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Poly(aspartic acid) with adjustable pH-dependent solubility

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ABSTRACT

Poly(aspartic acid) (PASP) derivatives with adjustable pH-dependent solubility were synthesized and characterized to establish the relationship between their structure and solubility in order to predict their applicability as a basic material for enteric coatings. Polysuccinimide, the precursor of PASP, was modified with short chain alkylamines, and the residual succinimide rings were subsequently opened to prepare the corresponding PASP derivatives. Study of the effect of the type and concentration of the side groups on the pH-dependent solubility of PASP showed that solubility can be adjusted by proper selection of the chemical structure. The Henderson–Hasselbalch (HH) and the extended HH equations were used to describe the pH-dependent solubility of the polymers quantitatively. The estimate provided by the HH equation is poor, but an accurate description of the pH-dependent solubility can be found with the extended HH equation. The dissolution rate of a polymer film prepared from a selected PASP derivative was determined by fluorescence marking. The film dissolved rapidly when the pH was increased above its pK_a. Cellular viability tests show that PASP derivatives are non-toxic to a human cell line. These polymers are thus of great interest as starting materials for enteric coatings.

Statement of Significance

Poly(amino acid) type biocompatible polymers were synthesized for future use as pharmaceutical film coatings. To this end, we tailored the pH-dependent solubility of poly(aspartic acid) (PASP). It was found that both the solubility and the pK_a values of the modified PASP depended strongly on composition. Fluorescent marking was used to characterize the dissolution of a chosen PASP derivative. In acidic media only a negligible amount of the polymer dissolved, but dissolution was very fast and complete at the pH values that prevail in the small intestine. As a consequence, enteric coatings based on such PASP derivatives may be used for drug delivery in the gastrointestinal tract.

1. Introduction

Use of conventional solid formulations can not only cause various side effects but also limits the bioavailability of drugs owing to the inability of these carriers to ensure targeted release of the active molecule in the gastrointestinal (GI) system [1]. Liberation of orally administrated drugs at the desired levels of the GI tract can be achieved by employing enteric tablet coatings based on pH-responsive anionic polymers [2,3]. Enteric coatings must be protective at the acidic pH of the stomach while dissolving easily at the elevated pH of the intestines. These coatings are generally made from polycarboxylic acids. Aqueous solubility of these polymers depends strongly on the pH because of the deprotonation of the dissociable groups above a well-defined pH [4].

Although, several polymers of natural origin, e.g. zein, shellac [5], cellulose acetate succinate [6], hydroxypropyl methylcellulose phthalate [7], hypromellose acetate succinate [8] and cellulose acetate trimellitate [9], are commonly used in enteric coatings, synthetic polyacrylates play a leading role in the market [1,10]. The reason for their extensive use is that their pH-dependent solubility can be controlled by the copolymerization of (meth)acrylic acids and properly chosen (meth)acrylic esters [11]. In contrast to polyacrylates, the solubility of polymers of natural origin cannot be adjusted precisely, because the functionalization of these polymers is complicated [12]. The disadvantage of polyacrylates is their relatively complex synthesis, which often requires toxic and environmentally harmful reagents (azobisisobutyronitrile, transition-metal activators such as copper, iron, or manganese, BuLi/pyridine,

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$Al(alkyl)_3/TiCl_3$, etc.) [13,14]. Furthermore, polyacrylates are not in general biodegradable [13]. However, in view of the enormous number of drug formulations currently in use, the need for biodegradable poly(carboxylic acid)s is pressing.

Poly(aspartic acid) (PASP) is a biopolymer that is a potential alternative of polyacrylates in enteric coatings [15]. The precursor polymer of PASP, polysuccinimide (PSI) reacts easily with primary amines, and for this reason PASP derivatives can be synthesized under mild reaction conditions with large chemical versatility [16–23]. Owing to the polyamide backbone, PASP derivatives are expected to be biocompatible and biodegradable [24,25]. The solubility of polyacrylates can be modified by introducing alkyl side groups [26], and we may therefore assume that the pH-dependent solubility of PASP can be controlled in a similar manner. Philippova et al. [27] found that swelling of polyacrylic acid gels could also be shifted towards higher pH values by using octyl, dodecyl, or octadecyl side groups in the repeating units. The pH-dependent solubility of PASP derivatives in water has not yet been investigated, but a few examples have been reported for the preparation of PASP containing alkyl and alkylaminoalkyl or hydroxyalkyl side groups [28–31]. Most of these polymers display temperature dependent solubility and/or gelation of the aqueous solution. Introduction of long alkyl side groups into the PASP chain (e.g. hexyl or hexadecyl) resulted in hydrophobic association of these groups and formation of micelles [32], liposomes [33] or nanoparticles [34,35]. Furthermore, the biodegradability of PASP is preserved with these modifications [36].

Despite the chemical versatility of PASP, it has not been used for the preparation of polymers with controllable aqueous solubility, which is the crucial requirement for enteric coatings. Accordingly, the present study focuses on the determination of the relationship between the structure and the solubility of PASP derivatives with a view to its possible application as an enteric coating. We synthesized PASP polymers substituted with short alkyl chains to obtain polymers with controllable pH-dependent solubility and dissolution rate. The Henderson–Hasselbalch (HH) [37] and the extended Henderson–Hasselbalch equations [38] were applied to obtain a quantitative description of the pH-dependence of the solubility of PASP polymers and to estimate their pK_a. The pH dependence of the dissolution rate and cytotoxicity were also determined to prove the potential of PASP derivatives as enteric coatings in controlled drug delivery in the GI tract.

2. Experimental

2.1. Materials

Imidazole (puriss), phosphoric acid (85%), L-tryptophan methyl ester hydrochloride (T), triethyl citrate and d_6-DMSO were purchased from Sigma–Aldrich. L-aspartic acid (99%), *n*-butylamine (B, 99%), dibutylamine (DBA, 99%), potassium chloride (99.5%), disodium hydrogen phosphate (a.r.), potassium dihydrogen phosphate (a.r.) and dimethyl sulfoxide (DMSO, 99.9%) were bought from Merck. Hydrochloric acid (HCl, 35%) was purchased from LachNer. Isopropyl-alcohol (99.9%), *n*-hexylamine (H, 99%) and sodium hydroxide (NaOH, a.r.) were bought from Reanal. DMEM cell culture medium, fetal bovine serum (FBS), L-glutamine, penicillin/streptomycin (PAA Laboratories), trypsin-EDTA solution (Sigma–Aldrich), MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Promega) were used for cytotoxicity tests. The water used for all reactions and the preparation of aqueous solutions was MilliQ grade from a water purification facility (Millipore Milli-Q Gradient, $\rho > 18.2\ \Omega m$). All reagents and solvents were used without further purification. Experiments were done at 25 °C unless otherwise indicated.

Buffer solutions for solubility measurements consisted of imidazole (pH = 8, c = 0.15 M) and their pH was adjusted by adding 1 M HCl. The ionic strength of the solutions was adjusted to the desired value by adding KCl (I = 0.15 M). The gastric fluid was simulated with an HCl solution (pH = 1.2) and the intestinal fluid by a phosphate buffer (PBS, pH = 6.8). The pH of the buffer solutions was checked with a pH/ion analyzer (Radelkis OP271/1).

2.2. Synthesis

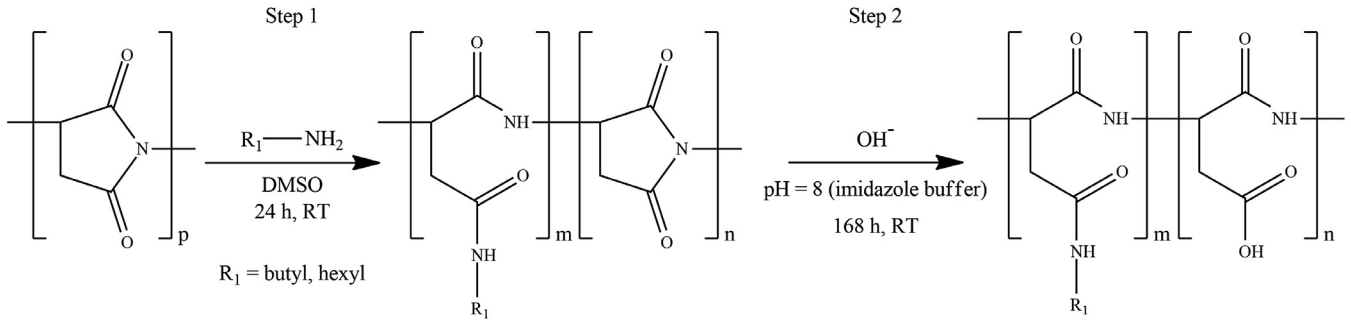
Polysuccinimide (PSI) was synthesized by thermal polycondensation of L-aspartic acid in a mixture of mesitylene and sulpholane at 160 °C (7 h) in the presence of 3 wt% phosphoric acid. PSI was purified by precipitation with DMF-H_2O (1:3) and dried in vacuum at 25 °C for 24 h.

^{1}H NMR of PSI (500 MHz, DMSO-d_6, δ) [ppm]: 5.12 (1H, CO-*CH*-CH_2-CO); 3.22 and 2.74 (2H, CO-CH-*CH_2*-CO). Average molecular weight of polysuccinimide (M_{PSI}) was determined by viscosimetry using a rolling ball viscometer (Anton Paar Lovis 2000). M_{PSI} was calculated from the intrinsic viscosity data using the Kuhn–Mark–Houwink equation. Constants for PSI ($K = 1.32 \times 10^{-1}$ and $\alpha = 0.76$) were taken from the literature [39]. M_{PSI} was determined to be 27.5 ± 0.2 kDa.

PSI was functionalized with alkyl side groups by the nucleophilic addition of *n*-butylamine or *n*-hexylamine onto the imide groups of the polymer chain (Scheme 1, Step 1). A typical procedure was as follows (e.g. PSI modified with 62.5 mol% *n*-hexylamine): 2.000 g of PSI (20.62 mmol succinimide unit) was dissolved in 18.0 g of DMSO and then 1.304 g (12.89 mmol) *n*-hexylamine was added dropwise to the PSI solution. The mixture was stirred at room temperature for 24 h and the polymer was precipitated by adding 50 ml of 10^{-4} M HCl. The filtered precipitate was washed with MilliQ water to reach neutral pH and dried under vacuum at 25 °C for 24 h. Finally, a white solid was obtained (average yield was between 75% and 85%). The polymers were stored at room temperature. ^{1}H NMR of PSI with 62.5 mol% *n*-butyl side group (500 MHz, d_6-DMSO, δ) [ppm]: 5.03 (1H, CO-*CH*-CH_2-CO, *f*), 4.55 (1H, CO-*CH*-CH_2-CO, *e*), 3.02 (1H, CO-CH-*CH_2*-CO, *g;* 2H, NH-*CH_2*-CH_2-CH_2-CH_3, *c*), 2.74 (1H, CO-CH-*CH_2*-CO, *g*), 2.62 (2H, CO-CH-*CH_2*-CO, *d*), 1.35 (2H, NH-CH_2-*CH_2*-CH_2-CH_3, *b′*), 1.24 (2H, NH-CH_2-CH_2-*CH_2*-CH_3, *b*), 0.85 (3H, NH-CH_2-CH_2-CH_2-*CH_3*, *a*) [Fig. 1]. ^{1}H NMR of PSI with 62.5 mol% *n*-hexyl side group (500 MHz, d_6-DMSO, δ) [ppm]: 5.02 (1H, CO-*CH*-CH_2-CO, *f*), 4.52 (1H, CO-*CH*-CH_2-CO, *e*), 3.01 (1H, CO-CH-*CH_2*-CO, *g;* 2H, NH-*CH_2*-CH_2-CH_2-CH_2-CH_3, *c*), 2.74 (1H, CO-CH-*CH_2*-CO, *g*), 2.62 (2H, CO-CH-*CH_2*-CO, *d*), 1.36 (2H, NH-CH_2-*CH_2*-CH_2-CH_2-CH_3, *b′*), 1.23 (6H, NH-CH_2-CH_2-*CH_2-CH_2-CH_2*-CH_3, *b,b″,b‴*), 0.85 (3H, NH-CH_2-CH_2-CH_2-CH_2-CH_2-*CH_3*, *a*) [Fig. 1].

Fluorescent marking was used to characterize the dissolution rate of the polymers. At first, PSI was modified with the methyl ester of a fluorescent amino acid, L-tryptophan. 1.940 g PSI (20.00 mmol succinimide unit) and 0.051 g (0.20 mmol) L-tryptophan methyl ester hydrochloride were dissolved in 10.0 g DMSO. When dissolution was complete, 1.0 g of 4 wt% H_3PO_4/DMSO solution and 0.10 g of (0.77 mmol) DBA were added dropwise, and the mixture was stirred for 48 h. Further steps were carried out in the same way as in the case of non-marked polymers.

PASP derivatives were prepared by opening the residual succinimide rings of the modified PSI polymers (Scheme 1, Step 2). 2.50 g of modified PSI was added to 1000 cm^3 of pH = 8 buffer (imidazole, I = 0.15 M) and the solution was stirred for 168 h. Modified PASP was precipitated by adding 300 ml of 10^{-4} M HCl, the precipitate was filtered and washed with 2 × 10 ml water, then dried under vacuum for 24 h (a slightly yellow solid was obtained, ~80%).



Scheme 1. Synthesis of alkyl side group decorated poly(aspartic acid).

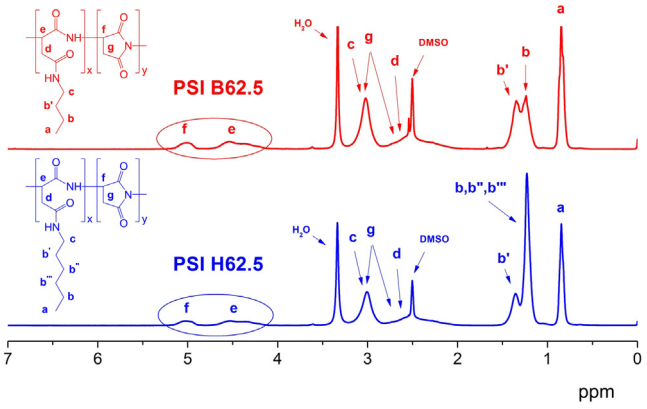


Fig. 1. ^{1}H NMR spectra of PSI with 62.5 mol% *n*-butyl (PSI B62.5) and *n*-hexyl (PSI H62.5) side group.

^{1}H NMR of PASP with 62.5 mol% *n*-butyl side group (500 MHz, d_6-DMSO, δ) [ppm]: 4.53 (1H, CO-*CH*-CH_2-CO, *e,e′*), 3.03 (2H, NH-*CH_2*-CH_2-CH_2-CH_3, *c*), 2.62 (2H, CO-CH-*CH_2*-CO, *d, d′*), 1.35 (2H, NH-CH_2-*CH_2*-CH_2-CH_3, *b′*), 1.24 (2H, NH-CH_2-CH_2-*CH_2*-CH_3, *b*), 0.85 (3H, NH-CH_2-CH_2-CH_2-*CH_3*, *a*). ^{1}H NMR of PASP with 62.5 mol% *n*-hexyl side group (500 MHz, d_6-DMSO, δ) [ppm]: 4.52 (1H, CO-*CH*-CH_2-CO, *e,e′*), 3.01 (2H, NH-*CH_2*-CH_2-CH_2-CH_2-CH_3, *c*), 2.62 (2H, CO-CH-*CH_2*-CO, *d, d′*), 1.37 (2H, NH-CH_2-*CH_2*-CH_2-CH_2-CH_3, *b′*), 1.23 (6H, NH-CH_2-CH_2-*CH_2-CH_2-CH_2*-CH_3, *b,b″,b‴*), 0.84 (3H, NH-CH_2-CH_2-CH_2-CH_2-CH_2-*CH_3*, *a*).

2.2.1. Abbreviations

We refer to the type and the concentration of the side groups in the abbreviations of the synthesized polymers throughout the paper as follows

- In the case of *PSI H50* PSI was modified with 50 mol% *n*-hexylamine (H).
- In the case of *PASP H50* PSI was modified with 50 mol% *n*-hexylamine (H) in and the residual succinimide rings were subsequently opened to form aspartic acid.
- In the case of *PASP T1H50* at first PSI was modified with 1 mol%, L-tryptophan methyl ester (T) then with 50 mol% *n*-hexylamine (H), and the residual succinimide rings were subsequently opened to form aspartic acid.

2.3. Characterization

2.3.1. Chemical structure

^{1}H NMR spectra of the polymers were recorded using a Bruker 500 MHz spectrometer with 128 scans in DMSO-d_6 (3 wt% polymer solution). TMS (0.03 V/V%) was used as internal standard. FTIR spectra were recorded using a Bruker Tensor 27 FTIR spectrometer on KBr pellets pressed (~1.5 mg polymer/250 mg KBr). 128 scans were recorded from 4000 to 400 cm^{-1} with a resolution of 2 cm^{-1} for each sample.

2.3.2. pH-dependent solubility

pH-dependent solubility of the polymers was investigated by acid-base titration. Supersaturated solutions of the PASP derivatives were prepared in imidazole buffer (pH = 8, I = 0.15 M) by hydrolysis of the corresponding PSI (168 h, 25 °C). The supernatant used as stock solution was removed after sedimentation. Different dilutions were prepared and used as analyte during the titration. The volume of analyte was 10 cm^3 for each measurement. 0.15 M HCl was used as a titrant and dosed by a Methrom Titrando 808 Dosing Unit (25 ± 2 °C). The endpoint was reached when the transparent solution became opaque. Solubility at a given pH (*S* [mg/100 g]) was determined as:

$$S = \frac{S_{max} \times V_0}{V_0 + V_b + V_t} \quad (1)$$

where S_{max} is the concentration of the polymer in the stock solution (concentration of the saturated solution at pH = 8), V_0 is the volume of the stock solution (cm^3), V_b is the volume of the diluent buffer (cm^3) and V_t is the volume of the titrant (cm^3). The polymer content of the stock solution was determined by the evaporating the solution to dryness. The solid content was corrected for the mass of buffer components.

2.3.3. Dissolution rate of polymers

The polymer of chosen composition (PASP T1H50) was dissolved in isopropyl alcohol at a concentration of 20 wt% and triethyl-citrate was used as plasticizer (20 wt% for the polymer). Subsequently an Erichsen BRID 284 film applicator (applied gap height 200 μm) was used to cast a polymer film from the solution onto a glass plate of width 24 mm. Prior to testing, the coated plate was dried at room temperature for 48 h. The thickness of the coating was measured with a micrometer.

The coated plate was immersed in 20 ml HCl (pH = 1.2, I = 0.15 M) for 120 min, then transferred into 20 ml of PBS (pH = 6.8, I = 0.15 M) and kept there for 6 h (T = 37 °C). The measurement was performed in a continuously stirred vessel (200 rpm). Samples of 2 ml were taken at predetermined time intervals, and replaced with 2 ml fresh buffer. The amount of dissolved PASP T1H50 was determined by fluorescence spectroscopy (Perkin Elmer LS55 fluorescence spectrometer; sample volume: 2 ml; λ_{ext} = 270 nm; λ_{em} = 344 nm).

2.3.4. Cell viability study

PC-3 human prostate cancer cell line was obtained from ATCC. PC-3 cells were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 μg/ml streptomycin and 100 U/ml

penicillin at 37 °C in humidified air atmosphere with 5% CO_2. The cytotoxicity of the PASP and PASP H50 polymers was evaluated by using MTT test performed as follows. Cells were collected from the culture flask by treating with trypsin-EDTA solution, then seeded in a 96-well plate at a density of 1000 cells per well in DMEM and cultured overnight. Aqueous polymer solutions with a starting concentration of 10 mg/ml were prepared and sterilized using a syringe filter (pore size: 0.2 μm). The culture medium in plate was replaced with fresh one and the polymer solutions were added to cells at final concentration 0–200 μg/ml. Sterile water was added instead of polymers as control.

Cells were cultured in the presence of polymers in standard conditions for 72 h, then the medium was replaced by the fresh one containing MTT reagent at a concentration of 0.5 mg/ml. Cells were additionally cultured for 3 h to allow them to reduce MTT into water insoluble product (formazan) followed by the medium discarding and formazan dissolution with 100 μl of DMSO per well. The absorbance of formazan solution in each well, which is proportional to the number of viable cells, was measured using an Infinite M200 PRO microplate analyzer (Tecan) at wavelength 555 nm. Cell viability was calculated as a percentage of control cells grown without polymers (100% viability).

2.3.5. Statistical analysis

Cellular viability, solubility and dissolution data were expressed as means ± standard error of triplicate samples. The statistically significant difference was evaluated by Student's *t*-test with a significance level of $p < 0.05$. The average error of ^{1}H NMR measurements was ±2%.

3. Results and discussion

3.1. Characterization

The modification of PSI by primary amines followed by the alkaline ring-opening of succinimide rings enabled us to synthesize a family of PASP derivatives with alkyl side groups. The idea that the pH-dependent solubility of the polymers can be controlled by the type and concentration of side groups, i.e., the structure-property correlations, was widely investigated in the case of these PASP derivatives.

PASP derivatives were synthesized from their corresponding PSI derivatives. It would also have been possible to use PASP as starting material, but the modification of carboxyl groups requires a more complex synthetic pathway, e.g., amide formation mediated by water-soluble carbodiimides, or the reaction of alkyl halogenides with the deprotonated form of the carboxyl acid group [34]. The reaction pathway chosen in this work can be performed at room temperature without any catalyst. The chemical structure of the resulting PSI and PASP derivatives was confirmed by ^{1}H NMR and FTIR spectroscopy (details in Supplementary Data).

Fig. 1 shows the ^{1}H NMR spectra of PSI derivatives containing *n*-butyl (B62.5 PSI), and *n*-hexyl (H62.5 PSI) side groups. The methylene peaks (g) of succinimide repeating unit appear at ~2.74 and ~3.02 ppm as inequivalent methylene protons. The broad peak at ~5.02 ppm (f) corresponds to the methyne proton of the succinimide ring. The characteristic peaks of the *n*-butyl [Fig. 1, PSI B62.5: 0.85 (a), 1.24 (b), 1.35 (b′) and 3.02 ppm (c)] and the *n*-hexyl group [Fig. 1, PSI H62.5: 0.85 (a), 1.23 (b, b″, b‴), 1.36 (b′) and 3.01 ppm (c)] are present in the spectra. The signal at ~4.52 ppm (e) is assigned to the methyne proton of the *N*-alkyl aspartamide units after the ring opening reaction. The degree of modification (the molar ratio of alkyl side groups to the repeating units, X_{calc}) was calculated from the integrated intensity of the peaks assigned to methyne protons of the succinimide unit (5.02 ppm, f) and to that of the methyne protons of the opened aspartic acid unit (4.52 ppm, e). Good agreement was found between X_{calc} and the feed composition (feed ratio of alkylamine molecules to succinimide units, X_{feed}) (Fig. 2). These results reveal that conversion of the reaction was complete in each case.

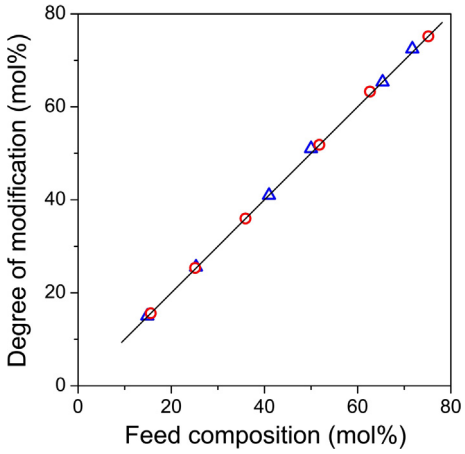


Fig. 2. Relationship between feed composition and the calculated degree of modification of the PSI derivatives containing (○) *n*-butyl or (△) *n*-hexyl side groups.

Fig. 3 shows the ^{1}H NMR spectra of PASP derivatives with *n*-butyl (PASP B62.5) and, *n*-hexyl (PASP H62.5) side groups. After the ring opening of succinimide groups, the methylene protons of the repeating units became equivalent (one signal at ~2.62 ppm (d, d′)). The broad peak at ~4.52 ppm (e, e′) corresponds to the methyne proton of the aspartic acid and the aspartamide repeating units. The broad peak at ~5.02 ppm (f) – corresponding to the methyne proton in succinimide rings (Fig. 1) – disappeared from the spectra of the PASP derivatives, indicating that ring opening of the succinimide units was complete. The degree of modification of PASP derivatives was calculated by comparing the integrated area of the peak at ~0.85 ppm (a) assigned to the methyl protons of the alkyl groups with the peak at ~4.52 ppm (d, d′), which belongs to the methyne protons of the aspartic acid and aspartamide repeating units. The results were identical to that of the corresponding PSI derivatives. This indicates that the hydrolysis triggered only the opening of the succinimide rings, but had no effect on the amide bonds of the aspartamide repeating units, thus demonstrating the hydrolytic stability of the side groups of each polymer (e.g. PSI B62.5: X_{calc} = 64%, PASP B62.5: X_{calc} = 63%; PSI H62.5: X_{calc} = 65%, PASP H62.5: X_{calc} = 65%).

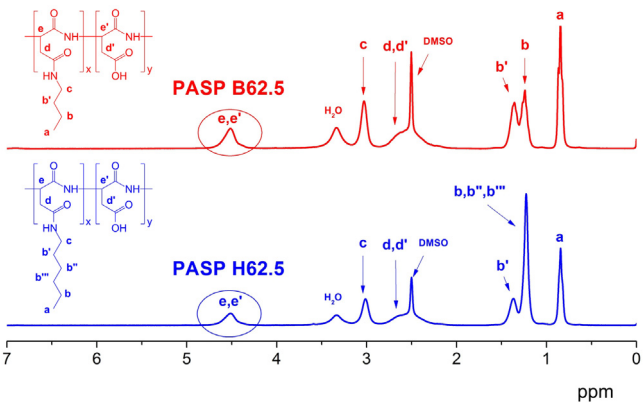


Fig. 3. ^{1}H NMR spectra of PASP derivatives with 62.5 mol% *n*-butyl (PASP B62.5) and *n*-hexyl (PASP H62.5) side group.

3.2. pH-dependent solubility of PASP derivatives with alkyl side groups

The solubility of polyelectrolytes depends strongly on pH owing to the presence of ionizable side groups. The carboxylic groups of polycarboxylic acids are deprotonated if the pH is above their pK_a value, resulting in increased solubility. Below the pK_a, the carboxyl groups are protonated and the solubility of the polycarboxylic acids is reduced due to the non-ionized state of the carboxyl groups. The solubility of PASP is excellent in the entire pH range, even in its protonated form (S > 10,000 mg/100 g at pH = 4). According to our hypothesis, the maximum solubility of the saturated solution of PASP at pH = 8, S_{max} is reduced by the introduction of alkyl side groups into the polymer.

The aqueous solubility of PASP derivatives was found to be much smaller than that of PASP (Fig. 4) and their solubility is quite limited even at alkaline pH (pH = 8). This moderate solubility of the PASP derivatives can be attributed to the incorporation of hydrophobic side groups. S_{max} changes by almost an order of magnitude on increasing the concentration of *n*-butyl side groups from 37.5 to 75 mol% and *n*-hexyl side groups from 25 to 62.5 mol%. The close correlation between solubility and the degree of modification enables us to synthesize PASP derivatives with controlled S_{max}.

Since the use of these polymers as enteric coatings requires adjustable pH-dependent solubility, the solubility was determined as a function of pH at each of the various compositions. A clear relationship was observed between the obtained solubility curves and the composition (Fig. 5). The maximum solubility decreases as the degree of modification increases, as shown also in Fig. 4. For each polymer a considerable change in solubility occurs over a narrow pH range (less than 2 units). The chemical structure of the polymers has a well-defined effect on the position of this significant transition. It shifts towards larger pH values with increasing degree of modification which can be explained by the presence of a hydrophobic molecular environment. The introduced alkyl side groups hinder the ionization of carboxylic acid groups which results in a decrease in their acidity [40]. Furthermore, the increase in degree of modification reduces the solubility of the polymers in water, even in their deprotonated form. Thus a higher degree of ionization is required to achieve the same solubility for different polymers. These two effects together result in the distinct solubility profiles. The solubility profiles are basically determined by the length of the side groups. Introduction of *n*-hexyl side groups instead of *n*-butyl groups shifts of the characteristic pH–range towards higher pH values and reduces the solubility at a given degree of modification. These results are consistent with data obtained for modified linear polyacids [41], and poly(acrylic acid) gels modified by alkyl side groups [27].

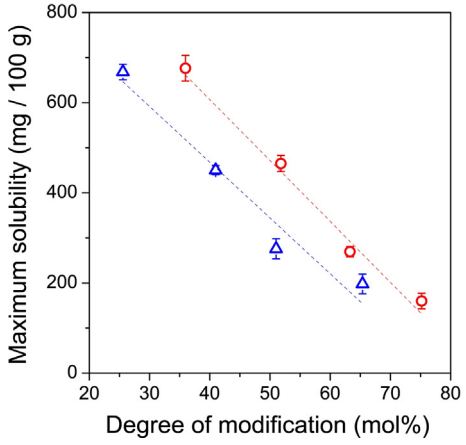


Fig. 4. Correlation between the degree of modification and the maximum solubility of alkylamine modified PASP derivatives containing (○) *n*-butyl or (△) *n*-hexyl side groups at pH = 8.

Although we proved that the pH-dependent solubility of PASP derivatives can be controlled by the type and concentration of the side groups, knowledge of a general relationship between the solubility profile and the chemical composition of the polymers would help enormously the efficient design of polymers with tailor-made aqueous solubility. We tested different models to describe the pH-dependent solubility of PASP derivatives.

For a carboxylic acid with a single ionizable group, the HendersonHasselbalch (HH) equation can be used to describe the pH-dependent solubility and to determine the pK_a value. With a modified HH equation the pK_a can be determined from the solubility data. If the solubility of the protonated form of the acid (S_{min}, intrinsic solubility) is much smaller than the solubility at a given pH (at least by two orders of magnitude), then S_{min} determines the solubility (S) of the acid in a wide pH range (Eq. (2)) [37]:

$$pH = pK_{a,HH} + \log\left(\frac{S - S_{min}}{S_{min}}\right) \quad (2)$$

where $pK_{a,HH}$ refers to the value of pK_a calculated from the model and corresponds to the point of inflection of the solubility profile. In Eq. (2) S is the solubility of polymer at a given pH and S_{min} is its intrinsic solubility, i.e. the solubility at a pH of which it is in its fully protonated form (in this study S_{min} is assigned to the smallest solubility of the polymer that can be determined by titration). The situation is more complex in the case of polyacids. Since the

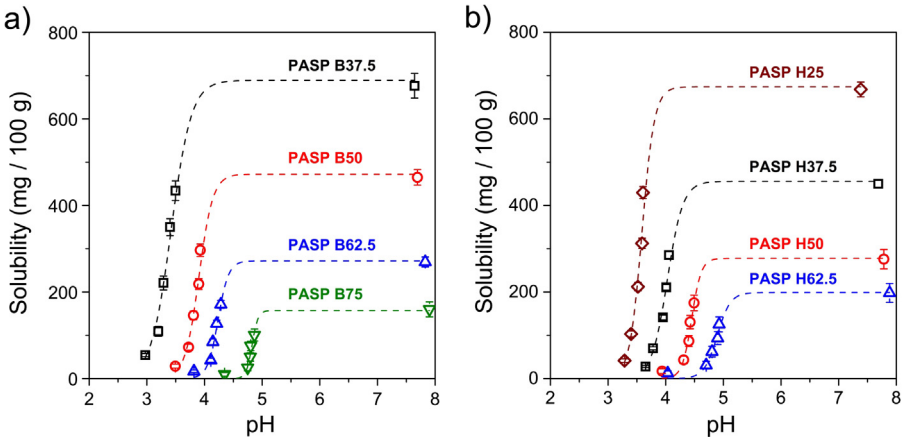


Fig. 5. pH-dependent solubility of PASP derivatives with different amounts of a) *n*-butyl and b) *n*-hexyl side groups. (Dashed curves are guides for the eye.)

dissociation of each ionisable group is affected by its neighbouring groups, the number of pK_a values is equal to the number of repeating units. The pK_a value depends on the degree of dissociation because this latter is inhibited by the increasing charge of the polyelectrolyte and particularly by the presence of neighbouring ionized groups. Thus, pK_a increases with increasing degree of dissociation and the HH equation must accordingly be extended to describe this change in pK_a (Eq. (3)) [38]:

$$pH = pK_{a,eHH} + n\log\left(\frac{S - S_{\min}}{S_{\min}}\right) \quad (3)$$

where n depends on the chemical composition of the polymer and on the ionic strength, while $pK_{a,eHH}$ is an apparent pK_a. This model takes into account the pH dependence of pK_a, but $pK_{a,eHH}$ does not necessarily coincide with pK_a at dissociation degree 50% – determined by potentiometric titration – because the value obtained at this point corresponds to the inflection of the solubility profile, which can be at different degrees of dissociation in the case of different polymer compositions as investigated by Bae et al. [42]. In our case, however, knowledge of apparent pK_a values is more important in order to be able to prepare polymers with tuneable pH-dependent solubility.

The HH equation gives accurate result only for molecules of low molecular weight and for pK_a values between 5 and 9 [37]. As described above, the extended HH equation gives a much better approximation in modelling the solubility of polyacids, and thus we can use this to describe the pH-dependent solubility of the synthesized PASP derivatives. However, there is a limitation of Henderson-Hasselbalch type equations, namely that these models are valid for compounds for which the solubility of the ionized form is much larger than the solubility of the uncharged species. As shown in Fig. 5, the synthesized PASP derivatives do not meet this requirement. It is expected that the pH-dependent solubility of our polymers cannot be described by this model above their pK_a. To eliminate this problem, we compared the calculated solubility based on the extended HH equation with the maximum solubility ($S_{\max}$) measured at high pH values, and then considered the calculated solubility (S) at a given pH as the minimum of the two. This procedure allows us to describe the pH-dependent solubility of the PASP derivatives in the entire pH range. We refer to this hereafter as the extended HH equation with limited solubility.

Measured solubility profiles were compared with the HH equation, the extended HH equation (Eqs. (2) and (3)) and the extended HH equation with limited solubility. For the HH equation, an additional linearization step was required to determine K_a (Eq. (4)):

$$\frac{K_a \times S_{\min}}{[H^+]} - S_{\min} = S \quad (4)$$

where $[H^+]$ is the proton concentration at a given pH. $S_{\min}$ was used as constants in Eqs. (2), (3) and (4). In Fig. 6 the pH-dependent solubility of PASP B62.5, which is representative of the other samples, is compared with the different models. Model fits of pH-dependent solubility of each PASP derivative with n-butyl or n-hexyl side groups are displayed in Figs. S4 and S5 of the Supplementary Data. The HH description of the solubility profiles is unsatisfactory. The extended HH equation yields better correlation and the fitted curves properly describe the solubility profiles of the polyacids investigated in this work. The accuracy of the fit can be attributed to the relatively simple structure (the acid groups plus side groups consisting of short linear alkyl chains) of these polyacids, which satisfies the requirements of the extended HH model [38]. However, the extended HH equation assumes infinite solubility of the fully deprotonated form. It follows that solubility cannot be determined above the calculated $pK_{a,eHH}$, i.e., this model is unable to describe the solubility over the entire pH range. The extended HH equation with limited solubility, by contrast, accurately models the solubility profile in the whole pH range investigated. The apparent pK_a values and n factors are summarized in Table 1. As it may be expected from the accuracy of the fits, the apparent pK_a values calculated from the extended HH equation ($pK_{a,eHH}$) differ notably from the $pK_{a,HH}$ values determined by the HH equation. In addition, a clear difference in tendency as a function of degree of modification is observed between the n factor derived from the extended HH equation, particularly in the case of PASP derivatives with n-butyl side groups. The large concentration of hydrophobic alkyl groups effectively isolates the aspartic acid units, thus eliminating the effect of neighbouring charged groups. As a consequence, the deprotonation of aspartic acid units is not inhibited by the other ionizable groups. Since the n factor incorporates the effect of the neighbouring groups, it decreases with increasing mole fraction of the side groups. For the case of PASP derivatives with n-hexyl side groups, only a loose correlation is observed between either n and the degree of modification. A more complex situation is expected here because of possible micelle formation as a result of longer side groups.

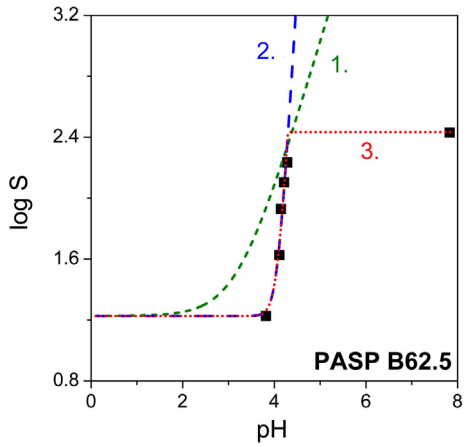


Fig. 6. pH-dependent solubility of a PASP derivative with 62.5 n/n% n-butyl side group (PASP B62.5) modelled by the HH (Eq. (2), short-dashed line, 1.), the extended HH (Eq. (3), long-dashed line, 2.) and the extended HH equation with limited solubility (dotted line, 3.).

Fig. 7 presents the $pK_{a,eHH}$ values of PASP derivatives calculated from the extended HH equation as a function of the degree of modification. The correlation is linear, indicating the validity of a linear free energy–structure equation. These Hammet type equations [43] can generally be written in the form

$$\Delta G^o_{subst} = \Delta G^o + \rho\sigma \quad (5)$$

Table 1
pK_a and n values of the PASP derivatives with alkyl side groups determined by the HH equation and the extended HH equation.

Sample name	X_{calc}	$pK_{a,HH}$	$pK_{a,eHH}$	n
PASP derivatives with n-butyl side groups				
PASP B37.5	36	2.5	3.2	0.33
PASP B50	52	2.8	3.7	0.27
PASP B62.5	63	3.2	4.1	0.21
PASP B75	75	3.7	4.8	0.14
PASP derivatives with n-hexyl side groups				
PASP H25	26	2.4	3.3	0.30
PASP H37.5	41	2.9	3.7	0.35
PASP H50	51	3.5	4.3	0.21
PASP H62.5	65	4.0	4.7	0.30

X_{calc}: calculated degree of modification; $pK_{a,HH}$: the pK_a from the HH equation; $pK_{a,eHH}$: apparent pK_a from extended HH equation; n: slope in the extended HH equation.

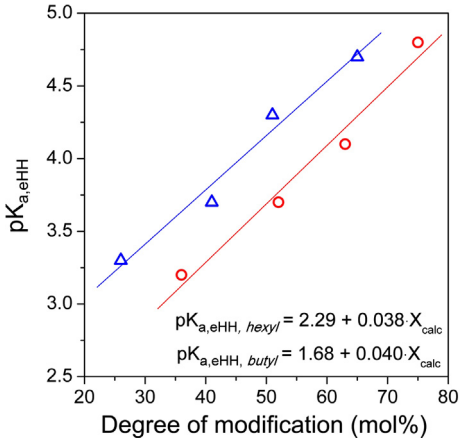


Fig. 7. Correlation between the degree of modification and $pK_{a,eHH}$ values of the PASP derivatives containing (○) *n*-butyl or (△) *n*-hexyl side groups.

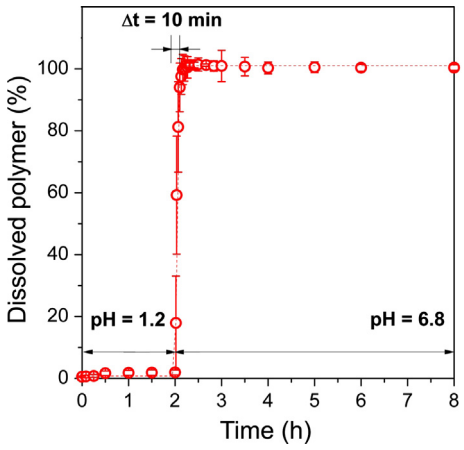


Fig. 8. PASP H50 practically insoluble at pH = 1.2 but dissolves quickly at pH = 6.8.

where ΔG^0_{subst} is the free energy of the given reaction of the substituted compound, ΔG^0 is that of the unsubstituted compound, σ is the substitution, while ρ is the reaction constant. The reaction here is the pH-dependent dissociation, which can be defined by the pK_a (Eq. (6)):

$$\Delta G^o\ (dissociation) = 2.703 pK_a \tag{6}$$

Using Eqs. (5) and (6) a linear correlation can be established between the degree of modification (X_{calc}) and pK_a:

$$pK_a = pK_{a,0} + CX_{calc} \tag{7}$$

where $pK_{a,0}$ is the extrapolated value of pK_a at zero degree of modification, and C characterizes the effect of side groups. A similar approach was used by Bae et al. [42] in the case of various sulphonamides, but the effect of chain length was not investigated in that work. The identical slope of the linear fits is remarkable, and it seems reasonable to conclude that PASP derivatives with desired apparent pK_a can be prepared by using different side groups in the proper concentration. Naturally, the validity of Eq. (7) is limited to the range investigated and cannot be used at low degrees of modification because the extrapolated $pK_{a,0}$ values differ in the case of PASP derivatives with butyl and hexyl side groups, which contradicts the expectation. Moreover, since the observations are valid only for poly(aspartic acid)s with poor solubility, Eq. (7) is not relevant at small X_{calc} values. The results presented above demonstrate that pH-sensitive solubility of the PASP can be adjusted exactly by choosing the type and concentration of side groups, and the solubility of these PASP derivatives can be modelled by using simple equations in a wide pK_a range.

3.3. Dissolution rate of a PASP derivative

The performance of PASP derivatives as enteric coating is determined not only by their equilibrium solubility, but also by their rate of dissolution at physiologically relevant pH values. Polymer coatings with poor solubility and low rate of dissolution in acidic media can protect the drug from the strongly acidic environment of the stomach, while fast dissolution is required in the more alkaline intestinal fluid.

The fluorescent L-tryptophan methyl ester (T) was grafted onto the polymer chain at low concentration (1 mol%) in order to characterize the dissolution rate of PASP polymers. In this approach we assume that the effect of alkyl groups is not influenced by the fluorescent ligands. To test the *in vitro* dissolution rate and the expected performance of the polymers as enteric coating, we chose a polymer with an apparent pK_a higher than the average pH in the stomach (pH = 1–2), but significantly lower than the pH in the duodenum (pH = 5.5–7). These requirements must be fulfilled to protect the drug in the stomach, but achieve fast dissolution of the coating in the intestines. Based on these considerations, PASP H50 marked with florescent ligands (PASP T1H50) was used in these experiments (pK_a = 4.3).

Fig. 8 shows the dissolution kinetics of a PASP T1H50 film (thickness: 31 ± 6 μm, $p < 0.05$). Less than 2% of the polymer was dissolved in acidic medium within 120 min, and we therefore expect efficient protection of the active molecule in the gastric fluid as well as the protection of the stomach from an irritative drug. At pH = 6.8 the film disintegrated and dissolved rapidly. Complete dissolution was achieved within 10 minutes, demonstrating the ability of the film to provide fast drug release in the intestinal tract. These results show that polymer films based on PASP derivatives are suitable for enteric tablet coatings.

3.4. Cell viability study

In our study, human prostate cancer cells (PC-3 line) were used as model cells to evaluate cellular compatibility of the PASP derivatives synthesized. An effect of medical materials on cell viability is a primary biocompatibility characteristic, which can be studied on different immortalized cells of cancer and embryonic origin, including PC-3 cells with established and reproducible properties

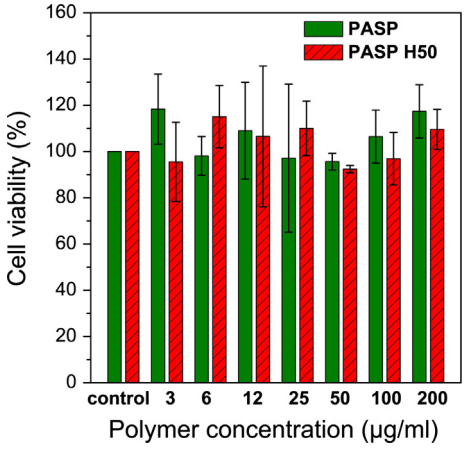


Fig. 9. Effect of non-modified PASP and PASP H50 on PC-3 cells viability. MTT assay, $p < 0.05$.

[44–46]. PC-3 cells are characterized by good surface adherence, contact inhibition and well-defined morphology, which make them convenient for cytotoxicity detection in a reproducible manner using conventional assays. The cell viability study was performed by MTT proliferation assay for PASP and PASP H50 used in dissolution tests. Neither polymer induced any significant decrease in cell viability upon culturing compared with control (100%) in the concentration range up to 200 mg/l (the maximum solubility of PASP H50) as shown in Fig. 9. These preliminary data indicate that the cytotoxicity of unmodified PASP is relatively low and also the lack of cytotoxic effect when *n*-hexyl groups are introduced into PASP backbone. Further toxicity studies of the polymers and their pharmaceutical formulations will be performed elsewhere.

4. Conclusion

PASP derivatives having significant concentrations of *n*-butyl or *n*-hexyl side groups were synthesized to determine the effect of type and concentration of alkyl side groups on the pH-dependent aqueous solubility of PASP. It was found that both the maximum solubility (S_{max}) and the pK_a values of the modified polymers can be controlled precisely by the chemical composition. The HH equation gives a poor representation of the solubility profiles, while the extended HH equation with limited solubility properly describes the pH-dependent solubility of the PASP derivatives. Strong correlation was found between the degree of modification and the pK_a values of the polymers. The results proved that the solubility of PASP can be tuned by proper selection of the type and concentration of substituent. Fluorescent marking was used to characterize the pH-dependent dissolution kinetics of a chosen PASP derivative. In acidic media (pH = 1.2) only a negligible amount of the polymer dissolved, but dissolution was very fast and complete at the pH values that prevail in the first tract of the small intestine, in the duodenum (pH = 6.8). This study shows that enteric coatings based on such PASP derivatives may be used in duodenum specific drug delivery. Moreover, targeted release into other parts of the gastrointestinal tract can be also achieved by the proper modification of PASP. Owing to their adjustable pH-dependent solubility and dissolution kinetics, as well as their biocompatibility, PASP polymers are good alternatives to polyacrylates in pharmaceutical film coatings. Further characterization of polymers and polymer films based on PASP derivatives with alkyl side groups including thermal stability, glass transition temperature as well as *in vitro* drug release properties will be performed.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actbio.2016.11.065.

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