Scientific Papers of the University of Pardubice, Series A; Faculty of Chemical Technology
24 (2018) 49–58.

University of Pardubice
Faculty of Chemical Technology

Preparation of hyaluronan nanoparticles using different cross-linking strategies

Jitka Kašparová[1], Lucie Korecká[1], Zuzana Bílková[1], Jiří Palarčík[2], Anna Lierová[3], Zuzana Šinkorová[3], and Lenka Česlová[4]*

[1] *Department of Biological and Biochemical Sciences, The University of Pardubice, CZ–532 10 Pardubice, Czech Republic*

[2] *Institute of Environmental and Chemical Engineering, The University of Pardubice, CZ–532 10 Pardubice, Czech Republic*

[3] *Department of Radiobiology, University of Defence in Brno, CZ–500 02 Hradec Kralove, Czech Republic*

[4] *Department of Analytical Chemistry, The University of Pardubice, CZ–532 10 Pardubice, Czech Republic*

Received: June 4, 2018; Accepted: July 28, 2018

Nowadays, the use of drug-delivery systems becomes more and more popular. The use of the supporting medium for drug substances may serve to delay and control the drug release. Biodegradable polymers, such as hyaluronic acid (HA), represent the most natural materials beneficially used for such systems. In this work, we focused on the preparation of hyaluronic acid nanoparticles using low-molecular weight HA (LMW-HA; 11 and 30 kDa) which is rarely used because the size of the obtained particles is usually higher than those of the high-molecular weight form. To verify the suitability of LMW-HA for the preparation of nano-scale particles, different cross-linking strategies were chosen. The hydrodynamic diameters of prepared nanoparticles and the structure compactness were then compared. Adipic acid dihydrazide was used as a conventional cross-linker which leads to monodisperse nanoparticles (186 nm, 255 nm resp.) and compact structure. The use of bis(3-aminopropyl)amine as a novel approach for covalent cross-linking of HA results in the particles with higher hydrodynamic diameters (592 nm, 317 nm resp.) and with less compact structure. Both strategies were then combined with a goal to reduce the hydrodynamic diameters to the nanoscale format and tighter structure. The results obtained has shown a slight improvement (208 nm, 286 nm resp.), but with negligible effect on the structure of particles.

Keywords: Hyaluronic acid; Nanoparticles; Cross-linking

* Corresponding author, ✉ Lenka.Ceslova@upce.cz

Introduction

Nanomaterials based on biocompatible and biodegradable materials are often used as drug delivery systems [1–3]. One of these materials is hyaluronic acid (HA) which occurs naturally in human or animal body. HA can reach different molecular weight based on a number of repeating disaccharide structure units consisting of D-glucuronic acid and *N*-acetyl-D-glucosamine connected via *β*-linkages in alternating position (1,3- and 1,4-). In human body, the high-molecular weight form (HMW-HA) attaining up to 8000 kDa is predominantly presented [4–6].

Since the hyaluronic acid had been discovered, its physiological aspects were extensively studied. Thanks to its hydrophilic, moisturizing, and immunosuppressive properties, HA is often used in pharmaceutical industry, especially in its HMW-HA form [7]. Less frequently occurring low-molecular weight forms (LMW-HA) or even oligosaccharides are responsible for immune response activation because these forms are produced in higher amounts under stress conditions by the HMW-HA degradation, serving as endogenous danger signals [5]. According to these contradictory effects of LMW- or HMW-HA forms, the selection of HA substrate could be crucial for designing of biodegradable nanomaterials with distinct physiological properties. As the molecular weight of HA predicts its behaviour in living organism, in the case of *in vitro* conditions, the molecular weight of HA predicts the size of the products formed. Regarding nanomaterials, there were observations that smaller nanoparticles can be prepared from HMW-HA forms like that of the LMW-HA type [8]. This work deals with the preparation of nanoparticles by different cross-linking strategies from LMW-HA (11 kDa and 30 kDa) that is usually less capable to form small particles in the nanoscale format. Our goal was to prepare the HA nanoparticles with hydrodynamic size as low as possible, ideally lower than 100 nm, which meets the criterion of nanoscale. Lower size of particles is beneficial for its easier administration, distribution in organism, influencing also a potential cellular uptake of nanoparticles. The main factors of cellular uptake and the effect of cytotoxicity are: (i) size and shape of nanoparticles, (ii) surface charge, and (iii) hydrophobicity. For phagocytes, the strong cytotoxic effect is exhibited by microparticles in contrast to nanoparticles [10]. Berkland and Fakhari (2013) have discovered that the nanoparticle size depends on the molecular weight of used HA. With increased molecular weight of the HA used, a lesser size of the prepared nanoparticles can be anticipated. The cited authors used the HA molecule with highest molecular weight of 1500 kDa, achieving the nanoparticles with size of about 87 ± 2.5 nm. As a LMW-HA, the HA with size 17 kDa was used for the preparation of nanoparticles with size of about 188 ± 24 nm [8]. As the most common cross-linking agent, bifunctional non-toxic agent, adipic acid dihydrazide, is usually recommended. Besides, the implementation of bifunctional cross-linker a bis(3-aminopropyl)amine as an alternative cross-linking agent was exploited in this work.

Materials and methods

Chemicals

Hyaluronic acid (HA; 11 kDa, 30 kDa) was product of Contipro (Dolni Dobrouc, Czech Republic). Adipic acid dihydrazide, N-(3-Dimethyl-aminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), bis(3-aminopropyl)-amine, hyaluronan lyase from Streptococcus pyogenes, and dialysis tubing cellulose membrane (avg. flat width 25 mm) were purchased from Sigma-Aldrich. Magnetic macroporous bead cellulose with fixed iminodiacetic acid (with diameter of 80–100 μm) was purchased from Iontosorb (Usti nad Labem, Czech Republic), acetone (reagent grade) supplied by Penta (Prague, CZ).

Preparation of hyaluronic acid nanoparticles using adipic acid dihydrazide

For preparation of HA nanoparticles, the already published protocol with use of adipic acid dihydrazide as crosslinking agent [8] was used with slight modifications. Briefly, 1.36 mL acetone was added to 2 mg HA (2.5 mg mL^{-1} solution) and stirred for 15 min at room temperature. Then, the addition of 1 mg of EDAC and 1 mg adipic acid dihydrazide followed when dosed dropwise and the mixture was stirred for 30 min at room temperature. Afterwards, the next addition of acetone was performed stepwise in three repetitions (0.81 mL per each step) with in-between 30 min incubation to reach a 300/80 acetone/water ratio (w/w). Surplus acetone was then removed by evaporation and the residual volume dialyzed against 0.9% NaCl overnight at room temperature while gently rotated. The hydrodynamic diameter and size distribution of the nanoparticles prepared were measured.

Preparation of hyaluronic acid nanoparticles using bis(3-aminopropyl)amine

Hyaluronic acid nanoparticles with bis(3-aminopropyl)amine (BAPA) as cross-linking agent were prepared by carbodiimide reaction. HA solution (2.5 mg/mL) was prepared by dissolution of solid HA in deionized water.

BAPA solution (1%; v/v) was adjusted to pH 5.5 by addition of 10 M NaOH. To prepare 3 mg nanoparticles, an amount of 1.2 mL HA solution was pre-activated by adding 2.5 mg EDC dropwise. Afterward, addition of BAPA (94 μL) followed — again, dropwise — during stirring. Subsequently, a 30 min incubation (gentle mixing) followed and dialysis against 0.9% NaCl to remove residual chemical reagents took place. The hydrodynamic diameter and size distribution of the nanoparticles prepared were measured.

Preparation of the double-crosslinked hyaluronic acid nanoparticles using adipic acid dihydrazide and bis(3-aminopropyl)amine

At first, the HA nanoparticles were prepared by the protocol described above using BAPA. Then, the prepared nanoparticles underwent the subsequent cross-linking with AAD to tighten and stabilize their structure following the above procedure. The hydrodynamic diameter and size distribution of the resultant nanoparticles were measured.

Immobilization of hyaluronan lyase onto magnetic beads

Hyaluronan lyase from *Streptococcus pyogenes* (SpHL) was immobilized by standard carbodiimide method with some modifications [9]. Magnetic beads (with macroporous bead cellulose and fixed iminodiacetic acid, 200 μL settled beads) were washed to remove storage stabilizers (10 times per 1 mL, deionized water). Afterwards, the addition of 0.4 M $CoCl_2$ followed by 90 min incubation, gentle agitation, and subsequent washing (5 times with deionized water) after incubations. For immobilization, the SpHL was applied in an amount of 100 μg per 200 μL settled beads in deionized water and incubating the mixture at 4 °C overnight. Finally, the magnetic beads were washed 10 times by deionized water. The enzyme activity was evaluated as 0.5 mU mL^{-1} settled beads and determined as amount of enzyme catalyzing the conversion of 1 μmol substrate per min (U), by using a spectrophotometric assay (with wavelength of 230 nm), assuming a molar absorption coefficient of 5.5×10^3 L mol^{-1} cm^{-1}.

Verification of structure compactness of hyaluronic acid nanoparticles by enzymatic degradation

Each portion of the nanoparticles prepared (0.25 mg) was treated with immobilized SpHL (50 μL, equal to app. 0.054 μU mL^{-1} settled beads) during 16 h incubation at 37 °C; reaction volume being 1.5 mL. Then, the immobilized SpHL was removed by application of magnetic field to achieve a pure solution of HA nanoparticles. Potential degradation products were monitored spectrophotometrically by employing a UV/Vis Biochrom spectrophotometer (model "Libra S22"; Biochrom, UK). Absorbance at 230 nm was monitored, corresponding to the product of enzymatic cleavage (disaccharide products with unsaturated bond). Simultaneously, a soluble native HA underwent the same procedure as a control sample. Briefly, the soluble HA in an amount of 0.25 mg was dissolved in 0.9% NaCl to total volume 1.5 mL with subsequent addition of 50 μL of SpnHl functionalized beads (16 h incubation at 37 °C).

Measurement of hydrodynamic diameter and size distribution

Hydrodynamic diameter and size distribution were measured using Dynamic Light Scattering (DLS) by the respective instrument (model "Zetasizer Nano ZS"; Malvern Instruments, UK). All the samples were measured in an amount of 0.25 mg of nanoparticles. The appropriate volume was adjusted by solvent to a total volume of 1.5 mL. Before measurement, all the samples were tempered to room temperature.

Results and discussion

The aim of this work was to prepare the nanoparticles of hyaluronic acid from rarely used low-molecular weight HA using different cross-linking strategies. As covalent cross-linkers, the two types of agents were used: the most conventional adipic acid dihydrazide and hitherto unusized bis(3-aminopropyl)amine. In the case of AAD cross-linking, nanoparticles based on 11 kDa or 30 kDa HA achieved hydrodynamic diameter measured by DLS at a value of 186 nm or 255 nm, respectively. Cross-linking with the use of BAPA led to formation of nanoparticles with a wider distribution of hydrodynamic diameters. From 11 kDa HA, the nanoparticles were prepared in the size of 592 nm, from 30 kDa HA then 317 nm (see Fig. 1). In this case, the dependency of particles size on the initial size of substrate was estimated by the first panel of experiments. Combination of two aforementioned cross-linking agents resulted in decrease in the size of original BAPA nanoparticles for both HA initial sizes. In case of 11 kDa, the size decreased from 592 nm down to 208 nm, whereas for 30 kDa from 317 nm to 286 nm.

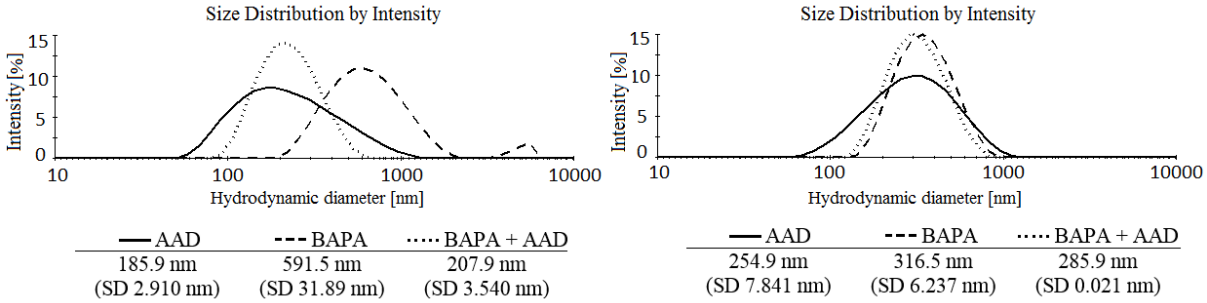

Fig. 1 *Hydrodynamic diameters of prepared nanoparticles by various protocols measured by DLS*

A) Initial HA size 11 kDa. B) Initial HA size 30 kDa. Solid line: cross-linking via adipic acid dihydrazide (AAD); dashed line: cross-linking via bis(3-aminopropyl)amine (BAPA); dotted line: cross-linking via bis(3-aminopropyl)amine (BAPA) in first step and followed with subsequent cross-linking via adipic acid dihydrazide (AAD). Inserted tables: hydrodynamic diameters of prepared nanoparticles. SD – standard error of mean (n = 6).

Prepared nanoparticles were then tested with respect to its structure compactness using the immobilized hyaluronan lyase (SpHL) as one of the enzyme which specifically degrades the HA. As a control of successful fragmentation, the soluble HA substrate was used. In case of HA cross-linked with AAD based on both 11 kDa and 30 kDa HA, the enzymatic treatment showed no changes in absorption spectrum; therefore, its structure showed high compactness and the surface smoothness. Otherwise, for the BAPA nanoparticles, the resistance toward enzymatic treatment was partial with an evident increase in absorption spectra at 230 nm, indicating a partial degradation of HA (see Fig. 2 and 3). In case of insufficient cross-linking of HA leading to incompactness of its structure, the free parts of HA chains having stuck out of the nanoparticle core are accessible to enzyme degradation. The double cross-linking by using BAPA + AAD led to the improved resistance toward enzyme treatment compared to BAPA nanoparticles themselves because a partial fragmentation was not as evident as in the case of BAPA nanoparticles.

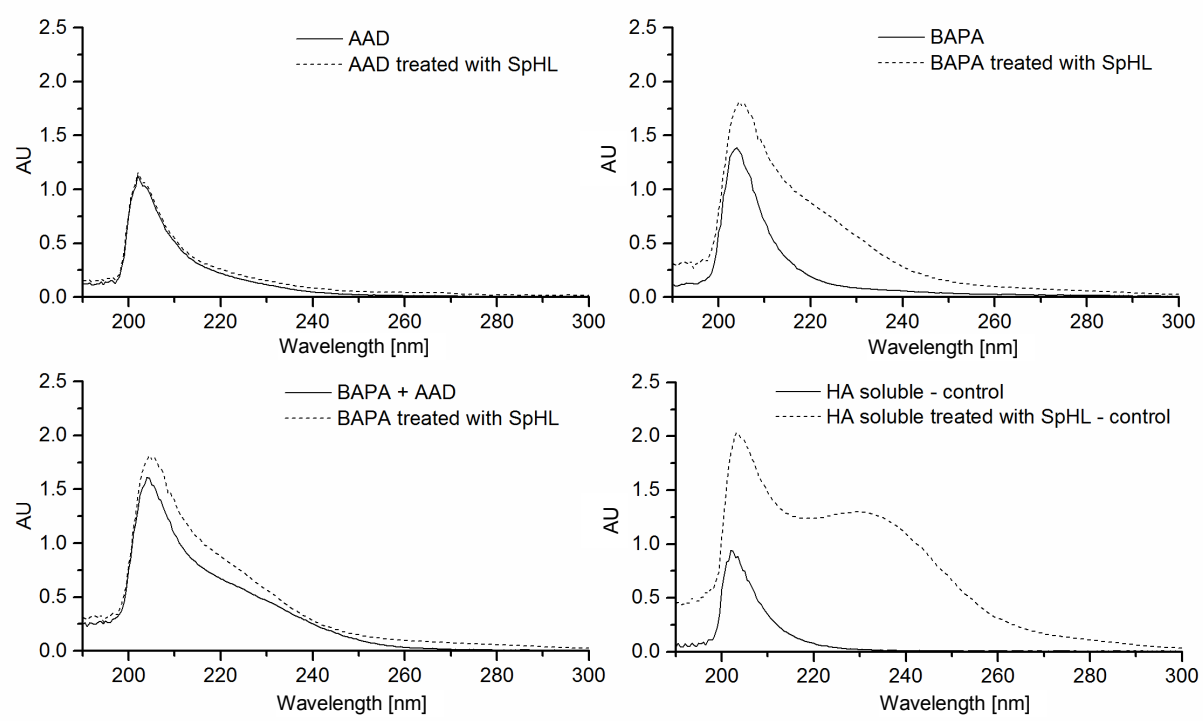

Fig. 2 *Verification of structure compactness of HA nanoparticles prepared from 11 kDa against enzymatic treatment with immobilized hyaluronan lyase using UV spectrometry*

Nanoparticles (250 μg) were treated with immobilized hyaluronan lyase (SpHL; 50 μL of settles beads, enzyme activity 0.06 μU mL^{-1} settles beads). Reaction was performed in 0.9% NaCl (1 mL total volume) overnight at room temperature. Solid lines: native nanoparticles; dashed lines: treatment with SpHL. As a control of successful enzymatic degradation, the soluble HA was used (250 μg).

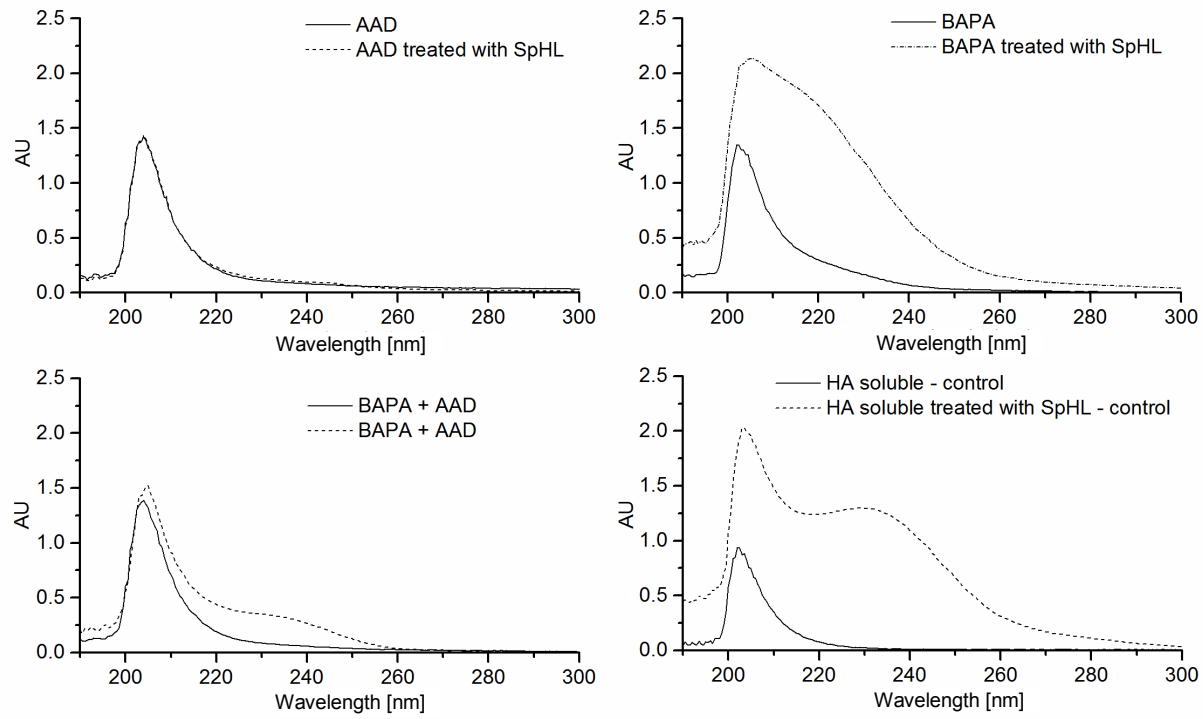

Fig. 3 *Verification of structure compactness of HA nanoparticles from 30 kDa against enzymatic treatment with immobilized hyaluronan lyase using UV spectrometry*

Nanoparticles (250 μg) were treated with immobilized hyaluronan lyase (SpHL; 50 μL of settles beads, enzyme activity 0.06 μU mL^{-1} settles beads). Reaction was performed in 0.9% NaCl (1 mL total volume) overnight at room temperature. Solid lines: native nanoparticles; dashed lines: treatment with SpHL. As a control of successful enzymatic degradation, the soluble HA was used (250 μg).

The hydrodynamic diameters of samples measured after enzymatic fragmentation confirmed the results of spectrophotometric measurements. The BAPA cross-linked nanoparticles (Fig. 4A and B), contained in its structure free chains of HA and cleaved by enzyme, when the structure of nanoparticles was in favour of higher sizes. The same results were achieved after subsequent cross-linking with AAD (Fig. 4C and D); hydrodynamic diameters changing as well.

These results suggested that only cross-linking with AAD had led to a compact structure with no accessible cleavage sites enabling the enzymatic degradation. In case of BAPA nanoparticles, the structure seemed to be less compact and having probably contained some free HA chains stuck out of the nanoparticle core accessible for the enzyme degradation and proved by the production of HA fragments using the spectrophotometric assay. Results of enzyme treatment confirmed that the compactness of nanoparticles was not complete due to the production of HA fragments monitored spectrophotometrically. This insufficient cross-linking was slightly improved by addition of AAD facilitating the subsequent cross-linking of the remaining free HA chains, which resulted in the decrease of hydrodynamic diameters. However, the spectrophotometric measurement confirmed the persisting ability of the enzyme to partially degrade the nanoparticle structure.

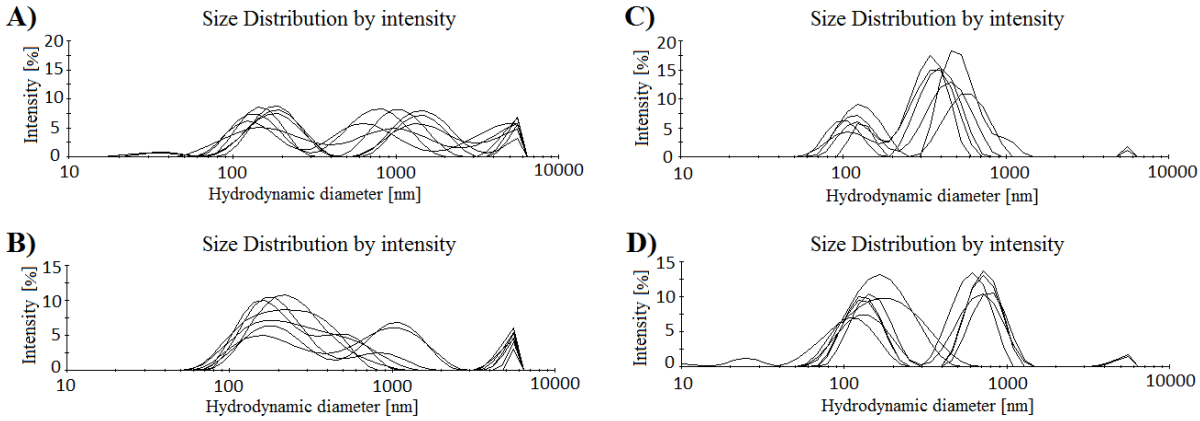

Fig. 4 *Monitoring of hydrodynamic diameters changes of prepared nanoparticles after enzymatic treatment with immobilized hyaluronan lyase*

A) Nanoparticles cross-linked via bis(2-aminopropyl)amine (BAPA), initial HA substrate size: 11 kDa; B) Nanoparticles cross-linked via bis(2-aminopropyl)amine (BAPA), initial HA substrate size: 30 kDa; C) Nanoparticles cross-linking via bis(3-aminopropyl)amine (BAPA) in first step and followed with subsequent cross-linking via adipic acid dihydrazide (AAD), initial HA substrate size 11 kDa; D) Nanoparticles cross-linking via BAPA in first step and followed with subsequent cross-linking via AAD, –initial HA substrate size 30 kDa

Conclusions

Preparation of the nanoparticles of hyaluronic acid (HA) was performed using low-molecular weight HA (LMW-HA; 11 kDa and 30 kDa). This initial size of HA substrate is rarely used for nanoparticles preparation and more challenging due to the obtainable higher size compared to that when using conventional high-molecular HA forms. Here, the nanoparticles were prepared by three methods / protocols differing in the cross-linking agent applied. As a conventional cross-linking agent used in numerous scientific papers, adipic acid dihydrazide (AAD) was used. As previously unused alternate cross-linking agent, bis(3-aminopropyl)amine (BAPA) was chosen. Third protocol combined BAPA and AAD — the latter as subsequent cross-linker — to tighten the structure and to decrease the final size of nanoparticles.

Our results have suggested that cross-linking via AAD is still the best option for achieving the monodisperse character of nanoparticles. With the use of LMW-HA substrate, the prepared nanoparticles ranged in hydrodynamic diameters in-between 186 nm and 592 nm (for 11 kDa HA substrate), or 255 nm and 317 nm (for 30 kDa HA substrate), respectively. The stability and compactness of the prepared nanoparticles by all the cross-linking strategies was monitored by enzymatic treatment with immobilized hyaluronan lyase, an enzyme responsible for specific HA degradation. AAD nanoparticles showed a resistance to enzymatic treatment while BAPA nanoparticles underwent a partial fragmentation evident from the corresponding absorption spectra, as well as

hydrodynamic diameters revealing the presence of more abundant populations of nanoparticles. After the subsequent cross-linking with AAD, the structure was much more compact, but some partial fragmentation could be observed as well. Thus, a step-by-step cross-linking of HA offered an option to modify the already prepared nanoparticles.

In drug-delivery systems, the subsequent cross-linking with two agents seems to be advantageous for possible locking of drugs in the structure of primary HA, which enables to attain a better resistance toward degradation in organism and may prolong the circulation and desired gradual drug release.

Acknowledgement

Financial supports from the University of Pardubice, Faculty of Chemical Technology (project SGS_005_2018) and from the European Union funding (projects CZ.02.1.01/0.0/0.0/17_048/0007421 and MSMT-20104/2017) are gratefully acknowledged.

References

[1] Hosseinkhani H., He W.-J., Chiang C.-H., Hong P.-D., Yu D.-S., Domb A.J., Ou K.-L.: Biodegradable nanoparticles for gene therapy technology. *Journal of Nanoparticle Research* **15** (20013) 1–15.

[2] Mahapatro A., Singh D.K.: Biodegradable nanoparticles are excellent vehicle for site directed *in-vivo* delivery of drugs and vaccines. *Journal of Nanobiotechnology* **9** (2011) 1–11.

[3] Ganesh S., Iyer A.K., Morrissey D.V., Amiji M.M.: Hyaluronic acid based self-assembling nanosystems for CD44 target mediated siRNA delivery to solid tumors. *Biomaterials* **34** (2013) 3489–3502.

[4] Cowman M.K., Lee H.-G., Schwertfeger K.L., McCarthy J.B., Turley E.A.: The content and size of hyaluronan in biological fluids and tissues. *Frontiers in Immunology* **6** (2015) 1–8.

[5] Stern R., Asari A, Sugahara K.: Hyaluronan fragments: An information-rich system. *European Journal of Cell Biology* **85** (2006) 699–715.

[6] Vigetti D., Karousou E., Viola M., Deleonibus S., De Luca G., Passi A.: Hyaluronan: Biosynthesis and signalling. *Biochimica et Biophysica Acta, General Subjects*. **8** (2014) 2452–2459.

[7] Stern R., Kogan G., Jedrzejas M.J., Šoltés L.: The many ways to cleave hyaluronan. *Biotechnology Advances* **25** (2007) 537–557.

[8] Berkland C., Fakhari A.: Hyaluronic acid particles and their use in biomedical applications. *WO 2013109959 A1* (2013).

[9] Přikryl P., Horák D., Tichá M., Kučerová Z.: Magnetic IDA-modified hydrophilic methacrylate-based polymer microspheres for IMAC protein separation. *Journal of Separation Science* **29** (2006) 2541–2549.

[10] Fröhlich E.: The role of surface charge in cellular uptake and cytotoxicity of medical nanoparticles. *International Journal of Nanomedicine* **7** (2012) 5577–5591.