence emission was observed in the absence of DP-456 (Supporting Fig. 4, BSA-553 + BSA-648), which confirms that the second FRET signal at 660 nm was due to the adsorption of the two BSA's to DP-456.

The DP-456 incubated with SALM-CS-553 showed a strong FRET emission at ~565 nm (Fig. 3B, DP-456 + SALM-CS-553) similar to that between DP-456 and BSA-553. This confirms that SALM-CS was able to coat the DP complex via electrostatic interaction. In contrast to the DP complex directly exposed to BSA, the DP-456 pre-incubated with SALM-CS-553 did not show the second FRET signal at ~660 nm upon the addition of BSA-648 (Fig. 3B, DP-456 + SALM-CS-553 + BSA-648). The absence of the second FRET signal suggests that BSA-648 was not present in proximity to SALM-CS-553: i.e., BSA-648 did not adsorb to the DP-456 pre-incubated with SALM-CS-553. This result corroborates the observation with the E100 polymer film and confirms that SALM-CS was able to prevent protein adsorption to the cationic surface.

Hemocompatibility of SALM-CS

Chitosan has been widely used in biomedical research due to the availability and bioadhesiveness.³⁹⁻⁴¹ However, there may be a concern for its use in parenteral applications, as chitosan is also known for the hemostatic activity and the ability to activate macrophages and cause cytokine stimulation.⁴² To study the safety of SALM-CS for systemic application, its compatibility with red blood cells (RBC) was assessed using the hemolytic assay³² and compared with the unmodified chitosan. The hemolytic assay measures the ability of a material to destabilize the cell membrane and cause hemoglobin release from the RBC.³² The hemolytic activity of a material is usually determined by the level of free hemoglobin in the supernatant of the RBC suspension treated with the material. However, this detection method was not suitable for unmodified chitosan, because the released hemoglobin adsorbed to the chitosan and formed red precipitates collected on the wall of a tube. Therefore, the effects of the unmodified chitosan and SALM-CS on the RBC were compared by visually monitoring the appearance of red precipitates and by counting the number of intact RBC on the microscope. As shown in Fig. 4A, unmodified chitosan resulted in visible red precipitates on the tube wall as well as a supernatant with slightly red tint, which was similar to the negative control (phosphate buffered saline, PBS, pH 7.4). Positive control (deionized water) caused complete cell lysis due to the osmotic pressure. In contrast, there were no detectable red precipitates or tint in the RBC incubated with SALM-CS at all levels (10-100 µg/mL). Similarly, the number of intact RBC after treatment with SALM-CS was significantly higher than those with the unmodified chitosan or PBS (Fig. 4B). The slightly red tint shown in PBS is likely to be a result of spontaneous lysis of RBC. In this regard, it is worthwhile to note the difference between SALM-CS and PBS, which suggests the protective effect of SALM-CS on RBC.

The safety of SALM-CS was further evaluated using the complement activation assay, which determines if a foreign material triggers the complement activation response in the plasma.³³ This assay detects the appearance of C3 fragments (a product of the complement activation cascade) in the plasma treated with a test material by Western blot. As shown in Fig. 4C, both cobra venom factor (positive control) and the unmodified chitosan (10 µg/mL) showed significant increase in band intensity of the C3 cleavage product as compared to the negative control (Ca²⁺/Mg²⁺-free Dulbecco's PBS, DPBS), consistent with an earlier report.⁴³ On the other hand, there was no significant difference between DPBS and SALM-CS at all levels of concentration (10-1000 µg/mL).

Tissue responses to intraperitoneally administered SALM-CS

Biocompatibility of SALM-CS was investigated by examining the responses of peritoneal tissues following intraperitoneal (IP) injection of the material. We previously found that the peritoneal cavity was sensitive to the presence of foreign materials, serving as an appropriate tissue model for evaluating the biocompatibility of a material.^{44,45} Solutions of SALM-CS or chitosan glutamate as well as PBS (pH 7.4) or glutamate buffer (pH 5) (mock treatment control for each chitosan) were administered to ICR mice by IP injection. The peritoneal tissues of mice treated with PBS, glutamate buffer, or two kinds of SALM-CS (An/Am ratio of 0.3 and 0.7) were grossly normal. On the other hand, for the mice injected with chitosan glutamate, opaque debris was evident in the peritoneal cavity. This difference was also observed on the histological level (Fig. 5). There were no important microscopic abnormalities in liver, spleen, and the abdominal wall of mice injected with PBS, glutamate buffer, or either kind of SALM-CS. In contrast, mice injected with chitosan glutamate had deposits of eosinophilic granular material (chitosan) on serosal surfaces of liver (Fig. 5) and spleen (not shown). The chitosan deposits were surrounded by chronic inflammation (macrophages, lymphocytes) and a thin layer of connective tissue. The serosal surfaces (arrow) peripheral to the deposits were slightly thickened with connective tissue,

inflammatory cells, and plump mesothelial cells. Peritoneal fluid of mice injected with PBS, glutamate buffer, or either kind of SALM-CS was unremarkable, composed of small macrophages and lymphocytes. On the other hand, peritoneal fluid from mice injected with chitosan glutamate contained many more large activated macrophages often with intracellular eosinophilic chitosan debris. Extracellular chitosan deposits were numerous and usually surrounded by macrophages.

SALM-CS compared favorably with the unmodified chitosan in hemocompatibility and tissue compatibility. Given that the biological activity of chitosan is mainly attributed to the positive charges carried by the amino groups,⁴² the benign nature of SALM-CS may be attributed to the fact that a fraction of amino groups of chitosan were converted to carboxyl groups in SALM-CS, resulting in a net negative charge at the physiological pH. In future studies, SALM-CS will be evaluated for the ability of SALM-CS to serve as a pH-responsive, removable stealth coating material for cationic nanocarriers. We envision that the SALM-CS with an IEP value of 6-7, thus negatively charged at physiological pH, would coat the cationic surface of a nanocarrier and prevent its opsonization. Once the SALM-CS-coated nanocarrier reaches the tumor stroma and faces the weakly acidic pH, the SALM-CS would be positively charged and removed from the cationic surface. The pH-responsiveness of SALM-CS and the simplicity of IEP control would uniquely qualify the SALM-CS as a promising polymer for surface modification of cationic nanocarriers.

Conclusions

Chitosan derivatives with pH-dependent charge profiles, which we call zwitterionic chitosans to signify the unique charge properties, were synthesized by amidation of chitosan with carboxylic acid anhydride such as succinic anhydride. The succinic anhydride-conjugated chitosan had an isoelectric point, which could be easily tuned from pH 4.9 to 7.1, and showed opposite charges below and above the isoelectric point. Due to the flexibility of controlling pH dependence, it is expected that zwitterionic chitosans would have a number of biomedical applications, where the ability to respond to delicate pH changes is required. The succinic anhydride-conjugated chitosan was able to inhibit the protein adsorption to the cationic surface at physiological pH, compatible with blood components, and well tolerated upon intraperitoneal injection. These results warrant further exploration of zwitterionic chitosan as a removable stealth coating for tumor-targeted nanocarriers to overcome the current limitation of PEG.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by National Institutes of Health Grant R21 CA135130, the Showalter Trust Award, the American Association of College of Pharmacy New Investigator Program Award, and BioActs (DKC Corporation, Korea). This work was also supported in part by a grant from the Lilly Endowment, Inc., to the School of Pharmacy and Pharmaceutical Sciences.

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Fig. 1.

^1H NMR spectra of SALM-CS prepared with different An/Am ratios. SALM-CS was dissolved in 2% CD_3COOD in D_2O at 70°C . % Degree of amidation = $m/(1+m)$, where m is the number of amidated amines and $1 + m$ the number of total amines in the parent chitosan.

Fig. 2. pH-dependence of (A) transmittance and (B) zeta potential of SALM-CS prepared with different An/Am ratios.

Fig. 3.

(A) BSA adsorption to a cationic polymer (Eudragit E100) film coated with SALM-CS (An/Am = 0.7). Data are averages and standard deviations of 3 measurements. **: $p < 0.01$ by the t-test. **(B)** The emission spectra of the DP-456 (top); DP-456 + SALM-CS-553, DP-456 + BSA-553 (middle); DP-456 + SALM-CS-553 + BSA-648, and DP-456 + BSA-553 + BSA-648 (bottom). The samples were excited with a 488-nm laser. † and ‡ indicate the first and the second FRET signal, respectively. The inset graph magnifies 625-700 nm range to clarify the difference in the second FRET signal. The number after each sample indicates the maximum absorption wavelength (nm) of the conjugated dye. For example, SALM-CS-553 is SALM-CS labeled with FPR-553.

Fig. 4.

(A, B) Hemolytic activity of SALM-CS. RBC were incubated with SALM-CS and the unmodified chitosan at the concentration range from 10 to 100 $\mu\text{g/mL}$ at 37 °C and pH 7.4 for 1 hour. (A) Pictures were taken after centrifugation of the tubes at 1000 rcf for 5 min. The pellets are RBC, and the red precipitates on the tube wall are the released hemoglobin adsorbed to the chitosan. PBS: phosphate buffered saline; DW: deionized water. (B) RBC counts after incubation with chitosans (100 $\mu\text{g/mL}$). CS: Unmodified chitosan. Cell counts were taken from pictures of 3 random fields and expressed as averages and standard deviations. * $p < 0.05$ by the t-test. (C) Complement activation assay. Bands indicate C3 fragments, a product of the complement activation cascade, in the plasma treated with the

following test materials: Lane 1, $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free DPBS; lane 2, cobra venom factor (CVF); lane 3, unmodified chitosan (CS) 10 $\mu\text{g/mL}$; lanes 4-6, SALM-CS (An/Am = 0.7) 10, 100, 1000 $\mu\text{g/mL}$. Data are averages and standard deviations of 3 measurements. * $p < 0.05$, **: $p < 0.01$ by the paired t-test.

Fig. 5.

Tissue reaction in liver (upper panel) and cytology of peritoneal fluid (lower panel) after IP injection of SALM-CS (An/Am=0.3 and An/Am=0.7) or chitosan glutamate. CH: chitosan; SMP: small macrophages; LMP: large macrophages. Hematoxylin and eosin stain: 100× (liver); 400× (peritoneal fluid).