University of Szeged, Faculty of Pharmacy
Department of Pharmaceutical Technology

Summary of Ph.D. thesis

Preparation and investigation of cross-linked hyaluronic acid nanoparticle systems

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1 Introduction

Nanoparticles and nanomedicines are increasingly applied in the manufacture of pharmaceuticals and cosmetic products. Nanocarrier-based nanomedicines are comprised of polymers, liposomes, dendrimers, quantum dots, nanotubes and their combinations. These systems form the basis for the introduction of personalized medicine and are powerful tools for the detection of diseases and diagnoses coupled with highly-effective targeted therapy.

Some major biopolymers are also used in medicine, such as alginic acid, hyaluronic acid, chitosan and polyglutamic acid, and nanosystems prepared from these biopolymers. Biopolymers are characterized by favourable biological properties, pharmaceutical technology and nanotechnological applications. The applied and intended pharmaceutical formulations will be described. One of the challenging aims of research at present is to achieve targeted drug delivery with minimum side-effects. The physical, chemical and pharmacokinetic properties of the active pharmaceutical ingredient can be changed through the use of nanotechnology. Combination therapy can be developed with nanotechnology systems. Nanotechnology is able to wrap up giant molecules via the covalent cross-linking of valuable groups. Certain nanoparticles are below 100 nm size and their original properties are not lost. The body does not handle them as foreign molecules and the nanomolecules display self-organizing ability to enhance stable and effective use.

This work describes and discusses the successful preparation and characterization of stable nanoparticulate systems based on hyaluronic acid (HA). The effects of the concentration of the HA, the cross-linking ratio and the applied medium were investigated. A stable cross-linked HA-based nanoparticulate semisolid preparation was also investigated in comparison with a hydrogel containing linear HA.

2 Aims

The aim of my work was to prepare and investigate a cross-linked hialuronic acid nanoparticle (CLHA) system produced through the reaction between HA and 2,2'(ethylenedioxy)bis(ethylamine), using a water-soluble carbodiimide (CDI) as condensation agent in aqueous medium.

In the first part of the investigation, the matrix form was synthetized in order to know the common effects caused by the different variables.
The main steps in our experiments were as follows.

- Parallel reactions under the same conditions and with the same parameters. In this case, the variables were the medium, the concentration of HA and the amount of the diamine and CDI. The standard parameters were the volume of the medium (50 ml), the dissolved HA was adjusted to the same pH, the preparation of the diamine solution, the mixing and stirring times, the temperatures applied, and the dialysis and freeze-drying methods.
- Confirmation of the existence of HA nanoparticles by transmission electron microscopy (TEM).

In the second part of the investigation, the manufacturing process was studied, as follows.

- Comparison of the particle sizes, particle size distributions (PSDs), molecule weight distributions and rheological measurements at different phases of the technology.
- Determination of the effects of the salt concentration on the size of the particles.
- Determination of the stability of these nanoparticle systems in different media and at different feed ratios.

In the third part of the investigation, HA-based nanoparticulate semisolid preparations were compared with a hydrogel containing linear HA.

- Measurements were made of the rheological properties, and hydration, and irritation effects.
- Skin penetration abilities were determined by using *in vitro*, *ex vivo* and *in vivo* skin models.

3 Materials and methods

3.1 Materials

HA (M_W = 4350 kDa) was obtained from Gedeon Richter Ltd. (Budapest, Hungary) in sodium salt form. Its quality met the Ph. Eur. 6th requirements. 2,2'-(Ethylenedioxy)bis(ethylamine) and 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide methiodide were purchased from Sigma-Aldrich Ltd. (Budapest, Hungary). The pH was adjusted with NaOH and HCl solutions. Millipore water was used throughout the study. Transcutol® and Labrasol® were from S&D Chemicals Ltd. (Budapest, Hungary). Glycerol 85% was from Molar Chemicals Ltd. (Budapest, Hungary).

3.2 Preparation of cross-linked hialuronic acid nanoparticle (CLHA)

The cross-linking reaction of HA was carried out in three different media (Table 1).

Table 1. The notations applied for the media throughout the study

Medium	water	NaCl (0.09% w/w)	NaCl (0.9% w/w)
Notation	A	B	C

The matrix end-products of the reactions were synthetized under different reaction conditions as concerns the medium applied, the concentration of HA and the cross-linking ratio. The nanoparticles obtained were identified by a three-notation system as follows: the initial letter refers to the medium and is followed by a number indicating the concentration of HA (mg/ml), and the final number indicating stoichiometric cross-linking ratio (feed ratio).

3.3 Methods

3.3.1 Dynamic light scattering (DLS)

The hydrodynamic diameter and size distribution of the CLHA nanosystems were measured with a Malvern–Zetasizer NanoZS90 instrument (Malvern Instruments Ltd., Worcestershire, UK). The measurements were made by photon correlation spectroscopy at a fixed angle of 173° at 25 °C.

3.3.2 Ultraviolet spectrophotometry

Transmittances of CLHA nanosystems were analysed with a HP-8453 Ultraviolet Spectrophotometer, at 500 nm in optically homogeneous quartz cells at 25 °C.

3.3.3 Transmission electron microscopy (TEM)

The size and morphology of the CLHA were determined with a JEOL2000 FX-II transmission electron microscope.

3.3.4 Gel permeation chromatography (GPC)

GPC is a type of size exclusion chromatography that separates analystes on the basis of size. The GPC column applied was the BioSuite 450 HR SEC.

3.3.5 Rheometry

Rheological measurements were carried out with a Physica MCR101 rheometer (Anton Paar, Austria). A cone-plate measuring device was used in which the cone angle was 1^{o}, and the thickness of the sample in the middle of the cone was 0.046 mm.

3.3.6 Hydration and irritation tests

The Corneometer® CM 825 (Courage and Khazaka Electronic GmbH, Cologne, Germany) is the instrument commonly used worldwide to determine the level of hydration of the skin surface. The Tewameter® TM 300 (Courage and Khazaka Electronic GmbH, Cologne, Germany) is the most generally accepted measuring device for the assessment of transepidermal water loss (TEWL).

3.3.7 *In vitro* API release and skin penetration measurements

Membrane diffusion and permeability studies were performed with a vertical Franz diffusion cell system (Hanson Microette TM Topical & Transdermal Diffusion Cell System, Hanson Research Corporation, Chatsworth, CA, USA).

3.3.8 Preparation of human epidermis

Excised human skin from patients who had undergone abdominal plastic surgery was used for the skin permeation studies. The epidermis was separated from the underlying dermis using the heat-separation technique. After excision, the subcutaneous fatty tissue was removed and

individual portions were immersed in water bath at 60 °C for 90 s and the epidermis was gently removed from the underlying dermis using forceps.

3.3.9 *In vivo* animal study

The modified skinfold chamber model was used. The experiments were performed on 15-week-old male hairless mice of strain SKH. The full skin was removed from one side of the skinfold. On the other side of the skinfold, the skin comprised complete epidermis, dermis and skin muscle. HA was determined by GPC.

4 Results

4.1 Results on the nanoparticle products

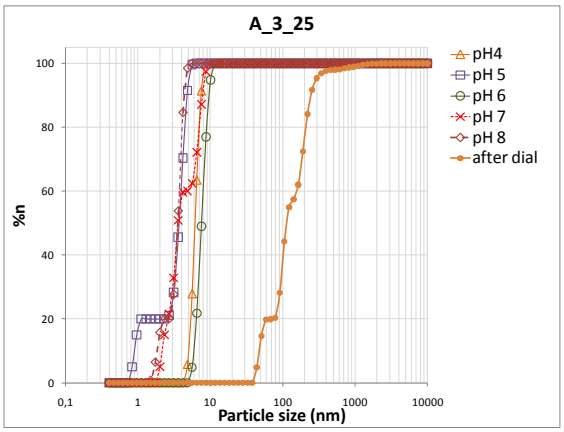
4.1.1 DLS results

The cross-linking process of HA can result in intramolecular and intermolecular cross-linking. A proportion of the CLHA nanoparticles were formed as small, individual particles; however, large particles were also produced. The large particles can be aggregates, associations caused by secondary interactions or intermolecular cross-linked particles. The hydrodynamic diameter of the nanoparticles from the reaction mixtures was therefore not monomodal, so the *Z*-average size was used to compare hydrodynamic size.

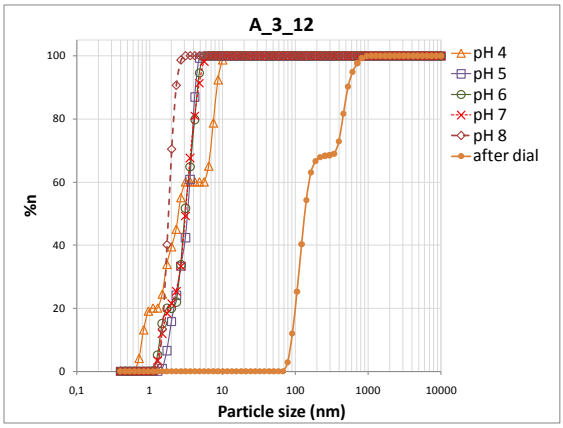
The end-point of Number Distribution Profile (NDP) for the reaction mixture of the matrix products shifts to smaller particles with increase of the concentration of HA at the same feed ratio (25%). The variation in size of the CLHA finds, with a higher concentration of native HA forming smaller nanoparticles. The explanation is that a higher concentration of HA leads to an increasing possibility of intermolecular cross-linking, thereby leading to more and more compact CLHA. This tendency indicates that the intermolecular forces between the nanoparticles are very low. Further, TEM showed that the HA nanoparticles were well dispersed with a spherical shape.

Figures 1a-c indicated that applied smaller feed ratio (12% or 7%) is sufficient to create an appropriate amount of intermolecular binding when an adequate concentration of HA is used. This is probably caused by the decrease in the feed ratio from 25% to 7%, and the setting of the pH for reaction mixtures does not significantly influence the cross-linked systems.

The overall charge of the CLHA is negative. The overall charge of the CLHA nanoparticles could be modified by adjusting the pH, but mutual repulsive forces and electric double layer were decreased by higher salinity.



a



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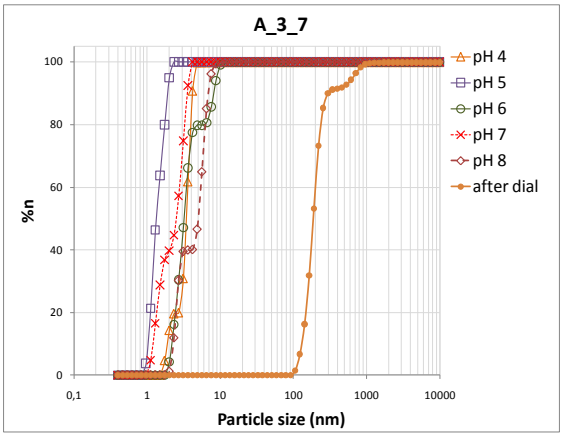

c

Figure 1. Effects of a smaller feed ratio of HA, pH and purification on the PSD of CLHA under the indicated reaction conditions: **a:** c_{HA} = 3 mg/ml, cross-linking ratio: 25%, prepared in water; **b:** c_{HA} = 3 mg/ml, cross-linking ratio: 12%, prepared in water; **c:** c_{HA} = 3 mg/ml, cross-linking ratio: 7%, prepared in water

Nevertheless, no significant correlations were established between the hydrodynamic diameters of swelled particles and the physico-chemical parameters of the reactions; the size and size distribution by number of all CLHA particles were below 20 nm.

However, we observed differences between the PSDs of the reaction mixture and the dialysed samples. The latter-mentioned profiles are indicated ‘after dial’ in Figure 1. Since the PSDs of all of the tested dialysed matrix products were similar and shifted towards larger particle sizes, it was clear that the smaller particles were lost when the reaction mixtures were dialyzed.

4.1.2 Hydrodynamic diameter (HD)

The reaction mixtures are not monomodal, so the *Z*-average size can only be used to compare results with samples measured via matrix products by the same technique. The HD was measured with DLS. The size of a particle was calculated from the translational diffusion coefficient by using the Stokes-Einstein equation:

$$\mathbf{HD} = \frac{kT}{3\pi\mu D} \quad (1)$$

where: HD = hydrodynamic diameter, D = translational diffusion coefficient, k = Boltzmann’s constant, T = absolute temperature, and μ = viscosity.

The translational diffusion coefficient D depends not only on the size of the particle “core”, but also the surface structure, and on the concentration and type of ions in the medium. A comparison of the A_1_25, A_2_25 and A_3_25 matrix products with the other matrix products reveals that the low conductivity medium water produces an extended double layer of ions around the particle, reducing the diffusion speed and resulting in a larger, apparent HD than in other media.

4.1.3 TEM results

TEM confirmed the existence of small nanoparticles (~10 nm) not only in the A_1_25 reaction mixture, but also in the A_3_7 mixture, where the HA concentration was higher and the feed ratio was lower. Increasing concentration of the native HA reduced the size of the dried particles to below 110 nm.

4.1.4 GPC results

GPC trace of HA shows with the broad size distribution of a linear biopolymer. We have compared GPC chromatograms of hyaluronan nanoparticles before and after dialysis. Before

dialysis the unimodal nanoparticulate system displayed a broad size distribution. The retention time for the nanoparticles increased; smaller particles were formed during the cross-linking reaction.

Following dialysis with cellulose dialysis tubes (molecular weight cut-off = 12,000 Da), the intensity of the GPC chromatogram of the purified reaction mixture was reduced at higher retention times. This suggests that smaller particles were formed during the cross-linking reaction, diffused through the membrane tube and were lost during the dialysis.

4.1.5 Transmittance results

The transmittance values were measured for the reaction mixtures of the different CLHA matrix products. These colloid dispersions are transparent or mildly opalescent systems. The transmittance values ranged between 91% and 99%. The results correspond with the Bouguer-Lambert-Beer law that transmittance values can be reduced due to increased concentration. Stable colloid particles were formed over a wide pH range of pH 3–8 in the different media during 3 weeks. The stability of these nanoparticles was not dependent on the medium or the feed ratio (from 7 to 25): no aggregation was found after three weeks.

4.1.6 Rheology

Viscosity, an important property of colloid systems, is related to the nature and the extent of the intermolecular interactions, and the entanglements of the polymer chains. The flow curves of the purified nanoparticle system run lower than that for the native HA, which proves that the successful cross-linking method resulted in small particles.

The comparison of the curve for the purified nanoparticle system and that for the unpurified nanoparticle system reveals that the curve for the reaction mixture runs lower than that for the dialysed sample, and the unpurified nanoparticle system contains small particles, which are missing from the purified sample.

The small particles produced in the applied cross-linking process can be lost during the purification, while the larger nanoparticles can remain in the purified system. The properties of these purified systems were determined by the larger particles.

4.2 Results on the semisolid linear HA and CLHA preparations

4.2.1 Results of rheological measurements

Rheological characteristics (viscosity and flow curve) are important properties of colloid systems. The gels applied to the skin had appropriate rheological properties. The shear stress of the cross-linked HA (CLHA) was lower than that of the linear HA, which confirmed the successful cross-linking and the formation of smaller particles (Figure 2).

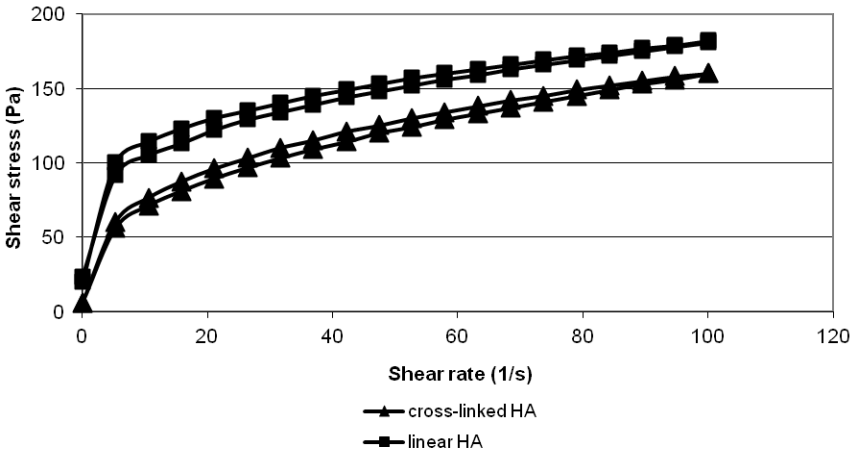

Figure 2. Flow curves of semisolid preparations containing linear HA or cross-linked HA

4.2.2 Results of hydration and irritation tests

Corneometry determines the water content of the skin surface, mainly the stratum corneum. It was found that there were no differences in the hydration effects of the CLHA and linear HA. Linear HA is known to hydrate the skin surface; after the chemical modification, the cross-linked HA retained the hydration effect (Figure 3). TEWL proved to be decreased after the application of the CLHA gel, which means that it undergoes longer hydration with no irritation effect on the skin (Figure 4).

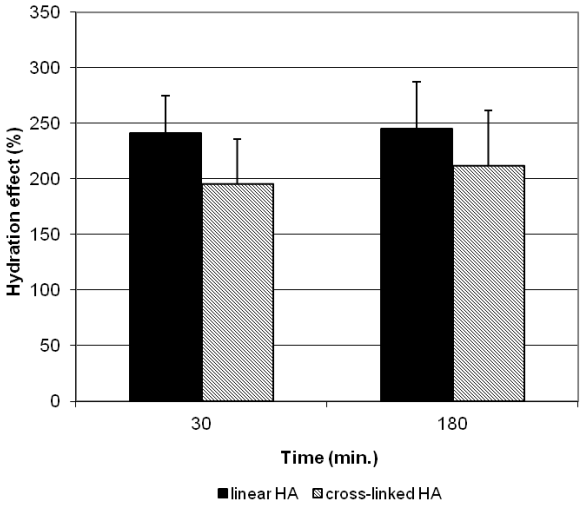

Figure. 3. Hydration effects of linear HA and cross-linked HA

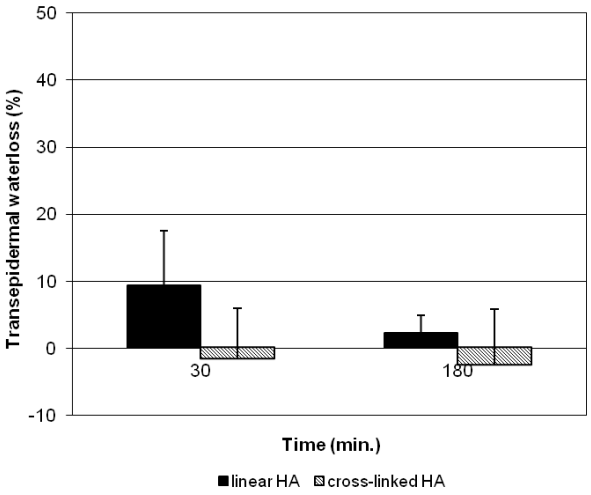

Figure. 4. Transepidermal water loss of linear and cross-linked HA

4.2.3 Diffusion, penetration and *in vivo* studies

Figure 5 depicts the cumulative amount per unit area of linear HA and CLHA that diffused through the synthetic.

It is clear that the diffusion of the CLHA was more intensive. After 10 h, a steady state was seen. The calculated result after 24 h: 1028 μg/cm^2. The linear HA also diffused through the synthetic membrane, but more slowly, and the amount diffused was significantly less, at 739

μg/cm^2 (after 24 h) The better diffusion of the cross-linked HA may be explained by the lower viscosity and the smaller particle size. The penetration through the human epidermis is illustrated in Figure 6.

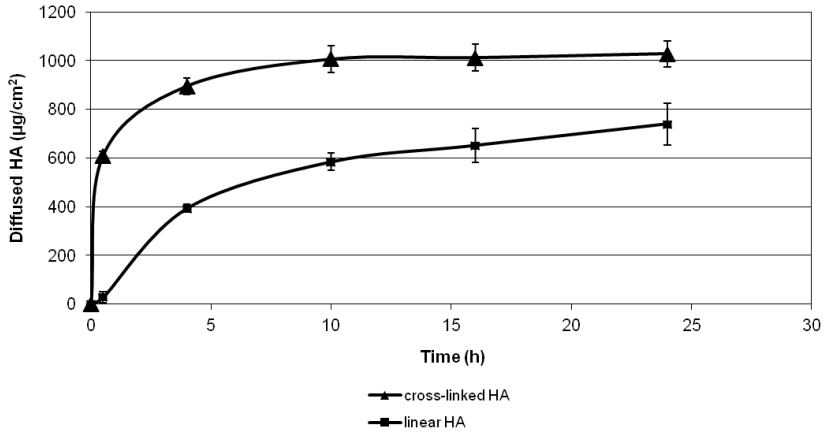

Figure 5. Cumulative amount of HA that diffused through a synthetic membrane

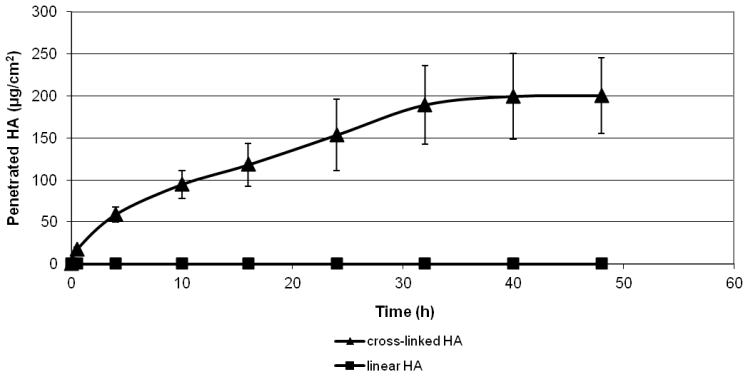

Figure 6. Cumulative amount of HA that penetrated through the human epidermis

There was no detectable penetration of linear HA during the observation period, whereas the level of penetration of CLHA was 200 μg/cm$^{2.}$

Figure 7 demonstrates the penetration of the CLHA and the linear HA as a function of time through living animal skin.

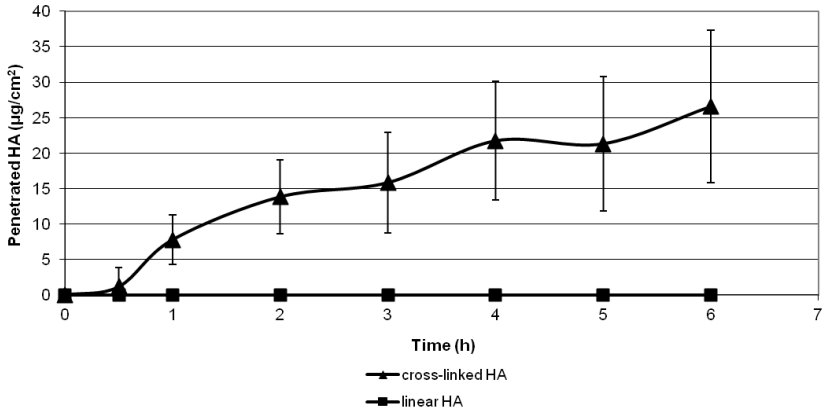

Figure 7. Demonstrates the penetration of the cross-linked and linear HA as a function of time through living animal skin.

Similarly as in the *in vitro* human epidermis study, there was no detectable penetration of linear HA. The quantity of CLHA that penetrated displayed a considerable elevation during the observation period; it increased steadily to about 26 μg/cm^2 by the end of the experiment.

5 Summary

The primary aim of my Ph.D. work was to prepare and investigate a crosslinked HA.

- The nanoparticles obtained were spherical with a size in the nanometric scale. Their physico-chemical properties, including the transmittance of an aqueous system containing nanoparticles and the hydrodynamic size of the HA nanoparticles, were controlled by varying the cross-linking density, the concentration of native HA and the parameters of the media.
- The TEM and rheology results proved the existence of systems of well-dispersed spherical HA nanoparticles.

The manufacturing process was studied.

- The DLS, transmittance and TEM results demonstrated that increase of the concentration of the native HA (from 1 mg/ml to 3 mg/ml) resulted in smaller product particles.

Comparisons of the particle sizes (TEM), particle size distributions (DLS) and molecular weight distributions in different phases of the technology led to the recognition of the loss of the smaller particles during the dialysis. The rheology results support the DLS and GPC results: the majority of the cross-linked nanoparticles are formed with a size less than 20 nm, but these particles can be lost during the purification.

- The electrical double layer and the measured HD were suppressed in media of higher conductivity. When the pH was increased, the HD displayed an increasing tendency. Narrower particle size range systems were observed at higher salinity, which did not change significantly when the pH was modified.
- The stability of these nanoparticles was not dependent on the medium and feed ratio (from 7 to 25%), and no aggregation was found after 3 weeks.

A semisolid HA-based nanoparticulate preparation was investigated in comparison with a hydrogel containing linear HA.

- This experimental work has revealed the advantages of CLHA over linear HA in dermal applications. The cross-linking of the linear HA molecule changes its rheological parameters. The shear stress decreases in response to cross-linking. The dynamic rheological measurements proved that the specific elastoviscous property of the HA gel was slightly changed after the cross-linking procedure.
- The corneometric tests revealed that the hydration effect was preserved. There was no irritant effect of the CLHA molecule in TEWL tests.
- The diffusion and penetration study demonstrated that the cross-linking of HA resulted in better diffusion through a synthetic membrane and better penetration through the human epidermis and living animal skin than when linear HA was used, where no penetration was observed. The cross-linking of HA makes it suitable for transdermal applications, promoting hydration in deeper layers of the skin and giving the possibility of a signal effect for HA production. CLHA can lead to the development of an API carrier system for deep penetration into the skin.

6 LIST OF PUBLICATIONS

I. M. Maroda, M. Bodnár, Sz. Berkó, J. Bakó, G. Erős, E. Csányi, P. Szabó- Révész, JF. Hartmann, L. Kemény, J. Borbély: Preparation and investigation of a cross-linked hyaluronan nanoparticles system. Carbohydr. Polymer (2011); 83: 1322-1329.
IF: 3.628

II. Csizmazia E., Berkó Sz., Maroda M., Szabó-Révész P., Csányi E.: A bőrön keresztüli hatóanyag permeáció modellezése és penetrációfokozók hatásának vizsgálata. Acta Pharmaceutica (2012); 82: 15-22.
IF: -

III. Sz. Berkó, M. Maroda; M. Bodnár, G. Erős, P. Hartmann, K. Szentner; P. Szabó-Révész, L. Kemény, J. Borbély, E. Csányi: Advantages of cross-linked versus linear hyaluronic acid for semisolid skin delivery systems Eur. Polym. J. (2013); 49: 2511-2517
IF: 2.739