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Design, synthesis, and evaluation of chitosan conjugated GGRGDSK peptides as a cancer cell-targeting molecular transporter

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ABSTRACT

Targeting cancer cells using integrin receptor is one of the promising targeting strategies in drug delivery. In this study, we conjugated an integrin-binding ligand (GGRGDSK) peptide to chitosan oligosaccharide (COS) using sulfo-SMCC as a bifunctional linker to afford COS-SMCC-GGRGDSK. The conjugated polymer was characterized by FT-IR, ^{1}H NMR, ^{13}C NMR, and SEM. COS-SMCC-GGRGDSK did not show cytotoxicity up to a concentration of 1 mg/mL in the human leukemia cell line (CCRF-CEM). The conjugate was evaluated for its ability to enhance the cellular uptake of a cell-impermeable cargo (*e.g.*, F′-G(pY)EEI phosphopeptide) in CCRF-CEM, and human ovarian carcinoma (SK-OV-3) cancer cell lines. Additionally, RGD modified and unmodified COS polymers were used to prepare nanoparticles by ionic gelation and showed particle size ranging from 187 to 338 nm, and zeta potential of 12.2–18.3 mV using dynamic light scattering. The efficiency of COS-NPs and COS-SMCC-RGDSK NPs was assayed for translocation of two synthetic cytotoxic agents ((2-(2-aminoethylamino)-4-(4-chlorophenyl)-6-(1H-indol-3-yl) nicotinonitrile (ACIN), and 2-(2-aminoethylamino)-6-(1H-indol-3-yl)-4-(4-methoxyphenyl)-nicotinonitrile (AMIN)) into CCRF-CEM and human prostate (DU-145) cancer cell lines. The results showed a dramatic reduction in the cell viability on their treatment with RGD targeted COS NPs in comparison to paclitaxel (PTX), free drug, and drug-loaded COS NPs.

1. Introduction

Over the past three decades, significant efforts have been devoted to develop efficient, nontoxic, drug delivery systems (DDSs) that offer selective translocation and controlled release of the cell impermeable molecular cargoes, such as drugs, proteins, antibodies, genes, and dyes, into the cell cytoplasm to perform their biological activity inside the targeted tissue. Cell-penetrating peptides (CPPs), nanoparticles, and polysaccharides including hyaluronic acid, dextran, water soluble cellulose derivatives, and chitosan derivatives have been used as tools for developing drug delivery systems [1–5].

Chitosan is a cationic polysaccharide composed of randomly distributed β-(1–4)-linked D-glucosamine and *N*-acetylated-D-glucosamine units. Chitosan and its derivatives are attractive biopolymers due to their biodegradability, low immunogenicity, and biocompatibility, which give them their favorable physicochemical and pharmacokinetic properties [6]. The primary amino groups (NH_2) on the C-2 position of chitosan D-glucosamine building block creates the positive charge on the chitosan surface that facilitates the intracellular translocation of its payload into the cells [7]. The ability of chitosan to pass through the cell membranes is due to the interaction of its protonated amino groups with the cell membrane phospholipids, which results in a reversible structural change and the opening of tide junction [8]. Moreover, the cationic nature of chitosan and its derivatives has inspired the investigators to use them as promising non-viral gene vectors because of their less cytotoxicity, immunogenicity and mutagenesis response in comparison with the viral vectors and polyarginine or polyethylene imine (PEI), and their ability in the formation of a stable complex with DNA and siRNA [9–12].

Since the utilization of chitosan is hindered by its poor solubility, chemical modification of chitosan is required to modulate its physicochemical and pharmacological properties [13]. These new derivatives increase the stability of the drug or gene/carrier complex, and the polymer ability in protecting its cargo from *in vivo*

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serum lytic enzymes, and lysosomal digestion inside the cells [14]. The tumor microenvironment such as pH, degree of vasculature, hypoxia, metabolites, receptors upregulation, has been utilized to achieve targeting for diagnosis, or treatment purpose [15–17]. Therefore, attaching a tumor targeting moiety to chitosan [18] such as folic acid [19], galactose [20], or homing peptides [21–23] has been used to direct the carrier system selectively to the site of action (organ or tissue). Tumors originated from epithelial cells overexpress heterodimeric glycoproteins consisting of α and β subunits, which combine to form various types of integrin receptors found in the extracellular matrix. These receptor proteins have high binding affinity for the ligands containing RGD peptide motifs [24–26]. RGD-based chitosan delivery systems have been widely used in bone tissue engineering and articular cartilage regeneration by enhancing the cell adhesion, growth, and differentiation. However, the use of integrin-targeting chitosan system as drugs and gene delivery to different tumor tissues is limited and still needs further exploration [27–30].

Herein, we report the synthesis of GGRDSK motif as integrin targeting RGD peptide and its immobilization on the surface of water-soluble chitosan oligosaccharide (COS) via a facile strategy using (sulfo-SMCC) cross-linking spacer. The efficiency of chitosan-RGD as a molecular transporter and the cellular uptake mechanistic study were evaluated on a panel of endothelial cancer cell lines including; leukemia (CCRF-CEM), ovarian (SK-OV-3), and prostate (DU-145) cancer cell lines as preliminary *in vitro* study models.

2. Materials and methods

2.1. Materials

All required organic solvents were purchased from Wilkem Scientific (Pawtucket, RI, USA). Coupling reagents, Rink amide MBHA resin, and Fmoc-amino acid building blocks were purchased from Chem-Impex International Inc. (Wood Dale, IL, USA). Chitosan oligosaccharide and other reagents and chemicals were purchased from Sigma-Aldrich Chemical Co. (Milwaukee, WI, USA).

2.2. Synthesis of linear peptide $HS\text{-}(CH_2)_2CONH\text{-}GGRGDSK\text{-}CONH_2$

In general, the peptide was synthesized by the solid-phase synthesis strategy employing *N*-(9-fluorenyl) methoxycarbonyl (Fmoc)-based chemistry and Fmoc-L-amino acid building blocks (Scheme 1). The sequence of the linear peptide of the sequence (thiopropionyl-GGRGDSK) was synthesized using Tribute automated peptide synthesizer (Protein Technology, Inc., Arizona). 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and 0.4 M *N*-methyl morpholine (NMM) in *N*,*N*-dimethylformamide (DMF) were used as coupling and activating reagents, respectively. Fmoc deprotection at each step was carried out using piperidine in DMF (20% v/v). The linear peptide carrying protected side chains was assembled on the Rink amide MBHA resin (loading 0.52 mmol/g) in 0.40 mmol scale. Then, the resin was washed with DMF (2 times, 10 min each) and DCM (3 times, 10 min each). After the peptide synthesis was completed the resin was washed with DMF (3 times, 10 min each) and dichloromethane (DCM, 3 times, 10 min each). The peptide was cleaved from the resin, and the side chain protecting groups were removed using the final cleavage cocktail (20 mL); reagent "R" containing (TFA/thioanisole/EDT/anisole (90:5:3:2 v/v/v/v) by shaking the mixture at room temperature for 6 h. The crude peptide was precipitated by adding cold diethyl ether (Et_2O, 50 mL × 2 times) and centrifuged for 10 min, then the precipitate was collected, dissolved in a mixture of water/acetonitrile, and purified by reversed-phase Hitachi HPLC (L-2455) equipped with a Waters XBridge™ BEH130 Prep C18 column with OBD™ 10 μm (19 mm x 250 mm) reversed-phase column. The purified peptide was lyophilized into powder for assay. High-resolution matrix-assisted laser desorption–ionization time-of-flight (MALDI-TOF/TOF) mass spectrometer (ABX SCIEX TOF/TOF) was used to confirm the structure of the final peptide. MALDI- TOF (*m*/*z*) for $C_{28}H_{50}N_{12}O_{11}S$ Calcd., 762. 3443; found 763.1235 $[M+H]^+$.

2.3. Synthesis of fluorescence-labeled GGRGDSK peptide (GGRGDSK(F′))

The synthesized GGRGDSK peptide (38.1 mg, 0.050 mmole) was dissolved in (1 mL) of anhydrous DMF and *N*,*N*-diisopropylethylamine (DIPEA, 6 equiv, 52.0 μL). 5(6)-Carboxyfluorescein *N*-hydroxy succinimide ester (35.5 mg, 0.075 mmole, 1.5 equiv) was dissolved in DMF (1 mL) and added dropwise to the solution of the peptide under stirring at room temperature in the dark for 24 h. Diethyl ether (Et_2O, 25 mL × 2 times) was added for precipitation; then the mixture was centrifuged for 10 min. The precipitate was collected, dissolved, and purified by HPLC (L-2455) equipped with a reversed-phase column as described above to yield GGRGDSK(F') (F′ = Fluorescein) that underwent lyophilization (Scheme 2). The structure of the final compound was confirmed by MALDI-TOF/TOF mass spectrometry. MALDI-TOF (*m*/*z*) $[C_{49}H_{60}N_{12}O_{17}S]$: Calcd, 1120.3920; Found, 1121.4078 $[M+H]^+$.

2.4. Synthesis of COS-SMCC

Chitosan (COS, 1 g, MWt = 4000–5000 Da, polydispersity (Mw/Mn) 1.25, with a degree of deacetylation = 90%) was dissolved completely in 100 mL of phosphate buffer solution (PBS), and the pH was adjusted to 7.5 Then, a solution of sulfosuccinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC, 43.6 mg, 0. 1 mmol in 10 mL of PBS) was added dropwise under stirring at room temperature with magnetic stirring for 24 h. The resulted COS-SMCC was purified using dialysis tube (MWCO 1000 Da) against distilled water for 3 days to remove the unreacted (Sulfo-SMCC) and any byproducts with continuous changing the milliQ-water 3 times/day and then freeze-dried affording product as a powder.

2.5. Conjugation of GGRGDSK to COS-SMCC

The COS-SMCC polymer (100 mg) was dissolved in (10 mL) of phosphate buffered solution at pH 6.5 and stirred for 30 min. Then, the linear peptide GGRGDSK (15.2 mg, 0.020 mmol) or GGRGDSK(F') (22.4 mg, 0.020 mmol) was dissolved in buffer solution and added dropwise to COS-SMCC polymer solution over 30 min. Then the reaction was continued under stirring at room temperature in dark for additional 48 h. The resulting CS-SMCC-peptide conjugates were purified using a dialysis membrane (MWCO 1000 Da) for three days against milliQ-water (3 times/day). Then, the solution of each copolymer conjugate was freeze-dried to obtain the powder of COS-SMCC-GGRGDSK or COS-SMCC-GGRGDSK(F′) (Scheme 3).

2.6. FT-IR

FT-IR spectra for COS, COS-SMCC, and COS-SMCC-GGRGDSK were obtained with (Alpha FT-IR Spectrometer/Platinum ATR from Bruker.