Reactive & Functional Polymers 84 (2014) 29–36

Contents lists available at ScienceDirect

Reactive & Functional Polymers

journal homepage: www.elsevier.com/locate/react

In situ oxidation-induced gelation of poly(aspartic acid) thiomers

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ARTICLE INFO

Article history:
Received 19 March 2014
Accepted 25 August 2014
Available online 6 September 2014

Keywords:
Poly(aspartic acid)
Thiolated polymers
Disulphide formation
Rheology
Injectable polymers

ABSTRACT

In situ gelling poly(aspartic acid) thiomers are investigated to demonstrate their potential application in the development of injectable formulations. The chemical stability of the thiomer solutions is measured against air to determine the maximum storage time of the solution before injection. Hydrogels exhibit considerably large storage moduli after the chemical oxidation of the low-viscosity thiomer solution. The gelation time can be controlled within 2–6 min, which is advantageous for injection because the thiomer solution and the oxidising agent can be mixed safely in a two-chamber system before injection into the desired site of the body.

1. Introduction

Thiolated polymers, referred to as thiomers, have inspired the development of numerous fields in polymer-based medicine [1–4]. The incorporation of thiol groups and disulphide linkages into biopolymers provides an easy means of control over the redox state of the designed systems by either external chemical agents or naturally occurring bioreductants, e.g., glutathione [5,6]. The permeation-enhancing effect, mucoadhesion, in situ gelling character and efflux pump inhibition of thiomers create the potential for an extremely wide range of applications [4,7,8].

Several naturally occurring polymers, e.g., chitosan [9], hyaluronic acid [10], cellulose [11], gelatine [12], collagen [13] and synthetic polymers, such as poly(acrylic acid) [14], are frequently used as thiomer pre-cursors. Nevertheless, the utilisation of a biocompatible and biodegradable synthetic polymer has the indisputable advantage of precise control over its molecular weight and structure. As a poly(amino acid), poly(aspartic acid) (PASP) exhibits all of these characteristics, and its properties can be adjusted within a wide range by the chemical modification of its pre-cursor polymer, polysuccinimide (PSI), under mild reaction conditions [15–17]. Recently, thiolation has been used to stabilise PASP-based nanoparticles [18–20]. However, the synthesis of gelling PASP thiomers has been only reported by our group, and the bulk hydrogels obtained by the oxidation of PASP thiomers have been applied as reduction-facilitated drug release vehicles [21]. The gelation of PASP thiomers was attributed to intermolecular disulphide formation between the pendant thiol groups. PASP thiomers have a considerable potential in injectable pharmaceutical applications for nasal, oral or vaginal drug delivery because the thiomer solution can be injected into the desired site while the prolonged residence time of the formulation is ensured by the formation of a cross-linked gel structure. The most important requirements for these injectable formulations are the following [8,22–24]: (1) the polymer and its solution must be free of any toxic compounds, (2) the pre-cursor solution of the gel must be a flowable sol to provide easy administration, (3) the polymer must have sufficient stability upon storage prior to injection, (4) the gelation time must be long enough to mix the pre-cursor solution and the oxidising agent safely without clogging (e.g., in a two-chamber syringe), (5) the gel formation should be fast enough to ensure that the formulation remains at the desired site and (6) the formed hydrogel should display considerable mechanical strength. The thiol content of the polymer should not decrease significantly within 30 min after the dissolution in water to fulfil requirement 3. The gelation time must be in the range of 1–5 min to fulfil requirements 4 and 5. The storage modulus must be at least several hundred pascals after gelation to fulfil requirement 6.

The goal of the present paper is to prove the genuine potential of PASP thiomers in injectable formulations. The chemical structure of the water-soluble polymer is carefully investigated by NMR spectroscopy to confirm the presence of cysteamine side chains and exclude irregular moieties, which might affect the gelation time or gel strength. The chemical stability of the polymer solution is investigated under atmospheric conditions, and its rheological behaviour is analysed before and after the oxidation-induced gelation by oscillatory shear experiments. The effect of

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http://dx.doi.org/10.1016/j.reactfunctpolym.2014.08.007
1381-5148/

composition on gelation time and gel strength is discussed to find a suitable composition for the water-based PASP formulation for further application as an injectable material.

2. Experimental

2.1. Materials

Imidazole (puriss. p.a., 99.5%), Ellman's reagent [5,5′-dithiobis(2-nitrobenzoic acid)] (98%) and deuterium oxide (99.9% D, containing 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid, sodium salt as an internal standard) were purchased from Sigma–Aldrich. S-aspartic acid (99%), dibutylamine (DBA, 99%), methanol (MeOH, 99.9%), cysteamine hydrochloride (97%), potassium chloride (99.5%), potassium dihydrogen phosphate (for analysis, ISO), disodium hydrogen phosphate heptahydrate (for analysis, ACS), sodium bromate (99%), dithiothreitol (DTT, for biochem.), sodium borohydride (for synthesis), mesitylene (for synthesis) and sulfolane (for synthesis) were obtained from Merck. Phosphoric acid (cc. 85%), hydrochloric acid (HCl, 35%) and dimethylformamide (DMF, pure) were purchased from Lach-Ner. Sodium hydroxide (a. r.) and sodium chloride (a. r.) were purchased from Reanal Hungary. Deionised water (Milli-Q reagent grade, $\rho > 18.2\ \Omega$ m, Millipore, USA) was used for the preparation of aqueous solutions. All reagents and solvents were used without further purification. The synthesis of modified and cross-linked polymers and all measurements were carried out at 25 °C unless otherwise indicated.

Buffer solutions of pH = 8 were prepared from imidazole (c = 0.1 M), and their pH was adjusted by 1 M HCl. The pH of the buffer solutions was checked with a pH/ion analyser (Radelkis OP-271/1, Hungary). The ionic strength of the solutions was adjusted to 0.15 M by KCl.

2.2. Synthesis

The pre-cursor polymer of PASP, polysuccinimide (PSI), was synthesised according to a previously reported procedure [21]. 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). The average molecular weight of the corresponding PASP polymer was determined using HPLC size exclusion chromatography and calculated to be M_w = 56.1 kDa, PDI = 1.07.

PASP thiomers were synthesised by the modification of PSI with cysteamine hydrochloride in DMF using dibutylamine (DBA) as a deprotonating agent (Scheme 1 and Table 1). A typical procedure was as follows (feed ratio of cysteamine to succinimide units, $X_{CYS,FEED}$, of 20%). First, 0.485 g of PSI (containing 5 mmol of succinimide repeating units) and 0.114 g (1 mmol) of cysteamine hydrochloride were dissolved in 9.143 g of DMF under a nitrogen atmosphere. DBA (340 μl, 0.258 g, 2 mmol) was added dropwise to the solution. Solutions of thiolated PSIs were cast into sheets with a thickness of 1.0 mm and converted into disulphide cross-linked PSI gels by atmospheric oxidation. After 2 days of oxidation, the PSI gels were placed into a mildly alkaline buffer solution of pH = 8. Water-swollen transparent (PASP) gels were yielded after 3 days of hydrolysis.

Table 1
Degree of modification and thiol content of the PASP thiomers.

Sample	$X_{CYS,FEED}$ [a] (%)	X_{CYS} [b] (%)	X_{SH+SS} [c] (%)	X_{SH} [d] (%)	$X_{SH,ac}$ [e] (%)
A	5	2.4	2.3	1.2	2.2
B	10	5.2	5.0	1.6	4.6
C	20	17.1	16.4	4.1	13.4

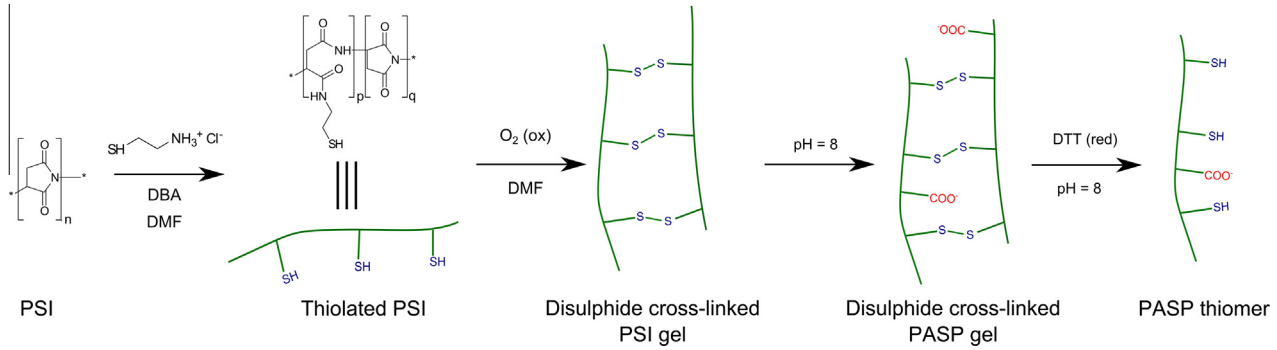
[a] $X_{CYS,FEED}$: feed molar ratio of cysteamine to succinimide units.
[b] X_{CYS}: degree of modification determined by NMR.
[c] X_{SH+SS}: sum of thiol and disulphide contents determined by Ellman's assay.
[d] X_{SH}: thiol content determined by Ellman's assay (pH = 6 during dialysis).
[e] $X_{SH,ac}$: thiol content determined by Ellman's assay (pH = 4 during dialysis).

PASP gels were dissolved by the addition of solid dithiothreitol (DTT) to prepare PASP thiomers. The molar ratio of DTT to thiol groups was 1:1. The dissolution of the gels was complete after 15 min. The reduced PASPs were dialysed (cut-off M_w = 12–14 kDa). The polymers were dialysed against either deionised water (pH ≅ 6) or a dilute solution of HCl (pH ≅ 4). The solid PASP thiomers were obtained by lyophilisation, and the polymers were stored at 8 °C for analysis and further use.

2.3. Chemical characterisation and stability

The chemical structure of PASP thiomers was confirmed by NMR. Sample solutions for NMR measurements were prepared by dissolving 20 mg of lyophilised PASP (sample B in Table 1) in 1000 μl of D_2O. The polymer was completely dissolved within 5 min. All spectra were recorded using a Bruker Avance 300 spectrometer (USA) operating at 300 MHz. The ^{1}H NMR spectrum was recorded with 128 scans, and the DEPT spectrum was acquired with 1024 scans. 2D spectra were recorded for the complete assignment of the peaks. Homonuclear (^{1}H–^{1}H) COSY spectrum was recorded with 16 scans, while heteronuclear (^{1}H–^{13}C) HSQC and HMBC spectra were recorded with 64 scans.

The thiol content of the polymers was determined by Ellman's assay. First, 20 μl of 10 mM Ellman's solution was added to a reaction mixture of 180 μl of the sample solution (pH = 8, $c_{thiomer}$ = 0.05 w/v%) and 1800 μl of an aqueous buffer solution (pH = 8, containing 1 mM ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) to avoid oxidation catalysed by traces of metal ions). The reaction was carried out at 37 °C for 20 min. Absorption spectra were recorded using a UV–VIS spectrophotometer (Specord 200, Analytic Jena, Germany). N-acetyl-cysteine was used as the



Scheme 1. Synthesis of poly(aspartic acid) (PASP) thiomers from polysuccinimide (PSI).