Biomaterials 30 (2009) 6629–6637

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

The characteristics, biodistribution and bioavailability of a chitosan-based nanoparticulate system for the oral delivery of heparin

Mei-Chin Chen [a], Hen-Sheng Wong [b], Kun-Ju Lin [c,d], Hsin-Lung Chen [b], Shiaw-Pyng Wey [c], Kiran Sonaje [b], Yu-Hsin Lin [e], Che-Yi Chu [b], Hsing-Wen Sung [b,*]

[a] Department of Chemical Engineering, National Cheng Kung University, Tainan City, Taiwan, ROC
[b] Department of Chemical Engineering, National Tsing Hua University, Hsinchu, Taiwan 30013, ROC
[c] Department of Medical Imaging and Radiological Sciences, Chang Gung University, Taoyuan, Taiwan, ROC
[d] Department of Nuclear Medicine and Molecular Imaging Center, Chang Gung Memorial Hospital, Taoyuan, Taiwan, ROC
[e] Department of Biological Science and Technology, China Medical University, Taichung, Taiwan, ROC

ARTICLE INFO

Article history:
Received 10 June 2009
Accepted 9 August 2009
Available online 19 September 2009

Keywords:
Anticoagulant
Drug delivery
Tight junction
Intestinal absorption
Paracellular pathway

ABSTRACT

Heparin is a potent anticoagulant; however, it is poorly absorbed in the gastrointestinal tract. In this study, we developed a nanoparticle (NP) system shelled with chitosan (CS) for oral delivery of heparin; the NPs were prepared by a simple ionic gelation method without chemically modifying heparin. The drug loading efficiency of NPs was nearly 100% because a significantly excess amount of CS was used for the CS/heparin complex preparation. The internal structure of the prepared NPs was examined by small angle X-ray scattering (SAXS). The obtained SAXS profiles suggest that the NPs are associated with a two-phase system and consist of the CS/heparin complex microdomains surrounded by the CS matrix. The stability of NPs in response to pH had a significant effect on their release of heparin. No significant anticoagulant activity was detected after oral administration of the free form heparin solution in a rat model, while administration of NPs orally was effective in the delivery of heparin into the blood stream; the absolute bioavailability was found to be 20.5%. The biodistribution of the drug carrier, ^{99m}Tc-labeled CS, in rats was studied by the single-photon emission computed tomography after oral administration of the radio-labeled NPs. No significant radioactivity was found in the internal organs, indicating a minimal absorption of CS into the systemic circulation. These results suggest that the NPs developed in the study can be employed as a potential carrier for oral delivery of heparin.

1. Introduction

Heparin is a potent anticoagulant, primarily through formation of a protease inhibitor complex with antithrombin III [1]. Clinically, it has been used to prevent deep vein thrombosis and peripheral arterial embolism and reduce the incidence of myocardial infarction and death in patients with unstable angina [1]. However, heparin is poorly absorbed in the gastrointestinal (GI) tract [2,3]. It, therefore, requires either intravenous (IV) or frequent subcutaneous administration, making administration on an outpatient basis problematic [4].

The oral route is considered to be the most convenient and comfortable means of drug administration for patients. Drug molecules can traverse the intestinal epithelium by a transcellular means involving endocytotic process or by passing between the adjacent cells through a process known as the paracellular pathway [5]. However, the intestinal epithelium is a major barrier to the absorption of hydrophilic macromolecules such as heparin as they cannot diffuse across the cells through the lipid-bilayer cell membranes, due to their high molecular weight and hydrophilicity [2,6]. A few technologies, based on using carrier molecules such as deoxycholic acid (DOCA) [2], have been recently proposed to overcome this limitation [7]. Studies have shown that these hydrophobic carriers enable the systemic absorption of heparin via transcellular delivery and their oral bioavailability was about 16.6% [8,9]. Nevertheless, the chemical modification of heparin with DOCA may cause a significant decrease in its relative bioactivity, compared to the raw heparin [10].

To facilitate paracellular transport of hydrophilic macromolecules, efforts have been made to induce transient opening of intercellular tight junctions [11,12]. It has been reported that chitosan (CS), a cationic polysaccharide, can adhere to epithelial

* Corresponding author. Tel.: +886 3 574 2504; fax: +886 3 572 6832.
E-mail address: hwsung@che.nthu.edu.tw (H.-W. Sung).

0142-9612/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2009.08.030

surfaces to impart transiently opening the tight junction between contiguous cells. Via this paracellular pathway, we developed a nanoparticle (NP) system shelled with CS for oral delivery of heparin in this study. The NPs were prepared by a simple ionic gelation method to form CS and heparin complex, without chemically modifying heparin.

Physicochemical characteristics of the prepared NPs in response to simulated GI media were examined by dynamic light scattering (DLS), transmission electron microscopy (TEM) and small angle X-ray scattering (SAXS). The change of transepithelial electrical resistance (TEER) for the tightness of Caco-2 cell monolayers was measured, and the paracellular transport of heparin was visualized by means of confocal laser scanning microscopy (CLSM). Additionally, the release profile of heparin from test NPs was studied *in vitro*, while their biodistribution and bioavailability after oral administration were investigated in a rat model.

2. Materials and methods

2.1. Preparation of low-molecular weight (low-MW) CS

In this study, a low-MW CS, obtained from the depolymerization of a commercially available CS, was used to prepare test NPs. Given a low-MW, the polycationic characteristic of CS can be used together with good solubility at a pH value close to physiological range [13]. Briefly, CS (MW 280 kDa) with a degree of deacetylation of approximately 85% (Challenge Bioproducts, Taichung, Taiwan) was treated with sodium perborate tetrahydrate ($NaBO_3$, Sigma–Aldrich, St. Louis, MO, USA) to produce low-MW CS according to a method described by Kubota et al. [14]. The depolymerized CS was precipitated with aqueous NaOH at pH 10–11. The weight average MW, M_w, of the depolymerized CS was determined by a gel permeation chromatography system equipped with a series of PL aquagel-OH columns (one Guard 8 μm, 50 × 7.5 mm and two MIXED 8 μm, 300 × 7.5 mm, PL Laboratories, UK) and a refractive index detector (RI2000-F, SFD, Torrance, CA, USA).

2.2. Preparation and characterization of test NPs

Test NPs were prepared by flush mixing of an aqueous heparin (1 mL, unfractionated heparin, Heparin Leo®, LEO Pharma, FL, USA), using a pipette (1 mL), into an aqueous CS (pH 6.0, 5 mL) at various weight ratios under magnetic stirring at room temperature. The obtained NP solution was then dialyzed (MWCO: 10000, Spectrum Laboratories, Laguna Hills, CA, USA) against deionized water for 3 days with water exchanges several times to remove the additives originally present in Heparin Leo®. The loading efficiency (LE) and loading content (LC) of heparin in test NPs were determined by assaying the amounts of free heparin left in supernatants using a toluidine-blue colorimetric method [15] and calculated using the equations listed below [16].

$$\mathrm{LE}(\%) = \frac{\text{total amount of heparin added} - \text{free heparin}}{\text{total amount of heparin added}} \times 100\%$$

$$\mathrm{LC}(\%) = \frac{\text{total amount of heparin added} - \text{free heparin}}{\text{weight of nanoparticles}} \times 100\%$$

The particle size, polydispersity index (PDI) and zeta potential of the prepared NPs were measured using a Zetasizer (3000HS, Malvern Instruments, Worcestershire, UK). The morphology and internal structure of NPs at different pH values simulating GI media were examined by TEM (JEOL, Tokyo, Japan) [16] and SAXS, respectively.

SAXS experiments were performed using a Bruker NanoSTAR SAXS instrument, which consists of a Kristalloflex K760 1.5 kW X-ray generator (operated at 40 kV and 35 mA), cross-coupled Göbel mirrors for CuK_α-radiation ($\lambda = 1.54$ Å) resulting in a parallel beam of about 0.05 mm^2 in cross section at the sample position and a Siemens multiwire type area detector with 1024 × 1024 resolution mode. All data were corrected by the empty beam scattering and the sensitivity of each pixel of the area detector. The area scattering pattern had been circularly averaged to increase the efficiency of data collection. The intensity profile was output as the plot of the scattering intensity (I) vs. the scattering vector, $q = 4\pi/\lambda \sin(\theta/2)$ (θ = scattering angle) [17].

2.3. In vitro drug release

The *in vitro* release of heparin from test NPs was evaluated in gradient pH media, simulating the GI environment, without (or with) enzymes at 37 °C under agitation at 150 rpm. The release media used without (or with) enzymes were: HCl solution at pH 1.2 (or with 0.1% by w/v pepsin) for 2 h; phosphate buffer at pH 6.6 for 2 h, pH 7.0 for 2 h and pH 7.4 for 18 h (or with 1.0% w/v pancreatin) [18]. At particular time intervals, the samples were taken out and centrifuged and the supernatants were used for a toluidine-blue colorimetric analysis [15]. The amount of heparin released was expressed as a percentage of the total heparin associated with test NPs as calculated from the LE.

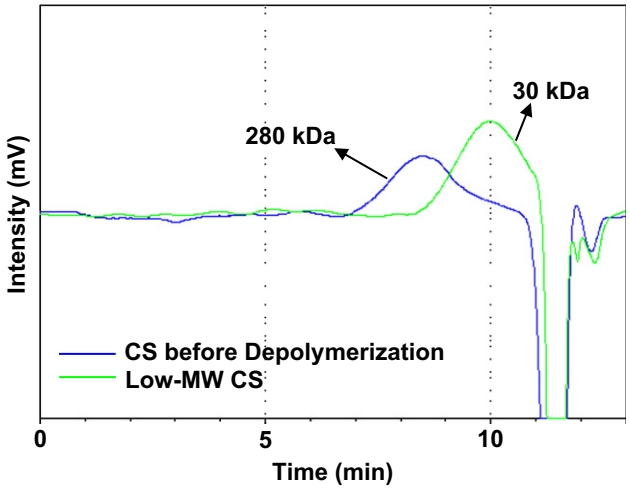

Fig. 1. Gel permeation chromatograms of chitosan (CS) before depolymerization and the low-molecular weight (low-MW) CS obtained in the study.

2.4. TEER measurements and CLSM visualization

Caco-2 cell monolayers were prepared on a tissue-culture-treated polycarbonate filter in Costar Transwell 6 wells/plates [16]. The transport medium containing test NPs (0.2 mg/mL) was introduced into the donor compartment of Caco-2 cell monolayers. Different pH values (pH 6.6, 7.0 or 7.4) were employed in the donor compartment to simulate the luminal pH conditions in the small intestinal segments (the duodenum, jejunum and ileum, respectively) [19], whereas the receiver compartment was maintained at pH 7.4, simulating the pH environment underneath the epithelial cells [20]. Hence, there was a pH gradient within the paracellular gap. The change of TEER for the tightness of cell monolayers was measured with a Millicell®-Electrical Resistance System (Millipore Corp., Bedford, MA, USA).

Cyanine 3-labeled CS (Cy3-CS) and fluoresceinamine isomer I-labeled heparin (FA-heparin) were synthesized according to the methods described in the literature [21,22] and used to prepare fluorescent NPs for the CLSM study. After addition of

Table 1
Effects of concentrations of chitosan (CS) and heparin used in the preparation of test nanoparticles on their particle size, polydispersity index (PDI) and zeta potential (n = 5).

	Particle Size (nm)				
CS/Heparin	0.0225%	0.0450%	0.090%	0.180%	0.360%
12.5 IU/mL	134.6 ± 15.3	156.2 ± 33.1	203.6 ± 38.4	232.1 ± 12.1	285.7 ± 14.5
25.0 IU/mL	82.9 ± 7.1	110.1 ± 5.4	149.9 ± 7.4	238.6 ± 18.2	277.6 ± 22.5
50.0 IU/mL	106.6 ± 4.1	91.9 ± 6.5	104.3 ± 1.2	133.1 ± 2.3	216.4 ± 14.7
100.0 IU/mL	157.8 ± 4.7	141.5 ± 8.7	115.6 ± 4.7	136.9 ± 7.3	168.9 ± 3.3
200.0 IU/mL	201.2 ± 7.4	200.9 ± 8.5	173.9 ± 2.3	160.7 ± 9.2	187.7 ± 5.2
	PDI				
CS/Heparin	0.0225%	0.0450%	0.090%	0.180%	0.360%
12.5 IU/mL	0.76 ± 0.07	0.82 ± 0.06	0.76 ± 0.09	0.72 ± 0.02	0.67 ± 0.02
25.0 IU/mL	0.33 ± 0.05	0.51 ± 0.02	0.61 ± 0.02	0.78 ± 0.05	0.76 ± 0.14
50.0 IU/mL	0.17 ± 0.01	0.27 ± 0.02	0.43 ± 0.04	0.54 ± 0.04	0.74 ± 0.13
100.0 IU/mL	0.13 ± 0.01	0.14 ± 0.02	0.22 ± 0.02	0.28 ± 0.06	0.57 ± 0.04
200.0 IU/mL	0.15 ± 0.03	0.15 ± 0.02	0.14 ± 0.02	0.18 ± 0.02	0.31 ± 0.03
	Zeta Potential (mV)				
CS/Heparin	0.0225%	0.0450%	0.090%	0.180%	0.360%
12.5 IU/mL	20.1 ± 2.6	17.5 ± 3.8	20.4 ± 2.9	24.6 ± 3.1	25.6 ± 2.1
25.0 IU/mL	15.8 ± 2.1	13.7 ± 1.3	24.1 ± 3.0	22.6 ± 1.6	25.5 ± 0.6
50.0 IU/mL	15.4 ± 1.5	22.6 ± 3.2	23.2 ± 1.5	22.1 ± 3.9	23.7 ± 4.2
100.0 IU/mL	16.7 ± 0.7	20.1 ± 0.9	21.5 ± 1.1	22.1 ± 1.1	25.2 ± 1.2
200.0 IU/mL	−25.6 ± 1.8	19.5 ± 1.1	19.3 ± 0.4	21.1 ± 0.9	23.2 ± 0.5