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Nanoparticle-induced tight-junction opening for the transport of an anti-angiogenic sulfated polysaccharide across Caco-2 cell monolayers

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ABSTRACT

Fucoidan has the ability to inhibit angiogenesis by human umbilical vein endothelial cells (HUVECs). However, a major clinical limitation is its poor oral availability because fucoidan is a hydrophilic macromolecule. In this study, an oversulfation reaction of fucoidan has been performed to enhance its anti-angiogenic activities. The synthesized, oversulfated fucoidan (OFD) was characterized by Fourier transform infrared spectroscopy. The oversulfate content of OFD was estimated to be 41.7% by using a $BaCl_2$ gelatin method. Nanoparticles (NPs) composed of chitosan (CS) and OFD were prepared by a polycation–polyanion complex method. The mean particle sizes of prepared CS/OFD NPs were in the range of 172–265 nm with a negative or positive surface charge, depending on the relative concentrations of CS to OFD used. The self-assembled NPs with pH-sensitive characteristics could be used as a pH-switched nanocarrier for oral delivery of the antiangiogenic macromolecule, OFD, in response to simulated gastrointestinal (GI) tract media. Evaluation of test NPs in enhancing the intestinal paracellular transport of OFD suggested that the NPs with a positive surface charge could transiently open the tight junctions between Caco-2 cells and thus increase the paracellular permeability. Tight-junction opening and restoration were examined by monitoring the redistribution of ZO-1 tight-junction proteins using confocal laser scanning microscopy (CLSM). The transported OFD significantly inhibits the tube formation of HUVECs via competitive binding of OFD and basic fibroblast growth factor (bFGF) to bFGF receptors (bFGFRs).

1. Introduction

Angiogenesis involves the formation of new blood capillaries and is required for physiological processes, including embryonic development, tissue regeneration and wound repair [1]. Angiogenesis is also required for invasive tumor growth and metastasis, and therefore suppression of the pathological neovascularization [2]. Unlike chemotherapy drugs, angiogenesis inhibitors target the tumor microenvironment rather than tumor cells through inhibiting the growth of the neovasculature rather than destroying it [3,4]. Because the regression of angiogenic vessels is a slow process, prolonged administration of an indirect angiogenesis inhibitor has become a promising approach for developing novel anticancer therapies [5,6].

The oral route is a convenient and comfortable means of drug administration for patients, especially for long-term administration of drugs. However, proteins and polysaccharides cannot easily transport across the cells through the lipid-bilayer cell membranes because the intestinal epithelium acts as a major barrier to hydrophilic macromolecules. Chitosan (CS) is a non-toxic biodegradable polycationic polymer with valuable properties which could be used in the biomedical and biotechnological fields and has been used for preparing pH-responsive nanoparticles for drug delivery [7,8]. To facilitate paracellular transport of hydrophilic macromolecules, CS-based microspheres and colloidal carriers have been developed for oral drug delivery [9–11]. Because CS has the ability to modulate reversible epithelial tight-junction opening [12–15], the CS-based NPs can adhere to and subsequently penetrate into the

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mucus of the intestinal tract via oral administration. After disintegration of the NPs, the released hydrophilic macromolecules were transported across the intestinal epithelial barrier and then entered the systemic circulation before reaching the site of action [16,17].

Fucoidans, which are sulfated polysaccharides extracted from marine brown seaweed, exhibit several biological behaviors such as antitumor [18,19], anti-angiogenic [20,21], antithrombotic and antiplatelet activities [22,23]. Fucoidans have also been used in tissue-engineering fields for mobilization of mesenchymal stem cells (MSCs) [24]. Previous studies indicated that the sulfate content and molecular weight had great influence on the anticoagulant and angiogenic activities of fucoidan [25,26]. Oversulfated fucoidan (OFD) was found to strongly inhibit basic fibroblast growth factor (bFGF) or vascular endothelial growth factor (VEGF)-induced vascular tube formation [27–29]. However, the bioavailability of fucoidan is poor. Only small quantities of fucoidan may cross the intestinal wall after oral administration [30,31].

In this paper, OFD was synthesized by introducing O-sulfate groups to fucose units on fucoidan. Polyelectrolyte complex nanoparticles prepared from CS and OFD have been developed for the purpose of oral antiangiogenic therapy. Physical and chemical characteristics of the prepared CS/OFD NPs in response to simulated GI media were examined by dynamic light scattering (DLS), transmission electron microscopy (TEM) and Fourier transform infrared spectroscopy (FT-IR). The change of transepithelial electrical resistance (TEER) for the tightness of Caco-2 cell monolayers and the paracellular transport of OFD was measured. Additionally, the inhibitory effects of the transported OFD on migration and tube formation by cultured human umbilical vein endothelial cells (HUVECs) were examined.

2. Materials and methods

2.1. Materials

Fucoidan was purchased from NOVA Pharma & Liposome Biotech Co., Ltd, Taiwan (Mw 80 kDa, from Fucus vesiculosus). CS (Mw 60 kDa) with a degree of deacetylation of ~85% was acquired from Koyo Chemical Co. Ltd (Japan). *N*,*N*-dimethylformamide (DMF), sulfur trioxide–trimethylamine complex (98%), fluoresceinamine isomer I (FA), MTT reagent and Hanks' balanced salt solution (HBSS) were purchased from Sigma–Aldrich (St Louis, MO). Matrigel was purchased from BD Biosciences (Becton, Dickinson and Company, USA). M199, fetal bovine serum (FBS), penicillin and trypsin–EDTA were obtained from Gibco (Grand Island, NY). *N*-hydroxy-succinimide (NHS)-functionalized cyanine 5 (Cy5–NHS ester) was obtained from Amersham Biosciences (Piscataway, NJ). All other chemicals were reagent grade and used without further purification.

2.2. Sulfation and characterization of polysaccharide and reaction

0.4 g of fucoidan was stirred in 30 ml of DMF until dissolution was complete. 2.0 g of sulfur trioxide–trimethylamine complex pre-dissolved in DMF was transferred into the fucoidan solution using a syringe. The reaction was continued at room temperature for 48 h. Subsequently, the reaction mixture was poured into 250 ml of ethanol to precipitate the final product. The polymer precipitate was filtered, re-dissolved in water and dialyzed against water for 72 h using a dialysis membrane (molecular weight cut-off 12,000, Spectra/Por® 2). The dialyzed solution was concentrated by rotary evaporation and re- precipitated by added into 250 ml of ethanol. After filtering, the product was dried at 50 °C overnight and stored in a desiccator until used. The chemical structures were analyzed by FT-IR (Perkin-Elmer Spectrum RX1 FT-IR System, Buckinghamshire, UK). The sulfate contents of fucoidans were determined by the $BaCl_2$ gelatin method using K_2SO_4 as a standard after hydrolyzing the polysaccharide in 0.5 M HCl at 105 °C for 5 h [29].

2.3. Preparation and characterization of NPs

CS/OFD NPs were prepared by a polycation/polyanion self-assembly method under magnetic stirring at room temperature. In brief, an aqueous OFD (1.0 mg ml^{-1}, 2 ml) was added by flush mixing with a pipette tip into an aqueous CS (1.2 mg ml^{-1}, 10 ml, in deionized (DI) water, pH 6.0). The self-assembled NPs were collected by ultracentrifugation at 15,000 rpm for 50 min. Supernatants were discarded and NPs were resuspended in DI water for further studies. The mean particle sizes and zeta potential values of NPs were measured using a Zetasizer (3000HS, Malvern Instruments Ltd, Worcestershire, UK) at distinct pH values (pH 1.2–7.4, simulating the pH environments in the GI tract) [32,33]. Because CS and fucoidan could not be digested by the intestinal enzyme, the media were used without the presence of enzyme. The mean particle size is an average of automatically repeated measures (10 times) of a single batch. The pH-dependent stabilities of NPs were also evaluated by turbidity measurement using a UV–vis spectrophotometer (Uvikon923, Kontron Instruments, Italy) at 500 nm. The morphologies of NPs at distinct pH values were examined by transmission electron microscopy (TEM). The test sample was prepared by placing a drop of the NP suspension onto a 400 mesh a carbon-coated copper grid. About 2 min after the deposition, the grid was tapped with a filter paper to remove surface water, followed by air-drying. The dried samples were observed by TEM (Hitachi H-600, Japan).

2.4. Loading and release of OFD

To determine the loading content (LC) and loading efficiency (LE) of OFD, the CS/OFD NPs nanoparticles were ultracentrifuged at 12,000 rpm and at low temperature (4 °C) for 30 min to prevent the degradation of fucoidans. The OFD concentration in the supernatant was measured by using a high performance GPC system (Waters, USA) equipped with a TSK Guard column (TSK Guard SW, 7.5 × 7.5 mm), followed by a primary column (SB-803HQ Shodex OH pak®, 6 μm SB-803 HQ 100 Å, 8.0 mm ID × 300 mm), high pressure pump (Waters 1525 binary pump) and a refractive index (RI) detector (Waters 2410 RI detector). Samples were eluted at a flow rate of 0.5 ml min^{-1} with salt solution (0.05 mol l^{-1} NaH_2PO_4, 0.05 mol l^{-1} Na_2HPO_4, 0.2 mol l^{-1} $NaNO_3$ and 0.02% NaN_3) as a mobile phase [19]. The columns are calibrated with dextran standards (Pharmacosmos, Denmark) with molecular weights of 5, 12, 25, 50, 80 and 150 kD and the standard curve was established using different concentrations of OFD solutions at concentrations ranging from 50 μg ml^{-1} to 200 μg ml^{-1}. The limit of detection (LOD) and the limit of quantification (LOQ) are 20 μg ml^{-1} and 50 μg ml^{-1}, respectively. The OFD LC and LE of NPs were determined as follows [12]:

$$\text{LC } (\%) = \frac{\text{total amount of OFD} - \text{free OFD}}{\text{weight of NPs}}$$

$$\text{LE } (\%) = \frac{\text{total amount of OFD} - \text{free OFD}}{\text{total amount of OFD}}$$

The release profiles of OFD from test NPs were investigated in distinct dissolution media (pH 1.5–7.4, simulating the pH environments in the GI tract) at 37 °C under agitation (100 rpm, DISTEK-2230A, North Brunswick, NJ). As reported in the literature, CS

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