Abstract
Poly(aspartic acid) (PASP) hydrogels were prepared by cross-linking polysuccinimide (PSI) with diaminobutane followed by the mild hydrolysis of the resultant PSI gels to PASP hydrogels. The composition dependence of the gelation time and the stiffness of bulk PASP hydrogels were determined by rheometry and compression tests, respectively. The composition of the prepared PASP nanogels was chosen based on the results on bulk PASP hydrogels. Prior to nanogel preparation stability of DMSO-in-oil (inverse) emulsions was tested as a function of the chemical quality of apolar phase, the concentration of the precursor polymer and the concentration of the surfactant. PASP nanogels in the size of a few hundred nanometers were prepared by the hydrolysis of PSI nanogels synthesized in inverse emulsion. PASP nanogels showed pH-dependent swelling and strongly negative surface charge at physiological pH values, thus they can be further developed to meet the specific criteria of different bio-related applications.

Keywords
hydrogel, nanogel, poly(aspartic acid), inverse emulsion, responsive polymer

1 Introduction
Hydrogels are commercialized in their bulk form in several medical devices and everyday applications, e.g. in implants, contact lenses, diapers [1]. Besides these macroscopic applications the living cells can be targeted with nanogels (with diameter of few hundred nanometers) [2-4] since they have a large capacity for the entrapment of bioactive molecules such as drugs, proteins, carbohydrates or DNA and can be taken up by the cells if proper ligands (e.g. recognition factors) are immobilized onto their surface [5, 6]. Well-known approaches for preparing nanogels include photolithographic and micro-molding techniques, microfluidics, free radical heterogeneous polymerization in dispersion, nanoprecipitation and emulsion techniques [2, 7-9]. Water-in-oil (inverse) emulsion technique is the most widely used since it does not require any complicated equipment and the size of the resultant nanogels can be easily controlled. The first step of this method is the emulsification of the solution of the precursor polymer or monomer in a continuous apolar phase with an oil-soluble surfactant. The stability of the emulsion against coalescence or Ostwald ripening plays an important role in controlling the size distribution of the forming nanogels. The second step is the chemical cross-linking of the precursor polymer trapped inside the droplets of the emulsion (or direct polymerization of the monomers). Cross-linking can be achieved by a suitable cross-linking molecule or by the reaction of functional groups of the polymer chains (e.g. intermolecular disulfide formation by the oxidation of thiol-groups [10]). Several examples are summarized in recent reviews of Matyjaszewski [2], Li [8] and Landfester [11] for the preparation of nanogels in the size range of 100-500 nm by inverse emulsion technique. Recently nanogels have been prepared mostly from natural polymers, e.g. chitosan, hyaluronic acid and alginate. In all cases the polar phase was water, while the most common apolar phases were hexane, cyclohexane or dichloromethane and the emulsions were stabilized usually with sorbitane monooleate (Span 80).

Natural polymers generally used in the preparation of nanogels are considered to be biocompatible and biodegradable but a huge drawback is their poorly reproducible structure and
molecular weight, which depends on the source of the polymer. Synthetic polymers provide better reproducibility and a control over molecular weight and structure, but they are not inherently biocompatible or biodegradable (e.g. limited biodegradability of polyacrylates [12]). Poly(aspartic acid) (PASP) as a synthetic poly(amino acid) is a well-known representative of biocompatible [13], biodegradable [14] and pH-responsive materials, thus it can combine the benefits of synthetic and natural polymers. The pre-cursor polymer of PASP, polysuccinimide (PSI) can be easily prepared from aspartic acid, a natural amino acid. Succinimide rings in the PSI are highly reactive, thus PASP derivatives and cross-linked PASP can be synthesized in various ways [10, 13, 15, 16]. There is no need for activating agents, e.g. carbodiimides [17], which simplifies the synthesis and the purification. The substantial benefit of using PASP instead its analogue, poly(glutamic acid) or polyacrylates is its easier synthesis and in contrast to polyacrylates, its biodegradability as well. The drawback of PSI is its solubility: it is soluble only in dimethyl sulfoxide (DMSO) and dimethylformamid (DMF), and this problem must be overcome in the nanogel synthesis. According to the studies of Imhof et al. [18], emulsions containing DMF can hardly be stabilized in contrast to emulsions with DMSO, thus we chose DMSO for the preparation of PASP nanogels. Only a few articles have reported the application of PASP in nanogel synthesis. Wu et al. [19] synthesized nanogels made of quaternized chitosan and PASP by ionotropic gelation technique for protein delivery. Li et al. [20] also reported the preparation of a novel nanogel system based on water-soluble chitosan, PASP and poly(ethylene glycol) by a coagulation method. Sumerlin et al. [21] synthesized nanogels from amphiphilic PASP derivatives by nanoprecipitation method for site-specific delivery in agriculture. In these cases nanogel formation was the result of ionic interactions, and nanogels were not stabilized with chemical cross-links. Chemically cross-linked poly(ethylene glycol)-block-poly(aspartic acid) (PEG-PASP) core-shell nanogels in the size range of 90-140 nm were prepared by Kim et al. [22] for pH-sensitive insulin delivery based on the self-assembly of PEG-PASP amphiphilic copolymers. Lee et al. [23] prepared lysosome-selective PEG-PASP nanogels for antitumor drug-delivery. In both cases the synthesis of the precursor polymers complicated the nanogel preparation and required the use of activating agents (e.g. carbodiimide) as well. Thus, there is still a need for a simple method to yield cross-linked PASP nanogels. The posterior cross-linking allows control over the concentration of net points in the polymer network, therefore over the swelling behavior.

Here we report a simple synthetic route to prepare pH sensitive PASP nanogels starting with the chemical cross-linking of its precursor polymer, PSI. The widely used inverse emulsion technique was chosen for the nanogel preparation. To overcome the problem of solubility of PSI, nanogels were prepared in a DMSO/dodecane emulsion that is a technique without precedent in the literature. The degree of swelling, the stiffness and the gelation time of bulk gels were determined. Nanogels were prepared of chosen composition and characterized by dynamic light scattering (DLS) and zeta potential measurements. Surface charge and pH-dependent response of the nanogels were also examined.

2 Materials and methods

2.1 Materials

L-Aspartic acid (99%), n-hexane (96.5%), n-heptane (99.5%), n-decane (99.5%), n-undecane (99%), n-dodecane (99%) and Span 80 were purchased from Merck (Hungary). Sodium tetraborate (99%), imidazole (99.5%), citric acid monohydrate (99.5%), phosphoric acid (85%); hydrogen chloride (37%) and potassium chloride (99.8%) were purchased from Reanal (Hungary). Dimethyl sulfoxide (99%), 1,4-diaminobutane (98%, DAB), sulfolane (99%) and mesitylene (98%) were purchased from Sigma-Aldrich. Ultrapure water (ρ > 18.2 MΩ·cm, Millipore) was used for aqueous solutions. All reagents and solvents were used without further purification. Synthesis and all the measurements were carried out at 25 °C.

2.2 Preparation of poly(aspartic acid) hydrogels cross-linked with diaminobutane

First, polysuccinimide (PSI, M_n = 56 kDa by size exclusion chromatography) was prepared by thermal polycondensation of aspartic acid as reported earlier [10]. In the synthesis of bulk gels PSI was cross-linked with DAB in dimethyl sulfoxide (DMSO) and subsequently the PSI gels were hydrolyzed to the corresponding PASP hydrogel at pH = 8 (Fig. 1). Hydrogels were prepared with different polymer (6, 8 and 10 w/w%) and cross-linker concentrations. The molar ratio of DAB to the repeating units of PSI (cross-linking ratio, X_DAB) was changed from 5 to 20%. Samples were named according to their composition, e.g. in the case of PASP-6-DAB-10 the hydrogel was prepared with a polymer concentration of 6 w/w% and X_DAB of 10%. In a typical procedure (PASP-6-DAB-10 hydrogel) 0.3000 g PSI was dissolved in 4.3905 g DMSO and 0.3095 g of 8.8 w/w% DAB (in DMSO) solution was added to yield DAB cross-linked PSI gels. Samples were prepared in cylindrical molds (diameter ≈ height ≈ 1.5 cm) for mechanical characterization and discs with a diameter of 10 mm were cut from PSI gel sheets with a thickness of 2 mm for swelling experiments. Gelation time was determined for gels prepared in 2 ml plastic vials. PSI gels were hydrolyzed in a large excess of imidazole buffer solution (pH = 8, I = 0.25 M) for 2 days to yield PASP hydrogels.

2.3 Preparation of PASP nanogels

PSI nanogels were prepared in inverse emulsion with PSI concentration of 6 w/w% and molar ratio of DAB to the repeating units of PSI of 10% (PSI-6-DAB-10). DMSO (polar phase) contained the PSI and the DAB, while dodecane (apolar phase)
contained the surfactant (Span 80). 10 ml of dodecane was measured into a 250 ml boiling flask and 0.5 g (5 w/V%) of Span 80 was added. Afterwards 10 ml of gel precursor solution was added to the dodecane. The resultant emulsion was stirred by an overhead stirrer at 500 rpm for 4 hours at room temperature, then poured into 500 ml of aqueous buffer solution (pH = 8) and stirred for additional 2 days to yield PASP nanogels (PASP-6-DAB-10). Dodecane was removed after phase separation using a separatory funnel. Nanogels were purified from buffer components by dialysis against water (cut off: 6-8 kDa).

2.4 Gelation time
The gelation time of bulk PSI gels was determined by visual observation using the tilting method in 2 ml Eppendorf tubes and by oscillation rheometry using an Anton Paar Physica MCR 301 Rheometer. For rheometry measurements cone-plate geometry with a diameter of 25 mm (CP25, cone angle was 1°) was used and the sample gap was set to 0.049 mm. 100 μl of the precursor solution containing PSI and DAB in DMSO was placed on the bottom plate of the rheometer immediately after mixing the solutions. The temperature was set to 25.0 °C and controlled by a Peltier system (PTD 200). Applied angular frequency (ω) was 10 rad/s with a deformation (γ) of 1%. Storage and loss moduli were followed over time and gelation time was defined as the time at the equality of the two moduli.

2.5 pH dependent degree of swelling
The degree of swelling of PASP hydrogels \((Q_m)\) is defined as the mass ratio of the swollen \((m_s)\) to the dried \((m_d)\) gel (Eq. (1)):

\[
Q_m = \frac{m_s}{m_d} \tag{1}
\]

PASP hydrogels were swollen in large excess of aqueous buffer solutions of citric acid \((2 \leq \text{pH} \leq 6)\), imidazole \((6 \leq \text{pH} \leq 8)\) and tetraborate \((8 \leq \text{pH} \leq 12)\) at ambient temperature. Two days were enough to reach the equilibrium. Ionic strength of buffer solutions was kept on 0.25 M by adding KCl. pH was adjusted by adding 1 M HCl or 1 M NaOH and measured with a Radelkis OP-271/1 pH/ion analyzer. The mass of the swollen hydrogels was measured after removing the excess swelling solution from the surface of the gel. The mass of the dried polymer gels was measured after removing the buffer components by immersing the gels into large amount of deionized water for 3 days with daily change of the water, finally drying them for two days under vacuum at ambient temperature.

2.6 Mechanical properties
The compressive elastic modulus \((G)\) of the hydrogels was determined by uniaxial compression tests using an Instron 5543 mechanical tester with a cell load of 5 N at room temperature. The force \((F)\) and the extent of compression \((\Delta h)\) of the cylindrical samples were recorded during the measurements, and the nominal stress \((\sigma_n)\) and compression ratio \((\lambda)\) were calculated by using the following equations:

\[
\lambda = \frac{\Delta h}{h_0} \tag{3}
\]

\[
\sigma_n = \frac{F}{A_0} \tag{2}
\]

where \((A_0)\) is the cross-sectional area of the undeformed hydrogel and \((h_0)\) is the original height. The maximum deformation was 10% of the original height and samples were compressed in a stepwise measurement with 0.1 mm in each step followed by a relaxation time of 8 s. The compressive elastic modulus was calculated by Eq. (4). Moduli were determined from the linear fit to the stress-strain function between 0 and 10% strain.

\[
\sigma_n = G(\lambda - \lambda^{-2}) \tag{4}
\]
2.7 Emulsion stability
The stability of emulsions was characterized as a function of the chemical quality of apolar phase, the concentration of the surfactant in the apolar phase and the PSI concentration in the polar phase. DMSO was the polar phase and hydrocarbons with different chain length were tested as apolar phase, such as \(n\)-hexane, \(n\)-heptane, \(n\)-decane, \(n\)-undecane and \(n\)-dodecane. The volume ratio of polar to apolar phase was 1:1 in each case, and experiments were done in Wassermann tubes with 2 ml sample volumes. As water-in-oil (W/O) emulsions can be stabilized by surfactants with low HLB (hydrophilic-lipophilic balance) number, a widely used surfactant, Span 80 (HLB = 4.3) was chosen [5]. The concentration of the surfactant in the apolar phase varied between 1 and 20 w/V%. Emulsions were shaken for 60 s intensively and their stability was characterized by the time when the height of the separated upper phase reached 10% of the height of the emulsion. The average error of emulsion stability tests was ±2 s.

2.8 Size distribution of nanogels
Size distribution and polydispersity of PASP nanogels were determined by dynamic light scattering (DLS). Dialyzed sample solutions were filtered through a nylon membrane filter with a pore size of 0.45 μm. To determine pH dependent size distribution, pH of the dialyzed sample solutions was adjusted by the addition of 1 M HCl or 1 M NaOH. DLS measurements were carried out on a Malvern Zetasizer Nano ZS instrument equipped with a 4.0 mW He-Ne laser operating at 173° (back scatter mode) on 633 nm. The hydrodynamic diameters \(d_H\) were calculated from diffusion coefficients using the Stokes–Einstein equation. All correlation functions were analyzed with the software supplied by the manufacturer.

2.9 pH-dependent zeta potential of PASP nanogels
Electrophoretic mobility was measured by a Brookhaven Zeta Potential Analyzer using the Zeta PALS (Phase Analysis Light Scattering) method. The pH of the dialyzed sample solutions was adjusted by the addition of 1 M HCl or 1 M NaOH. Smoluchowski equation (Eq. (5)) was used to calculate the zeta potential:

\[
\xi = \frac{\mu_E \eta}{\varepsilon}
\]

where \(\xi\) is the zeta potential, \(\mu_E\) is the electrophoretic mobility, \(\eta\) is the viscosity of water and \(\varepsilon\) is the permittivity of water. The standard error of the measurements was calculated out of 5 runs each time, and each run consisted of 10 cycles.

Fig. 2 a) Simultaneous increase in the dynamic moduli around the gel point of the PSI in DMSO (PSI-6-DAB-10), gelation time was defined as the time at the equality of the two moduli and b) gelation time of PSI bulk gels cross-linked with DAB as a function of cross-linking ratio was determined by rheometry (solid symbols) and visual observation (open symbols).

3 Results and discussion
3.1 Characterization of bulk poly(aspartic acid) hydrogels
To find the suitable gel composition for the synthesis of nanogels, first bulk PASP hydrogels with different polymer concentration and cross-linking ratio were studied. The gelation time of bulk PSI gels was determined by oscillation rheometry. Storage \((G')\) and loss \((G'')\) moduli were followed over time as shown in Fig. 2a, and gelation time was defined as the time at the equality of the two moduli [24]. The gelation times derived from rheological measurements are in good agreement with the values determined by visual observation (Fig. 2b).

Gelation time varied in a wide interval from 1 minute to half an hour and was highly dependent on the composition: it decreased sharply with increasing cross-linker concentration, \(X_{DAB}\) and decreased with increasing PSI concentration. The minimum cross-linking ratio at the lowest polymer concentration was 6.7%.
Figure 3a shows the nominal stress during the compression of PASP-6-DAB-10 hydrogel. The curves tend to an almost straight line in all cases, and the compressive elastic modulus was calculated from the slopes. The elastic moduli of PASP hydrogels as a function of polymer concentration and cross-linking ratio are shown in Fig. 3b. Stiffness of PASP hydrogels increased with increasing the PSI concentration and/or increasing $X_{\text{DAB}}$. Mechanical properties could easily be controlled by the composition of the polymer network and the elastic modulus could be changed in wide interval from ~3 kPa (very soft) to ~120 kPa (rather stiff). According to our experience, PASP hydrogels with elastic modulus larger than 5 kPa are self-standing and mechanically stable (Fig. 3b). Hydrogel could not be obtained with the lowest PSI concentration (6 w/w%) and $X_{\text{DAB}}$ (5%). PASP hydrogel with the highest PSI concentration (10 w/w%) and $X_{\text{DAB}}$ (20%) was rigid and fragile. It broke during the compression test, thus no elastic modulus was determined.

In order to prove the pH responsive character, the degree of swelling of PASP-10-DAB-10 hydrogel was measured as a function of environmental pH (Fig. 4). The degree of swelling had an abrupt change at around pH = 4, this value is close to the $pK_a$ values of $\alpha$ and $\beta$ aspartic acid (3.25 and 4.35 respectively [25]). In this interval, the concentration of deprotonated repeating units increases dramatically, resulting in better water-solubility of the polymer thus in higher degree of swellings. We expected similar pH-dependent swelling behavior from the nanogels.

![Figure 3a](image1.png)

**a)** Determination of compressive elastic modulus (sample PASP-6-DAB-10) and **b)** compressive elastic modulus of PASP-DAB hydrogels with different composition

![Figure 4](image2.png)

**Fig. 4** Degree of swelling of PASP-10-DAB-10 hydrogel as a function of pH

### 3.2 Synthesis and characterization of the nanogels

PSI nanogels were prepared in an inverse emulsion. Prior to the preparation of nanogels stability of emulsions was studied as a function of the chemical quality of apolar phase, concentration of the surfactant in the apolar phase and the PSI concentration in the polar phase. DMSO was chosen to be the polar phase, since PSI only soluble in DMSO and DMF, and emulsions containing DMF were hardly stabilized [18]. Linear hydrocarbons with different chain length were tested as apolar phase. The emulsions were stabilized with Span 80. The effect of the chain length of the $n$-alkanes of the apolar phase and the concentration of the surfactant on the emulsion stability were tested with emulsions containing DMSO without PSI as the polar phase (Fig. 5a). Emulsion stability increased with increasing chain length of the $n$-alkanes. The most stable emulsion was obtained with 5 w/V% Span 80 and above this concentration the stability did not show any improvement. To make the cleaning process easier the surfactant concentration had to be kept as low as possible. Dodecane with 5 w/V% Span 80 was chosen as the apolar phase for the preparation of the PSI-DAB nanogels. Afterwards the effect of PSI concentration on the time of phase separation was studied. The time of phase separation in the case of 6, 8 and 10 w/w% PSI concentration were 41, 34 and 32 s, respectively, so we can conclude, that emulsions with higher...
PSI concentrations were less stable (Fig. 5b). Thus, the lowest polymer concentration, 6 w/w% was chosen for the preparation of nanogels, as below this polymer concentration gelation did not occur in bulk experiments. If we compare these results with the mechanical data obtained from bulk PASP hydrogels, in the case of PSI concentration of 6 w/w%, the minimum cross-linking ratio is 10% to obtain hydrogel with satisfying stiffness (G > 5 kPa), thus nanogels with the composition of PASP-6-DAB-10 hydrogels were prepared.

Monodisperse PASP nanogels in the size range of 200-400 nm (Fig. 6a) were successfully prepared. This size range enables the nanogels to be internalized by cells via endocytosis [26]. PASP-6-DAB-10 nanogels – similarly to bulk hydrogels – showed characteristic pH dependent degree of swelling (Fig. 6b), due to polyelectrolyte character of the polymer main chain. Carboxyl groups are protonated below the pK\textsubscript{a} value of aspartic acid repeating units (pK\textsubscript{a} = 4.2) and deprotonated above it. At acidic pH values the repeating units are neutral, consequently the volume of PASP nanogels is smaller. We can establish that diameter of the nanogels changed abruptly between pH = 3 and 4, as shown in Fig. 6b.

The surface charge of nanogels plays an important role both in vitro and in vivo [27]. The positively charged nanogels can easily be internalized by cells, since they can bind electrostatically with the negatively charged cell membranes. However, positively charged nanogels often interact strongly with serum components, which causes severe aggregation and rapid clearance from circulation and limits their in vivo application. In contrast, the negatively charged carriers show potential for protein resistance and also exhibit prolonged circulation time for in vivo applications. The Zeta potential of PASP nanogels was negative in almost the entire pH range as shown in Fig. 7. Below pH = 4 the zeta potential was close to zero, because of the protonated, neutral form of carboxylic groups. Above pH = 4 zeta potential decreased abruptly and showed a strongly negative value (−20 to −30 mV) as the carboxylic groups became deprotonated. The strongly negative zeta potential of PASP nanogels at the pH range of the human cells (6.8 ≤ pH ≤ 7.4) and blood (7.2 ≤ pH ≤ 7.6) can be beneficial for the possible in vivo applications.
Acknowledgement

This research was supported by the OTKA Foundation (PD76401) and by the New Széchenyi Plan (TÁMOP-4.2.1/B-09/1-2010-0002). The authors thank András Deák and Eszter Fülop in the Institute of Technical Physics and Materials Science, MFA, Research Centre for Natural Sciences, HAS for supplying the DLS instruments and their guidance.

References

[1] Calo, E., Khutoryanskiy, V. V. "Biomedical applications of hydrogels: A review of patents and commercial products." European Polymer Journal. 65, pp. 252–267. 2015. https://doi.org/10.1016/j.eurpolymj.2014.11.024

[2] Oh, J. K., Drumright, R., Siegwart, D. J., Matyjaszewski, K. "The development of microgels/nanogels for drug delivery applications." Progress in Polymer Science. 33(4), pp. 448–477. 2008. https://doi.org/10.1016/j.progpolymsci.2008.01.002

[3] Hamidi, M., Azadi, A., Rafiei, P. "Hydrogel nanoparticles in drug delivery." Advanced Drug Delivery Reviews. 60(15), pp. 1638–1649. 2008. https://doi.org/10.1016/j.addr.2008.08.002

[4] Kingsley, J. D., Dou, H., Morehead, J., Rabinow, B., Gendelman, H. E., Destache, C. J. "Nanotechnology: A focus on nanoparticles as a drug delivery system." Journal of Neuroimmunology. 13(3), pp. 340–350. 2006. https://doi.org/10.1016/S0165-5728(06)00324-4

[5] Chacko, R. T., Ventura, J., Zhuang, J., Thayumanavan, S. "Polymer nanogels: a versatile nanoscopic drug delivery platform." Advanced Drug Delivery Reviews. 64(9), pp. 836–851. 2012. https://doi.org/10.1016/j.addr.2012.02.002

[6] Zha, L., Banik, B., Alexis, F. "Stimulus responsive nanogels for drug delivery." Soft Matter. 7(13), pp. 5908–5916. 2011. https://doi.org/10.1039/C1SM01307B

[7] Motornov, M., Roiter, Y., Tokarev, I., Minko, S. "Stimuli-responsive nanoparticle and capsules for integrated multifunctional intelligent systems." Progress in Polymer Science. 35(1-2), pp. 174–211. 2010. https://doi.org/10.1016/j.progpolymsci.2009.10.004

[8] Li, Y., Maciel, D., Rodrigues, J., Shi, X., Tomás, H. "Biodegradable polymer nanogels for drug/nucleic acid delivery." Chemical Reviews. 115(16), pp. 8564–8608. 2015. https://doi.org/10.1021/acs.chemrev.5b00317

[9] Csetneki, I., Faix, M. K., Szilágyi, A., Kovács, A. L., Németh, Z., Zrínyi, M. "Preparation of magnetic polystyrene latex via the miniemulsion polymerization technique." Journal of Polymer Science Part A: Polymer Chemistry. 42(19), pp. 4802–4808. 2004. https://doi.org/10.1002/pola.20300

[10] Gyarmati, B., Vajna, B., Némethy, A., László, K., Szilágyi, A. "Redox and pH-responsive cysteamine-modified poly(aspartic acid) showing a reversible sol-gel transition." Macromolecular Bioscience. 13(5), pp. 633–640. 2013. https://doi.org/10.1002/mabi.2012000420

[11] Landfester, K., Musyanovych, A. "Hydrogels in Miniemulsions." Chemical Design of Responsive Microgels. 234, pp. 39–63. 2010. https://doi.org/10.1007/12_2010_68

[12] Herth, G., Schornick, G., Buchholz, F. L. "Polyacrylamides and Poly(Acrylic Acids)." In: Ullmann's Encyclopedia of Industrial Chemistry. pp. 1–16. 2015. https://doi.org/10.1002/14356007.a21_143.pub2

[13] Gyarmati, B., Mészár, E. Z., Kiss, L., Deli, M. A., László, K., Szilágyi, A. "Supermacroporous chemically cross-linked poly(aspartic acid) hydrogels." Acta Biomaterialia. 22, pp. 32-38. 2015. https://doi.org/10.1016/j.actbio.2015.04.033

[14] Roweton, S; Huang, SJ; Swift, G. "Poly (aspartic acid): synthesis, biodegradation, and current applications." Journal of environmental polymer degradation. 5(3), pp. 175-181. 1997.

[15] Némethy, A., Solti, K., Kiss, L., Gyarmati, B., Deli, M. A. Csányi, E., Szilágyi, A. "pH- and temperature-responsive poly(aspartic acid)-l-poly(N-isopropylacrylamide) comouted hydrogel." European Polymer Journal. 49(9), pp. 2392–2403. 2013. https://doi.org/10.1016/j.eurpolymj.2013.02.015

[16] Gyarmati, B., Krisch, E., Szilágyi, A. "In situ oxidation-induced gelation of poly(aspartic acid) thiomers." Reactive & Functional Polymers. 84, pp. 29-36. 2014. https://doi.org/10.1016/j.reactfunctpolym.2014.08.007

Fig. 7 Zeta potential of PASP-6-DAB-10 nanogels as a function of pH

4 Conclusion

The gelation time of PASP hydrogels cross-linked with dianinobutane (PASP-DAB) was determined. The hydrogels showed sufficient mechanical stability and their stiffness varied depending on the composition. Proper composition for nanogel preparation was chosen by a minimum criterion of stiffness. Prior to nanogel preparation stability of emulsions with different compositions was studied. Given the solubility of PSI, DMSO was chosen as the polar phase. Based on the emulsion stability tests, n-dodecane was selected as the apolar phase for the nanogels synthesis. The PASP-DAB nanogels were prepared by inverse emulsion technique in the size range of 200–400 nm. The size of the PASP nanogels showed strong pH dependence, similarly to the bulk PASP hydrogels. Zeta potential of the PASP nanogels at the pH range of the human body was strongly negative, which can be beneficial for the possible in vivo applications. The prepared nanogels may have several applications in biomedical areas. Characterization of the biocompatibility of the gels and introducing surface ligands to enhance biomolecular recognition are the most important future directions in our research work.
[17] Hua, J., Li, Z., Xia, W., Yang, N., Gong, J., Zhang, JF., Qiao, CS. "Preparation and properties of EDC/NHS mediated crosslinking poly(gamma-glutamic acid)/epsilon-polylysine hydrogels." *Materials Science & Engineering C-Materials for Biological Applications*. 61, pp. 879–892. 2016. https://doi.org/10.1016/j.msec.2016.01.001

[18] Imhof, A., Pine, D. J. "Stability of nonaqueous emulsions." *Journal of Colloid and Interface Science*. 192(2), pp. 368–374. 1997. https://doi.org/10.1006/jcis.1997.5020

[19] Wang, TW., Xu, Q., Wu, Y., Zeng, AJ., Li, MJ., Gao, HX. "Quaternized chitosan (QCS)/poly (aspartic acid) nanoparticles as a protein drug-delivery system." *Carbohydrate Research*. 344(7), pp. 908–914. 2009. https://doi.org/10.1016/j.carres.2009.02.018

[20] Shu, S. J., Zhang, X. G., Teng, D. Y., Wang, Z., Li, C. X. "Polyelectrolyte nanoparticles based on water-soluble chitosan-poly(L-aspartic acid)-polyethylene glycol for controlled protein release." *Carbohydrate Research*. 344(10), pp. 1197–1204. 2009. https://doi.org/10.1016/j.carres.2009.04.018

[21] Hill, M. R., Mackrell, E. J., Forsthoefer, C. P., Jensen, S. P., Chen, M. S., Moore, G. A., He, Z. L. L., Sumerlin, B. S. "Biodegradable and pH-Responsive Nanoparticles Designed for Site-Specific Delivery in Agriculture." *Biomacromolecules*. 16(4), pp. 1276–1282. 2015. https://doi.org/10.1021/acs.biomac.5b00069

[22] Park, C. W., Yang, H. M., Lee, H. J., Kim, J. D. "Core–shell nanogel of PEG–poly(aspartic acid) and its pH-responsive release of rh-insulin." *Soft Matter*. 9(6), pp. 1781–1788. 2013. https://doi.org/10.1039/C2SM26865E

[23] Oh, N. M., Oh, K. T., Yoon, Y. S., Lee, D. K., Cha, K. H., Lee, D. H., Lee, E. S. "Poly(L-aspartic acid) nanogels for lysosome-selective antitumor drug delivery." *Colloid and Surface B-Biointerfaces*. 101, pp. 298–306. 2013. https://doi.org/10.1016/j.colsurfb.2012.07.013

[24] Winter, H. H., Mours, M. "Rheology of polymers near liquid–solid transitions." In: Neutron Spin Echo Spectroscopy Viscoelasticity Rheology. Springer, 134, pp. 165–234. 1997.

[25] Kokufuta, E., Suzuki, S., Harada, K. "Potentiometric titration behavior of polyaspartic acid prepared by thermal polycondensation." *Biosystems*. 9(4), pp. 211–214. 1977. https://doi.org/10.1016/0303-2647(77)90005-3

[26] Rejman, J., Oberle, V., Zuhorn, I. S., Hoekstra, D. "Size-dependent internalization of particles via the pathways of clathrin- and caveola-mediated endocytosis." *Biochemical Journal*. 377, pp. 159–169. 2004. https://doi.org/10.1042/BJ20031253

[27] Mailander, V., Landfester, K. "Interaction of nanoparticles with cells." *Biomacromolecules*. 10(9), pp. 2379–2400. 2009. https://doi.org/10.1021/bm090266r