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Methylated 4-*N*,*N* dimethyl aminobenzyl *N*,*O* carboxymethyl chitosan as a new chitosan derivative: Synthesis, characterization, cytotoxicity and antibacterial activity

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ABSTRACT

Chitosan, as a biocompatible polymer, is very attractive for biomedical applications. Continues studies are performing for improving its physicochemical features in order to make it more suitable for such approaches. In this study, methylated 4-*N*,*N* dimethyl aminobenzyl *N*,*O* carboxymethyl chitosan (MABCC) was synthesized,as a new chitosan derivative, in three steps. The investigations were carried out using FTIR, NMR, TGA and zeta potential measurement. Antibacterial and cell viability assessments were performed on four bacterial strains and two cell lines respectively. FTIR and NMR results showed that all substitution reactions were successfully carried out. Zeta potential of MABCC at various pH especially alkaline pH was greater than chitosan and it revealed increasing the solubility of the derivative. Antibacterial activity of MABCC was extremely greater than chitosan especially in Gram positive bacteria.Furthermore,it had no significant cytotoxicity against MCF-7 and Skov-3 cell lines in comparison to chitosan. These findings confirm that this new derivative can be introduced as a suitable compound for biomedical purposes.

1. Introduction

Naturally derived polymers have gotten much attention in medical sciences and biotechnology in recent decades (Porto, 2012). Chitosan is one of these polymers which plays a progressive important role in such studies, because of its unique properties like biodegradability, biocompatibility, antimicrobial activity, nontoxicity, low immunogenicity, accessibility and inexpensiveness(Avadi et al., 2004; Cheung, Ng, Wong, & Chan, 2015; Jain, Kumar, & Dutta, 2015; Upadhyaya, Singh, Agarwal, & Tewari, 2013). Presence of functional groups like hydroxyl and amino groups in structure of chitosan allows it to be chemically modified in order to improve its physical characteristics such as solubility and electric charge (Li, Yang, & Yang, 2015; Samadi, Mohammadi, Yousefi, & Majdejabbari, 2016). Among several modifications, quaternization of chitosan is a well-studied method that leads to better solubility in physiological pH and increase of its positive charge. Improvement of these

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features can enhance antimicrobial activity and cellular uptake of chitosan derived systems in gene or drug delivery(Cheung et al., 2015; Garaiova et al., 2012). Trimethylation of amino groups in chitosan structure is one of the most simple methods of quaternization which has been studied by several researchers (El-Sherbiny, Salih, & Reicha, 2015; Germershaus, Mao, Sitterberg, Bakowsky, & Kissel, 2008a; He, Yin, Tang, & Yin, 2013; Hu, Tang, & Yin, 2014). Chitosan solubility improvement can also be achieved by carboxymethylation as a hydrophilic modification. Carboxymethyl chitosan has a lot of biomedical applications such as wound healing, tissue engineering, bio imaging and drug/gene delivery (Anitha et al., 2011; Chen & Park, 2003; Upadhyaya et al., 2013). Sincequaternization and carboxymethylation can increase antibacterial activity,some researches were undertaken into synthesize trimethylcharboxymethylchitosans(Geisberger, Gyenge, Maake, & Patzke, 2013; Patrulea, Applegate, Ostafe, Jordan, & Borchard, 2015; Xu, Xin, Li, Huang, & Zhou, 2010). In addition of hydrophilic moieties, chitosan could be modified by hydrophobic substitution such as aromatization. This modification can improve some chitosan properties which are important in gene/drug delivery applications. For instance aromatized derivatives of chitosan can form smaller gene containing nanoparticles because of strong interaction between polymer and nucleic acid. Moreover, these derivatives can facilitate the dissociation of gene from nanocomplexes in target site. (Holappa, Nevalainen, Soininen, Másson, & Järvinen, 2006; Jiao et al., 2011; Sajomsang, Ruktanonchai, Gonil, Mayen, & Opanasopit, 2009; Sajomsang, Tantayanon, Tangpasuthadol, & Daly, 2008).

So, those chitosan derivatives which containing both hydrophilic and hydrophobic moieties can be very attractive in drug/gene delivery studies.

In this study we synthesized methylated 4-*N,N* dimethyl aminobenzyl *N,O* carboxymethyl chitosan as an innovative amphiphilic chitosan derivative in order to profit the advantages of both hydrophilic and hydrophobic moieties. Antibacterial activity of this new derivative was tested on two gram negative and two gram positive bacteria and also cytotoxicity was determined in two cell lines of breast and ovarian cancers.

2. Materials and methods

2.1. Materials

Chitosan with aMw of 120 kDa and a 95% degree of deacetylation, was purchased from Primex company (Iseland). Sodium borohydride, ninhydrin powder, XTT powder (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) and PMS (phenazinemethosulfate) powder were purchased from Sigma(USA). Dialysis tube with molecular weight cut off of 12,000–14,000 g/mol was purchased from Sigma Aldrich (USA). 4*N,N* dimethylaminobenzaldehyde, acetic acid, monochlorobromic acid, *N* methyl pyrolidone (NMP), EDTA, triethylamine and iodomethane were purchased from Merk (Germeny). DMEM cell culture medium, fetal bovine serum (FBS) and trypsin were purchased from Gibco (USA). Escherichia coli (ATCC25922), Pseudomonas aeruginosa (ATCC27853), Staphylococcus aureus (ATCC29213) and Staphylococcus epidermis (ATCC1435), MCF-7and Skov-3 cell line supplied by American Type Culture Collection (ATCC).

2.2. Synthesis and characterization

2.2.1. Synthesis of 4-N,N dimethyl aminobenzyl chitosan

1 g chitosan was suspended in 200 mL distilled water and then in order to increase the solubility, a few drops of glacial acetic acid were added to the suspension. The pH of the solution was adjusted to about 5. When all of the chitosan was dissolved in the media, 1.3 g 4-*N,N* dimethylaminobenzaldehyde that was dissolved in 20 mL methanol was added to the chitosan solution stepwise. The solution was stirred at 25 °C for 48 h. Sodium borohydride (600 mg) was added to the reaction mixture in 3 steps. After 1 h, precipitate was filtered and washed twice with acetone and then dissolved in the aqueous solution of 1% acetic acid and dialyzed for 3 days (First day against 1% acetic acid solution and then against distilled water). At last dialyzed solution was lyophilized.

2.2.2. Methylation of 4-N,N dimethyl aminobenzyl chitosan

The precipitant of previous step was dissolved in 100 mL solution of 1% acetic acid. Then this solution was gradually added to 100 mL sodium bicarbonate solution (2% w/vin H_2O:MeOH, 40:60 v/v). Because of the alkaline pH of the mixture, the polymer was precipitated. After that, it was filtered and suspended in 20 mL NMP and the mixture was stirred at 25 °C for 12 h. Then 2 mL triethylamine was added to the reaction media. Iodomethane (10 mL) was added in two steps during 1 h. Stirring of the mixture was continued under reflux condition at 50 °C for 48 h and another 5 mL of iodomethane was added in 24th hour. After that, 300 mL acetone was added to the solution to precipitate the methylated polymer. To substitution of iodide with chloride, the precipitance was dissolved in 50 mL of 15% w/v aqueous sodium chloride solution. Then the solution was dialyzed for 3 days (First day against 1%aqueous sodium chloride solution and then against distilled water). The dialyzed solution was freeze dried.

2.2.3. Carboxymethylation of methylated 4-N,N dimethyl aminobenzyl chitosan

Lyophilized compound of the previous stage was suspended in 70 mL isopropanol at 45 °C under reflux condition overnight. Then 3.5 mL of 50% aqueous NaOH solution was added to the mixture gradually in 20 min. After 45 min, 0.18 g monobromoacetic acid over a period of 25 min was added to the reaction medium and stirring continued for 24 h at 60 °C under argon atmosphere. Next, pH of the solution was adjusted to 7 by adding HCl and then water and isopropanol were removed by rotary evaporator. Subsequently the precipitant was dissolved in 1% aqueous HCl solution and dialyzed for 3 days (First day against 1%aqueous HCl solution and then againstdistilled water). The dialyzed solution was freeze dried.

2.2.4. FTIR spectra

FT-IR spectra were recorded by PerkinElmer Spectrum version 10.4.1 Fourier Transform Infrared (FTIR) spectrometer. All samples were prepared as potassium bromide pellets.

2.2.5. ^{1}HNMR spectra

^{1}H NMR spectra were measured by Bruker 500 MHz spectrometer. The samples were dissolved in D_2O.

2.2.6. Ninhydrin test

Ninhydrin test was run to quantify the primary amino groups of the derivative which didn't take part in the reactions. Firstly 150 μg/mL glycine stock solution as a standard solution was prepared and diluted into five concentrations. 1 mL of ninhydrin reagent (8% w/v in acetone) was added to 4 mL of each tube of the standard, sample (90 μg/mL)and blank (water) solution. Both sample and standard were dissolved in 0.2 M acetate buffer. After mixing the tubes, they were placed in boiling water for 15 min. Followed by adding 1 mL ethanol into each tube, their absorption were analyzed by Bio Photometer at 550 nm (Meyer, 1957).