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Intracellular delivery and anti-cancer effect of self-assembled heparin-Pluronic nanogels with RNase A

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ABSTRACT

A novel self-assembled nanogel was prepared for the intracellular delivery of ribonuclease A (RNase A) and the anti-cancer efficacy of RNase A delivery was investigated. The physical properties of self-assembled heparin-Pluronic (HP) nanogels incorporating RNase A (HPR nanogels) were characterized by dynamic light scattering (DLS), ξ-potential, and transmission electron microscopy (TEM). RNase A showed a strong affinity for the HP nanogel, resulting in a high loading efficiency (>78%) and significantly decreased hydrodynamic size (from 89 to ~29). HPR nanogels were efficiently internalized into HeLa cells and localized in the cytosol as well as the nucleus. In the mechanism study of cellular uptake, treating with methoxy β-cyclodextrin (Mβ-CD) decreased the uptake efficiency of HP nanogel, indicating that internalization occurs via caveolae/lipid-raft mediated endocytosis. Localization in the nucleus most likely occurred because the conjugated heparin facilitated nucleus penetration. The cytotoxicity of HPR nanogels was significantly increased when the RNase A concentration was increased, which resulted from the degradation of single stranded RNAs in the cytosol and the nucleus due to the intracellular localization of the HPR nanogels. These results demonstrate that self-assembled HP nanogels are a remarkable vehicle for intracellular protein delivery and hold promise for use as cancer chemotherapeutics.

1. Introduction

The intracellular delivery of exogenous proteins for therapeutic purposes has received much attention in pharmaceutical fields [1,2]. For the effective delivery of proteins, many studies have attempted to utilize nano-sized carrier systems such as liposomes, biodegradable nanospheres, electrostatic complexes, and hydrogel nanoparticles (called nanogels) [3,4]. Although many nanocarrier systems for protein delivery have been investigated, most of systems have exhibited problems such as denaturation or aggregation of proteins, low encapsulation efficiency, or inefficient intracellular uptake. Recently, the conjugation of cell penetrating peptides (CPP) to nanocarriers has been examined as a potential strategy to improve the intracellular uptake of proteins or other therapeutic agents. However, these systems still have several limitations that need to be resolved such as the instability of CPP and the specificity to cells [5].

Nanogels, which are nano-sized polymeric networks, have several advantages including relatively simple formulation procedure, high drug loading capacity, high stability and stimuli-responsive properties [6–9]. In addition, nanogels also have the appropriate size for intracellular delivery of therapeutic molecules with respect to cellular uptake via endocytosis and the enhanced permeation and retention (EPR) effect [10]. In our group, various types of nanogel-like structures have been developed using physical self-assembly, including chitosan-Pluronic [11,12] and heparin-conjugated micelles [13–15]. In these systems, the therapeutic agents were physically entrapped inside the polymeric network of nanoparticles during hydrogel formation, resulting in the formation of relatively small-sized (~50 nm), swelled nanoparticles that displayed a narrow size distribution. An example of these nanogels includes polymeric complexes between Pluronic-grafted polysaccharides and proteins or small molecules, in which the amphiphilic Pluronic chains are assembled via hydrophobic interaction, which are supported by hydrogen bonds or electrostatic interactions between the polyelectrolytes and/or proteins.

Heparin, a highly negatively charged glycosaminoglycan (GAG), has a binding affinity for various therapeutic proteins including cytokines and growth factors [16]. Because of this unique feature, heparin holds promise for use in the effective and stable delivery of proteins. In addition, the therapeutic activities of heparin can be utilized for intracellular delivery using heparin derivatives, which include regulatory effects in cancer, vascular, rheumatoid and other diseases [17–20]. In addition to these effects, our interest has been

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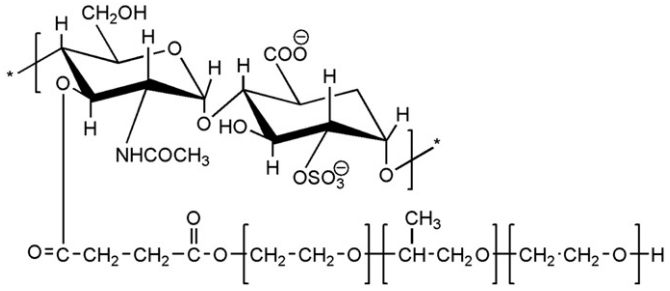

Fig. 1. Chemical structure of heparin-Pluronic (HP) conjugate.

focused on the extra- or intracellular actions of heparin, which are involved in cellular uptake and nucleus penetration [21].

In this study, a novel self-assembled nanogel composed of a heparin-Pluronic (HP) conjugate was developed to enable delivery of RNase A to intracellular regions and its anti-cancer effect was assessed (shown in Fig. 1). HP nanogels show extraordinary features in terms of drug loading, intracellular fate and hydrogel properties, compared with other nanoparticles. Specifically, HP nanogels exhibited high drug loading efficiency via simple method, high uptake efficiency, and less-swollen nanogel with a decreased size after drug loading. RNase A was used as a model protein, which can bind to heparin, hydrolyzing single stranded RNA without sequence specificity and thereby inducing cytotoxic effect. The therapeutic potential of HP nanogels was demonstrated by transmittance electron microscopy (TEM), dynamic light scattering (DLS), formulation efficiency, cellular uptake, and cytotoxicity tests.

2. Materials and methods

2.1. Chemicals

Pluronic® F127 ($M_n = 12{,}600$) was purchased from BASF Korea (Seoul, Korea). Heparin sodium (150 unit/mg) was obtained from Acros (Pittsburgh, USA). Ribonuclease A (RNase A) and dimethylaminopyridine (DMAP) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Dicyclohexylcarbodiimide (DCC) was purchased from Fluka (Ronkonkoma, NY, USA). Triethylamine and dimethylsulfoxide (DMSO) was obtained from Kanto Chemical Co. (Tokyo, Japan). Fluorescence isothiocyanate (FITC) and Alexa Fluor® 860 fluorescence dyes were purchased from Invitrogen (Carlsbad, CA, USA). All other chemicals were of analytical grade and used without further purification.

2.2. Preparation of RNase A-loaded HP nanogels

2.2.1. Synthesis of HP nanogels

For the synthesis of HP, the end group of Pluronic was modified to a carboxyl group as previously described [22]. Briefly, Pluronic (F127, 2.778 mmol) was diluted in 350 ml of 1, 4-dioxane. Succinic anhydride (3.3336 mmol), DMAP (2.778 mmol) and triethylamine (2.778 mmol) were then added to the solution. After stirring the mixture for 24 h, the mixture was filtered and precipitated in diethyl ether. The product was lyophilized for 2 days. Carboxylated Pluronic was conjugated to heparin using EDC and NHS coupling agents as shown in Fig. 1 [23]. Briefly, carboxylated Pluronic (6.75 g) was dissolved in 0.05 M MES buffer, followed by the addition of EDC and then NHS to the solution. After stirring for 30 min, a heparin solution (1 g) was mixed in the solution and stirred for 24 h. The mixture was then filtered using a glass filter and dialyzed (Spectrapore, molecular weight cut-off = 50 kDa) for 4 days, followed by lyophilization for 3 days. The direct dissolution method was used to prepare the HP nanogel. Ten milligrams of HP was dissolved in 5 ml of D.I. water at 4 °C. After HP was clearly dissolved, the temperature was increased to 37 °C. The solution was then stirred for 24 h and filtered (pore size = 0.2 μm, Advantec, Toyo Roshi Kaisha, Ltd., Japan). The final product was acquired as a powder by lyophilization. The obtained HP conjugate was characterized by ^{1}H NMR, IR, and TGA and the amount of heparin is determined by the toluidine blue colorimetric assay that has been commonly used for heparin determination (data not shown).

2.2.2. Conjugation of fluorescence probes to HP nanogel and RNase A

The synthesized HP nanogel (100 mg) was dissolved in DMSO (80 ml). DCC (9.6 mg), DMAP (3.2 mg) and FITC (10 mg) were then added to the solution and stirred at 37 °C for 24 h. The resulting product was purified by dialysis (molecular weight cut-off = 3.5 kDa) for 4 days. A powder was obtained by freeze-drying. RNase A was labeled with Alexa Fluor® 633 using an Alexa Fluor 633 protein labeling kit according to the manufacturer's instruction.

2.2.3. Preparation of RNase A-loaded HP (HPR) nanogels

The HP conjugate (100 mg) was poured into D.I. water at 4 °C. Various concentrations of RNase A were added to the aqueous HP solution as shown in Table 1. The mixture was stirred at 37 °C for 24 h. The final product was obtained by filtration and lyophilization. The loading efficiency of RNase A was determined using the Micro BCA chromogenic assay. In brief, 100 μl of different RNase A-loaded HP nanogel solutions (HPR, 1 mg/ml) were placed in 96-well plate. A mixture of BCA agents (100 μl) was added to the samples and incubated at 37 °C for 1 h. The absorbance was measured using a multi-plate reader at 605 nm.

2.3. Characterization of HPR nanogels

2.3.1. Dynamic light scattering and ζ-potential

To measure the size of the HPR nanogels, dynamic light scattering (DLS) measurements were performed with a Malvern Zetasizer Nano Z instrument (Malvern Instruments, Ltd., UK). HPR nanogel dispersions were prepared in D.I. water at 37 °C at a concentration of 1 mg/ml. The hydrodynamic diameter of the HPR nanogels was calculated using a Laplace inversion program (CONTIN). The ζ-potential was obtained by using a disposable capillary cell in automatic mode on the same instrument.

2.3.2. TEM measurement

The surface morphology of HP nanogels was imaged by transmittance electron microscopy (TEM, CM-30 Philips). TEM images were acquired at an operating voltage of 300 kV. Specimens were prepared by dropping a solution of HP nanogels on a cupper grid, followed by air drying for 1 min and negative staining with phosphotungstic acid (1 wt%).

2.4. In vitro RNase A release test

The release of RNase A from HP nanogel was measured using the dialysis method. Ten milligrams of the HPR nanogels were dissolved in 1 ml of phosphate buffered saline (PBS) solution (pH 5.0 and pH 7.5) at 37 °C and placed in a dialysis membrane (Spectrum

Table 1
Formulation results and hydrodynamic properties of HPR nanogels.

Samples	Formulation (HP:RNase A)	Loading efficiency (%)	Loading amount (μg/mg of HP)	ζ-potential (eV)	Diameter (nm)
HP	—	—	—	−10.3 ± 0.94	89.1 ± 8.4
HPR-1	100:1	99.3 ± 2.80	9.92 ± 0.280	−9.8 ± 3.97	29.5 ± 0.7
HPR-5	100:5	78.0 ± 3.47	39.01 ± 0.347	−12.4 ± 1.29	29.4 ± 2.7
HPR-20	100:20	79.1 ± 3.01	158.17 ± 0.301	−26.9 ± 1.05	27.0 ± 13.5