STRUCTURAL ELUCIDATION OF SODIUM HYALURONATE GEL FOR INTRA-ARTICULAR APPLICATION

PhD thesis

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Introduction

One third of Europeans suffers at least once in their life from rheumatic diseases and furthermore all fifth people stands longer time under such treatment. By contrast, a disproportionately small number of products are marketed for intra-articular (i.a.) treatments. Several forms of rheumatic diseases are also known but only in a few cases there is the possibility of complete recovery or to prevent the disease. Besides surgical treatments nowadays a number of drugs are available for the systemic drug therapy. However, it is not significant in the joint injectable (intra-articular) forms of the locally applied active ingredients. The compositions of synovial fluid space are very similar to those of the serum and they are in constant contact with the bloodstream, but it is difficult to have a systemic drug therapy without unnecessarily high load of the organization. By using intra-articular applications drugs can be directly injected into the joint, which can ensure a high local drug concentration and low systemic effect. The non-invasive therapies are more comfortable drug delivery for the patients but in the long run the intra-articular therapy causes less damage to health such as digestive tract and renal vascular side effects.

Human joint is one of the best units of the nature. Its role is to maintain stability and movement of the skeleton, accompanied by precise function through the life, even under extreme load capacity. However, the secret to this ongoing development of renewed bone is the cartilage and synovial fluid. For the joints function without friction an extremely smooth surface is required. This ensures the cartilage which is stably and elastically covers the bone. The synovial fluid as a lubricant protects the cartilage by supplying with nutriment and oxygen.

One of the most important cartilage protectors is the hyaluronic acid (HA). It is the lubricant for the cartilage. Basically, the HA is responsible for the viscoelasticity of the synovia. The result of the viscoelasticity is that in case of low-frequency movement of the joint the viscous liquid works as a lubricant and in case of major mechanical stress the viscous liquid can store mechanical energy elastically.

Healthy joints contain approximately 2.26 g/l HA. If the joints are in motion, HA has a low viscosity in the synovial fluid; at rest, it is very viscous. HA macromolecular chains are built from D-glucuronic acid and *N*-acetyl-D-glucosamine disaccharides. A molecule of 10 million Da contains 25,000 disaccharide units in the chains, which are held together by hydrophobic bonds. The polysaccharide chains are linear and unbranched and roll up into a coil conformation. These coils can straighten, and this behaviour is the mechanism of action behind viscosupplementation. The length of the polysaccharide chains and the M_w of the HA are very different in various tissues. In normal tissues, a molecule of HA (10 million Da) has a thickness of 1 nm and a length of 25 μm. In the biomatrix, HA has an M_w in the range of 6 to 12 million Da. The molecular weight of HA is approximately 7 million Da in healthy joints and 4.8 million Da in unhealthy joints. The viscoelastic properties of HA under 1 million Da are negligible because of the M_w in inflamed joints (4.8 million Da). For that purpose, cross-linked HA is in demand (like Hylan G-F 20, with an M_w of 6-7 million Da) for intra-articular injections. Due to the chemical structure of the polymer, HA shows interesting rheological properties.

Parenteral compositions also i.a. belong to the pharmaceutical formulations with the most stringent requirements. For the i.a. compositions the sterility is essential. The sterility of solutions can be achieved by wet and dry heat if during the process all of the active ingredients and excipients remain stable. The sterility of the other intra-articular dosage forms can be achieved with adequate preparation of the materials and by keeping the fully aseptic conditions. Sterility for the injection dosage forms of the HA derivatives can be achieved only by sterile filtration of the solution or with the application of sterile solid HA treated by gamma irradiation (5 – 10 kGy) or with gas sterilization. Thermal effect or treatments, pressure, ultrasonic effects, filtration, mixing or using syringe can destroy the HA chains. These chain scissions cause the reduction of the M_w and accordingly the reduction of the viscosity.

NaHA is a very unstable molecule. If its solution is warmed to 100 ºC, the bonds between the chains are damaged, and the M_w and viscosity decrease. Experiments with "ready-to-use pre-filled" HA syringes showed that by autoclaving for 20 min, the M_w decreased from 1.4 million Da to 0.8 million Da. This is the reason that sterilisation of HA in solution is performed by sterile filtration, and the solid HA by gamma radiation

(5-10 kGy). However, data from simulated sterilisation conditions suggest that higher concentrations of NaHA may have a protective effect on the stability of the long-chain molecule, such that heat treatment would not denature the hydrogel.

Freeze-drying process (lyophilisation) is a treatment to get chemically stable and sensitive substances more sustainable and it should be taken into consideration that it is also able to reduce the number of the bacteria in the formulation because of the very low temperature during the treatment. Earlier data indicated that lyophilisation of hyaluronates as free acid, in contrast to the sodium salt form, can have a detrimental effect on their physical characteristics. These data indicate that the free acid form of medium molecular mass hyaluronate exhibited marked changes in molecular mass during lyophilisation whereas the NaHA appeared unchanged.

Despite the experiences concerning the thermal instability of NaHA under heat sterilisation, the primary aim was to track the changes in the macro and supramolecular structure of NaHA gels caused by the heat effect of autoclaving. In addition, the correlation between the free volume changes of NaHA macromolecules and the rheological properties of NaHA gels of various concentrations was determined.

Aims

The aim of this research work was to formulate sodium hyaluronate gels of different concentrations for intra-articular application. Another purpose was to clarify the microstructural changes due to the manufacturing process namely the effect of heat sterilization and freeze-drying.

The objectives of the research were as follows:

- *(i)* formulation of NaHA hydrogels intended for parenteral (intra-articular) administration,
- *(ii)* studying the effect of heat sterilization of NaHA hydrogels considering the microstructural changes (changes in the polymeric free volume and free volume distribution) and viscoelasticity,

(iii) evaluation of the effect of freeze-drying on the microstructure and rheological behaviour of NaHA hydrogels

Methods

Preparation of gel samples

Pharmaceutical grade NaHA (M_w=1.500 kDa) was obtained from Gedeon Richter Ltd., Hungary. NaHA gel samples of 10 mg/ml concentration were prepared allowing 24 hours for swelling in sodium-phosphate buffer adjusted to the physiological range (218–286 mOsm/l, pH= 7.44–7.45).

Heat sterilization of gels

The samples were studied without any further treatment at two hours and at one week after autoclaving (Boxer Autoclave 30075LR, Boxer Laboratory Equipment Ltd, UK) at 121 °C for 20 min, 103.4 kPa. The untreated and heat sterilised gels were dried at a temperature of 22 ± 0.5 °C and a relative humidity of 55 ± 2 %.

Freeze-drying of gels

The freeze-drying was performed in Scanvac CoolSafe 100-9 Pro type equipment (LaboGene ApS, Lynge, Denmark) equipped with a 3 shelf sample holder unit, recessed into the drying chamber. The process was controlled by a computer program (Scanlaf CTS16a02), the temperature and pressure values were recorded continuously.

The temperature of the drying chamber was between (-96) °C – (-92). Freezing of the samples was performed in the drying chamber.

Rheological measurements

Rheological measurements were carried out with a Kinexus Pro rheometer (Malvern Instruments Ltd, UK). The measured data were registered with rSpace for Kinexus Pro 1.3 software. A cone and plate geometry was used for the measurements. The gap between the cone and plate of sample placement was 0.03 mm. The temperature of the samples was controlled within 37±0.1 °C by a Peltier system.

For the analysis of viscoelasticity, the storage (G') and loss modulus (G'') of shear were plotted against the frequency, and their points of intersection were analysed.

Surface morphology (SEM)

The surface morphology of the raw and freeze-dried NaHA was investigated by scanning electron microscopy (Hitachi S4700, Hitachi Scientific Ltd., Japan). A sputter-coating apparatus (Bio-Rad SC 502, VG Microtech, Uckfield, UK) was applied to induce electric conductivity on the surface of the samples. The air pressure was 1.3-13.0 mPa. Briefly, the samples were sputter-coated with gold-palladium under an argon atmosphere, using a gold sputter module in a high vacuum evaporator (0.1 Pa), and the samples were examined with the SEM instrument set at 10 kV (current 10 μA).

Positron annihilation lifetime spectroscopy

For positron lifetime measurements, a positron source made of carrier-free $^{22}NaCl$ was used. Its activity was around 10^6 Bq and the active material was sealed between two very thin Ti foils. Lifetime spectra were measured with a fast-fast coincidence system based on BaF_2 /XP2020Q detectors and Ortec® electronics. Every spectrum was recorded in 3050 channels of an analyzer card for 1800 s and each contained about $1.5x10^6$ coincidence events (in the case of gels:3800 s and $2x10^6$ coincidence events). Three parallel spectra were measured at each concentration to increase reliability. After summarizing the parallels, spectra were evaluated by the RESOLUTION computer code; the indicated errors are the deviations of the lifetime parameters obtained. Three lifetime components were found in all the samples. The MELT code was used to extract lifetime distributions from the spectra. These latter evaluations were used to characterize the size distribution of free volume holes in the samples through ortho-positronium (o-Ps) lifetime.

Determination of water content by Karl Fischer method

The water content was determined using a Karl Fischer titrator (787 KF Titrino type, Metrohm AG, Herisau, Switzerland). Prior to the titration of the sample the water equivalency factor of Hydranal is determined using sodium tartrate (Hydranal water standard, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). The water equivalency factor was determined with 6 parallel measurements and the average of the results was used as the calibration for the samples. The solvent was extra dry methanol, which was titrated to the electrometric end-point with Karl Fischer reagent (Hydranal-composite 5, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) before the measurement. Approximately 100 mg sample was accurately weighed and immediately transferred to the titration vessel. After dispersing the sample (1 min at 15000 rpm) it was titrated with Karl Fischer reagent until the end-point. Each sample was analyzed in triplicate.

Structural analysis by X-ray powder diffraction (XRPD)

XRPD spectra were recorded with a Bruker D8 Advance X-ray diffractometer (Bruker AXS GmbH, Karlsruhe, Germany) system with Cu Kλl radiation (λ = 1.5406 Å) over the interval 3° - 40° (2θ). The measurement conditions were as follows: target, Cu; filter, Ni; voltage, 40 kV; current, 40 mA; time constant, 0.1 s; angular step 0.007°.

Thermoanalytical characterization

Thermogravimetric (TG) analysis of NaHA was registered out on a METTLER Toledo TGA/DSC 1 (Mettler-Toledo AG, Greifensee, Switzerland) system, in nitrogen atmosphere with a flow rate of 50 ml/min, at a heating rate of 10 °C/min in the temperature range of 25 - 300 °C with sample weight of 8 - 15 mg in capsules of aluminum as sample containers.

The differential scanning calorimetry (DSC) measurements were carried out on a METTLER Toledo 821e DSC (Mettler-Toledo AG, Greifensee, Switzerland) system. Under the Ar atmosphere with a flow rate of 100 ml/min samples with weight of 3-5 mg were measured at a heating rate of 10 °C/min in a temperature range of 25 - 300 °C and

also in capsules of aluminum as sample containers. The measured data were registered with STAR^e software.

Results

NaHA hydrogels with three various concentrations and with composition of sodium phosphate buffer for parenteral application were examined.

The supramolecular changes of NaHA hydrogels were investigated before and after sterilization. Autoclaving caused structural changes in the molecule, which depends on the polymer concentrations. The sterilized hydrogels showed partially irreversible structural alteration.

After autoclaving a regeneration process is occurred for the structure. However, the initial structure has not recovered and therefore autoclaving is not a suitable sterilization method. The free volume changes tracked by PALS and the rheological measurements showed that the possibility of heat sterilization should not necessarily be excluded.

PALS has proven to be a sensitive mean to explore structural alteration in the polymer molecule including NaHA.

In the course of freeze-drying, supramolecular ordering and highly porous structure of freeze-dried NaHA gel was formed due to the presence of phosphate salts which enabled fast gelling ability during reconstitution with water.

There was no amorphous to crystalline transition of NaHA with phosphate salts. Supramolecular ordering did not cause functionality – related alteration, viscoelasticity was preserved for reconstituted hydrogels.

Conclusions

In summary, parenteral sodium hyaluronate (NaHA) hydrogels and its structural changes after different treatments were investigated. In the thesis the effect of heat sterilization on the macro- and microstructural characteristics of various sodium hyaluronate gels of animal origin was evaluated. Along with the functionality-related

characteristics (viscoelasticity, ability for reconstitution) the positron annihilation lifetime spectroscopy (PALS) was applied for the tracking of the microstructural changes of the macromolecule before and after freeze-drying of the gels.
The combination of different testing methods enabled a comprehensive characterization of NaHA hydrogels.

The obtained novel results are the followings:

NaHA hydrogels have been successfully formulated with different concentrations for parenteral purposes with special focus on the requirements of the intra-articular administration.

The hydrogels after autoclaving showed partially irreversible structural alteration, depending on the concentration, which were confirmed by viscometric and PALS measurements. Consequently the possibility of heat sterilization should not necessarily be excluded.

The freeze-dried hydrogels showed fast gelling ability in the presence of sodium phosphate salts in contrast to the freeze-dried hydrogels prepared only with water, which can be explained by an ordered ladder-like macroporous structure. The found phenomenon was responsible for the fast reconstitution which was followed by the changes of the free volume holes with PALS.

The clarification of the structural changes of different NaHA gels after heat sterilization and freeze drying could contribute in the determination of proper manufacturing process conditions in order to reserve the required functionality-related characteristics (viscosupplementation) of the intended dosage form.

List of own publications

Publications relevant to the dissertation

1. Szabó A, Zelkó R, Antal I. (2011) Reumás megbetegedések kezelése intraartikuláris készítménnyel. Acta Pharm Hung, 81: 77-86.

2. Szabó A, Szabó B, Balogh E, Zelkó R, Antal I. (2012) Módosított hatóanyagleadású intraartikuláris készítmények. Acta Pharm Hung, 82: 69-74.

3. Szabó A, Szabó B, Balogh E, Zelkó R, Antal I. (2013) Structural elucidation of hyaloronic acid gels after heat sterilization. Polym Test, 32: 1322-1325.

4. Krüger-Szabó A, Aigner Z, Balogh E, Sebe I, Zelkó R, Antal I. (2015) Microstructural analysis of the fast gelling freeze-dried sodium hyaluronate. J Pharm Biomed Anal, 104: 12-16.

Other publications

5. Szabó A. (2011) Magizzunk vagy ne magizzunk, avagy hogyan csinálják a németek?. Gyógyszerészet, 55: 606-610.

List of oral presentations relevant to the dissertation

1. Szabó A, Szabó B, Balogh E, Zelkó R, Antal I.
   Intraartikuláris készítmények fejlesztési lehetőségei
   XVII. Gyógyszertechnológiai és IX. Gyógyszer az ezredfordulón Konferencia
   Siófok, Hungary, September 27-29, 2012

List of poster presentations relevant to the dissertation

1. Szabó A, Zelkó R, Balogh E, Antal I.
   Development and physical characterization of intra-articular injection containing piroxicam and sodium hyaluronate
   8th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology, Istanbul, Turkey, March 19-22, 2012

2. Krüger-Szabó A, Szabó B, Zelkó R, Balogh E, Antal I.
   Structural elucidation of hyaluronic acid gels after heat sterilisation
   9th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology, Lisbon, Portugal, March 31-April 3, 2014

3. Krüger-Szabó A, Aigner Z, Sebe I, Balogh E, Zelkó R, Antal I.
   Piroxikámot és nátrium-hialuronátot tartalmazó intraartikuláris injekciós készítmény formulálási vizsgálata
   XV. Congressus Pharmaceuticus Hungaricus, Budapest, Hungary, April 10-12, 2014