In situ gelation of thiolated poly(aspartic acid) derivatives through oxidant-free disulfide formation for ophthalmic drug delivery

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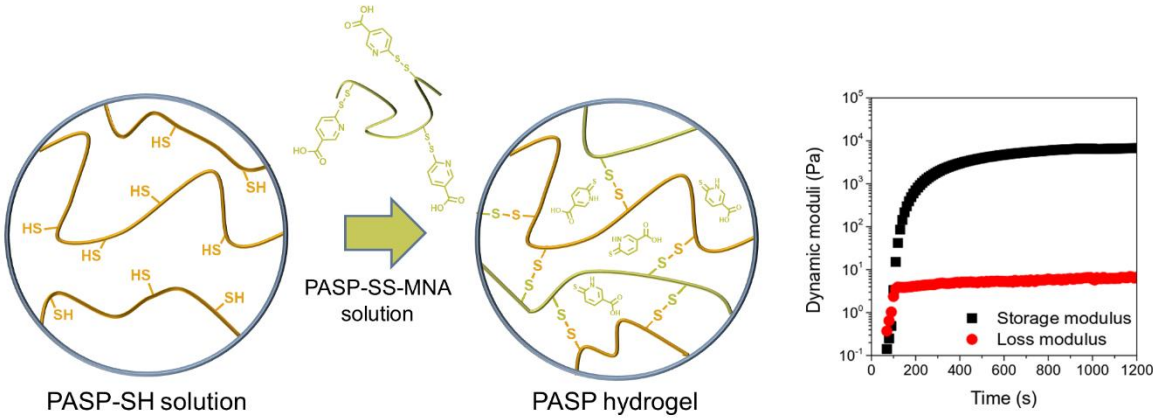
Graphical Abstract



Highlights

− S-protected, preactivated derivatives of thiolated poly(aspartic acid) were prepared.

− The polymers form covalent bonds with mucin protein by thiol-disulfide exchange reaction.

− Aqueous solutions of S-protected poly(aspartic acid) and thiolated poly(aspartic acid) yields chemically cross-linked hydrogels with no additional reagents.

− The hydrogels with no residual thiol groups reach their equilibrium swelling degree at physiological pH.

− The presence of free thiols causes the dissolution of the hydrogels as a result of a series of thiol-disulfide exchange reactions.

− Ofloxacin can be encapsulated into the poly(aspartic acid) hydrogel, and it is released with a sustained kinetics.

Abstract

Efficient topical treatment of ocular diseases requires a prolonged residence time of drug formulations. An *in situ* gelling, mucoadhesive system can provide improved residence time while keeps the installation of the formulation easy and accurate due to its low initial viscosity. We synthesized a two-component, biocompatible water-based liquid formulation showing *in situ* gelation. S-protected, preactivated derivatives of thiolated poly(aspartic acid) (PASP-SS-MNA) were synthesized by coupling of the free thiol groups of thiolated poly(aspartic acid) (PASP-SH) with 6-mercaptonicotinic acid (MNA). The amount of protecting groups was found to be 242, 341 and 530 μmol/g depending on the degree of thiolation of PASP. The interaction between PASP-SS-MNA and mucin was proven indicating the mucoadhesive properties. Disulfide cross-linked hydrogels were formed *in situ* without an oxidizing agent by mixing the aqueous solutions of PASP-SS-MNA and PASP-SH. The gelation time was controlled between 1 and 6 min, while the storage modulus was as high as 4 to 16 kPa depending on the composition. Swelling experiments showed that hydrogels with no residual thiol groups are stable in phosphate-buffered saline at pH = 7.4 whereas the presence of free thiol groups leads to the dissolution of the hydrogel with a rate depending on the excess of thiol groups. Biological safety of the polymers and 6-mercaptonicotinic acid was confirmed on Madin-Darby Canine Kidney cell line. Furthermore, a prolonged release, compared to a conventional liquid formulation, of ofloxacin was observed at pH = 7.4, supporting the potential of the developed biopolymers in ophthalmic drug delivery.

Keywords

Thiolated poly(aspartic acid); Oxidant-free *in situ* gelation; Preactivated; Mucoadhesion; Thiol-disulfide exchange

1. Introduction

Bioavailability of topically administered ophthalmic drugs can be significantly improved by mucoadhesive dosage forms. As their residence time is prolonged compared to conventional dosage forms, passive diffusion of the active pharmaceutical ingredient (API) is more efficient, by their use the protective mechanisms of the eye including dilution by tears, lachrymal drainage, the retention of the active compound by corneal barrier and other losses can be decreased [1,2]. The efficacy of mucoadhesive polymeric excipients is improved by their thiolation [3–6] which can provide mucoadhesive property even to low molecular weight polymers [7] due to the formation of covalent disulfide bonds with the cysteine-rich subdomains of mucin glycoproteins.

Thiolated polymers are sensitive to atmospheric oxidation in aqueous media at a pH where sufficient amount of thiolate anions are present. The rate of the oxidation depends on the pK_a of the thiol groups but the charge of the neighbouring functional groups on the polymer also plays a significant role [5,8,9]. Thus, a thiol pK_a, that promotes disulfide formation at the site of action at physiological pH, has a negative impact on the shelf-life of the material and vice-versa. In order to prevent such premature oxidation while improving reactivity, thiol groups can be protected as an asymmetrical disulfide by attaching a thiopyridyl subunit [10,11]. This kind of coupling facilitates disulfide formation with mucin thiols by fast thiol-disulfide exchange reaction because of the electron withdrawing effect of the π-system of the aromatic ring, thus the thiopyridyl group can be easily cleaved off [5].

The first step in the synthesis of preactivated derivatives is the coupling of conventional mucoadhesive polymers with thiol-bearing side groups e.g. by carbodiimide chemistry. Chitosan can be coupled with thioglycolic acid [12]; pectin [13], and hyaluronic acid [10]; or poly(acrylic acid) [14] can be coupled with cysteine. This step is followed by the attachment of the thiopyridyl protecting group onto the free thiol groups through thiol-disulfide exchange reaction with the excess of the dimer of a thiopyridyl type compound such as 2-mercaptonicotinic acid [11,13], 6-mercaptonicotinic acid [15], and 6-mercaptonicotinic amide [16,17]. Another strategy is the preparation of a S-protected small molecule in advance followed by the modification of the polymer [10,11,13,17]. Biological safety of S-protected polymers is undoubtedly crucial, since the protecting group is always released as a side-product of the thiol-disulfide exchange reaction with the mucin glycoproteins. Thus, assessing biocompatibility not only of the polymer but also of the protecting group is of utmost

importance. S-protected polymers can be excipients in tablets [13,17], semi-solid formulations [11,12] and nanoparticles [14].

Residence time of ocular drug formulations can be further improved by *in situ* gelling liquid polymer solutions that are able to undergo a sol-to-gel transition at the site of action [18–23]. The transition can be triggered by a certain physical (e.g. changes in the pH [24,25] or the temperature [19,26] at the surface of the eye) or chemical stimulus (e.g. cross-linking by genipin [27]). They are as easy to administer as an eye drop due to their low viscosity, but the rapid increase in their viscosity and the formation of cohesive polymer network complemented by the mucoadhesive property of the polymer ensures the prolonged residence time of such formulation.

Polysaccharides and synthetic polyacrylates are generally used for the preparation of drug formulations discussed above but the application of such polymers raises concerns over sustainability and environmental load. Synthetic poly(amino acid)s as degradable and water-soluble polymers are potential alternatives for fossil-based synthetic polymers and natural polymers due to their biomass origin and chemical versatility [28]. Poly(aspartic acid), a synthetic poly(amino acid), has high potential in biotechnology [29] and biomedical applications [30,31] such as drug delivery vehicles [32–35], film coatings [36], electrospun matrices [37,38], and scaffolding materials [39]. Its versatility is based on the reactivity of its precursor anhydride, polysuccinimide (PSI). PSI can be modified with primary amines under mild reaction conditions, and the PSI derivatives can be hydrolysed to their corresponding PASP derivatives, therefore a wide range of polymers can be synthesized. As it is a synthetic polymer, its structure and molecular weight can be precisely controlled and can be produced on large scale [30]. The protein-like structure of the PASP derivatives indicates its biocompatibility [35,36,40] and biodegradability [28,30,39,41–44]. These benefits led us to investigate the potential of poly(aspartic acid) derivatives as mucoadhesive excipients [45–47]. In our earlier studies mucoadhesive and *in situ* gelling properties of thiolated poly(aspartic acid) derivatives were investigated. The aqueous solution of PASP derivatives showed self-cross-linking because of intermolecular disulfide formation upon oxidation. According to adhesion tests performed on porcine conjunctive tissue, the thiolation had a beneficial effect on mucoadhesive properties [46].

In this study, synthesis method of a thiol-protected, preactivated derivative of thiolated poly(aspartic acid) (PASP-SS-MNA) and its application as an *in situ* gelling mucoadhesive hydrogel system is reported. PASP-SS-MNA was synthesized by coupling thiolated

poly(aspartic acid) (PASP-SH) with 6-mercaptonicotinic acid protecting group. Disulfide cross-linked hydrogels were prepared *in situ* without an oxidizing agent by mixing the aqueous solutions of PASP-SS-MNA and PASP-SH. The disulfide cross-linked PASP derivatives formed stable, cohesive chemical hydrogels, and the S-protected moieties of PASP-SS-MNA were able to readily react with the thiol groups of mucin proteins. Chemical structure, viscoelastic and swelling behaviour of the hydrogels were thoroughly tested. Toxicity of the polymers and the 6-mercaptonicotinic acid leaving group is also assessed by MTT assays performed on Madin-Darby Canine Kidney (MDCK) cells. Release profile of ofloxacin from the hydrogel matrices was studied to demonstrate the effect of the chemical network on the drug diffusion.

2. Materials and methods

2.1. Materials

L-aspartic acid (extra pure, 99%), Ellman's reagent (5,5′-dithiobis(2-nitrobenzoic acid), 98%), and monobasic potassium phosphate (KH_2PO_4, 99.5%) were purchased from Merck. D_2O (99.9 atom% D, containing 0.05 wt% 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt), crystalline phosphoric acid (H_3PO_4, 99%), 6-mercaptonicotinic acid (MNA, technical grade, 90%), ofloxacin and mucin from porcine stomach (Type II) were bought from Sigma-Aldrich. 6,6'-dithionicotinic acid (>98%) was purchased from TCI Chemicals. Cysteamine (95%), dibasic sodium phosphate monohydrate ($Na_2HPO_4 \cdot H_2O$), ethylenediaminetetraacetic acid disodium salt dihydrate ($Na_2EDTA \cdot 2H_2O$), dithiothreitol (DTT, 99%), hydrochloric acid (HCl), methanol, monobasic sodium phosphate dihydrate ($NaH_2PO_4 \cdot 2H_2O$, 99%), *N*,*N*-dimethylformamide (DMF), sodium chloride (NaCl), sodium hydroxide (NaOH) and citric acid were bought from Reanal (Hungary). Methanol and acetonitrile were HPLC grade and purchased from Molar Chemicals (Hungary). For cytotoxicity tests, Triton X-100, Dulbecco's Modified Eagle Medium (DMEM), Dulbecco's Phosphate Buffered Saline (DPBS), dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (UK).

Purity of all reagents was "for analysis" unless otherwise noted, and they were used without further purification. Ultrapure water ($\rho > 18.2$ MΩ cm, Millipore) was used for the preparation of aqueous solutions. Syntheses and measurements were carried out at 25 °C

unless otherwise noted. The composition of the buffer solutions used can be found in the Supplementary Data.

2.2. Syntheses

2.2.1 Polysuccinimide

Polysuccinimide (PSI) was synthesized by thermal polycondensation of L-aspartic acid in a solvent free reaction. L-aspartic acid (20 g) and crystalline phosphoric acid (20 g) was reacted in a rotary evaporator at 10 mbar and 180 °C for 7 h. The crude product was then cooled to room temperature and dissolved in 300 ml DMF. The PSI was precipitated in 600 ml of water, then the precipitate was washed with water and methanol. The resulting white product was dried overnight at 40 °C (yield: 95%). Its chemical structure was confirmed by ^{1}H NMR (300 MHz, DMSO-d_6, δ: 5.10 ppm (d, 1H, CH); 3.20 and 2.75 ppm (s,s, 2H, CH_2). The viscosity average molecular weight of PSI was 31.5 kDa determined by a rolling ball viscometer (Anton Paar Lovis 2000, solvent: 0.1 M LiCl in DMF, Mark–Houwink constants: a = 0.76; $K = 1.32 \cdot 10^{-2}$ ml/g [48]).

2.2.2. Thiolated poly(aspartic acid)

Thiolated poly(aspartic acid) (PASP-SH) was synthesized by the addition of cysteamine side groups onto polysuccinimide and subsequent hydrolysis (Fig. 1a), as described earlier [46]. Polymers were prepared with different degree of modification ($X_{CEA\text{-}feed}$ is defined as the feed molar ratio of cysteamine to succinimide repeating units). The synthesized PASP derivatives are listed in Table 1. In a typical procedure ($X_{CEA\text{-}feed}$ = 5%), 1.0 g PSI (10.3 mmol of succinimide repeating units) and 39.7 mg cysteamine (0.502 mmol) was dissolved in 9.0 g DMF in a glass vial and 39.7 mg (0.26 mmol) DTT was added. Nitrogen was bubbled through the reaction mixture for 1 h and it was stirred for 72 h. The reaction mixture was precipitated in 100 ml 1 M HCl and washed with 1 M HCl and finally with water until neutral pH. Nitrogen was bubbled through 150 ml of 0.1 M aqueous NaOH for 1 h. The precipitate was dispersed in the NaOH solution and was stirred for 2 h with constant nitrogen bubbling, and a clear solution was obtained. The solution was neutralized using 1 M HCl, dialysed against water, concentrated in a rotary evaporator (30 mbar, 35 °C) to ca. 15 ml and freeze-dried to obtain the final product. The average yield was 70%.

2.2.3. S-protected thiolated poly(aspartic acid)

S-protected thiolated poly(aspartic acid) (PASP-SS-MNA) was synthesized by thiol-disulfide exchange reaction of thiolated poly(aspartic acid) (PASP-SH) and 6,6'-dithionicotinic acid

(DTNA) (Fig. 1b). PASP-SH polymers with three different degrees of modification were prepared ($X_{CEA\text{-}feed}$ = 5%, 10% and 20%, see Table 1) with the method described in the previous section; after the neutralization, the PASP-SH solution was concentrated to ca. 5 ml. The DTNA solution was prepared by dispersing DTNA (4-fold excess of DTNA to the feed amount of thiol groups, which is 0.64 g (2.1 mmol), 1.27 g (4.1 mmol), and 2.54 g (8.2 mmol), respectively) in water (10, 20, 40 ml, respectively), then 1 M NaOH was added dropwise, set the pH of the solution to 8 and dissolve the DTNA. The PASP-SH solution was added dropwise to the vigorously stirred solution of DTNA using a syringe pump with a flow rate of 2 ml/h. After the addition of the polymer, the mixture was stirred for 12 h, then it was dialysed against water, concentrated in a rotary evaporator and freeze-dried. The products were off-white, yellowish in colour and odourless and they were easily soluble in water.

2.3. Chemical characterization of the polymers

2.3.1. ^{1}H NMR spectroscopy

Structure of the polymers was confirmed by ^{1}H NMR. Sample solutions for the measurements were prepared by dissolving 25 mg of the freeze-dried polymer in 750 µl of D_2O. Spectra were recorded by using a Bruker Avance 300 MHz spectrometer with 128 scans.

2.3.2. Thiol content of PASP-SH polymers (Ellman's assay)

The molar amount of free thiol groups per gram polymer was determined by Ellman's assay ($X_{SH\text{-}Ellman}$). 200 µl of aqueous solution of the freeze-dried polymer sample (nominal thiol concentration was 0.5 mM) was diluted to 1800 µl with phosphate buffer. Then, 200 µl of 1 mM solution of Ellman's reagent was added and the reaction was carried out at 37 °C for 20 min. Optical absorbance spectra of the solutions were collected using a Cary 60 spectrophotometer (Agilent, United States). A calibration curve was determined using *N*-acetylcysteine standard in the concentration range of 0.02−0.10 mM (λ = 412 nm; ε = 13 880 l mol^{-1} cm^{-1}; R^2 = 0.9999).

2.3.3. Coupled mercaptonicotinic acid content of PASP-SS-MNA polymers

The molar amount of covalently attached S-protecting mercaptonicotinic acid (MNA) groups per gram polymer ($X_{MNA\text{-}UV}$) was determined by UV-Vis spectroscopy. A polymer solution with an MNA concentration of ca. 0.5 mM was prepared in PBS containing 100 mM dithiothreitol. The solution was stirred for 4 h to reduce the disulfide groups resulting in the

release of the MNA. Then, the solution was diluted ten times and the optical absorbance spectrum was collected (Cary 60, Agilent, United States). A calibration curve for MNA was determined with DTNA reduced with 100 mM DTT in PBS in the concentration range of $7.8 \cdot 10^{-3}$–0.25 mM (λ = 343 nm; ε = 10 997 l mol^{-1} cm^{-1}; R^2 = 0.9999).

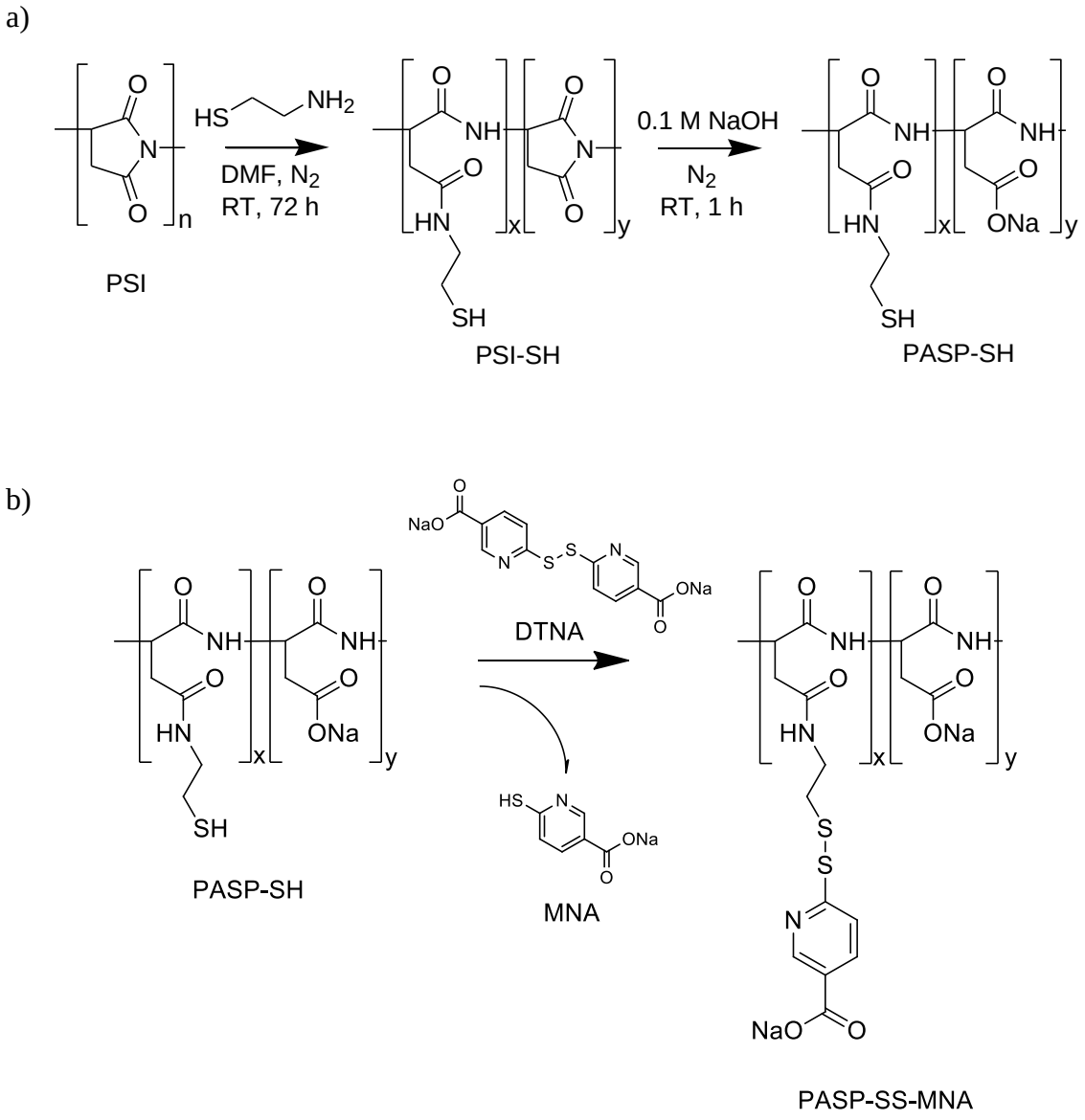


Fig. 1. (a) Synthesis of thiolated poly(aspartic acid). (As a product of the ring opening reaction of polysuccinimide, both α- and β-poly(aspartic acid) derivatives can form. To ease the interpretation, only α repeating units are shown in figures.) (b) Synthesis of S-protected thiolated poly(aspartic acid) (PASP-SS-MNA).

2.4. Interaction between polymer and mucin

2.4.1. Ellman's assay of mucin

Thiol content of porcine gastric mucin was determined by Ellman's assay. 100 mg mucin was dispersed in 9 ml phosphate buffer solution. Then, 1 ml of 1 mM solution of Ellman's reagent was added and the reaction was carried out at 37 °C for 20 min. The resulting mixture was filled in a centrifuge tube and centrifuged for 10 minutes at 13500 G. The supernatant was then removed and filtered with a syringe filter (0.22 μm). Optical absorbance spectra of the filtered solutions were collected with Cary 60 spectrophotometer (Agilent, United States). Thiol content was calculated based on the calibration curve in Section 2.3.2.

2.4.2. Evaluation of MNA release during interaction of mucin and S-protected PASP

Thiol-disulfide exchange reaction between the PASP-SS-MNA polymers and the mucin was proven by measuring the MNA groups cleaved off. 100 mg mucin was dispersed in 900 μl PBS, then 100 μl of 20 w/V% PASP-SS-MNA in PBS was added. The mixture was equilibrated for 30 min. 100 mg mucin was dispersed in 1 ml PBS as control sample. The samples were diluted to 10 ml, centrifuged (13500 G, 10 min) and filtered with a syringe filter (0.22 μm), then optical absorbance spectrum was collected using the control sample as background. The MNA content was determined based on the calibration curve in Section 2.3.3.

2.5. Characterization of *in situ* forming hydrogels

2.5.1. Preparation of the hydrogel samples

Hydrogels were prepared *in situ* without an oxidizing agent by mixing the 10 wt% solutions of PASP-SS-MNA and PASP-SH prepared in PBS at 25 °C. The mixtures were poured into disc shape moulds (d = 6 mm; h = 1 mm) covered with a glass slide to prevent solvent evaporation and the gelation took for 12 h.

2.5.2. Rheology of gelation

Gelation was monitored by oscillation rheology using an Anton Paar Physica MCR301 rheometer (Austria) with cone-plate geometry (CP-25, cone angle: 1°, sample gap: 0.049 mm;

T = 25 °C). 10 wt% solutions of the freeze-dried PASP-SS-MNA and PASP-SH polymers were prepared in PBS. 50 µl of the two solutions were then mixed on the plate of the rheometer to initiate gelation. The change of dynamic moduli was monitored in oscillatory mode at constant strain and angular frequency (γ = 1%, ω = 10 rad s^{-1}). After 20 min, the frequency dependence of the moduli (γ = 1%, ω = 1−100 rad s^{-1}) of the resulting gels was also recorded.

2.5.3. Swelling of the hydrogel samples

Gravimetric method was used to study the swelling behaviour of the hydrogels. Mass of the hydrogel discs was measured after removing them from the moulds (preparative mass), then they were placed in PBS buffer and their mass were measured at predetermined times. Relative degree of swelling was defined as the percentage ratio of the swollen mass of the hydrogels to the preparative mass.

2.6. Cytotoxicity assays of the polymers

Madin-Darby Canine Kidney (MDCK) cells were seeded on 48-well plates (Corning Incorporated Costar 3548, tissue culture treated plates) in a sterile lab and checked by an inverse optical microscope the day before the toxicity assay. DMEM was used as the growth medium (450 µl for each well). Poly(aspartic acid), thiolated polymers and polymers with activated thiol groups as well as 6-mercaptonicotinic acid were tested. Known cytotoxic agent Triton X-100 was used as the positive control while DPBS was used as the negative control. All of the materials were dissolved in DPBS at a concentration of 10 mg/ml. 50 µl of samples were added directly onto the seeded 48-well plates and 6 parallels were used for each composition. Samples were incubated for 4 h at 37 °C in GalaxyR CO_2 incubator. After incubation the medium from the wells was aspirated carefully from the cells and replaced with 450 µl of fresh DPBS. Finally 50 µl of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]/DPBS solution (c = 5 mg/ml) was added to each well and plates were shaken for 5 min and incubated for 3 h at 37 °C. The medium from each well was aspirated carefully with a pipette and 500 µl of DMSO was added to dissolve formazan. Samples were shaken for another 10 min. A purple colour was observed in some samples, indicating the activity of enzymes in the living cells that reduce MTT to formazan dyes, absorbance was directly correlated with cell quantity. Absorbance for all plates was measured at 560 nm with a background reference at 670 nm, using a Tecan Safire 2 plate reader.

2.7. Release of ofloxacin

Ofloxacin release measurements were performed using a vertical Franz diffusion cell system (HansonMicroette Plus™). Precursor polymer solutions of the gels was prepared by dissolving 15 mg of solid PASP-SS-MNA in 135 µl of 3 mg/ml ofloxacin solution in PBS and dissolving 15 mg PASP-SH in 135 µl of 3 mg/ml ofloxacin in PBS. The two solutions were mixed and the mixture was transferred onto a synthetic membrane (Porafil membrane filter, pore size of 0.45 µm, Macherey-Nagel GmbH) in the donor phase of the diffusion cell. The membrane was previously impregnated with PBS. The acceptor phase was also PBS, thermostatically controlled at 35 °C. Measurements were performed for 24 h. A sample of 0.8 ml was taken at given time intervals from the acceptor phase and it was replaced with fresh PBS by the autosampler. 270 µl of ofloxacin (3 mg/ml) eye drop was used as reference sample. The amount of released ofloxacin was measured with a Shimadzu Nexera UHPLC/HPLC system using reverse phase HPLC method. The column was Phenomenex Gemini-NX C18 with dimensions of 5 µm, 250 * 4.6 mm, and used at 25 °C. The separation was evaluated with isocratic elution using 7% acetonitrile and 15% methanol in 0.4 M citric acid solution. The injection volume was 20 µl and the flow rate was 1.2 ml/min. The time of analysis was 8 min, and the retention time of ofloxacin was 4.5 min. The detection wavelength was 287 nm.

2.8. Statistical analysis

All experiments and measurements were performed in triplicate unless otherwise noted.

Statistical analysis was carried out with GraphPad Prism 7 software. MNA release (section 2.4.2) was evaluated by ANOVA with Bonferroni post-tests. Drug release experiments (section 2.7) were evaluated by repeated measures ANOVA, followed by Bonferroni post-tests. A level of $p \leq 0.05$ was taken as significant, $p \leq 0.01$ as very significant and $p \leq 0.001$ as highly significant.

The statistical analysis of cytotoxicity results was performed in three steps. First, the MDCK cell viability in the presence of the polymer samples was analysed with multiple linear regression. Second, the cell viability results were compared by means of one-way ANOVA. Finally, planned comparisons were conducted between the negative control (DPBS) and MNA, as well as between negative control and the united group of polymers.

3. Results and discussion

3.1. Synthesis and chemical characterization of the polymers

S-protected, preactivated derivative of thiolated poly(aspartic acid) (PASP-SS-MNA) polymers was successfully synthesized in a three-step reaction pathway. First, polysuccinimide was modified with cysteamine to incorporate thiol groups. In the second step, the unreacted succinimide rings were opened to aspartic acid units by alkaline hydrolysis. Finally, free thiol groups of the PASP-SH were coupled with MNA groups by thiol-disulfide exchange reaction with 6,6'-dithionicotinic acid, the dimer of MNA. The reaction steps and the proposed chemical structure of the products is illustrated in Fig. 1.

Three PASP-SS-MNA polymers with different MNA content were prepared. The chemical structure of the polymers and the conversion of the reactions steps were characterized by ^{1}H NMR spectroscopy and UV-Vis spectroscopy. The ^{1}H NMR spectrum of a PASP-SS-MNA sample is shown in Fig. S1. The ratio of incorporated cysteamine side groups ($X_{CEA\text{-}NMR}$) and the ratio of coupled MNA groups ($X_{MNA\text{-}NMR}$) to the total number of repeating units were calculated from integrated intensities with the formulas shown in the Supplementary Data. According to the results listed in Table 1, cysteamine addition took place with about 80% conversion as $X_{CEA\text{-}NMR}$ were 3.8%, 8.2%, and 16.2% compared to the degree of modifications ($X_{CEA\text{-}feed}$) of 5%, 10%, and 20%, respectively. In the final products, 3.5%, 6.5%, and 10.0% MNA coupled repeating units are present ($X_{MNA\text{-}NMR}$) meaning that a part of the incorporated free thiols could be coupled with MNA.

The incomplete coupling of MNA to thiol groups indicates a side reaction between the MNA coupled groups and free thiol groups resulting in disulfide formation. PASP-SH was added slowly to a large excess of DTNA to exclude this side reaction but the presence of highly thiolated derivatives induced undesired thiol-disulfide exchange reactions causing a decreased conversion. The presence of this side reaction was supported by the decreasing conversion of MNA coupling with increasing thiol content. The conversion of MNA coupling was 92%, 79%, and 62% for PASP-SH polymers with $X_{CEA\text{-}NMR}$ of 3.8%, 8.2%, and 16.2%, respectively. The ratio of coupled MNA was also analysed by UV-Vis spectroscopy ($X_{MNA\text{-}UV}$). The covalently attached MNA side groups were cleaved off the polymer backbone using a reducing agent, DTT and MNA was quantified photometrically. According to Table 1, the PASP-SS-MNA polymers contained 242, 341, and 530 μmol MNA/g polymer, respectively. Samples without the addition of DTT did not display free MNA. Ellman's analysis did not

show any free thiols in the polymer meaning that during preparation every thiol group react either with DTNA to produce an MNA coupled disulfide or with an already coupled disulfide producing intra- or intermolecular disulfide bonds of polymers formed by two cysteamine groups (side reaction).

Table 1 Chemical characterization of S-protected thiolated poly(aspartic acid) and thiolated poly(aspartic acid) polymers

Sample name	$X_{CEA\text{-}feed}$ [%]	$X_{CEA\text{-}NMR}$ [%]	$X_{MNA\text{-}NMR}$ [%]	$X_{MNA\text{-}UV}$ [μmol/g]	$X_{SH\text{-}Ellmann}$ [μmol/g]
PASP-SH178	5	4.0	-	-	178
PASP-SH545	10	8.6	-	-	545
PASP-SS-MNA242	5	3.8	3.5	242	0
PASP-SS-MNA341	10	8.2	6.5	341	0
PASP-SS-MNA530	20	16.2	10.0	530	0

$X_{CEA\text{-}feed}$: the feed molar ratio of cysteamine to succinimide repeating units
$X_{CEA\text{-}NMR}$: the molar ratio of cysteamine-modified repeating units to all repeating units based on the corresponding areas of NMR peaks (Formula 1 in the Supplementary Data)
$X_{MNA\text{-}NMR}$: the molar ratio of MNA coupled repeating units to all repeating units based on the corresponding areas of NMR peaks (Formula 2 in the Supplementary Data)
$X_{MNA\text{-}UV}$: the molar amount of MNA groups per gram polymer determined by UV-Vis spectroscopy
$X_{SH\text{-}Ellman}$: the molar amount of free thiol groups per gram polymer determined by Ellman's analysis

3.2. Interaction between polymer and mucin

Preactivated thiolated polymers can react with the free thiol groups of the mucin proteins forming disulfide bonds which can contribute to their mucoadhesive properties [5]. First, free thiol content of the lyophilized mucin particles was determined using Ellman's assay to quantify thiol groups available for interaction with PASP-SS-MNA polymers. The mucin contains 1.79 μmol/g of free thiol groups (Fig. 2). The reaction between PASP-SS-MNA and mucin can be monitored by measuring the amount of MNA molecules released as a by-product of the thiol-disulfide exchange reaction. The amount of MNA should be equal to the amount of free thiol groups on mucin regardless the degree of modification of PASP-SS-MNA taking into account the large excess of preactivated thiol groups on the polymer compared to the thiol content of mucin. The results support the hypothesis that the amount of MNA formed in the reaction is approximately the same, and the difference is not significant

($p > 0.999$ in all cases). This proves that the thiol groups of the mucin formed disulfide bonds with the PASP-SS-MNA polymers. The amount of released MNA is higher than the free thiol measured by Ellman's analysis ($p \leq 0.01$ in all pairwise comparisons) which might be due to the incomplete conversion of the Ellman's reagent.

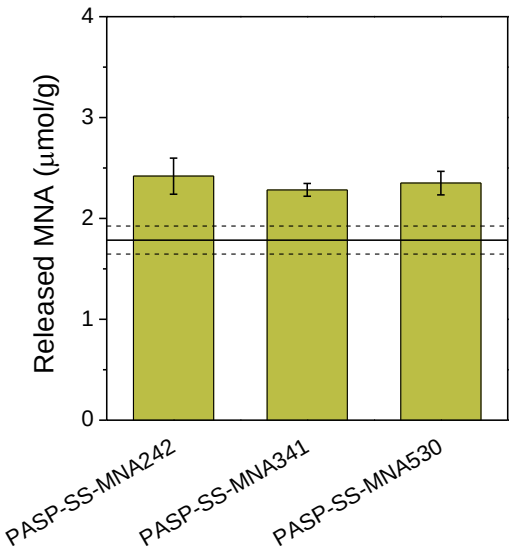


Fig. 2. Amount of released 6-mercaptonicotinic acid in the reaction of PASP-SS-MNA polymers and mucin. Horizontal lines represent the free thiol content of mucin (solid line), and its standard deviation (dashed line) determined by Ellman's assay.

3.3. Characterization of *in situ* hydrogels

PASP-SS-MNA polymers were synthesized to serve as an *in situ* gelling drug delivery system. *In situ* gelling property without an oxidizing agent is ensured by the S-protected and activated polymer side groups. These MNA side groups can react with free thiol containing compounds in a quick thiol-disulfide exchange reaction in aqueous solution at pH 7.4. Hence, by mixing the aqueous solution of PASP-SS-MNA and PASP-SH, intermolecular disulfide bonds are formed without any oxidizing agent, and the low-viscosity polymer mixture turns into a stiff cohesive hydrogel as it is shown in Fig. 3.

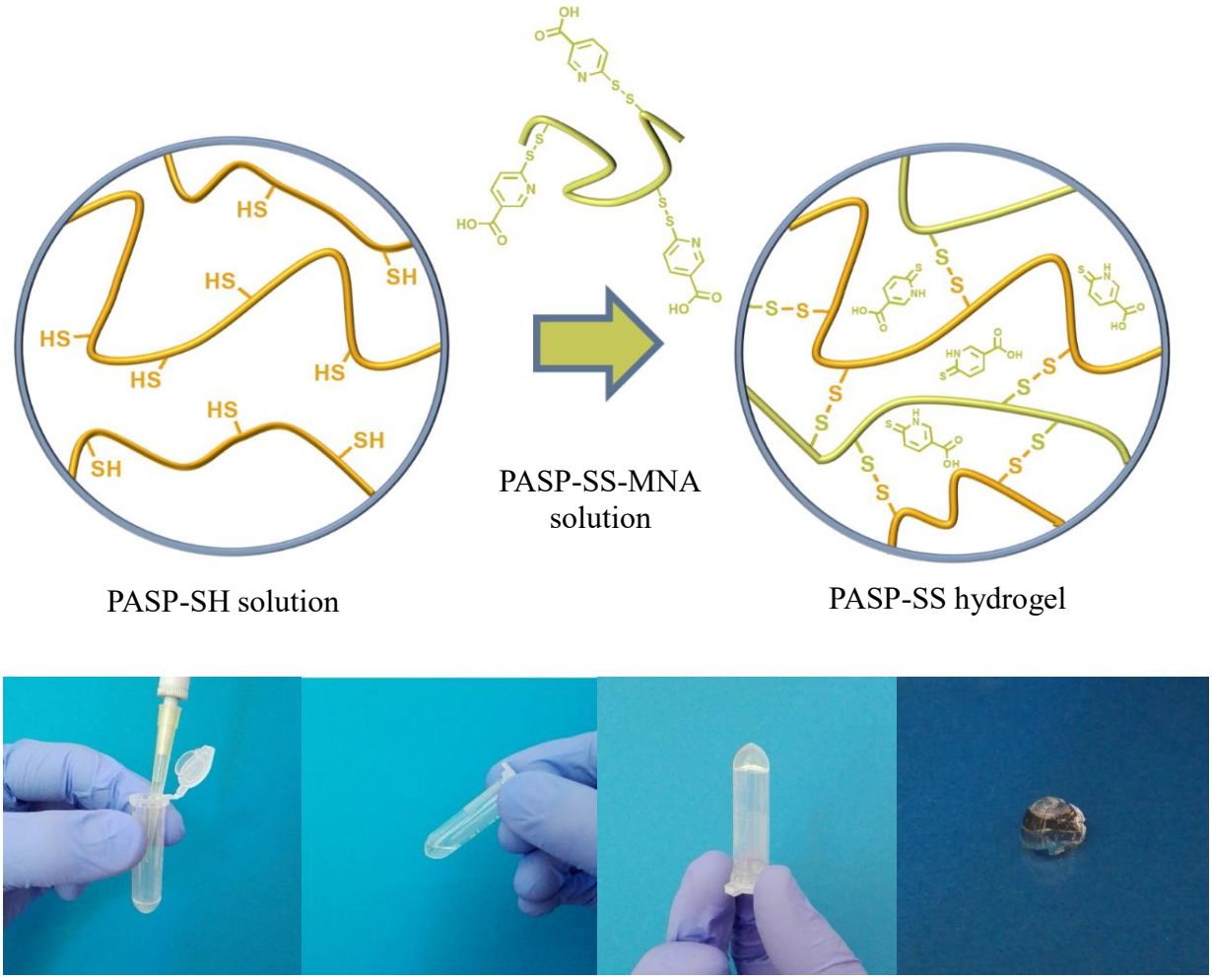


Fig. 3. Cross-linking reaction of thiolated poly(aspartic acid) and S-protected thiolated poly(aspartic acid)

PASP-SS-MNA polymers with three different MNA contents were prepared, and each was reacted with PASP-SH with two different thiol contents ($X_{SH\text{-}Ellman}$) to produce six different hydrogels as it is summarized in Table S2. In principle, three hydrogel samples (MNA242SH178, MNA341SH178 and MNA530SH178) have unreacted MNA groups (MNA:SH molar ratio > 1) while the other three (MNA242SH545, MNA341SH545, MNA530SH545) have free thiol groups in the cross-linked matrix (MNA:SH molar ratio < 1). It was expected that all these compositions produce cohesive hydrogels but they show significantly different properties due to the different MNA:SH molar ratio.

3.3.1. Rheology of gelation

The kinetics of gelation and the viscoelastic properties of the hydrogels listed in Table 2 were monitored by oscillation rheometry. As a representative example, in course of the gelation of MNA242SH545 (Fig. 4a) both storage and loss moduli are low right after mixing, but as the chemical reaction proceeds, storage modulus increases by several orders of magnitude indicating the formation of cross-links, hence an elastic polymer hydrogel. On the frequency sweep tests (Fig. 4b), the storage modulus found to be independent of frequency in the frequency range tested confirming the presence of a stiff, chemically cross-linked network. The nature of the resulting curves was similar in the case of all hydrogel compositions. Gelation time of the compositions was determined as the inflexion point of the time-dependent storage modulus [46] and the stiffness was characterized by the storage modulus determined after 20 min from the frequency sweep curves at 10 rad s^{-1}. In the case of the hydrogel samples which contain MNA groups in excess (MNA242SH178, MNA341SH178, MNA530SH178), the concentration of the netpoints is limited by the same thiol content of each gel (Fig. 4c). This would lead to the formation of hydrogels with similar storage moduli. According to the results, storage modulus values are indeed in the same order of magnitude, however, a slight increase in experienced with increasing MNA content. Gelation time of these gels shortens remarkably with MNA content, because the larger concentration of MNA groups causes higher reaction rate. The larger concentration of MNA leads to increasing storage modulus. In the case of gel samples that contain thiol groups in excess (MNA242SH545, MNA341SH545, MNA530SH545), the reaction rate increases with increasing MNA content (Fig. 4d), as expected, leading to decreasing gelation times, and storage modulus increases due to the larger concentration of netpoints.

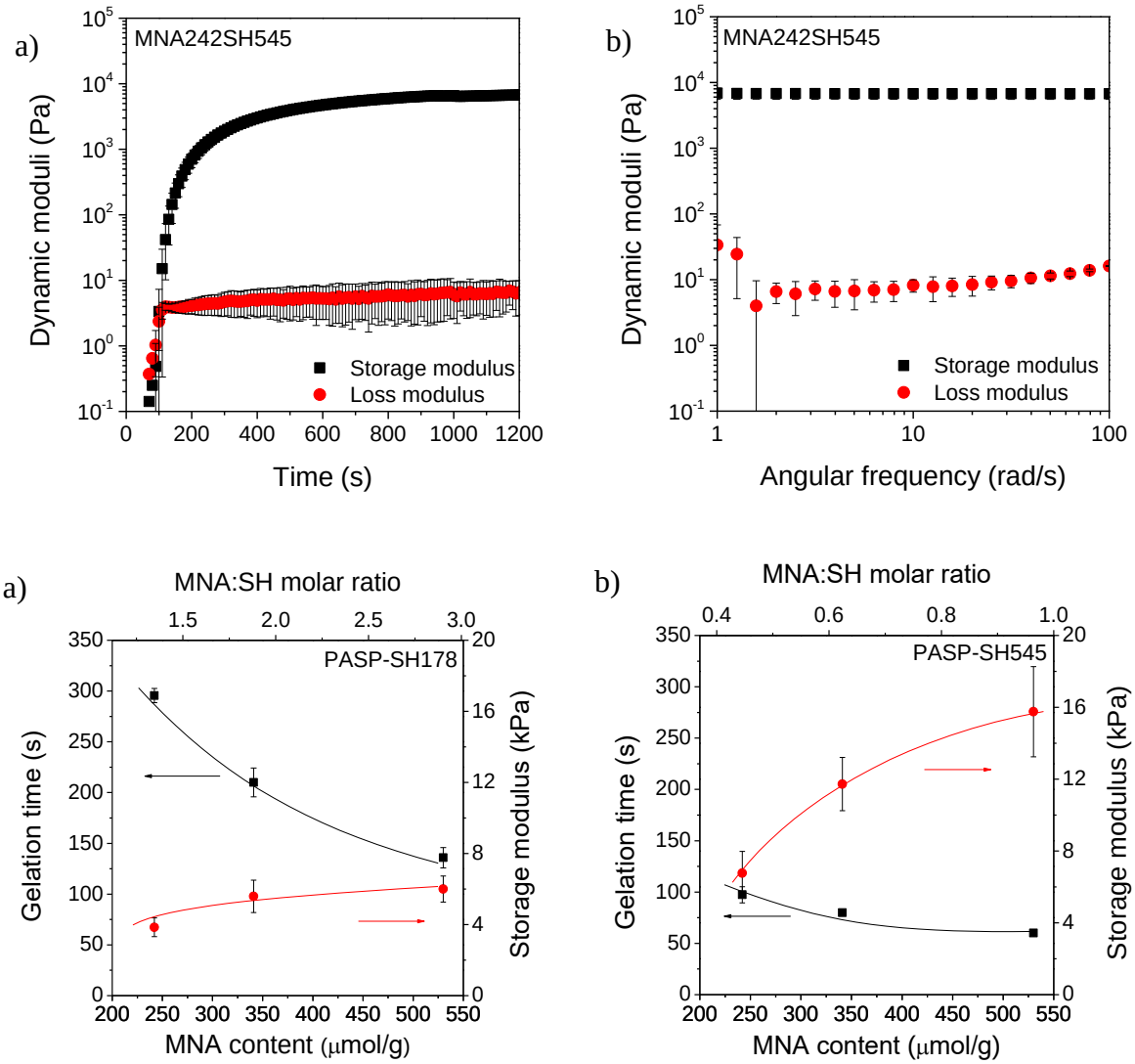


Fig. 4. Dynamic moduli as a function of (a) time (γ = 1%, ω = 10 rad s^{-1}) and (b) angular frequency (γ = 1%) (MNA242SH545, $c_{polymer}$ = 10 wt% in pH = 7.4 PBS). Gelation time of and storage modulus of hydrogel compositions with (c) MNA:SH molar ratio > 1 and (d) MNA:SH molar ratio < 1 as a function of MNA content ($c_{polymer}$ = 10 wt% in pH = 7.4 PBS).

3.3.2. Swelling of the hydrogel samples

The poly(aspartic acid) hydrogels, in general, display pH-dependent swelling owing to the change of protonation of carboxyl groups around their pKa values [49]. First, the possible effect of coupled MNA and thiol groups on the kinetics of swelling was studied in PBS, in which high water uptake is expected due to the deprotonated state of repeating units. Hydrogel samples with an MNA:SH molar ratio > 1 (Fig. 5a) reached their equilibrium degree of swelling within 6 h and they were stable in the buffer solution for at least two

weeks. The degrees of swelling of these samples are approximately the same due to the equal number of netpoints. Hydrogel samples with an MNA:SH molar ratio < 1 (Fig. 5b), show a markedly different kinetics of swelling. The gel with the highest thiol content (MNA242SH545) do not reach swelling equilibrium, but its degree of swelling gradually increased until the gel became so soft that its mass could not been measured and finally dissolved in about 8 h. MNA341SH545 gel shows a similar progress in the initial stage of swelling, but this sample finally reaches an equilibrium degree of swelling after one day. MNA530SH545 gel, which contains only a small amount of free thiol groups, reaches its equilibrium degree of swelling in about 8 h. The kinetics of swelling of these compositions (MNA:SH molar ratio < 1) is in strong correlation with the amount of residual free thiol groups in the samples. In PBS buffer (pH = 7.4) both thiol-disulfide exchange reaction and thiol oxidation occur at atmospheric conditions. We assume that the dissimilar kinetics of swelling of these gels is caused by a series of thiol-disulfide exchange reactions (Fig. 5c).

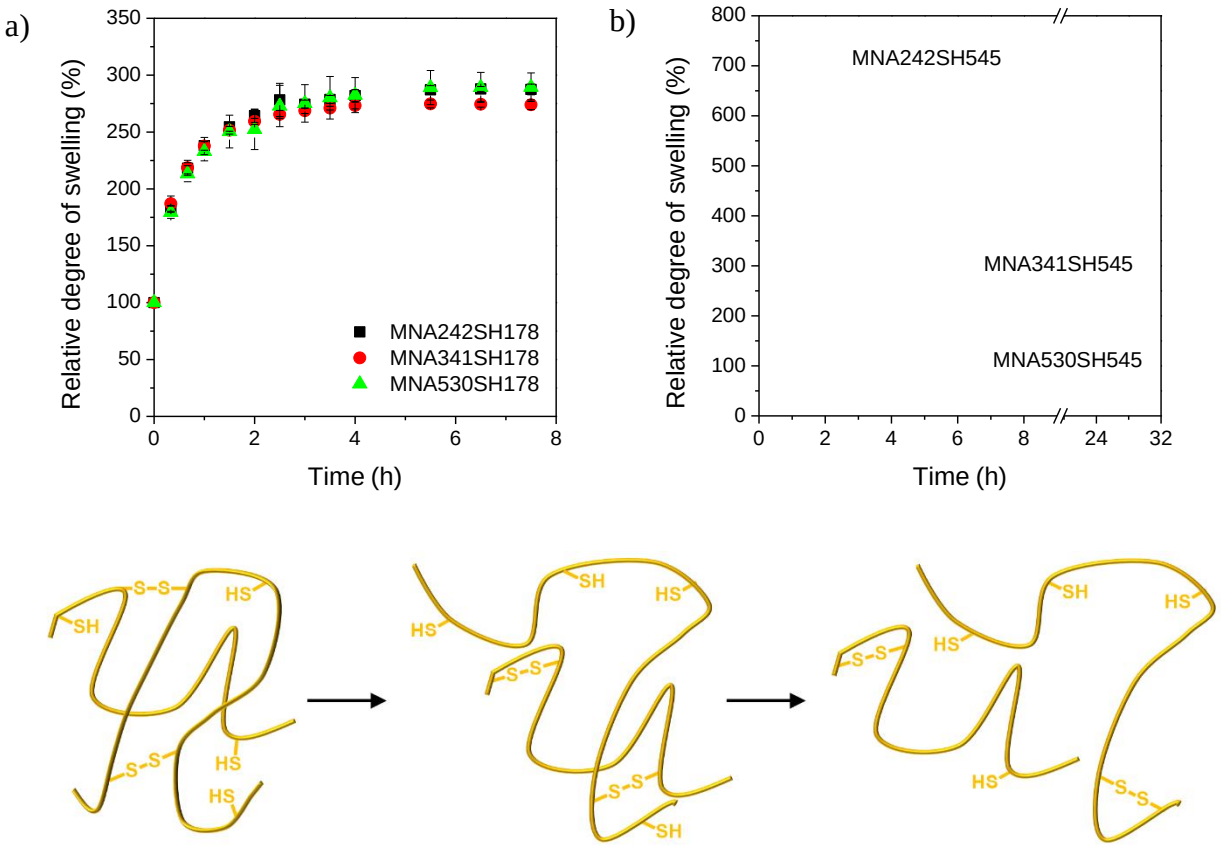


Fig. 5. Kinetics of swelling of hydrogel samples (a) MNA:SH molar ratio > 1 and (b) MNA:SH molar ratio < 1 in pH = 7.4 PBS ($c_{polymer}$ = 10 wt%). (c) Mechanism of the dissolution of hydrogels with a large excess of thiols through a series of thiol-disulfide exchange reactions.

Although the overall amount of disulfide bonds does not change in the process, intermolecular linkages can be converted into intramolecular ones reducing the concentration of chemical bonds between the different polymer chains and also chain entanglements. Ultimately, the concentration of effective netpoints decreases finally resulting in the dissolution of the gel. The entire process is driven by the high solubility of PASP chains in aqueous medium, and the thiol-disulfide exchange reaction rate is high due to the high concentration of thiolate anions at pH = 7.4. As a parallel reaction, a part of the thiol groups can be oxidized by air under the experimental conditions used preventing dissolution of gel samples with lower initial thiol content. To confirm our hypothesis, kinetics of swelling was also measured at pH = 2 in citric acid buffer solution when no thiolate anions are present and thiol-disulfide exchange reaction occurs only to a minor extent (Fig. S2 in the Supplementary Data). It was found that every gel sample reached its equilibrium degree of swelling and was stable. The degree of swelling of every sample at pH = 2 is markedly lower than that of in PBS (pH = 7.4) due to the pH-responsive nature of the poly(aspartic acid) backbone [49]. Below the pK_a values of PASP (3.3 and 4.5 for α and β repeating units, respectively [49]), the COOH pendant groups are in their protonated form making the polymer backbone less hydrophilic, and the gels shrink. Furthermore, kinetics of swelling of a stable gel sample with no residual thiol groups (MNA341SH178) was measured in the presence of 0.01 M acetylcysteine in PBS (Fig. S3 in the Supplementary Data) and it was found that the thiol groups added externally induce the dissolution of the gel and confirms that the dissolution of the gels was induced by cleavage of disulfide bonds.

3.4. *In vitro* cytotoxicity

In our study, MDCK cell line was used to determine the *in vitro* cytotoxicity. This *in vitro* toxicity assay provides a high-throughput cellular cytotoxicity model system to monitor acute toxicity for screening potentially harmful compounds. The cells were treated with thiolated polymers, polymers with activated thiol groups and the MNA leaving group. The *in vitro* cytotoxicity of the compounds were characterized by the MDCK cell viability as shown in Fig. 6. As control samples, DPBS and Triton X-100 were used for 100% and 0% viability, respectively. PASP as a biocompatible polymer [36,40] without thiol- or disulfide-containing functional groups was used as a reference polymer, and exhibited a cell viability close to 100%. Polymers with free thiol groups also showed viability values around 100% regardless of thiol content. The cell viability values for polymers with activated thiol groups varied

between 90 and 100% indicating they are not toxic to MDCK cells. MNA itself did not reduce cell viability indicating the absence of toxic effect when gel is formed by mixing of thiolated and MNA-containing polymers. According to the results of one-way ANOVA, no significant difference is found among the cytotoxicity results of DPBS, MNA and the united groups of the polymers ($p = 0.9190$). Compared to DPBS, cell viability is not significantly altered in the presence of MNA or the polymers (p-values are 0.8730 and 0.8574, respectively).

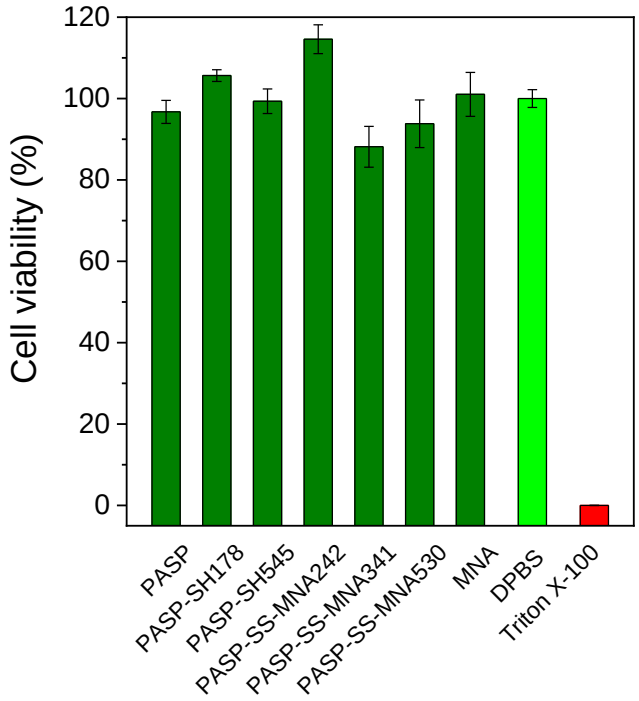


Fig. 6. Cell viability of PASP, thiolated PASP, polymers with activated thiol groups and MNA on MDCK cell line at a concentration of 1 mg/ml

3.5. Release of ofloxacin

Application of an *in situ* gelling mucoadhesive dosage form enables prolonged drug release as the residence time is increased. Drug release measurements were conducted to determine the release profile of the *in situ* PASP hydrogels and the effect of a cohesive chemical hydrogel matrix on the rate of the drug diffusion. Hydrogels were prepared *in situ* (compositions can be found in Table 2) on the donor side of a Franz-diffusion cell by mixing PASP-SS-MNA and PASP-SH solutions containing ofloxacin. The release of ofloxacin from a commercially available eye drop was used as reference. According to Fig. 7 and Table S3, the release of ofloxacin from the eye drop is substantially faster than that of from the hydrogels. In the case

of the eye drop 90% of the ofloxacin diffused to the acceptor side in only 2 h, while the hydrogels only released less than 60%. We can conclude that the presence of the hydrogel matrix slowed the diffusion of the drug, thus a sustained release profile was obtained. Statistical analysis confirmed that the reference significantly differs from the hydrogels in the first 12 h of the experiment ($p < 0.001$ for each pairwise comparisons). The calculations also show that the difference between the hydrogel formulations is not significant ($p > 0.9999$ for all pairwise comparisons) meaning that the different cross-linking densities or the presence of either MNA or free thiol groups (thus structural differences) in the hydrogels do not affect the release profile.

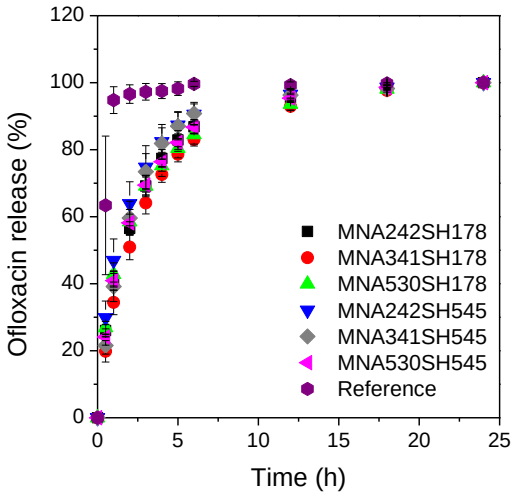


Fig. 7. Release profile of ofloxacin from *in situ* forming PASP hydrogels ($c_{polymer}$ = 10 wt%, in pH = 7.4 PBS, T = 35 °C).

4. Conclusions

In this study, a synthesis method of S-protected and preactivated thiolated poly(aspartic acid) (PASP-SS-MNA) was developed. PASP polymers with a large amount of S-protected moieties (up to 530 μmol/g) were prepared by coupling thiolated poly(aspartic acid) with 6-mercaptonicotinic acid group through thiol-disulfide exchange reaction. It was shown that because of the S-protected moieties, the polymer forms covalent disulfide bonds with the thiol groups of mucin glycoproteins indicating mucoadhesive properties. The PASP-SS-MNA can be potentially used as an *in situ* gelling ophthalmic drug delivery system, as its low viscosity aqueous solution forms stiff, cohesive hydrogels upon mixing with PASP-SH due to the disulfide formation between the polymers without the addition of any oxidizing agent.

Gelation time and stiffness of the hydrogels can be controlled by the degree of modification of the polymers. Biological safety of the polymers and 6-mercaptonicotinic acid was assessed on MDCK cell line and no harmful effects were found. The cohesive hydrogel matrix ensured the sustained release of ofloxacin compared to a liquid formulation. Hence, the two component *in situ* gelling system of PASP-SS-MNA and PASP-SH has the potential for application in ophthalmic drug delivery.

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