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Facile synthesis of multilayered polysaccharidic vesicles

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ABSTRACT

In this study, we developed facile synthesis method of multilayered polysaccharidic vesicles (hereafter termed 'mPSVs') using polysaccharides such as starch, hyaluronate (HA), and glycol chitosan (GC) via simple chemistry and using enzymatic reactions among polysaccharides. The enzymatic degradation of the HA shell by hyaluronidase (HYAL) enzyme contributed to accelerate the release of protein/peptide from the mPSVs. The mPSVs containing folate ligand and apoptotic cell death-inducing D-$(KLAKLAK)_2$ peptide were effectively accumulated in in vivo KB tumor cells, primarily owing to passive tumor penetration via the enhanced permeability and retention (EPR) effect and active targeting via specific binding to folate receptors expressed on KB tumor cells. These mPSVs resulted in a significant increase in the in vivo tumor inhibition. This vesicle system is expected to exhibit great potential as an advanced platform technology for biomedical applications involving small molecular drugs with protein/gene targets.

1. Introduction

Cells are the basic structural and functional units of all living organisms, modulating diverse biochemical functions. Nano-structural designs mimicking these cells have been developed as part of a broader effort to develop a new type of colloidal system [1–5]. Bilayered vesicles similar in structure to the cell membrane are produced from lipids or synthetic polymers and have been utilized as a bio-vector for the encapsulation of biofunctional high-molecular-weight compounds [6–8]. However, their limited functionality and poor immobilization of biofunctional compounds usually result in relatively low effectiveness in the field of drug delivery, cosmetics, and tissue engineering. Moreover, the transfer of biofunctional compounds into cells is inefficient, and the targeting to specific tissues is difficult. Despite recent attempts involving the use of polypeptides or proteins, there have been few examples of well-constructed vesicles [9,10]. A complementary approach that is efficiently applicable for biomedical purposes is to create next-generation vesicular colloids with the goal of achieving desirable physicochemical properties in vitro/in vivo [6–10]. Among the various biopolymers that are currently available, polysaccharides are biocompatible and biodegradable, high-molecular-weight carbohydrate molecules that are favorable for bulk encapsulation and various chemical reactions [11]. Furthermore, polysaccharides or oligosaccharides, which are plentiful within the cell membrane, nucleus, and cytoplasm, regulate a variety of functions in cell/cell, cell/matrix, and cell/molecule interactions in multicellular organs or cellular organelles [11–13]. There have been tremendous efforts to modulate natural cell interactions or metabolisms using polysaccharides or oligosaccharides [11–13]. However, little attention has been given to the design of a vesicle-like supra-structure using these polysaccharides or oligosaccharides.

Herein, we report the facile synthesis of multilayered polysaccharidic vesicles (hereafter termed 'mPSVs') using polysaccharides such as starch, hyaluronate (HA), and glycol chitosan (GC) or poly(L-lysine)-*g*-poly(ethylene glycol) [poly(Lys)-*g*-PEG]. In particular, we utilized a multilayered polysaccharide prepared via a chemical grafting reaction between starch and hyaluronate (HA) in the presence of cystamine (with a thiol linkage) as a coupling agent (Supplementary Fig. S1, Fig. 1). After fabricating self-assembled polysaccharidic nanoparticles [inner core (starch), electrostatic-interacted shell: HA and GC or poly(Lys)-*g*-PEG] using HA grafted with starch (HA-*g*-starch) and GC [or poly(Lys)-*g*-PEG], the amylase treatment of the nanoparticles allows the presence of a vacant inner core (resulting from the degradation starch molecules) within the fabricated particles. It is worth noting that the step-wise enzymatic degradation tailor-made for biomimetic vesicles in this study is an important property in creating new colloidal systems and improving their potential for pharmaceutical application. In particular, when compared to prior synthetic vesicle (using amphiphilic lipids or polymers) technologies in the field of drug delivery, our vesicles can be simply fabricated using biocompatible and biodegradable polysaccharides or polypeptides without a critical consideration about the molecular weight balance of hydrophilic/hydrophobic segments [1–5]. Furthermore, our vesicles are expected to provide efficient drug release properties due to the enzymatic degradation of HA layer in hyaluronidase-rich cellular conditions [1,3], which is comparable with the conventional vesicles having a relatively impermeable amphiphilic bilayer [1–5].

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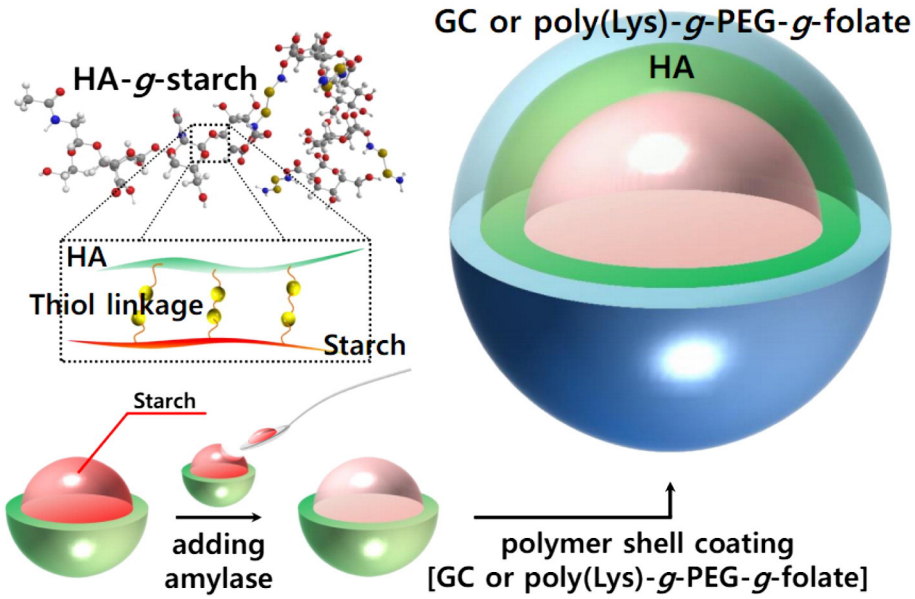

Fig. 1. Schematic concept and preparation of the proposed multilayered polysaccharidic vesicles (mPSVs).

2. Materials and methods

2.1. Materials

Hyaluronate (HA, Mw = 40 kDa), starch, glycol chitosan (GC, Mw = 50 kDa), poly-L-lysine [poly(Lys), Mw = 5 kDa], monocarboxylated poly(ethylene glycol) (PEG, Mw = 2 kDa), succinic anhydride, 4-dimethylaminopyridine (DMAP), N,N′-dicyclohexylcarbodiimide (DCC), N-hydroxysuccinimide (NHS), triethylamine (TEA), pyridine, cystamine, folate, dimethyl sulfoxide (DMSO), α-amylase (from porcine pancreas), hyaluronidase type-2 (HYAL-2), fluorescein isothiocyanate (FITC), bovine serum albumin (BSA), and RPMI-1640 medium were obtained from Sigma-Aldrich (USA). Chlorin e6 (Ce6) was purchased from Frontier Scientific Inc. (USA). D-$(KLAKLAK)_2$ and D-$(KLAKLAK)_2$ tagged with tetramethylrhodamine (TAMRA) [D-$(KLAKLAK)_2$ and D-$(KLAKLAK)_2$-TAMRA), >95% purity] were purchased from Peptron Inc. (Korea). Fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Welgene Inc. (Korea). A Cell Counting kit-8 (CCK-8) was obtained from Dojindo Molecular Technologies Inc. (Japan).

2.2. HA-g-starch synthesis

Starch (2 mM) was pre-activated using succinic anhydride (5 mM), DMAP (5 mM), DCC (10 mM), and NHS (10 mM) in DMSO (20 mL) containing TEA (2 mL) and pyridine (1 mL) at room temperature for 8 h and reacted with excess cystamine (50 mM) at room temperature for 24 h. This synthesis yielded functionalized starch with disulfide bond and free amine groups (Supplementary Fig. 1). The free amine groups of functionalized starch (2–10 mM) were reacted with the NHS groups of the HA (prepared after the pre-activation process of the carboxyl group of HA using DCC and NHS in DMSO at room temperature for 8 h) (20 mM) in DMSO (10 mL) at room temperature for 24 h (Supplementary Fig. S1). The solution was purified via dialysis (using Spectra/Por® MWCO 100K membrane) against fresh DMSO to remove any unconjugated chemicals. The resulting solution containing HA grafted with starch (HA-g-starch) was freeze-dried for 2 days.

2.3. Poly(Lys)-g-PEG-g-folate synthesis

Monocarboxylated PEG (2 mM) pre-activated with DCC (3 mM) and NHS (3 mM) was grafted to free amine groups of poly(Lys) (2 mM) in DMSO (10 mL) containing TEA (2 mL) and pyridine (1 mL) at room temperature for 24 h, yielding poly(Lys) grafted with PEG [poly(Lys)-g-PEG]. The solution was purified via dialysis (using Spectra/Por® MWCO 10K membrane) against fresh DMSO to remove any unconjugated chemicals and byproducts. The resulting solution was freeze-dried for 2 days. Subsequently, residual free amine groups of poly(Lys)-g-PEG (1 mM) were reacted with folate (1 mM) [pre-activated using DCC (2 mM) and NHS (2 mM)] in DMSO (10 mL) containing TEA (2 mL) and pyridine (1 mL) at room temperature overnight. The solution was purified via dialysis (using Spectra/Por® MWCO 5K) against fresh DMSO to remove unconjugated chemicals. The resulting solution was freeze-dried for 2 days. From the ^{1}H NMR spectra of poly(Lys)-g-PEG-g-folate conjugates, we estimate that 8.8×10^{-1} mol of PEG and 4.56 mol of folate were grafted to 1 mol of poly(Lys), as determined from δ 4.33 ppm [–CH in poly(Lys)], δ 3.32 ppm ($–CH_2$ in PEG), and δ 6.64 ppm ($–CH_2$ in folate) (Supplementary Fig. S3).

2.4. mPSV fabrication

HA-g-starch (1 mM) dissolved in DMSO (10 mL) was added to a round-bottom flask. The solvent in the round-bottomed flask was removed using a rotary evaporator (EYELA, N-1000) to form a thin film on the surface of the flask. The film was rehydrated in PBS (pH 6.0, 5 mL) containing amylase (10 μg/mL) using a sonicator (60 Hz for 5 min). The solution was then mixed with GC [or poly(Lys)-g-PEG-g-folate] (1 mM) at 14,000 rpm for 30 s. The resulting solution was dialyzed using a Spectra/Por® MWCO 100K membrane to remove non-complexed polymers, free amylase, and starch degradates. The yield of mPSV (III)–(VI) fabrication was 63 ± 8 wt.%, as calculated after lyophilization. The residual starch concentration in the mPSVs was calculated after measuring the concentration of starch degradates suspended in the supernatant of the mPSV solution centrifuged at 20,000 rpm for 10 min, using the phenol–sulfuric acid method.

2.5. Characterization of mPSVs

The morphology and shell thickness of mPSVs was confirmed using a transmission electron microscope (TEM; JEM 1010, Japan). mPSVs were mounted onto carbon-coated copper grids and examined using a TEM operated at 60 kV and a CCD camera (SC1000 Orion, USA). The 'width' refers to the short dimension of the vesicle shell, whereas the