Biomaterials 33 (2012) 4204–4219

Contents lists available at SciVerse ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Depolymerized chitosans functionalized with bPEI as carriers of nucleic acids and tuftsin-tethered conjugate for macrophage targeting

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ARTICLE INFO

Article history:
Received 13 December 2011
Accepted 21 February 2012
Available online 13 March 2012

Keywords:
Depolymerized chitosan
bPEI
In vivo gene delivery
Tuftsin
Confocal microscopy

ABSTRACT

Development of efficient and safe nucleic acid carriers (vectors) is one of the essential requirements for the success of gene therapy. Here, we have evaluated the gene transfer capability of chitosan-PEI (CP) conjugates prepared by conjugating low molecular weight branched polyethylenimine (LMWP) with depolymerized chitosans (7 and 10 kDa) via their terminal aldehyde/keto groups. The CP conjugates interacted efficiently with nucleic acids and also showed higher cellular uptake. These conjugates on complexation with DNA yielded nanoparticles in the size range of 100–130 nm (in case of C7P) and 115–160 nm (in case of C10P), which exhibited significantly higher transfection efficiency (~2–42 folds) *in vitro* compared to chitosans (high and low mol. wt.) and the commercially available transfection reagents retaining cell viability almost comparable to the native chitosan. Of the two CP conjugates, chitosan 7 kDa-LMWP (C7P) displayed higher gene transfer ability in the presence and absence of serum. Luciferase reporter gene analysis in male Balb/c mice receiving intravenous administration of C7P3/DNA polyplex showed the maximum expression in their spleen. Further, tuftsin, a known macrophage targeting molecule, was tethered to C7P3 and the resulting complex, i.e., C7P3-T/DNA, exhibited significantly higher gene expression in cultured mouse peritoneal macrophages as compared to unmodified C7P3/DNA complex without any cytotoxicity demonstrating the suitability of the conjugate for targeted applications. Conclusively, the study demonstrates the potential of the projected conjugates for gene delivery for wider biomedical applications.

1. Introduction

In recent years, chemical vectors based on synthetic and natural polymers have emerged as efficient gene carriers for diverse biomedical applications including treatment of acquired and infectious diseases [1–4]. Amongst them, chitosan is the only natural cationic polysaccharide that has a capacity to strongly bind DNA in an acidic medium [5–7]. It is a linear, biodegradable and non-toxic polymer that generates low immune response [8,9]. Over the years, this polysaccharide has been considered as an attractive gene delivery vector [10–12]. However, low transfection efficiency of chitosans (low and high mol. wt.) has limited their applications *in vitro* and *in vivo*, which is mainly attributed to their strong binding to DNA, leading to a delay in the release of pDNA in cytoplasm [13]. This inefficient unpackaging from endosomes gradually leads to degradation of nucleic acids by the enzymes present in the cytosol [14]. In addition, high molecular weight chitosans (HMWC) are prone to form aggregates, which make them less soluble at physiological pH and their high viscosity make them unsuitable for *in vivo* gene delivery [15–17]. Therefore, modifications have been incorporated in the polysaccharide backbone through easily modifiable primary amino and hydroxyl groups. In a recent report from our laboratory, chitosan was converted into its chlorohydrin derivative, which was found to have improved solubility at physiological pH [18]. Chitosan chlorohydrin was subsequently conjugated with low molecular weight linear polyethylenimine (lPEI) to improve the buffering capacity of the resulting copolymer, which showed improved efficiency for gene delivery. Further, backbone modifications of chitosan involve labour intensive and multistep chemical synthesis [18]. Such procedures are expected to affect the

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doi:10.1016/j.biomaterials.2012.02.035

biodegradability of the resulting chitosan derivatives [18]. Alternatively, in an attempt to overcome the limitations of the HMWCs, several studies have been carried out on depolymerized chitosans, which are generally produced by enzymatic [19], oxidative [20], or ultrasonic degradation [21], to delineate the effect of molecular weight on transfection efficiency. Low molecular weight chitosans (LMWCs, 10–50 kDa) with improved solubility seem to be promising as gene delivery reagents *in vitro* and *in vivo* [22], however, contradictory results on efficiency of transfection have also been reported in the literature [13,23,24].

We hypothesized that the proposed chitosan conjugate should work as an efficient and biodegradable gene delivery agent compared to the previously reported one [18,22,23]. In this context, our strategy was to improve the solubility and buffering capacity of the chitosan for rapid endosomal escape keeping its backbone intact.

Taking advantage of the increased solubility of depolymerized chitosans, our strategy was to conjugate depolymerized chitosans through their terminal reactive groups to varying amounts of LMWP, which is known for its non-toxic properties [25,26]. The buffering capacity was also brought to the resulting depolymerized chitosans by amalgamating the proton sponge properties of LMWP. These conjugates were tested for their improved buffering capacity and enhanced endosomal escape rate concomitant with their higher transfection efficiencies and reduced cytotoxicity than the commercially available transfection reagents. Further, one of the conjugates, C7P3, was validated by *in vitro* delivery of siRNA, intracellular trafficking and *in vivo* transfection efficiency in Balb/c mice. In addition, for targeting specific cell type, we modified the chitosan-PEI conjugate, C7P3, to examine its efficacy for wider applications. Since tuftsin has been reported as a macrophage targeting molecule [27], we explored this possibility by targeting tuftsin-grafted C7P3 (C7P3-T/DNA) to reach macrophages in comparison to unmodified C7P3/DNA complex.

2. Materials and methods

2.1. Cell culture and materials

LMWP, branched PEI 25 kDa (HMWP), agarose, deoxyribonuclease I (DNase I), sodium cyanoborohydride ($NaCNBH_3$), sodium nitrite ($NaNO_2$), potassium persulphate ($K_2S_2O_8$), 3-(4,5 dimethylthazol-2-yl)-2,5 diphenyl tetrazole bromide (MTT) reagent, trypsin/ethylenediaminetetraacetate (EDTA), tetramethylrhodamine isothiocyanate (TRITC), and bovine serum albumin (BSA) were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO). High molecular weight chitosan (HMWC) was purchased from Fluka (Buchs, Switzerland). NMR spectra were obtained from JEOL-DELTA2 400 spectrometer (Tokyo, Japan) operating at 400 MHz using D_2O as a solvent. The qualitative functional group analysis in depolymerized chitosan was carried out by FTIR (a single beam Perkin Elmer, Spectrum BX Series, Massachusetts, USA) with the following scan parameters: scan range, 4400–400 cm^{-1}; number of scans, 16; resolution, 4.0 cm^{-1}; interval, 1.0 cm^{-1}; unit, %T. Commercially available transfection reagents, viz., Superfect™, GenePORTER 2™, Lipofectamine™, and Fugene™, were purchased from Qiagen (Courtaboeuf Cedex, France), Genlantis (San Diego, CA), Invitrogen (Carlsbad, CA) and Roche (Branchburg, NJ), respectively. Fetal bovine serum (FBS) was obtained from Gibco (Carlsbad, CA) and YOYO-1 was purchased from Invitrogen (Carlsbad, CA).

The cell lines, viz., HEK293, CHO, HeLa and A549 used in this study, were obtained from National Centre for Cell Sciences, Pune, India, and maintained as per the standard protocols. HEK293, CHO and HeLa cells were cultivated in Dulbecco's modified Eagle's culture medium (DMEM) from Sigma–Aldrich (St. Louis, MO), supplemented with 10% FBS, while A549 were cultivated in DMEM F-12 (Sigma–Aldrich) containing 10% FBS, 0.2% $NaHCO_3$, 1% antibiotic-antimycotic. Cultures were maintained at 37 °C in a humidified 5% CO_2 environment.

2.2. Animals for in vivo gene expression studies

In vivo studies were carried out on male Balb/c mice (6–7 weeks old, 25 ± 3 g), obtained from the animal breeding colony of Indian Institute of Toxicology Research (IITR), Lucknow (U.P., India) and were acclimatized under the standard laboratory conditions. The animals were cared humanely as per the guidelines laid down by the Animal Ethics Committee of the Institute.

2.3. Preparation of primary cultures of mice peritoneal macrophages (MPMΦ)

Male Balb/c mice (5–6 weeks old, 20 ± 2 g) obtained from the animal breeding colony of Indian Institute of Toxicology Research (IITR), Lucknow (U.P., India) were acclimatized and cared as described above. Bacillus Chalmette Guerin (BCG) stocks were thawed and resuspended at a density of 10^7 CFUs in 0.5 ml of sterile PBS without Ca^{+2}/Mg^{+2}. The peritoneal macrophage activation was done by challenging Balb/c mice with BCG (10^7 CFUs in 0.5 ml) intraperitoneally and left for five days. Six animals were sacrificed on day 6 and abdomen of each mouse was soaked with 70% ethanol. Then a small incision was made along the midline with sterile scissor. Abdominal skin was retracted manually to expose the intact peritoneal wall and PBS (10 ml) was injected in the peritoneal cavity essentially spleen side. All the peritoneal fluid after aspiration was centrifuged at 400 × g for 10 min at 4 °C and the supernatant was discarded. The pelleted cells were resuspended in RPMI medium containing 10% fetal bovine serum (FBS), 0.2% sodium bicarbonate ($NaHCO_3$), 1% antibiotic-antimycotic (Gibco BRL, USA) with gentle tapping the bottom of the tube and pipetting up and down several times. Cells were counted on a hematocytometer and ~1 × 10^6 cells were seeded in 6-well tissue culture plates. The cells were allowed to adhere to substrate by culturing them for 1–2 h in humidified 5% CO_2 environment at 37 °C. Non-adherent cells were removed by gently washing the cells with PBS (3 × 1 ml) and the remaining adhered macrophage cells were maintained as such. The adherent cells are comprised of more than 95% of macrophages.

2.4. Synthesis and characterization of depolymerized chitosans

2.4.1. (a) Depolymerization of chitosan with sodium nitrite

HMWC was depolymerized following a procedure described previously [28]. Briefly, HMWC (1 g) was suspended in 1 N hydrochloric acid (100 ml) at 25 ± 2 °C and after complete dissolution, pH of the solution was brought to 1.6 by adding a solution of sodium hydroxide followed by drop-wise addition of sodium nitrite (1 M, 1 ml) with vigorous stirring. After 1 h, it was cooled to 4° C and a solution of sodium hydroxide was added to bring the pH of the reaction mixture to 7.2. Subsequent addition of ethanol (500 ml) resulted in the precipitation of depolymerized chitosan, which was collected on a Buchner funnel and after thorough washing with ethanol (3 × 50 ml), it was dissolved in MilliQ water (25 ml) and dialyzed extensively (3.5 kDa cut-off) against water for 3 days with intermittent changes (6 × 12 h) of water. Finally, the lyophilization of depolymerized chitosan (7 kDa, C7) resulted in a white powder in ~85% yield.

2.4.2. (b) Depolymerization of chitosan with potassium persulfate

Persulfate-mediated depolymerization of chitosan was carried out following a procedure reported by Hsu et al. [29]. HMWC (1 g) was completely dissolved in 2% hydrochloric acid (100 ml) at 25 ± 2 °C. It was then heated to 70° C and potassium persulfate (500 mg) was added. After 10 min, the solution was cooled to 4° C, pH adjusted to 7.2 with a solution of sodium hydroxide and ethanol was added to precipitate out the depolymerized chitosan. The precipitated chitosan (10 kDa, C10) was processed, as described above, to obtain ~89% yield.

2.5. Conjugation of depolymerized chitosans (C7 and C10) with LMWP

To an aqueous solution of LMWP (1.47 mg, 34 μmol, 1 mg/ml) was added a solution of the depolymerized chitosan (7 kDa or 10 kDa, 50 mg, 0.31 mmol, 10 mg/ml in water) and kept on stirring for 2 h at 25 ± 2 °C. Subsequently, sodium cyanoborohydride ($NaCNBH_3$, 100 mg) was added and the reaction stirred for an additional 8 h at 25 ± 2 °C followed by an extensive dialysis against deionized water (6 × 12 h). After dialysis, the solution was lyophilized to obtain C7P1 in ~83% yield. Likewise, C7P2 (LMWP, 68 μmol, 2.94 mg), C7P3 (LMWP, 102 μmol, 4.41 mg), C7P4 (LMWP, 136 μmol, 5.88 mg), C10P1 (LMWP, 34 μmol, 1.47 mg), C10P2 (LMWP, 68 μmol, 2.94 mg), C10P3 (LMWP, 102 μmol, 4.41 mg) and C10P4 (LMWP, 136 μmol, 5.88 mg) were synthesized and lyophilized to obtain in ~78–83% yield. The conjugation of LMWP with depolymerized chitosans was determined by ^1H NMR.

2.6. Preparation of chitosan-LMWP-Tuftsin (C7P3-T) conjugate

Protected tuftsin (3.2 μmol) was dissolved in a mixture of dry N,N-dimethylformamide (0.5 ml) and triethylamine (50 μl). N-Hydroxysuccinimide (3.8 μmol) and EDAC (4 μmol) were added and the reaction mixture was stirred for 3 h at 25 ± 2 °C to generate tuftsin-NHS. To an aqueous solution of C7P3 (20 mg, 500 μl of MilliQ water), the above solution of tuftsin-NHS (110 μl, for 1% substitution) was added and the reaction mixture allowed to stir at 25 ± 2 °C. After 4 h, piperidine (0.5 ml) was added and the reaction mixture was agitated for 2 h, which was then dried on a rotary evaporator and the residue was suspended in trifluoroacetic acid: water (9:1, v/v, 2 ml). After stirring for 1 h, it was concentrated on a rotary evaporator. The residue was suspended in 10% aqueous sodium bicarbonate solution and