FINAL REPORT

Final report of the research "Poly(aspartic acid) and polyaspartamide polymers for ophthalmic drug delivery" supported by the National Research, Development and Innovation Office, identification number FK 125074

1. Introduction

The main goal of the project was to synthesize aspartic acid based polymer matrices for drug delivery which may provide better therapeutic efficiency and improved patient compliance compared to those of currently available formulations. One of our main topics was the synthesis of nanofibrous polyaspartamide matrices. In the first step of this topic, we explored the structure-property correlations in a wide range of compositions. Based on the knowledge gained here, we prepared polymer matrices for the release of drugs. The other topic of our project was the synthesis of mucoadhesive polymers. The aqueous solutions of thiol-containing polymers synthesized via various pathways presumably interact with the mucin glycoprotein. We assumed that polymers showing mucoadhesion would adhere to the mucosal membrane of the eye. The polymer matrices with favorable characteristics were evaluated by drug release measurements. The most promising polymers of the project were investigated by cytotoxicity tests. The development of potential formulations may begin as the continuation of this project.

2. Structure-property correlations

The molecular weight of polysuccinimide (PSI) used for the preparation of polyaspartamide and poly(aspartic acid) (PASP) derivatives as well as hydrogels determines the properties of the formulations developed (e.g., electrospun matrices). To improve mechanical properties, we attempted to increase the molecular weight of PSI based on the literature data, particularly patents on the preparation methods of high-molecular-weight PSI. The molar amount of the catalyst and aspartic acid was varied to optimize the synthesis. The reaction product was characterized by nuclear magnetic resonance (NMR) and rolling ball viscometry. We found a feed composition that enabled us to prepare higher molecular weight polymers than our previous works. The result is supported by the reduced gelation time and increased stiffness of the gels made of this polymer.

Several advantages of polyaspartamides have not yet been exploited due to incomplete knowledge of structure-property relationships. We synthesized various polyaspartamides to establish the correlation between thermal properties and chemical composition. The structures of polyaspartamides were confirmed by NMR and FTIR measurements. The glass transition

temperature (Tg) of the PSI cannot be detected below its decomposition temperature. However, results of differential scanning calorimeter analysis showed that opening of the succinimide rings by hydrolysis or by the introduction of side groups induced a significant decrease in the Tg, which depended strongly on chemical composition. The thermal stability of the polymers strongly depended on the polymer composition: a clear correlation was found between the decomposition temperature and the concentration of the introduced side groups. Nonetheless, each polymer displayed high decomposition temperature, which can be beneficial in various methods, e.g., processing in molten state, spin coating, or other techniques.

3. *In vitro* safety of poly(aspartic acid) and polyaspartamide derivatives

Despite the high application potential of PASP and polyaspartamide derivatives, data on their biological activity have only been sporadically reported. *In vitro* safety evaluation of a chosen range of polymers was done in cooperation with Pharmidex Pharmaceutical Services Ltd. We focused on the biological safety of thiolated polymers and cationic polyaspartamides. A free radical damage assay was carried out to reveal the contribution of such polymers in the development of oxidative stress. PASP did not induce any lipid peroxidation, while thiolated or most cationic polymers had a slight antioxidant, protective effect. The biological activity on the cellular level was investigated by testing hemolysis and cell toxicity, performed on rat red blood cells and MDCK cells, respectively. Most polymers had negligible hemolytic or cytotoxic effects, excluding some cationic polymers with measurable and structure-dependent hemolysis and reduced cell viability. To model the effect of these polymers on metabolic pathways, their enzyme inhibition effect was determined on human CYP enzymes. Most derivatives did not affect enzymatic activity, but one polyaspartamide had a strong inhibiting effect. A strong correlation was established between the chemical structure, particularly the presence of thiol groups as well as the concentration of amine groups, and the biological activity of the polymers. These findings can help the design of newer derivatives with sufficient biological safety.

4. Effect of side groups on the hydrolytic stability of thiolated and disulfide cross-linked polyaspartamides

We described the controlled degradation of cysteamine-modified polyaspartamides bearing *N*,*N*-dimethyl-2-aminoethyl (DME), *N,N*-dimethyl-3-aminopropyl (DMP), or 2-hydroxyethyl (HE) side groups. The hydrolytic stability of polyaspartamides was studied at pH = 7.7 and at pH = 5.5 to model extracellular and endosomal conditions, respectively. All derivatives were stable at pH = 5.5, whereas the presence of DME side groups induces degradation depending

on the ratio of DME in polymer composition at pH = 7.7. The degradation rate could be adjusted by varying the DME, DMP, and HE content of polyaspartamides. Results of mass loss tests upon dialysis and molar mass change detected by size exclusion chromatography suggest that primarily main-chain degradation occurs in the presence of DME groups. Disulfide cross-linked hydrogels formed upon oxidation of thiol groups in polyaspartamides, and the stability of the hydrogels could also be controlled by their composition. The analysis of water-soluble degradation products by NMR showed that the dissolution of the hydrogels could not be explained only by main-chain degradation. The rearrangement of disulfide linkages also decreases the concentration of effective cross-linking points. The composition- and pH-dependent degradation of these polyaspartamides may find applications in biomedical fields. (B. Gyarmati, A. Mammadova, G. Stankovits, D. Barczikai, A. Szilágyi: Periodica Polytechnica-Chemical Engineering 2021, 65(2), 183-191. & B. Gyarmati, A. Mammadova, D. Barczikai, G. Stankovits, A. Misra, M.S. Alavijeh, K. László, A. Szilágyi: Polymer Degradation and Stability 2021, 188, 109577.)

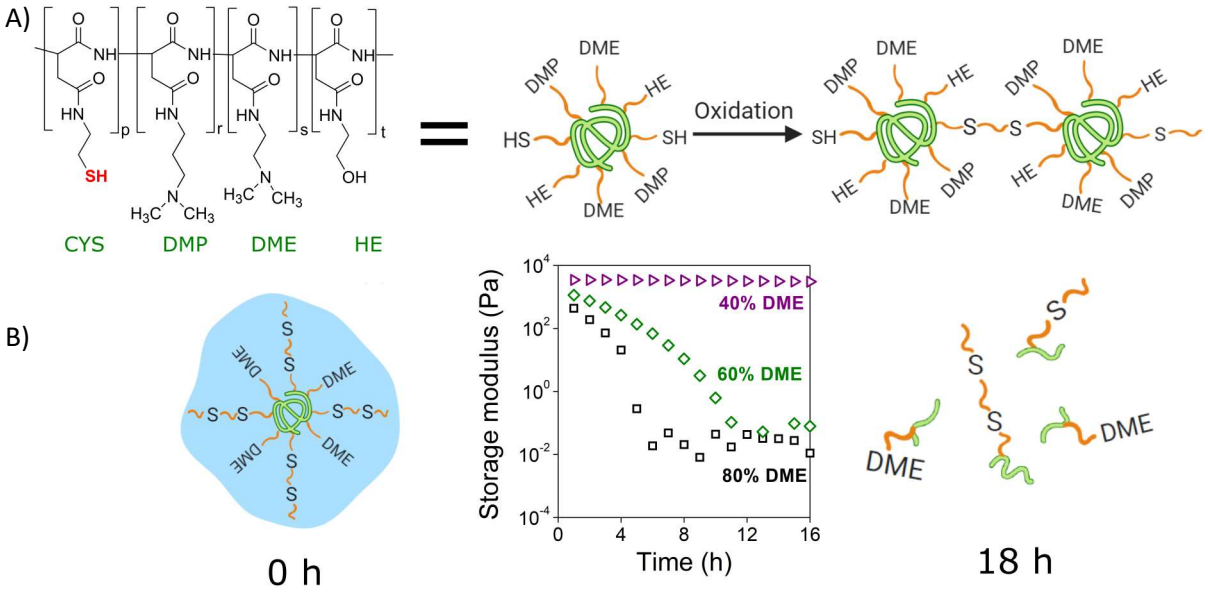

Figure 1. (A) General structure of thiolated polyaspartamides synthesized. (B) The change of storage modulus of hydrogels over time.

5. Fast-dissolving nanofibrous matrices prepared by electrospinning of polyaspartamides

Polymer matrices prepared by solvent electrospinning have great potential for drug delivery, but the complicated synthesis and functionalization of the currently used polymers results in limited chemical diversity. Owing to their versatile chemistry, polyaspartamides have recently attracted increased interest in various biomedical uses, such as drug delivery systems and scaffolding materials. The solubility of these polymers in organic solvents and water at specific pH values can be fine-tuned by their chemical composition, which was exploited to fabricate fast-dissolving electrospun matrices in ethanol with no additives. Various side groups were tested to control the solubility of the polymers as well as the morphology and moisture uptake of the matrices produced. Tertiary amine groups were immobilized to ensure high solubility around neutral pH, while modification with alkyl side groups limited moisture uptake. Finally, 3-(diethylamino)propyl and *n*-butyl side groups were used in equal amounts. The effect of viscosity, surface tension, and specific conductivity of polymer solutions on the fiber morphology was determined to optimize the conditions for preparing fibers with a narrow size distribution and large specific surface area. The polymer withstood the electrospinning process without any chemical degradation, as determined by FTIR, and was thermally stable. Furthermore, the matrices exhibited a glass transition temperature above room temperature. The complete release of vitamin B_{12} was observed within one minute due to the fast dissolution of the matrices in simulated salivary fluid at pH = 6.8, supporting the potential of the developed materials in oral drug delivery. (C. Németh, B Gyarmati, J. Gacs, D.V. Salakhieva, K. Molnár, T. Abdullin, K. László, A. Szilágyi: European Polymer Journal 2020, 130, 109624.)

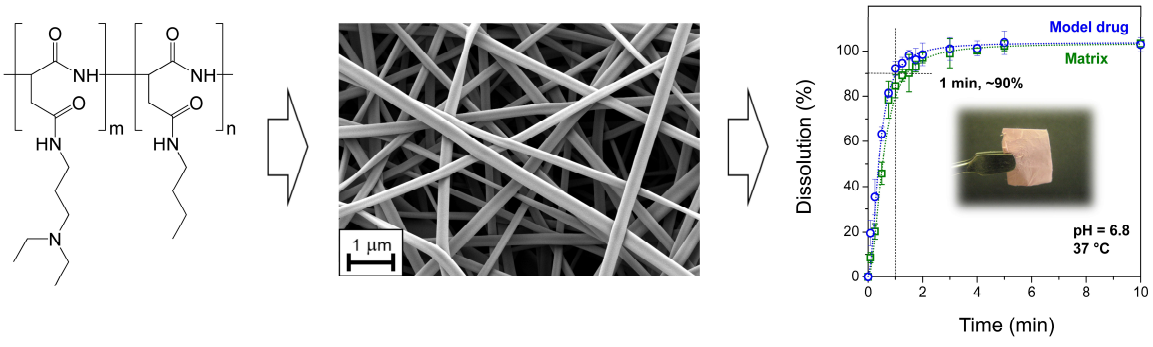

Figure 2. Nanofibrous matrices were successfully prepared from cationic or nonionic polyaspartamides by solvent electrospinning. The complete release of the model drug was observed within one minute due to the fast dissolution of the matrices in simulated saliva.

Rifampicin is one of the most effective antibiotics. The stability of rifampicin is low in aqueous solutions in the presence of oxygen. Its eye drops are prepared in pharmacies extempore with special care and stored in a freezer until application. We have targeted the synthesis of polyaspartamides, which can be the starting materials of a formulation in ophthalmic drug delivery. Polyaspartamides were synthesized by reacting PSI with amino-alcohols and subsequently with *n*-alkyl-amines. Polyaspartamide nanofibers containing rifampicin were fabricated by electrospinning, and ethanol was used as a solvent to avoid the degradation of the active compound. The morphology of electrospun matrices proved that uniform nanofibers could be produced with submicron-sized fiber diameters depending on the chemical composition. The chemical structure of polyaspartamides had a significant effect on the release rate of active pharmaceutical ingredients, opening up new possibilities for improving therapeutic efficiency with solid dosage forms. (D. Balogh-Weiser, C. Németh, F. Ender, B. Gyarmati, A. Szilágyi, L. Poppe: book chapter, InTech Open Access Publisher, ISBN: 978-1-78923-581-4, 2018, 135-147.)

6. Mucoadhesive interactions between synthetic polyaspartamides and porcine gastric mucin on the colloid size scale

Synthetic polyaspartamides with various functional side groups, including primary, secondary, tertiary amines, or carboxyl groups, were designed to explore the effect of chemical composition on polymer-mucin interactions. Since the molecular weight of the polymers and the degree of modification were identical for each derivative, the role of the functional groups could be evaluated. Chitosan was used as a control sample due to its strong interaction with mucin, primarily through electrostatic forces. Mucoadhesive interactions of the polymers with the aqueous dispersion of commercially available porcine gastric mucin were probed on the colloid size scale using various methods, including turbidimetric titration, dynamic light scattering, and zeta potential measurements. The charge of the polymers and the type of amine groups had a pronounced effect on the interactions. The interactions were further analyzed by partially screening them with either sodium chloride or urea. The results obtained allow us to classify these polymers in terms of *in vitro* mucoadhesive strength, which can be useful in designing mucoadhesive formulations. (B.Á. Szilágyi, A. Mammadova, B. Gyarmati, A. Szilágyi: Colloids and Surfaces B: Biointerfaces 2020, 194, 111219.)

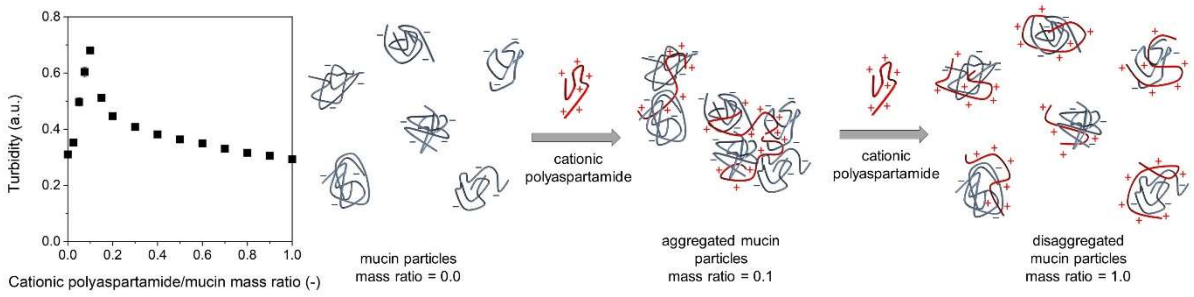

Figure 3. (A) Turbidimetric titration of porcine gastric mucin dispersion. (B) Changes in the colloidal size during the titration.

7. A robust mucin-containing poly(vinyl alcohol) hydrogel model for the *in vitro* characterization of mucoadhesion of solid dosage forms

The evaluation of mucoadhesive properties of dosage forms is crucial in the development of drug delivery systems. Mucoadhesion testing at a macroscopic scale needs a robust, convenient *in vitro* method. The most common method for solid formulations such as tablets is the tensile method. During the measurement, the formulation is brought into contact with mucosal tissue, and the force-displacement profile during detachment is measured. *Ex vivo* methods suffer from poor reproducibility, ethical problems, and difficulties in preparing and storing biological samples.

In our work, a synthetic poly(vinyl alcohol) (PVA) based mucosa-mimetic hydrogel has been developed. PVA serves as a hydrophilic inert matrix in which lyophilized mucin protein is dispersed to represent the properties of mucous membranes. We synthesized mucin-containing PVA hydrogel substrates (Muc/PVA) and mucin-free PVA hydrogels to measure the adhesion of polymer tablets. To preserve the functionality of mucin, the freeze-thaw method was used for gelation to avoid chemical cross-linking. Particle size distribution suggested a coating effect of PVA, which ensured even distribution of mucin particles in the matrix. The presence of mucin was confirmed also by FTIR measurements. Mechanical properties of mucin-containing and mucin-free PVA hydrogels were similar according to oscillatory rheology measurements. The adhesion of common mucoadhesive polymers (slightly cross-linked poly(acrylic acid), PAA and hydroxypropylmethylcellulose, HPMC) was tested with outstanding reproducibility on individual batches of hydrogels and qualitative agreement with ex vivo literature data. These polymers do not show attractive interactions with mucin according to turbidimetric titration,

unlike chitosan, a mucoadhesive polymer with positively charged functional groups potentially interacting with mucin. Chitosan showed enhanced adhesion on Muc/PVA substrate compared to its adhesion on mucin-free PVA, whereas HPMC as a neutral polymer displayed similar adhesion strength on both surfaces. PAA, as a negatively charged polymer, was less adhesive on Muc/PVA surface than on mucin-free PVA. These results could prove the electronic theory of mucoadhesion supported by the negative zeta potential of mucin and PAA, positive zeta potential for chitosan, and zero for HPMC in an aqueous medium. (B. Gyarmati, G. Stankovits, B.Á. Szilágyi, D.L. Galata, P. Gordon, A. Szilágyi: Colloids and Surfaces B: Biointerfaces 2022, 213, 112406.)

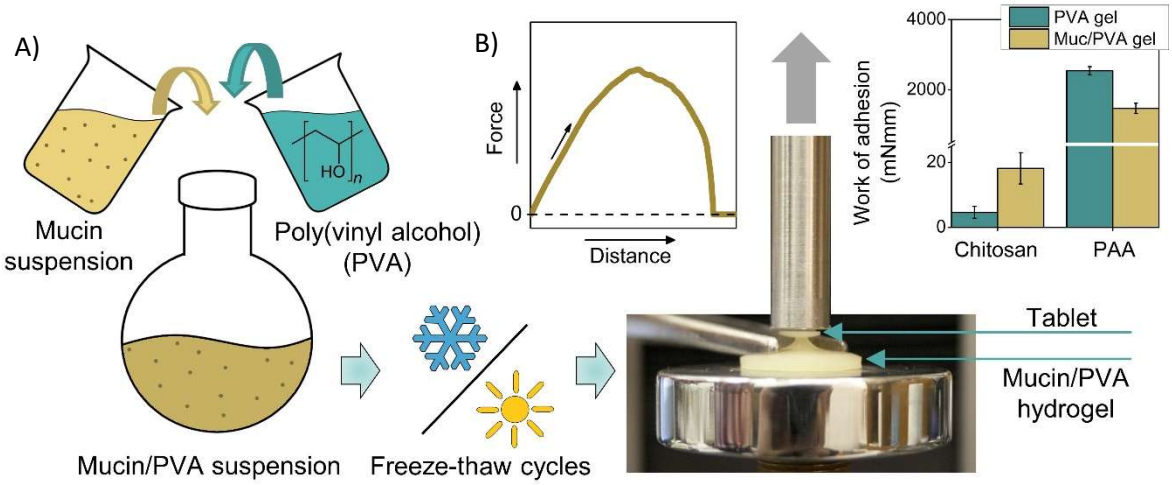

Figure 4. (A) The freeze-thaw method was used for the gelation. (B) On anionic Muc/PVA hydrogel higher adhesion was measured for cationic chitosan compared to the adhesion on mucin-free PVA gel, for anionic PAA showed stronger adhesion on pure PVA.

8. Thiolated cationic poly(aspartamides) with side group-dependent gelation properties for the delivery of anionic polyelectrolytes

In situ gellable polymers have potential applications as injectable formulations in drug delivery and regenerative medicine. Herein, thiolated cationic polyaspartamides were synthesised with two different approaches to correlate side group structure with gelation properties, gel strength and drug release kinetics. Cysteamine (CEA) was used as a thiolating agent to prepare thiolated cationic polyaspartamides groups with short thiolated side goups. As a new pathway, thiolactone chemistry was integrated with cationic modification of polyaspartamides to prepare thiolated derivatives with longer, flexible side groups using *N*-acetyl-*DL*-homocysteine (NAH) thiolactone. Both type of thiolated polyaspartamides could be converted into stiff hydrogels under mild reaction conditions through the oxidation-induced intermolecular disulfide formation. We confirmed that the longer side groups largely

accelerated gelation and the stiffness of the resultant hydrogels was higher than that of the CEA-modified counterparts. Both gelation time and stiffness could be adjusted by the degree of thiolation. PASP derivatives with a controlled concentration of anionic groups were entrapped in the hydrogels during the *in situ* gelation. We confirmed the electrostatic interaction between the linear anionic PASP derivatives and the cationic thiolated polyaspartamides. The release kinetics of PASP derivatives was followed by the fluorescent labelling of the encapsulated polymers, which showed that fully anionic PASP was entrapped completely in the hydrogels. In contrast, the reduction of the concentration of anionic groups caused a partial release of PASP derivatives. NAH and CEA-modified cationic polyaspartamide hydrogels showed distinct release rates indicating the interplay of electrostatic interactions and the chemistry of thiolated side groups. The developed *in situ* gellable materials have potential applications as injectable, fast gelling formulations to deliver macromolecular drugs of various charges, and possible non-enzymatic degradation of cationic poly(aspartamides) can be combined with these properties for further benefits. (A. Mammadova, B. Gyarmati, K. Sárdi, A. Paudics, Z. Varga, A. Szilágyi: J. Mater. Chem. B, 2022, 10, 5946–5957.)

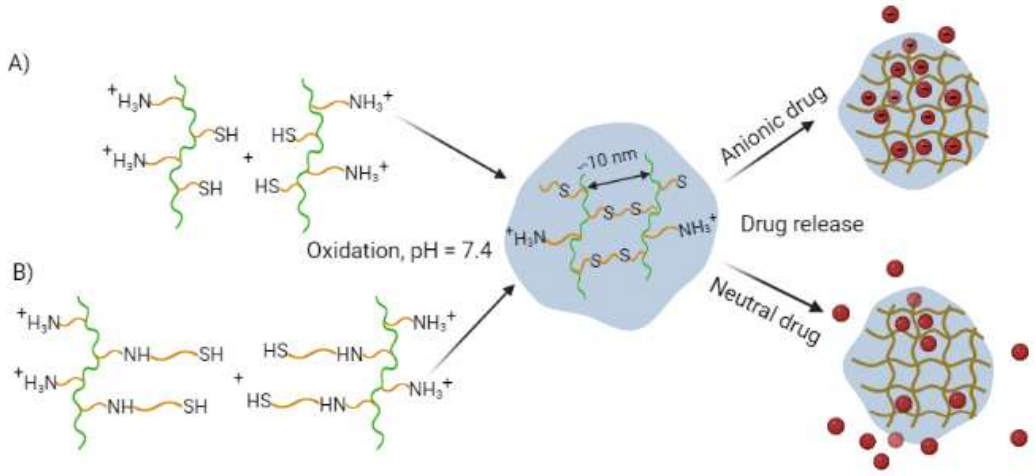

Figure 5. The effect of the side group structure on oxidation-induced gelation and release of anionic and neutral model drug. (A) Thiolation by CEA; (B) thiolation using NAH thiolactone.

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

In the topical treatment of ocular diseases, an *in situ* gelling mucoadhesive system can provide improved residence time while keeping the formulation installation easy and accurate due to its low initial viscosity. We aimed to synthesize a two-component, biocompatible water-

based liquid formulation of PASP derivatives showing *in situ* gelation upon mixing the components. Thiol-protected, preactivated derivative of thiolated PASP (PASP-SS-MNA) was synthesized by coupling of the free thiol groups of thiolated PASP (PASP-SH) with 6-mercaptonicotinic acid (MNA). The chemical structure of PASP-SS-MNA was confirmed by 1H NMR spectroscopy. The amount of protecting groups was determined by UV-Vis spectroscopy after releasing MNA groups and 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 in its aqueous dispersion was proven, indicating the mucoadhesive properties. Disulfide cross-linked hydrogels were formed *in situ* with no oxidizer 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 of the hydrogels. Hydrogels with a controlled amount of residual thiol − free or protected − groups can be prepared *in situ*. Swelling experiments showed that hydrogels with no residual thiol groups are stable in phosphate-buffered saline at pH = 7.4. In contrast, the presence of free thiol groups leads to the dissolution of the hydrogel at a rate depending on the thiol concentration. The biological safety of the polymers and 6-mercaptonicotinic acid was confirmed on Madin-Darby Canine Kidney (MDCK) cell line. Furthermore, compared to a conventional liquid formulation, a prolonged release of ofloxacin was observed at pH = 7.4, supporting the potential of the developed biopolymers in ophthalmic drug delivery. (B.Á. Szilágyi, B. Gyarmati, E.L. Kiss, M. Budai-Szűcs, A. Misra, E. Csányi, K. László, A. Szilágyi: Colloids and Surfaces B: Biointerfaces, under review.)

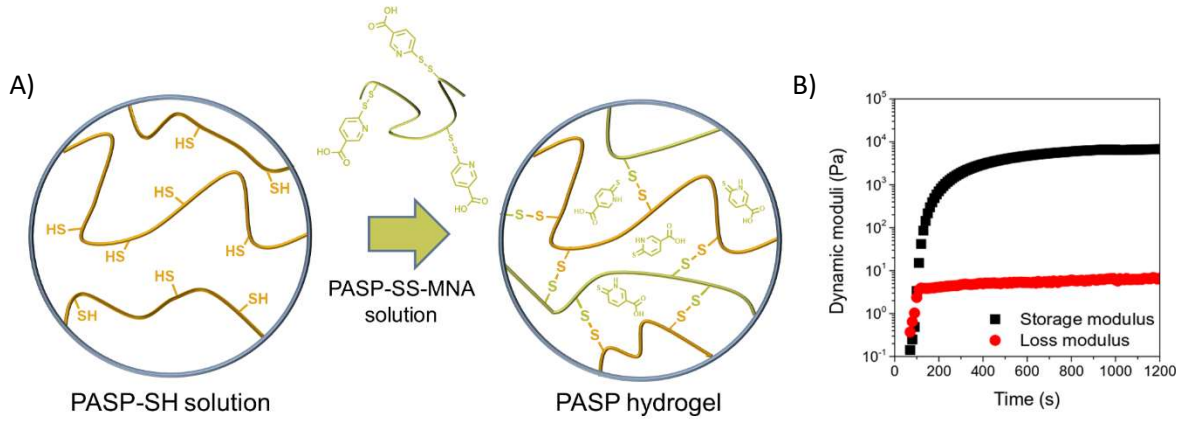

Figure 6. (A) The aqueous solutions of S-protected PASP and PASP-SH form chemically cross-linked hydrogels without additional reagents. (B) Dynamic moduli as a function of time.

10. Cyclodextrin-modified, thiolated poly(aspartic acid) as self-gelling formulation of lipophilic ophthalmic drugs: prednisolone and dexamethasone

Poor bioavailability of traditional eye drops might be overcome by using mucoadhesive thiolated polymer dosage forms providing a longer residence time as thiol groups can form disulfide linkages with cysteine-rich subdomains of the mucus layer. PASP-SH shows *in situ* gelling properties by oxidation of thiol groups to disulfide linkages or thiol/disulfide exchange reactions. A further goal was to increase the solubility and residence time of lipophilic ophthalmic drugs (prednisolone and dexamethasone) in hydrophilic mucoadhesive ophthalmic formulations.

PASP derivatives were synthesized with dual functionality to improve the solubility of lipophilic drugs and to achieve *in situ* gelation. First, amine-modified β-cyclodextrin (CD) was attached to PSI. Second, thiol functionalities were added by the reaction of cysteamine and succinimide rings. Finally, the PSI derivatives were hydrolyzed to the corresponding PASP derivatives to get water-soluble polymers.

Phase-solubility studies confirmed the complexation ability of CD-containing PASP derivatives. *In situ* gelation and the effect of the CD immobilization on this behavior were characterized by rheological measurements. It was established that the chemical bonding of the cyclodextrin molecule did not affect the complexation of the lipophilic drugs. In addition, the aqueous solution of the thiolated derivatives could be converted to stiff hydrogels within a few minutes. The storage modulus and complex viscosity displayed a more than 1000-fold increase during gelation, suggesting the possible increase of residence time on mucosal surfaces. The chemical immobilization of cyclodextrin modified the diffusion profile of prednisolone, and prolonged drug release was observed. (M. Budai-Szűcs, E.L. Kiss, B.Á. Szilágyi, A. Szilágyi, B. Gyarmati, S. Berkó, A. Kovács, G. Horvát, Z. Aigner, J. Soós, E. Csányi: Polymers 2018, 10(2), 199.)

In the case of dexamethasone, the solubilizing effect of CD was confirmed by kinetic solubility measurements, whereas *in vitro* corneal permeability assay (corneal-PAMPA) measurements were performed to determine *in vitro* permeability and flux values. The effect of the PASP derivatives on permeation strongly depended on chemical composition and polymer concentration. In conclusion, PASP derivatives were found to be promising excipients for the development of eye drops, but several factors must be considered to achieve optimal viscosity increase or *in situ* gel formation for longer residence time, enhanced permeability, and improved bioavailability. (B. Gyarmati, G. Dargó, B.Á. Szilágyi, A. Vincze, R. Facskó, Mária

Budai-Szűcs, E.L. Kiss, L. Szente, A. Szilágyi, G. T. Balogh: Eur. J. Pharm. Biopharm. 2022, 174, 1–9.)

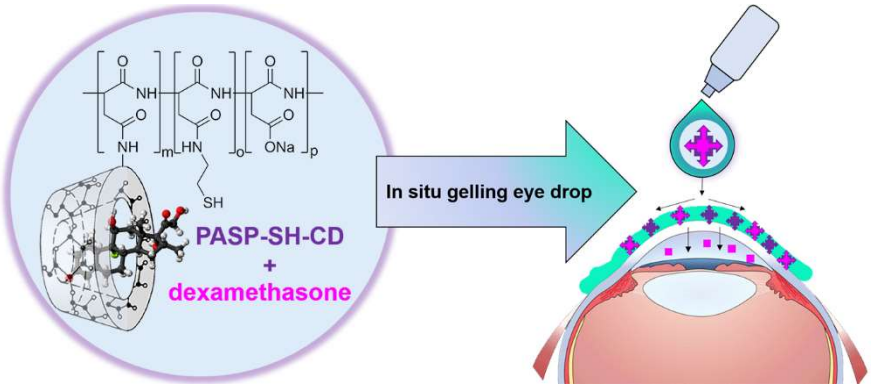

Figure 7. Thiolated and cyclodextrin-functionalized self-gelling PASP solubilizes dexamethasone. These PASP derivatives are promising excipients for the development of eye drops.

11. Composite beads of silica gel, alginate, and poly(aspartic acid) for the immobilization of a lipase enzyme

As a spin-off of our research with thiolated polymers, a method was developed to immobilize Candida antarctica lipase B (CaLB). CaLB was first adsorbed onto mesoporous silica gel, followed by the entrapment of the enzyme-loaded particles in the interpenetrating network of PASP-SH and sodium alginate cross-linked by Zn^{2+} ions to obtain spherical beads. Finally, the enzyme and the particles were stabilized by covalent cross-linking with a bisepoxide cross-linker, which established chemical bonds between the thiol groups of PASP-SH, the amine groups on the silica gel particles, and the functional groups of the CaLB. We proved that the activity of the enzyme was preserved while reusability was increased significantly in comparison with CaLB physically adsorbed on silica gel particles. The thermal stability of the immobilized enzymes was significant. (E. Krisch, D. Balogh-Weiser, J. Klimkó, B. Gyarmati, K. László, L. Poppe, A. Szilágyi: Express Polymer Letters, 2019, 13(6), 512-523.)

12. Other results

We successfully purchased a spectrofluorometer (FS5 Spectrofluorometer, Edinburgh Instruments) which have been replaced the previous spectrofluorometer in our department. In addition to our own work, we have already successfully used the device in other projects. NKFI grant ID was included in the acknowledgments in the following publications: A. Paudics, S. Farah, I. Bertóti, A. Farkas, K. László, M. Mohai, G. Sáfrán, A. Szilágyi, M. Kubinyi: Applied Surface Science, 2021, 541, 148451. & A. Paudics, D. Hessz, M. Bojtár, B. Gyarmati, A. Szilágyi, M. Kállay, I. Bitter, M. Kubinyi: Molecules 2020, 25(21), 5111.

13. Conclusions

Within this project, the following results are highlighted:

− The hydrolytic stability of thiolated cationic polyaspartamides is controlled by side group ratio. Linear thiolated and disulfide cross-linked cationic polyaspartamides are mainly degraded by main-chain fragmentation. The rearrangement of intermolecular disulfide bridges disintegrates cationic polyaspartamide hydrogels.

− Nanofibrous matrices from cationic and neutral polyaspartamides were prepared by solvent electrospinning using ethanol, a green and pharmaceutically accepted solvent. We showed that drug molecules could be encapsulated into the polyaspartamide matrices, and the release rate of the active molecules can be tuned by the polymer composition.

− We found mucin particles aggregate in aqueous dispersion upon adding cationic polyaspartamides due to a bridging effect. The phenomenon is primarily caused by the electrostatic interaction between the positively charged side groups of polyaspartamides and mucin particles bearing a net negative charge. The strongest interaction was found in the case of the polyaspartamide with primary amine pendant groups.

− We synthesized mucin-free PVA and mucin-containing PVA hydrogel substrates to measure the adhesion of polymer tablets.

− As a new pathway, thiolactone chemistry was integrated with cationic modification of polyaspartamides to prepare thiolated derivatives with longer, flexible side groups using *N*-acetyl-*DL*-homocysteine (NAH) thiolactone. The developed *in situ* gellable materials have potential applications as injectable, fast-gelling formulations to deliver macromolecular drugs of various charges.

− We developed a synthesis method to prepare S-protected PASP-SH derivatives by conjugating free thiol groups of the polymer with 6-mercaptonicotinic acid. The aqueous solution of S-protected PASP yields cohesive, chemically cross-linked hydrogels upon the addition of the aqueous solution of thiolated poly(aspartic acid) with no additional reagents. Prolonged release of ofloxacin was observed from the hydrogels.

− PASP derivatives modified with thiol and β-cyclodextrin moieties simultaneously were synthesized. The polymers formed an inclusion complex with lipophilic drugs such as prednisolone and dexamethasone. The stability constant of the complex formation of the polymer does not differ significantly from that of the β-cyclodextrin small molecule. The β-cyclodextrin moieties did not hinder the sol-gel transition of the polymer.