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Chitosan–DNA–rectorite nanocomposites: Effect of chitosan chain length and glycosylation

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ABSTRACT

Two fully deacetylated chitosans with low- and high molecular weight (LMW and HMW) and their glycosylated derivatives substituted with the trimer GlcNAc–GlcNAc–Mannose (AAM) were intercalated into rectorite to prepare polymer/clay nanocomposites. The interlayer distance of rectorite depended on the amount of chitosan and the structure of intercalated chitosan. The largest interlayer distance of 3.35 nm was obtained when the mass ratio of LMW non-substituted chitosan to rectorite was 2:1. The four chitosan–rectorite nanocomposites were loaded with DNA and their dispersion stability and DNA retention capability were evaluated. Similarly as for DNA–chitosan polyplexes, the colloidal stability of the chitosan–DNA–rectorite nanocomposites increased with the increasing molecular weight of the intercalated chitosan and was improved by glycosylation. The nanocomposite of HMW and glycosylated chitosan did not aggregate in phosphate buffered saline (PBS) at pH 7.4 and retained a size of approximately 200 nm diameter. The DNA retention capability of the nanocomposites was also dependent on the structure of the intercalated chitosan. Nanocomposites based on the LMW and glycosylated chitosan required high amount of the polymer (corresponding to amino: phosphate ratio of 60) to retain the loaded DNA. The in vitro transfection study revealed that the chitosan/rectorite composites were able to deliver DNA to human cells, albeit with reduced efficacy compared to chitosan/DNA nanoparticles.

1. Introduction

Biopolymer–clay nanocomposites have recently attracted much attention, exhibiting remarkable improvements of material properties when compared to clay or polymers alone (Viseras et al., 2008; Zeng & Yu, 2008). Clay materials have been extensively applied for drug delivery due to the good intercalation capacity of the clay particles (Aguzzi, Cerezo, Viseras, & Caramella, 2007). Clay minerals such as montmorillonite, illite, and kaolinite are well known to protect DNA from nucleases during natural bacterial genetic transformation (Demaneche, Jocteur-Monrozier, Quiquampoix, & Simonet, 2001; Lin, Chen, Cheng, & Kuo, 2006). The transformational ability of clay is believed to play a role in evolution and genetic exchange among unrelated organisms (Kawase et al., 2004). Clay minerals have already been used safely in human applications, such as anti-diarrheal medicine, antacids, and cosmetics (Aguzzi et al., 2007). Rectorite (REC) is a regularly interstratified clay mineral with alternate pairs of dioctahedral mica-like layer (nonexpansible) and dioctahedral montmorillonite-like layer (expansible) in a 1:1 ratio.

To modify or improve the affinity of the natural clays for the bioactive molecules, such as DNA, polymers may be added. Although various polymers have been used in polymer–clay composites, biopolymers are particularly interesting for drug and gene delivery applications.

Cationic polymers which condense plasmid DNA (pDNA) through electrostatic interactions to form polyplexes have emerged as safer, though less efficient, options for gene transfer (Montier, Benvegnu, Jaffres, Yaouanc, & Lehn, 2008). Chitosans, a family of cationic and linear polysaccharides derived from chitin, have been employed as polycationic carriers for pDNA in gene delivery systems with promising results (Kim et al., 2007). As generic chitosans are only soluble at pH-values below 6 and will precipitate upon increase of pH to neutral values (Varum, Anthonsen, Grasdalen, & Smidsrod, 1991), their application in biological systems at physiological pH is challenging. To overcome this obstacle, different derivatives of chitosan have been prepared. Glycosylation of chitosan was shown to improve the colloidal stability of the formulation as well as gene delivery efficacy (Hashimoto, Morimoto, & Saimoto, 2006; Issa et al., 2006; Strand, Issa, Christensen, Varum, & Artursson, 2008).

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It has been suggested that a key to successful transfection with chitosan is to achieve a subtle balance between DNA protection and intracellular DNA release (Koping-Hoggard et al., 2004; Strand, Danielsen, Christensen, & Varum, 2005). Consequently, all variables that affect the stability of polyplexes such as chain length, the fraction of acetylated units (F_A), glycosylation and so on were found to influence gene transfer efficacy (Issa et al., 2006; Koping-Hoggard et al., 2004; Romoren, Pedersen, Smistad, Evensen, & Thu, 2003; Strand et al., 2008).

Chitosan has also been extensively used in combination with inorganic materials such as gold (Liu, Chen, & Liu, 2008; Liu, Peng, Yang, Wu, & He, 2008), TiO_2 (Yuan, Venkatasubramanian, Hein, & Misra, 2008), Fe_3O_4 (Yuan, Ji, Fu, & Shen, 2008; Yuan, Venkatasubramanian, et al., 2008) and clay (Liu, Chen, et al., 2008; Liu, Peng, et al., 2008; Wang, Du, & Luo, 2008; Wang, Pei, Du, & Li, 2008). Liu (Liu, Chen et al., 2008) reported drug release behavior of chitosan–montmorillonite nanocomposite hydrogels following electrostimulation. In our previous study (Wang, Du, et al., 2008; Wang, Pei, et al., 2008), quaternized chitosan/montmorillonite nanocomposite nanoparticles were prepared, and it was found that certain montmorillonite loadings on quaternized chitosan can enhance the drug encapsulation efficiency and decrease the drug release rate. Also, our previous study revealed that REC is nontoxic to cells and can be used as a non-viral gene delivery system after the combination with quaternized chitosan (Wang, Du, et al., 2008; Wang, Pei, et al., 2008). As the nanocomposites are known to combine the physical and chemical properties of both inorganic and organic material, we sought to combine the advantages of the trisaccharide-substituted chitosan with rectorite.

In this study, we have prepared and characterized four different chitosan–rectorite nanocomposites, where both the structure and amount of intercalated chitosans were varied. Specifically, we have compared nanocomposites based on two fully deacetylated chitosan (low/high molecular weight) and their 2-acetamido-2-deoxy-β-D-glucopyranosyl-(1 → 4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1 → 4)-2,5-anhydro-D-mannofuranose(A-A-M) substituted analogues. After characterization, we loaded the nanocomposites with DNA and compared their dispersion stability and DNA retention ability. Finally we have investigated the transfection efficacy of chitosan–DNA–rectorite nanocomposites and compared them with DNA–chitosan polyplexes.

2. Experimental

2.1. Materials

Three fully deacetylated chitosans ($F_A < 0.001$ as determined from their proton NMR-spectra (Varum et al., 1991) were prepared by heterogeneous de-N-acetylation of shrimp chitin as previously described (Ottøy, Vårum, & Smidsrød, 1996), converted to HCl salts, and lyophilized. The measured intrinsic viscosity (η)-values of the chitosans at pH 4.5 and ionic strength 0.1 M were: 49, 220 and 440 ml/g. The molecular weights (M_w) and molecular weight distributions were analyzed by size-exclusion chromatography with refractive index (RI) and a multiangle laser light scattering detector (SEC–MALLS). All samples were dissolved in MQ water (5–7 mg/ml) and filtered through 0.22 μm syringe filter (Millipore). The column used was TSK 3000, and sample was eluted with 0.2 M ammonium acetate (pH 4.5) at a flow rate of 0.5 mL/min. For simplicity, the three chitosans are denoted as ChLo (M_w 20 kDa), ChMe (M_w 67 kDa) and ChHi (M_w 146 kDa). The characteristics of the chitosans used in the study are given in Table 1. Calcium rectorite (Ca^{2+}-REC) refined from the clay minerals was provided by Hubei Mingliu Inc. Co. (Wuhan, China). Sodium nitrite, ammonium acetate and sodium cyanoborohydride were obtained from Merck.

Table 1
Characterization of chitosans used in this study. Intrinsic viscosity [η], molecular weight (M_w), polydispersity index (PDI) and degree of substitution (d.s.).

Sample	d.s. (%)	[η] ml/g	M_w (g/mol)	PDI (M_w/M_n)
ChLo		49	20,000	1.7
ChMe		220	67,000	1.5
ChHi		440	146,000	1.7
TriChLo	9.3			
TriChHi	8.6			

D_2O (99.96% D atom) was purchased from Isotech Inc. Agarose (A 9539) and ethidium bromide (EtBr, E 1510) were purchased from Sigma–Aldrich DNA. The plasmid pBR322 (~4.4 kbp, Promega) was used in this study.

2.2. Preparation of fully N-acetylated trimers

The trimer 2-acetamido-2-deoxy-β-D-glucopyranosyl-(1 → 4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1 → 4)-2,5-anhydro-D-mannofuranose (A-A-M) was prepared as earlier described (Tommeraas, Varum, Christensen, & Smidsrod, 2001). The oligomers were separated by size-exclusion chromatography (SEC).

2.3. Preparation of trimer-substituted chitosan

Two of the chitosans (ChLo and ChHi) were substituted by reductive N-alkylation with the trimer A-A-M as previously described (Tommeraas et al., 2002). The characterization of the trimer-substituted chitosans using ^1H NMR spectroscopy revealed a degree of substitution (DS) of 9.3% and 8.6%. These chitosans are referred to as TriChLo (M_w 20 kDa) and TriChHi (M_w 146 kDa).

2.4. Quantification of chitosan adsorbed to rectorite

The REC sample was dispersed in distilled water and the clay suspension was left for 24 h after vigorous stirring for 30 min. Three chitosans were dissolved in water to obtain a 0.5% (w/v) solution, and were then added slowly into the REC suspension at the mass ratios of chitosan to rectorite of 0.1:1, 0.3:1, 0.5:1, 2:1 and 4:1. The adsorption proceeded at 60 °C for 2 days. The resulting solutions were centrifuged at 14,000 rpm for several times until no free chitosan in the supernatant was detected, and the sediments were collected and freeze-dried at −60 °C. Finally, the samples were applied to elemental analysis (C, N, H) on a Flash Elemental Analyzer 1112 (ThermoQuest, Milan, Italy) to determine the chitosan amount adsorbed on rectorite.

2.5. Preparation of chitosan/rectorite nanocomposites

The chitosan–rectorite nanocomposites were prepared as described above at the mass ratios of chitosan to rectorite of 1:1 and 2:1. The nanocomposites were freeze-dried at −60 °C and ground to powder. The overview of prepared nanocomposites and their designation is given in Table 2.

2.6. Characterization of chitosan/rectorite nanocomposites

The X-ray diffraction (XRD) experiment was performed using a D8 Advance diffractometer (Bruker, USA) with Cu target and Kα radiation ($\lambda = 0.154$ nm) at 40 kV and 50 mA. The scanning rate was 0.05°/min and the scanning scope of 2θ was 1–10° at room temperature.

Ultrathin films (50 nm) for transmission electron microscopy (TEM) analysis were prepared by cutting from the epoxy block with the embedded nanocomposites sheet at room temperature