Functional Stimuli-Responsive Nanogel-Particles for Oral Peptide Delivery: Preparation, Drug-Release Behaviors and In Vitro Cellular Interaction

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ABSTRACT

Novel nanogel particles composing of thermosensitive poly(*N*-isopropylacrylamide) core and pH-sensitive surrounding layer of poly(methacrylic acid) grafted with poly(ethylene glycol) (CSNPs) were developed as an oral peptide delivery device. The CSNPs exhibited both temperature- and pH-sensitive swelling behaviors, allowing drug-loading by equilibrium partitioning under mild conditions such as low temperature and neutral pH, and pH-dependent controlled-release of vancomycin (a model peptide drug). Additionally, the CSNPs were found to show no cytotoxic effect on the Caco-2 cells and to possess an ability to open the tight junctions of the cell monolayers which is a requisite for enhanced permeation of peptide drug across the small intestine.

Keywords: nanogel, stimuli-responsive polymer, oral peptide delivery, controlled-release, caco-2 cells

1 INTRODUCTION

Significant advances in biotechnology and biochemistry have led to the discovery of a large number of bioactive molecules, such as peptides and proteins. However, bioavailability of peptides and proteins as therapeutic agents after oral administration is very low in general due to epithelial barriers of the gastrointestinal tract and gastrointestinal degradation by digestive enzymes. This requires a delivery system with specific abilities such as protecting the peptides from harsh manufacturing and physiological environment and enhancing the drug absorption at the preferable sites in the gastrointestinal tract.

One approach of achieving such specific functions is to use stimuli-responsive polymers. We have designed thermo- and pH-sensitive nanogel particles with core-shell structure (CSNPs) as a functional particulate device for oral peptide delivery [1]. The employed core and shell components for the CSNPs are poly(*N*-isopropylaclyramide) (p(NIPAAm)) hydrogel and poly(methacrylic acid) grafted with poly(ethylene glycol) monomethacrylate (p(MAA-*g*-EG)), respectively. As is well known, p(NIPAAm) hydrogel swells below 32˚C while it collapses above 32˚C [2]. P(MAA-*g*-EG) is a pH-sensitive polymer which collapses in acidic media and swells in neutral media [3]. Additionally, the swollen p(MAA-*g*-EG) possesses mucoadhesive property [4] as well as proteolytic enzyme inhibitory effect [5], which are favorable for enhancing peptide absorption through the small intestine. The proposed performances of the CSNPs thus designed are as follows: (1) Peptides can be loaded at such mild conditions as low temperature and neutral pH where both core and shell components swell; (2) Both components remain collapsed in the gastric fluid around body temperature, so that peptides can be protected from enzymatic degradation; (3) In the small intestine, the swollen p(MAA-*g*-EG) shell can exhibit both mucoadhesive properties and peptide-transport enhancing effect in the mucosal cell lining, while the p(NIPAAm) core remains shrunken, allowing the peptide release in a prolonged manner.

The aim of the present study is to prepare the CSNPs by a free radical dispersion polymerization and to examine their functionalities such as drug-loading, controlled-release ability, cytocompatibility and cellular tight junction opening effect.

2 EXPERIMENTS

2.1 Preparation of Nanogel Particles

Nanogel particles were synthesized by a semi-continuous two-stage photo-initiated free radical dispersion polymerization technique. In a typical experiment, NIPAAm (1.5 g), tetraethylene glycol dimethacrylate (TEGDMA, cross-linking agent, 0.06 g) and 1-hydroxycyclohexyl phenyl ketone (an initiator, 0.06 g) were dissolved in an aqueous SDS solution. This solution was exposed to UV-light (Photocure 200, Hamamatsu Photonics, Japan) with a wavelength of 365 nm for 30 min at 70˚C in order to prepare colloidal p(NIPAAm) hydrogel cores. Subsequently, 75 g of an aqueous solution containing specific amounts of methacrylic acid (MAA) and methoxy-terminated poly(ethylene glycol) monomethacrylate (PEGMA, number of ethylene glycol unit; 23) was added to the reactor at flow rate of 1 g/min to prepare p(MAA-g-EG) shell layers on the colloidal p(NIPAAm) cores. The resultant nanogel particles were dialyzed against distilled water using cellulose acetate tubing for 5 days to remove unreacted substances.

2.2 Characterization of Nanogel Particles

The yield of CSNPs was determined gravimetrically. Morphological observation of CSNPs was carried out by FEI/Philips CM-10 transmission electron microscopy.

Equilibrium swelling volume ratios of the CSNPs were assessed as follows: The particle sizes of CSNPs at 20˚C and 40˚C in distilled water or at pH 3.0 and 7.0 in Teorell-Stenhagen buffer solutions with a constant ionic strength of 0.1 M at 25˚C were measured by a dynamic light scattering method using a Horiba LB-500. Under assumptions that the CSNPs are spherical and that the swelling occurs isotropically, the ratio of the equilibrium swelling volumes at the different temperatures (V_t) or pH values (V_p) were estimated and each parameter was used as a measure of temperature- and pH-sensitivity upon the volume change.

2.3 Drug Loading and Release Studies

Vancomycin hydrochloride (VCH, Mw 1486) was used as a model peptide drug. Drug loading was accomplished by an equilibrium partition technique. One hundred mg of freeze dried CSNPs were added to phosphate buffered saline solution containing VCH (100 mg/mL) and allowed to stand at 4˚C for 24 h. Then, the CSNPs were warmed to 40˚C to be collapsed, washed with 5 mL of pre-warmed 0.1 M HCl under ultra-sonication at 40˚C and centrifuged at 1000 rpm for 20 min to remove unloaded VCH. This washing procedure was repeated three times. The VCH-loaded CSNPs were freeze dried and stored for further studies.

Release studies were conducted in the first (pH 1.2) and second (pH 6.8) fluids of Japanese Pharmacopoeia using a 6-ml volume cylindrical glass cell under gentle agitation with a magnetic stirring bar. Fifty mg of the freeze dried CSNPs containing VCH were charged into 5 mL of the release media. At specific time points, 0.3 mL of the sample was withdrawn and replaced with an equal volume of fresh media. After the centrifugal filtration of the samples, the concentration of released VCH was determined spectrophotometrically at 282 nm.

2.4 Cell Studies

Caco-2 cells, a colon carcinoma derived from human, were employed since the cells spontaneously differentiate and possess tight junctions, which are characteristics of the cells lining the intestine and thus serve as a good model for studying the effect of nanogel particles on the tight junctions of the small intestine. Cell viability after 2.5 h of contact time with the nanogel particles was assessed by an aqueous non-radioactive cell proliferation assay reagent (CellTiter96®, Promega) based on an NADPH reactive assay. Cell viability (%) was expressed as the ratio of the NADPH activity in the treated groups to that in the control (untreated) groups.

The integrity of the cell monolayers was determined by measurement of transepithelial electrical resistance (TEER) to evaluate the extent to which the cell tight junctions opened [6]. The nanogel particles were applied to the apical side of the cell monolayers grown on the Costar 6-well transwell plates, incubated for 3 h and then removed from the wells. At specific time points, the TEER was measured by a Millicell®-ERS voltohm meter.

3 RESULTS AND DISCUSSION

Characteristics of CSNPs in various formulations were summarized in Table 1. The yields of CSNPs were considerably varied, ranging from 82 to 97%. There was no obvious correlation between the yields and the formulation variables of CSNPs.

Morphological observation of the CSNPs thus prepared was carried out by transmission electron microscopy (TEM). TEM image of the CSNPs with core-shell ratio of 45:10 and MMA/EG unit molar ratio of 1:1 is shown in Figure 1 as a representative example of the series of CSNPs. The CSNPs were found to be spherical, monodispersed particles with a relatively distinct core-shell structure. This is an invaluable asset for fully utilizing the functions of p(NIPAAm) and p(MAA-*g*-EG).

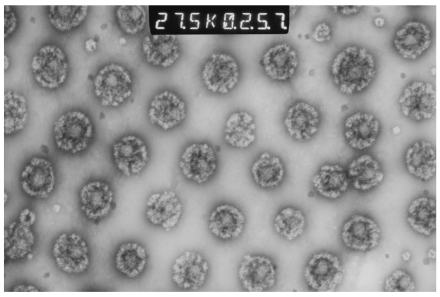

Figure 1: TEM images of CSNPs

The equilibrium particle size and swelling ratio of a series of nanogel particles are listed in Table 1. The simple p(NIPAAm) nanogel particles showed V_t of 128.2 and V_p of 1.1, indicating that they could swell in response to only temperature change. In contrast, the CSNPs exhibited both thermo- and pH-sensitive swelling behaviors, as evidenced from their V_t and V_p values. The magnitude of the volume change upon temperature and pH changes strongly depended on core-shell weight ratio and MAA-EG unit molar ratio. With decrease in core-shell weight ratio or increase in MAA-EG unit molar ratio, the V_t tended to decrease while the V_p increased.

The significant reduction of V_t would be ascribed from the restricted swelling of p(NIPAAm) core by the presence of highly entangled p(MAA-*g*-EG) chains in the shell layers. Another possibility for the decreased thermo-sensitivity is penetration of the shell component(s), mainly MAA with smaller molecular size than PEGMA, into the

Table 1: Characteristic of nanogel particles.

Core-shell weight ratio	MAA/EG molar ratio	Yeild (%)	Equilibrium particle size (nm)				*Vt*	*Vp*
			20℃	40℃	pH 3.0	pH 7.0		
100:0	-	97.4	775 ± 10	153 ± 4	775 ± 9	778 ± 10	128.2	1.1
50:10	1:1	83.9	349 ± 11	167 ± 5	349 ± 2	709 ± 15	9.1	8.4
50:10	5:1	92.6	269 ± 2	169 ± 7	268 ± 8	692 ± 14	4.0	17.3
45:10	1:1	97.2	327 ± 17	172 ± 2	301 ± 10	674 ± 14	7.0	11.3
45:10	2:1	83.3	276 ± 4	174 ± 2	271 ± 1	706 ± 10	4.1	17.7
45:10	3:1	85.0	243 ± 2	170 ± 5	253 ± 2	722 ± 17	3.0	23.4
45:10	4:1	96.0	261 ± 10	185 ± 6	259 ± 10	732 ± 24	2.8	22.5
45:10	5:1	84.1	244 ± 2	176 ± 1	238 ± 4	733 ± 5	2.7	29.2
40:10	1:1	83.9	310 ± 5	171 ± 1	313 ± 8	747 ± 13	5.9	13.6
40:10	5:1	82.2	233 ± 15	175 ± 4	241 ± 13	786 ± 8	2.4	34.7

p(NIPAAm) core during polymerization of the shell components. This migration of the shell component(s) would lead to formation of 'semi-interpenetrating network' in parts. As a result, thermo-sensitivity of the p(NIPAAm) core remarkably declined. This hypothesis is supported by the fact that the particle size of CSNPs at 20˚C in the buffer of pH 7.0, where both p(NIPAAm) core and p(MAA-*g*-EG) shell swell, was compatible to or even smaller than that of p(NIPAAm) core, despite that the shell component was added to the surface of p(NIPAAm) core. In contrast, the increased V_p values in the CSNPs might be due to the increase of carboxylic group in number, resulting in much electrostatic repulsion between p(MAA-g-EG) chains at higher pH and/or firm polycomplex formation at lower pH.

In a preliminary experiment, it was found that the redispersibility of lyophilized CSNPs in water was strongly affected by MAA-EG unit molar ratio (MAA/EG). Among a series of prepared here, the CSNPs with the MAA/EG of 5:1 showed an excellent redispersible property (data not shown). Therefore, the drug loading and release studies were carried out using this formulation. The VCH content in the CSNPs was found to be ranged from 2.1 to 6.5%, depending on the core-shell weight ratio, but no clear correlation was observed between the drug content and the core-shell weight ratio of the CSNPs. This suggests that there might be no specific interaction between VCH molecules and CSNPs.

Figure 2 shows release profiles of VCH from the CSNPs with the core-shell weight ration of 45:10 and MAA/EG molar ratio of 5:1. The CSNPs showed pH-dependent release behaviors. Release of VCH was restrained in the first fluids (pH 1.2) compared with the second fluids (pH 6.8). However, complete suppression of release was not achieved in the first fluid. Biphasic release profiles with the 'burst-type' release of VCH at the initial stage were observed in the second fluid where the pH-sensitive shell layer swelled. This rapid release of VCH is attributed to the presence of VCH molecules loaded into the shell layer during the loading process. Namely, the VCH molecules should be distributed into both core and shell in the loading process, because neither p(NIPAAm) core nor p(MAA-g-EG) shell would be specifically interactive with VCH molecules. In neutral pH, therefore, the VCH molecules distributed into the shell layer were rapidly released from the swollen shell layer, while the VCH molecules existing in the core were released gradually because of the collapse of p(NIPAAm) core at 37˚C.

The cell viability versus particles concentration of the simple p(NIPAAm) nanogel particles and the CSNPs with core-shell weight ratio of 45:10 and MAA/EG molar ratio of 5:1 is shown in Figure 3. The results were expressed in terms of the amount of NADPH produced by the cells still functioning in the culture. More than 90% of the cells remained viable, indicating a good cytocompatibility of the CSNPs. Unlike traditional absorption enhancers being applied in the form of solution, it is reasonable to consider that the present CSNPs would be hardly absorbed into the Caco-2 cell monolayer because of their infinitely large macromolecular size. It was also experimentally confirmed that MAA/EG unit molar ratio at the fixed core-shell weight ratio of 45:10 did not show any significant effect on the cell viability (data not shown).

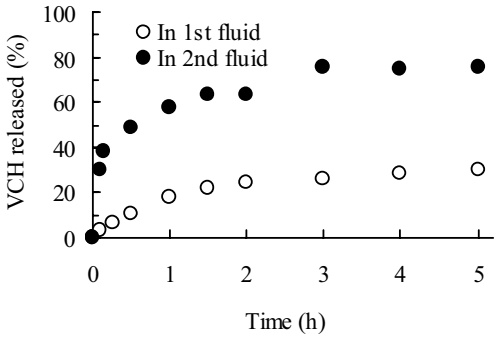

Figure 2: Release profiles of VCH from CSNPs.

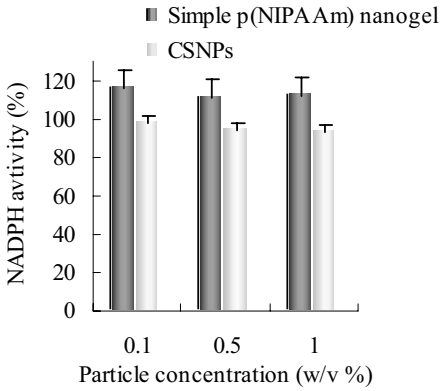

Figure 3: Viability of Caco-2cells after 2.5 h of contact time with simple p(NIPAAm) nanogel particles and CSNPs.

Figure 4 shows time course changes of TEER value of the Caco-2 cell monolayers after exposure to the simple p(NIPAAm) nanogel particles and the CSNPs (core-shell weight ratio of 45:10 and MAA/EG unit molar ratio of 5:1). No significant decrease of TEER was observed in the simple p(NIPAAm) nanogel particles, compared with the control group which was not exposed to the nanogel particles. In contrast, application of the CSNPs gave rise to a significant reduction of TEER. This indicates that the integrity of the Caco-2 cell monolayers became weak. It is well known that cellular Ca^{2+} concentration have a very important role to maintain the integrity of cell monolayers. Most probably, complexation or binding of extracellular Ca^{2+} with the swollen p(MAA-*g*-EG) shell layers of CSNPs was responsible for this effect. The TEER value after 3 h of contact time with the CSNPs reached 68%, but it recovered within several hours after removing the CSNPs at 3 h. From this result, it was confirmed that the CSNPs possessed an ability to open the tight junctions in a temporary manner and did not provide fetal damage on the Caco-2 cell monolayers.

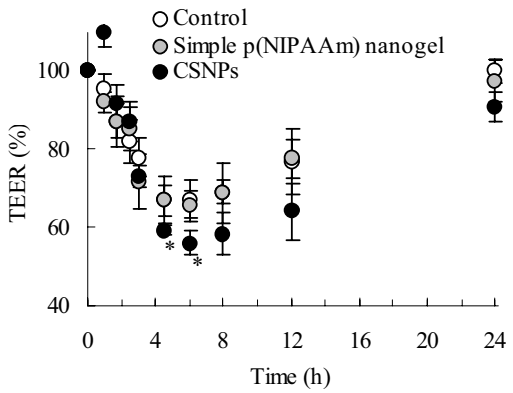

Figure 4: Time course changes of TEER of Caco-2 cell monolayers in contact with nanogel particles (mean±S.D., n=3-7, p<0.05*).

4 CONCLUSIONS

Novel stimuli-responsive nanogel particles composed of p(NIPAAm) core and p(MAA-*g*-EG) shell layer were prepared as an oral peptide delivery device and their several characteristics were evaluated. Through the present study, it was found that the nanogel particles with core-shell structure possessed peptide-loading capacity of around 7%, an ability to release peptide drugs in a pH-dependent manner, a good cytocompatibility, and cellular tight junction opening effect.

5 ACKOWLEDGEMENT

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