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A new chitosan–thymine conjugate: Synthesis, characterization and biological activity

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ABSTRACT

Conjugation of chitosan with nucleobases is expected to expand its not only antimicrobial activity but also anti-cancer activity. Here, we report the synthesis of a novel chitosan–thymine conjugate by the reaction between chitosan and thymine-1-yl-acetic acid followed by acylation. The synthesized conjugate was characterized by FTIR, XRD, ^{1}H NMR, TGA and SEM. The microbiological screening results demonstrated the antimicrobial activity of the conjugate against bacteria viz., *Escherichia coli*, *Staphylococcus aureus*, and fungi viz., *Aspergillus niger*. The chitosan–thymine conjugate also inhibited ($p < 0.05$) the proliferation of human liver cancer cells (HepG2) in a dose-dependent manner but had no cellular toxicity in non-cancerous mouse embryonal fibroblast cells (NIH 3T3). Thus, the chitosan–nucleobase conjugate may open a new perspective in biomedical applications.

1. Introduction

During the past few decades, there has been an increasing interest in the utilization of nucleobase conjugates with various natural and synthetic biopolymers. Phenanthridinium–nucleobase conjugates [1], metallocene–nucleobase conjugates [2], symmetrical and unsymmetrical α,ω-nucleobase mono- & bis-amide conjugates [3], cyclodextrin–DNA conjugate [4], ferrocene–bis(nucleobase) conjugates [5], neamine–nucleoside conjugates [6], DNA-peptide conjugates [7], peptide–nucleobase conjugates, nucleobase PNA conjugates [8] are few examples that can inhibit the expression a specific DNA or mRNA molecule, inducing a blockade in the transfer of genetic information from DNA to protein either by antigene or antisense strategy. Single stranded domains of DNA and RNA play an essential role in a number of processes in living cells including those involving viruses. Nucleobase-containing peptides, also referred to as nucleopeptides, represent a promising class of molecules of important biomedical significance presenting a peptide-like backbone conjugated to nucleobases through different linker moieties. Recently, Gross et al. have synthesized a bimetallic ruthenocene dicobalt-hexacarbonyl alkyne peptide bioconjugate [9]. Given these benefits, we are interested in modifying the chitosan with nucleobases for their utilization in various biomedical applications. Numerous works have been published on the chemical modification of chitosan [10–18]. This polymer is still being modified to produce various derivatives with improved properties. We have recently demonstrated that chitosan–chloroquinoline conjugate has also potent antimicrobial activity [19]. Several previous studies have synthesized various thymine derivatives and reported potent anti-cancer effect [20–23]. More recently, Manna et al. found that, modification of hyaluronic acid with thymine, by the LBL process, imparted them an anticancer activity [16]. The conjugation of a nucleobase to chitosan may therefore, expand its biomedical utility to include both antimicrobial and anti-cancer action. Chitosan is readily soluble in various acidic solvents, which limits its applications [17]. It has high antimicrobial activity against many pathogenic and spoilage microorganism, including Gram-positive and Gram-negative bacteria and fungi. The exact antimicrobial mechanism of chitosan is still unclear, but several mechanisms have been proposed. The most feasible hypothesis is the leakage of cellular proteins and other intracellular constituents caused by the interaction between the positively charged chitosan and negatively charged microbial cell membranes. Other mechanisms proposed are the inhibition of microbial growth and toxin production by the chelation of essential metals and nutrients, spore components, as well as the penetration of the nuclei of the microorganisms, which leads to the interference of mRNA and protein synthesis. The principle that underlines the anticancer activity of chitosan–nucleobase is conceptually very simple and straightforward. Being an analogue of natural nucleobases, chitosan–nucleobase conjugate may be incorporated into the nuclear DNA during DNA synthesis and into mRNA during transcription. Since chitosan–nucleobase lacks the 3′OH group that is required for the attachment of additional nucleotides, its

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incorporation into DNA or mRNA would induce strand breakage by chain termination leading to cell cycle arrest [24]. Given that cancer cells generally divide faster and have shorter cell cycle, they are likely to be affected by the chitosan–nucleobase more than the non-cancerous or slow dividing cells [25]. The specificity against cancer cells may further be increased by conjugating the chitosan with a polynucleotide whose sequence is complementary to that of oncogene or its mRNA product. In this case, the sequence specific nucleobase of chitosan–nucleobase conjugates will interact with the DNA/mRNA of target site by complementary base pairing (Thymine/Uracil with Adenine and Cytocine with Guanine) and thereby, would inhibit the DNA synthesis and/or mRNA transcription and translation of the cancer causing gene. However, the chitosan–thymine conjugate has not been reported so far as per our knowledge. With this view the present work is directed to the synthesis, characterization and evaluation of the biological activities of the new biopolymer nucleobase conjugate.

2. Experimental

2.1. Materials

The chitosan powder was a product of Qingdao Yunzhou Biochemistry Co. Ltd., China, average molecular weight about <5000 g/mole (Mv) and a degree of deacetylation (DD) of 90%. Thymine (Sigma–Aldrich), KOH (Sigma–Aldrich), bromoacetic acid (Sigma–Aldrich), conc. HCl (Samchun Chemicals, Korea), DCC (Flucka analytical), acetic acid, and ethanol (Dae Jung, Korea) were used without further purification. The antimicrobial test strains, *Escherichia coli* MTCC 739, *Staphylococcus aureus* MTCC 3160, and *Aspergillus niger* MTCC 3537, were arranged from IMTECH, Chandigarh. The purity of all the synthesized compounds has been checked by TLC using silica gel with different solvent systems.

2.2. Synthesis of thymine-1-yl acetic acid

In a 250 mL of round bottomed flask thymine (20 mmol) was dissolved in a solution of potassium hydroxide (80 mmol) in 15 mL of water. While this solution was warmed in a 42 °C in water bath, a solution of bromoacetic acid (35 mmol) in 10 mL of water was added over 30 min. After this, the reaction was stirred for 2 h at this temperature. It was allowed to cool to room temperature (15 °C) and the pH was adjusted to 5.5 with conc. HCl. The solution was then cooled in a refrigerator for 2 h. Precipitate formed was removed by filtration. The solution was then adjusted to pH 2 with conc. HCl and put in a freezer for 5 h. The resulted white precipitate product (Scheme 1) was isolated by filtration, washed with water 2–3 times and dried in vacuum oven at 35 °C for 7 h. The yield 82%, White solid, and m.p. 253–255 °C was obtained according to procedure as described elsewhere [26].

2.3. Synthesis of chitosan–thymine conjugate

To synthesize chitosan–thymine conjugate 100 mg of chitosan powder dissolve in 1% (w/v) hydrochloric acid. The mixture was vigorously stirred by a magnet stirrer at room temperature until the polymer was completely dissolved. 0.294 g thymine-1-yl acetic acid solution was added into the chitosan solution and stirred for 2 h. Then, 0.355 g DCC dissolved in 20 mL of 95% ethanol was added to induce the acylation reaction. The mixture was then stirred for 8 h at room temperature (15 °C). When the reaction finished, the product was then filtered by vacuum and washed with 95% ethanol to remove excessive DCC. The final product (Scheme 1) was dried in vacuum oven at 35 °C for 4 h.

2.4. Measurements

Fourier transform infrared (FT-IR) spectra were recorded on JASCO FT-IR 300E device using KBr. ^{1}H NMR spectra of the samples were recorded on a Bruker 600 MHz NMR spectrometer using tetramethylsilane (TMS) as an internal standard and CD_3COOD and DMSO as a solvent. XRD pattern of the samples were recorded on X-ray diffractometer (D/Max2500VB+/Pc, Rigaku, Japan) with CuKα characteristic radiation (wavelength $\lambda = 0.154$ nm) at a voltage of 40 kV and a current of 50 mA. The scanning rate was 3°/min and the scanning scope of 2θ was from 2° to 45° at room temperature (25 °C). Thermogravimetric analysis (TGA) was carried out in a TA Q 50 system TGA. The samples were scanned from 0 to 800 °C at a heating rate of 10 °C/min under flow of nitrogen. The surface morphology was analyzed by scanning electron microscopy (SEM) JEOLJSM-6490LA. All the spectra were taken in room temperature.

2.5. Antimicrobial activity assay

The antimicrobial activity of the chitosan–thymine conjugate was evaluated by the agar plate disc-diffusion method [27]. Briefly, the solution (0.1%, 0.05% and 0.01%) of the chitosan–thymine conjugate was absorbed in sterilized discs and placed on the lawn cultures of selected microorganisms to assess their antimicrobial activity against one Gram-positive (*S. aureus*), one Gram-negative (*E. coli*) bacteria and one fungus (*A. niger*). The solution (0.1%) of the chitosan only and (0.1%) of thymine only was used for antimicrobial activity. The agar plates were incubated at 37 °C for 24 h and diameters of the inhibitory zone of clearance (mm) surrounding the discs were measured to estimate the antimicrobial activity.

2.6. Assays for cellular cytotoxicity, proliferation, and viability

Mouse embryonic fibroblast cell line (NIH 3T3) and human liver cancer cell line (HepG2) were cultured in Dulbecco's modified Eagle's medium (DMEM, high glucose formulation; Gibco BRL, Grand Island, NY) supplemented with 10% (v/v) fetal bovine serum (Hyclone, Logan, UT), MEM nonessential amino acids (Gibco BRL), 50 μM 2-mercaptoethanol (Sigma–Aldrich Co., St Louis, MO), and chitosan–thymine conjugate (0, 5, 50, 100 μM) for seven days at 37 °C in a humidified atmosphere of 5% CO_2 in air. Non-treated cells and those treated with pure chitosan or thymine (100 μM) were used as controls for comparison. The cells were plated in 24-well plates at an initial seeding density of $2–4 \times 10^4$ cells/mL in triplicates and were evaluated for the rate of cellular toxicity and proliferation by counting the total number of cells every 24 h, as we described earlier [28]. The population doubling time was calculated with the equation $Y_{end} = Y_{start} \times 2(t/T)$, where T is the population doubling time, Y_{start} is the initial cell count, and Y_{end} is the cell count at the end of culture period (t). The rate of cell proliferation (r) was calculated with the equation $r = (\log NH - \log NI)/T2 - T1$, where NH is number of cell harvested, NI is number of cells initially seeded, T1 is the time at seeding (h), and T2 is the time till harvesting (h) [29]. Viability of cells was evaluated based on the esterase enzyme activity and plasma membrane integrity upon FDA (3′6′diacetyl fluorescein diacetate) assay as described earlier [28]. Briefly, cells were washed in Dulbecco's phosphate-buffered saline (DPBS) for 1 min followed by incubation with 2.5 μg/mL FDA stain for 1 min. Stained cells were then washed in PBS to remove the traces of the dye and observed under UV illumination of an epifluorescent microscope fitted with FITC filter set (excitation: 460–490 nm; emission; 515–550 nm; dichromatic: 505 nm). Live cells emitted green fluorescence while dead ones were non-fluorescent. Viability was calculated as the number of green cells/total number of cells × 100. All experiments were repeated three times.