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Thiolated methylated dimethylaminobenzyl chitosan: A novel chitosan derivative as a potential delivery vehicle

Shirin Hakimi[a], Elaheh Mortazavian[b], Zohreh Mohammadi[c,*], Fatemeh Yazdi Samadi[c], Hamidreza Samadikhah[d], Sadegh Taheritarigh[e], Niyousha Rafiee Tehrani[f], Morteza Rafiee-Tehrani[g]

[a] Faculty of Pharmacy, International Campus, Tehran University of Medical Sciences, Tehran, Iran
[b] Medicinal plants Research Center, Institute of Medicinal Plants, ACECR, Karaj, Iran
[c] Nanobiotechnology Research Center, Avicenna Research Institute, ACECR, Tehran, Iran
[d] Department of Nanobiotechnology, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran
[e] Department of Plant Breeding and Biotechnology, Faculty of Plant Production, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran
[f] Faculty of Medicine, Tehran University of Medical Sciences, Tehran, Iran
[g] Department of Pharmaceutics, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran

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ABSTRACT

Chitosan is a natural mucoadhesive, biodegradable, biocompatible and nontoxic polymer which has been used in pharmaceutical industry for a lot of purposes such as dissolution enhancing, absorption enhancing, sustained releasing and protein, gene or drug delivery. Two major disadvantages of chitosan are poor solubility in physiological pH and low efficiency for protein and gene delivery. In this study thiolated methylated *N*-(4-*N*,*N*-dimethylaminobenzyl) chitosan was prepared for the first time in order to improve the solubility and delivery properties of chitosan. This novel chitosan derivative was characterized using ^{1}H NMR, Ellman test, TGA and Zetasizer. Cell toxicity studies were performed on Human Embryonic Kidney 293 (Hek293) cell line using XTT method, to investigate the potential effect of this new derivative on cell viability. ^{1}H NMR results showed that all substitution reactions were successfully carried out. Zeta potential of new derivative at acidic and physiological pHs was greater than chitosan and it revealed an increase in solubility of the derivative. Furthermore, it had no significant cytotoxicity against Hek293 cell line in comparison to chitosan. These findings confirm that this new derivative can be introduced as a suitable compound for biomedical purposes.

1. Introduction

Chitosan is a natural non-toxic polymer which its biocompatibility and biodegradability properties in addition to low cost make it a good candidate for using in pharmaceutical and medical industry [1,2]. Existence of functional groups in chitosan structure has been led to its wide use in delivery and targeting purposes including drug, protein, gene and vaccine delivery [3–5]. However this polymer has also unfavorable properties such as poor water solubility at physiological pH which could result in some limitations in protein, gene or drug delivery [6]. Nowadays, many researchers are trying to improve the chitosan properties by conjugating of different molecules to the polymeric backbone. Trimethylated chitosan (TMC) [7], PEGylated chitosan [8], *N*-acetylated chitosan [9], Galactosylated chitosan (GC) [10], *N*-dodecylated chitosan [11] and *N*-alkylated chitosan [12] are some examples of these investigations. Thiolated chitosan is another derivative which shows an interesting mucoadhesive, permeation and cellular uptake properties in gene and drug delivery studies both in vitro and in vivo [13,14]. To improve both water solubility and drug/gene delivery efficacy in addition to decrease cytotoxicity, a methylated *N*-(4-*N*,*N*-dimethylaminobenzyl) derivative of chitosan was developed successfully by Rojanarata et al.[6,15]. This structure demonstrated desirable solubility and delivery properties with low cytotoxicity.

In this study, to profit the desirable properties of both thiolated and aminobenzyl chitosan, a new generation of chitosan derivative called thiolated methylated *N*-(4-*N*,*N*-dimethylaminobenzyl) chitosan (M-Bz-Cs-Cys), was synthesized and characterized. More-

* Corresponding author.
E-mail address: z.mohammadi@avicenna.ac.ir (Z. Mohammadi).

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over the cytotoxicity study of this new derivative was investigated to evaluate whether this derivative is appropriate for the delivery purposes.

2. Materials and methods

2.1. Materials

Low molecular weight chitosan (95% DA)(Premix), acetic acid, 4-*N*,*N*-dimethylbenzaldehyde, *N*-methyl pyrolidone, iodomethane, sodium hydroxide and sodium chloride were purchased from Merck (Darmstadt, Germany). Sodium borohydride ($NaCNBH_3$) was provided from Aldrich (UK), Dialysing tube with a molecular cutoff of 12,000 Da (D0405) was obtained from Sigma (USA), polyethylenimine (PEI), MW 25 kDa, were purchased from Aldrich, Germany. 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2*H*-Tetrazolium-5 Carboxanilide (XTT) was purchased from Sigma-Chemical Co., USA. Dulbecco's modified Eagle's medium (DMEM), Trypsin–EDTA, penicillin–streptomycin antibiotics and fetal bovine serum (FBS) were obtained from GIBCO-Invitrogen (USA). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), N hydroxysuccinimide (NHS), L-cysteine hydrochloride (Cys), dimethyl sulphoxide (DMSO), HEK293 cell line (human embryonic kidney cells) was from the American Type Culture Collection (ATCC, Rockville, MD, USA), Ellman's reagent (DTNB, 5,5 – dithiobis(2-nitrobenzoic acid)) purity ≥98%, obtained from Sigma–Aldrich.

2.2. Methods

2.2.1. Synthesis of (4-N,N-Dimethylaminobenzyl) chitosan

(4-N,N-Dimethylaminobenzyl) Chitosan was prepared as previously reported [15]. 2.00 g of chitosan dissolved in 1% acetic acid (pH 4, 200 mL), 4.00 g 4-*N*,*N*-dimethylamino benzaldehyde was added and stirred at room temperature for 24 h, with the final adjusted pH at 5.0 (using 1 N NaOH). Then 4.00 g $NaCNBH_3$ was added to the solution. The solution was stirred at room temperature for another 24 h (pH was adjusted to 7.0 using 1 N NaOH). The solution was precipitated and dried.

2.2.2. Synthesis of methylated N-(4-N,N-Dimethylaminobenzyl) (M-Bz-Cs)

2.00 g of dried precipitate obtained from the previous step was dispersed in 100 ml *N*-methyl Pyrrolidone (NMP) and stirred at room temperature for 24 h. Then 15 ml of iodomethane was slowly added in 5-ml portions over a 3-h period. The solution was refluxed at 50 °C for 72 h. The purified compounds obtained by precipitation in 450 mL of acetone, were dissolved in 20 ml of water. This solution was dialyzed against NaCl 10% (w/v) for 3 days and then precipitated by adding 450 ml of acetone to obtain the chloride salt. The final compounds were subjected to further analysis (Scheme 1).

2.2.3. Synthesis of thiolated (M-Bz-Cs)

Cys was used to modify M-Bz-Cs. As mediated by EDAC/NHS, amide bonds were formed between the carboxyl groups on Cys and residual primary amino groups on chitosan. On this regard, 4.00 g of Cys and 2.00 g of M-Bz-Cs were dissolved in 2 mL of a 200 mM solution of EDAC and NHS in water. This mixture was stirred for 5 h in darkness at room temperature (pH 5.0). Then a 5-day dialysis was applied to the obtained TMC-Cys at 4 °C against HCl (pH 5.0) and the resultant polymeric solution, which included (M-Bz-Cs)-Cys, was lyophilized and stored at 4 °C for further investigations (Scheme 2).

2.2.4. ^1H NMR analysis

The ^1H NMR spectrum was recorded in D_2O using a 500 MHz spectrometer (Brucker AC500, USA) and the degree of quaternization was calculated. The chemical shift was adjusted with external standard (dioxane in D_2O).

2.2.5. Determination of thiolation degree of synthesized polymer

To determine the amount of thiol groups and degree of modification Ellman's test had been used according to its standard procedure [16]. Briefly, Ellman's reagent, Tris solution, and sample were mixed together in appropriate amounts and its absorbance was measured at 412 nm using UV–vis spectrophotometer (Cary100, Varian Company).

2.2.6. FTIR spectra

FT-IR spectra were recorded by PerkinElmer Spectrum version10.4.1 Fourier Transform Infrared (FTIR) spectrometer. All samples were prepared as potassium bromide pellets.

2.2.7. Thermogravimetric analysis (TGA)

Thermogravimetric analysis curves of chitosan, M-Bz-Cs and thiolated (M-Bz-Cs)resulted molecules were obtained in Nitrogen atmosphere, using a TGA (TGA ʒ 50 SHIMADZU), with a heating rate of 10 °C/min, from room temperature to 595 °C.

2.2.8. Zeta potential measurements

Zetasizer ZS (Malvern Instruments Ltd., Malvern, UK) were used to determine the surface charge of chitosan derivative and unmodified chitosan in two acidic and physiological pHs (5, 7.2). Filtered distilled water (0.22 micron membrane filter) was used for solving the polymer.

2.2.9. Evaluation of cytotoxicity

2.2.9.1. Cell culture. Human Embryonic Kidney 293 cells (Hek 293) were seeded in DMEM media supplemented with FBS (10% v/v) and penicillin/streptomycin (100 Units/ml) at 37 °C in a 5% CO_2 incubator.

2.2.9.2. XTT assay. Cytotoxicity of polymers was determined using XTT assay. 24 h before performing the test, cells were seeded into 96-well plates at the density of 10000 cells/100 μl medium per well. Test cells, treated with the different concentrations of chitosan, M-Bz-Cs or PEI (as control). cells were incubated for another 24 h; Then fresh XTT (1 mg/ml in pre warmed (70 °C) serum-free medium) and PMS (5 mM (1.53 mg/ml) in PBS) were mixed together and were added to each well at the final amount of 50 μg of XTT and 0.38 μg of PMS per well. The cells were incubated for 4 more hours at 37 °C in a 5% CO_2 incubator. Finally the absorbance was measured at 450 nm using ELISA reader (ELx800, Biotek).

2.3. Statistical analysis

Statistical analysis was performed using Student's *t*-test and the level of statistical significance was considered at $p < 0.05$.

3. Results and discussion

3.1. ^1H NMR analysis Of M-Bz-Cs

The ^1H NMR spectra of chitosan and synthesized quaternized aromatic derivatives have been shown in Figs. 1 and 2, respectively. In chitosan spectra the signal at *d* 1.8 ppm, indicates N acetyl group proton. The signals at δ 2.7–4.0 ppm are referred to the protons of sugar skeleton of chitosan. The peak at δ 5.4 ppm is assigned to anomeric hydrogens. In Fig. 2, the sharp peaks at 2.1 and 2.8 ppm represent methyl protons of (–N (CH_3)–) and (–N + $(CH_3)_2$–), respectively, related to aliphatic amine of methylated (aminobenzyl) chitosan, indicating formation of quaternized derivative [1,6]. Sharp peaks at 3.3 and 3.6 ppm indicate methyl

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