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In situ facile-forming PEG cross-linked albumin hydrogels loaded with an apoptotic TRAIL protein

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ABSTRACT

The key to making a practicable hydrogel for pharmaceutical or medical purposes is to endow it with relevant properties, i.e., facile fabrication, gelation time-controllability, and *in situ* injectability given a firm basis for safety/biocompatibility. Here, the authors describe an *in situ* gelling, injectable, albumin-cross-linked polyethylene glycol (PEG) hydrogel that was produced using a thiol–maleimide reaction. This hydrogel consists of two biocompatible components, namely, thiolated human serum albumin and 4-arm PEG_{20k}-maleimide, and can be easily fabricated and gelled *in situ* within 60 s by simply mixing its two components. In addition, the gelation time of this system is controllable in the range 15 s to 5 min. This hydrogel hardly interacted with an apoptotic TRAIL protein, ensuring suitable release profiles that maximize therapeutic efficacy. Specifically, tumors (volume: 278.8 mm^3) in Mia Paca-2 cell-xenografted BALB/c *nu/nu* mice treated with the TRAIL-loaded HSA-PEG hydrogel were markedly smaller than mice treated with the hydrogel prepared via an amine-N-hydroxysuccinimide reaction or non-treated mice (1275.5 mm^3 and 1816.5 mm^3, respectively). We believe that this hydrogel would be a new prototype of locally injectable sustained-release type anti-cancer agents, and furthermore offers practical convenience for a doctor and universal applicability for a variety of therapeutic proteins.

1. Introduction

Hydrogels have attracted growing attention for a variety of pharmaceutical and biomedical applications, such as, drug delivery, cell therapy, and tissue engineering [1]. For drug delivery hydrogels, injectability is mandatory to avoid the complications of frequent surgical implantation, and thus, *in situ* gelation is an important property of injectable hydrogels. Accordingly, several approaches have been exploited to achieve gelation, for example, using specific chemical reactions or sensitivity to light, pH, or temperature [2–6]. However, these methods suffer from the disadvantages of potential toxicity, untimely/slow gelation, or require additional devices [7,8]. In this context, hydrogels that are rapidly produced *in situ*, that is, hydrogels that are produced rapidly by simply mixing solutions containing components, offer a more efficient, attractive, clinical option.

The high water contents and soft inner natures of hydrogels make them useful for the delivery of therapeutic proteins susceptible to physical or chemical denaturation [1,9,10]. Nonetheless, many of the hydrogels produced have shown instability and concomitant bioactivity loss because of their unfavorable gelation chemistries [11,12]. In particular, interactions between proteins and gel components are frequently caused by undesirable chemical reactions [13]. In general, protein drugs possess many functional surface groups, which can interact unpredictably with polymeric structures and adversely affect drug release patterns [14].

In recent years, human serum albumin (HSA) has been regarded as a versatile functional material in drug delivery for the purpose of (i) protracting circulation of drugs or (ii) drug targeting. Particularly, HSA can be safely used as a biocompatible carrier because its degraded fractions or metabolites are non-toxic and non-immunogenic [15]. On the basis of these advantages, the utility of HSA has been expanded to the hydrogel systems. Many research articles have reported that albumin-based hydrogels are useful in modulating sustained drug release and in preparing 3D cell scaffold [16–18].

In our previous study, we developed a four-arm PEG cross-linked hyaluronic acid hydrogel containing PEGylated TRAIL. Despite the remarkable antitumor efficacy, the system did not have *in situ* gelation ability (gelation time: ~4 h) and thus PEGylated TRAIL should be loaded after the gel formation, which required additional several hours [19]. Herein, we present a new injectable polyethylene glycol (PEG)-cross-linked human serum albumin (HSA) hydrogel, which is biocompatible and biodegradable, produced *in situ* in a predictable time frame (i.e., <

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60 s), and reduces the interactions with loaded protein drugs. In order to demonstrate the merits of the devised hydrogel system, we used the antitumor protein TRAIL (TNF-related apoptosis inducing ligand) as a model drug. The hydrogel consists of two basic components, namely, HSA and PEG, which are known to be safe and compatible with pharmaceutical use in man. Hydrogels made of PEG are regarded as standard in this field due to their many practical merits, for example, they have low protein adsorptions, negligible immunogenicities, and are easily fabricated and incorporated [20]. Specifically, HSA was added as a macromolecular cross-linker to ensure biocompatibility and functionality, because it has been well shown that cross-linkers, such as, glutaraldehyde, formaldehyde, glyoxal, isocyanate, and genipin, are toxic and unsafe [21–24]. In addition, the physicochemical properties, release profiles, and *in vivo* antitumor efficacies in a mouse model pancreatic cancer of the TRAIL-loaded PEG-cross-linked HSA hydrogel were examined.

2. Materials and methods

2.1. Materials

An active TRAIL variant possessing trimer-forming zipper sequences at its N-terminus hTRAIL (114–281) was produced in *Escherichia coli* using pET23dw-ILZhTRAIL expression vector [19]. After amplification of *E. coli*, TRAIL expression was induced with isopropyl-β-D-1-thiogalactopyranoside (IPTG, 1 mM) for 7 h at 27 °C. Harvested cells were then lysed by sonication in PBS (pH 7.4, 20 mM), and cell extracts were centrifuged at 12,000 rpm at 4 °C for 20 min. Supernatant was collected and purified by Ni-affinity chromatography, stepwise washing with 5 and 10 mM imidazole buffer, and elution with 20 mM imidazole buffer (pH 7.5). Human serum albumin (HSA, ~99%, 66.5 kDa) and 4-arm polyethylene glycol maleimide (4-arm PEG-Mal; Mw 20 kDa) were purchased from Sigma-Aldrich (St. Louis, MO) and NOF Corporation (Tokyo, Japan), respectively. FITC Annexin V staining kits and the Cy5.5 NHS ester dye were from BD Pharmingen™ (Heidelberg, Germany) and GE Healthcare (Piscataway, NJ), respectively. All other reagents were obtained from Sigma-Aldrich, unless otherwise specified.

2.2. Animals

BALB/c *nu/nu* mice (nude mice, males, 5 weeks old) were purchased from Hanlim Experimental Animal Laboratory (Seoul, Korea). Animals were cared for in accordance with the guidelines issued by the National Institute of Health (NIH) regarding the care and use of laboratory animals (NIH publication 80–23, revised in 1996). Animals were housed in groups of 6–8 under a 12-h light/dark cycle (lights on 6 am), allowed food and water ad libitum, and acclimatized for 2 weeks. This study was approved by the Ethical Committee on Animal Experimentation at Sungkyunkwan University.

2.3. Fabrication of HSA-SH/PEG-MAL hydrogel

HSA-SH/PEG-MAL hydrogel was prepared as previously described method with slight modification [25,26]. Human serum albumin (HSA, 40 mg) was dissolved in 0.2 mL of PBS (pH 8.0, 10 mM) and mixed with predetermined equivalents of 2-iminothiolane (2-IT = Traut's reagent; 9–24 M equivalents (eq.) with respect to HSA) and allowed to react for 90 min. The number of thiols per a thiolated HSA was measured by using the Ellman method [27], and the molecular mass of HSA-SH (1:12) was measured by using matrix-assisted laser desorption and ionization time of flight mass spectrometry (MALDI-TOF MS). The reaction mixture was then dialyzed versus 10 mM PBS (pH 7.4) using a Centricon-10 concentrator (MwCO: 30 kDa) and stored at a final concentration of 100 mg/mL at 4 °C until needed. 4-arm PEG-maleimide dissolved in PBS (PEG-MAL, 12 mg) was mixed with an aliquot of thiolated HSA (40 mg) at different final concentrations (200–1200 μM). Thioether linkage-based HSA-SH/PEG-MAL hydrogel was formed under the conditions established for optimal *in situ* gelation (e.g., HSA:2-IT = 1:12; HSA-SH and PEG-MAL, each 400 μM). When required, PEG-MAL was dissolved in 1 mL of TRAIL solution (3 mg) and mixed with an aliquot of the thiolated HSA to load TRAIL into the hydrogel. In order to monitor gelation times, 1 M equivalent of PEG-MAL was mixed with different molar ratios (0.8–1.2 M eq. versus PEG-MAL) of thiolated HSA (HSA:2-IT = 1:12) at a final concentration of 400 μM.

Gelation times were determined using the vial tilting method. After mixing components of suggested hydrogel (HSAs and PEG-MAL or PEG-NHS), no flow or no significant movement of gel or gel materials within each time point upon inverting the vial was regarded as a gel state, as described previously [28]. The HSA-SH/PEG-MAL hydrogel was prepared within 6 h after making thiolated HSA to prevent aggregation between thiol groups in HSA-SH. Separately, as a control hydrogel, a hydrogel formed via amide linkage formation was prepared by mixing naïve HSA and 4-arm PEG-NHS (PEG-NHS) under the same conditions.

2.4. Evaluation of TRAIL release from hydrogels

TRAIL-loaded hydrogels (HSA-NH_2/PEG-NHS or HSA-SH/PEG-MAL hydrogels) prepared from 0.5 mL of pre-hydrogel solutions under optimal conditions were suspended in 3 mL of PBS (pH 7.4, 10 mM) in glass vials, which were then gently rotated at 37 °C. At pre-determined times, supernatants were collected and stored at −70 °C until needed, and vials were replenished with the same volume of fresh PBS. Collected supernatants were purified by Ni-affinity chromatography by stepwise washing with 5 and 10 mM imidazole buffers and elution with 20 mM imidazole buffer. TRAIL amounts released from each hydrogel were determined by reversed-phase high performance liquid chromatography on a PLRP-S 300A column (8 μm, 150 × 4.6 mm; Agilent Technologies, CA, USA).

2.5. Observation of rhodamine-tagged TRAIL in hydrogels

TRAIL (10 mg) was conjugated with 1.5 M equivalents of rhodamine-NHS (Pierce, Rockford, IL, USA) in 10 mM phosphate buffer (5 mL, pH 7.0) for 3 h at room temperature. Mixtures were dialyzed for 1 h vs. 10 mM PBS (pH 7.4) using a Centricon-10 concentrator (MwCO:30 kDa) and stored at 3 mg/mL at 4 °C until needed. Rhodamine-tagged PEG-TRAIL (3 mg) was loaded into 1.5 mL aliquots of pre-hydrogel solutions for each hydrogel using the method mentioned above. The hydrogels were carefully washed three times with 10 mM PBS (pH 7.4) and incubated at 37 °C for 5 days. The distributions of rhodamine-tagged TRAIL from hydrogel surfaces (0 μm) to a depth of 150 μm (in 30 μm intervals) were visualized by confocal laser scanning microscopy (CLSM; Carl Zeiss Meta LSM510, Germany) to investigate the interaction between TRAIL and the hydrogels. In addition, rhodamine-tagged TRAIL loaded hydrogels (HSA-NH_2/PEG-NHS or HSA-SH/PEG-MAL) in 10 mL of 10 mM PBS (pH 7.4) were gently agitated at 37 °C, and at predetermined times, photographs of hydrogels were taken to visualize release patterns.

2.6. In vivo monitoring of TRAIL release from HSA-SH/PEG-MAL hydrogel

TRAIL was labeled with 1.5 M equivalents of Cy5.5 NHS ester dye (15 μg) at pH 8.0. Unreacted dye was removed using a Centricon 10 concentrator (Millipore, Beverly, MA, USA). HSA-SH/PEG-MAL or HSA-NH_2/PEG-NHS hydrogel (150 μL of pre-hydrogel solution) containing Cy5.5-modified TRAIL (300 μg as a TRAIL) were directly injected into BALB/c *nu/nu* mice by subcutaneous injection, and whole body fluorescences were visualized at 0, 3, 12, 48, 72, and 120 h post-injection using an *in vivo* imaging system (Optix MX3, ART Advanced Research Technologies Inc., USA).