STRUCTURAL ELUCIDATION OF SODIUM HYALURONATE GEL FOR INTRA-ARTICULAR APPLICATION

PhD thesis

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Abbreviations

$^{22}NaCl$	sodium-22 radionuclide NaCl
ATR	attenuated total reflectance
AUC	area under the curve
Bq	Becquerel
Da	dalton
DMARD	disease-modifying antirheumatic drugs
DSC	differential scanning calorimetry
EDTA	ethylenediaminetetraacetate
FTIR	Fourier Transform Infrared
FWHM	full width at half maximum
G´	storage modulus of shear
G´´	loss modulus of shear
H_2O	water
HA	hyaluronic acid
HPC	hydroxypropyl cellulose
i.a.	intra-articular
IgG	immunoglobulin G
IL	interleukin
IR	infrared
MALS	multi-angle light scattering
M_w	molecular weight
NaHA	sodium hyaluronate
NMR	nuclear magnetic resonance
NSAID	non-steroidal antiinflammatory drugs
NSARD	non-steroidal antirheumatic drugs
o-Ps	orto-positronium
PALS	positron annihilation lifetime spectroscopy
PMMA	polymethyl methacrylate
polyHEMA	poly-(2- hydroxyethyl)-methacrylate
p-Ps	para-positronium

PVA	polyvinyl alcohol
QbD	Quality by Design
rpm	revolutions per minute
SEC	size-exclusion chromatography
SEC-MALS	size-exclusion chromatography multi-angle light scattering
SEC-MALS-RI	size-exclusion chromatography multi-angle light scattering and refractive index
SEM	scanning electron microscopy
TBF-α	tumor necrosis factor alpha
TG	thermogravimetric
UV	ultraviolet
XRPD	X-ray powder diffraction

1. 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 (Eular, 2011). By contrast, a disproportionately small number of products are marketed for intra-articular 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 (Gerwin et al., 2006). 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 (Edwards et al., 2007).

1.1. Intra-articular delivery systems

1.1.1. Anatomy of articular joint

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. The synovial fluid (synovia) is produced by the synovial membrane which covers the connective tissue in the joint capsule. The capsule

prevents the exit of the synovial fluid from the articular cavity. The joint is held together by the ligament (Figure 1) (Röder, 2010).

Sufficient oxygen and nutrients to absorb into the cartilage from the joint fluid it is essential to move. Thereby resulting pressure effect that from the cartilage depleted synovial fluid is pressed out and is replaced with fresh fluid. So the cast synovial fluid enters the bloodstream through the synovial membrane and fresh nutrients and oxygen from the blood comes into the synovial.

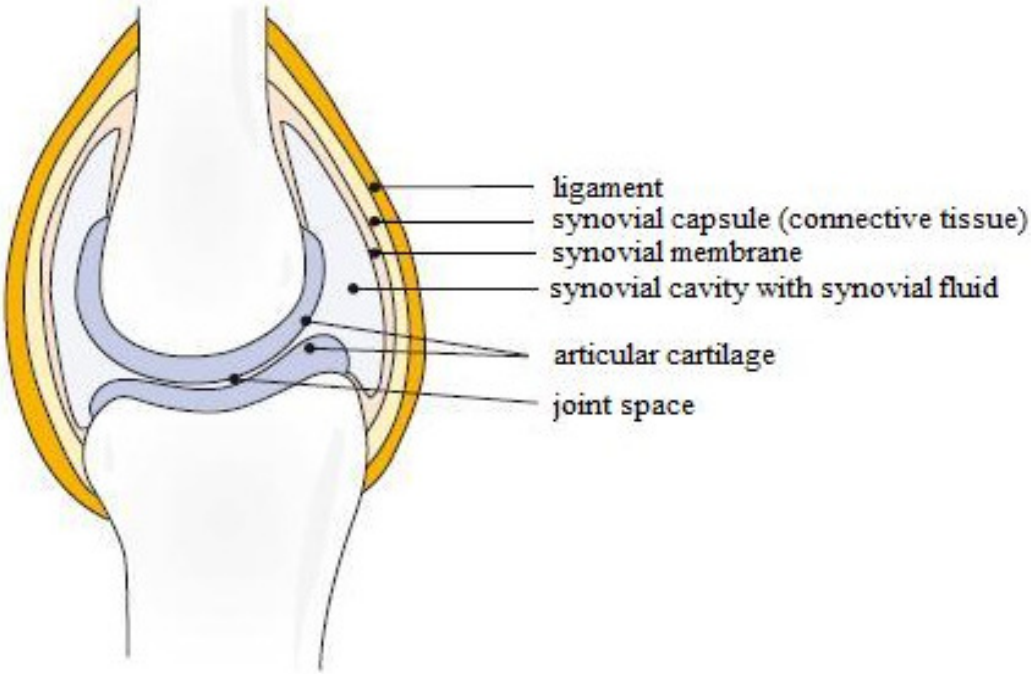

Figure 1 Schematic illustration of healthy joint (Röder, 2010)

Intra-articular (i.a.) preparations go directly into the synovial fluid. Therefore it is important to know the exact composition of the synovial fluid (Table 1). The synovia is none other than the blood dialysate (Zeidler, 1986). The composition and content of electrolytes and low-molecular materials is identical to those of serum. In the healthy joint the synovial fluid amounts approximately 0.5-2 ml (Dewire et al., 2001; Mason et al., 1999). The synovial fluid is changed continuously and rapidly (approx. 2 hours) and one of the key components of the synovia is only renewed every 38 hours (Mason et al., 1999).

Table 1 Composition of healthy synovial fluid (Zeidler, 1986)

Physical data and materials	Mean value	Extreme range
Physical data		
Volume (ml/normal knee joint)	1.1	0.13-4.0
Relative density	-	1.008-1.015
Osmolarity (mmol/l)	296	292-300
pH	7.434	7.31-7.64
Water (g/kg)	-	960-988
Solids (g/kg)	34	12-48
Cells (per µl)	-	13-180
Inorganic materials		
Carbon dioxide (mmol/l)	-	19.3-30.6
Chloride (g/l)	3.81	3.08-4.89
Phosphate	corresponds to the contents of the serum	
Potassium (mg/l)	156	-
Sodium (g/l)	3.13	-
Calcium (mg/l)	-	48-96
Iron (µg/kg)	290	-
Copper (µg/kg)	275	-
Zinc (µg/kg)	176	-
Organic materials		
Glucose	corresponds to the contents of the serum	
Urea (mg/l)	150	-
Uric acid (mg/l)	39	-
Total protein (g/l)	13.1	10.4-15.8
Albumin (g/l)	8	6-10
Globulin (g/l)	0.5	-
Hyaluronic acid (g/l)	2.26	1.45-2.94
Chondroitin sulfate (mg/l)	42	-
Lipids :		
Cholesterol	71	-
Phospholipids	138	130-152

Basically, the hyaluronic acid (HA) is responsible for the viscoelasticity of the synovia (Balazs et al., 1970; Balazs, 2009). 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. In the inflamed joints larger quantities (up to 100 ml) of synovial fluid can be produced wherein the hyaluronic acid lose its viscosity by reduction of the concentration and the molecular weight (Marshall et al., 1997). The viscosity of the synovial fluid of a healthy joint exceeds 300 mPa × s. However, after the outbreak of rheumatic symptoms the viscosity drops below 300 mPa × s (Dewier et al., 2001, Simkin, 1985).

1.1.2. Rheumatic diseases

Rheumatic diseases can be arthrosis (wear of joint) or arthritis (inflammation of joint).

Arthrosis, osteoarthrosis and degenerated articular rheumatism class in articular wear diseases (Röder, 2010). Presumably, arthritis is caused by the imbalance between the demand and supply of nutrients. Other possible reasons are that the synovial fluid gets only a few nutrients or because of the lack of movement an inadequate nutrient circulation arises. If the cartilage does not get the proper quantity of nutrients from the joint fluid, cartilage cells die. The results are that the cartilage is torn into fibers and loses from the smooth surface. With any further movement a piece of cartilage drops out and the bone protected membrane becomes thinner (Figure 2) (Röder, 2010). The bone forms bulges below the cartilage which cause the breaking off further pieces of cartilage. The immune system treats these pieces in the synovial fluid as a foreign body which so the inflammation caused triggers a protective reaction. As a result, materials having a disastrous effect on cartilage are sent out which leads to rapid death of the cartilage cells. Because of the pain from inflammation patients move even less which leads to even less nutrient emissions. The resulting circulus vitiosus is difficult to curb. Arthrosis affects most often the load bearing joints such as the hip (coxarthrosis) or knee (gonarthrosis) joint.

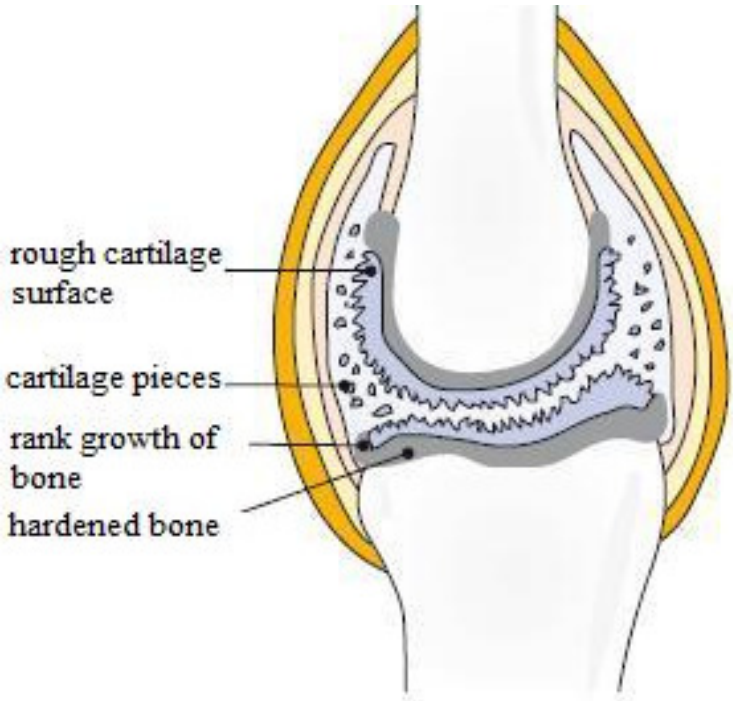

Figure 2 Schematic illustration of rheumatic joint caused by wear (Röder, 2010)

The inflamed articular disease is the arthritis. The most prevalent form is the chronic polyarthritis that calls most often rheumatoid arthritis. The inflammation becomes recognizable early because of the redness, warmth and swelling of the joint. At the beginning of the disease a high fever can occur in several times. Autoimmune reactions trigger the inflammation which results that the immune system replaces cartilage with foreign body material or it also attacks. The causes of the autoimmune reaction are not yet known to science but presumed causes could be bacteria, viruses or mechanical cell damage. During activation of the immune system B lymphocytes produce antibodies (lgG) which induce new auto antibodies (rheumatoid factor). The lgG and the rheumatoid factor form an immune complex which is phagocyted by the granulocytes and cells of the synovial membrane. As a result cytokines released to ensure the maintenance of the inflammatory reaction. Cytokines in the rheumatic joint can be divided into three groups (Mutschler, 2001):

- proinflammatory cytokines such as interleukin (IL)-1, -2, -6, -8, -15 and -18 or tumor necrosis factor alpha (TNF-α) regulate the inflowing and activation of the inflammatory effector cells (for exp. T-cells) and the proliferation of the chondrocytes and fibroblasts;

- anti-inflammatory cytokines such as IL-4, -10 and -13 inhibit purposefully the proinflammatory cytokines;
- anti-cytokine protein such as the soluble TNF-α-receptor or the IL-1-receptor regulate the inflammation by catching the proinflammatory cytokine.

In the joint of the patient with rheumatoid arthritis all of the three types of cytokine can be found, but during the diseases it shifts the balance to the cytokines. Then the occurring inflammation starts from the synovial membrane (synovitis) which at first of all becomes thick, forming bumps and fibrous, stringy tissue projects forward (Figure 3) (Röder, 2010). The resulting tissue called pannus. In the pannus tissue a large number of antibodies can be found to lead to the destruction of cartilage. The rheumatoid arthritis can occur in all joints. Firstly, in the most cases it is formed in joints of hand and finger, but later it can spread in the knee and hip joints. The symptoms can be very strong especially at night and in the morning. Another (rarely occurring) two forms of inflammatory rheumatic diseases are known: psoriasis arthritis and Bechterew's disease. In both cases, the diseases are triggered by autoimmune reactions.

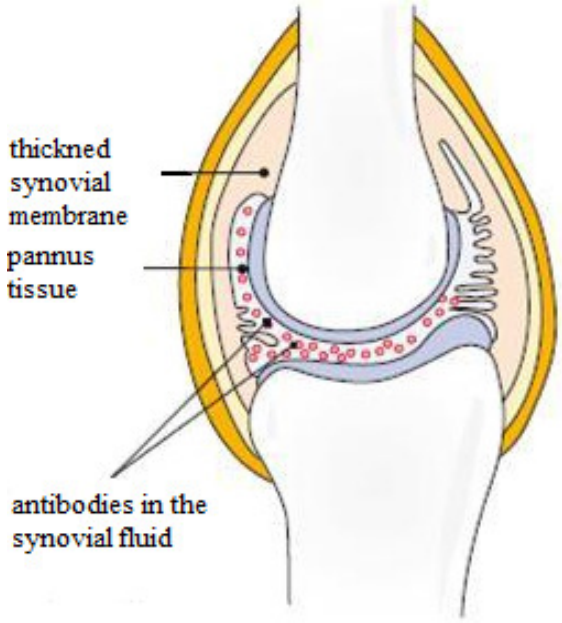

Figure 3 Schematic illustration of rheumatic joint caused by inflammation (Röder, 2010)

1.1.3. Drug therapies of rheumatic diseases

In recent decades therapies and strategies have been pretty changed. During the drug therapies of rheumatic diseases the patient can have a shorter or longer period asymptomatically. The attenuation of pain is the most important aspect. Drug therapy especially considering the groups of active ingredients the following options are available:

- non-steroidal anti-inflammatory drugs (NSAID);
- non-steroidal antirheumatic drugs (NSARD);
- glucocorticoids;
- synthetic disease-modifying antirheumatic drugs (synthetic DMARD);
- biological disease-modifying antirheumatic drugs (biological DMARD);
- cartilage protector.

Cartilage protectors are the following substances: chondroitin sulfate, glucosamine, carrageenan and hyaluronic acid. They possess cartilage building characteristics. One of the most important is the HA. It is the lubricant for the cartilage and responsible for the viscosity of the synovial fluid. Due to the viscoelastic properties of HA, by the different moving of the joint (exp. sport), HA can have very different viscosity values. Thanks to this property, HA is a very versatile molecule.

There are a growing number of such active ingredients marketed as parenteral, solid or semisolid forms for rheumatic diseases. At this point it should be mentioned that parenteral biologicals make a greater number. According to an outlook to 2020 worldwide the Rx drug sales add up to $1 trillion and the biological make up 52 % (Evaluate, 2014).

1.1.4. Intra-articular injections

1.1.4.1. General about intra-articular injections

Oral compositions (e.g. glucocorticoids, NSARD) have a great disadvantage for the treatment of rheumatic diseases: unpleasant systemic side effects may occur when taking. Undoubtedly, because of the poor bioavailability other active ingredients (e.g. HA) are used only for parenteral form. These problems can be avoided by using of i.a. injections Thus, due to the natural endowments of the joints the i.a. form is a very optimal input gate. In most of the cases the hand, knee and foot joints are affected and their good accessibility favors the intra-articular injections. From the synovial fluid only a little active ingredient enters the bloodstream and therefore systemic side effects are negligible. A very important drawback is the extremely high risk of infection during the use of i.a. preparations, especially for several consecutive injections (Albert et al., 2006). However, the risk of infection is reducible by compliance with the standards, using sterile components and thorough disinfection of the injection site (Schumacher, 2003).

1.1.4.2. Modified release intra-articular drug delivery systems

During the injections, there is the risk for infection therefore the number of injections in a year should be reduced. Also a long-term drug exposure should be achieved with an injection, and if it is possible, a combination of active ingredients should be used. Many different active ingredients could have a long-term drug exposure with carrier systems like micro- and nanoparticles (Szabó A. et al., 2012), liposomes (Foong et al., 1988; Lopez-Garcia et al., 1993; Elron-Gross et al., 2009), hydrogels (Réeff et al., 2012; Shah, 2001) and physically activated delivery systems like thermoresponsive (Betre et al., 2006; Shamji et al., 2007; Kusanagi et al., 2007; Aly, 2008) or magnetically modulated carriers (Butoescu et al., 2009). Almost every third knee articular injection is inaccurate (Brandt et al., 2003), which unnecessarily exposes patient to invasive therapy without improving the condition.

1.1.5. Requirements of intra-articular delivery systems

Parenteral compositions also i.a. belong to the pharmaceutical formulations with the most stringent requirements. The following requirements shall be taken into account:

- sterility;
- pyrogen-free;
- isotonic;
- physiological pH;
- viscosity;
- compatibility of active ingredients and excipients;
- contamination free;
- compatibility with the packaging material;
- stability during storage.

The injectable suspension is one of the most complex pharmaceutical formulation considering their stability, production and application. By parenteral suspensions several factors should be taken into consideration (Liebermann et al., 1996):

- dispersibility /sedimentation rate;
- stability;
- particle size;
- syringeability.

For the intra-articular 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 (Gerwin et al., 2006). Sterile filtration is not feasible by formulations containing particles or by solid, semisolid forms.

Ensuring isotonicity of the preparations is just as important as the sterility. Hypotonic or hypertonic parenteral preparations can cause pain or tissue damage (Bauer et al., 2002).

Therefore, it is very important to achieve an appropriate isotonic value which must be between 225 and 430 mOsm/kg.

The pH value should be set to the physiological pH (7.4). Greater deviations from the physiological value could cause pain. The minor differences can be compensated by the synovial fluid.

For the applicability of the product the viscosity plays a very important role. The higher viscosity can cause strong pain to the patients or the optimal syringeability could be difficult or even totally blocking. Syringeability is described by the Hagen-Poiseuille equation (Fry, 2014):

$$F = \frac{128Q\mu LA}{\pi D^4} \qquad \text{(Eq. 1)}$$

where:

F = syringe stopper (plunger) force;

Q = volumetric flow rate;

μ = dynamic viscosity;

L = needle length;

D= needle bore diameter;

A = syringe plunger area.

The syringe stopper or plunger force is dependent on numerous factors, including the volumetric flow rate, the dynamic viscosity, the needle length and bore diameter and the syringe plunger area, the relevant tissue.

The parenteral manufacturing industry pays great attention to particulate contamination. Particulate contaminations in parenterals are risk factors and can lead to damage to patients. Contaminations can be caused by interactions between the formulation, product packaging, packing materials, formulation ingredients, manufacturing process and environment (Langille, 2013). Quality by Design (QbD) is an important approach by parenteral manufactures. Quality is ensured by planning or design. The crises, risks and problems to get particulate contaminations can be avoided by well management of the different processes. The collaboration with logistic partners,

suppliers, technical engineering, glass manufacturers and the development of further testing methods (final visual inspections, measurement of changes in conformation that could lead to aggregation, using instruments for sub visible particles etc.) to avoid contaminations, is indispensable (Challener, 2014). QbD pays also attention to speed up the processing time and to reduce the costs.

The formulations could lose their stability during storage. Therefore some compositions are marketed as a dry powder which must be dispersed in a solvent before use (Gerwin et al., 2006). On the pharmaceutical market some products can be found in ready-to-use pre-filled syringe. The application of pre-filled injections avoids the critical point when the content of the vial is pulled up into the syringe which has new infection risks (Ayral, 2001). On the other hand the pre-filled syringes are also contributor to the increased incidence of problems with visible particulate contaminations (Challener, 2014). The inadequate time and temperature storage can also cause particulate contaminations in the parenteral preparations.

In the case of intra-articular suspensions the good and rapid and the slow sedimentation have an important role in the correct dosage of drugs. The remaining active ingredient(s) in the syringe can have serious consequences for the therapy.

The stability of the suspension is good if the particles are dispersible and do not agglomerate.

Several scientific articles were published about the optimum size of particles of intra-articular suspensions. Results of phagocytose of suspended parts are very different. So it should be noted, most of the literature suggests the avoidance of phagocytosis. Thus, the particles must be at least between 5 - 15 μm that exceeded the size of the macrophages and thereby prevent the annexation (Howie et al., 1993).

In parenteral considering i.a. injections a high number of excipients can be used safely. Water works as a solvent or dispersant. In most cases for isotonic sodium chloride, mannitol or sorbitol can be used. Sodium hydroxide, sodium hydrogen phosphate, hydrochloric acid, phosphoric acid or citric acid adjust the optimum pH value. In some of the products other excipients can be found: polysorbate 80 such a surfactant, sodium EDTA such a stabilizer, propylene glycol or polyethylene glycol such cosolvents and preservatives (benzyl alcohol, methyl- or propyl-4-hydroxy

benzoate and cetylpyridinium chloride) (Gerwin et al., 2006). For the stabilization of suspensions croscarmellose sodium, hypromellose or gelatine can be used.

There are a small number of intra-articular injections on the pharmaceutical market (Table 2). Mainly, glucocorticoids are available for the i.a. administration. However, an increasing number of publications have been published in the topic of i.a. delivery systems with NSAI and NSAR active ingredients.

Table 2 Some commercially available i.a. products (without HA products)

Commercial name	Company	Formulation	Drug	Concentration (mg/ml)
CELESTAN ® Depot	MSD SHARP & DOHME GMBH	suspension	betamethasone	2.7
CELESTAN ® solubile	MSD SHARP & DOHME GMBH	solution	betamethasone	4
Delphicort ®	RIEMSER Pharma GmbH	suspension	triamcinolone	25; 40
dexa-clinit ®	Hormosan	solution	dexamethasone	4
Dexamethason-mp ®	medphano Arzneimittel GmbH	solution	dexamethasone	4
Diprosone ® Depot	MSD SHARP & DOHME GMBH	suspension	betamethasone	2
Fortecortin ® Injekt	Merck Serono	solution	dexamethasone	4; 8
Lederlon ®	RIEMSER Pharma GmbH	suspension	triamcinolone	5; 20
Lipotalon ®	Recordati Pharma GmbH	liposome	dexamethasone	2.5
Prednigalen ®	GALENpharma GmbH	suspension	prednizolone	10; 25; 50
Prednisolut ®	mibe GmbH Arzneimittel	solution	prednizolone	5; 12.5; 25; 50

In the last years the number of HA/sodium hyaluronate (NaHA) i.a. formulations increased (Table 3). Most of the preparation is in the form of a viscous solution, also known as hydrogel. HA is an innovative active ingredient with a lot of possible applications.

Table 3 Some commercially available i.a. HA products

Commercial name	Company	Conc. (mg/ml)	Excipients
ARTROject ®	ORMED GmbH	5	NaCl, Na_2HPO_4, NaH_2PO_4, H_2O
Curavisc ®	curasan AG	10	NaCl, Na_2HPO_4, NaH_2PO_4, H_2O
Fermathron ®	Biomet Deutschland GmbH	10	NaCl, Na_2HPO_4, NaH_2PO_4, H_2O
GO-ON ®	Rottapharm|Madaus GmbH	10	NaCl, Na_2HPO_4, NaH_2PO_4, H_2O
GO-ON ® matrix	Rottapharm|Madaus GmbH	20	sorbitol
Hya-ject ® / -mini	ORMED GmbH	10	NaCl, Na_2HPO_4, NaH_2PO_4, H_2O
Hya-ject ® plus	ORMED GmbH	20	mannitol, NaCl, Na_2HPO_4, NaH_2PO_4, H_2O
Hyalart ® /D	MEDA Pharma GmbH & Co. KG	10	NaCl, Na_2HPO_4, NaH_2PO_4, H_2O
Hyalubrix ®	MEDA Pharma GmbH & Co. KG	15	NaCl, Na_2HPO_4, NaH_2PO_4, H_2O
Monovisc ®	Plasmaconcept AG	22	NaCl, H_2O
Orthovisc ® / -mini	Plasmaconcept AG	15	NaCl, H_2O
Ostenil ® / -mini	TRB Chemedica AG	10	NaCl, Na_2HPO_4, NaH_2PO_4, H_2O
Ostenil ® plus	TRB Chemedica AG	20	mannitol, NaCl, Na_2HPO_4, NaH_2PO_4, H_2O
Recosyn ®	Recordati Pharma GmbH	10	NaCl, Na_2HPO_4, H_2O
Recosyn ® -m.d.	Recordati Pharma GmbH	10	NaCl, Na_2HPO_4, H_2O, citric acid
Recosyn ® -forte	Recordati Pharma GmbH	20	NaCl, Na_2HPO_4, NaH_2PO_4, H_2O, Na_2CO_3
Sinovial ® / -Mini / -HighVisc	IBSA Farmaceutici Italia Srl	8; 16	NaCl, H_20, sodium phosphate (unknown)
Synvisc ® / -One	Sanofi-Aventis DL GmbH	8	NaCl, Na_2HPO_4, NaH_2PO_4, H_2O
Viscoseal ®	TRB Chemedica AG	5	NaCl, Na_2HPO_4, NaH_2PO_4, H_2O

Nowadays, it is very difficult to classify which formulations or applications are medical devices or drugs. This special emphasis is made on the regulatory issues of HA, too. The different countries have different opinion about the regulatory. There is no specification about the concentration, M_w, type of the application, viscosity, condition etc. of HA to classify to the medical devices or to the drugs.

1.2. Hyaluronic acid

1.2.1. History

NaHA is the sodium salt of HA. There is a wide variety of names for HA which refers to the versatility of the material (Dicker et al., 2014). In 1934 Meyer et al. isolated it and called it HA because of his supposed mild acid properties. In the physiological medium, HA is present as a polyelectrolyte salt, calls “hyaluronate” with a cation, mostly with sodium, like “sodium hyaluronate”. Balazs et al. (1986) used the name “hyaluronan”, which form can be always used.

They are of importance in epidermal pathology, tissue engineering, ophthalmic surgery, drug delivery systems, pulmonary and joint pathologies and aesthetic surgery (Price et al., 2007). HA is the most important substance in the synovial fluid of articular joints to be found in the extracellular matrix of the synovium. It is the lubricant for the cartilage and responsible for the viscosity of the synovial fluid. Healthy joints contain approximately 2.26 g/l HA (Zeidler, 1986). It is a normal process that for people over the age of 30 years starts to lose HA production in body. Therefore, characteristics and formulations of HA are increasingly important in pharmaceutical technology.

The history of HA goes back to 1934 when Karl Meyer purified HA from the vitreous of bovine eyes (Meyer et al., 1934). In 1958, Meyer et al. identified the structure of HA. At the beginning of the researches the biggest challenge was the biological purity of HA. It was very difficult to remove all of the impurities from the molecule which can cause inflammation, irritation or allergy. In the late 1960s the use of the “non-inflammatory fraction of sodium hyaluronate” was started for therapeutic proposes. In the mid-1970s 1 % HA solution (2 – 3 million Da) was used in veterinary medicine. In 1992 the first HA with 6 million Da was usable for therapeutic applications (Balazs, 2009).

The first NaHA product for i.a. pathway with the name Hyalart®/Hyalgan® was marketed by Fidia in Italy in year 1986 (Fidia Pharma, 2015). It was used for the osteoarthritis of the knee.

1.2.2. Structure

HA macromolecular chains are built from D-glucuronic acid and *N*-acetyl-D-glucosamine disaccharides (Figure 4) (Kogan et al., 2007). A molecule of 10 million Da contains 25,000 disaccharide units in the chains, which are held together by hydrophobic bonds (Romagnoli et al., 2008). The polysaccharide chains are linear and unbranched and roll up into a coil conformation. The intramolecular hydrogen bonds have a big role in the conformation of HA. In solution the surrounding water molecules of HA causes another formation of hydrogen bonds. According to the concept of Sheehan et al. (Sheehan et al., 2001) the hydrogen bonds are not responsible for the stiffening of the molecule. There is a little difference in hydrogen bond energy whether the molecule is hydrogen bonded to itself or to water molecules. The different conformations are deal subsequently.

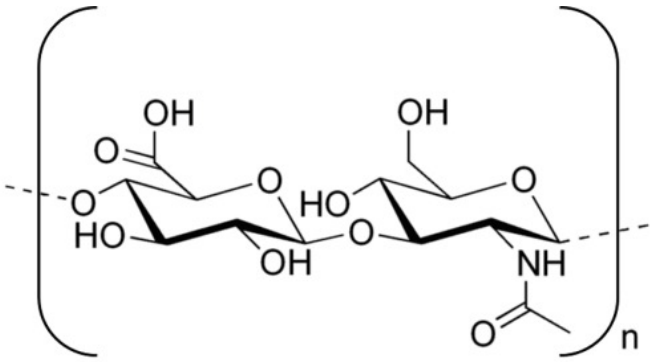

Figure 4 Structural formula of HA

The length of the polysaccharide chains and the M_w of HA are very different in the various tissues. In normal tissues, a molecule of HA (10 million Da) has a thickness of 1 nm and a length of 25 µm (Romagnoli et al., 2008). In the biomatrix, HA has an M_w in the range of 6 to 12 million Da (Balazs, 2009). The molecular weight of HA is approximately 7 million Da in healthy joints and 4.8 million Da in unhealthy joints (Wohlrab et al., 2004). The viscoelastic properties of HA under 1 million Da are

negligible because of the M_w in flamed joints (4.8 million Da). For that purpose, cross-linked HA is in demand (such as Hylan G-F 20, with an M_w of 6-7 million Da) for i.a. injections (Migliore et al., 2010).

HA can have very different molecular weights, from hundreds up to millions of Daltons, it gets interesting to determine the molecular weight of Ha.

Size-exclusion chromatography (SEC) is used for polymer molecular weight characterization, but it not suitable for the determination of the molecular weight of HA. It requires specific calibration standards. Multi-angle light scattering (MALS) detector can directly determine the molecular weight of a polymer without calibration standards. If SEC is combined with a refractometer as a concentration detector and a viscometer for measuring intrinsic viscosity (SEC-Triple), it becomes a powerful technique to characterize HA and other polymers (Sabagh et al., 2015).

Šoltés et al. (2002) successfully used SEC-MALS coupled with capillary viscometer for the molecular characteristics of some commercial high-molecular-weight HA. Caspersen et al. (2014) studied the kinetics and mechanism of depolymerisation of solid sodium hyaluronate at elevated temperatures and various pH. To develop an improved correlation between M_w and intrinsic viscosity for sixty HA batches with M_w ranging from 0.4 to 2.3 MDa, SEC with dual detection of multi-angle light scattering and refractive index (SEC-MALS-RI) was used.

1.2.3. Biological attributes

Naturally, the HA molecules is synthesized inside the cells, for example, by endothelial cells, adventitious cells or oocytes (Romagnoli et al., 2008). In the synovium it is produced by synoviocytes, fibroblasts and chondrocytes. In the nature, the most of the HA amount can be found in rooster comb (7500 μg/ml), in human umbilical cord (4100 μg/ml), in human joint fluid (1400 – 3600 μg/ml), in bovine nasal cartilage (1200 μg/ml) and human vitreous body (140 – 340 μg/ml). The whole list of the tissues and body fluids with different HA content was published by Shiedlin et al. (2004). Approximately 11-17 g HA can be found in a human being (Laurent et al., 1991).

HA can be animal or bacterial origin. The most animal origin HA is produced from rooster comb and the bacterial origin HA can be prepared from the capsules of streptococcus bacterium. Therefore, each product has slightly different rheological properties (Manna et al., 1999). The drawback of the animal HA is the impurities. The bacterial HA has no problem with biological purity and that is why it has a better and repeatable quality. In the European Pharmacopoeia there is a request concerning the amount of the bacterial endotoxin or pyrogens. These substances have to be limited in parenteral products at most 0.5 and by intra-articular or intra-ocular parenteral preparations 0.05 I.E. pro mg NaHA (Ph. Eur. 8., 2014).

1.2.4. Physical and chemical properties

In the physiological medium, HA is present as a hyaluronate salt, negatively charged and highly hydrophilic (Vasi et al., 2014). In general, it has the cation sodium [$(C_{14}H_{20}NNaO_{11})_n$], but other cations are possible (like zinc, potassium, magnesium, calcium) too. In solution HA has a much stiffened random coil structure. HA can have different conformations, which depend on the pH and the compounds of the solution. In 1983 Sheehan et al. (1983) reported about a left-handed four-fold helical structure by sodium hyaluronate and a three-fold helix by calcium hyaluronate. In 2001, Sheehan et

al. published results with potassium hyaluronate and their different helical structure (studied by X-ray fiber diffraction) (Figure 5). Mainly the electrolytes and the solvents have an effect on the chain flexibility of HA (Gribbon et al., 2000). Calcium and manganese has a greater impact on the flexibility of HA chains, while the chains are less flexible with sodium and potassium counter ions (Sheehan et al., 1983; Gribbon et al., 2000).

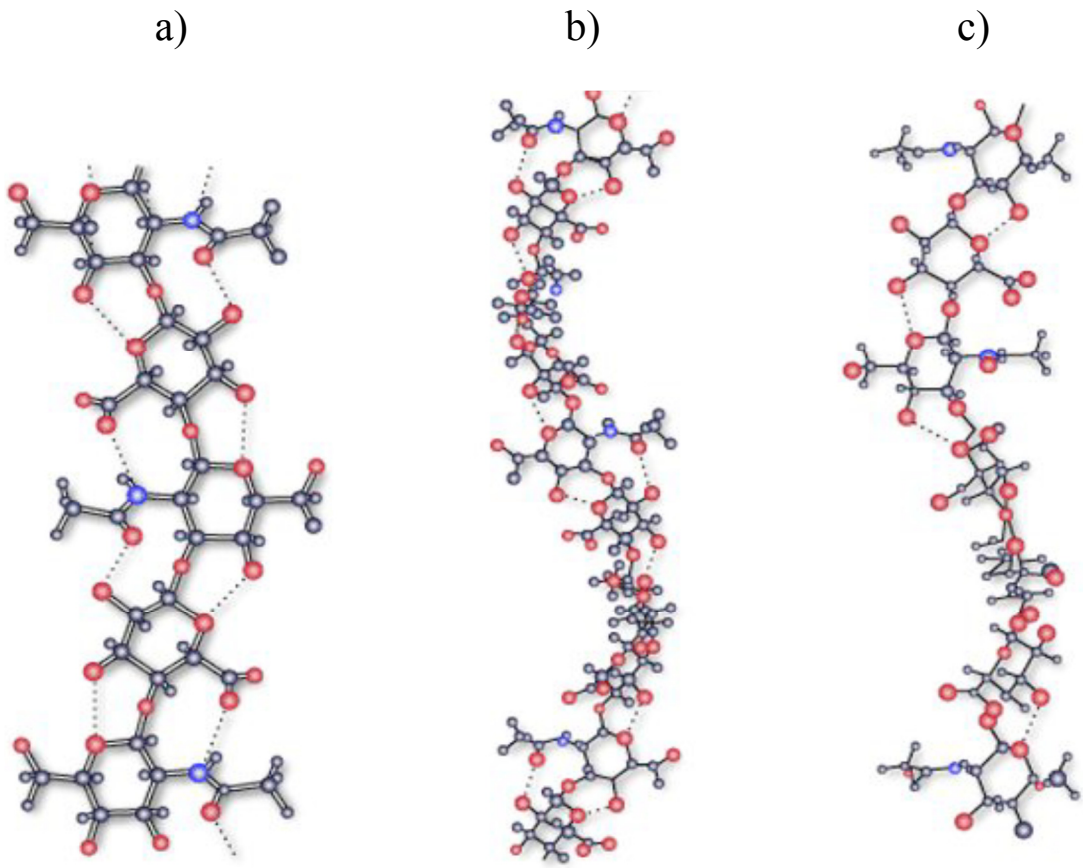

Figure 5 X-ray fiber diffraction of potassium hyaluronate a) 2-fold helix conformation with an axial rise per disaccharide of 0.98 nm; b) left-handed 4-fold helix conformation with an axial rise of 0.84 nm per disaccharide and c) left-handed 4-fold helix of axial rise of 0.95 nm per disaccharide (Sheehan et al., 2001)

The pH of the i.a. formulations has to have about 7.4 (as the physiological pH value of the synovial fluid) or slightly lower, but not below pH ~ 5.5 (Gerwin et al., 2006). HA solution has pH between 6 and 7.2, therefore it is ideal for i.a. treatments. By decreasing pH (< 3) the carboxyl groups are protonated and HA form a water-insoluble gel (Wohlrab et al., 2004).

Under physiological condition HA with the carboxyl anion is able to bind a large amount of water. That is why the HA of 2 % in solution can bind 98 % water by forming a gel (Wohlrab et al., 2004). This gel - under physiological condition - shows viscoelastic and pseudoplastic characteristics and it is a non – Newtonian liquid.

In inflamed joints (e.g. in osteoarthritis, rheumatoid arthritis), the low viscosity is caused by increase of the volume of the synovial fluid and the reduction of the HA concentration and M_w (Marshall et al., 1997). M_w of HA has a significant effect on its half-life time. HA is metabolized very fast in the most tissues. The half-life time of HA with 6 million Da is about 13.2 h (Wohlrab et al., 2004). In different tissues HA has another length of the polysaccharide-chains, another M_w and that is why another half-life time. HA has a longer half-life time in vitreous of the eyes then in articular joints. But this quantity depends on the replacing of the vitreous humor and the synovial fluids, too. Larsen et al. (Larsen et al., 2008) summarized the synovial disappearance half-lives and molecular weights of various solutes (lidocaine, diclofenac, hydrocortisone, hyaluronan, albumin). Hyaluronan has the longest half-life time in the synovial fluid, it is about 21.8 – 26.3 hours with 3×10^6 g/mol molecular weights.

Various HA derivatives can be achieved by chemical modification. Carboxyl groups of HA is especially suitable for this preparation. HA receptors and hyaluronidase recognize the carboxyl groups of HA (Banerji et al., 2007). Therefore, these derivatives can modify the enzymatic degradation of the molecule and accordingly the effect of HA. Mainly carboxyl and hydroxyl groups of HA are used for chemical modification, for example, with carbodiimide, divinyl sulfone, glycidyl ether or dialdehyde (Oh et al., 2010). In order to produce hydrogels, HA derivatives are in strong demand, too. There are three types of HA whereby hydrogels are got, direct cross-linking of HA, cross-linking of HA derivatives and cross-linking between two different kinds of HA derivatives (Oh et al., 2010).

Maroda et al. (2011) prepared and characterized HA nanoparticles formed by amidation with an amine as a cross-linking agent in aqueous media. Cross-linked nanoparticles have a size less than 20 nm but during the purification it can be lost.

HA has an also great mucoadhesive property (Liao et al., 2005). HA creates bonds with the mucin in the mucous membrane and it causes longer residence time. Because of these mucoadhesive properties, HA is able to increase the absorption capacity of different drugs (Lim et al., 2000).

1.2.5. Stability

HA is a very unstable and heat sensitive molecule, the molecular mass and the viscosity may be decreased due to the damage of bonds in the polymeric chains (Bailey et al., 1968). If HA in solution is warmed up to 100 ºC, the bonds between the chains are get damaged, and the M_w and viscosity decrease. That is the reason why sterilizer with high temperature cannot be used for HA.

Thermal effect or treatments, pressure, ultrasonic effects, filtration, mixing or using syringe can destroy the chains. These chain scissions cause the reduction of the M_w and accordingly the reduction of the viscosity (Bailey et al., 1968).

It is well known that hyaluronates are susceptible to degradation under a variety of conditions such as acid hydrolysis (Cleland, 1977; Longas et al., 1981), oxidative depolymerisation reactions (Matsumura et al., 1966; Kvam et al., 1993), and sonication (Kubo et al., 1993).

But there is also another natural destroyer for HA, it is the degradation enzyme, hyaluronidase. Hyaluronidase breaks down HA very fast in the articular joints. The speed of the degradation depends on the M_w and the structure of the HA. Improved stability of HA can be achieved with cross-linking. Cross-linked HA has a higher half-life time and can stay longer in the articular joints.

1.2.6. Hyaluronic acid-based hydrogel

Gels are one-phase systems, the most common group, in which the gel medium is the water, are the hydrogel. The gelling materials can be different polymers, like polymethyl methacrylate (PMMA), poly-(2-hydroxyethyl)-methacrylate (polyHEMA) or polyvinyl alcohol (PVA) (Li et al., 2009). The polymers of the hydrogels are held together by physical, ionic or covalent bonds and can absorb water (Davidovich-Pinhas et al., 2010; Sriamornsak et al., 2008). The gel carriers for i.a. uses play a dual role in articular therapy: on the one hand gel carriers ensure longer residence time for active ingredients in the joints compared to the drug solution forms, and on the other hand through their viscosity they maintain the motility of joints such an artificial lubricant. Despite the many known artificial polymer systems in the development of pharmaceutical formulations, the endogenous HA and its various derivatives are the most preferred gelling material. Thus, the HA hydrogels used to help restore the physiological HA concentration, which increase the reduced viscosity. At present, in the therapeutic practice, only hyaluronic acid-based gels are applied (Orthovisc®, Monovisc®, Synvisc®, Biovisc®, Hyalgan®, Synocrom®, Synocrom Forte®) (Szabó A. et al., 2011). In *in vitro* studies, Réeff at al. (2012) with a HA gelling agent a release of several days (active ingredient clonidine) was reached. They used glyceryl monooleate as excipient for slowly soluble viscous phase in the synovial fluid (Shah et al., 2001).

1.2.7. Studies on the characteristics

1.2.7.1. Viscometry

Due to the chemical structure of the polymer, HA shows interesting rheological properties. The coils of HA can straighten, and this behavior is the mechanism of action behind viscosupplementation (Balazs et al., 1970). The aqueous solutions of high-molecular-weight HA exhibit shear-dependent viscosity and frequency-dependent elasticity (Šoltés et al., 2002).

The viscoelastic behavior of HA in solution changes with exposure to different shear rates. The shear rate is dependent on the dynamic elastic and viscous moduli. The crossover point of these two moduli can be used for the rheological characterization of HA.

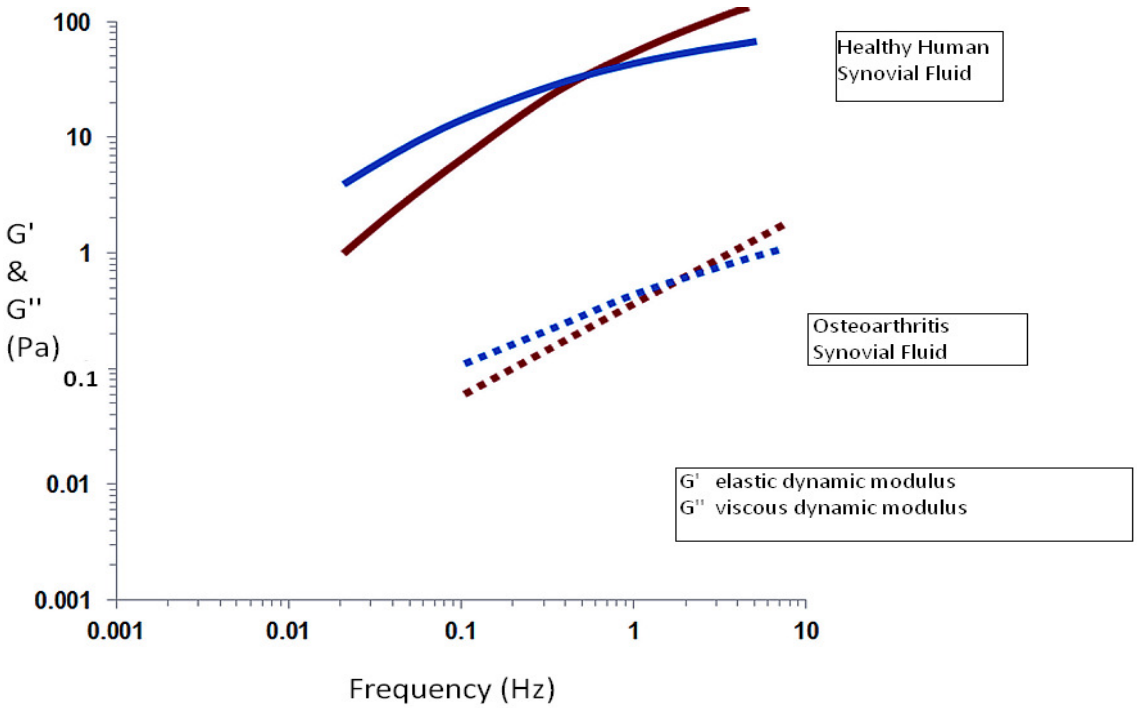

Figure 6 The viscoelastic behavior of HA and background of the viscosupplementation (brown: G' elastic dynamic modulus, blau: G" viscous dynamic modulus; based on Balazs, 2009)

Above this frequency, the solution has elastic properties, and below this frequency it shows viscous behavior (Figure 6) (Balazs, 2009). This network, when sheared by flow or oscillation movements, can dissipate the energy in viscous flow or store it as elastic deformation (Gibbs et al., 1968). These rheological properties of HA provide viscous protection and elastic shock absorption in the joints (Phillips et al., 2013). The more suitable viscosupplementation product is the one with an elastic component similar to or greater than that of healthy young synovial fluid (Phillips et al., 2013).

In 1990 Hassan et al. have also used a simple viscometric method to quantify mucin-polymer bioadhesive bond strength. The mucoadhesive capacity was measured by a rheometer and this rheological behavior between the polymer and the mucin can be described by the following equation:

$$\eta_b = \eta_t - \eta_m - \eta_p \qquad \text{(Eq. 2)}$$

where:

η_b = bioadhesive viscosity component;

η_t = viscosity of the mucin-polymer containing samples;

η_m = individual viscosity coefficients of the mucin;

η_p = individual viscosity coefficients of the bioadhesive polymer.

1.2.7.2. Thermal analysis

The thermal degradation of NaHA was evaluated by thermogravimetric analysis (Villetti et al., 2002) which indicated that low thermal stability can be observed for the polyanionic NaHA.

1.2.7.3. X-ray diffraction

Guss et al. (1975) examined the molecular conformations and interactions in two sodium salts of HA. The results of X-ray diffraction showed that the counterions form strong intermolecular interactions with neighbouring HA molecules. Water molecules are attracted by the charged sites to form hydration shells. Guss et al. with X-ray experiments convinced that increasing the amount of water present in the hydration shells causes the reduction of the mechanical stiffness of the HA molecule.

Sheehan et al. (1983) used X-ray fiber diffraction to study of conformational changes in hyaluronate induced in the presence of sodium, potassium and calcium cations. The effects of pH, humidity, temperature, ionic strength and hydrogen ionic activity were studied in physiological situations. X-ray fiber diffraction method is a monitoring technique to illustrate the conformational changes. The results showed that the conformations of the chains are held together by stronger or weaker intra-chain hydrogen bonds. HA has in the presence of counterion sodium a left-handed four-fold helix form and in the presence of calcium a three-fold helical structure. These results were confirmed by the results of NMR studies, too (Almond et al., 2006).

1.2.7.4. Spectroscopy

Lee et al. (1993) reported about the tightly bound water (primary water) of hydration in Li- and NaHA. Brillouin spectroscopy was used to examine dynamic coupling with the water of hydration and phase transitions in HA. Their results were that the relaxation time of the water relaxation mode is approximately the same in Li- and NaHA and the coupling to the biopolymer depends on the biopolymer itself.

Ultraviolet spectroscopic studies of Lee et al. (1995) examined the physical properties of hyaluronate films. Taken together, two results of electronic states were reported; the elastic moduli of Li- and NaHA decrease over the entire range of hydration (this indicates that the long-range Coulombic interactions are very sensitive to the water content) and the changes in the UV spectrum of a NaHA film (before and after being

cycled through the phase transition between 84 and 92 % relative humidity) were found to be irreversible.

Infrared (IR) spectroscopy is used for the identification and classification of compounds and because of this unique characterization, it is often called “fingerprint” of the molecules. This experimental spectroscopy is very often used for polymers. In 1954 Orr et al. used conventional IR spectroscopy for partial assignments of bands of HA and other hyaluronates.

In 1976 Cael et al. recorded spectra with Fourier Transform Infrared Spectroscopy (FTIR) for two different crystalline forms of NaHA. It is an improved technique for the analysis of solid, liquid and gas. A review of research papers about the variety of molecules (mono- and oligosaccharides, polysaccharides) examining by FTIR have been presented (Kačuráková et al., 2001). HA-phosphate fiber mats were examined by Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR) spectroscopy (Brenner et al., 2013). HA fiber mats were produced with glycerol phosphate, sodium phosphate and tripolyphosphate salts. All display the characteristic carbohydrate peaks for HA.

Gilli et al. (1994) studied NaHA and its oligomers by FTIR spectroscopy in the amorphous solid phase and in aqueous solution. Dřímalová et al. (2005) depolymerised HA by ultrasonic, microwave irradiation and conventional heating, tested the effect of pH and oxidants on HA. This degradation and the molecular weight changes of HA were studied by FTIR spectroscopy. The results are in agreement with the previous IR study on HA from Gilli et al. (1994), the overall spectral pattern has not changed by decreasing the molecular mass of HA. Dřímalová et al. also reported about NMR studies with the HA samples. The NMR and also FT-IR spectral analyses indicated that HA in the studied whole molecular weight range retained almost the backbone of the parent polysaccharide independently on the degradation method used.

The first time Lahajnar et al. (1986) reported on the proton nuclear magnetic resonance (NMR) spectroscopic study of water absorbed in solid, oriented NaHA. The results indicate a hydration similar to that of many other fibrous biopolymers at comparable relative humidity levels. Investigations using NMR in order to characterize the accurate solution conformation were mainly unsuccessful because of the overlap presents of the NMR spectra (Holmbeck et al., 1994; Donati et al., 2001).

Even so, Almond et al. (2006) successfully presented results about the solution conformation of HA determined by computer modeling (molecular dynamics simulations) and high-field nuclear magnetic resonance (NMR). The resulting data are used to test molecular dynamics simulations containing explicit water and ions at the atomic level. The results of this new description of the solution conformation of HA - a left-handed four-fold helix structure - are similar to that observed for potassium, sodium and calcium HA fibers by X-ray diffraction (Sheehan et al., 1983). The experiments of Almond et al. also pointed out that the intramolecular hydrogen bonds in HA exist in aqueous solution but they are constantly in motion and in rapid interchange with water-mediated interactions. This simulation can be used as a basis for constructing molecular models to understand the interaction between HA and proteins, while also explaining the viscoelastic properties of HA.

1.2.7.5. Positron annihilation lifetime spectroscopy

Variation of the free volume with temperature in the sol-gel transition of 2 % (w/w) κ-carrageenan showed the sol-gel transition to be an onset of the decreasing percentage of the free volume (Wakabayashi et al., 1996). Positron annihilation lifetime spectroscopy (PALS) (Figure 7) (Szabó B. et al., 2011; Szabó B. et al. 2012) is able to give important information about the free volume properties of polymers related to their molecular dynamics, volume relaxation and physical ageing.

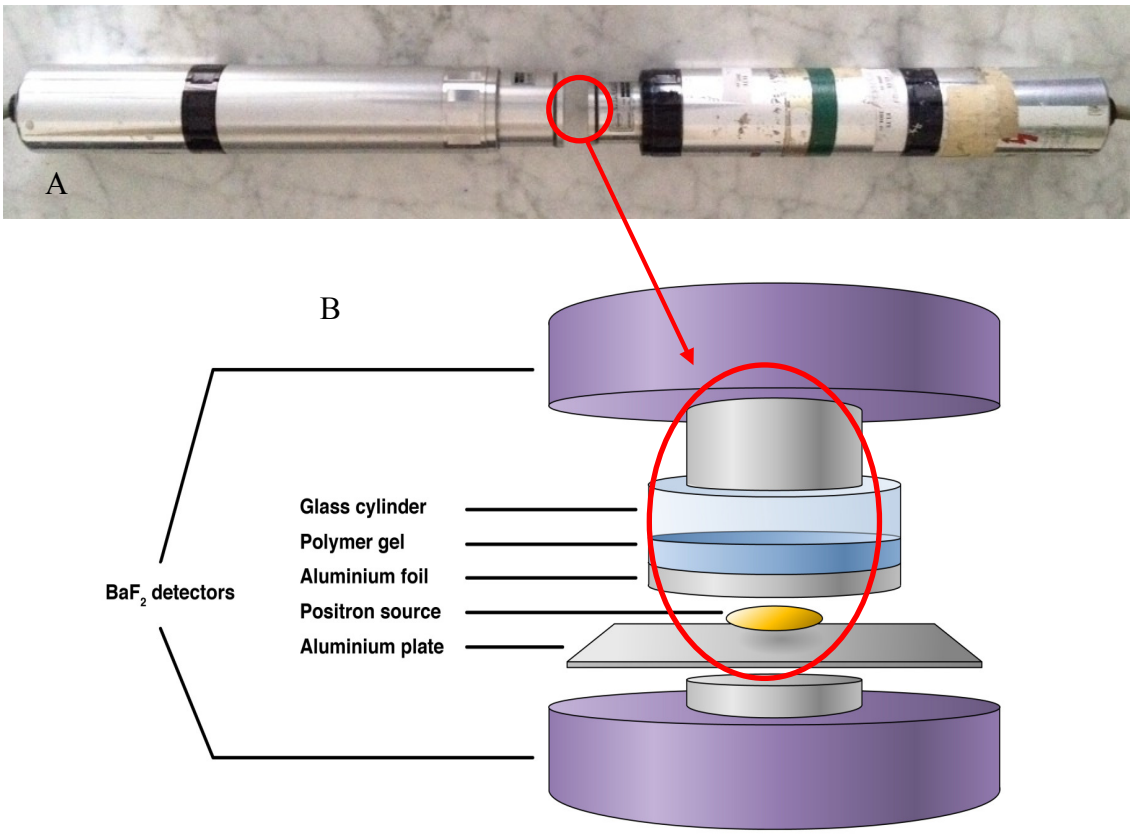

Figure 7 Method of PALS (A - real construction of PALS; B - schematic construction of PALS (Szabó B. et al., 2012))

Sebe et al. (Sebe et al., 2012) have summarized the principles and the functions of PALS: the method is based on the annihilation of the electrons and positrons with high-energy γ-radiation. The electrons and positrons work as particle and antiparticle pairs and they destroyed mutually and finally they disappear or annihilate. During this process, the total mass of particle and antiparticle mutate to energy (photons without resting mass) according to Einstein equation $E = mc^2$ (m is the mass of the particle, c is the speed of light and E is the released energy). This process produces energy without any side products, with 100 % efficiency.

During the annihilation of positron and electron resulting generally two photons leave each other at an angle of 180°. This phenomenon can be used for research purposes. Before the annihilation of positron and electron there is another intermediate form, the positronium atom. There are two forms of positronium atom: if the positron and electron spin are the same (+1/2 or -1/2) (the spin of positronium atom is ± 1), then called orto-positronium (o-Ps) or if the spins are contrary (the spin of positronium atom is 0), then called para-positronium (p-Ps). The life of p-Ps is too short, it annihilates in vacuum in 125 ps during resulting two photons, but the o-Ps survives (because the contrary spins inhibit the annihilation). But, after a time the inhibition is not strong enough and the o-Ps annihilate during resulting three photons. Its lifetime in vacuum is 142 ns. In polymers this lifetime can reduced to a few ns, because of the pick-off annihilation with an electron during resulting two photons as by p-Ps. This pick-off annihilation can be used to define the cavity size of polymers. The smaller the cavity of the polymer is, where the o-Ps passes, the faster it meets electron with contrary spin (Sato et al., 2008).

According Doppler-effect the velocity of the emitting source (in this case the electron during the annihilation) and the produced radiation (in this case the photons) may be measured. If in the polymer electron has energy (because of its velocity), it is added to the energy of produced photos. If the energy of annihilated photons can be measured, the momentum of electrons in the polymer cavity can be described. Generally, ^{22}NaCl is used as positron source and two scintillation detector with BaF_2 are needed. The detectors must be placed opposite each other as close as that goes (between the detectors is the polymer (Figure 7). The time between the two detectors sign is the lifetime of the positron. The resulting curve can be evaluated with different computer programs.

Only one publication in the past affects the topic hyaluronic acid examined with PALS. Süvegh et al. (2000) have tested the positron lifetime technique as a tool of the structure study of sodium and zinc hyaluronates. By zinc hyaluronate the positron lifetime significantly increased comparing sodium hyaluronate. And normally it would mean that the free volume between the chains of zinc hyaluronate has also increased. But this was not the case because in prior rheological studies (Burger et al., 2001) it was demonstrated that the free volume values in zinc hyaluronate decreased comparing

sodium hyaluronate and the o-Ps lifetime of zinc hyaluronate increased. The study reported about the different bonds: sodium salt has intermolecular hydrogen-bridge-bonds and the exchange of cations (sodium to zinc) results intramolecular bonds. In the latter the chains curl up and that is why the free volume values decrease. This indicates that the intramolecular hydrogen-bridge-bonds are tauter and the o-Ps cannot access to the electrons so easy.

1.2.8. Formulation aspects of hyaluronic acid

1.2.8.1. Sterilization

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) (Wohlrab et al., 2004).

Experiments with "ready-to-use pre-filled" HA syringe showed that by autoclaving for 20 min, the M_w decreased from 1.4 million Da to 0.8 million Da. By this gamma ray intensity the number of the chain scissions is acceptable and the results are repeatable.

Barbucci et al. (2002) investigated the sterilization of lyophilised cross-linked HA (synthesized from 50 % with COOH groups cross-linked HA). It was sterilized with steam, γ-rays and ethylene oxide. The swelling ability and morphology of HA were not modified by sterilization with ethylene oxide and γ-rays. The steam sterilization caused modifications of this type of HA.

However, data from simulated sterilization conditions suggest that higher concentrations of HA may have a protective effect on the stability of the long-chain molecule, such that heat treatment would not denature the hydrogel (Ali et al., 2009).

Furthermore, Novozymes Biopharma was dedicated to providing a Bacillus-derived high-quality HA. No animal-derived raw HA provides superior heat stability for more efficient sterilization process (Novozymes, 2012). The investigations of Szabó A. et al. (2013) showed that the heat sterilization modified both the micro- and macrostructures of the NaHA (animal derived) gels depending on the concentration and therefore it is not a suitable method to achieve the sterility.

1.2.8.2. Freeze-drying

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. However, the freeze-drying and subsequent rehydration of thermosensitive polymer gels, like hydroxypropyl cellulose (HPC), could alter the microstructural properties of the gels in a way that leads to rapid shrinking rates (Kato et al., 2004).

Earlier data indicated that lyophilisation of hyaluronates as the free acid, in contrast to the sodium salt form, can have a detrimental effect on their physical characteristics (Doherty et al., 1994). The data of Doherty et al. (1994) indicate that the free acid form of medium molecular mass hyaluronate exhibited marked changes in molecular mass during lyophilisation whereas the NaHA appeared unchanged.

Measurements showed that the evolution of carbohydrate radicals on freeze-drying of HA is more than three times that of NaHA, which explained that HA is structurally less stable than NaHA (Tokita et al., 1997). Further, the calculations of Tokita et al. suggest that HA is much more labile against hydrogen abstraction as compared to NaHA.

The results of Wedlock et al. (1983) suggest that the structural stability of HA against a distortion induced by freeze-drying is lower than that of NaHA. Concerning the stability of hyaluronate during freeze-drying, significant concentration effects would occur with respect to the hyaluronate, and it is possible that a combination of concentration effects and shifts in the effective pH of the amorphous reaction matrix could contribute to the apparent changes in molecular mass. Furthermore, this hypothesis concerning and pH effects is consistent with the observed stability of NaHA to lyophilisation as the pH of those solutions was near neutral (Doherty et al., 1994).

The changes of the NaHA microstructure were studied by positron annihilation lifetime spectroscopy (PALS) in the course of the lyophilisation that gives information about the free volume of the polymer systems. This measurement can be used for the analysis of amorphous materials like NaHA (Zelkó et al., 2006).

2. Aims

Many people have joint problems and therefore, the intra-articular delivery system provides a very promising solution for the therapies. An increasing number of HA injections appears on the international market, and this point shows the interest in this product. This molecule is also very manifold but the handling and the working with them are not so easy for the pharmaceutical industries. Because of the sensitivity of the molecule many processing and manufacturing processes are not cleared up.

The aim of this research work was to study the formulation of 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) to formulate 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

3. Materials and methods

3.1. Materials

3.1.1. Active ingredient

NaHA of low molecular weight (M_w = 1,500 kDa) has an animal origin, and is produced from rooster comb. The pharmaceutical grade of NaHA was obtained from Gedeon Richter Ltd., Budapest, Hungary. The water content of the raw NaHA was 9,5 ± 1,37 %.

3.1.2. Excipients

Na_2HPO_4 and NaH_2PO_4 were used as isotonic sodium phosphate puffer (19.543 g Na_2HPO_4 x 2 H_2O and 2.425 g NaH_2PO_4 x 0 H_2O ad 1000 ml water). The puffer had pH 7.554 and an osmolarity of 286 mOsm/kg. The pharmaceutical grade was obtained from VWR International Ltd., Debrecen, Hungary.

3.2. Methods

3.2.1. Preparation of hydrogels

NaHA hydrogels of 5, 7.5 and 10 mg/ml concentration were prepared. At first the polymer was swelled in sodium phosphate buffer during 24 hours. Table 4 shows the measured parameters (concentration, pH, osmolarity) during the production.

Table 4 Product parameters of NaHA hydrogels

Samples	Autoclaving	Freeze-drying
Concentration (mg/ml)	5, 7.5, 10	5, 7.5, 10
pH	7.44 - 7.45	7.50 - 7.55
Osmolarity (mOsm/kg)	218 - 286	294 - 311

In the second part of the thesis 10 mg/ml NaHA hydrogels were prepared with water (without sodium phosphate puffer). The polymer was swelled in water during 24 hours. The pH and the osmolarity were 6.416 and 0.006 Osm/kg.

In both of the case, the hydrogels mixed with a glass rod at room temperature and stored foil sealed in refrigerator for 24 hours. After 24 hours the evaporated water was completed and again homogenized by a glass rod.

3.2.2. Heat sterilization

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 minutes, 103.4 kPa. The untreated and heat sterilized gels were stored at a temperature of 22 ±0.5 °C and a relative humidity of 55 ± 2 %.

3.2.3. Freeze-drying

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.

3.2.4. Thermal analysis

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 aluminium 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 aluminium as sample containers. The measured data were registered with STAR^{e} software.

3.2.5. Reconstitution

For the reconstitution 5 g raw NaHA and 5-5 g the freeze-dried NaHA samples were dissolved in 5 ml water. The water was very slowly added by injection. It was measured how much time the dissolution and the hydration needed to get a hydrogel. The timescale was noticed during 2 min.

3.2.6. 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 diameter of the cone was 50 mm. The quantity of the samples was 0.57 ml. 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 analyzed.

3.2.7. Surface morphology

The scanning electron microscopy (SEM) 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).

3.2.8. Positron annihilation lifetime spectroscopy

The source was covered by very thin foils. Due to the exponential penetration of the radiation, a larger intensity and smaller variation of the o-Ps lifetime could be obtained than with regular foil of 20-25 μm. Approximately 150 μl (2-3 drops) from each homogeneous polymeric gel was poured onto the foil. The solvent evaporation was prevented by a cylindrical metal cover.

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 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 different channels of an analyzer card for different time and each contained about different coincidence events (Table 5). The number of coincidence counts was inevitably slightly lower than normal because of the increased distance between the two detectors. The detectors were arranged vertically in this experimental setup. An aluminium plate was placed on the lower detector. Although a great number

of positrons were lost using this arrangement, the significant part of the signal remained intact. As the aluminium does not produce an o-Ps component, any signal in that certain lifetime range came exclusively from the gel (Szabó B. et al., 2012). The temperature during the measurements was 22 ± 0.5 °C.

Three parallel spectra were measured at each concentration to increase reliability. After summarizing the parallels, spectra were evaluated by the RESOLUTION computer code (Kirkegaard et al., 1981); the indicated errors are the deviations of the lifetime parameters obtained. Three lifetime components were found in all the samples. The MELT code (Shukla et al., 1993) 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 o-Ps lifetime.

Table 5 Different process parameters for PALS measurements

	Sterilization	Freeze-drying
Foils for active materials	Kapton	Ti
Channels	4096	3050
Time (s)	9000	1800
Coincidence events	1.5×10^6	10^6

3.2.9. Karl Fischer water content determination

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.

3.2.10. X-ray powder diffraction

Structural analysis was examined 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°.

4. Results

The investigations were focusing to examine the influence of heat sterilization and NaHA concentration on the micro- and macrostructure of NaHA hydrogels. NaHA hydrogels of different concentrations were prepared and heat sterilized. The microstructures of the polymer gels were characterized by PALS based on their o-Ps lifetime values and distributions, while their macrostructures were characterized by rheological measurements. It was believed, that the heat sterilization modifies both the micro- and macrostructures of the gels and the NaHA concentration is also observed to influence the hydrogel structure.

After the investigation of the sterilization, the freeze-drying ability of the hydrogels was examined. However the freeze-drying is not a sterile procedure, it could make simpler and speed up the production process and the application. But then each ingredient should be sterilized in accordance with the properties of materials and these should be freeze-dried under aseptic conditions.

Aqueous NaHA gels were prepared and the obtained samples were freeze-dried. It has been observed how fast the gelling ability is meanwhile preserved their initial viscoelasticity even after reconstitution. The microstructure of gels obtained from raw substance and freeze-dried NaHA samples was characterized with PALS and X-ray diffraction patterns, while their functionality-related macrostructural properties were tested based on their rheological behavior. Furthermore, thermogravimetric, differential scanning calorimetry measurements, surface morphology and Karl Fischer water content determination were used for the tracking of the structural alterations.

These properties may be advantageous in the development of a freeze-dried NaHA injection dosage form.

4.1. Heat sterilization

4.1.1. Formulation and characterization of sodium hyaluronate gels

At the beginning of these studies, some product was summarizing from the present pharmaceutical market (Table 2 and 3). The concentrations of the sodium hyaluronate product were researched but there was no information about the M_w of the formulations. Therefore, NaHA with M_w = 1,500 kDa was used and three different concentrations (5.0, 7.5 and 10.0 mg/ml) was taken.

The samples with the three different concentrations were prepared and were heat sterilized at 121 °C for 20 min. Additionally, the samples were studied without any further treatment at two hours and at one week after autoclaving. The untreated and heat sterilized gels were dried at a temperature of 22 ± 0.5 °C and had a relative humidity of 55 ± 2 %. The untreated (without autoclaving), treated after two hours and one week (at two hours and at one week after autoclaving), untreated dried and treated dried samples have been compared in the next chapters.

4.1.2. Structural elucidation of sodium hyaluronate gels

4.1.2.1. Positron annihilation lifetime spectroscopy

On first time the untreated NaHA gels and the treated NaHA gels at two hours and at one week after autoclaving were compared. The Figure 8 (A-C) shows the o-Ps lifetime distributions of these samples of various concentrations. 7.5 mg/ml NaHA gels (Figure 8B) showed the smallest changes in the o-Ps lifetime values before and after heat sterilization.

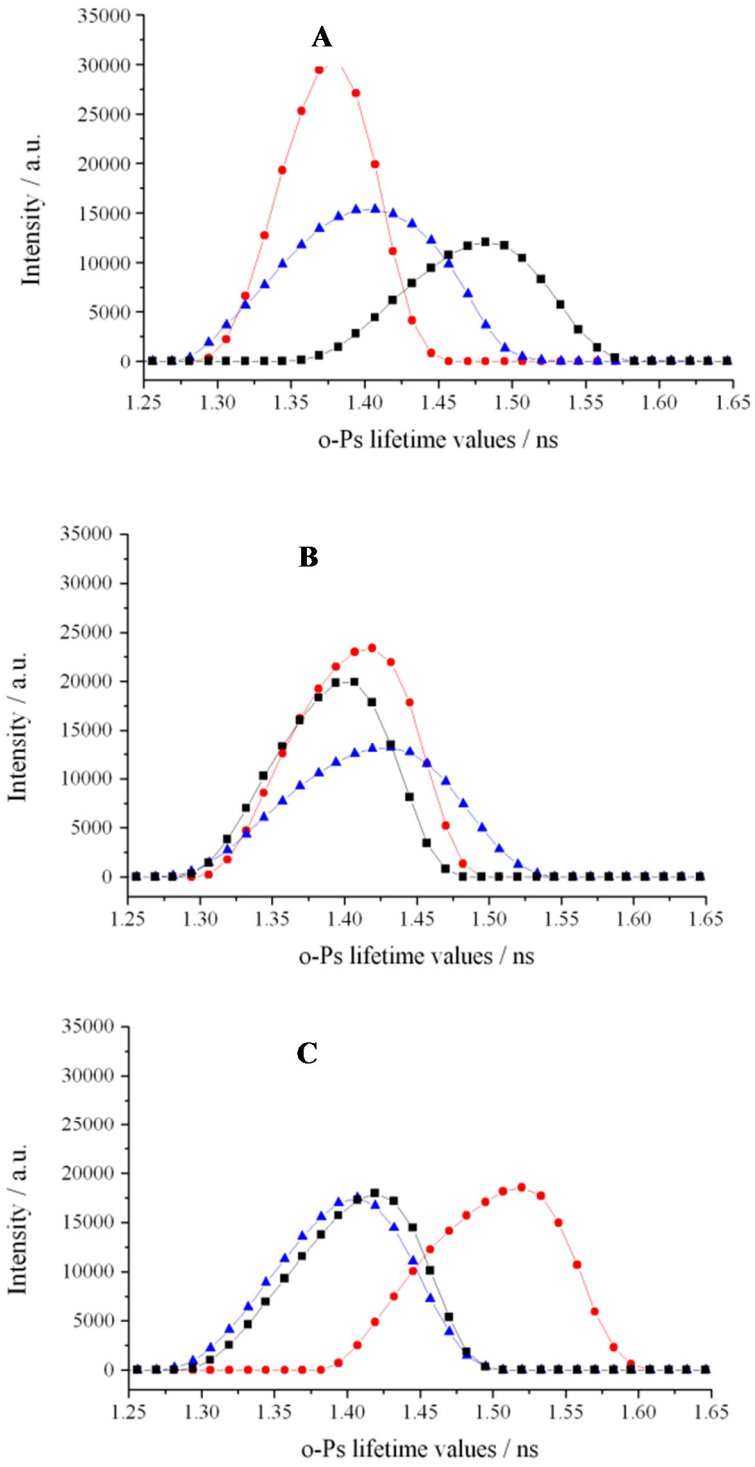

Figure 8 A-C Lifetime distribution curves of the HA gels of different concentrations (black: untreated sample, red: treated sample; blue: one week after the treatment) A – 5.0 mg/ml HA, B – 7.5 mg/ml HA, C – 10.0 mg/ml HA (Szabó A. et al. 2013)

Table 6 also represents the changes of the o-Ps lifetime values of the gels and dried samples before and after heat sterilization. At 5.0 mg/ml NaHA concentration, a close-packed structure is formed while in the case of 10.0 mg/ml NaHA, it is the formation of a more open structure, thus the distance between the NaHA chains increases. The sample with 7.5 mg/ml concentration has the least distribution which shows the structural stability of the hydrogel after sterilization.

At 10 mg/ml NaHA, the structural change was reversible; after one week of storage, a chain relaxation occurred (Figure 8C).

Table 6 Summarised results from RESOLUTION computer code (o-Ps lifetimes in ns) (Szabó A. et al. 2013)

Concentrations	Untreated	Treated	After 1week	Dried	Treated, dried
5.0 mg/ml	1430 ± 15	1380 ± 11	1389 ± 11	1102 ± 37	1093 ± 40
7.5 mg/ml	1393 ± 11	1396 ± 11	1405 ± 13	1095 ± 21	1094 ± 25
10.0 mg/ml	1400 ± 12	1465 ± 12	1390 ± 12	1118 ± 23	1131 ± 16

Figure 9 illustrates the o-Ps lifetimes distributions of 5.0, 7.5 and 10.0 mg/ml dried samples before and after autoclaving. The effect of the heat treatment on the dried samples was different. However, in case of the 7.5 mg/ml sample, the microstructure was not significantly changed by the heat treatment.

Table 7 presents the uniformity of the free volumes based on full width at half maximum (FWHM) of the fitted Gaussian curves. In case of 7.5 mg/ml NaHA gels, the least distribution is discernible.

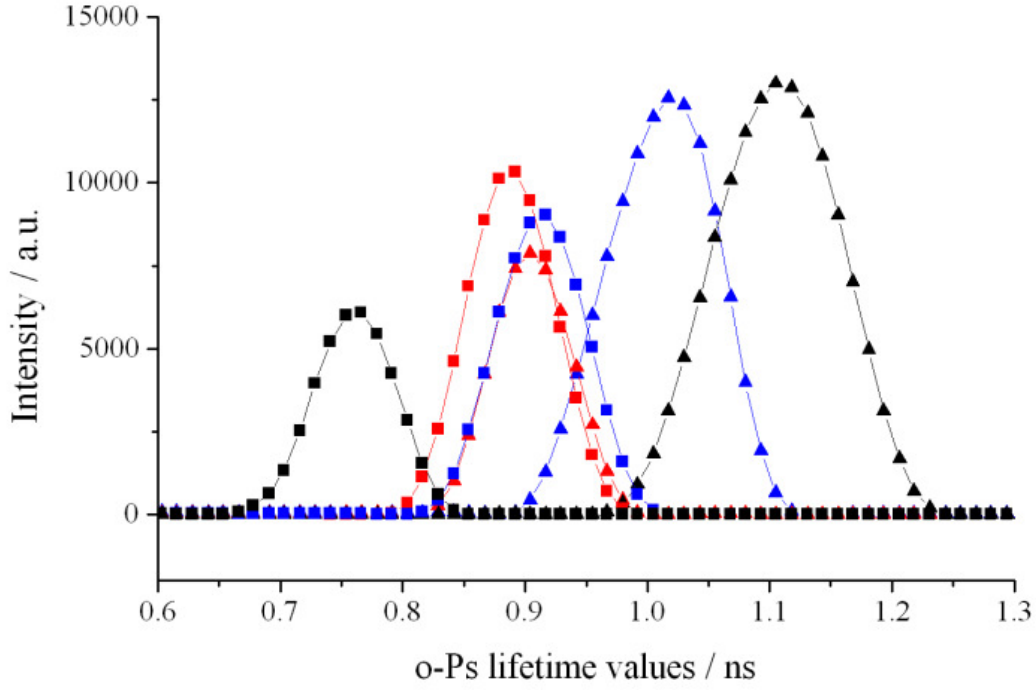

Figure 9 Lifetime distribution curves of the dried samples of different concentrations (black: 5.0 mg/ml, red: 7.5 mg/ml, blue: 10.0 mg/ml; square: untreated dried samples, triangle: treated dried samples) (Szabó A. et al., 2013)

Table 7 o-Ps distribution curve parameters (center and FWHM) in ns (Szabó A. et al., 2013)

Sample	Untreated		Treated		After 1 week	
	Center	FWHM	Center	FWHM	Center	FWHM
5.0 mg/ml	1.4820	0.1070	1.3820	0.0732	1.4070	0.1244
7.5 mg/ml	1.4070	0.0912	1.4190	0.0951	1.4320	0.1299
10.0 mg/ml	1.4190	0.1002	1.5200	0.1125	1.4070	0.1041

4.1.2.2. Viscoelasticity

Viscometric studies were accomplished to get information about the microstructural characteristics of the samples. The rheological behavior of the NaHA gels is demonstrated as a function of increasing frequency.

Figure 10 shows the viscoelastic curves of the 7.5 mg/ml NaHA gel before and after treatment. The autoclaving did not cause significant changes of the transition point.

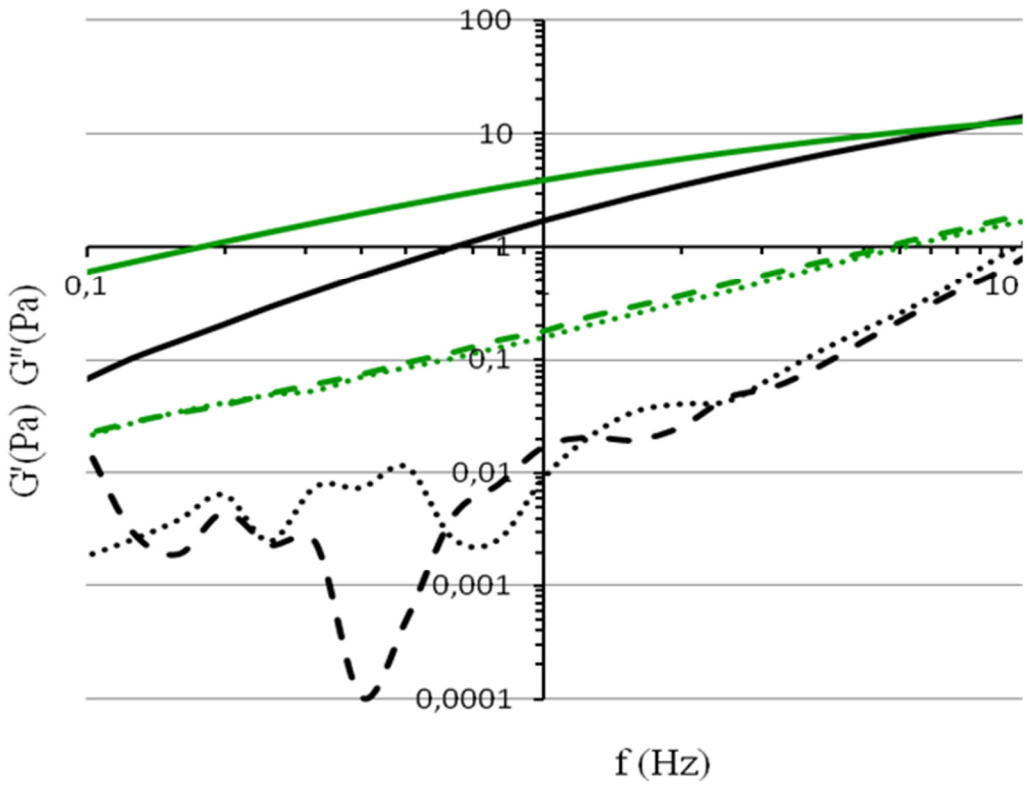

Figure 10 Viscoelastic curves of the 7.5 mg/ml NaHA gels (black: storage modulus; green: loss modulus; line: untreated sample; dashed line: treated sample at two hours after autoclaving; dotted line: treated sample at one week after autoclaving) (Szabó A. et al., 2013)

The elastic and viscoelastic properties of NaHA at three different concentrations were compared in Figure 11. The autoclaving degraded the gel structure in case of the 5 mg/ml sample, the decrease of the viscoelasticity can be observed. The viscoelastic nature of the two other gels containing 7.5 and 10.0 mg/ml NAHA was maintained. The temperature dependence does not play a role.

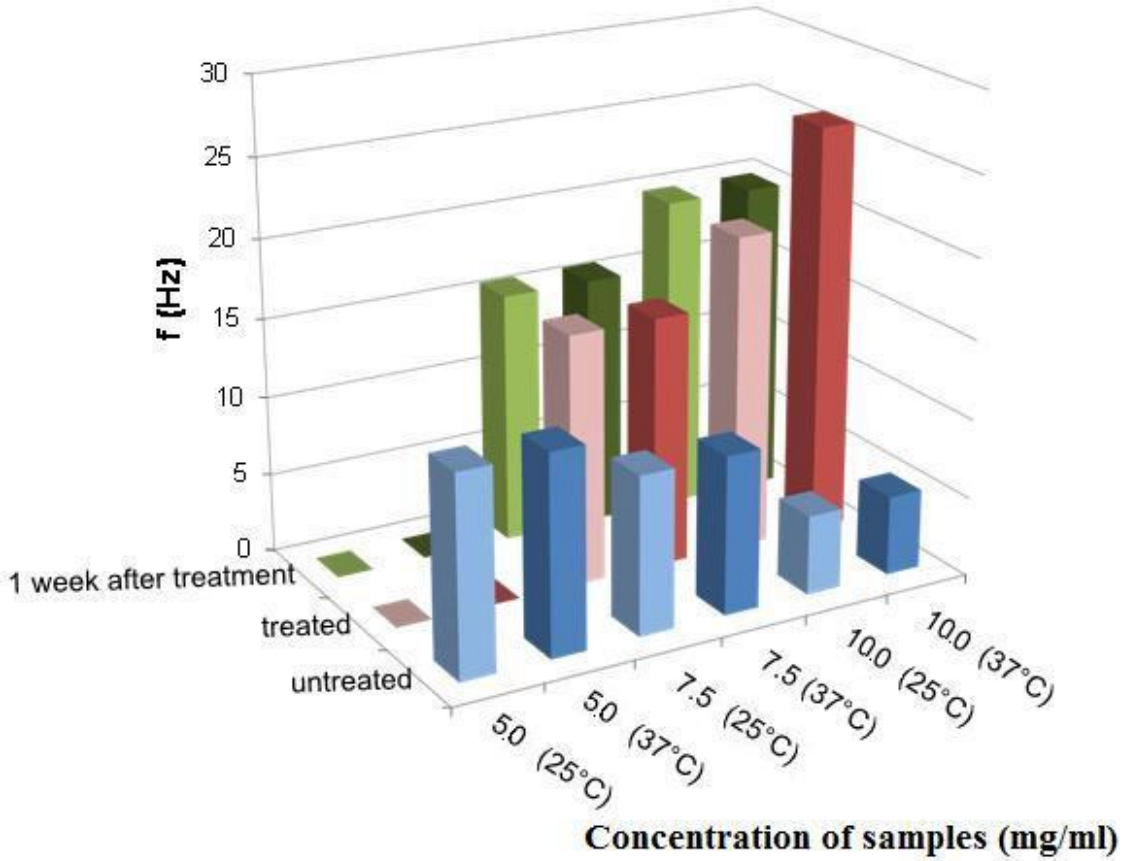

Figure 11 Effect of the HA concentration and the heat sterilization on the intersection of the viscoelastic curves of the samples (blue columns: untreated samples; red columns: treated samples; green columns: samples one week after the treatment) (Szabó A. et al., 2013)

4.2. Freeze-drying

4.2.1. Formulation and characterization of sodium hyaluronate gels

In the first series all of the samples with three different concentrations (5.0, 7.5, 10.0 mg/ml) in sodium phosphate buffer were tested by PALS and the viscoelastic properties were obtained.

In the next series of experiments three different formulations were examined to get explanation what happens with the NaHA structure in the presence of phosphate buffer after freeze-drying (raw NaHA, freeze-dried NaHA gel of 10 mg/ml prepared with water and freeze-dried NaHA gel of 10 mg/ml prepared with sodium phosphate buffer).

Thereafter the gels were freeze-dried. The temperature of the drying chamber was between (-96) °C – (-92) °C. Freezing of the samples was performed in the drying chamber. The process parameters are shown in Table 8 and in the next diagram (Figure 12).

Table 8 Process parameters of freeze-drying of hydrogels

Process	Time (hour:min)	Product temperature (°C)	Shelf temperature (°C)	Pressure (hPa)
freezing	00:00	21	27	69
	00:01 - 00:30	27 - 21	27 - 17	725 - 707
	00:31 - 01:04	20 - 0	16 - (-7)	690 - 673
	01:05 - 02:06	-1 - (-8)	-8 - (-20)	673 - 656
drying	02:07 - 20:38	-8 - (-35)	-20 - 19	43 - 0.02
secondary drying	20:41 – 28:11	-34 - (-19)	34 - 41	0.02 - 0.017

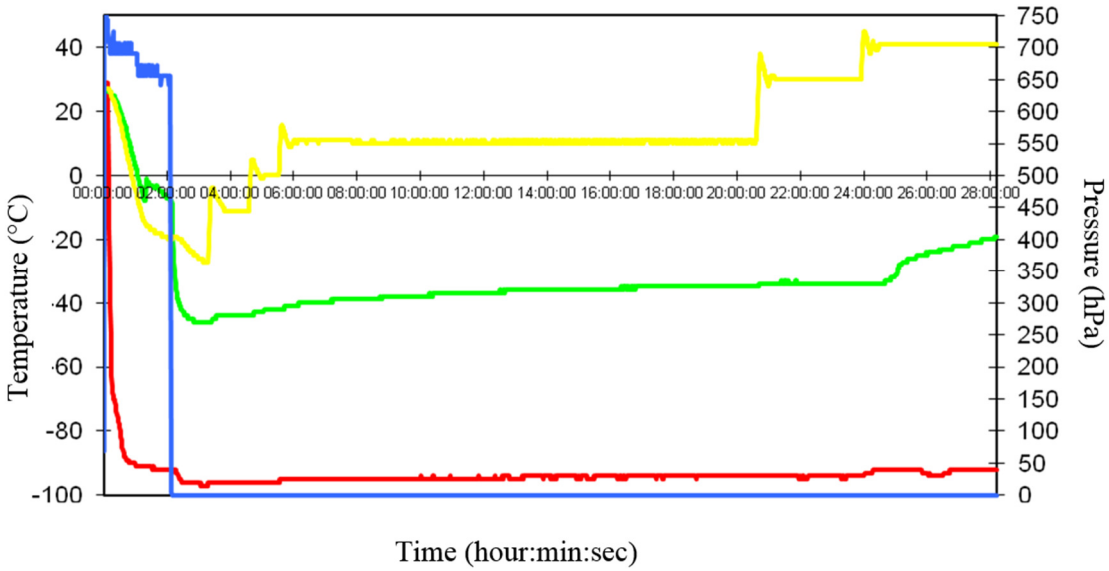

Figure 12 Diagram to the freeze-drying process of hydrogels (red: chamber temperature, green: product temperature, yellow: shelf temperature, blue: pressure)

Figure 13 shows the NaHA hydrogels after freeze-drying prepared with sodium phosphate buffer and with water. The sample with phosphate puffer has a higher volume which can be concluded from the very porous structure.

Figure 13 Freeze-dried 10.0 mg/ml NaHA hydrogel samples with sodium phosphate buffer (left) and with water (right) (each glass ampoule contain 5 g of the sample)

4.2.2. Structural elucidation of sodium hyaluronate gels

In the first series the following experiments were performed: PALS measurements with hydrogels and freeze-dried NaHA hydrogels with 5.0, 7.5 and 10.0 mg/ml in sodium phosphate buffer.

Figure 14 shows the o-Ps lifetime values of hydrogel samples. The most significant supramolecular change, indicated by the greatest difference in the o-Ps lifetime values, can be observed in the case of hydrogels of the lowest NaHA (5.0 mg/ml) concentrations. The smallest difference in the supramolecular change is by the NaHA hydrogel with 10.0 mg/ml concentration.

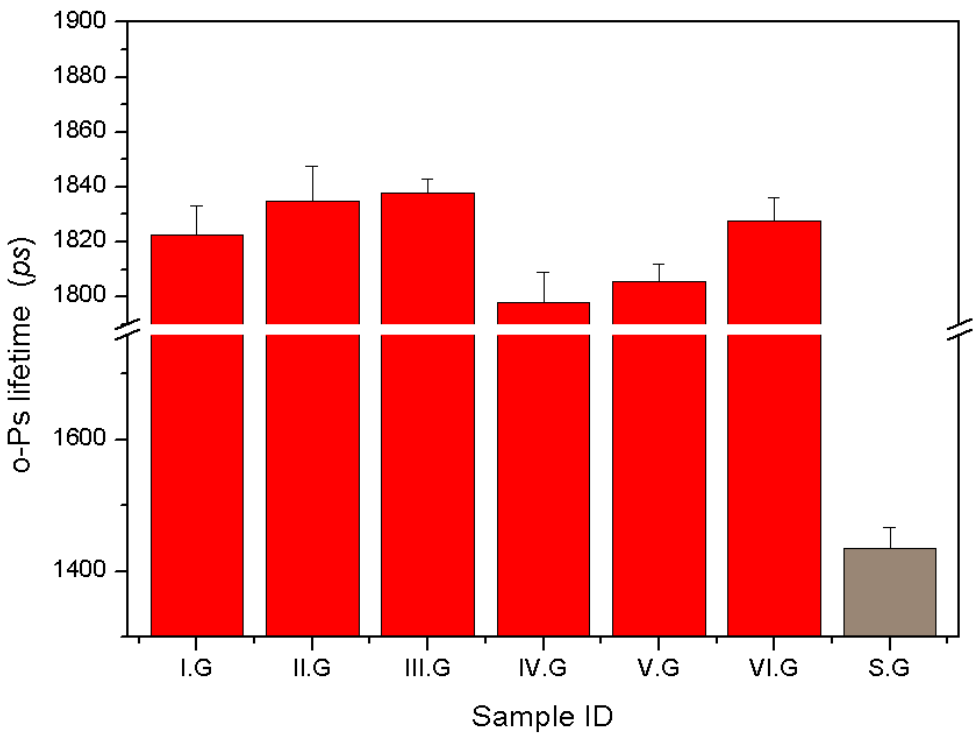

Figure 14 o-Ps lifetime values of various gel samples (I.G - III.G: NaHA hydrogels of 5.0, 7.5, 10.0 mg/ml polymer concentrations in sodium phosphate buffer; IV.G - VI.G: freeze-dried NaHA hydrogels of 5.0, 7.5, 10.0 mg/ml polymer concentrations in sodium phosphate buffer; S.G: NaHA raw substance (powder))

Support for the previously obtained results, furthermore NaHA hydrogel with 10.0 mg/ml was examined. The following experiments were performed: raw substance NaHA, freeze-dried NaHA hydrogel of 10.0 mg/ml prepared with water and freeze-dried NaHA hydrogel of 10.0 mg/ml prepared with sodium phosphate buffer. The structural alteration was investigated.

4.2.2.1. Reconstitution

The water content of the samples was determined by Karl Fischer titration. In all three cases the values were very similar: raw substance: 9.5 ± 1.37%; freeze-dried NaHA gel prepared with water: 10.36 ± 1.41%; freeze-dried NaHA gel prepared with phosphate buffer: 9.35 ± 1.76%.
During reconstitution or gelling process, 5 ml water was added by injection to 5 g sample. The time which needed to get a gel from the freeze-dried substance was measured. Despite of the similar water content values, the gelling of NaHA was much different during reconstitution.

The timescale of gelling from raw substance and freeze-dried samples was demonstrated in Figure 15. The reconstitution of the freeze-dried NaHA gel containing sodium phosphate buffer was completed within 2 min. In comparison with that the gelling process of the freeze-dried gel prepared with water was not carried out after 2 min. It required longer time. In contrast to the freeze-dried gels, the complete gelling process of the NaHA raw substance required several hours.

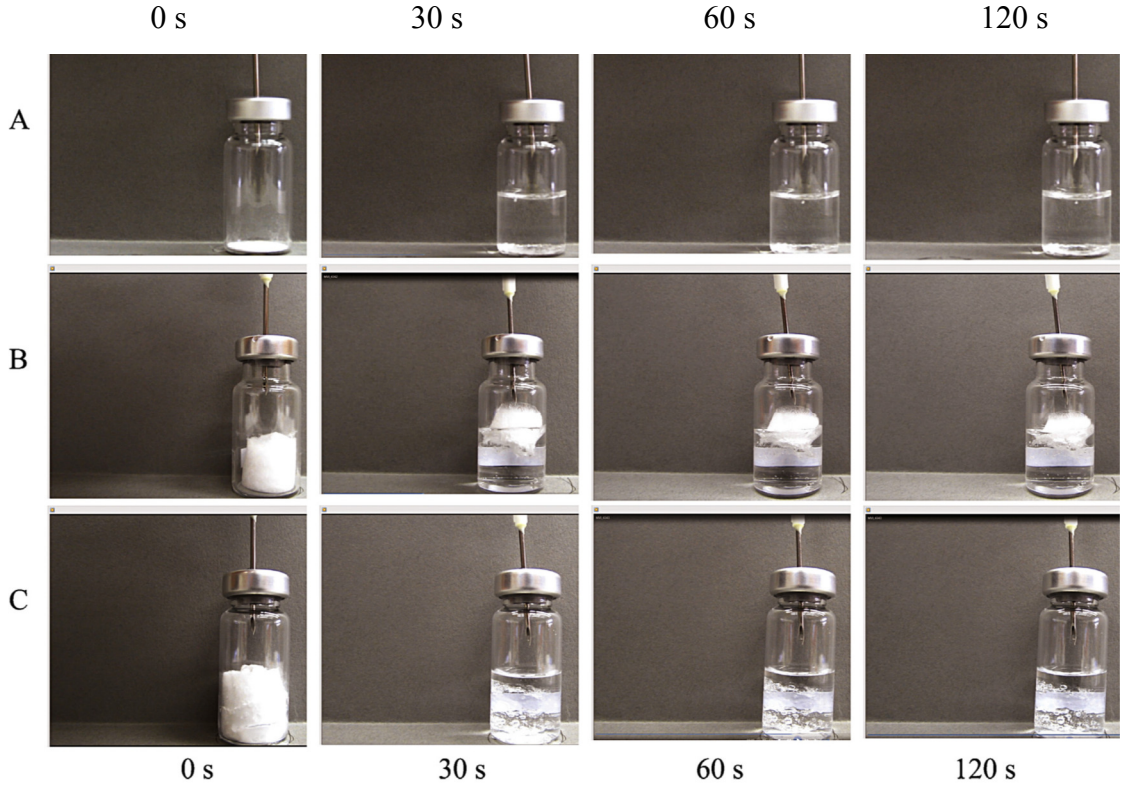

Figure 15 Tracking of the dissolution and gelling of freeze-dried NaHA (10.0 mg/ml) during reconstitution with water (A – raw substance in powder form; B – freeze-dried NaHA prepared with water; C – freeze-dried NaHA prepared with phosphate buffer) (Krüger-Szabó et al., 2015)

4.2.2.2. Viscoelasticity

Figure 16 demonstrates the effect of freeze-drying on the viscoelastic behavior of NaHA hydrogels prepared from water or from sodium phosphate buffer in contrast to NaHA gel prepared from the raw substance.

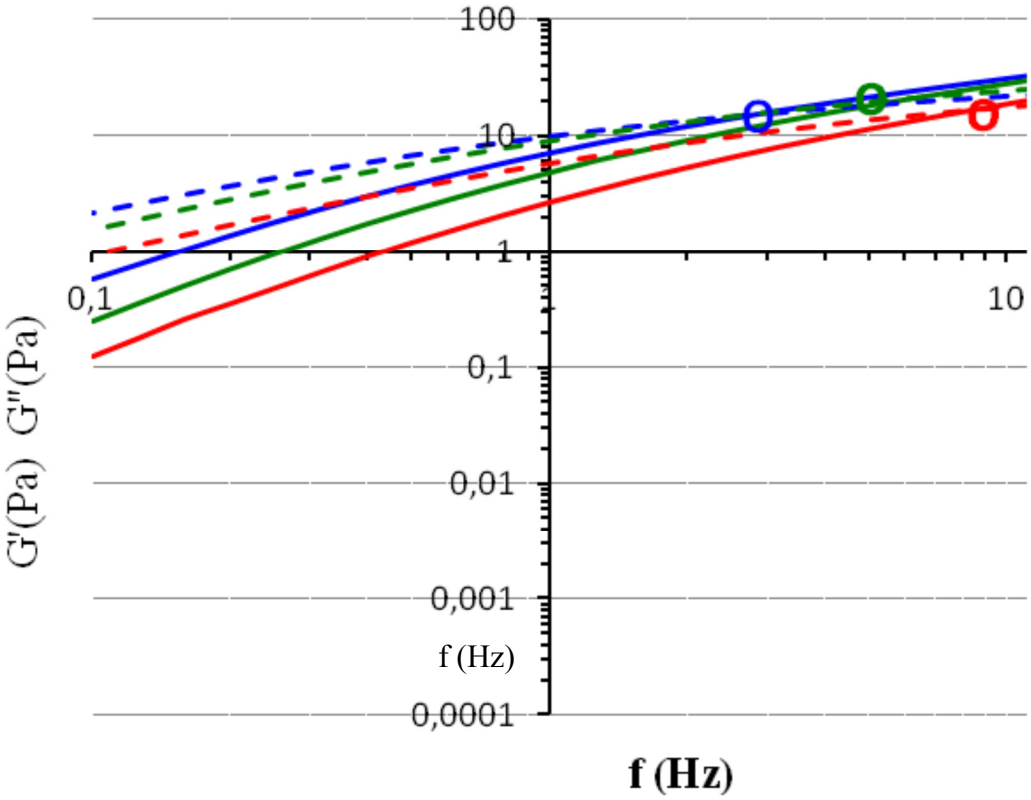

Figure 16 Viscoelasticity of NaHA gels (10.0 mg/ml) prepared from both the raw substance and the reconstituted freeze-dried gels (blue: reconstituted freeze-dried gel prepared with water; red: reconstituted freeze-dried gel prepared with phosphate buffer; green: gel prepared from raw substance; line: storage modulus; dashed line: loss modulus)

In case of all three samples, the transition point was not significantly changed, which means that the samples held their viscoelastic properties.

4.2.2.3. Thermal analysis

The already mentioned lower o-Ps lifetime values and the consequent smaller free volume holes of the reconstituted freeze-dried gels indicate that less water remained, which is shown by TG curves (Figure 17). It can be seen that the raw NaHA and freeze-dried NaHA in water contain more water than the freeze-dried NaHA in buffer. At the temperature of 240 °C NaHA shows a total degradation.

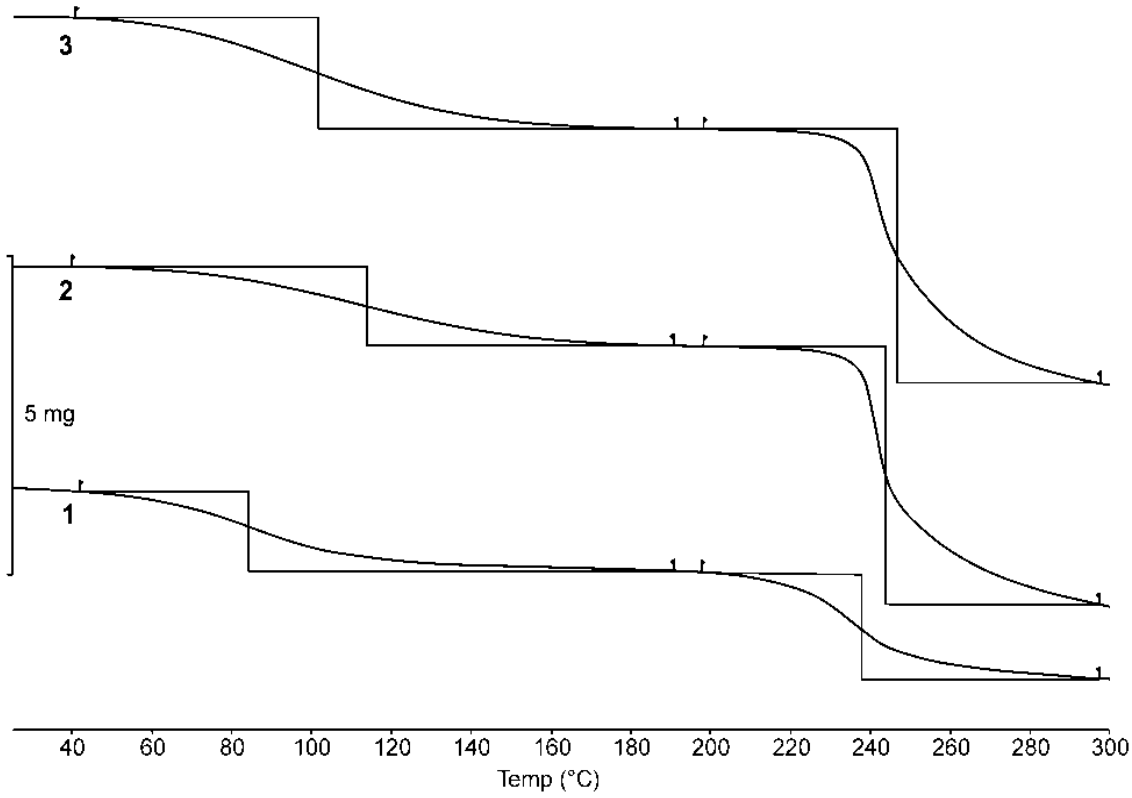

Figure 17 Effect of freeze-drying on the TG curves of 10.0 mg/ml freeze-dried NaHA in phosphate buffer (1), 10.0 mg/ml freeze-dried NaHA in water (2) and raw NaHA (3)

DSC was used to identify the amount of water and the binding processes between hyaluronates and water (Takigami et al., 1993; Takigami et al., 1995). The DSC curves (Figure 18) for 10.0 mg/ml freeze-dried NaHA in buffer, 10.0 mg/ml freeze-dried NaHA only in water and raw NaHA were registered up to 300 °C. The endothermic peak of the DSC curves presents the loss of the moisture content until ca. 160 °C. Afterwards NaHA disintegrates with the exothermic peak at the temperature of 240 °C by raw NaHA and a little bit earlier by freeze-dried NaHA gels.

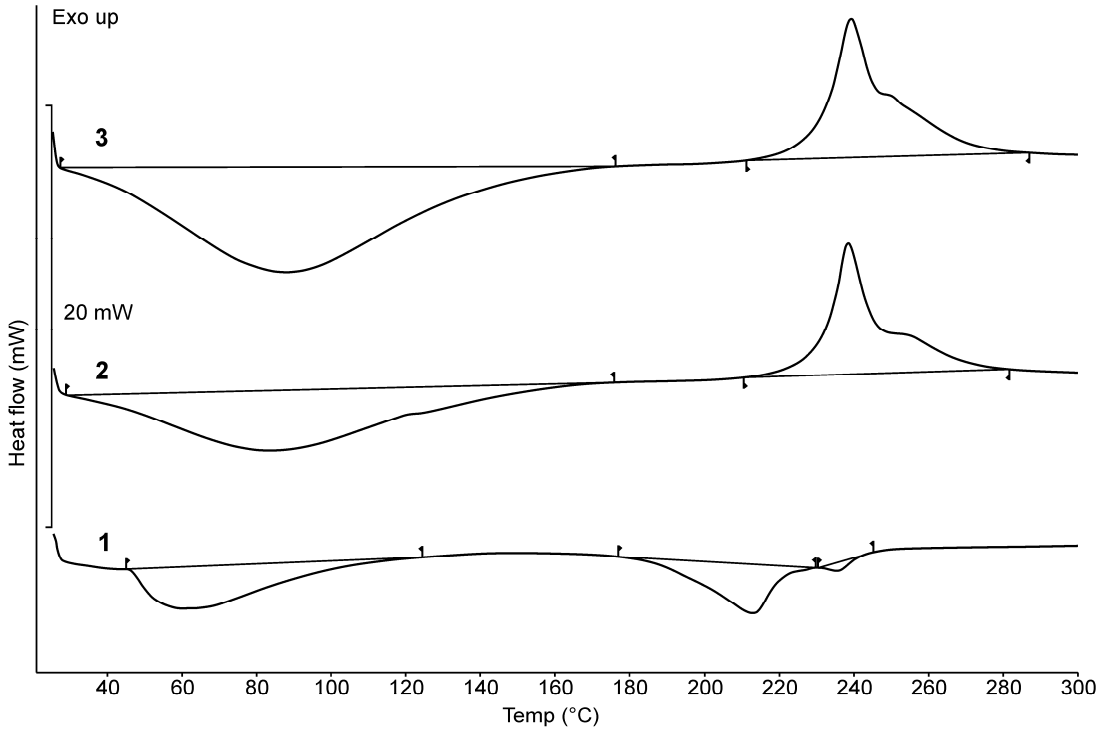

Figure 18 Effect of freeze-drying on the DSC curves of 10.0 mg/ml freeze-dried NaHA in phosphate buffer (1), 10.0 mg/ml freeze-dried NaHA in water (2) and raw NaHA (3)

4.2.2.4. XRPD

Figure 19 demonstrates the X-ray diffraction pattern of the different samples. After freeze-drying no amorphous-crystalline transition of NaHA can be observed. The only samples that showed a crystalline character were the two types of sodium hydrogen phosphates.

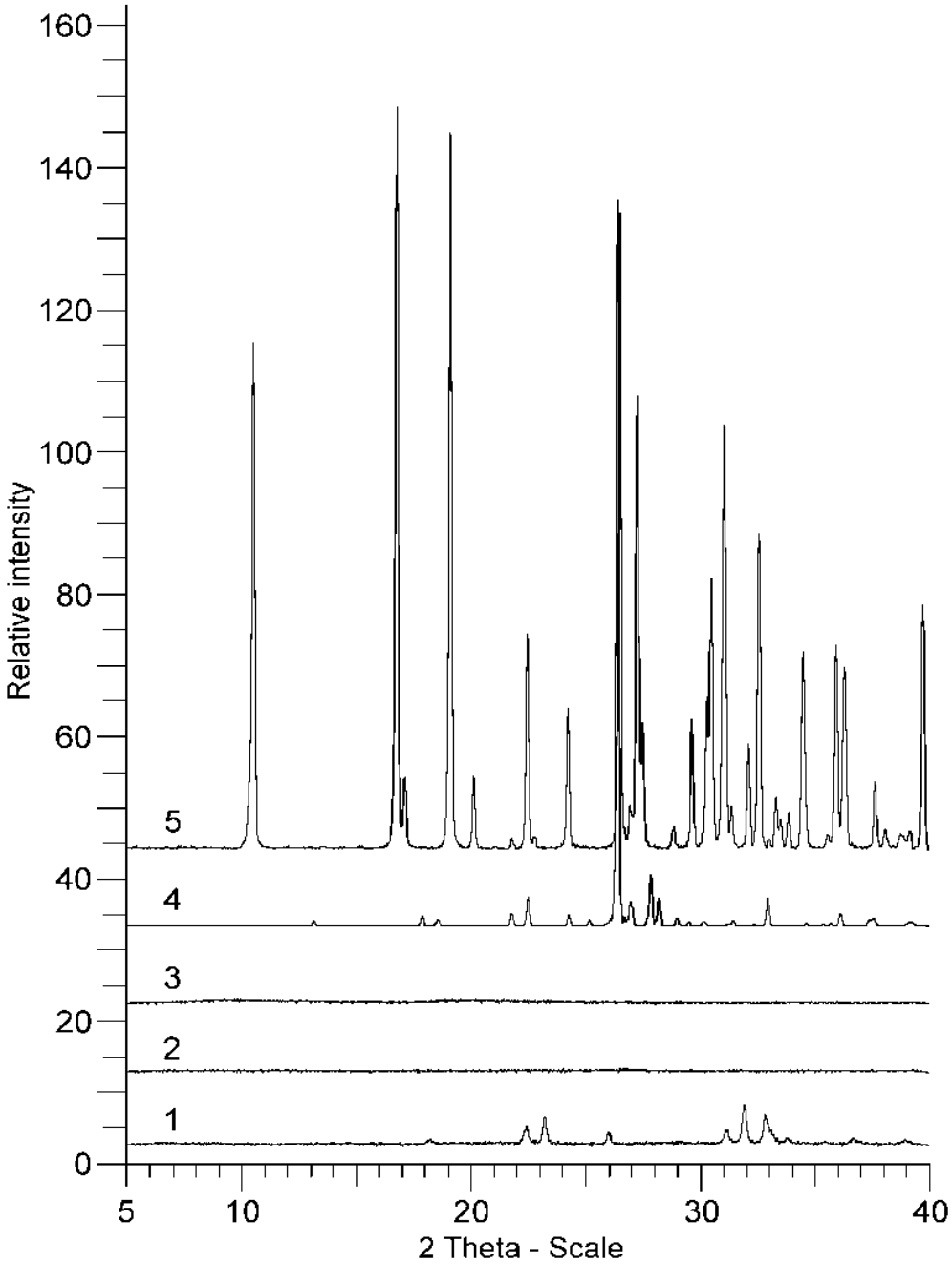

Figure 19 X-ray powder diffraction pattern of various samples (1 – freeze-dried NaHA prepared with phosphate buffer; 2 – freeze-dried NaHA prepared with water; 3 – NaHA raw substance; 4 – monobasic sodium hydrogen phosphate; 5 – dibasic sodium hydrogen phosphate) (Krüger-Szabó et al., 2015)

4.2.2.5. SEM

Figure 20 A-C visualizes the changes in the microstructure of the NaHA samples after freeze-drying. The SEM photos highlight the visible alteration of the structure.

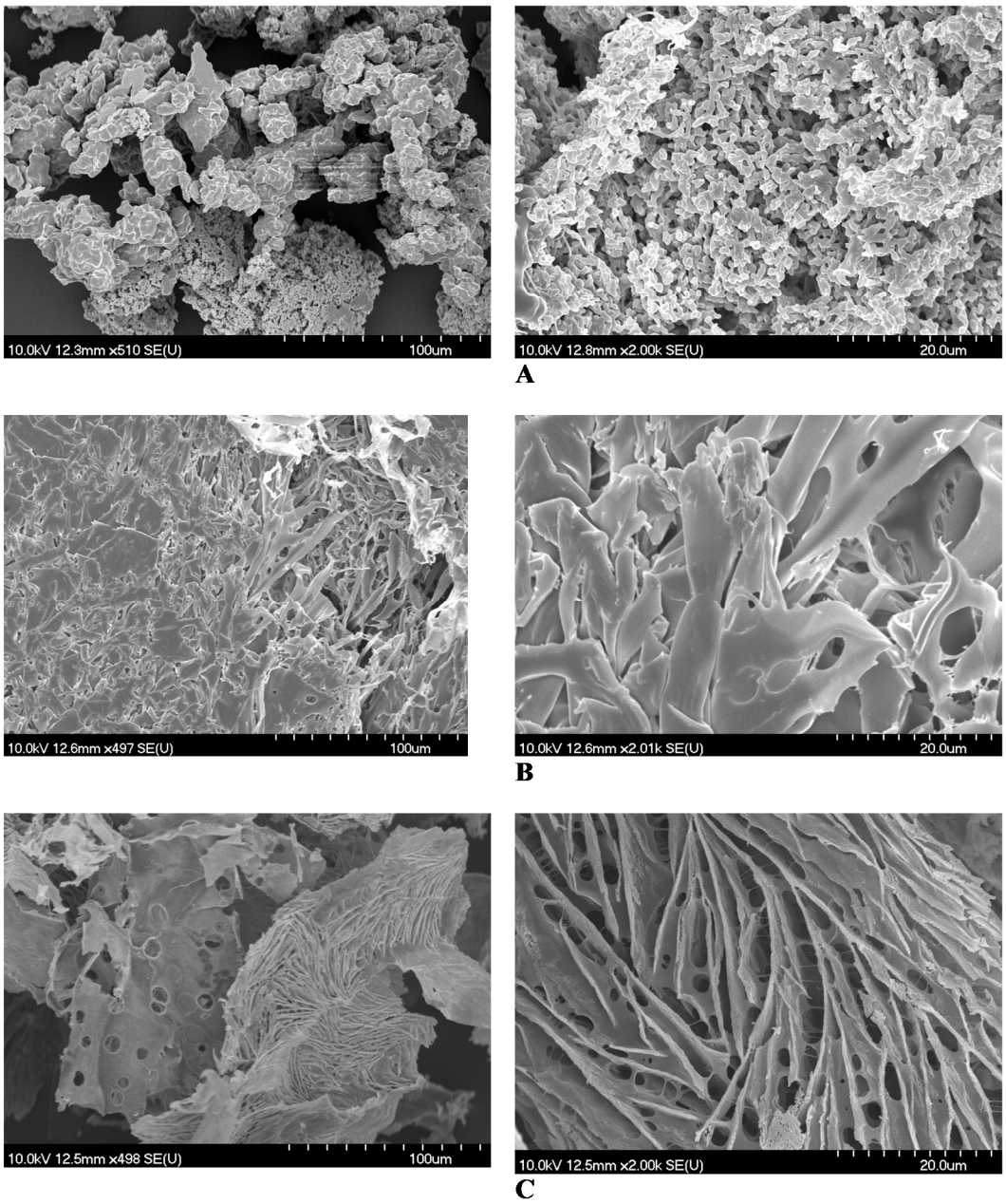

Figure 20 SEM photos of NaHA samples (A – raw substance; B – freeze-dried NaHA gel of 10 mg/ml concentration prepared with water; C – freeze-dried NaHA gel of 10 mg/ml concentration prepared with phosphate buffer) (Krüger-Szabó et al., 2015)

The raw substance (Figure 20 A) showed aggregated micron sized small particles. Figure 20 B-C demonstrated continuous porous matrix after freeze-drying. An internal "ladder-like" structure with a very noticeable porosity can be observed in Figure 20 C, in which NaHA gel was prepared with sodium phosphate buffer and afterwards it was freeze-dried.

4.2.2.6. o-Ps lifetime values

Figure 21 shows the decreased o-Ps lifetime values and the consequent lower free volume holes. By the freeze-dried NaHA gel prepared with phosphate buffer, this could be explained with the increasing number of the pores and the reduction of their size.

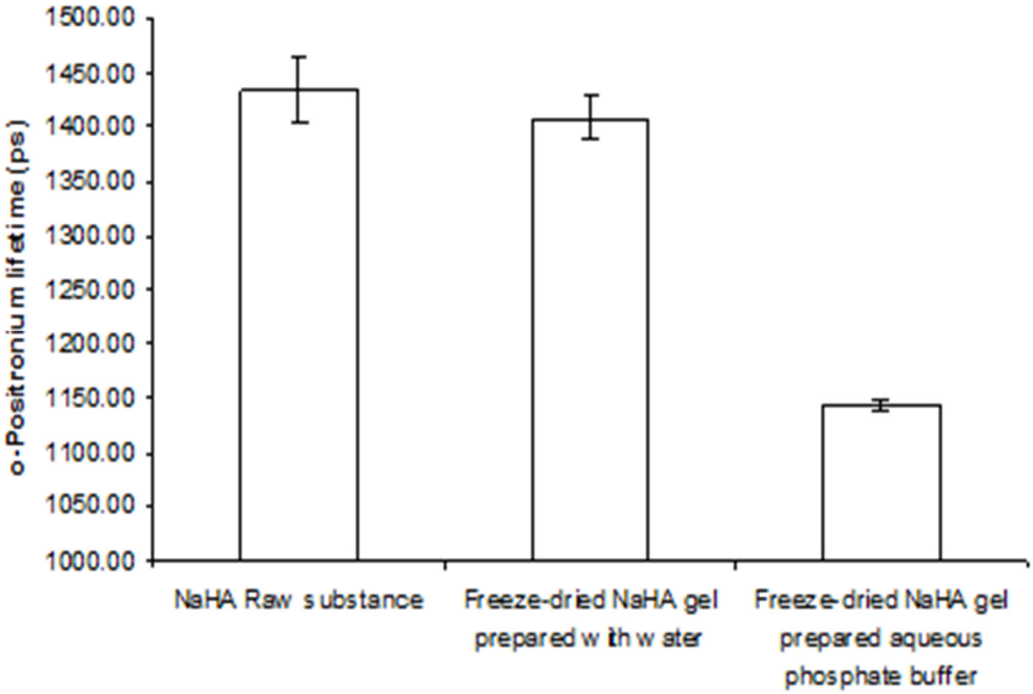

Figure 21 o-Ps lifetime values of various NaHA samples (10.0 mg/ml) (Krüger-Szabó et al., 2015)

5. Discussion

5.1. Effect of the heat sterilization

Based on some literature data (Ali et al., 2009; Novozymes, 2012), that is already succeeded NaHA to sterilize with heat. The aim of this step was to learn more about the alteration of the structure of NaHA by heat sterilization.

The results from the autoclaving process indicated that the energy of heat sterilization modified the supramolecular structures of NaHA depending on its concentration in the aqueous gel system.

After heat sterilization and measurements by PALS, a close-packed structure was showed by 5.0 mg/ml NaHA sample, while a more open structure by 10.0 mg/ml NaHA concentration. At 7.5 mg/ml NaHA, there was no significant change in the polymer structure, in contrast to the other two concentration studied. At 10.0 mg/ml NaHA, a similar effect was found in the dried samples as in the gels, while 5.0 mg/ml NaHA formed a more closely packed polymeric structure and could absorb more water, thus resulting in the increase of o-Ps lifetime values. Because the PALS method cannot distinguish between bound and free water, and in the course of the treatment the water content was not changed in hydrogels, no increase in the o-Ps lifetime values of 5.0 mg/ml NaHA hydrogels can be seen.

The least variable distribution is also obtained with 7.5 mg/ml NaHA gels, and it indicated a supramolecular structural stability of these hydrogels after heat sterilization.

The viscometric studies showed that the transition point was not significantly changed by the heat treatment, which is in good agreement with the free volume distribution results.

Due to the heat sterilization, the viscosity of 10.0 mg/ml NaHA hydrogel decreased, thus dramatically increasing the frequency of the viscoelastic transition. Despite the shift in the intercept, the autoclaving has no irreversible effect on the viscoelasticity.

Based on these results, the possibility of heat sterilization should not necessarily be excluded, but for the pharmaceutical industries it is not an optimal solution.

5.2. Effect of the freeze-drying

The handling and working with NaHA for injections should be speeded up with freeze-drying. Freeze-drying has already proven as an outstanding industrial process for sensitive substances. These experiments were investigated to get data about the alterations of the structural properties of NaHA after freeze-drying.

The results of PALS measurements indicate that with increasing NaHA concentrations (5.0, 7.5 and 10.0 mg/ml) a denser polymer system can be obtained resulting in higher water-binding and consequent swelling ability in the course of gel formation. The freeze-drying process evaporated not only the physically bound water but ordered the supramolecular polymer structure, as well (Quinn et al., 1963). The latter can be confirmed with the decrease of the o-Ps lifetime values of gels obtained with the redissolution of the freeze-dried samples. The effect of the polymer concentrations on redissolved freeze-dried samples showed similar tendency to the original gels of corresponding concentrations without freeze-drying.

Support for these obtained results, 10.0 mg/ml NaHA gels were examined in more detail. Raw NaHA and samples prepared with water or with sodium phosphate buffer were used.

Hyaluronates have a special affinity for water. They show helical structure, which size is depending on the presence of cations (sodium, potassium, calcium) and the packing of the double helical units is stabilized by water molecules (Sheehan et al., 1983).

However, the reconstitution of the samples with the same concentration but with other excipients showed very different hydration properties.

After freeze-drying the reconstitution test showed a great gelling ability by the freeze-dried NaHA gel prepared with buffer. The redissolution process was much faster than by the other samples without buffer.

The results of Lee et al. (1995) showed the importance of relative humidity of NaHA and the hydration degree. It indicates that the interactions are very sensitive to the water content. Lahajnar et Rupprecht (1986) reported also about the hydration similar to that of many other fibrous biopolymers at comparable relative humidity and the intramolecular dipolar interaction of the water molecules.

There are three types of water: non-freezing water, freezing-bound water and free water. The amount of water bonds in hyaluronates are greater than in the other polymers that were investigated (sodium carboxylmethyl cellulose, sodium xanthan etc.) (Phillips et al., 2013).

The viscometric studies demonstrated the effect of freeze-drying on the viscoelastic behaviour of NaHA and showed that the transition point remained almost the same. The viscoelastic curves of the freeze-dried samples strengthened that the supramolecular ordering did not cause functionality-related alteration in the polymer after gelation. NaHA retained its viscoelastic property after freeze-drying.

The TG measurements showed that at the temperature of 240 °C a total degradation of NaHA happened, which is also similar to the results obtained in the literature (Villetti et al., 2002; Vasi et al., 2014).

The results from the DSC investigations correlated with the DSC curves in the literature. The smaller AUC of the endothermic water peak of the thermogram also approved the microstructurally detected phenomenon. The DSC curves of the samples present a typical amorphous substance type with no interference on the chain conformation (Vasi et al., 2014).

X-ray diffraction pattern of the samples referred to that after lyophilisation. No amorphous-crystalline transition of NaHA can be observed which means that the different rate of gelling cannot be explained by the new formation of disordered structure.

The SEM photos showed also that this disordered and elongated texture comes to an agreement with Harding et al. (1992). The possible explanation of this phenomenon could be the supramolecular interaction between the polymer chains and the phosphate salts.

The decreased o-Ps lifetime values and the consequent lower free volume holes confirmed the more ordered polymer structure due to the presence of phosphate salts.

These results are in good agreement with previous studies, which confirmed that HA cross-linking via phosphates could also potentially occur (Brenner et al., 2013). The addition of phosphate salts also facilitated the formation of ordered structure of HA that enabled the successful preparation of electrospun fiber mats. Similar phenomenon was observed in the case of interaction of phosphate salts with chitosan polymer, where phosphate groups increased the cross-link density and accelerated the gelation (Ruel-Gariepy et al., 2000). The possible formation of the cross-linked network within the chains of hyaluronic acid in the presence of phosphates can hinder the crystal growth of water in the aqueous sodium hyaluronate solution in the course of the freeze-drying process, thus resulting in more pores with smaller average size. The latter was visualized on the SEM photos as ladder-like pore structure. Milosĕv et al. (2013) investigated HA stimulation by calcium phosphate in simulated physiological solution and used to analyse the composition by X-ray photoelectron spectroscopy. It is hypothesized that calcium promotes the cleavage of HA to smaller units, which then react chemically with calcium phosphate.

As a consequence, the highly porous structure enabled faster water absorption in the course of gelling to obtain redissolved NaHA.

The freeze-drying is definitely a danger for the HA molecule. The biggest danger is the depolymerisation, which not only destroys the structure but also affects the solubility of the product (Wedlock et al., 1983). The raw starting HA is highly soluble in water and buffers. However, if the degree of the polymerization decreases, the solubility in water and in buffer of the obtained product decreases, too. To prevent this, different counterions can be used. The counterions have a protective effect for HA against depolymerisation. The obtained structure helps the reconstitution, too.

The other influencing factor of the reconstitution is the cross-linking (Chen et al., 2014). The cross-linking agent causes a special ordering in the molecule and therefore the reconstitution-ability increases.

6. Conclusions

The supramolecular changes of NaHA hydrogels after heat sterilisation can be tracked using a PALS method based on the o-Ps lifetime values and distributions. The latter indicated the concentration dependence of the structural ordering, as interpreted by free volume holes. The results enabled the determination of the optimum polymer concentration, which allowed only reversible microstructural changes. The results were in good agreement with the viscoelasticity data, consequently the possibility of heat sterilisation should not necessarily be excluded.

As a result of freeze-drying of NaHA supramolecular ordering was observed in the presence of phosphate salts and consequently highly porous structure was formed, which enabled fast gelling ability during reconstitution with water. The viscosity curves of the freeze-dried samples strengthened that the supramolecular ordering did not cause functionality-related alteration in the polymer after gelation. This phenomenon could be advantageous in the preparation of injection immediately before the application.

Practical relevance of the work

The combination of macro- and microstructural characterization of NaHA hydrogels of various concentrations and buffer solutions enabled both the optimization of the preparation process and the composition of the intra-articular injections.

These preliminary screening examinations could contribute to the selection of the required type and amount of excipients as well as the technological procedures.

7. Summary

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:

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

ii. 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.

iii. 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.

8. Összefoglalás

Parenterális módon alkalmazható nátrium hialuronátot tartalmazó hidrogélek különböző kezelések hatására végbemenő szerkezetváltozásainak feltárásával foglalkoztam. Állati eredetű nátrium hialuronát géleket hővel sterileztem; illetve fagyasztva szárításos módszerrel nátrium hialuronátot formuláltam parenterális célokra a makro- és mikroszerkezeti vizsgálatok eredménye alapján.

Az injekció formulálás körülményeinek meghatározásához különböző fizikai-kémiai vizsgálati módszerek kombinációját alkalmaztam.

Az eredményeim egyértelműen bizonyították:

i. Különböző koncentrációjú NaHA hidrogéleket sikeresen formuláltam parenterális célra, kifejezetten az intraartikuláris alkalmazás szempontjait figyelembe véve.
ii. A hidrogélek a hősterilezés hatására, koncentrációjuktól függően különböző mértékű, részben irreverzibilis szerkezetváltozást mutattak, amelyet mind a makro-, mind a mikroszerkezet-vizsgáló módszerek eredményei alátámasztottak. Következésképpen a hősterilizés lehetőségét nem szükséges minden esetben elvetni.
iii. A nátrium-foszfát sókat tartalmazó hidrogélek fagyasztva szárításával kapott minták rendezett, létraszerű makroporózus szerkezetük következtében gyorsabb újragélesedést mutattak, mint a csak vízzel készültek. A gyors rekonstitúció hátterét makro- és mikroszerkezeti vizsgálatokkal jellemeztük.

A NaHA gélek hősterilezése és fagyasztva szárítása során végbemenő szerkezeti változások feltárása hozzájárulhat a megfelelő gyártástechnológia kialakításához annak érdekében, hogy a készítmény hatékonyságának (viszkoszupplementáció) alapjául szolgáló funkcionális tulajdonságát biztosítsuk.

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10. List of own publications

10.1. 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.

10.2. Other publications

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

10.3. List of Presentations

1. Szabó A, Szabó B, Balogh E, Zelkó R, Antal I. (2012) Intraartikuláris készítmények fejlesztési lehetőségei. XVII. Gyógyszertechnológiai és IX. Gyógyszer az ezredfordulón Konferencia. 27-29.09.2012, Siófok.

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