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Amino acid based polymer hydrogel with enzymatically degradable cross-links

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ABSTRACT

The synthesis of a chemically cross-linked polymer hydrogel consisting exclusively of amino acids is described in this paper. A natural amino acid, aspartic acid was polymerized to polysuccinimide which was cross-linked by a tetrapeptide sequence designed for proteolytic degradation, and then the corresponding poly(aspartic acid) hydrogel was obtained by alkaline hydrolysis. The hydrogel dissolved in the presence of trypsin. According to *in vitro* cellular assays, the degradation products of the hydrogel cross-linked with the peptide were non-cytotoxic and non-cytostatic. The sustained release of an encapsulated macromolecular model drug, FITC-dextran, was triggered by the degradation of the hydrogel induced by trypsin. These results suggest the potential application of the trypsin-responsive hydrogel for drug delivery in the small intestine.

1. Introduction

Polymer hydrogels are applied as biosensing materials [1], scaffolds for tissue engineering [2] or as drug delivery vehicles [3] owing to their large water uptake, biocompatibility and responsive properties. A wide variety of polymers, cross-linkers and cross-linking methods are in our hand to design hydrogels with tailor-made properties like hydrogels responsive to pH, temperature, redox potential or other external stimuli [4–8]. Yet the most attractive stimulus for biomedical applications is the concentration gradient of biomolecules, like enzymes, in the human body. The most important advantages of using enzymes over other stimuli are their high selectivity, inherent biocompatibility and their availability at the site of action, *i.e.* they do not need to be added externally [9]. The possible responses to the presence of enzymes include swelling [10–13], gelation [14–18] or degradation. The degradation of polymer hydrogels can be classified into at least two different categories, such as the degradation of the polymer backbone [19,20] or that of the cross-linkers [16,21–39]. The third possibility, cleavage of pendant groups from the backbone chains may or may not result in the dissolution of the hydrogel [40]. Various enzymes have been reported to induce such a response, *e.g.* dextranase [20], horseradish peroxidase [14,17,18] or various proteases [16,21–30,32–39]. The hydrogels responsive to enzymes can be used as scaffolds with controlled rate of degradation [16,21–23,32,39] or in controlled drug delivery exploiting the change in the expression or activity of the enzymes at certain disease sites [27–29,35,36,41,42].

Hydrogels showing enzymatic response can be prepared by using peptide cross-linkers. The peptide sequence can be designed for selective cleavage by a protease enzyme available at the chosen therapeutic site. The presence of trypsin is a particularly attractive trigger for site-specific drug delivery systems. Trypsin-degradable hydrogels have been already reported in the literature [26,29,42], and they can be utilised for the protection of bioactive molecules (*e.g.* therapeutic proteins) in the stomach and for the release of them in the small intestine. Van Dijk and co-workers [26] reported the synthesis of a hydrogel by the use of the Cu(I)-catalyzed 1,3-dipolar cycloaddition of a trypsin-responsive bis-azido peptide and a star-shaped alkyne derivatized PEG. The degradation of the resulting hydrogel was induced by trypsin. Horava and co-workers [29] recently reported a pH- and enzyme-responsive microgel of poly(methacrylic acid)-*g*-poly(ethylene glycol) cross-linked with an oligopeptide composed of arginine (Arg, R), glycine (Gly, G) and lysine (Lys, K) for targeted cleavage by trypsin and resistance to degradation by pepsin. The hydrogel can be used for the oral administration of haematological factor IX for the treatment of haemophilia B. Enzymatically degradable hydrogels are often synthesized by the co-polymerization of acrylated peptide derivatives [21,32–37]. Various

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click reactions are also used such as the Michael addition of cysteine thiols with vinyl-sulfone [16,22,39], maleimide [23] or norbornene functionalised poly(ethylene glycol) [24,38]. Copper catalyzed Huisgen cycloaddition of azido-terminated PEG and alkyne-terminated peptides [25] or alkynated PEG and peptide azides [26] can be used as well. The carboxyl groups of the polymer and the terminal amine groups of the peptides can be coupled by *p*-nitrophenyl ester activation [27] or carbodiimide chemistry [28–30,42]. The drawback of all these methods is that they require the derivatization of the polymer before the cross-linking reaction, and the acrylate polymers are not biodegradable.

Apart from polymer hydrogels, enzyme-responsive physical gels have also been reported in the literature. Pochan and Schneider prepared a series of synthetic oligopeptides which are able to fold into β-hairpin, then self-assemble into a β-sheet rich network to form a self-supporting hydrogel [43]. The oligopeptides composed of 20 amino acids include a six residue sequence that is cleavable by matrix metalloproteinase-13, thus, these peptides have a potential as extracellular matrices for facilitating repair of damaged tissues [44].

In this work, a polymer hydrogel with trypsin-induced degradation was prepared exclusively from amino acids. The polymer backbone of the hydrogel is a pH-responsive poly(aspartic acid) (PASP) [5,6], a synthetic polymer, which is biocompatible [45] and biodegradable [46] due to its protein-like poly(amino acid) structure. PASP has a robust synthesis method allowing precise control over molecular structure [47,48]. The precursor anhydride of PASP, polysuccinimide (PSI), reacts with primary amines under mild reaction conditions. Thus, PSI can be cross-linked by any peptide bearing amine groups at both termini, which means that no activation of the peptide is required before cross-linking. In the present work, a tetrapeptide, composed of phenylalanine (Phe, F), arginine (Arg, R) and lysine (Lys, K), abbreviated as FRFK, was used as trypsin-degradable cross-linker. Degradation, biocompatibility, and drug release properties of the hydrogel were studied in comparison with a non-degradable poly(aspartic acid) hydrogel.

2. Experimental

2.1. Materials

Imidazole (99%), potassium chloride (KCl), sodium chloride (NaCl), dibasic sodium phosphate dihydrate ($Na_2HPO_4{\cdot}2H_2O$), mesitylene (99%), phosphoric acid (85% H_3PO_4), methanol, dibutylamine (DBA, 99%), and dithiothreitol (DTT, 99%) were purchased from Reanal (Hungary). Monobasic potassium phosphate (KH_2PO_4, 99.5%) L-aspartic acid (extra pure), sulpholane (99%), cystamine dihydrochloride (CA, 98%), and HPLC-grade acetonitrile (MeCN) were bought from Merck. Hydrochloric acid (HCl), dimethyl sulfoxide (DMSO), dichloromethane (DCM), dimethylformamide (DMF), and diethylether were purchased from Molar Chemicals (Hungary). Deuterated dimethyl sulfoxide (DMSO-d_6, 99.9 atom% D, containing 0.03 v/v% tetramethylsilane), trypsin (from porcine pancreas, Type IX-S, 13,000–20,000 BAEE units/mg protein), and FITC-dextran (M_w = 70 kDa; FITC:glucose = 1:250) were bought from Sigma-Aldrich.

All amino acid derivatives (Fmoc-Phe-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Lys(Boc)-OH), 2-chlorotrityl-chloride resin, coupling reagents (*N*,*N*′-diisopropylcarbodiimide (DIC), 1-hydroxybenzotriazole (HOBt)) and scavenger (triisopropylsilan (TIS)) were purchased from IRIS Biotech GmbH (Germany). Piperidine and trifluoroacetic acid (TFA) were Fluka products.

The imidazole buffer (pH = 8; I = 0.15 M) was prepared by dissolving 6.81 g imidazole and 10.39 g KCl in 1 dm^3 of water, the pH was adjusted with 1 M HCl. The phosphate buffered saline (PBS) solution of pH = 7.4 was prepared by dissolving 8.00 g of NaCl, 0.20 g of KCl, 1.44 g of $Na_2HPO_4{\cdot}2H_2O$ and 0.12 g of KH_2PO_4 in 1 dm^3 of water, the pH being adjusted with 0.1 M HCl. The pH of the buffer solutions was checked with a Radelkis OP-271/1 pH/ion analyser.

For the *in vitro* cell culturing assays, RPMI-1640 medium, gentamicin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and fetal calf serum were obtained from Sigma-Aldrich. The 96-well microtest plates were the products of Sarstedt (Hungary).

All reagents were used without further purification and their quality was "for analysis" unless otherwise noted. Experiments were performed at room temperature (T = 25 °C) unless otherwise indicated. Ultrapure water (ρ > 18.2 MΩ·cm, Millipore) was used for the preparation of aqueous solutions.

2.2. The synthesis of polysuccinimide

Polysuccinimide (PSI) was synthesized by the thermal polycondensation of aspartic acid in a mixture of mesitylene and sulpholane (7/3 weight ratio) at 160 °C with 85% H_3PO_4 as catalyst (the molar ratio of H_3PO_4 to aspartic acid was 16%). After 7 h, the resultant polymer was filtered and dissolved in DMF, then precipitated with excess amount of water. The precipitate was washed with water and MeOH and dried at 80 °C. The chemical structure of PSI was confirmed by ^{1}H NMR (300 MHz, DMSO-d_6, δ: 5.10 (d, 1H, CH); 3.20 and 2.75 (s,s, 2H, CH_2)). PSI was hydrolysed to poly(aspartic acid) (PASP) in a mildly alkaline solution (imidazole buffer, pH = 8, 24 h), the solution was dialysed against water and the solid PASP was obtained by freeze-drying. The average molecular weight of the resultant PASP was determined by HPLC size-exclusion chromatography (SEC). A Nucleogel GFC-300 column was used (molecular weight range of 1–100 kDa) with PBS eluent. The average molecular weight of PASP was calculated to be M_w = 56.1 kDa with a polydispersity index (PDI) of 1.07.

2.3. Synthesis of the peptide cross-linker

TFA salt of FRFK was synthesized by solid phase methodology using Fmoc-chemistry on 2-chlorotrityl-chloride resin [49] using diisopropylcarbodiimide/1-hydroxybenztriazole (DIC/HOBt) as coupling reagent. The protecting group of arginine and lysine were 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) and tert-butyloxycarbonyl (Boc) groups, respectively. The tetrapeptide was cleaved off the resin in a solution containing 95% TFA, 2.5% water and 2.5% triisopropylsilane by stirring the solution for 2 h. The resin was removed by filtration, the filtrate was dropped into cold ether to precipitate the peptide. The ether was distilled three times to remove free TFA. The crude products were purified by semi-preparative reversed-phase high-performance liquid chromatography (RP-HPLC) and the purified compounds were characterized by analytical RP-HPLC (Fig. S1) and electrospray ionization ion trap mass spectrometry (ESI-IT-MS). The molecular mass of the FRFK tetrapeptide was 596.5 Da (Fig. S2).

2.4. Synthesis of poly(aspartic acid) hydrogel cross-linked with the tetrapeptide

The poly(aspartic acid) hydrogel cross-linked with the tetrapeptide (PASP-FRFK) was prepared by dissolving 14.6 mg (150 μmol) PSI and 20.6 mg (25 μmol) FRFK·2TFA in 106.7 mg DMSO, then 8.1 mg (62.5 μmol) dibutylamine (DBA) was added to initiate gelation, and the solution was vortexed. The gelation was carried out overnight, then the gel was placed into imidazole buffer (pH = 8) to hydrolyse unreacted succinimide rings. The buffer was replaced daily for 3 days. PASP-FRFK was stored in PBS (pH = 7.4) for further experiments. PASP-FRFK gel loaded with the model drug was prepared by dissolving PSI and FRFK in DMSO along with 2.91 mg FITC-dextran (20 w/w% of the polymer) before the gelation. An oscillatory rheometer (Anton Paar Physica MCR 301, Austria) with plate-plate geometry (PP25, diameter 25 mm, measuring gap 2.75 mm, normal force 0.5 N) was used to assess the mechanical properties of the hydrogel. Frequency sweep measurements were performed in the range of linear viscoelasticity with a strain of 1% in the angular frequency range of ω = 1–100 rad/s. The temperature