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Preparation of low molecular weight N-maleated chitosan-*graft*-PAMAM copolymer for enhanced DNA complexation

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ABSTRACT

Low molecular weight N-maleated chitosan-*graft*-PAMAM (polyamidoamine) copolymer was prepared through N-maleated chitosan (NMC) by Michael type addition reaction to enhance its solubility in water as well as its cationic character for enhancement of DNA complexation. FTIR, ^{1}H NMR, XRD and GPC were used to characterize the graft copolymers. The copolymer showed better DNA complexation ability at low N/P ratio than that of chitosan due to increased surface charge density by the incorporation of PAMAM molecule on to chitosan backbone. The copolymer can effectively protect the DNA toward anionic surfactant. In vitro release study showed efficient DNA release occurred at physiological pH (pH 7.4). In vitro cell cytotoxicity test indicated toward less cytotoxicity of NMC-*graft*-PAMAM copolymers compared to that of 25 kDa PEI. Thus, the synthesized NMC-*graft*-PAMAM copolymers have great potential of finding application in drug and gene delivery.

1. Introduction

Chitosan (CTS) is a naturally occurring linear binary cationic polysaccharide consisting of D-glucosamine and N-acetyl-D-glucosamine repeating units linked by a β (1→4) glycosidic bond. It is obtained by alkaline hydrolysis of chitin. Chitosan has gained considerable attention in pharmaceutical and biomedical research such as wound dressing, skin grafting template, hemostatic agent, hemodialysis membrane, drug delivery and gene delivery vehicle [1–4] due to its favorable biological properties such as low toxicity, good biocompatibility and biodegradability. Although, the backbone of chitosan consists of hydrophilic functional groups, it is insoluble in water and in most of the organic solvents such as dimethyl sulfoxide, dimethyl formamide, alcohols and pyridine. The insolubility of chitosan in aqueous and organic solvents is a result of its crystalline structure due to extensive intramolecular and intermolecular hydrogen bonding between the chains and sheets [5]. The literature reported pK_a value of chitosan is 6.5 [6]. Therefore, it is only soluble in acidic aqueous solutions such as formic, acetic, pyruvic, 10% citric and lactic acids where the pH < 6.5 because, the amine groups of chitosan is protonated. Although, chitosan dissolves in aqueous medium at pHs less than or equal to 6.5, acidic solutions may not be desirable in many of the applications of chitosan such as cosmetics, food and biomedicines. Hence, chemical modifications of chitosan become necessary to improve its water solubility. Many researchers already prepared water soluble chitosan derivatives via quaternization of the amino groups of chitosan, acylation, via introduction of PEG and carboxymethyl group in chitosan [7–11]. There are many chemical modifications to improve the physicochemical and biochemical properties of chitosan [12]. Among them, graft copolymerization technique has extensively been used for modification of chitosan with synthetic polymers to get novel materials [13].

On the other hand, dendrimers are relatively a new class of synthetic polymers with highly branched and nanospherical well-defined architectures, precise molecular weight and multivalent functionalization sites [14–17]. Among the dendrimers, cationic poly(amidoamine) (PAMAM) dendrimer have recently been used as effective macromolecules in biomedical applications (i.e., the delivery of active pharmaceuticals, imaging agents or gene delivery) [18–20]. PAMAM dendrimers are uniform in size with a high density of primary amino groups at the surface and are highly soluble and stable in aqueous solution. It is reported that PAMAM dendrimers are nonimmunogenic and can mediate the enhanced delivery of diverse nucleic acids [21,22].

It is well known that chitosan is a nontoxic biopolymer and it has unique glucosamine repeating units in its polysaccharide backbone. Due to the presence of primary amine group in its backbone, chitosan in acidic solution is cationic, which helps it to encapsulate and deliver the genetic materials due to electrostatic interaction. On the other hand, PAMAM dendrimers are hydrophilic macromolecules with a high density of primary amine groups. But, the dendrimers have severe toxicity [23]. Therefore, the chemical combination of chitosan with PAMAM dendrimers may provide novel biomaterial

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with improved water solubility, higher charge density and lower toxicity. Sashiwa et al. [24,25] prepared chitosan-*graft*-PAMAM copolymers through tetraethylene glycol spacer by reductive N-alkylation, but till date, no report is available about N-maleated chitosan-*graft*-PAMAM copolymer.

In this work, Michael type addition reaction was used to prepare N-maleated chitosan-*graft*-PAMAM copolymer to improve water solubility, low toxicity and DNA complexation ability. For this purpose, we firstly prepared low molecular weight chitosan by oxidation process. Thereafter, full generation PAMAM dendrimers (G1–G3) were grafted onto chitosan through N-maleated chitosan by Michael type addition reaction. In comparison to the graft copolymer of Sashiwa et al. [24,25], the graft copolymer reported in this paper is unique with respect to mode of preparation and properties. All the related properties such as its solubility, complexation with DNA, in vitro cytotoxicity and in vitro DNA release study were investigated in details in this paper.

2. Materials and methods

Chitosan [MW 222 kDa and degree of deacetylation (DDA) 84%] was purchased from Acros Organics, USA. Methylacrylate (MA), ethylenediamine (EDA), methanol and sodium nitrite ($NaNO_2$) were obtained from Merck, India. Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin, trypsin, fetal bovine serum (FBS) were purchased from Himedia Laboratories Pvt. Ltd., India. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and agarose were obtained from Sisco Research Laboratories Pvt. Ltd., India. pGL3 control vector (5.25 kb) containing SV-40 promoter, resulting in strong expression of luc^+, was purchased from Promega (Madison, WI, USA). The plasmids were propagated in *Escherichia coli* (*E. coli*) and the plasmid DNA (pDNA) was isolated with QIAGEN Midiprep pDNA isolation Kit (USA) according to the manufacturer's instructions. Its purity was confirmed by spectrophotometry (A_{260}/A_{280}) and its concentration was determined from the absorbance at 260 nm. Vero cell line was kindly donated by Dr. Gopal Chakraborty, Department of Biotechnology, University of Calcutta. All other reagents were analytical grade and were used directly without further modification.

2.1. Preparation of low molecular weight chitosan

The low molecular weight chitosan was prepared by oxidative degradation with $NaNO_2$ at room temperature according to the previous report [26]. Briefly, 1% (w/w) chitosan was dissolved in 1% acetic acid solution under magnetic stirring. When chitosan was completely dissolved, the appropriate amount of $NaNO_2$ in 1% acetic acid was added drop wise over a half an hour period with vigorous stirring and the reaction was performed at room temperature for another 3 h. The reaction mixture was subsequently neutralized with 1 N NaOH to pH 7.4 to complete precipitate of chitosan. The precipitate was recovered by centrifugation, washed several times with deionized water and lyophilized for three days.

2.2. Synthesis of PAMAM dendrimers

PAMAM dendrimers were prepared following the previously reported procedure [27]. Briefly, a freshly distilled EDA solution (0.083 mol) in 20 mL methanol was added drop wise to a stirred methylacrylate (MA) solution (0.407 mol) in 20 mL methanol at 0 °C under nitrogen atmosphere over a period of 2 h. The final mixture was stirred for 30 min at 0 °C and then allowed to warm to room temperature and stirred for a further 24 h. After that, the solvent was removed under reduced pressure at 40 °C using a rotary evaporator and the resulting colorless oil dried under vacuum (10^{-1} mm Hg, 50 °C) overnight to give half generation (0.5 G) PAMAM dendrimer (30 g, 89.5%). ^{1}H NMR ($CDCl_3$) δ_H: 2.34 ppm (8H, t, $C\bar{H}_2CO_2CH_3$), 2.42 ppm (4H, s, $C\bar{H}_2N$), 2.66 ppm (8H, t, $NC\bar{H}_2$), 3.64 ppm (12H, s, $CO_2C\bar{H}_3$).

A solution of G0.5 PAMAM dendrimer precursor (10 g, 0.025 mol) in 20 mL methanol was carefully added to a vigorously stirred solution of EDA (1.248 mol) in 100 mL methanol at 0 °C under nitrogen atmosphere over a period of 2 h. After complete addition, the mixture was stirred for 96 h at room temperature. The solvent was then removed under reduced pressure at 40 °C using a rotary evaporator and gave the colorless oil of tetra-amine terminated G1 PAMAM dendrimer (11.2 g, 86.8%). ^{1}H NMR ($CDCl_3$) δ_H: 2.3 ppm (8H, p, $C\bar{H}_2NH_2$) 2.47 ppm (8H, t, $N\bar{H}_2$), 2.57 ppm (4H, s, $C\bar{H}_2N$), 2.61 ppm (8H, t, $C\bar{H}_2CONH$), 2.69 ppm (8H, t, $C\bar{H}_2CH_2CONH$), 3.29 ppm (8H, bq, $CONHC\bar{H}_2$), 7.85 ppm (4H, bt, $CONH$).

The next generation PAMAM dendrimers (G1.5, G2, G2.5 and G3) were prepared by repeating the above procedures. Selected data for **G1.5** PAMAM: Yield: 89.3%; IR υ_{max}/cm^{-1}: 1731 (C=O of ester group), 1638 (C=O of CONH group) 3280 (N–H); ^{1}H NMR ($CDCl_3$) δ_H: 2.35 ppm (8H, t, $C\bar{H}_2CONH$), 2.43 ppm (16H, t, $C\bar{H}_2CO_2CH_3$), 2.57 ppm (16H, t, $C\bar{H}_2CH_2COCH_3$), 2.61–2.31 ppm (20H, m, other $C\bar{H}_2$), 3.71 ppm (24H, s, $CO_2C\bar{H}_3$), 7.34 ppm (4H, bt, $CONH$).

Selected data for **G2** PAMAM: Yield: 87.4%; IR υ_{max}/cm^{-1}: 1637 (C=O of CONH group), 1546 (N–H of NH_2); ^{1}H NMR (D_2O) δ_H: 2.27 ppm (16H, bp, $C\bar{H}_2NH_2$), 2.48 ppm (16H, t, $N\bar{H}_2$), 2.63–2.49 ppm (52H, m, other $C\bar{H}_2$), 2.68 ppm (8H, t, $C\bar{H}_2N$), 3.28–3.11 ppm (24H, bq $NC\bar{H}_2$), 7.9 ppm (12H, bt, $CONH$).

Selected data for **G3** PAMAM: Yield: 84.4%; IR υ_{max}/cm^{-1}: 1640 (C=O of CONH group) 1546 (N–H of NH_2); ^{1}H NMR (D_2O) δ_H: 2.28 ppm (32H, bp, $C\bar{H}_2NH_2$), 2.46 ppm (32H, t, $N\bar{H}_2$), 3.01–2.89 ppm (24H, bm, $C\bar{H}_2N$), 2.83–2.59 ppm (116H, bm, other $C\bar{H}_2$), 3.5–3.21 ppm (56H, bm, $NHC\bar{H}_2$), 7.95 ppm (28H, bm, $CONH$).

2.3. Preparation of N-maleated chitosan (NMC)

NMC was prepared according to the previous report [28]. At first, chitosan was purified by dissolving 1 g of chitosan in 50 mL of 1% acetic acid solution, precipitated with 1 M NaOH solution and the precipitate was collected by filtration and then washed with water to pH 7. The purified chitosan obtained from the above was dispersed in 75 mL of DMSO with constant stirring. Then, 2 g maleic anhydride in DMSO solution was added into above solution. The mixture was reacted at 60 °C for 8 h. The reaction product was cooled to room temperature and subsequently precipitated in 250 mL of acetone, filtered, washed with acetone and diethyl ether and then lyophilized for three days to get N-maleated chitosan.

2.4. Preparation of NMC-graft-PAMAM copolymer

To graft PAMAM with NMC, 0.2 g NMC was dissolved in 20 mL of 0.25% sodium hydroxide solution. The aqueous solution of PAMAM dendrimers (3 g) of different generations (full generations, G1–G3) was then added into the above solution. The mixture was stirred and the reaction was carried out at room temperature for 4 days. Then, hydrochloric acid was added in the mixture with stirring until the pH value reaches 7.0. The product was then dialyzed (molecular weight cut off, MWCO: 3500 Da) against distilled water for 3 days and then lyophilized for 3 days.

2.5. Characterization of NMC-graft-PAMAM copolymer

The molecular weight of the depolymerized chitosan and graft copolymers were measured by gel permeation chromatography (GPC) equipped with a Waters PC2 separation module and Waters 2414 refractive index detector. Waters empower software was used to calculate the molecular weight based on a universal calibration curve generated by PEG standards (Sigma–Aldrich) of narrow