Università degli Studi di Ferrara

DOCTORAL COURSE IN

"CHEMISTRY"

CYCLE XXXIII

DIRECTOR Prof. Alberto Cavazzini

Design, synthesis, characterization and in vitro evaluation of new cross-linked Hyaluronic acids for Pharmaceutical, Nutraceutical and Cosmetic applications

Scientific/Disciplinary Sector (SDS) CHIM/08

Candidate

Dott. Sciabica Sabrina

Supervisor

Prof. Manfredini Stefano

Co-Supervisors

Dr. Anna Baldisserotto (Unife)

Prof. Silvia Vertuani (Unife)

Dr. Luisa Canilli (Istituto Ganassini Spa)

Dr. Massimo Terno (Istituto Ganassini Spa)

Years 2017/2020

ACKNOWLEDGMENTS

I wish to acknowledge Professor Alessandra Semenzato and her collaborator Dott. Giovanni Tafuro (University of Padua, Department of Drug Science) for the kindful collaboration in performing rheology studies and for providing all the necessary informations for the full characterization of the synthesized material.

I would also like to thank professor Daniela Traini and her research group, the Woolcock Institute of Medical Research and Discipline of Pharmacology of Sydney, in particular to Dr. Larissa Gomez, for conducting the studies on the biological activity of the products carried out in this project.

ABBREVIATION

ALI: air-liquid interface

BDDE: butanediol-diglycidyl ether

CD44: cluster of differentiation-44

CDMT: 2-chloro-4,6-dimethoxy-1,3,5-triazine

COOH Carboxylic group

DCF: dichlorofluorescin

DCFH-DA: 2′,7′-dichloro- fluorescein diacetate

DL%: drug loading

D_2O: Deuterium oxide

DSC: differential scanning calorimetry

DVS: dynamic vapour sorption

ECM: extracellular matrix

EDC: N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride

EE%: encapsulation efficiency

FBS: foetal bovine serum

FDA: Food and Drug Administration

G': elastic modulus

G": viscous modulus

GAGs: glycosaminoglycans

GlcA: D-glucuronic acid

H-bonds: hydrogen bonds

H_2O_2: hydrogen peroxide

HA: hyaluronic acid; hyaluronate; hyaluronan

HA- SAP MS: native hyaluronic acid microspheres encapsulating sodium ascorbyl phosphate

HA-Arg: Arginine cross-linked hyaluronic acid

HA-Arg-SAP MS: Arginine cross-linked hyaluronic acid microspheres encapsulating sodium ascorbyl phosphate

HA-Orn: Ornithine cross-linked hyaluronic acid

HA-Orn-SAP: Ornithine cross-linked hyaluronic acid microspheres encapsulating sodium ascorbyl phosphate

HARE: hyaluronan receptor for endocytosis

HAS: hyaluronan synthases

HCl: Hydrochloric acid

HMW: high molecular weight

HYAL: hyaluronidases;

IL-6: interleukin-6

IL-8: interleukin-8

IR: infrared

LMW: low molecular weight

LYVE-1: lymphatic vessel endothelial hyaluronan receptor 1

MSs: Microspheres

MTS: methyl tetrazolium salt

MW: molecular weight

NAOH: Sodium hydroxide

NMM: N-methyl morpholine

NMR: Nuclear magnetic resonance spectroscopy

MWCO: Molecular Weight Cut-off

NHS: N-hydroxysuccinimide

PBS: phosphate buffer saline; phosphate buffered saline;

RH: relative humidity

RHAMM: receptor for HA-mediated cell motility

ROS: reactive oxygen species

SAP: sodium ascorbyl phosphate

SEM: scanning electron microscop

TBA: tetrabutylammonium

TGA: thermal gravimetric analysis

TLR: Toll-like receptor;

UI: international unit of enzymatic activity

UV: ultraviolet Y%:

yield.

Index

THESIS ABSTRACT

Hyaluronic acid (HA) is an excellent biomaterial which thanks to its peculiar properties, such as biocompatibility, biodegradability, mucoadhesiveness, hydration and viscoelasticity is currently one of the most attractive polymers for many biomedical applications. Unfortunately, many of its potential applications are limited due to its short half-life as it is rapidly degraded by the hyaluronidase enzymes.

In order to improve its half-life and consequently increase its performance, in this PhD thesis, native HA was modified through cross-linking reactions in which the polymer chains are stabilized by covalent bonds using natural and biocompatible aminoacids as cross-linkers agents, such as Arginine and Ornithine to overcome the potential toxicity of commonly used synthetic molecules.

CDMT / NMM was used as activating agent and the structure of the new products was characterized by ^{1}H NMR and FT-IR to confirm the occurence of the chemical modification. The morphology of the compacted and interconnected structure characterized by cages of variable size was studied by SEM. The thermal behaviour (TGA and DSC) and the rheological properties were analized and the results had shown a stable thermal profile with a rheological behaviour liquid-like and a weak-gel profile for HA-Arg and HA-Orn respectively. Swelling studies suggested a dependence on the degree of modification and pH value.

Finally, the Gel Permeation Chromatography data did not reveal significant changes in molecular weight but a lower recovery value indicated that the cross-linking process has occurred.

It was also demonstrated by an in vitro degradation test that the products presented an enhanced resistance profile towards enzymatic digestions therefore the cross-linking process has reduced the susceptibility of HA to the digestive action of enzymes.

An evaluation of the biological profile of HA-Arg and HA-Orn was studied as possible coadjuvants therapy for the treatment of lung inflammation.

In particular, in vitro studies were performed to evaluate the release of IL-6 and IL-8, antioxidant capacity, cytotoxicity by MTS assay, and evaluation of the integrity of the epithelial barrier. The studies were performed on Calu-3 and H441 cells evaluating the treatment of HA-Arg and HA-Orn individually and in combination with sodium ascorbyl phosphate (SAP). None of the investigated treatments appeared cytotoxic and inflammatory and antioxidant activity was promisingly and different based on the cell line tested. HA-Arg and HA-Orn were employed for the production of microspheres by emulsificationsolvent evaporation, as potential carrier of SAP: optimization studies led to the realization of MSs with spherical morphology and smooth surface.

An in vitro release study showed that the drug was released from microspheres in controlled manner.

This work has been object of the patent application N° 102019000024117 of the 16th December 2019.

Chapter 1

1 INTRODUCTION

1.1 Chemical structure and physicochemical properties

HA is a unique non-sulfated glycosaminoglycan (GAG) that was first isolated it by Meyer in 1934 from the vitreous body of bovine's eyes (Meyer & Palmer, 1934)
HA consisting of N-acetyl-D-glucosamine and D-Glucuronic sugars linked by ß 1,3glycosidic bonds (Weissmann & Meyer, 1954a) (Weissmann et al., 1954b)
to form units of disaccharides which are connected repeatedly by 1-4 bond to form polymers of different length (Figure 1).

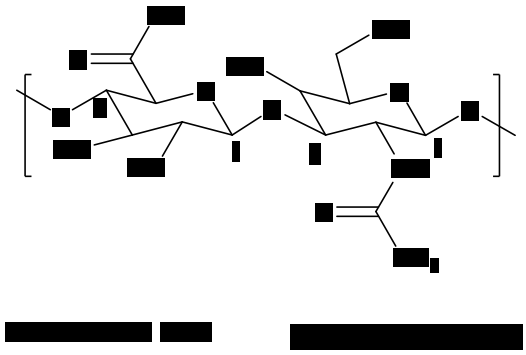


Figure 1. Structure of the disaccharide repeating unit in HA.

The number of repeated disaccharide units that compose hyaluronan molecule is proportional to the molecular weight (MW) of the whole polymer and can reach a value of 10^8 D depending on physiological role in living organism (Weiss et al., 2000; Girish & Kemparaju, 2007; Knopf-Marques, et al., 2016; Kogan et al., 2007)
The HA MW plays a key role on biological activities as well as the viscolelastic properties in fact, in acqueous solution, HA form a stable tertiary structure (Scott & Heatley, 1999) which is compacted by inter and intra-molecular hydrogen bonding, hydrophobic interactions and self-association in order to establish an extended network depending on molecular weight (MW) and concentration.

At physiological pH values, the pKa of carboxylic function on the unbranched chain is equal to 3-4 therefore HA is negatively charged associated with counterions.

In aqueous solution, the negative charge on the carboxyl groups makes the structure highly hygroscopic and therefore capable of absorbing large quantities of water molecules up to 1000 times its original weight (Laurent , 1970; Turino & Cantor, 2003) to form a hydrogel

network (Allemann & Baumann, 2008,; Kablik, et al., 2009). Consequently, HA is capable of profound effects on the hydratation and the physical properties of the biological tissue in which it is concentrated, playing an important homeostatic role.

The structural organization of HA is strongly influenced by the pH value: acidic or basic aqueous media, strongly compromise the integrity of the chains due to hydrolysis reaction of glycosidic bonds (Ghosh et al., 1993; Maleki et al.,2008).

Because of its hygroscopic properties, hyaluronan significantly influences hydration and the physical properties of the extracellular matrix and it's involved in cell proliferation, migration and differentiation.

1.2 HA occurrence in living organism and physiological functions

In the human body, HA is ubiquitous, but it is mainly present in the extracellular matrix (ECM) of connective tissues (Laurent & Fraser, 1992; Schiraldi et al., 2010) where it performs both structural functions, which derive from the viscoelastic properties and specific functions, for example, it's involved in the process of wound repair, cartilage regeneration and ocular homeostasis. In humans the greatest amount of HA is present in the skin containing almost 50% of the total content within the adult body where a concentration of 0.2g / kg is estimated (Romagnoli & Belmontesi, 2008; Volpi et al., 2009).

Considerable amounts of HA resident also in the synovial joint fluid (3–4 mg/mL), eye vitreous body (0.1 mg/mL) and in the umbilical cord (4 mg/mL) where is the highest hyaluronan content among all tissue (Laurent & Fraser, 1992; Schiraldi et al., 2010; Robert et al., 2010; Sobolewski et al.,1997).

The turnover of HA is fast (5 g/day) and is finely regulated through enzymatic synthesis and degradation (Volpi et al., 2009).

As it was the simplest of GAGs , HA is not sulfated and it is not synthesized by Golgi enzymes but is produced in the inner side of the plasma membrane without any binding to a protein core and it can form non-covalent complex with proteoglycans (such as aggrecan, versican) (Le Baron, Zimmermann, & Ruoslahti, 1992; Delpech, et al.,1989), structural

glycoproteins such as collagen, elastin, integrins, fibronectin (Turley & Moore, 1984) (Isemura et al., 1982), and other GAG (King et al., 1991) contributing to keep the ECM structure stable.

It is widely diffused in many animal tissue, for example, in rooster comb can be found extraordinarily source (7.5 mg/ml) and also some Streptococcal bacteria may contain HA (Balazs et al., 1993; DeAngelis et al., 1998; De Oliveira et al., 2016; Schiraldi et al., 2010)

1.3 Biology of HA

1.3.1 HA synthesis in the human body

In vertebrates HA is synthesized by three types of hyaluronan synthase (HAS) : HAS1, HAS2 and HAS3 (Weigel et al., 1997) isoforms that differ mainly in the chain length of the hyaluronan molecules produced and their ease of release from the cell surface.

Overexpression of these genes has a profound control on HA molecular weight, which correlated with the levels of UDP-sugars and in particular, UDP-GlcNAc (Chen et al., 2009).

After its biosynthesis, HA is secreted into extracellular space where due to its hygroscopic properties, significantly influences hydration and the physical properties of the extracellular matrix.

Despite sharing in 50-71% of their amino acid sequence, the three isoforms of hyaluronic synthase enzymes are distinguished by different kinetic characteristics and biochemical properties which influence the molecular weight of HA produced. HAS1 and HAS2 proteins are responsible for the synthesis of high molecular weight HA (HMW-HA) and HAS2 is the more active of the two, whereas HAS3 protein possesses the highest activity and produces low molecular weight HA (Girish & Kemparaju, 2007). An alteration of the regulation mechanism of HAS genes can lead to the triggering of pathological events linked to an abnormal production of HA such as malignant transformation and metastasis (Adamia et al., 2005; Adamia et al., 2013; Heldin et al., 2018; Toole, 2004).

1.3.2 HA-receptor interaction

HA through the interaction with its binding proteins act as signaling molecule and is involved in the processes of cell proliferation, migration and differentiation (Hemshekhar et al., 2016). These binding proteins are called hyaldherins and comprise structural matrix proteins and cell-surface receptors that bind with high affinity to HA.

Five type of receptors are currently known and among these, the CD44 (cluster of differentiation-44) receptor family is the main and therefore better characterized.

Multiple isoforms of CD44 have been identified that bind not only with HA but also other ligands and are present in almost all types of human cells (Vigetti et al., 2014a).

Interacting with HA, the receptor induces the initiation of signaling pathways that control different cell biological processes including receptor-mediated hyaluronan internalization/degradation, angiogenesis, cell migration, proliferation, aggregation and adhesion to ECM components. An over-expression or anomalous activation of CD44 plays important roles in disease progression, inflammation, tissue regeneration and neoplastic

transformation including prostate, colon, breast, and endometrial cancers (Girish & Kemparaju, 2007; Toole, 2004; Mattheolabakis et al. 2015)

Another HA receptor structurally different from CD44 is RHAMM (Receptor for Hyaluronan-Mediated Motility). RHAMM has two isoforms which differ in location on cell surfaces or in the cytosol and nucleus (Zhang et al., 1998; Leach & Schmidt, 2004). HARHAMM interaction is involved both in cell migration by binding the protein of the cytoskeleton resulting in the activation of protein kinases (Aya & Stern, 2014), and in cell division by binding with the intra-cellular receptor which modulates the cell cycle and the formation of the mitotic spindle (Girish and Kemparaju, 2007; Knopf-Marques et al., 2016; Cyphert et al.,2015).

Therefore, RHAMM plays an important role in the inflammatory process and in tissue repair due to its control of different signaling pathways on macrophages and fibroblasts cells (Vigetti et al., 2014b).

Other cell surface hyaldherine are HARE (HA receptor for endocytosis) involved in the clearance of GAGs from circulation and LYVE (lymphatic vessel endhothelial hyaluronate receptor) that controls HA turnover and is implicated in the regulation of lymphangiogenesis and intercellular adhesion. (Girish and Kemparaju, 2007; Knopf-Marques et al., 2016; Vigetti et al., 2014b).

HA can activate another complex receptor involved in inflammatory responses in acute lung injury, the Toll-like receptors such as TLR2 and TLR4 (Jiang et al., 2005).

LMW-HA promotes pro-inflammatory cytokines (Cyphert et al., 2015) by acting as an agonist of this receptor while HMW-HA attenuates inflammation creating a thick, viscous covering around cells that inhibits surface signaling (Maharjan et al., 2011). When an inflammatory process occurs due to factors such as ROS, pH alterations or the presence of pathogens, an alteration of the synthesis and degradation processes occurs, which can damage the protective coating of HA pericellular barrier and therefore the accessibility to the cell receptor (Ebid et al., 2014).

1.3.3 HA degradation in the human body

It is well known that the turnover of HA in vertebrate tissues is fast, due to a degradation process to which it is continuously subjected (Brown et al., 1991). In the body, the degradation of HA occurs through two main mechanisms, an enzymatic type by a family of enzymes called hyaluronidase (HYAL) and another non-enzymatic type which includes degradative reaction by the action of free radicals (ROS).

HYAL have a predominant control on the metabolism of hyaluronan. In general, HYAL are endoglycosidases that randomly cleavage the β-N-acetyl-D-Glucosaminidic linkages of chains on polymer leading to smaller oligosaccharides and low-molecular-weight HA that exhibit pro-angiogenic properties (Mio & Stern, 2002; Stern et al., 2007).

In the human genome, there are six HYAL gene sequences clustered on chromosome 3p21.3 (HYAL 1, HYAL 2, HYAL 3) and 7q31.3.5 (HYAL4, PH-20/SPAM1and HYALP1) and their expression appears to be tissues specific. HYAL 1 and HYAL 2 are the most widely expressed hyaluronidase in human somatic tissues and have an optimal activity at acidic pH (≤4) (Lepperdinger et al., 1998; Lokeshwar, et al., 2001).

HYAL 1 was the first human HYAL to be characterized and is expressed in liver, heart, spleen, kidney, and bone marrow (Csóka et al., 1999). It not only regulates the cell cycle progression and apoptosis but as the main HYAL expressed in cancer it also regulates tumor growth and angiogenesis (Stern, 2008). HYAL 2 is highly espressed in many human tissue specially in spleen (Csóka et al., 1999; Lepperdinger et al., 1998), it is also the only hyaluronidase founded in platelets (De la Motte et al., 2009)

HYAL 1 and HYAL 2 conduce the degradation of hyaluronan in a concerted and successive manner. The mechanisms of hyaluronan catabolism are not yet detailed, but they are in agreement with a hypothetical model that predicts that HYAL 2, fixed on the outer side of the cell surface by anchoring, performs a first fragmentation of HA into oligosaccharides of about 20 kDa (approximately 25 disaccharide units). The fragments generate from this incomplete fragmentation are internalized by endocytosis, delivered to endosomes and finally completely degraded into tetrasaccharide units (800 Da) by HYAL 1 (Cyphert et al., 2015). The biological properties of these HA fragments are different from those of the larger precursor molecules: those with HMW possesses antiinflammatory, antiangiogenic, and immunosuppressive properties while LMW fragments are highly angiogenic, inflammatory, and immunostimulatory.

Molecular modelling studies suggest the carboxylic groups of HA as the recognition site of HYAL-2 enzymes (Banerji et al., 2007).

Free radical species like superoxide, hydrogen peroxide, nitric oxide, peroxynitrite are also known to degrade HA through oxidation.

In the organism, this radicals are the result from inflammatory processes, tissue damageous or tumorigenesis (Girish & Kemparaju, 2007; Soltés et al., 2006) and mediate the hydrolysis of HA through the cleavage of glycosidic bonds determining HA fragmentation (Volpi et al., 2009; Rees et al., 2004).

Temperature and pH values are other factors that can involve the degradation of HA throught mechanisms of acidic and alkaline hydrolysis or thermal degradation. Viscosity has been shown to decrease exponentially in function of temperature. Being a primary constituent of the interstitial barrier, the degradation of HA reduces the viscosity and this determines an increase in the permeability of the tissues (Necas et al., 2008)

In fact, some bacteria, such as Staphylococcus aureus, Streptococcus pneumoniae and Clostridium perfringens, produce hyaluronidase as a means of increasing mobility through the body's tissues and as an antigenic disguise that prevents their recognition by phagocytes of the immune system (Ponnuraj & Jedrzejas, 2000; Lokeshwar et al., 2002; Kim et al., 2005; Girish & Kemparaju, 2006). Every day about one third of the 15 g of hyaluronan present in the body of an adult human being weighing 70 kg is replaced, infact due to a degradation process to which it is continuously subjected the half-life of HA in vivo reaches only a short period of maximum of 1-2 days in the skin epidermis, 1-3 day in the eyes and about 1–3 weeks in cartilage tissue (Brown et al., 1991). By contrast the filler injected has a half-life of only 2.5–4.5 min, determined in rabbit.

1.4 Mechanisms of action of HA in relation to its MW

HA is involved in several biological functions in human both as signaling active molecule involved in cell mobility, proliferation and differentiation and as passive structural molecule due to its physico-chemical properties. HA due to its hygroscopy and viscoelasticity behaviour acts as a structuring and moisturizing agent contributing to stabilization of ECM structure but can also act as a signaling molecule by selectively binding with cellular receptors CD44, RHAMM, HARE or LYVE-1 and promotes the production of cellular

physiological substances, such as cytokines, prostaglandin E2 and metalloproteinases (MMPs). However, the biological effects of HA are believed to be strongly MW dependent. HMW HA was described to be antiangiogenic, and plays an important role in inflammation, tissue injury and repair, wound healing and immunosuppression (Cyphert et al., 2015; Girish and Kemparaju, 2007; Jiang et al., 2011), moreover it is also involved in the maintenance protecting of tissue cartilage due it osmotic lubricating and viscoelasticity property (Tamer, 2013). In contrast LMW HA derived from environmental and pathological conditions, such as rheumatoid arthritis and pulmonary diseases including asthma, pulmonary fibrosis and hypertension, was reported to promote pro-angiogenic activities, ECM remodelling (Heldin et al. 2018) and to induce proinflammatory processes.

In particular, HA oligomers, encourage the production of inflammatory cytochine, chemokines, nitric oxide and growth factors (McKee et al., 1997; Cyphert et al., 2015; Girish and Kemparaju, 2007; Jiang et al., 2011) which can activate various signaling mechanisms for cancer progression.

It is evident that the MW HA performs a fundamental regulatory function in several of both physiological and pathological processes. Therefore, a proper balance between

synthesis and degradation is essential as it determines not only the quantity and size of HA but also the related biological effects.

1.4 Hyaluronic acid chemical modification

HA is a unique macromolecule employed in cosmetic, medical and pharmaceutical products due to its biological and viscoelastic properties. Chemical modifications of HA can be performed aiming to enhance half-life and modulate or control the desired therapeutic action. A crucial part to its modification consists in maintaining the original properties such as biocompatibility, biodegradability and mucoadhesivity of linear HA and at the same time acquiring different physicochemical properties to obtain more performing products (KnopfMarques et al., 2016). In fact, as previously reported, HA has very limited

applications due to the degradative action of hyaluronidase enzymes and therefore HA should be stabilized to ensure a prolonged half-life in the human body after administration.

For this purpose, derivatization strategies are used that mainly involve two sites of functional groups of HA: the carboxyl group (-COOH) on the D-glucuronic acid and hydroxyl group (-OH) present on N-acetyl D-glucosamine. There is also the possibility of exploiting a third functionality wich is an amino group ($-NH_2$) after de-acetylation of the N-acetyl group, although the required conditions can cause the hydrolysis of the polymer. There is also the possibility of exploiting a third functionality which is an amino group ($-NH_2$), after deacetylation of the N-acetyl group, although the required conditions can cause hydrolysis of the polymer. Several hydroxyl groups are available for functionalization (C_2, C_3 and C_5) but being these secondary they are less accessible than primary alcohol (–OH) in C_6.

According to the desired objective, it is possible to operate two types of reaction on HA: the first is a conjugation, in which a molecule is grafted onto a functionality of the polymer, the second is a cross-linking consisting of the connection of different chains by means of covalent bonds using a chemical cross-linker (Schanté et al., 2011).
Generally, the conjugation reaction is used to bring the therapeutic effect (Luo & Prestwich, 1999), introduce new reactive groups on the HA scaffold or to produce a drug vehicle (Drobnik, 1991; Schantè et al.; 2011; Choi, et al., 2009), while cross-linking is mostly used

to increase mechanical and rheological properties with consequent reduction of the degradation rate. It's possible to carry out both chemical reaction in water or in organic solvent but in the latter case, a conversion step of the HA in its tetrabutylammonium salt (HA-TBA) is required (Magnani et al., 2000). Several chemical modifications of the three functional groups of HA are shown below.

1.4.1 Modification of HA carboxylic groups

The carboxyl group thanks to its versatility allows a wide range of chemical reactions to form ester or amide derivatives.
For ammidation reaction, a critical step for the derivatizationof -COOH is its activation which requires the use of activating agents.
HA amidation can be carried out using N-(3-dimethylaminopropyl)-N-ethylcarbodiimide (EDC) in combination with N-hydroxysuccinimide (NHS) or hydroxybenzotriazole (HOBt)

to reduce side reactions (Figure 2 a.) (Bulpitt & Aeschlimann, 1999). This carbodiimide has the advantage of being usable in water and not leading to cleavage of HA chain but is highly pH dependent and requires a high amount of reagents.

Another effective amidation method, is based on the use of 2-chloro-dimethoxy-1,3,5triazine (CDMT) as activator together with the N-methyl morpholine (NMM) as a chloride ions neutralizer (Figure 2 b.) (Bergman et al., 2007) or (4-(4,6-dimethoxy-1,3,5-triazin-2yl)-4-methylmorpholinium (DMTMM) (D' Este et al., 2014). The reactions are conduct in mild conditions usually in a mixture of water and acetonitrile, without being pH dependent and can be performed requiring lower amounts of coupling agent achieving high grafting yields without side reactions. Additionally, triazine coupling agents are milder than carbodiimide compounds, less allergenic, and safer (Kaminski, 1985; Rayle & Fellmeth, 1999).

Another reaction that first requires the conversion of HA in HA-TBA can be performed in anhydrous organic solvent (DMSO and DMF) using 2-chloro-1-methylpyridinium iodide (CMPI) as activating agents in association to triethylamine (TEA) to neutralize the released iodide ion (Figure 2 c.).

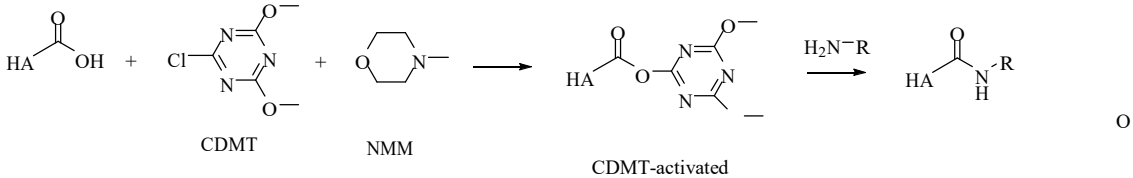
[redacted]

O

R

N N

OH b.



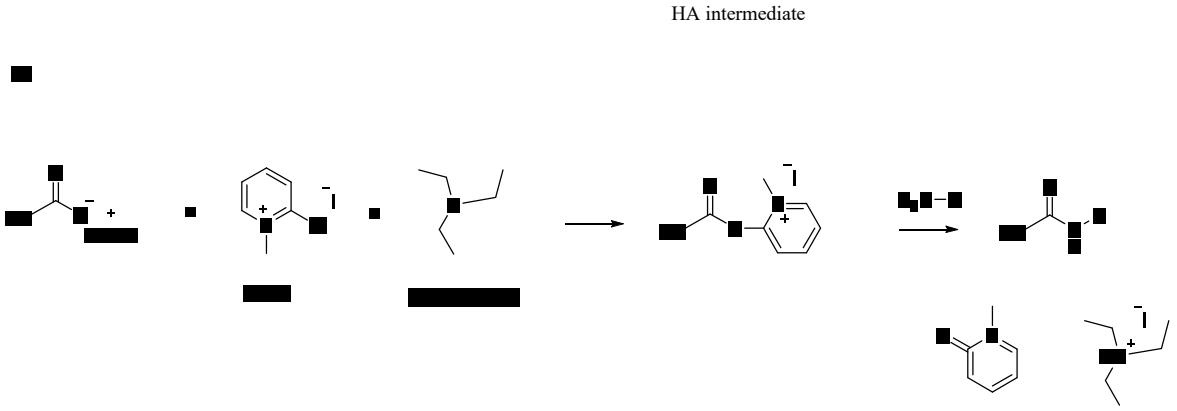


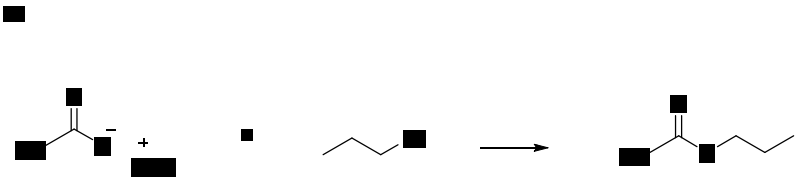
Figure 2. HA amidation mechanism with EDC/NHS and EDC/HOBt (a), CDMT/NMM (b) and CMPI (c).

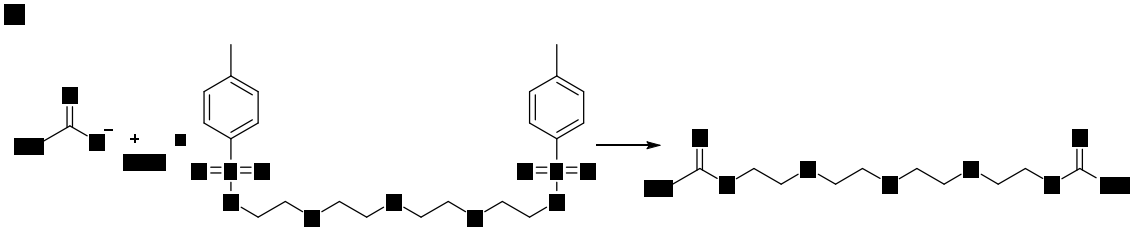
This method also confirms its greater effectiveness as it uses low amounts of reagents compared to the previous method with carbodiimides. However, this reaction is not as quick as amidation and longer purification steps are required.

Additionally, -COOH can be modified by esterification or by Ugi condensation.

HA esters bonds can be performed starting from HA-TBA using esterifying agent such as alkyl halides (Figure 3 a.) (Della Valle & Romeo, 1989; Pelletier et al., 2001) or tosylate activation (Figure 3 b.) (Huin-Amargier et al., 2006). Moreover –COOH can undergo esterification also using diazomethane (Figure 3 c.) (Hirano et al., 2005) and epoxides (Prata et al., 2010).

Instead, the Ugi condensation (Figure 3 d.) uses a diamine linkages to form a final acylamino amide bond and linkages between two HA chains. The method is performed in water at pH values of 3 in presence of formaldehyde, known to be carcinogenic (Maleki et al., 2007).





c.

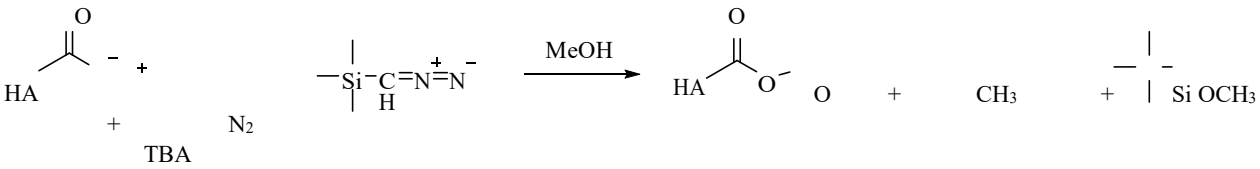


Figure 3. HA esterification using alkyl halides (a), tetraethylene glycol ditosylate (b), trimethylsilyl diazomethane (c) and Ugi condensation reaction (d).

1.4.2 Modification of HA hydroxyl groups

The primary hydroxyl group on HA is the most reactive compared to the other, in fact its pKa value is about 9 and it is reasonable to assume that the reaction takes place mainly on it. The strategies for its derivatization comprise esterification, etherification, oxidation and carbamate formation (Figure 4).

The use of epoxides such as 1,4-Butanediol diglycidyl ether (BDDE) under alkaline conditions or divinyl sulfone (DVS) are the most commonly used approaches to form ether bonds with the HA hydroxyl groups. Another common cross-linker that leads to the formation of hemiacetal bonds at acidic pH is Glutaraldehyde (GTA) which, being toxic, requires special handling and purification conditions (Crescenzi et al., 2003; Tomihata & Ikada, 1997).

The hydroxyl groups of HA can also be converted into esters by reaction with activated compounds. For example, acyl-chloride activated carboxylate compound (Toemmeraas & Eenschooten, 2007) or anhydrides (Seidlits et al., 2010) by chloroacylation with thionyl chloride under alkaline conditions.

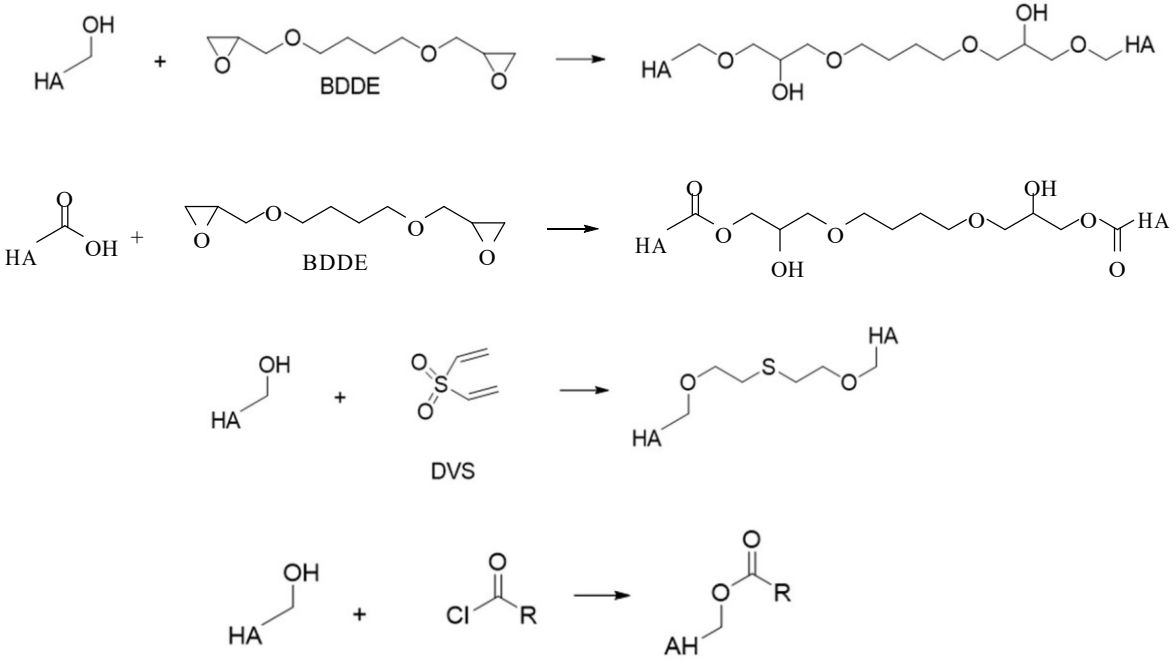
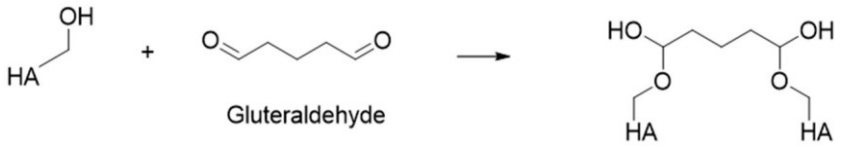


Figure 4. Ether derivatization by modifications of the hydroxyl group (–OH) of HA.



A further option for hydroxyl groups is the the activation using cyanogen bromide to synthesize carbamate derivatives. The reaction carried out in water with a high pH value (about 10) leads to a high degrees of substitution in just one hour of reaction. (Mlcochová et al., 2006).

1.4.3 Modification of HA N-acetyl group

Although it is not a common approach, it is possible deacetylate the N-acetyl group on HA using hydrazine sulfate (Figure 5) (Bellini & Topai, 2000) or enzymes (Platt & Szoka, 2008): both methods lead to free amino groups that can react to form amide bonds by methods described above.

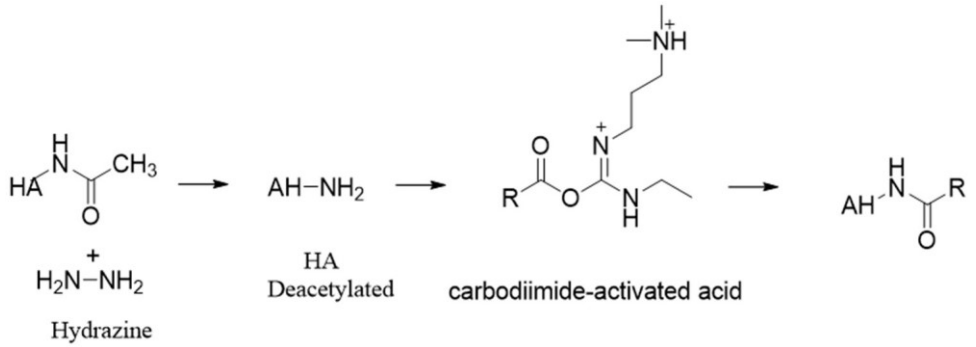


Figure 5. HA deacetylation and amidation.

The drawback that limits this practice is that deacetylation alters the structural modification and therefore also the biological properties typical of HA. It has recently been reported that this modification leads an alteration in the interaction with the CD44 receptor (Bhattacharya et al., 2017).

1.4.4 HA cross-linking

Generally, to improve the mechanical behaviour of HA and prolong its half-life, it can be subjected to cross-linking strategies via condensation reactions, enzymatic cross-linking, disulfide cross-linking, click chemistry, polymerization, etc. to form double links of different chains.

The most common approaches to cross-link HA usually targets the carboxylic group for esterification or amidation or the hydroxyl group of the HA chain structure employing various chemical cross-linkers such as carbodiimide, divinyl sulfone (DVS) and 1,4butanediol diglycidyl ether (BDDE) as described above.

The degree of cross-linking is directly proportional to the number of molecules forming double bonds wich play an important role in the viscoelastic properties of HA. Therefore, it is important to evaluate the desired degree of cross-linking for the development and

commercialization of new HA products, especially for the biomedical and cosmetic application.

A polymer with high degree of cross-linking corresponds to the slower degradation rate and consequently it remains longer in the body. The result of chemical cross-linking is a threedimensional HA hydrogel which could retain water within its cross-linked network, but does not dissolve readily in water.

However, these are molecules of synthetic origin which are not always accompanied by a high safety profile due to the potentially formation of toxic degradation products.

Therefore, recently, the use of bioactive molecules to cross-linked HA chains has been proposed with the aim to obtain biomaterials with improved physical-chemical characteristics avoiding the loss of biocompatibility of native HA. The biocompatibility and safety profile of HA derivatives can be exploited in the biomedical field as well as aesthetics and bioengineering, as multifunctional activities (Bowman et al., 2018; Gelardi et al., 2013; Kaur et al., 2006; Knopf-Marques et al.,2016; Nobile et al., 2014).

As an alternative to chemical cross-linking, several authors have also described the synthesis of cross-linked HA by photocross-linking (Bencherif et al., 2008; Baier et al., 2003) where generally a methacrylate-HA product was subjected to UV light and consequently crosslinked by free radical polymerization.

The resulting photo-crosslinked hydrogels showed bicompatibility, and good mechanical properties related to the degree of modification, which depends on the type of photoinitiator and the time of exposure to light as well as the concentration.

Another interesting non-covalent approach to HA-based hydrogel design is represented by physical cross-linking. The method does not use chemical cross-linkers and consists in the functionalization of the HA with hydrophobic molecules, from the hydrophobic interaction of which hydrogels are obtained.

Alternatively, from the interactions between two opposite charged polymers it is possible to form complexes by electrostatic interactions (Borzacchiello et al., 2010; Palumbo et al., 2006; Gulrez et al., 2011). Most of the amphiphilic derivatives of HA belong to this category of non-covalent hydrogels and behave like gels.

Since the network is formed by reversible interactions, it can easily undergo changes in particular conditions of mechanical stress or alterations in chemical-physical conditions (Rosiak & Yoshii, 1999).

1.5 Applications of HA and its derivatives

Over the past decade, HA due to its safety profile and the unique physical and chemical properties has proven to be one of the most attractive materials for application in various biomedical fields including pharmaceutical, food and cosmetic applications. The following are the most common applications of HA in ophthalmic, wound healing, soft tissue regeneration and cosmetics fields.

1.5.1 Ophthalmology

As one of the main components of the eye, HA is commonly used in ophthalmology to protect and lubricate the delicate eye tissues, supplement vitreous humour and particularly in cataract surgery to replace lost vitreous fluid and to maintain the space and avoid the collapse of the eye tissue during the operation.

On the other hand, HA is the main ingredient of many artificial tears that are produced with HA at different molecular weights and at different polymer concentrations. Therefore, HA-based ophthalmic products are completely biocompatible and do not trigger foreign body reactions (Schanté et al., 2011).

Eye drops with hyaluronan derivatives with improved mechanical and biological properties have recently been formulated (Laffleur & Dachs, 2015).

1.5.2 Wound healing

Since HA has both structural and regulatory functions in the processes of wound repair and re-epithelialization (Weigel et al., 1986) as well as in the stimulation of collagen production, it has been considered as an appropriate candidate to support skin tissue regeneration.

Therefore, it is often included alone or in combination with other therapeutic agents in topical formulations for the treatment of skin irritations and wounds including abrasions and burns as well as post-surgical, metabolic and vascular ulcers.

Generally, these products are used not only in the dermatological field but also in ophthalmology, otolaryngology rhinology and dentistry (Aya & Stern, 2014)

Cross-linked HA hydrogel films have also been shown to accelerate the wound healing process by promoting the re-epithelialization that typically occurs after damage (Borzacchiello et al., 2000).

1.5.3 Soft tissue regeneration

HA is an important structural element of the skin to which it provides hydration, elasticity and volume but unfortunately its concentration decrease drastically with age causing the formation of wrinkles (Robert et al., 2010; Baumann, 2004).

For this reason, HA-based dermal fillers (DF) in recent years have aroused wide interest in the field of cosmetic surgery to restore lost volumes and correct facial imperfections of the dermis (Gatej et al., 2005).

There are several types of HA-based DFs on the market, but they are usually cross-linked HA-based that exhibit network behaviour that can persist in the body. The corrective effect is reversible and lasts from three to 24 months (Muhn et al., 2012).

1.5.4 Cosmetics

In the cosmetic fields, HA and its derivatives are widely used as an anti-aging active ingredient to restore hydration and elasticity of the skin depending on the molecular weight and concentration. HMW is able to form film on the skin surface which reduces transcutaneous evaporation of water and protects the stratum corneum while the medium molecular weight acts mainly by binding moisture from the environment.

A significant improvement in wrinkle reduction has been observed as a result of the application of low molecular weight which may be due to its better ability to penetrate the skin. Furthermore, HA is also found included in sunscreen products for its potential ability

to protect the skin from harmful radiation due to its possible scavenging properties (Hašová et al., 2011; Trommer et al., 2003).

1.5.5 Drug delivery systems

Hyaluronic acid is one of the most promising polymeric materials for the construction of drug delivery systems in fact it has been extensively studied in the ophthalmic field (Järvinen & Urtti, 1995; Langer et al., 1997; Moreira et al., 1991; Herrero-Vanrell et al., 2000), for nasal administration (Morimoto et al., 1991) and for parenteral drug delivery (Drobnik, 1991; Luo & Prestwich, 1999; Peer et al.; 2003; Eliaz & Szoka, 2001, Brown & Jones, 2005). HA can be a vehicle for the controlled release of active molecules alone or together with other natural polymers such as collagen (Segura et al., 2005), gelatin (Hoare & Kohane, 2008), chitosan (Hamman, 2010) and synthetic ones such as PLGA (Lee et al., 2009). Since it has several functional groups available for conjugation, HA can be conjugated directly to the drug to constitute the pro-drug.

For example, it has been conjugated to anti-inflammatory molecules for the intra-articular therapy of osteoarthritis and also to curcumin to increase its solubility and stability (Manju & Sreenivasan, 2011). Furthermore, since HA shows a high affinity for binding to tumor cells that overexpress the CD44 receptor, it has been chemically anchored to nanoparticles containing chemotherapeutic agents such as doxorubicin (Eliaz & Szoka, 2001; Eliaz et al., 2004).

Alternatively, HA can be used as a drug carrier system for the development of liposomes, microsphere, and micelles to improve the pharmaceutical properties and the drug release profile.

The greatest advantage of these systems is their excellent biocompatibility and ability to sustain drug delivery for varying periods of time.

One of the most promising and extensively studied groups of parenteral controlled release formulations for peptides, proteins and, recently, plasmid DNA are biodegradable particulate systems, such as microspheres.

In general, these formulations consist of a polymeric matrix containing an active ingredient uniformly dispersed. It can be prepared by several techniques including emulsion method followed by solvent evaporationand spray-drying.

For example, ofloxacin-containing HA microspheres prepared for pulmonary use showed a better pharmacological profile than free drug and other methods of administration (Hwang et al., 2008). Furthermore, it has been reported that HA microspheres are also promising for the delivery of plasmid DNA and monoclonal antibodies in gene transfer (Yun et al., 2004). Generally, drug release of drug from macromolecules occurs both by degradation of the polymer and by controlled diffusion through the pores of the polymer matrix and can be influenced by many factors, such as the preparation technique, the properties of the drug and the composition of the polymer. The cross-linking of hyaluronic acid in order to decrease the mesh size as well as decrease the swelling balance of polymer results in a slower release due to both the slower diffusion of the drug through the polymer matrix and to the reduced erosion of the polymer matrix which contains it.

In the third chapter of this thesis, cross-linked products used for the development of microspheres for the prolonged release of encapsulated drug will be described.

Chapter 2

2 AIMS OF STUDY

Hyaluronic acid is a polymer with unique biological properties that has acquired great interest over the years, finding application in various fields such as the pharmaceutical, cosmetic and biomedical ones.

Due its high hygroscopy it is able to retain large quantities of water providing hydration, volume and protection of the various tissues of the organism.

Although HA is an excellent biomaterial many of its potential applications are limited due to its short half-life since after interaction with the CD44 receptor present on the cell membrane it is internalized and rapidly degraded by hyaluronidases.
In order to prolong its half-life and consequently increase its performance, native HA is usually modified through cross-linking reactions in which the polymer chains are stabilized by covalent bonds using chemical cross-linkers to obtain a material less susceptible to chemical hydrolysis and enzymatic, showing improved mechanical properties.

In the literature are reported various methods that generally use cross-linkers of synthetic origin including divinylsulfone (DVS), 1,4-butanediol diglycidyl ether (BDDE) (La Gatta et al., 2013), carbodiimide (Schanté et al, 2011) etc., capable of forming a three-dimensional network structure with enhanced mechanical resistance but which does not have excellent biocompatibility characteristics for human tissuesand therefore potentially dangerous to cause adverse reactions (Sung et al., 1998; Ferretti et al., 2006; Tan et al., 2009).
Therefore, several studies are being focused on obtaining new hydrogels with relatively safe for the body compartments increased biocompatibility and biodegradability.
In the present PhD thesis, two new cross-linked hyaluronic acids have been developed and patented, through a useful cross-linking technique and the use of natural cross-linking agent such as arginine and ornithine methyl ester. These amino acids are physiologically present in the human body and, therefore, are considered extremely biocompatible.
The development and obtaining of these new raw materials subsequently shifted the final purpose leading to investigate a potential cosmetic, pharmacological and nutraceutical

application not only of the products obtained as active ingredients but also as possible drug carriers.

In particular, this manuscript is organized in five chapter;

The Chapter 1 of this manuscript includes a general introduction to hyaluronic acid and describes its structural and biological properties, chemical derivatization strategies are provided including applications such as medical, pharmaceutical and cosmetic as an active molecule, filler and system carrier.

In Chapter 2, the main objectives and structure of manuscript are described.

Chapter 3 provides the results and discussion section divided into four main studies

The first study describes the design and synthesis process applied to produce the two crosslinked hydrogels (HA-Arg and HA-Orn) with the use of arginine and ornithine methyl ester as cross-linker and a physico-chemical characterization that confirms that the occurrence of chemical modifications has been provided.

The second study provides an evaluation of the in vitro degradation rate products crosslinked with Ornithine and Arginine.

In third study, the cytotoxicity and bioactivity of HA-Arg e HA-Orn, alone and in combination with an ascorbic acid derivative (SAP), for the potential treatment of inflammatory lung diseases were evaluated.

Finally, the fourth study describes the formulation of microspheres as a delivery system produced using HA-Arg e HA-Orn in comparison to native HA with and without SAP loading to investigate its potential drug release mechanisms as carrier for biomedical and pharmaceutical applications.

Chapter 4 includes the experimental part presented in the materials and methods section.
Chapter 5 summarizes the main conclusions concerning this manuscript.
In chapter 6, the references cited throughout the dissertation are presented.

This work has been object of the patent application N° 102019000024117 of the 16th December 2019.

Chapter 3

3 RESULTS AND DISCUSSION

3.1 Cross-linking hyaluronic acid with arginine and ornithine methyl ester

Hyaluronic acid is an important starting molecule for the production of new biocompatible and biodegradable polymers. It is a polymer can be chemically modified both by conjugation and by cross-linking through amidation or esterification of the carboxylic functional groups on glucuronic unit or hydroxylic moiety of N-acetyl-glucosamine component for hydrogel creation.

The establishment of covalent bonds in several points of the hyaluronic acid chains creates three-dimensional networks with improved mechanical properties and greater resistance to enzymatic degradation than native HA.

Today, there are several products on the market that boast as claim ingredient the crosslinked hyaluronic acid that use as cross-linking molecules only compounds of synthetic origin such as DVS (divinylsulfone) and BDDE (1,4-butanediol diglycidyl ether) etc.; their use is able to make the cross-linked hyaluronic acid more resistant from a mechanical point of view but which does not have excellent biocompatibility characteristics for the tissues organism and potentially able to cause adverse reactions.

Many of them are in fact recognized as mutagenic (De Boulle et al., 2013) and toxic molecules but considered acceptable by the FDA because they are mainly related to the polymeric component and present only in traces in their free form, therefore negligible. At the same time, however, it has been shown that they promote inflammation in a concentration-dependent manner (De Boulle et al., 2013; Lai, 2014) and are therefore dangerous when released from the polymer lattice as a result of the hydrogel hydrolysis process.

Thereore, this chapter explores the use of aminoacidic cross-linkers such as ornithine and arginine that have never been reported in the literature. HA was cross-linked with Arginine and Ornithine methyl ester through a procedure that involves the formation of a triazine derivative with CDMT with the aim of obtaining hyaluronic acid derivatives with implemented characteristics of bioactivity and resistance and with the aim to produce new raw material with a safe profile related to biocompatibility and safety of the cross-linker used.

In fact, Ornithine and Arginine are molecules naturally present in the human body where they are part of the Natural Moisturizing Factor (NMF) (Arezki et al., 2017) and are therefore considered biocompatible and non-toxic.

They are two interesting amino acids for a double aspect:

- are physiologically active components present in the body and used in the nutraceutical and cosmetic fields as functional ingredients as they are recognized as anti-aging, immunostimulants and promoters of tissue repair.

- they have a chemical structure that allows them to be used as as cross-linkers as they have homo-bifunctional aminic residues;

It is well known that Arginine is the substrate of nitric oxide synthesis, and is involved in angiogenesis and cell proliferation, as well as an indirect precursor of collagen synthesis via the proline pathway (Gad, 2010; Witte & Barbul, 2003).

Ornithine is the major metabolite of arginine in the urea cycle and shares many of the biopharmacological effects of Arginine:

- prevents the skin aging;
- performs a moisturizing and trophic action of the skin: through the production of nitric oxide, the blood flow increases, which transports nutrients and water to the cells;
- accelerates the wound healing process especially in the case of burns; □ improves skin elasticity.

Due to the effect on safety and health effects of Arginine, Ornithine and HA they can be suitable for the synthesis of innovative hyaluronic acid derivatives with potential multiple applications in the medical, pharmaceutical and cosmetic fields both as an active ingredient and as a starting material for the formulation of delivery systems.

For the synthesis of the two new cross-linked, the reaction was carried out in a solvent mixture of water and acetonitrile in a 3: 2 ratios. The reaction initially involves the activation of the carboxylic group on the glucuronic component in the formation of an activated intermediate (BKaminska et al., 1999) (Kunishima et al., 2001) which secondly, it undergoes the nucleophilic attacks of amino groups with the formation of an amide bonds between the chains of linear hyaluronic acid (Kaminski, 1994).

Among the various procedures reported in the literature, the chosen method involves the use of CDMT, a recently reported activator of the carboxylic group which has shown to proceed with high efficiency and no side reactions. This water-soluble coupling reagent usually allows higher degree of amidation and purer reaction products than carbodiimides (Kunishima et al., 1999; Gennari et al., 2016) and it is also believed to be less allergenic and safer than carbodiimide compounds.
To evaluate the cross-linking efficiency and to corfirm the occurence of the chemical modification several analyses were performed including nuclear magnetic resonance spectroscopy (NMR) and infrared spectroscopy (IR), swelling capacity, surface morphology, thermal and rheological behaviour.

3.2 Synthetic procedures

The Synthesis of each product was initially attempted using CDMT / NMM and EDC / NHS as condensing agents in order to setup the ideal synthetic procedure for HA cross-linking with a high degree of modification and good yield. For the EDC / NHS route initially the same stoichiometric ratio between HA : EDC : Aminoacid was used and NHS was used in the same stoichiometric amount for EDC. In the absence of detectable results, stoichiometric ratios were then increased but with poor results. Therefore, the use of CDMT / NMM is reported below as a condensing agent that has led to the production of cross-linked polymer with Arginine and Ornithine.

3.2.1 Synthesis of cross-linked HA-Arg and HA-Orn

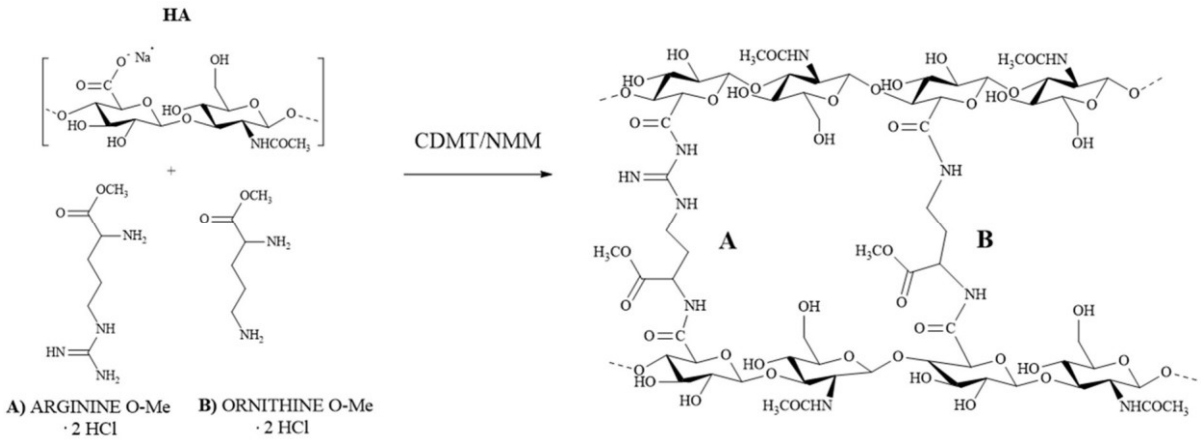
HA cross-linked products were successfully prepared by amidation of the carboxyl groups through a synthetic procedure adapted from the Bergman method (Bergman et al., 2007) using as cross-linkers the amino acid Ornithine and Arginine methyl ester to form diamidic bonds between the chains of hyaluronic acid. To identify the ideal reaction conditions and

achieve the highest degree of modification, synthesis attempts have also been performed using EDC/NHS as a condensing agent at different combinations between HA, condensing agent and amino acid Arginine methyl esters ratios. From the scale up study conducted, CDMT, in association with NMM, was found to be the most potent activator. Therefore, the synthesis of HA-Arg and HA-Orn were carried out at room temperature with an excess of both activating agent and amino acid, precisely in ratio of HA : CDMT: Amino acid of 1 : 3 : 1.5 was used to covalently attach the amine moiety onto the native HA. NMM was used in the same stoichiometric amount to CDMT.

The reaction was carried out in a mixture of solvents composed of water deionized and acetonitrile in a ratio 3:2 for an optimal solubilization of the reagents. The reaction initially involves the activation of the carboxylic acid with CDMT to form the intermediate activated HA and then, N-methyl morpholine (NMM) was added to the mixture in equimolar ratio to CDMT to neutralize the chloride ions formed (Scheme 1).

A purification step by exhaustive dialysis was conducted until the resulting hydrogels were completely purified of any detectable residual cross-linker agent. Therefore, each product was dialyzed 24 hours against water, 48 hours against 0.1 M NaCl solution and finally 48 hours again against water. The solutions were then lyophilized to obtain white spongy products.

To confirm the absence of traces of unreacted aminoacids, the polymers were analyzed with the ninhydrin colorimetric test.



Scheme 1. Synthesis scheme of HA crosslinked with a) Arginine methyl ester and b) Ornithine methyl ester using CDMT/NMM to amidic bond formation.

3.2.2 Physicochemical characterization methods

3.2.2.1 Nuclear magnetic resonance spectroscopy (NMR)

NMR spectroscopy is the most common tool for a first characterization of the extent of the chemical modification occurred on native HA.

More precisely it is possible to quantify the degree of modification by calculating the integration of a signal belonging to the cross-linker with respect to the characteristic peak of the native HA at 1.9 ppm belonging to the protons of methyl proton group of N-acetyl glucosamine.

The ^{1}H-NMR spectra of native HA and purified cross-linked products HA-Arg and HA-Orn are shown below (Figure 6) and showed the expected signals. The spectra of hyaluronic samples gave enlarged signals and this is due to the viscosity of the product in solution. The peaks of the methyl groups of the Arginine and Ornithine side chains were sected because not overlapping the peaks of the native HA. Therefore, this allows an easy calculation of the degree of modification. The new signals detected in the HA-Arg spectrum correspond to the methyl groups of Arginine in position γ (1.52 ppm), β (1.67 ppm), δ (3.19 ppm) and to the methoxyl group -OCH_3 (3.61 ppm). Also HA-Orn displays three characteristic peaks: two

wide signals at 1.56 ppm (-CH_2 γ),1.72 ppm (-CH_2 β) and the metossilic group at 2.89 ppm. In all spectra the multiplet of signals in the region from 3.2 to 3.9 ppm corresponds to the protons of the component of HA component. However, HA also showed a large signal at 4.4 ppm belonging to the two anomeric protons. In the spectra of HA-Arg and HA-Orn this signal was overlapped on the signals of the methyl groups in α at 4.4 and 4.5 ppm, respectively. From the calculation of the ratio between the two reference signals, it was found that the degree of modification was estimated around 45% for HA-Arg and 35% for HAOrn: these values correspond to the amount of Arginine and Ornithine cross-linked with native HA. To validate the value of the degree of substitution obtained, before recording the NMR spectrum, each sample, was tested through the qualitative ninhydrin assay to verify the absence of free amines in the final products.

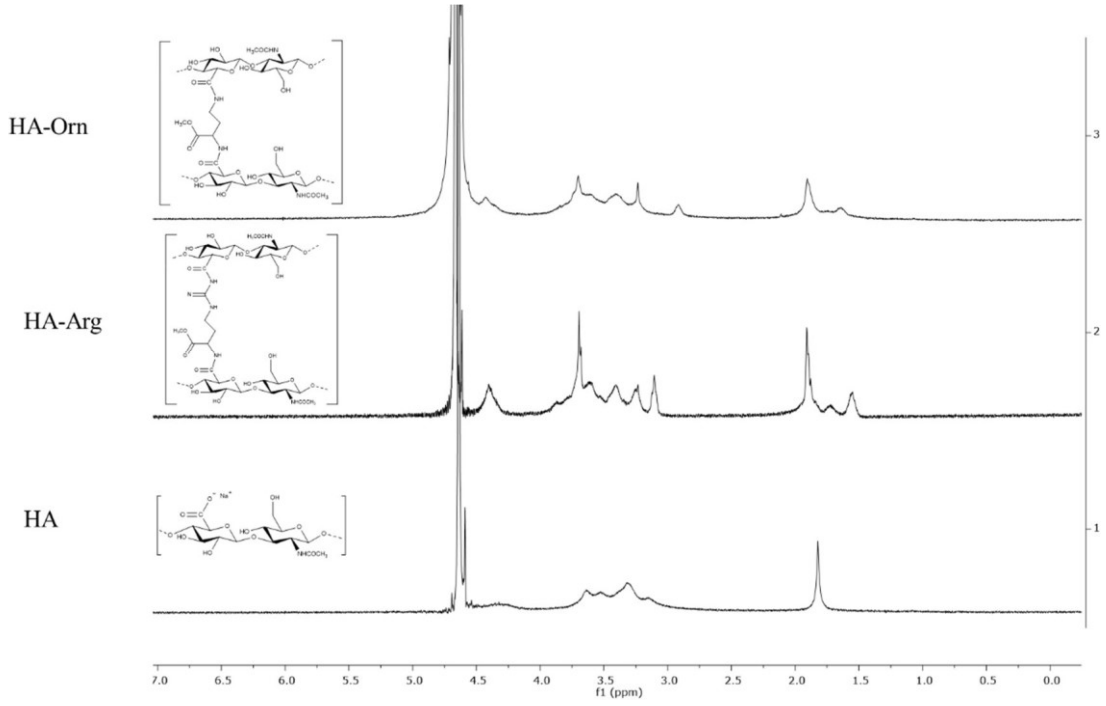


Figure 6. ^{1}H-NMR spectrum of native HA and cross-linked HA-Arg and HA-Orn recorded in D_2O.

3.2.2.2 IR spectroscopy

Infrared (IR) spectroscopy is another common characterization technique often used to determine the type of bond that forms during the modification of the HA.

The difference in the IR spectra between HA native and cross-linked HA-Arg and HA-Orn are reported in Figure 7. HA shows main absorption bands at 3267 cm^{-1} corresponding to O–H and N-H groups stretching; a double band at 1617 cm^{-1} and 1409 cm^{-1} attributed to the asymmetric and symmetric C=O stretching modes of the planar carboxylate groups and at 1010 cm^{-1} indicating C-OH stretching vibrations. The most evident difference in the spectra of HA-Arg end HA-Orn that can be osserved was: 1) the appearance of C=O amide bands at 1636 cm^{-1} and 1640 cm^{-1} respectively, wich correspond to the new amide bonds formed between the diamines of the aminoacids and the carboxylate group of native HA as a

consequence of the cross-linking reaction. 2) There is also the disappearance of the band related to the carboxylate groups at 1600 -1400 cm^{-1}, an increase in the intensity of the NH group peak at about 3200 cm^{-1} and the appearance of NH bending band at 1515 cm^{-1} were observed. 3) Another new band is evident around 1720 cm^{-1} relating to the methyl ester groups present on amino acids. The signals of the non-derivatized HA groups remain in the spectrum of the cross-linked products. These results agree with the cross-linking reaction and provide further evidence of the desired bond formation.

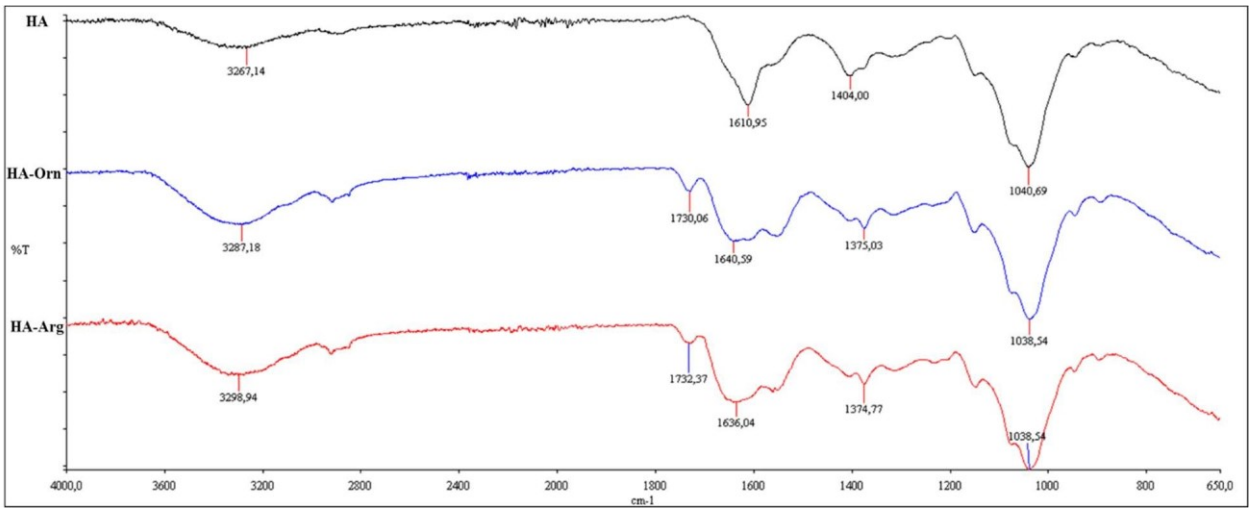


Figure 7. FTIR spectra of native HA and cross-linked HA-Arg and HA-Orn.

3.2.3 Thermal analysis: Differential Scanning Calorimetry (DSC)

Chemical cross-linking leads to the change of different chemical-physical characteristics with respect to the original compound which can be correlated to a different thermal behaviour.

The thermal behaviour of HA-Arg and HA-Orn was investigated by Differential scanning calorimetry (Ford & Timmins, 1989) a thermal analysis able to provide information on their hydration and thermal resistance properties (Collins & Birkinshaw, 2007; 2008; Kafedjiiski et al., 2007).

The DSC tecnique is based on the different temperature demand between the reference and the sample as well as the measurement of the heat absorbed or released by the system when the temperature changes following the occurrence of some structural change in it.

DSC thermograms showing the influence of the heating rate on the thermal behaviour of linear HA, HA-Arg and HA-Orn respectively are shown in Figure 8. The data obtained are reported in details in Table 1 reporting the important temperatures peaks.

The HA thermogram shows two peaks: the first is an endothermic peak around 101 °C and suggests the temperature at which the sample dehydration occurs, followed by an exothermic peak attributable to the sample decomposition temperature at 238 °C.

The profile of the thermograms of the two cross-linked HA-Arg and HA-Orn indicated that they are similar shape to native HA but with lower onset temperatures values; It is possible to observe a broad endothermic peaks respectively at 99.1 °C e 97.4 °C associated with the loss of moisture content (residue after lyophilization) followed by exothermic peaks at

temperature below (230 °C and 232 °C respectively), corresponding to the decomposition of HA-Arg and HA-Orn.

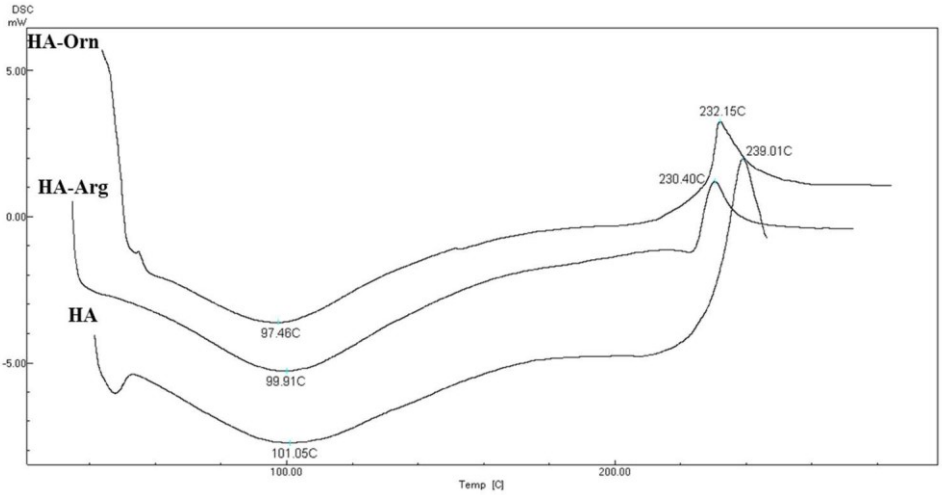


Figure 8. DSC thermograms of native HA, HA-Arg and HA-Orn.

	Native HA	HA-Arg	HA-Orn
Dehydration temperature (°C)	101.05	99.91	97.46
Decomposition temperature(°C)	239.01	230.40	232.15

Table 1.Comparison of temperature values of the endothermic and exothermic peaks of HA, HA-Arg and HA-Orn.

The different values of the thermodynamic parameters obtained for both cross-linked with respect to linear HA are attributable to an alteration of the original system. The shift of the endothermic peak in the cross-linked products reflects a lower water retention therefore to a reduction of the calorimetric energy necessary for it removal. Furthermore, as can be seen from the comparison, the exothermic peaks of HA-Arg and HA-Orn have lower temperature of about 7 °C and 9 °C than native HA due to the presence of aminoacids as components of the new structure.

This is because the aminoacids Arginine and Ornithine methyl esters, having a lower melting point temperature than the polymer to which they are linked reduce the overall decomposition temperature of the new materials.

A physical mixtures of Arginine methyl ester and Ornithine methyl ester, respectively, with HA was used to compare the thermal behaviour with the corresponding covalently crosslinked products (data not shown). In the thermal profile of physical mixtures, the degradative behaviour of the aminoacids prevails over that of the polymer, not allowing to continue the thermal evaluation.
These observations show that the the cross-linking has clearly produced a new material with a different structural organization from the original system.

3.2.4 Rheology

A Controlled Shear Rate test was conducted on cross-linked hyaluronic acid dispersions prepared at a concentration of 2% w/w of raw material, to analyse the viscosity trend in function of the shear rate applied, ranging from 0.001 to 1000 s^{-1}. Both samples showed a shear-thinning behaviour, since the viscosity values gradually decreased under shear application (Figure 9). The sample HA-Orn reached higher values of viscosity, as a result of the firmer interactions between the material's cross-linked chains and the solvent, forming a three-dimensional network, in which the aqueous solvent is trapped. The viscosity at rest (η_0) calculated for HA-Orn with the Carreau-Yasuda model, was of 9578.38 Pa.s. The viscosity rapidly decreased in function of shear rate as as shown in Figure 9: the polymeric chains started to align toward the flow direction. The sample HA-Arg showed lower viscosity values of more than three magnitude orders compared to HA-Orn. The viscosity at rest calculated with the Carreau-Yasuda model for HA-Arg was of 3.98 Pa.s. It also showed a less marked shear-thinning behaviour: at lower shear rate values, the viscosity of HA-Arg remained almost constant, showing a large Newtonian plateau region. As the shear rate increased at about 1 s^{-1} the viscosity progressively decreased and the system started to flow.

The viscoelastic behaviour of both samples was investigated through the rheological analyses in oscillatory flow conditions. By increasing the strain values from 0.01 to 1000%,

at a fixed oscillation frequency of 1 Hz, an Amplitude Sweep test was conducted. This test allowed identifying the Linear Viscoelastic Region (LVER), in which the elastic G' and the viscous G'' moduli were not dependent on strain showing a constant trend (Figure 10).

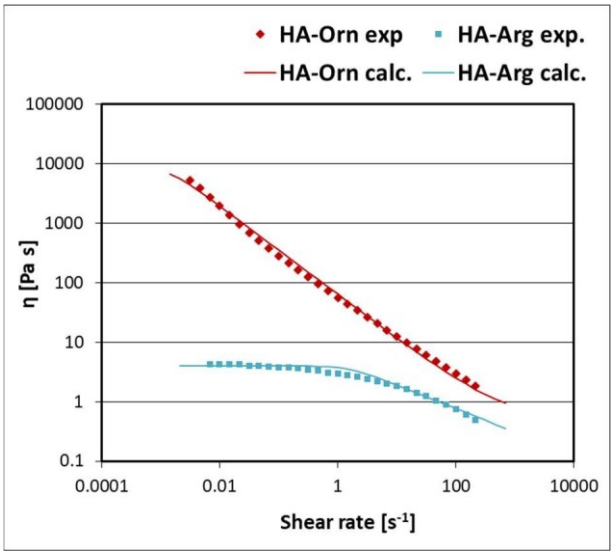


Figure 9. Viscosity trend in function of the shear rate values for the water dispersions of HA-Orn and HAArg prepared with 2% w/w of raw material. Flow curves fitted with the Carreau−Yasuda model.

According to the flow conditions tests, the two dispersions at 2% w/w of cross-linked hyaluronic acid showed very different viscoelastic properties. The HA-Arg solution had a liquid-like behaviour, with G'' always dominating over G', in the entire range of amplitude strain investigated. HA-Orn was characterized by higher moduli values than HA-Arg, with G' over G'' in the LVER, indicating a predominantly elastic behaviour of the material due to the highly swollen network formed in water.

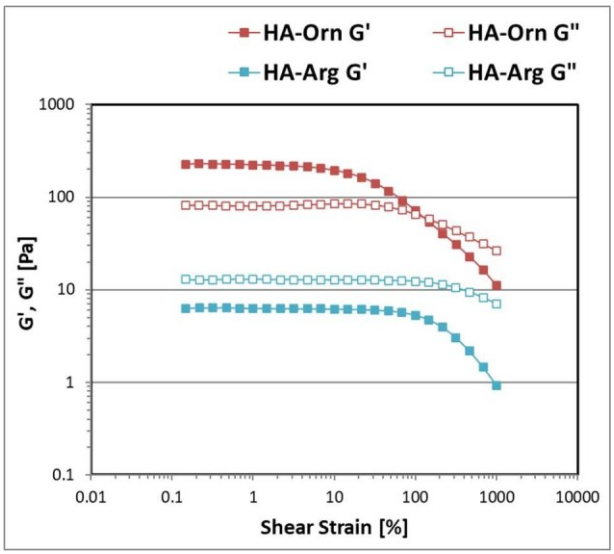


Figure 10. Elastic G′ and viscous G″ moduli in the function of shear strain for the water dispersions of HAOrn and HA-Arg prepared with 2% w/w of raw material.

After this region, increasing the amplitude strain, G′ progressively decreased until it intercepted, at just about 100% of strain, the viscous modulus G″ in the critical strain point ($\gamma_{G'=G''}$), with consequent inversion of the moduli, thus indicating the breakage of the microgel structure.

A frequency sweep test was conducted by taking a fixed value of amplitude strain from the LVER (1%), and gradually decreasing the oscillation frequency from 10 Hz to 0.01 Hz. Figure 11 (panel a) shows the Complex modulus G* trends in function of the oscillation frequency applied on the samples HA-Orn and Ha-Arg prepared with a concentration of 2% w/w of cross-linked hyaluronic acids dispersed in water. G* is a useful property to measure the rigidity of a material's soft solid structure and to quantify its overall resistance to a deformation below the critical strain value. This parameter is an indicator of some physicalmechanical properties such as the firmness and the flexibility of the material (Equation 1).

$$|\square * | = \sqrt{\square' + \square''} \qquad \text{Equation 1.}$$

As expected, HA-Orn showed higher values of G* than HA-Arg. The curve is indeed collocated between the second and the third decades and scarcely dependent on the applied

frequency. On the contrary, the G* values of HA-Arg were strongly dependent on the changes in frequency since the curve dramatically decreased with decreasing values of frequency. Moreover, higher values of the damping factor tanδ, which represents the ratio between the viscous modulus and the elastic modulus, were registered for the HA-Arg dispersion (Figure 11 panel b). Tan□ values >1 indicated a liquid-like behaviour of HA-Arg dispersion and a greater dissipative capacity, while tan□ values <1 indicated a prevalence of the elastic modulus. The tanδ values of HA-Orn sample had a constant trend and were less than 1. This rheological profile describes a typical weak-gel pattern, where the storage G′ modulus dominated over the loss G″ modulus throughout all the investigated range of frequencies. The elastic components always dominated over the viscous ones and the system's structure was held by physical bonds between the macromolecules. This was probably due to the presence of a higher cross-linking degree that determined the formation of a stiffer gellified system.

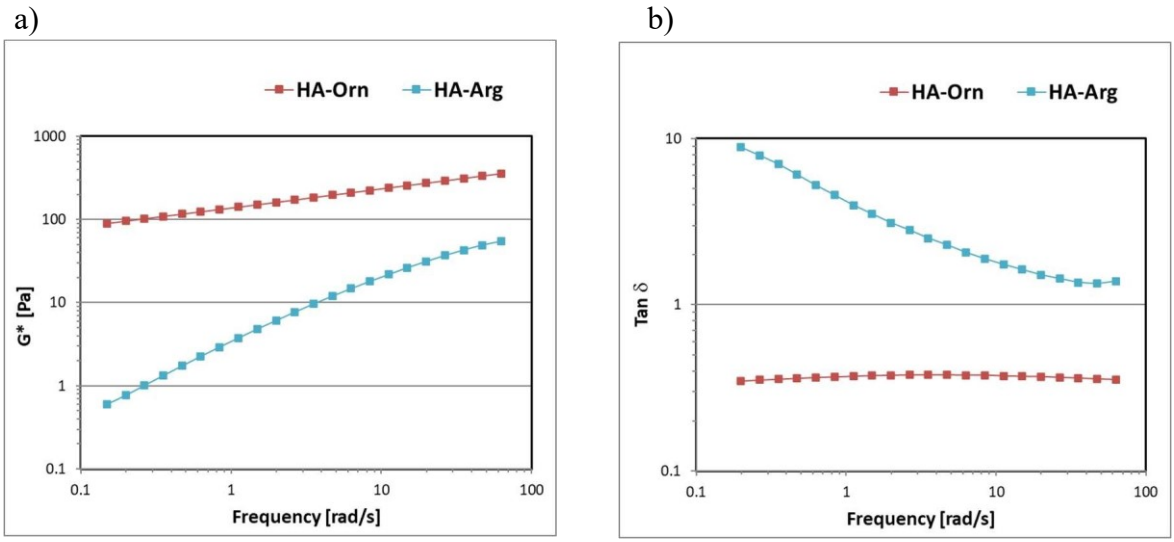


Figure 11. Trend of complex (G*) moduli (a) and damping factor (tan δ) (b) in function of the frequency for the water dispersions HA-Orn and HA-Arg prepared with 2% w/w of raw material.

3.2.5 Swelling ratio measurement

The Swelling degree (SD) is a gravimetric measure used for the study of the cross-linking density of the hydrogel in order to evaluate the water content that the sample is capable of absorbing. It is an essential parameter for an assessment of adhesive and cohesive properties, as well as drug release. Controlling of the rate and extent of hydration is necessary to promote prolonged adhesion and the cross-linking strategies are used to achieve purpose (Smart, 2005).

As reported in the literature high swelling values are related to a lower cross-linking density (Flory & Rehner, 1943).

Figure 12 shows the 24 hours swelling at room temperature of cross-linked HA-Arg and HA-Orn samples performed over a range of pH values representative of some biological fluids (4,5–9,5) (Schneider et al., 2007; Shukla et al., 2007).

Linear hyaluronic acid, not being cross-linked, dissolves rapidly when immersed in an aqueous medium forming a solution, consequently it is not possible to measure the swelling on this sample.

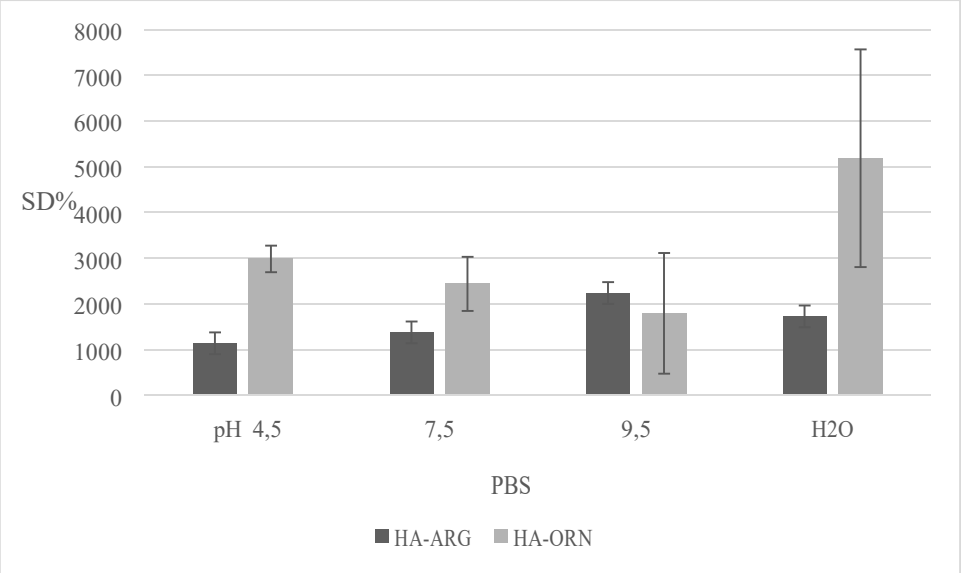


Figure 12. Swelling degree (SD)% of HA-Arg and HA-Orn at different pH value.

The results obtained by HA-Arg and HA-On are in agreement with the swelling values typical of hydrogels and show an almost opposite behaviour.

In fact, while the swelling property of HA-Arg increases with increasing pH, on the contrary, in the HA-Orn it decreases suggesting the influence of the ionic strength of the buffer on it.

The highest swelling of HA-Orn is observed when the sample is immersed in distilled water, possibly due to electrostatic repulsion between the negatively charged groups of the anionic showed higher swelling values than HA-Arg with each pH value analyzed and this is in agreement with the lower replacement rate compared to HA-Arg (see previous section 3.2.2.1).

As far as HA-Arg is concerned, the highest swelling value is reached immersed in PBS at pH 9.5, probably due to a greater affinity for the basic medium which allows a greater distention of the derivatized HA chains; it is known that at basic pH there is a more consistent breakage due to the lower rigidity of the polymeric backbone which can derive from a general hydrolysis of the H-bond network.

The results described suggest that the hydrogels exhibit a certain degree of pH reactivity and this property may be important because the swelling degree of cross-linked hydrogels can be modulated by the pH of the medium in which it is placed and this can be useful for a potential application in drug release.

3.2.6 Scanning Electron Microscopy (SEM)

To observe the morphology of the scaffold, cross-linked lyophilized hydrogels were studied by scanning electron microscope (SEM) which offers relevant information on the compactness and interconnection of the microstructure.

The Figure 13 shows SEM surface images of native HA and of the HA-Arg HA-Orn cross-linked.

Native HA images (Figure 13, panel a1, a2) showed a filamentous and irregular structure, while on the contrary the hydrogels acquired a new structural organization. A common feature of HA-Arg and HA-Orn products consists in the interconnectivity of these circular pores along the entire polymer matrix which establishes a solid structure with respect to linear HA preventing it form rapidly dispersion by hydration in aqueous media.

A spherical three-dimensional structure can be observed for both cross-linked. HA-Orn (Figure 13, panel c1, c2) exhibits a denser and evenly distributed structure with a smaller pore size than HA-Arg. The latter, in fact (Figure 13, panel b1, b2) shows a different morphological structure and a different dimensional distribution of the pores, differences that probably derive from the length of the cross-linking molecule used.

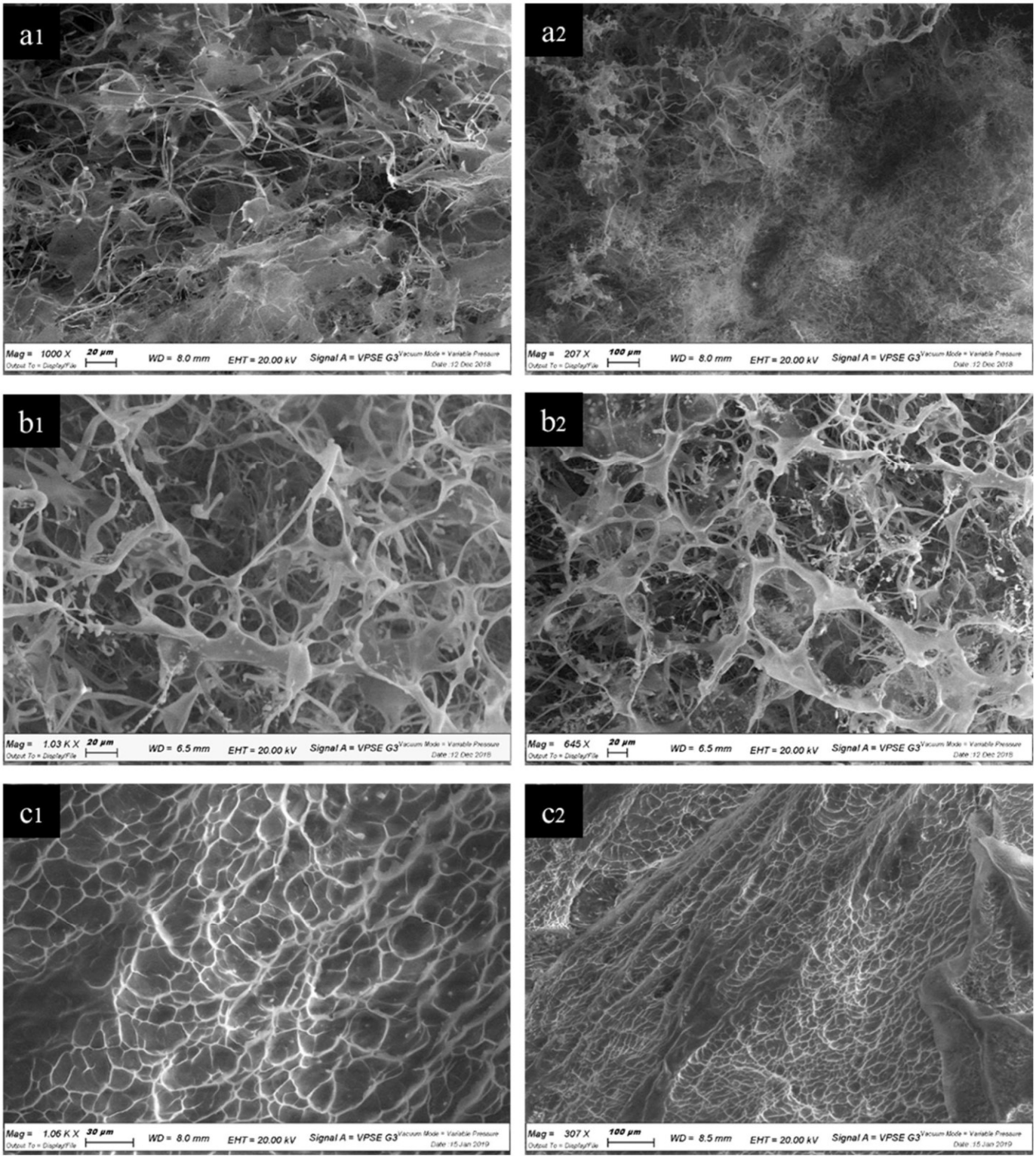


Figure 13. SEM images of HA (panel a1, a2), HA-Arg (panel b1, b2) and HA-Orn (panel c1, c2).

3.2.7 Molecular weight

Molecular weight is a relevant qualitative parameter for the characterization of HA-based products and to evaluate their possible applications in the commercial fields (Liu, Liu, Li, & Du, 2011). The molecular weight of HA can reach values ranging from 10^6 to 10^8 Da depending on the enzyme that catalyzed its synthesis (Stern et al., 2007) but it is know that HA is susceptible to various stages of chemical derivatization, in particular freeze-drying and exposure to alkaline or acidic conditions (Maleki et al., 2008; Tokita et al., 1997; Ghosh et al.; 1993) and preserving the integry of the HA chain is a challenge in chemical derivatization processes.

Gel Permeation Chromatography (GPC) is an analytical tool used to determine the molecular weights of polymers and their distribution therefore solutions of linear polymer HA and derivatives HA-Arg, HA-Orn were analyzed making use of the facilities available at the laboratoratories of the Lamberti Group (Milan) and the results were summarized in Table 2.

The molecular weight is calculated on the soluble fraction of the sample using the specific refractive index (dn / dC) which is the ratio of the detector response of the refractive index to the concentration of the sample. The dn / dC values of native HA solutions are generally between 1.430 and 1.670 ml / g depending on the solvent and the measurement parameters used (Bergman et al., 2007; Hokputsa et al., 2003, Mendichi et al., 1998)

The molecular weights were obtained by comparison with pullulan standards.

HA HA-Arg HA-Orn

Mw x 10^6 Da	1.04	0.91	0.95
Mn x 10^6 Da	0.80	0.70	0.56
Mw/Mn	1.29	1.30	1.68
Recovery	74.3%	0.42%	0.12%

Table 2. Results obtained by GPC analysis (Lamberti spa).

As shown in Table 2, the following values of MW have been obtained: HA (1.04 × 10^6g/mol), HA-Arg (0.91 × 10^6 g/mol), HA-Orn (0.985 × 10^6 g/mol).

The molecular weight value of native HA was slightly underestimated but remains in agreement with the supplier's claim. As for HA-Arg and HA-Orn, there was a slight decrease in molecular weight compared to the starting molecule: this can be related to the synthesis procedure used (CDMT / NMM) which involved steps of dissolution, stirring, dialysis and freeze-drying. The drying and freezing steps most likely caused the degradation of a portion of the expected polymer.

Overall, however, there is no significant variation in the molecular weight, and this is due to the fact that the cross-linked polymer, as a whole, is not soluble and cannot be eluted and thus the data collected relate to the soluble portion injected. From the recovery it is possible to extrapolate an indication of the degree of cross-linking of the samples, calculated from the parameter dn / dc which provides a quantitative indication of the dissolved polymer chromatographed and after being injected. The degree of recovery for HA-Arg (0.42%) and HA-Orn (0.12%) compared to native HA (0.69%) together with increased resistance to filtration suggests that a process of cross-linking had occurred and was more consistent for HA-Orn than for HA-Arg. However, further experiments or alternative evalutation methods are required to exactly attribute the molecular weight value of cross-linked products obtained.

3.3 In vitro degradation of HA-Arg and HA-Orn

Due to the high clinical and commercial value, cross-linked hyaluronic acids have been the focus of increasing scientific interest, thus, the research for new cross-linked HA products and new production methods led to improved mechanical performance to a wider range of applications.

The cross-linking process is a chemical strategy aimed to increase the stability of the native polymer by reducing the susceptibility to enzymatic degradation, in order to develop a unique material with a longer half-life, greater mechanical strength and a longer residence time after administration into the body (Collins & Birkinshaw, 2007; Tomihata & Ikada, 1997).

The development of mechanical strength (i.e. the cross-linking density) is important, because it plays an important role in tissue morphogenesis and in the drug release control.

The evaluation of the cross-linking efficiency and the availability of data on the susceptibility to enzymatic degradation of the two new cross-linked (HA-Arg and HA-Orn) are fundamental information because they greatly influence the biodegradability of the hydrogels and thus the specific fields of application.

Several in vitro techniques have been reported in the literature to monitor the degradation rate properties of cross-linked HA hydrogels, including viscosity change, water content variation and colorimetric dosage (Sall & Férard, 2007). Among the latter, one of the main methods applied to evaluate the cross-linking efficiency is the Carbazole assay through which it is possible to study the degradation behaviour. Since hyaluronic acid is a polymer composed of D-glucuronic acid and N-acetylglucosamine, when subjected to degradation it breaks down releasing its individual monomeric components.

Therefore, the in vitro degradation of the samples was performed by hyaluronidase (HAase) activity to mimic the in vivo conditions and tested using the Carbazole colorimetric assay to examine the D-glucuronic acid content generated during incubation.

The assay was conducted on both HA-Arg and HA-Orn cross-linked products compared to native HA according to the method originally described by Bitter et al. (Bitter & Muir, 1962). The degradation was quantified in terms of the increase in the fraction of soluble sample in the medium in which it is placed.

3.3.1 Samples Degradation by Hyaluronidase Enzyme Solution

The study of enzymatic degradation was performed on HA and cross-linked HA-Arg and HA-Orn by bovine testicular hyaluronidase (HAse) up to 24 hours.

Freeze-dried samples were previously cut into discs and inserted in vials containing PBS (10 ml, pH 7.4, 10Mm) with HAse (50U/ ml) and incubated at 37 °C.

At predetermined time intervals, an aliquot of the supernatant was withdrawn, and its degradation was stopped by boiling the sample. The solutions were then suitably diluited, filtered and tested by Carbazole assay to quantify the extent of degradation during enzymatic incubation as discussed below.

3.3.2 The Carbazole assay

The method is based on the quantitative colorimetric reaction between Carbazole and GlcA and includes two consecutive steps:

1) hydrolysis of the HA supernatant with 0.025M sodium tetraborate in sulfuric acid to generate GlcA and glucosamine monomers;

2) development of the colorimetric reaction between GlcA. and Carbazole (0.125% in ethanol.

Both reactions require a boiling step to complete.

A blank control was prepared with only phosphate buffer and carbazole reagent to confirm that no purplish red products are detected in the absence of GlcA.

The assay was performed on the supernatants of HA, HA-Arg and HA-Orn withdrawn from their respective degradation solutions and the GlcA content was quantified using a calibration curve of the GlcA standard at different dilutions (2.3-120 μg /ml) and recording the absorbance value of the solutions at 523nm (Figure 14).

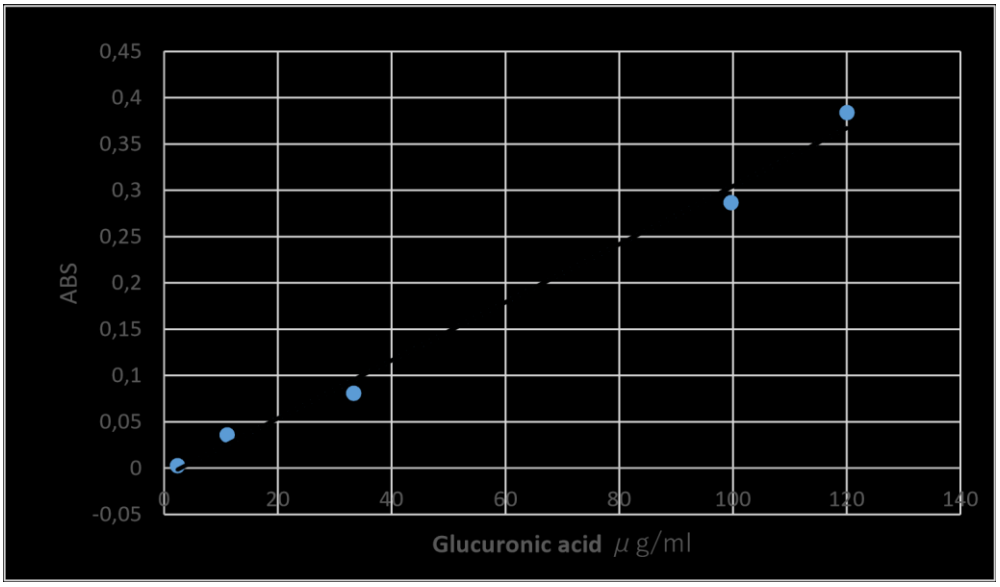


Figure 14.Example of calibration curve of Glucuronic acid standards.

The linear equation was obtained from the calibration curve with a correlation coefficient R^2 of 0.9920± 0.00395. The linear equation was employed to quantify the amounts of GlcA produced after one-day enzymatic digestion in the HA-Arg and HA-Orn hydrogels samples compared to native HA.

3.3.3 In vitro enzymatic degradation

In Figure 15, the degradation rate of native HA and cross-linked HA-Arg and HA-Orn hydrogels is reported.

As shown, the amount of glucuronic acid released from the native HA sample is greater than the cross-linked samples for each of the times tested. Its maximum value is reached at the time of 240 min (159.532 μg /ml) and then remained almost unchanged, which means that the glucuronic acid produced by the degradation of 15 mg of hyaluronic acid has reached its maximum value.

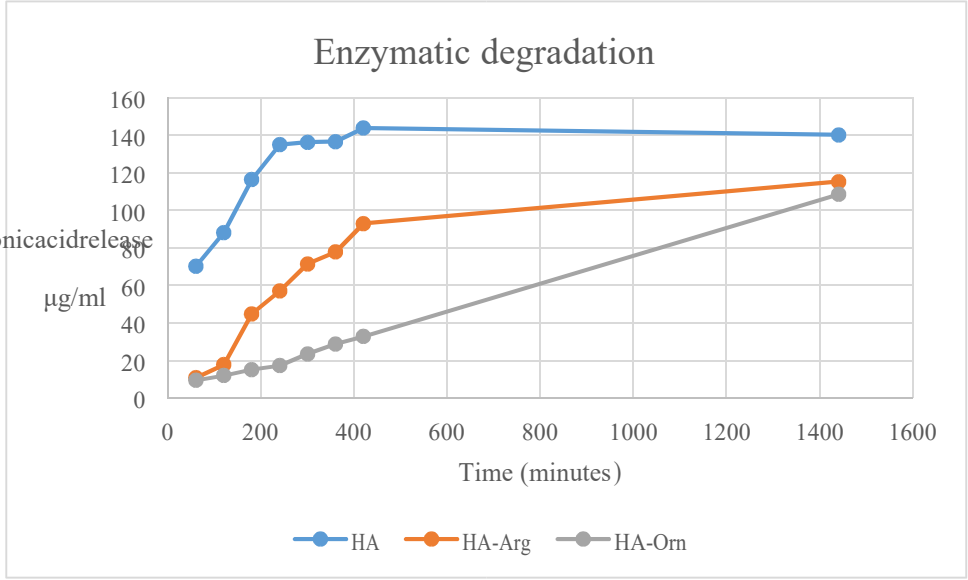


Figure 15. Glucuronic acid released from in vitro degradation of HA, HA-Arg and HA-Orn in PBS, pH 7.4 at 37 °C in the presence of 50U/ml of hyaluronidase. Each curve is the result of at least three different experiments.

A confirmation of the complete degradation of the sample is also found at the macroscopic level where a completely clear and homogeneous solution could be observed. On the other hand, complete degradation did not occur even after the longest time tested (24 h of incubation), for the two cross-linked hydrogels. In fact, both the cross-linked HA showed up to 24h visually detectable insoluble fractions with a released GlcA value of 125.1 µg/ml and 113.5 µg/ml for HA-Arg and HA-Orn respectively.

HA-Arg after 2 hours of degradation has released a GlcA content equal to 14% (18.51 µg /ml) compared to the final amount released while the native HA has released 57.2% (91.42 µg /ml) of the total content. As for HA-Orn, the GlcA content released at the same time is 15.9% compared to the content detected at 24h. The slower release of the two cross-linked is also sustained at a macroscopic level as it was possible to observe the swollen disc suspended inside the tube not yet degraded.

Moreover, the absence of the plateau effect suggest that the degradation process is not yet finished at 24 hours.

The higher degradation rate of HA-Arg compared to HA-Orn, might be due to the greater interactions between the highly swollen hydrogel and the enzymatic solution probably because the latter is able to more easily penetrate into the network due to the larger pore size, as evidenced by the morphological structure analyzed by SEM (3.2.6 section).

However, both hydrogels showed a slower release of GlcA than native HA and this can be attributed to the efficiency of the cross-linking process which stabilizes the HA chains and provides greater resistance to the polymer from enzymatic attacks.

This significantly reduced biodegradation rate as compared to unmodified HA is a prerequisite for the versatile future clinical applications of HA-Arg and HA-Orn in wound healing and tissue repair. Furthermore, these new polymers appear to be very promising materials for the development of effective delivery systems.

3.4 Application of arginine and ornithine cross-linked hyaluronic acid and sodium ascorbyl phosphate on the treatment of oxidative stress and anti-inflammatory lung diseases

Considering that hyaluronic acid is a natural anti-inflammatory and antioxidant, it represents a multifunctional agent for the treatment of certain lung diseases including airway diseases with a predominant inflammatory component such as rhinosinusitis, asthma, chronic obstructive pulmonary disease, cystic fibrosis and primary ciliary dyskinesia (Furnari et al., 2012; Garantziotis et al., 2016; Gavina et al., 2013, Petrigni & Allegra, 2006).

All these lung diseases are characterized by periodic or chronic inflammatory processes as result of releasing inflammatory cytokines from the cells such as IL-6 and IL-8. These inflammatory mediators result in an increase of ROS and hence an increase in oxidative stress in the lungs (Rahman & Adcock, 2006) that play a key role in the regulation of wound healing. For these reasons, to arrest the propagation of inflammatory cytokines and activation of the innate immunity system, we resort to the use of anti-inflammatory and antioxidant biomolecules such as vitamins, polyphenols and active plant compounds considered potential candidates for a new adjuvant therapeutic approach for the treatment of inflammatory lung diseases.

Among the different molecules present in nature, the use of vitamin C has been described as a potent antioxidant substance to treat lung disease as it is able to reduce oxidative stress (Larsson et al., 2015). It is also able to restore the function of the epithelial airway barrier, to regulate the immune system (Li & Li, 2016) and it plays therapeutic roles as reducing the acute pulmonary inflammatory response (Silva Bezerra et al., 2006).

Recently the use of polymers such as HA has also been reported in airway treatment because of its unique ability to retain water and regulate fluid balance in the lung interstitium. However, due to its anti-inflammatory properties and tissue repair properties, the integration of exogenous high molecular weight hyaluronic acid can provide important results in reducing inflammation and, consequently, disease symptoms (Máiz Carro & MartínezGarcía, 2020).

The use of HA, as a therapeutic agent for the treatment of airway pathologies, is interesting because it opens up the possible development of a new class of therapeutic agents resulting from the combination with molecules active for application in pulmonary therapy.

In this part of the study, safety and the biological activity were evaluated: specifically, HAArg and HA-Orn were studied alone and in combination with a stable vitamin C salt (SAP) with high antioxidant properties and able to promote the collagen synthesis. For this reason, an in vitro study was conducted on lung adenocarcinoma derived epithelial Calu-3 and H441, representative of both the upper and lower lung regions (bronchial and alveolar).

Additionally H441 and Calu-3 cells possess the characteristics of alveolar epithelial cells, including the high expression of tight junction proteins (Foster et al., 2000; Sakagami, 2006; Sakamoto et al., 2015; Togami et al., 2016). In particular, cytotoxicity was evaluated by MTS assay and epithelial barrier integrity, instead, anti-inflammatory and antioxidant properties were studied by measuring the levels of cytokines IL-6 and IL-8 and reactive oxygen species (ROS).

The aim of the study was to highlight the biological activity of cross-linked HA-Arg and HA-Orn alone and/or in combination with sodium ascorbyl phosphate (SAP). Finally, they were compared to the single and combinated HA for the potential use in treatment of inflammatory lung diseases.

3.4.1 MTS cytotoxicity assays

The cytotoxicity of free SAP and the polymers HA, HA-Arg, HA-Orn at different concentration, alone or in combination with SAP at a 1 : 3 ratio, was evaluated on Calu-3 and H441 cells via cell metabolic activity (MTS assay). As shown in Figure 16 (panel a and

b) no cytotoxicity occurs when cells were exposed to single polymers HA, HA-Arg and HAOrn at all concentrations assessed (0.09-0.3%) and to their combination (1 : 3) with SAP for both cell lines.

However, when SAP is in combination with HA, HA-Arg and HA-Orn, a significant increase in cell proliferation was observed for HA above 0.075% (w/v) and for both cross-linked HAArg and HA-Orn above 0.037% (w/v) on Calu-3 cells. In particular, when combined with HA-Orn, SAP increased cellular viability more prominently ($P<0.05$).

The same proliferation effect occurs on H441 cells for native HA in combination with SAP. However, when combined with both cross-linked HA (HA-Arg+SAP and HA-Orn+SAP) no significant increase in H441 proliferation was observed when compared with cell no treatment. It therefore emerged that between the two cell lines there is a difference in terms of viability: the effect of SAP on the H441 is less evident when combined with the two crosslinked, much more evident when combined with HA.

These differences could depend on the mechanism of action and the type of interaction, which can be different depending on the cell line. However, both HA-Arg and HA-Orn maintain a consistently improved cell viability profile at all concentrations tested compared to HA.

Overall, the results showed that all the combinations of hyaluronan - SAP were considered safe for Calu-3 epithelium and H441 even at the highest concentration tested.

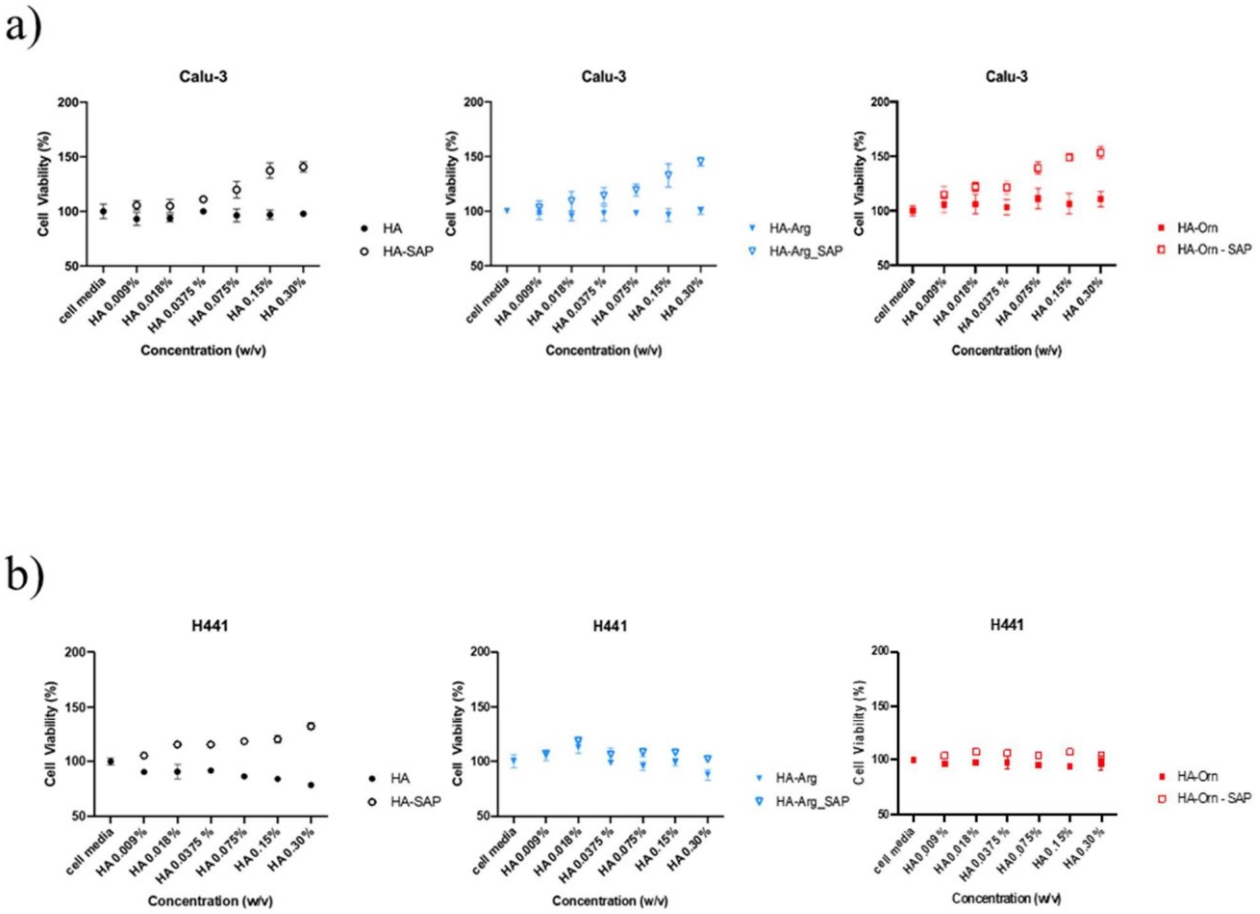


Figure 16. Viabilities of Calu-3(a) and H441 (b) cells evaluated using MTS assay after 24 h of treatment with sample solutions.

3.4.2 Evaluation of epithelial barrier integrity

The epithelial barrier integrity test was conducted as a secondary measure of the cytotoxic profile of samples, as tight junctions are proteins that are essential in the health of lung epithelia cells. To evaluate the influence of treatments, transepithelial cell permeability was performed using an ALI (Air Liquid Interface) set up by a sodium fluorescein (Na-Flu) as paracellular transporter marker: Na-Flu transport to the receiving chamber indicates loss of epithelial barrier integrity.

Na- Flu permeation test was performed on paracellular transport mechanism across epithelial Calu-3 and H441 cells as representative of both upper and lower lung regions (bronchial and alveolar). Figure 17 (panel a.) shows no significant difference ($P > 0.05$) for the Papp values calculated for Calu-3 cells treated with HA + SAP, HA-Arg + SAP, HA-Orn + SAP, SAP. Similar results were observed for the alveolar cell lines H441 (Figure 17 panel b.) with similar Na-Flu Papps across all treatments. This observation confirms the results obtained in the cytotoxicity MTS assay, where no toxic effect of the solutions was observed on the epithelial cells under the concentrations studied.

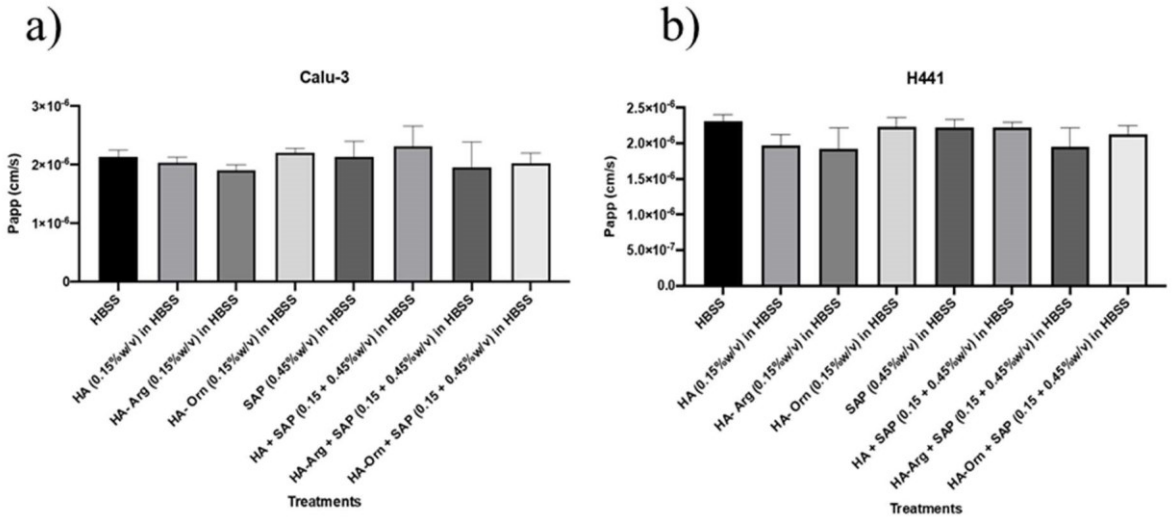


Figure 17. The paracellular permeation of Flu-Na across the calu 3(a) and H441 (b) cells after 4H treatment with 0.15–0.45% (w/v) hyaluronan-SAP, 0.45% (w/v) SAP, 0.15% (w/v) HA, HA-Arg, and HA-Orn solutions compared to cells incubated with HBSS vehicle alone. Experiments were conducted in triplicate.

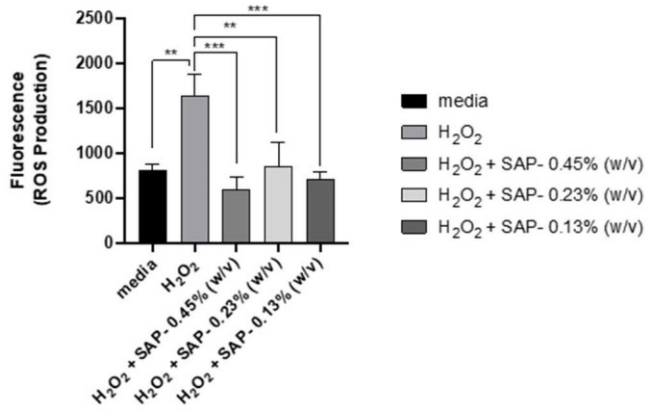
3.4.3 Antioxidant effect

Reactive oxygen species (ROS) play a fundamental role in regulating the normal wound healing response. When an imbalance between endogenous and exogenous pro-oxidant species occurs, caused for many different stimulus (i.e. dust, cigarette smoke, pollutants, viruses) an oxidative stress condition occurs with harmful effects to the tissue (MacNee, 2001).

To test the effect of formulations as potential antioxidants, intracellular ROS were quantified after the treatment of Calu-3 and H441 cells with hydrogen peroxide to lead to an oxidative state.

ROS levels of cells treated with 0.15–0.45% (w/v) HA + SAP, combination were compared with ROS levels detected in cells exposed to 0.45% (w/v) SAP, 0.15% (w/v) HA, HA-Arg and HA-Orn, cells not stimulated (negative control) and stimulated with H_2O_2 (positive control).

a)



b)

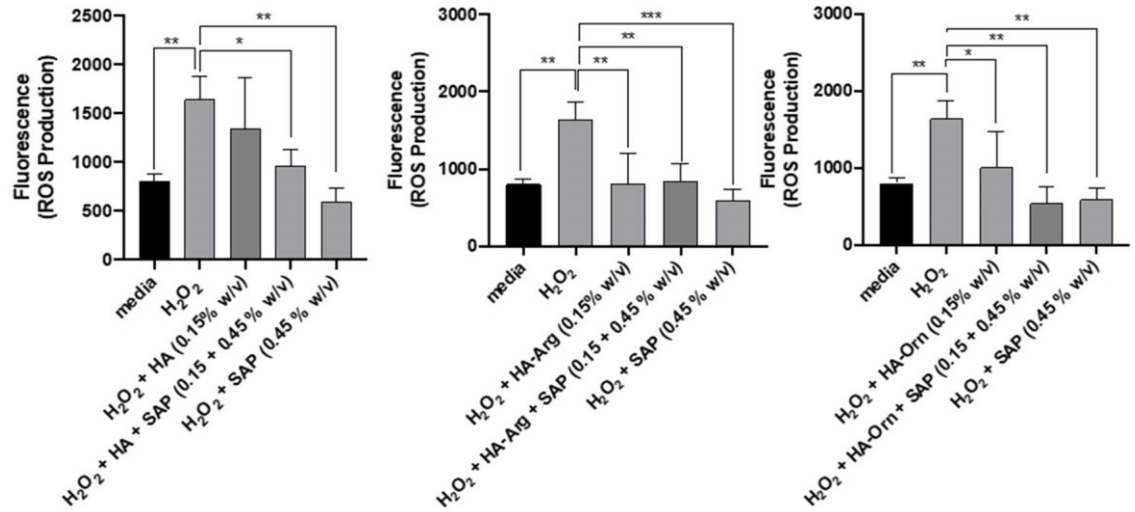


Figure 18. Oxidative effects of different concentration of SAP (a),0.15–0.45% (w/v) hyaluronan-SAP, 0.45% (w/v) SAP, 0.15% (w/ v) HA, 0.15% (w/v) HA-Arg and 0.15% (w/v) HA-Orn solutions, and controls (b)(untreated cells, 0.03% H_2O_2 on intracellular ROS production (%) in Calu-3 cells. Data represent mean ± standard deviation (n=3). Asterisks indicate significant differences from untreated cells control with *P < 0.05, **P < 0.01 and ***P < 0.001.

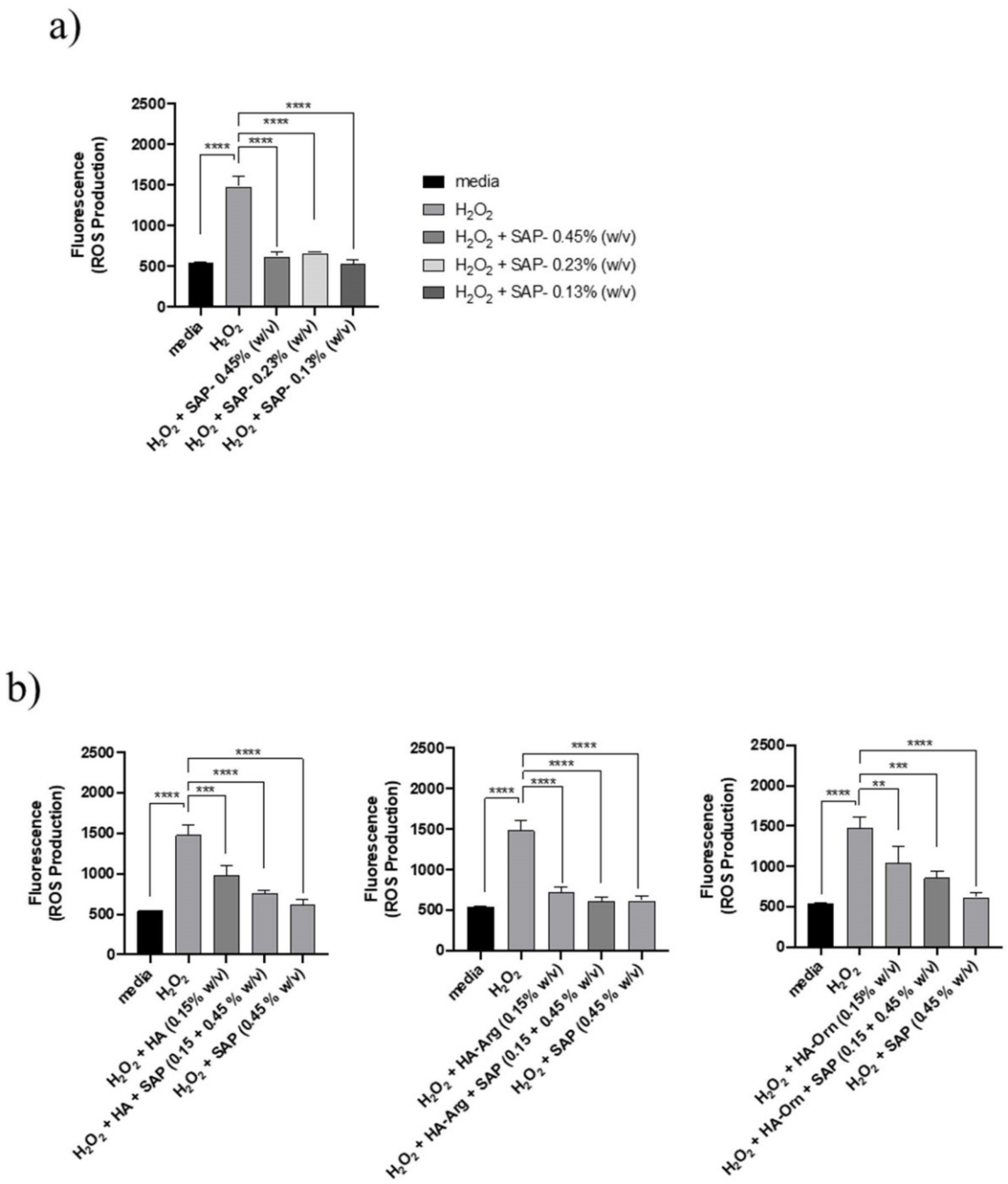


Figure 19. Oxidative effects of different concentration of SAP (a),0.15–0.45% (w/v) hyaluronan-SAP, 0.45% (w/v) SAP, 0.15% (w/ v) HA, 0.15% (w/v) HA-Arg and 0.15% (w/v) HA-Orn solutions, and controls (b). in H441cells Data represent mean ± standard deviation (n=3). Asterisks indicate significant differences from untreated cells control with *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.

As shown in Figure 18 and Figure 19, all treatments did not induce oxidative stress on Calu3 and H441 cells, on the contrary they reduced ROS production in 0.03% H_2O_2 stimulated cell. In particular, SAP significantly reduced ROS production on both stimulated Calu 3 and H441 cells (Figure 18 panel a. and Figure 19 panel a).

More precisely, in Calu-3 cells the combination HA-Orn + SAP showed the highest antioxidant effect with a significantly greater decrease in ROS release than those given by the association HA-Arg + SAP and HA + SAP as well as SAP free.
On H441 cells the best results were obtained by HA-Arg + SAP which showed an interesting antioxidant activity even if slightly lower than that of SAP alone.
However, treatment of the cells with the polymer alone showed greater antioxidant activity for HA-Arg than HA-Orn and HA in this order.

3.4.4 Pro-inflammatory markers expression

The expression of inflammatory cytokines such as IL-6 and IL-8 is a biological response influenced by many both genetic and exogenous factors and represent the principal mechanisms involved in the progression of many lung diseases (Kleniewska & Pawliczak, 2017; MacNee, 2001; Moldoveanu et al., 2009; Wouters et al., 2007).
On the basis of this information and considering our previously obtained data (Fallacara et al., 2018) the anti-inflammatory activities of HA cross-linked products were measured in vitro by the evaluation of IL-6 and IL-8 markers on Calu-3 and H441 cells, after 24h treatment with HA, HA-Arg and HA-Orn and their combination with SAP.
As can be seen in Figure 20 (panel a) HA-Arg with and without SAP significantly decreased ($P<0.05$) the IL-6 and IL-8 levels when compared to control on Calu-3 cells.
For HA-Orn there was an increase in IL-6 and IL-8 production compared to control even when associated with SAP. However, this data needs further confirmation.
Differently from the results observed on Calu-3 cells, on H441 cells (Figure 20 panel b), both HA-Arg and HA-Orn cross-linked polymers tested alone, as well as in combination with SAP, significantly reduced the IL-6 levels when compared to control.
The most prominent results were obtained by HA-Arg +SAP and HA-orn + SAP which consistently reduced IL 6 levels as compared to the untreated control.
Regarding IL-8 levels on H441 cells, HA and HA + SAp showed inductive effect as compared to the control. Wherease the most significative value was showed by the HA-Orn + SAP that

able to reduce the IL-8 levels significantly. HA-Arg, alone and in combination showed a promising margin of reduction of IL-8 levels.

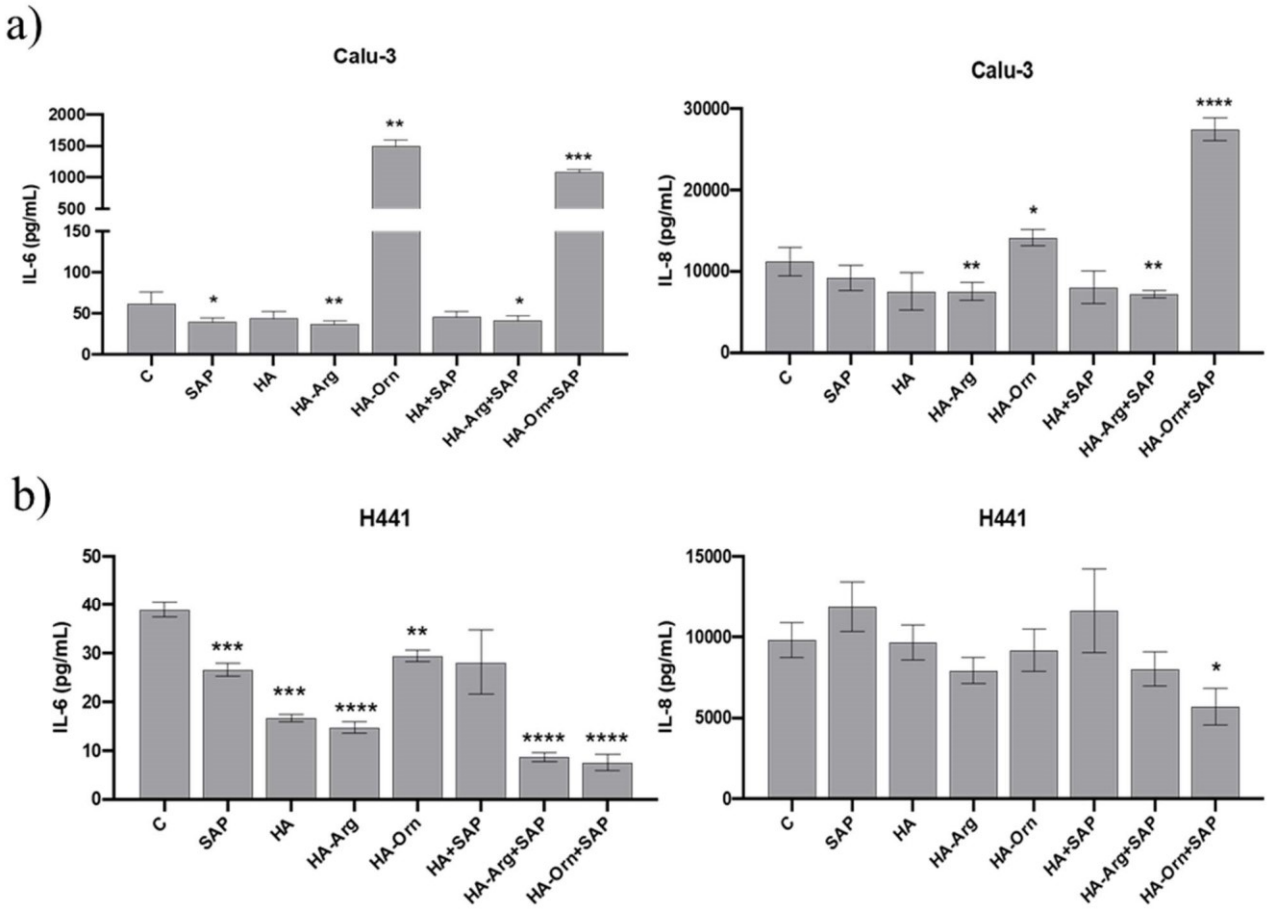


Figure 20. Concentration of IL-6 and IL-8 inflammatory cytokine in Calu-3(a) and H441(b) cells exposed for 24 h to 0.15+0.45% (w/v) HA+SAP, HA-Arg+SAP, HA_Orn+SAP, 0.45% (w/v) SAP, 0.15% (w/v) HA, HA-Arg and HA-Orn solutions, evaluated in comparison to untreated cells (control). Data represent mean ± standard deviation (n = 3). Asterisks indicate significant difference from untreated cells control (*P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001)

3.5 HA-Arg and HA-Orn based Cross-linked microspheres: preparation and evaluation of matrix for the controlled release of SAP

In the recent years, there has been considerable interest in the development of a controlled or sustained drug delivery system using polymeric microspheres (MSs).

The use of biodegradable and mucoadhesive polymers, for the formulation of delivery systems such as microsphere, offers several advantages including the prolongation of the release over time of the release of the drug contained (Abd El-Hameed & Kellaway, 1997; Esposito et al., 2005; Kulkarni et al., 2016) and a faster tissue repair process (Degim, 2008).

Hyaluronic acid is one of the most used materials to formulate controlled release systems thanks to its mucoadhesive characteristics, biocompatibility and biodegradability.

In particular, when cross-linked, it is able to form a three-dimensional network capable of swelling and modulating the release of the loaded active ingredient, such as antibacterial molecules (Luo et al., 2000) anti-inflammatory substances (Manconi et al., 2017) proteins and antibodies (Egbu et al., 2018).

Moreover, several studies show that HMW HA owns intrinsic therapeutic potential and results anti-inflammatory in mammals (Gomez-Gaete et al., 2008; Hwang et al., 2008; Allegra et al., 2012) as involves inflammation, oxidative stress and epithelial remodelling.

Therefore, a carrier composed of HMW HA cross-linked loaded with an active ingredient with anti-inflammatory activity represents a promising multifunctional system with intrinsic therapeutic potential for the treatment of inflammatory diseases.

Considering that inflammation and oxidative stress represent the principal mechanisms underlying the development and progression of many diseases, the use of anti-inflammatory and antioxidant compounds as adjunctive therapy could be a promising approach to restore and maintain normal viable functions.

Therefore, in this study, was investigated the use of the new cross-linked biopolymers HAArg and HA-Orn, in comparison to HA as new systems for the drug delivery of SAP, a pro-drug of vitamin C with improved physico-chemical stability.

Microspheres were prepared with a systematic study useful to achieve ideal conditions for the production of improved microspheres using the technique of emulsion with solvent

evaporation. The microparticles obtained were investigated on the morphological properties, drug loading, encapsulation efficiency and sustainability of the drug release in comparison to native HA microspheres.

3.5.1 Dynamic Vapour Sorption: DVS

At first, to characterize the polymers drugs stability and evaluate their effect on the MSs formulation, a study was conducted on HA, HA-Arg and HA-Orn under different humidity conditions (0-90% relative humidity, RH) by dynamic vapour sorption (DVS).

The resulting moisture sorption-desorption isotherms are shown in Figure 21 and confirm the typical trend of a hygroscopic HA-based polymer.

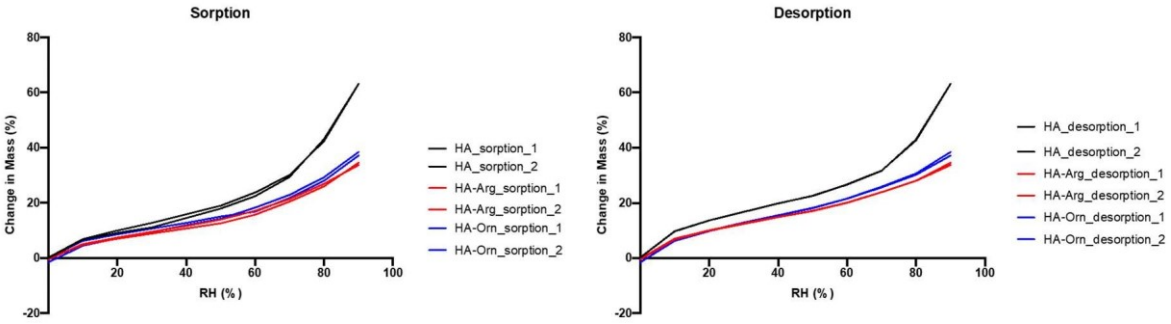


Figure 21. DVS isotherms of two cycles of moisture sorption and desorption of HA (black lines) and HA-Arg (red line) and HA-Orn (blue line).

The HA results are in agreement with what previously observed by Panagopoulou et al. (Panagopoulou et al., 2013); the water content linearly increased up to 29.3 % in response to RH increment from 0 to 70 %. Then, moisture uptake increased more rapidly in the RH range 70-90%, leading to a water content of 63.1%. This rapid increase of moisture uptake is likely due to its prominent humidity retaining capacity. Regarding the two HA crosslinked no drastic increments of the isotherm were observed but rather a slow and gradual increase for the whole range of RH humidity analysed. The first sorption curve of HA-Arg and HA-Orn from 0 to 70 %; were characterized by an increase of water content up to 20.5% and 21.8% respectively; whereas at 90% RH a final water content values of 34.5% and

37.1% was observed for HA-Arg and HA-Orn, respectively, demonstrating that this behaviour is in line with what was expected for the modification induced by cross-linking, i.e. that the cross-linking process leads to a reduction in the ability of the polymer to absorb water (Ghosh et al., 2005; Guilherme et al., 2005, Noh et al., 2006).

The HA desorption curves, as well as those of HA-Arg and HA-Orn, showed a very similar profile with respect to their absorption curve. Minimal hysteresis phenomena were observed which indicates that despite being moisture sensitive, this effect is reversible.

These results indicate that, notwithstanding the modification due to the cross-linking process, the HA-Arg and HA-Orn polymers maintained part of the water-retaining ability as HA and hence the related properties. At the same time the cross-linking process made them less susceptible in terms of response to variations of humidity, as observed by the lower water sorption compared to HA but without drastic altering between their sorption e desorbption isothermal moisture, that exclude permanent changes in the structure of the polymers. These observations support the suitability of HA-Arg and HA-Orn to behave as stable HA-based polymers for the production of a formulation that can be further exploited, due to its stability, in the field of drug delivery.

3.5.2 Thermal analysis:TGA

To characterize thermal stability of the drug (SAP) and polymers used to produce microspheres (HA, HA-Arg, HA-Orn) a Termogravimetric analysis using TGA was conducted.

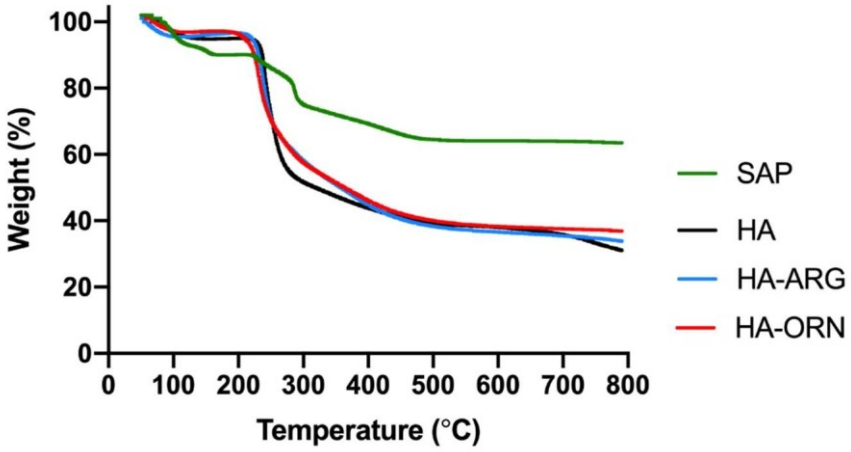


Figure 22. TGA thermograms of SAP, HA, HA-Arg and HA-Orn.

As shown in Figure 26, the weight percentage of SAP loss occurred in two stages: the first is between 30 and 225 °C with a decrease in weight of indicatively 11% w/w probably due to the moisture loss of the drug, and the second, from 225 °C to 800 °C, shows the drug decomposition with subsequent production of degradation products leading to a carbon residue of approximately 65% of the initial mass.

For the polymers HA, HA-Arg and HA-Orn there was a similar degradation pattern occurred with two main phases of weight-loss.

The first weight loss in the TGA curve of HA occurred from 30 to 238 ° C with a weight decrease of about 13% that can be attributed to dehydration, a process due to the highly hydrophilic nature of the HA, concomitantly to a possible degradation of some oligomers (Kantappa & Lee, 2017).

The second transition of 238–300°C was characterized by an intensive loss of mass up to 50% attributable to the decomposition of the backbone chains (Kafedjiiski et al., 2007). A comparable TGA profile was observed for the two cross-linked polymers characterized by a

weight loss of 5 % w/w of weight loss in the first region (30-220 °C) and 32% w/w in the second (220-300 °C) for HA-Arg while for HA-Orn it was 5% w/w in the first region (30209 °C) and 41% w/w in the second (209-296 °C).

Furthermore, HA showed a third-stage of weight loss between 300 to 800 °C , mainly due to the degradation of Na-hyaluronate into Na_2CO_3 (K. Kafedjiiski et al., 2007), which led to a carbon yield of 31 %. A third stage also occurred for HA-Arg and HA-Orn with carbon yields of 33% (300-800 °C) and 36 % w/ w (296-800 °C), respectively. Therefore, both cross-linked samples showed slightly higher thermal stability than the native HA.

3.5.3 Production of HA, HA-Arg and HA-Orn microspheres encapsulating or not SAP

The microspheres, wheter encapsulating SAP or not, were prepared using the emulsion solvent evaporation method adapted by Lim et al (Lim et al., 2000).

It is known that various parameters, correlated to the method or dependent on the starting polymeric solutions can influence the morphology of the microspheres and the yield of the process. To overcome this drawback, a systematic approach was carried out to understand the influence of the applied variables on the characteristics of the MSs produced in order to optimize the shape and surface of the particle (Table 3).

As a first approach, the influence of the different ratio between polymer and active was evaluated while maintaining the concentration of emulsifier constant. The second approach was to evaluate the effect of changing the concentration of emulsifier added to the oil phase. 1% polymer solutions were used for the production of MSs (HA and HA-Arg) with and without SAP. It was not possible to work at the same concentration with HA-Orn due to the viscosity of the solution at that percentage, which prevents dripping in the oil phase through the volumetric pump. Therefore, the MSs HA-Orn with and without SAP were prepared starting from a 0.5% solution.

Polymer	Polymer:SAP	Span 80 (%)	Morphology
HA	-	0.1	Several aggregates
		1	Spherical shape
HA-SAP	4:1	0.1	Agglomerates
		1	Spherical shape
	1:1	0.1	Agglomerates
		1	Irregular shape
HA-Arg	-	0.1	Agglomerates
		1	Spherical shape
HA-Arg-SAP	4:1	0.1	Agglomerates
		1	Spherical shape
	1:1	0.1	Collapsed
		1	Wrinkle spheres
HA-Orn*	-	0.1	Agglomerates
		1	Spherical shape
HA-Orn*-SAP	4:1	0.1	Several agglomerates
		1	Spherical shape
	1:1	0.1	Collapsed
		1	Agglomerates

Table 3. Variables of polymer: drug ratio and emulsifier concentration applied to produce MSs starting 1% polymer solution (* starting polymer solutions 0.5%).

Each of the microspheres obtained from this preliminary study was characterized by the SEM through which it was possible to highlight the morphological aspect. The best results, in morphological terms, were obtained with a low active content (polymer: active ratio 4: 1)

and a higher emulsifier content (1%). These conditions have been shown to be ideal for all polymers, leading to smooth surface spherical particles also for both HA-Arg and HA-Orn.

This is probably due to the fact that the use of the lowest emulsifier concentration (0.1%, w / w) and high concentration on active ingredient (ratio polymer : active equal to 1 : 1) induce an increase in size drops in the dripping phase that tend to merge together.

In fact, the presence of an active compound in the solution may also affect the viscosity of the solution to be emulsified in an unpredictable manner. In particular, the addition of a high concentration of SAP in the solution significantly increases its pH with a consequent lowering of the total viscosity of the solution. The combination of these two factors led to the recovery of collapsed particles. In Figure 22, is reported an example of HA MSs obtained with a spherical shape but agglomerates and an example of collapsed HA MS.

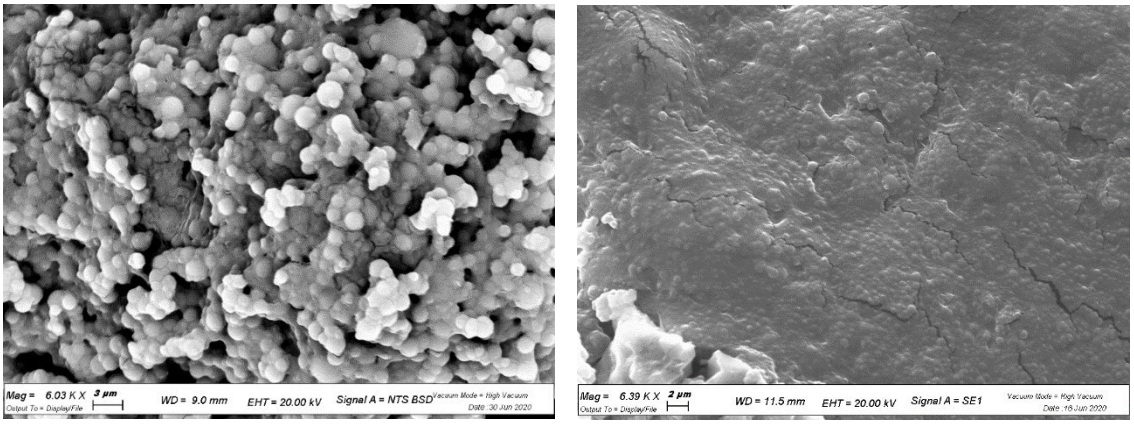


Figure 23. Example of and collapsed microspheres.

Taking into account the results of this pre-formulation study, aqueous solutions of polymer and drug in a 4: 1 ratio and containing 1% oil phase emulsifier were selected as the standard condition to produce optimized MSs which were used for subsequent studies.

3.5.4 Characterization of the surface morphology of HA microspheres

The microspheres produced under optimized conditions were then studied by scanning electron microscopy (SEM) and a dimensional range was provided and shown in Figure 23. From a morphological point of view, all the microspheres showed a spherical regular shape and a smooth surface. No free or incorporated drug crystals were observed on their surface. In some points, a slight aggregation between the single particles could be observed probably due to the centrifugal separation process providen by the preparation procedure.

The SEM analysis consented a characterization of the dimensional range (images not shown): it emerged that the size of the microspheres is influenced by the type of polymer used. In fact, unloaded HA MSs are small in size, in the range of 1.678 μm and 2.445 μm, as well as for HA-Arg with a size between 1.470 μm and 1.642 μm.

Whereas, the particle sizes of MSs HA-Orn are significantly larger with values ranging from 4.178 μm to 5.865 μm. This could be attributable to the different molecular weight of the polymers, a parameter whose value is directly correlated to an increase in the viscosity and viscoelasticity of the solutions (Falcone, 2006; Ré et al., 2004). These conditions are generally related to the production of larger droplets which increase the formation of larger microspheres (Esposito et al., 2005). However, also the drug (SAP) loaded MSs results in further increase of particle size as previously found in other studies (Esposito et al., 2001; Cortesi et al., 2002), showing higher dimensional range : 2.340 μm -5.998 μm for MSs HASAP, 1.445 μm - 4.479 μm for HA-Arg-SAP and 4.198 μm -11.00 μm for HA-Orn-SAP. This behaviour can possibly be related to the influence of SAP on the properties of solutions, wich, as previously mentioned, increasing of pH value. This condition, as reported elsewhere (Gatej et al., 2005; Maleki et al., 2008) reduces the viscosity of the final solution that and causes an increase in droplet size within w/o emulsion precursors and therefore the resulting microspheres.

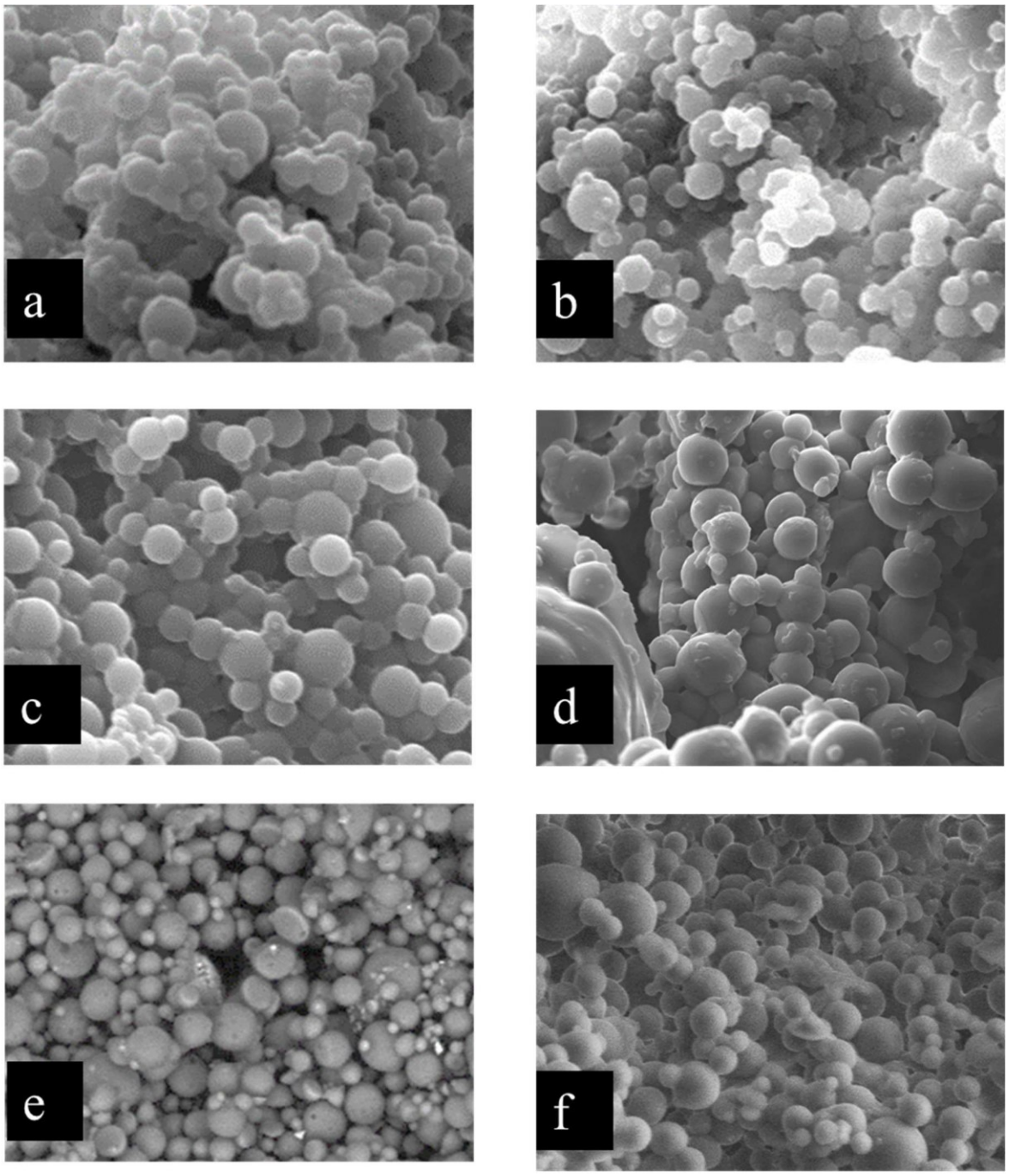


Figure 24. SEM micrographs of hyaluronan MSs optimized after metallization: HA (a), HA - SAP (b), HA-Arg (c), HA-Arg– SAP (d), HA-Orn (e) and HA-Orn-SA P (f).

3.5.5 IR

To understand if an interaction between polymer and active ingredient had occurred inside the microspheres and to highlight the stability of the cross-linked polymers subjected to the preparation process, an IR analysis was conducted.

Figures 24 and 25 show the spectrum obtained by infrared spectroscopy with Fourier transform (FT-IR) of the MSs formulations loaded with SAP or not.

SAP was characterized by the broader peaks of OH group at 3506.91 cm^{-1} and 3155.50 cm^{1}, C=O functional group stretching vibration at 1720.07 cm^{-1}, C=C stretching vibration at 1585.66 cm^{-1}, C-H stretching at 1413.88 cm^{-1}, C-O-C stretching vibration at 1112.19 cm^{-1} and multiple phosphate group vibration in the region between 662 cm^{-1} and 1062 cm^{-1} (Khan et al., 2016). Unloaded HA, HA-Arg and HA-Orn MSs showed all the distinctive peaks of the raw material used for their formulation, maintaining the initial IR profile unchanged (see chapter 1 section IR 3.2.2.2).

Specifically, the characteristic peaks of HA's MSs appear at 1607 cm^{-1}, 1405 cm^{-1} and 1034 cm^{-1} corresponding respectively to double peaks of the C=O stretching of the carboxylate anionic groups (-COO-) and to the C-OH stretching.

While in the MSs produced with cross-linked HA-Arg and HA-Orn, it was possible to identify the most important characteristic peak of the amide bond at about 1640 cm^{-1} that derived from the carboxylic groups. Furthermore, in the loaded HA-Arg and HA-Orn MSs, it was possible to verify that the peak due to O–H stretching vibration appears at higher energy values, indicating that both intra- and inter-molecular hydrogen bonds in the HA molecules decreased after SAP encapsulation.

The IR profile of all types of loaded MSs produced the maintenance of the bands belonging to raw polymer used: these were the main visible bands for the microparticles, since the polymer was the more abundant component. Although no SAP–related bands are detectable from the IR spectrum due to the reduced presence relative to the polymer and the complete signal overlap in the microsphere, the absence of new bands indicates that there was no new bond between the polymer and SAP. Therefore, it can be said that the employed did not affect on the stability of the polymer and on its degradation.

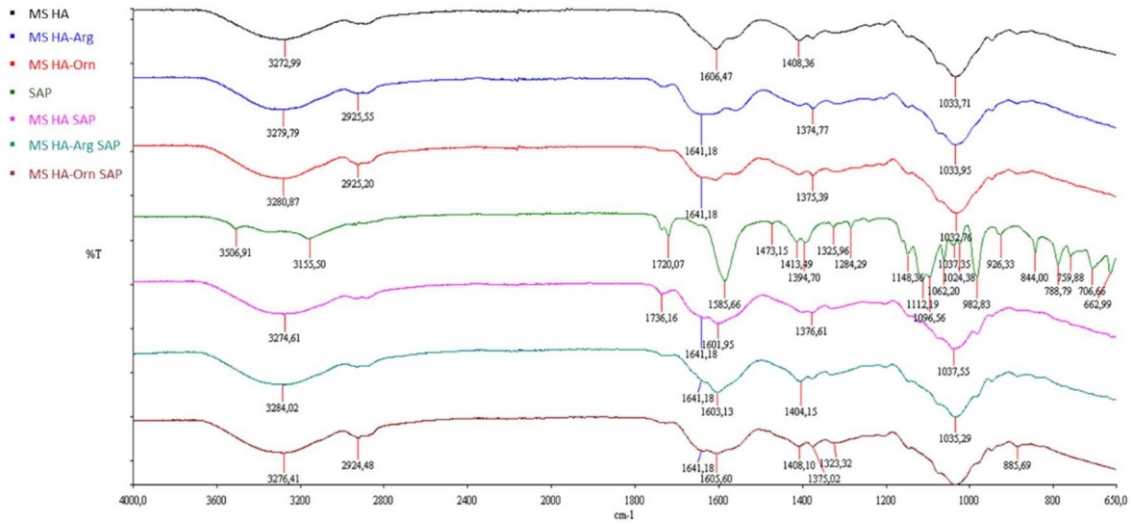


Figure 25. FT-IR spectra of MSs of HA, HA-Arg, HA-Orn samples, empty and loaded.

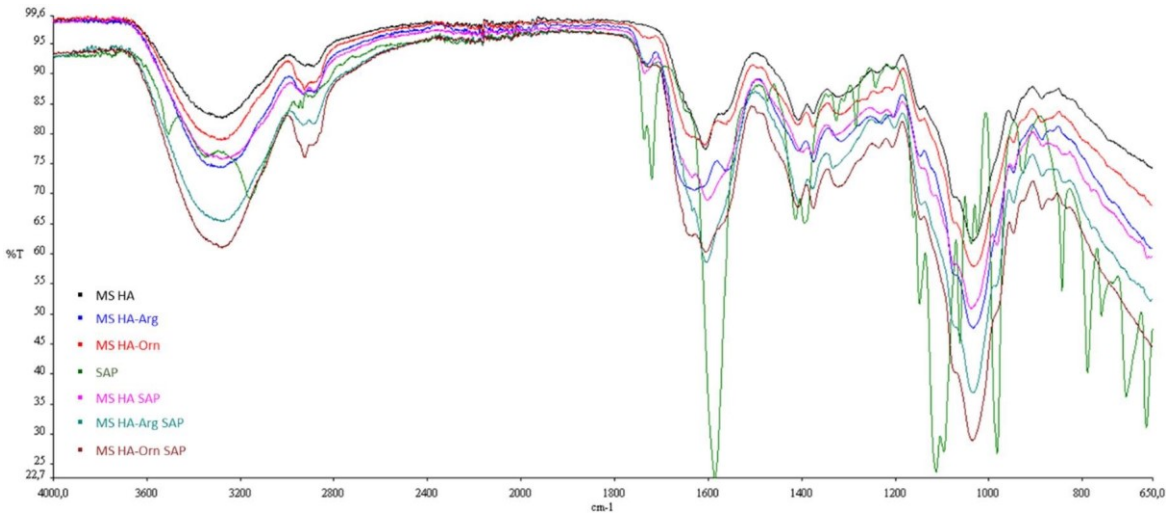


Figure 26. Overlapped FT-IR spectra of MSs of HA, HA-Arg, HA-Orn samples, empty and loaded.

3.5.6 MSs yield, drug loading and encapsulation efficiency

The effects of the HA type (native or cross-linked) and the presence of SAP on yield, drug load and encapsulation efficacy of the obtained microspheres are shown in Table 4.
The highest yield % was obtained for HA MSs (94.6 ± 6.3 %) and HA-Orn-SAP (94.00 ± 1.6%), while comparable values were observed for HA-SAP (75.61 ± 1.5%) and HA-Orn (75.4 ± 2.8%).

A lower particle recovery (Y%) occurred for HA-Arg and HA-Arg SAP with 57.9 ± 2.7 and 60.52 ± 3.5 respectively, probably due to the bioadhesivity of the polymers which led to the loss of a part of the formulation on the homogenizer head.

Polymer	Solution %	SAP	Span 85(%)	Y%	DL%	EE%
HA	1	-	1	94.6 ± 6.3	-	-
HA SAP	1	4:1	1	75.61 ± 1.5	15.6 ± 0,9	60.04 ± 1.89
HA-Arg	1	-	1	57.9± 2.7	-	-
HA-Arg SAP	1	4.1	1	60.52± 3.5	22.03± 1.4	64.5±3.67
HA-Orn	0.5	-	1	75.4±2.8	-	-
HA-Orn SAP	0.5	4:1	1	94.0± 1.6	11.6±2.5	43.5±2.32

Table 4. Effect of polymer type and SAP presence on yiels, drug loading and encapsulation efficacy of MSs producted (n = 3, ± SD).

DL % and EE% were acceptable for HA-SAP MSs (15.6 ± 0.9% and 60.04 ± 1.89%, respectively) and for HA-Arg SAP MSs (22.03 ± 1.4% and 64.5 ± 3.67%, respectively). As regard HA-Orn SAP MSs, significantly lower percentages were observed (11.6 ± 2.5% and 43.5 ± 2.32% respectively. These percentage values were probably related to the higher molecular weight of the polymer as well as to its molecular structure characterized by cages smaller than HA-Arg which could avoid the complete dispersion, in the preparation phase of the aqueous solution. However, this drawback could be overcome by increasing the dissolution time of SAP in the polymer solution to obtain an optimal dispersion before

dripping step or alternatively by dispersing the polymer in a solvent which can provide complete relaxation of the chain before the introduction of the drug.

3.5.7 In vitro drug release: Dialysis

Evaluation of drug release from biodegradable polymeric microparticles is essential for testing the safety and stability of the microspheres (Weng et al., 2020) although it requires long-term studies under in vivo or in vitro conditions. As a preliminary study, in order to investigate the potential characteristics of the microspheres produced as carrier, an in vitro drug release analysis was conduct.

In particular, the test performed with the dialysis method, a widely employed delivery technique in which the microspheres are retained in a membrane having a molecular weight cut-off (MWCO) smaller than the size of microspheres and greater than the drug molecule (SAP: 322.05 g/mol), placed in an external receiving medium.

However, the drug concentration in the receiver compartment may not timely reflect the actual free drug concentration released by the microparticles as the drug must first diffuse from the matrix to the compartment medium within the membrane and then diffuse to the external medium, inevitably delaying the real times of diffusion.

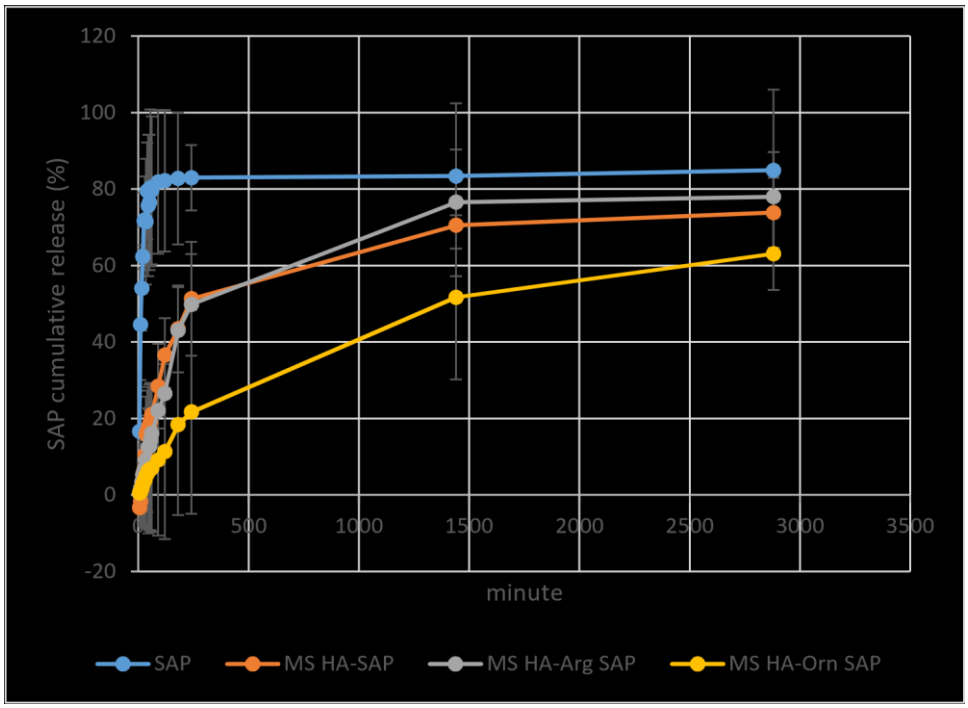


Figure 27. Cumulative in vitro SAP release profiles of MSs HA – SAP (orange curve), MSs HA - Arg - SAP (grey curve) and MSs HA – Orn – SAP (yellow curve) in comparison to free SAP (blue curve), using dialysis method. Error bars represent standard deviation (n=3).

As shown in Figure 27 the free SAP release profile appeared faster than the SAP release from MSs and within 90 minutes it was fully released into medium.

For the loaded microspheres, the SAP release rate at the same time (90 min) was 28 ± 2.4%, 22 ± 2.4% and 9 ± 1.1% for HA, HA-Arg and HA-Orn respectively. No burst effect was observed, therefore a homogeneous deep dispersion of SAP in the microsphere without surface deposits can be assumed. The lowest drug release occurred from the HA-Orn MSs which showed a significantly slower trend of the other MSs. At the longest time analyzed (48h) the release rate of SAP from HA- SAP MSs, as well as HA-Arg was completed while HA-Orn-SAP MSs were still being released. This can easily be related to the higher molecular weight and the implemented mechanical strength of the HA-Orn polymer as could be expected from the molecular weight studies and rheological analyses conducted previously which results in a better and longer entrapment period of the drug during the MSs formulation.

Delayed release is important because it contributes to a prolonged therapeutic effect, with a consequent decrease in the frequency of administration, better patient compliance and reduced cost.

This performance HA-Orn-SAP MSs could be attributed to a release controlled not only by the drug diffusion but also by reduced degree of polymer dissolution and swelling compared to HA –SAP MSs.

Chapter 4

4 EXPERIMENTAL SECTION

4.1 General Materials and Methods

Sodium salt Hyaluronate (HA) isolated from Streptococcus equi with average MW 1.2 MDa was purchased from Caldic (Origgio, Varese, Italy).

Arginine methyl ester (H-Arg-OMe · 2HCl) and Ornithine methyl ester (H-Orn-OMe · 2HCl) were purchased from Bachem. 2-chloro-1-methylpyridinium N-methylmorpholine (NMM), NaCl and acetonitrile were purchased from Sigma–Aldrich. 2-chloro-dimethoxy-1,3,5triazine (CDMT) purchased from TCI. The phosphate buffer saline (PBS) of different pH value was purchased from sigma Aldrich and adjusted with HCl or NaOH 0.5 M prepared in the laboratory. Sodium ascorbyl phosphate (SAP) was purchased from DSM Nutritional Products Ltd. (Segrate, Milano, Italy). Sorbitan monooleate (Span 80®) was provided by Acef (Fiorenzuola D'Arda, Piacenza, Italy).

The resulting products were purified by extensive dialysis within a dialysis membrane tube cut-off 3,5 KDa purchased from Termo Fisher Scientific Italia.

Lyophilization was performed using aa Lio-5P lyophilizer during overnight.

A physical mixture of Arginine methyl ester and ornithine methyl ester with HA were used to compare the chemical shift difference with the corresponding covalent bonding product.

4.2 Chemistry

4.2.1 Synthetic Procedures

A first series of experiments was carried out using EDC/NHS as condensing agent in a stoichiometric ratio 1:1 between the amount of EDC/NHS and the repeating units of HA.

Under these conditions, the use of EDC/NHS led to a degree of substitution that was not detectable by NMR. Therefore, a new synthesis set was performed using a molar excess (4:1), but also with poorly detectable NMR results. It was decided to continue the study

using the initial stoichiometric ratio of 1:1 with the repeating units of HA, which was already sufficient to give significant substitution, but using CDMT/NMM. However, an increasing scale up study has been undertaken to provide the best degree of substitution without overuse for both the cross-linking agent and amino acid.
The synthetic procedures referring to the use of CDMT / NMM in molar excess 3: 1 with HA, and in stoichiometric ratio 2: 1 with amino acids, have been reported below.

4.2.1.1 Synthesis of cross-linked HA-Arg

264 mg of HA sodium salt (0.66 mmol of carboxylate group) were uniformly dissolved in 52 ml of deionized water before the addition of 35 ml of acetonitrile. The homogeneous solution was cooled in an ice bath for 0.5 hours and 347.5 mg of CDMT (1.98 mmol) were then added. One hour later, 258.5 mg of Arg-OMe· 2 HCl (0.99 mmol) were added. The pH value was adjusted at 6.5 with NaOH 0.5M and then 217.8 μL of NMM (1.98 mmol) were added and the reaction was allowed to proceed overnight under constant stirring (about 400 rpm). The Ha-Arg product was then transferred into a dialysis membrane and purified by dialysis against H_2O for 24h, against aqueous NaCl (0.1 M) for 2 days and finally against water for 2 days and lyophilized to give HA-Arg as white spongy in yield 76% (290 mg).

HA-Arg :^{1}H NMR (400 MHz, D_2O) δ ppm: 4.40 (m, 3H, O–CH–O; – CH α), 3.95-3.10 (m, 15H, C–CH–O; –OCH_3), 3.107 (m, 2H, CH_2δ), 1.97 (s, 3H, CO–CH_3–N), 1.80–1.43(m, 4H, C–CH_3–CO and –CH_2).

4.2.1.2 Synthesis of cross-linked HA-Orn

264 mg of HA sodium salt (0.66 mmol of carboxylate group) were uniformly dissolved in 52 ml of deionized water before the addition of 35 ml of acetonitrile. The homogeneous solution was cooled in an ice bath for 0.5 hours and 347.5 mg of CDMT (1.98 mmol) were added. One hour later, 261.9 mg of Orn-OMe· 2 HCl (0.99 mmol) were added. The pH value was adjusted at4 with HCl 0.5M and then 217.8 μL of NMM (1.98 mmol) were added and the reaction was allowed to proceed overnight under constant stirring (about 400 rpm). The HA-Orn product was then purified by dialysis against H_2O for 24h, against aqueous NaCl

(0.1 M) for 2 days and finally against water for 2 days and lyophilized to give HA-Orn as white spongy in yield 73% (280 mg).

HA-Orn: ^{1}H NMR (400 MHz, D_2O) δ ppm: 4.43 (m, 3H, O–CH–O;– CH α), 3.97-3.09 (m, 15H, C–CH–O;–OCH_3), 2.92 (m, 2H, CH_2δ), 1.97 (s, 3H, CO–CH_3–N), 1.80–1.56 (m, 4H, C–CH_3–CO and –CH_2).

4.2.2 Molecular characterization

4.2.2.1 Spectroscopic analysis

For ^{1}H-NMR spectroscopy, the samples were dissolved in D_2O, at a concentration of 5–8 mg/ mL and spectra were registered with Varian spectrometers and Mercury Plus-400 at 400 MHz at room temperature (average of 64 scans). Chemical shifts are measured with respect to residual solvent peaks as an internal standard set to D_2O and expressed in parts per million (ppm).
UV spectrophotometric analyses were carried out on a UV-31 SCAN ONDA (Giorgio Bormac S.r.l. Carpi (MO) Italy); FTIR measurements were performed on dry samples (MSs) using a FTIR spectrophotometer PerkinElmer Spectrum 100 with ATR method (attenuated total reflectance) scanned from 4000 to 650 cm-1, at room temperature.

The samples were placed directly on the crystal and FTIR spectra were obtained after 8 scans with a resolution of 1 cm^{-1}. Background spectra were subtracted from the sample ones.

4.2.2.2 Ninhydrin test

HA-Arg and HA-Orn solutions prepared in deionized water (5-6 mg/ml) were spotted on TLC Chromatography plates by graduated capillary tubes, dried, sprayed with Ninhydrin solution (0.25g of Ninhydrin in 12.5 ml of Ethanol 95%) and then heated at 110 °C for 5

min. As expected, a yellow spot were visually observed indicative of the presence of secondary amino groups (Sinhababu & Basu, 2013) (Basak, et al., 2005).

4.2.2.3 Differential Scanning Calorimetry (DSC)

To study how the chemical modification could influence the interactions between the polymer chains and the water content, a thermoanalytical tests were carried out on lyophilizates of HA, HA-Arg and HA-Orn. The heat transition within the HA solutions was determinated by a differential scanning calorimeter (DSC) (DSC60A, calibrated with a pure indium standard). Amount of 2 to 6 mg of samples were placed in hermetically sealed aluminum pans and heated from 25°C to 300°C at 10°C /min. Measurements were performed in an inert nitrogen atmosphere purged at a flow rate of 45.0 mL/min.
The endothermic and exothermic peaks were elaborated using TA-60 WS system software (Shimazu corporation Kyoto, Japan).

4.2.2.4 SEM morphological analysis

The morphology of freeze-dried samples of HA and HA, HA-Arg, HA-Orn was observed using a field emission scanning electron microscope (Zeiss EVO 40XVP, Carl Zeiss Pty Ltd., Oberkochen, Germany), with a voltage of acceleration of 20 kV. The freeze-dried powder samples were deposited on carbon sticky tabs, analyzed in variable pressure mode and scanned and photographed randomly.

4.2.2.5 Swelling Measurement

The swelling capacity of HA-Arg and HA-Orn, (Sw), has been defined as the ratio between the weight of the inflated gels (Ws) after extensive dialysis against aqueous media at different pH values and the weight of the dry nets (Wd). In particular, 100 mg of dry lyophilized samples were exactly weighed before to be put in dialysis tube against 300 ml of PBS at pH values equal to 4.5, 6.5 and 9 value, and deionized H_2O at 25°C. The weight of the each swollen samples after 24 hours was weighted to calculate Sw by applying the following equation:

$$\% SD = W_s/W_d$$ Equation 2

4.2.2.6 Rheological measurement

The rheological analyses have been performed with the rheometer Physica MCR-101 from Anton-Paar. The instrument was equipped with a PP50-P2 parallel plate geometry with serrated surfaces and the gap between the surfaces of the plates was fixed at 1 mm. Each sample was loaded between the rheometer plates and the material in excess was wiped off with a spatula. A Peltier system ensured the maintenance of a controlled temperature of 23 ± 0,05°C. Before the measurements, all samples were allowed to rest at the initial temperature for 1 min. The rheological tests were conducted in triplicate.

A Controlled Shear Rate (CSR) test was conducted in continuous flow conditions, recording the viscosity (η) values of the samples at increasing shear rates ranging from 0.001 to 1000 s^{-1}. The flow curves obtained were fitted with the Carreau-Yasuda mathematical model (Equation 3), that well describes the shear-thinning behaviour and allows calculating the zero-shear viscosity (η_0) i.e.: the material's viscosity at rest (Yasuda et al., 1981).

•

$$\square = \square\infty\square + \blacksquare \quad (\square 0 - \square\infty)\square \cdot$$

Equation 3

The rheological tests in oscillatory flow conditions were conducted to highlight the viscoelastic behaviour of the samples, measuring the trends of the storage (G') and the loss (G'') modules (Tafuro et al., 2020). An Amplitude Sweep (AS) test was performed at a fixed value of frequency (1 Hz) and varying the strain (γ) from 0.01 to 1000%. This test allowed identifying the Linear Viscoelastic Region (LVR) i.e.: the strain region in which the moduli values remain stable and the sample does not undergo irreversible changes. A Frequency Sweep (FS) test was conducted at a fixed value of strain, taken from the LVR previously identified, and increasing the values of frequency in a range that went from 10 to 0.01 Hz. This test was performed to study the materials' inner structure and physical stability (Tadros, 2004). The damping factor (tan δ), calculated from the ratio between the loss modulus G″

and the storage modulus G′, was used as an indication to describe which of the solid-elastic and liquid-viscous components is the preponderant one.

4.2.2.7 Molecular weight measurement by GPC

The Mw evaluation was performed on HA and cross-linked HA-Arg and HA-Orn by size exclusion chromatography SEC-MALS (Size Exclusion Chromatography - Multi Angle Laser Scattering detector using Malvern GPC Max with Malvern TDA detector and Malvern Viscotek Omnisec chromatographic software. The instrument was equipped with two columns (7.8 mm ID × 30 cm, 13 µm TSKgel® GMPWXL, separation range ~1,000 to 5 × 106 g/mol, PN 0008025) with pre-column manteined at 50°C. The samples (about 8 mg) were diluted in 40 ml the mobile phase (0.1M $NaNO_3$ + 0.02% sodium azide) to obtain a concentration of about 0.2% and stirred for 48h. They were then filtered on Millipore MillexGV Hydrophilic PVDF 0.22 µm 0.02% and 200µl injected at flow rate of 1 mL/min into a chromatographic system calibrated with pullulan dn/dc 0.16 (dn/dc sample 0.160).

4.2.2.8 Measurements of Water Content of the materials

The relative moisture sorption of HA, HA-Arg, and HA-Orn, after exposure to different humidity levels (0-90% RH) was analysed by Dynamic Vapour Sorption (DVS) (DVS-1, Surface Measurement Systems Ltd., London, UK). Samples were added to aluminium sample pans and placed in DVS sample chamber. Each sample was exposed to two cycles of 0-90% relative humidity (25°C) in increments of 10% RH increments. The equilibrium

moisture content in each humidity phase was determined as a change in the ratio of mass to time (dm/dt) of 0.0005% min^{-1}.

4.2.2.9 Thermo gravimetric analysis

The thermal stability of the raw materials was evaluated using a Mettler Toledo instrument (Thermogravimetric analyzer, TGA/DSC 1 STARe system). Dry samples (5-10 mg) were placed on alumina crucibles (70 µL) and analyzed in the temperature range from 30 to 800 °C, at a heating rate of 10 °C/min, under nitrogen atmosphere.

4.3 Enzymatic degradation test with hyaluronidase

4.3.1 Preparation of the samples

Linear HA and HA-Arg and HA-Orn cross-linked were prepared for the enzymatic degradation test by forming solid disks approximately 1 mm thick. In particular, the samples were first diluted to 1.5% in distilled water until a homogeneous solution was obtained, then 1 ml of each solution was poured into preformed spherical molds with a diameter of 10 mm and lyophilized. The dehydrated samples were then pressed into the mold that housed them to form dense discs weighing 15 mg.

4.3.2 In vitro degradation

In vitro tests were performed to evaluate the degradation rate of HA-Arg and HA-Orn crosslinked compared to native HA.

A stock solution of hyaluronidase was prepared from a starting solution of 500 Units/mL of HAase in PBS. This solution was diluted to a concentration of 50 Units/ml and the samples were inserted and kept at 37° C for the different test times to allow the release of glucuronic acid at the different incubation times. In order to verify the absence of degradation

phenomena due to temperature, a degradation control was carried out on each sample test without HAse at the same temperature.

4.3.3 The Carbazole assay

Briefly, 200 µl of collected supernatant was added to 3 ml of 0.025M sodium tetraborate (Sigma) in sulfuric acid in the tubes. The samples were vortexed and then boiled for 10 min at 100° C. After cooling, 100 µl of 0.0125% Carbazole reagent in EtOH are added and boiled again for 15 min so that the reaction with the carbazole takes place and the purple colour develops with an intensity proportional to the GlcA content in each sample. The GlcA content after degradation was then quantified by UV-Visible spectrophotometer and the absorbance was recorded at 523 nm.
A blank control was prepared with only phosphate buffer and carbazole reagent to confirm that no purplish red products are detected in the absence of GlcA.
The degradation was quantified as the increase in the fraction of soluble sample in the medium in which it is placed.

4.4 HA, HA-Arg and HA-Orn microspheres loaded or not with SAP

4.4.1 Production of MSs

Microspheres of HA, HA-Arg and HA-Orn, with and without SAP loading, were prepared by solvent evaporation technique of a water-in-oil (w/o) emulsion:
Acqueous polymer solutions were prepared by dissolving 100 mg or 50 mg of polymer in 10 ml distilled water, to obtain 1% or 0.5% solution, respectively, by continuous magnetic stirring (250 rpm) at room temperature until it became a transparent solution. Likewise, for SAP loaded microspheres 100 mg, 50 mg, 25 mg or 12.5 mg di SAP were added at in the

polymer solution to form polymer solution containing drug (aqueous phase) at different ratio. The polymeric solution prepared with and without Sap was then added dropwise using peristaltic pump (MASTERFLEX C/L mod.77122-24 flow rate: 0,91 mL/min) into 100 g of mineral oil containing 1% (w/w) or 0.1% of sorbitan monooleate as oil phase emulsifying agent under moderate magnetic stirring (400 rpm).

The polymer solutions thus obtained were then added dropwise (flow rate: 0.91 mL/min) into 100 g of mineral oil and then emulsified at 1000 rpm for 30 minute, using a Silverson L5M-A Laboratory Mixer (Silverson Machines, Buckinghamshire, United Kingdom), with an appropriate fine emulsor screen workhead.

The aqueous phase dispersed in the emulsion was completely evaporated by maintaining the system under moderate agitation at a temperature of 37 ± 2°C overnight.

The microparticles suspended in the oil phase were then separated by centrifugation at 4000 rpm for 30 min (Centrifuge Neya 10R, Giorgio Bomarc, Carpi (MO) – Italy) the pellets were then washed with hexane until traces of oil were removed and then centrifuged again.

The collected MSs were finally dried in an oven at 37 ± 2°C for 12 hours.

4.4.2 SEM morphological analysis of MSs

The structural conformation (shape and surface) of HA and cross-linked HA MSs containing or not SAP was examined using a field emission scanning electron microscope (Zeiss EVO 40XVP, Carl Zeiss Pty Ltd., Oberkochen, Germany), with an acceleration voltage of 20 kV. The solid form samples were randomly scanned and photographed after deposition on carbon sticky tabs with and without metallization.

4.4.3 Loading Efficiency Determination of SAP content

To determine the final SAP concentration in the MSs, 10 mg of each microsphere sample was completely dissolved into 100mL of PBS (pH 7.4 0.01M) and measured by ultraviolet (UV) spectroscopy (UV-31 SCAN ONDA, Giorgio Bormac S.r.l. Carpi (MO) Italy) at the 258 nm (SAP □max) using a calibration curve of SAP in H_2O in a linear range of 0.5- 64 μg/ml with the $□^2 = 0.9987$.

The percent MS yield (Y%), drug loading (DL%) and drug entrapment efficiency (EE%) were deteminated in triplicate using equations 4, 5, and 6, respectively and the results were reported as the mean ± standard deviation (SD):

□% = _____ · 100 Equation 4

□□% _____
=
·100 Equation 5

□□% _____
=
· 100 Equation 6

4.4.4 In vitro release assays from microspheres

Microparticles release studies of the microparticles were conducted in triplicates using the dialysis membrane methods. The different weights of MSs containing an estimated equal amount of SAP (about 5 mg) were transferred into a preconditioned dialysis bag (Slide-ALyzer G2, regenerated cellulose dialysis tubing) with an MWCO of 3.5 kDa. Dialysis membranes were immersed into 300 mL of release medium (PBS, pH=7.4,0.01M,) at room temperature under magnetic stirring at 100 rpm. At predefined time intervals up to 48h, 1mL of the release medium was withdrawn and replaced with an equal volume of fresh PBS. The amount of SAP released was quantified by UV spectroscopy using a SAP calibration curve as a standard. The analysis was performed in triplicate and the results were averaged and expressed as % cumulative release with respect to the initial weight of SAP loaded into the particles.

4.5 Biological assays

4.5.1 Cell culture

Two lung epithelial cell lines were used to characterise the effect of Arginine (Arg) and Ornithine (Orn) cross-linked HA on the lung epithelia: Calu-3 and H441 cells. These cell lines were chosen due to their vast use as respiratory cell models due to their ability to grow in an air-liquid interface, produce tight junction, and mucus, similar to the physiologic conditions observed in the lung.

Cells were cultured in 75 cm^2 flasks and maintained in humidified 95% air, 5% CO_2 atmosphere, at 37°C. Calu-3 was cultured in Dulbecco's Modified Eagle's Medium/F-12 supplemented with 1% (v/v) non-essential amino acids, 1% (v/v) 200mM L-glutamine solution, and 10% (v/v) foetal bovine serum (FBS); while H441 cells were cultured in RPMI1640 Medium, supplemented with 200nM dexamethasone (Sigma) and 1% (v/v) insulintransferrin-selenium supplement (100x, Gibco). Media was changed 2-3 times a week until confluency was reached. After reaching confluency cells were passaged using trypsin at 1:3 and 1:7 ratio for Calu-3 and H441 cells, respectively.

To establish an air-liquid interface, both cell lines 3 x 10^4cells were seeded per well of a Transwell polyester insert containing 100 µL in the apical chamber and 600 µL in the basal chamber. The medium from the apical chamber was removed after 24h from seeding and every day afterwards until an ALI was achieved, while the medium from the basal chamber was replaced every second day up to 14 days of culture. For H441, a differentiation medium was used on the basal chamber from day 2 from seeding the cells consisting of RPMI-1640 Medium supplemented with 200nM dexamethasone (Sigma) and 1% (v/v) insulintransferrin-selenium supplement (100x, Gibco).

4.5.2 Cytotoxicity using MTS

Cells were seeded at density of 50,000 cells/well in a 96 well plate. After 24h, the media was removed and replaced with 100 µL the raw materials prepared in HBSS. After 24h incubation, 20 µL of MTS reagent was added to the wells and read after 3h at 490 nm. The solutions were prepared as a serial dilution from 0.30% (w/v) for HA, HA-Arg and HAOrn and from 0.9% (w/v) for SAP. For the treatments combining HA, Ha-Arg or HA-Orn and SAP, serial dilutions occurred from 0.3-0.009% (w/v) for HA/SAP. DMSO 20% was used as control for cells death.

4.5.3 Effect on the epithelial barrier integrity

Maintenance of the epithelial barrier integrity is an indicative of product cytotoxicity, as tight junctions are proteins that are essential in the health of lung epithelia cells. The effect of the materials on the epithelial barrier of the cells grown in an ALI culture (14 days), was assessed by the addition of 100 µL of test solution to the apical chamber. Cells were incubated for 24h, followed by measurement of permeability of the cell layer to sodiumfluorescein (Na-Flu, 2.5 mg/mL), a paracellular transporter marker, over 4h.

Treatments consisted of HA, HA-Arg and HA-Orn at 0.15% (w/v), SAP at 0.45% (w/v), and HA + SAP (0.15 + 0.45% w/v), HA-Arg + SAP (0.15 + 0.45% w/v) and HA-Orn + SAP (0.15 + 0.45% w/v). Experiments were conducted in triplicate, and results were compared to cells incubated with HBSS vehicle alone.

The apparent permeability coefficient (Papp) of Flu-Na across the epithelial cell layer was calculated according to a simplified diffusion equation (Equation 7):

$$Papp = (VBasal / AMembrane \times CApical) \times (dC/dt) \quad \text{Equation 7}$$

Where:

VBasal =the basal volume,

AMembrane = the area of the inserts membrane,

CApical = the initial Flu-Na concentration a dC/dt

= the flux of Flu-Na.

4.5.4 Antioxidant effect

The effect of SAP alone and in combination with HA and cross-linked HA was assessed using DCFDA dye. Cells were seeded in 96 well plate at 50,000 cells/well and allowed to attach overnight. After media removal, cell surface was washed twice with PBS and dyed with DCFDA (prepared in SF media) at 5 µM for 30 minutes at 37°C. The dye excess was removed, and to assess their potential antioxidant effect, ROS production was stimulated using hydrogen peroxide at 0.03% (v/v) for 10 minutes followed by addition of the treatments (HA, HA-Arg and HA-Orn at 0.15% (w/v), SAP at 0.45% (w/v), and HA + SAP (0.15, 0.45% w/v), HA-Arg + SAP (0.15, 0.45% w/v) and HA-Orn + SAP (0.15, 0.45% w/v), prepared in SF media). The cell fluorescence levels were measured after 10 min, and 1h at 480/520nm excitation/emission. N-acetyl cysteine (NAC) at 5 µM was used as an antioxidant control. Cells without dye (no DCFDA), and without stimulus (DCFDA, without H_2O_2) were also included as controls for the assay.

4.5.5 Effect on Inflammatory markers

The effect of the raw materials on the expression of inflammatory markers on Calu-3 and H441 cells was assessed after 24 hours exposure to the materials. The potential use of these materials as an anti-inflammatory treatment was also assessed after an inflammation-like state was induced in the cells. In a 12-well plate, $5x10^5$ cells were seed and allowed to attach for 24 hours. Cells were stimulated with LPS from E. coli at 5 µg/ml.

4.6 Statistical Analysis

All the results were reported as mean ± standard error mean of at least three separate determinants. The statistical software, GraphPad Prism (version 8.2.1) was used to

determine One-Way or Two-Way ANOVA to test for significance for each experiment. Significance was determined as $p < 0.05$.

Chapter 5

5 CONCLUSIONS

With the aim of developing new hyaluronic acid-based product characterized by biocompatibility, safety and resistance to enzymatic degradation, two new cross-linked products were synthesized using CDMT as activating agent and the amino acid Arginine and Ornithine methyl ester as cross-linker agents.

The physico-chemical characteristics of the two new synthesized products were determined by ^{1}H NMR (Nuclear magnetic resonance) spectroscopy and Fourier-transform infrared spectroscopy. The results confirmed the occurrence of the cross-linking process and the formation of desired products from the formation of the new C=O amide bond in the HAArg and HA-Orn products, with satisfactory degrees of modification for both aminoacids used.

The thermal degradation of the HA-Arg and HA-Orn cross-linked was analyzed by DSC: the thermograms showed that the samples maintain the thermal behaviour typical of HAbased polysaccharides with endothermic and exothermic peaks typical of a new polymer structure.

The reological analisys highlighted the difference between the cross-linked products: HAArg had a liquid-like behaviour while HA-Orn showed a rheological profile attributable to a weak-gel.

The swelling ratio showed that the values of the HA-Arg and HA-Orn hydrogels were significantly variable depending on the type of cross-linking and pH: this behaviour gives them an additional characteristic that makes them exploitable as pH responsiveness polymer for a potential application in drug delivery.

The morphological analysis has shown that the cross-linkers compact the HA polymeric network, forming a cage structure whose mesh size depends on the cross-linking amino acid used.

These first evidences have allowed us to affirm that HA-Arg and HA-Orn are interesting products not only as active molecules, as they are synergistically enriched by the bioactivity and biocompatibility of the amino acids Arginine and Ornithine as cross-linkers, but also as innovative polymeric systems for the constitution of drug delivery systems.

Furthermore, these results encouraged us to start other studies in order to explore CDMT/NMM on cross-linking of HA using other natural cross-linked agents with interesting preliminary results.

In order to validate the effective enhanced enzymatic resistence of HA-Arg and HA-Orn, as an expression for the degree of cross-linking efficiency, an evaluation of the degradation

rate of HA-Arg and H-Orn was conducted. In fact, because the use of Arginine and Ornithine methyl ester as cross-linking agents is new, their behaviour in terms of improved stability and preserved biocompatibility had not yet been demonstrated.

An in vitro degradation study was performed on HA-Arg and HA-Orn using hyaluronidase, and the Carbazole assay was used to verify the extent of digestion over time. The results showed that HA-Arg and HA-Orn, with the cross-linking process, had acquired a significantly lower rate of degradation than native HA. On the other hand, HA-Orn is more resistant than HA-Arg, with a degradation rate that was found to be less than half that of HA-Arg. Therefore, it can be concluded that both cross-linked showed greater resistance to the enzymatic digestion to hyaluronidase. This behaviour demonstrates that the effectiveness of the cross-linking of aminoacids to the carboxylic group of the HA, involved in the binding with the cross- linker, but also that the porous structure limits access and action of degrading enzymes.

This versatile property is a fundamental prerequisite for the future uses of HA-Arg and HAOrn as novel polymers for different applications to the biomedical fields (i.e. wound healing, tissue repair, skin anti-ageing, and delivery of active molecules).

However, further in vivo enzymatic degradation studies are needed to better assess the potential of these newly developed cross-linked hyaluronan products.

Since the use of naturally occurring molecules for the treatment of pathologies and disturbs related to oxidative stress is known, the potential of a salt of vitamin C (SAP), described as a powerful antioxidant substance in the cosmetic and food supplements fields, was investigated.

For this reason, to evaluate their safety and biological activity, HA-Arg and HA-Orn alone, and in combination with SAP, were tested on alveolar (H441) and lung epithelial (Calu-3) cells in order to identify a possible adjuvant treatment in pulmonary anti-inflammatory therapy.

In vitro tests demonstrated that the cross-linked polymers HA-Arg and HA-Orn maintained cell viability and the integrity of the epithelial barrier at all concentration values explored on both cell lines, either alone and in combination with SAP and can therefore be considered safe.

Only on Calu-3 cells, the association to SAP showed an increase in cell proliferative for HAArg and HA-Orn.

The anti-inflammatory activity expressed as the levels of IL-6 and IL-8 released showed a good anti-inflammatory effect of HA-Arg alone and in combination with SAP on both cell

lines, and of HA-Orn in combination with SAP on the H441 cell line. The best effect expressed by the association of both cross-linked HA with SAP indicating that the combination of these compounds could be useful, allowing to work synergistically for the treatment of chronic inflammatory diseases in which inflammation and oxidative stress are present.

A systematic approach was then adopted for the preparation of microspheres based on the cross-linked HA-Arg and HA-Orn and antioxidant (SAP) as a delivery system by using the emulsification-solvent evaporation technique. The results obtained from this study showed how by optimizing the parameters affecting the formulation, optimized products suitable for the delivery of drugs can be obtained.

The microparticles obtained under optimized conditions (low concentration of active and high concentration of emulsifying agent) were examined by FT-IR to study the interactions between drug and microparticles. This analysis confirmed the stability of the polymers after the formulation process and the absence of the formation of new bonds between SAP and the polymers. The microspheres were then examined using the SEM technique which revealed a spherical shape and smooth surface.

Yield%, DL%, and EE% were evaluated for the MSs producted under the conditions of optimal process parameters. An in vitro dialysis release study was performed which showed a more extensive drug release for HA-Orn-SAP compared to HA-Arg-SAP and HA-SAP.

This underlined the mechanical properties implemented by cross-linking on HA-Orn which makes it an efficient material for use in the formulation of microsystems that can be prepared in the future with the inclusion of drugs of different nature. These systems could be improved to further slow down the release of the drug and for therapeutic application.

The preliminary release study showed that the MSs formulated with HA-Orn loaded with SAP were able to sustain a longer release than the MSs formulated with native HA. The reported data provided preliminary evidence that the prepared MSs could be adaptive systems for the treatment of inflammatory diseases resulting from an oxidative stress condition, for the administration of water-soluble anti-inflammatory drugs. However, further investigations are needed to understand its potential also in other application fields, therefore bioactivity and cytotoxicity studies are underway on other cell lines of interest.

Overall, a promising framework emerged from this preliminary study relating to the preparation of carrier systems of pharmaceutical interest using an HA-based polymer derivative to be considered effective and safe. Finally, the synthetic approach developed resulted solid and promising for the extension to other cross-linkers, making this approach

a valuable tool in the preparation of multifunctional molecules caged in a polymeric transport system or directly bound with the transport system. In addition, the assembly of microspheres that may in turn contain active principles is particularly attractive in approaches to pulmonary diseases in the form of sprays or powders especially in today's epidemiological context, characterized by the pandemic emergency from Covid-19. Such a topical treatment, especially if early, could significantly reduce the resolution time of the disease.

This work has been object of the patent application N° 102019000024117 of the 16th December 2019.

6 REFERENCES

Abd El-Hameed, M., & Kellaway, I. (1997). Preparation and in vitro characterisation of mucoadhesive polymeric microspheres as intra-nasal delivery systems. Eur. J. Pharm. Biopharm., 44, 53-60.

Adamia, S., Maxwell, C., & Pilarski, L. (2005). Hyaluronan and hyaluronan synthases:potential therapeutic targets in cancer. Curr. Drug Targets Cardiovasc.

Haematol. Disord., 5, 3-14.

Adamia, S., Pilarski, P. B., & Pilarski, L. (2013). Aberrant splicing, hyaluronan synthases and intracellular hyaluronan as drivers of oncogenesis and potential drug targets. Curr. Cancer Drug Targets, 13, 347-361.

Allegra, L., Della, P., & Petrigni, G. (2012). Hyaluronic acid: perspectives in lung diseases. Handb Exp, Pharmacol., 207, 385.

Allemann, I., & Baumann, L. (2008). Hyaluronic acid gel (Juvederm) preparations in the treatment of facial wrinkles and folds. Clinical Interventions in Aging, 3(4), 629634.

Arezki, N., Williams, A., Cobb, A., & Brown, M. (2017). Design, synthesis and characterization of linear unnatural amino acids for skin moisturization. Int. j. cosmet. sci., 39(1), 78-82.

Aya, K., & Stern, R. (2014). Hyaluronan in wound healing: rediscovering a major player. Wound Repair Regen., 22, 579-593.

Baier Leach, J., Bivens, K., Patrick Jr, C., & Schmidt, C. (2003). Photocrosslinked hyaluronic acid hydrogels: Natural, biodegradable tissue engineering scaffolds. Biotechnology and Bioengineering , 82(5), 578-589.

Balazs, E., Leshchiner, E., Larsen, N., & Band, P. (1993). Applications of hyaluronan and its derivatives. Gebelein, C.G. (ed) Biotechnological polymers., 41-65.

Banerji, S., Wright, A., Noble, M. M., Campbell, I., Day, A., & Jackson, D. (2007). Structures of the Cd44-hyaluronan complex provide insight into a fundamental carbohydrate-protein interaction. Nat. Struct. Mol. Biol., 14, 234–239.

Basak, B., Bandyopadhyay, D., Patra, M., Banerji, A., Chatterjee, A., & Banerji, J. (2005). Role of Sulfur Compounds in the Detection of Amino Acids by Ninhydrin on TLC Plate. Journal of Chromatographic Science, , 43, 104-105.

Baumann, L. (2004). Dermal fillers. J. Cosmet. Dermatol., 3(4), 249-250.

Bellini, D., & Topai, A. (2000). Amides of hyaluronic acid and the derivatives thereof and a process for their preparation. Google Patents.

Bencherif, S., Srinivasan, A., H. F., H. J., Matyjaszewski, K., & Washburn, N. (2008). Influence of the degree of methacrylation on hyaluronic acid hydrogels properties. Biomaterials, 29, 1739-1749.

Bergman, K., Elvingson, C., Hilborn, J., Svensk, G., & Bowden, T. (2007). Hyaluronic acid derivatives prepared in aqueous media by triazine-activated amidation.

Biomacromolecules, 8, 2190-2195.

Bhattacharya, D., Svechkarev, D., Souchek, J., Hill, T., Taylor, M., Natarajan, A., & Mohs, A. (2017). Impact of structurally modifying hyaluronic acid on CD44 interaction. J.Mater. Chem. B., 5, 8183-8192.

Bitter, T., & Muir, H. (1962). A modified uronic acid carbazole reaction. Analyt. Biochem., 330-334.

Kaminska, J. E., Kami´nski, Z. J., & Góra, J. (1999). Acyloxy-4,6-dimethoxy-1,3,5triazine – A new reagent for ester synthesis. 4, 593–596.

Borzacchiello, A., Mayol, L., Schiavinato, A., & Ambrosio, L. (2010). Effect of hyaluronic acid amide derivative on equine synovial fluid viscoelasticity. J Biomed Mater Res A, 92(3), 1162-1170.

Borzacchiello, A., Netti, P. A., Ambrosio, L., & L, N. (2000). Hyaluronic acid derivatives mimic the rheological properties of vitreous body. New Frontiers in Medical Sciences: Redefining Hyaluronan, Elsevier, 192-202.

Bowman, S., Awad, M., Hamrick, M., Hunter, M., & Fulzele, S. (2018). Recent advances in hyaluronic acid based therapy for osteoarthritis. Clin. Transl. Med. , 7, 6.

Brown, M., & Jones, S. (2005). Hyaluronic acid: a unique topical vehicle for the localized delivery of drugs to the skin. JEADV, 19(3), 308-318.

Brown, T., Laurent, U., & Fraser, J. (1991). Turnover of hyaluronan in synovial joints:elimination of labelled hyaluronan from the knee joint of the rabbit. Exp Physiol., 76, 125-134.

Bulpitt, P., & Aeschlimann, D. (1999). New strategy for chemical modification of hyaluronic acid: preparation of functionalized derivatives and their use in the formation of novel biocompatible hydrogels. J. Biomed. Mater. Res., 47, 152-169.

Chen, W., Marcellin, E., Hung, J., Nelsen, & L.K. (2009). Hyaluronan Molecular Weight Is Controlled by UDP-N-acetylglucosamine Concentration in Streptococcus zooepidemicus. The J. of Biol.Chem., 284(27), 18007–18014.

Choi, K., Min, K., Na, J., Choi, K., Ki, K., Park, J., . . . S.Y., J. (2009). Self-assembled hyaluronic acid nanoparticles as a potential drug carrier for cancer therapy: synthesis, characterization, and in vivo biodistribution. J. Mater. Chem, 19, 4102–4107.

Collins, M., & Birkinshaw, C. (2007). Comparison of the effectiveness of four different crosslinking agents with hyaluronic acid hydrogel films for tissue-culture applications. J. Appl. Polym. Sci., 104, 3183–3191.

Collins, N., & Birkinshaw, C. (2008). Physical properties of crosslinked hyaluronic acid hydrogels. J. Mater. Sci.: Mater. Med., 19, 3335-3343.

Cortesi, R. E. (2002). Production of lipospheres as carriers for bioactive compounds. Biomaterials, 23, 2283-2294.

Crescenzi, V., Francescangeli, A., Taglienti, A., Capitani, D., & Mannina, L. (2003). Synthesis -and partial characterization of hydrogels obtained via glutaraldehyde crosslinking ofacetylated chitosan and of hyaluronan derivative. Biomacromolecules, 4, 1045-1054.

Csóka, A. B., Scherer, S. W., & Stern, R. (1999). Expression analysis of six paralogous human hyaluronidase genes clustered on chromosomes 3p21 and 7q31. . Genomics, 60, 356-361.

Cyphert, J., Trempus, C., & Garantziotis, S. (2015). Size matters: molecular weights pecificity of hyaluronan effects in cell biology. Int. J. Cell Biol., 563818.

D' Este, M., Eglin, D., & Alini, M. (2014). A systematic analysis of DMTMM vs EDC/NHS for ligation of amines to hyaluronan in water. Carbohydr. Polym., 108, 239-246.

De Boulle, K., Glogau, R., Kono, T., Nathan, M., Tezel, A., Roca-Martinez, J., . . . Stroumpoulis, D. (2013). A review of the metabolism of 1,4-butanediol diglycidyl ether-crosslinked hyaluronic acid dermal fillers. Dermatol. Surg., 39(1), 758–1766.

De la Motte, C., Nigro, J., Vasanji, A., Rho, H., Kessler, S., Bandyopadhyay, S., . . . Stern, R. (2009). Platelet-derived hyaluronidase 2 cleaves hyaluronan into fragments that trigger monocyte mediated production of proinflammatory cytokines. Am. J. Pathol., 174, 2254-2264.

De Oliveira, J., Carvalho, L., Gomes, A., Queiroz, L., Magalhães, B., & Parachin, N. (2016). Genetic basis for hyper production of hyaluronic acid in natural and engineered microorganisms. Microb. Cell Fact., 15, 119.

DeAngelis, P., Jing, W., Drake, R., & Achyuthan, A. (1998). Identification and molecular cloning of a unique hyaluronan synthase from Pasteurella multocida. J. Biol. Chem., 273, 8454-8458.

Degim, Z. (2008). Use of microparticulate systems to accelerate skin wound healing. J. Drug Target. , 16, 437-448.

Della Valle, F., & Romeo, A. (1989). Esters of hyaluronic acid. US4851521.

Delpech, B., Delpech, A., Bruckner, G., Girard, N., & Maingonnar, C. (1989). Hyaluronan and hyaluronectin in the nervous system. Evered D, Whelan J, editors. The biology of hyaluronan: Ciba Foundation Symposium, 143, 208–232.

Drobnik, J. (1991). Hyaluronan in drug delivery. Advanced Drug Delivery Reviews, 7(2), 295–308.

Drobnik, J. (1991). Hyaluronan in drug delivery. Advanced Drug Delivery Reviews, 7(2), 295–308.

Ebid, R., Lichtnekert, J., & Anders, H. (2014). Hyaluronan is not a ligand but a regulator of toll-like receptor signaling in mesangial cells: role of extracellular matrix in innate immunity. ISRN Nephrol., 714081.

Egbu, R., Brocchini, S., Khaw, P., & Awwad, S. (2018). Antibody loaded collapsible hyaluronic acid hydrogels for intraocular delivery. Eur. J. Pharm. Biopharm., 124, 95-103.

Eliaz, R., & Szoka, F. (2001). Liposome-encapsulated doxorubicin targeted to CD44: a strategy to kill CD44-overexpressing tumor cells. Cancer. Res., 61, 2592–2601.

Eliaz, R., Nir, S., Marty, C., & Szoka, F. (2004). Determination and modeling of kinetics of cancer cell killing by doxorubicin and doxorubicin encapsulated in targeted liposomes. Cancer Res., 64, 711–718.

Esposito, E., Cortesi, R., Luca, G., & Nastruzzi, C. (2001). Pectin based microspheres: a preformulatory study. Annals of the New York Academy of Sciences, Bioartificial Organs III: Tissue Sourcing,Immunoisolationand clinical trials, 944, 160-179.

Esposito, E., Menegatti, E., & Cortesi, R. (2005). Hyaluronan-based microspheres as tools for drug delivery: a comparative study. nt. J. Pharm., 288, 35-49.

Falcone, S. P. (2006). Rheological and cohesive properties of hyaluronic acid. J. Biomed. Mater. Res. A, 76, 721-728.

Fallacara, A., Marchetti, F., Pozzoli, M., Citernesi, U. R., Manfredini, S., & Vertuani, S. (2018). Formulation and Characterization of Native and Crosslinked Hyaluronic Acid Microspheres for Dermal Delivery of Sodium Ascorbyl Phosphate:A Comparative Study. Pharmaceutics, 10(4), 254.

Ferretti, M., Marra, K. K., Defail, A., & Chu, C. (2006). Controlled in vivo degradation of genipin crosslinked polyethylene glycol hydrogels within osteochondral defects. Tissue Engineering, 12(9), 2657–2663.

Flory, P., & Rehner, J. (1943). Statistical mechanics of cross-linked polymer networks II.Swelling. J. Chem. Phys., 11, 521.

Ford, J., & Timmins, P. (1989). Pharmaceutical Thermal AnalysisTechniques and Applications. Ellis Horwood, Chichester.

Foster, K., Avery, M., Yazdanian, M., & Audus, K. (2000). Characterization of the Calu-3 cell line as a tool to screen pulmonary drug delivery . International Journal of Pharmaceutics, 208, 1-11.

Furnari, M., Termini, L., Traverso, G., Barrale, S., Bonaccorso, M., Damiani, G., . . . Collura, M. (2012). Nebulized hypertonic saline containing hyaluronic acid improves tolerability in patients with cystic fibrosis and lung disease compared with nebulized hypertonic saline alone: a prospective, randomized, double-blind, controlled study. . Ther. Adv. Respir. Dis., 6, 315-322.

Gad, M. A. (2010). nti-aging effects of l-arginine. Journal of Advanced Research, 1, 169–177.

Garantziotis, S., Brezina, M., Castelnuovo, P., & Drago, L. (2016). The role of hyaluronan in the pathobiology and treatment of respiratory disease. Am. J. Physiol. Lung Cell Mol. Physiol., 310, L785-L795.

Gatej, I., Popa, M., & Rinaudo, M. (2005). Role of pH on hyaluronan behavior in aqueous solution. Biomacromolecules, 61-67.

Gavina, M., Luciani, A., Villella, V., Esposito, S., Ferrari, E., Bressani, I., . . . Raia, V. (2013). Nebulized hyaluronan ameliorates lung inflammation in cystic fibrosis mice. Pediatr. Pulmonol., 48, 761–771.

Gelardi, M., Iannuzzi, L., & Quaranta, N. (2013). Intranasal sodium hyaluronate on the nasal cytology of patients with allergic and nonallergic rhinitis. Int. Forum Allergy Rhinol. , 3, 807-813.

Gennari, A., Pelliccia, M., R., D., Kimber, I., & Tirelli, N. (2016). Mannosylation Allows for Synergic (CD44/C-Type Lectin) Uptake of Hyaluronic Acid Nanoparticles in Dendritic Cells, but Only upon Correct Ligand Presentation . Adv. Healthcare Mater., 5(8), 966-976.

Ghosh, K., Shu, X. M., Prestwich, G., & Rafailovich, M. e. (2005). Rheological characterization of in situ cross-linkable hysluronan hydrogels. Biomacromolecules, 6(5), 2857–2865.

Ghosh, S., Kobal, I., Zanette, D., & Reed, W. (1993). Conformational contraction and hydrolysis of hyaluronate in sodium hydroxide solutions. Macromolecules, 26(17), 4685–4693.

Girish, K. S., & Kemparaju, K. (2007). The magic glue hyaluronan and its eraser hyaluronidase: A biological overview. Life Sciences, 80(21), 1921–1943.

Girish, K., & Kemparaju, K. (2006). Inhibition of Naja naja venon hyaluronidase: Role in the management of poisonous bite. Life Sciences, 87, 1433–1440.

Gomez-Gaete, C., Tsapis, N., Silva, L., Bourgaux, C., Besnard, M., B. A., & al., e. (2008). Supramolecular organization and release properties of phospholipid-hyaluronan microparticles encapsulating dexamethasone. Eur J Pharm Biopharm., 70(1), 116–26.

Guilherme, M., Campese, G. M., Radovanovic, E., Rubira, A. F., Feitosa, J. P., & Muniz, E. (2005). Morphology and water affinity of superabsorbent hydrogels composed of methacrylated cashew gum and acrylamide with good mechanical properties. Polymer, 46, 7867–7873.

Gulrez, S., Al-Assaf, S., & Phillips, G. O. (2011). Hydrogels: Methods of Preparation, Characterisation and Applications. Progress in Molecular and Environmental Bioengineering - From Analysis and Modeling to Technology Applications.

Hamman, J. (2010). Chitosan based polyelectrolyte complexes as potential carrier materials in drug delivery systems. Mar. Drugs, 8, 1305-1322.

Hašová, M., Crhák, T., Safránková, B., Dvořáková, J., Muthný, T., Velebný, V., & Kubala, L. (2011). Hyaluronan minimizes effects of UV irradiation on human keratinocytes. Arch. Dermatol. Res.(303), 277-284.

Heldin, P., Lin, C., Kolliopoulos, K., Chen, Y., & Skandalis, S. (2018). Regulation of hyaluronan biosynthesis and clinical impact of excessive hyaluronan production. Matrix Biol., doi: 10.1016/j.matbio.2018.1001.1017.

Heldin, P., Lin, C., Kolliopoulos, K., Chen, Y., & Skandalis, S. (2018). Regulation of hyaluronan biosynthesis and clinical impact of excessive hyaluronan production. Matrix Biol., doi: 10.1016/j.matbio.2018.1001.1017.

Hemshekhar, M., Thushara, R., Chandranaya, S., .Sherman, L., Kemparaju, K., & Girish, K. (2016). Emerging roles of hyaluronic acid bioscaffolds in tissue engineering and regenerative medicine. Intern. J.of Biol. Macromol., 86, 917-928.

Herrero-Vanrell, R., A., F.-C., Frutos, G., & Cadorniga, R. (2000). Enhancement of the mydriatic response to tropicamide by bioadhesive polymers. J. Ocul. Pharmacol.

Ther., 16(5), 419–428.

Hirano, K., Sakai, S., Ishikawa, T., Avci, F., Linhardt, R., & Toida, T. (2005). Preparation of the methyl ester of hyaluronan and its enzymatic degradation. Carbohydr. Res., 340, 2297-2304.

Hoare, T., & Kohane, D. (2008). Hydrogels in drug delivery: progress and challenges. Polymer, 49(8), 1993-2007.

Hokputsa, S., Jumel, K., Alexander, C., & Harding, S. (2003). A comparison of molecular mass determination of hyaluronic acid using SEC/MALLS and sedimentationequilibrium. European Biophysics Journal, 32(5), 450–456.

Huin-Amargier, C., Marchal, P., Payan, E., Netter, P., & Dellacherie, E. (2006). New physically and chemically crosslinked hyaluronate (HA)-based hydrogels for cartilage repair. J. Biomed Mater. Res., A 76, 416–424.

Hwang, S., Kim, D., Chung, S., & Shim, C. (2008). Delivery of ofloxacin to the lung and alveolar macrophages via hyaluronan microspheres for the treatment of tuberculosis. J. Control. Release, 129(2), 100–106.

Isemura, M., Yosizawa, Z., Koide, T., & Ono, T. (1982). Interaction of fibronectin andits proteolytic fragments with hyaluronic acid. J. Biochem., 91, 731–734.

Järvinen, K., T., J., & Urtti, A. (1995). Ocular absorption following topical delivery. Adv. Drug Dev. Rev., 16(1), 3-19.

Jiang, D., Liang, J., & Noble, P. (2011). Hyaluronan as an immune regulator in human diseases. Physiol. Rev., 91, 221-264.

Jiang, D., Liang, J., Fan, J., Yu, S., Chen, S., Luo, Y., . . . Noble, P. (2005). Regulation of lung injury and repair by Toll-like receptors and hyaluronan. Nat. Med., 11, 1173–1179.

Kablik, J., Monheit, G., Yu, L., P.H.D., Chang, G., & Gershkovich, J. (2009). Comparative physical properties of hyaluronic acid dermal fillers. Dermatology Surgery, 35, 302-312.

Kafedjiiski, K., Jetti, R., Föger, F., Hoyer, H., Werle, M., Hoffer, M., & Bernkop-Schnürch, A. (2007). Synthesis and in vitro evaluation of thiolated hyaluronic. International Journal of Pharmaceutics, 343 , 48–58.

Kafedjiiski, K., Jetti, R., Föger, F., Hoyer, H., Werle, M., Hoffer, M., & BernkopSchnürch, A. (2007). Synthesis and in vitro evaluation of thiolated hyaluronic acid for mucoadhesive drug delivery. Int. J. Pharm., 343, 48-58.

Kaminski, Z. J. (1985). 2-Chloro-4,6-disubstituted-1,3,5-triazines a novel group of condensing reagents. Tetrahedron Letters, 26(24), 2901–2904.

Kaminski, Z. J. (1994). he concept of superactive esters. International Journal of Peptide and Protein Research, 43(3), 312–319.

Kantappa, H., & Lee, J. (2017). Functional hyaluronic acid conjugates based on natural polyphenols. J. of Ind. and Eng. Chem., 54, 44-51.

Kaur, K., Singh, H., & Singh, M. (2006). Repair of tympanic membrane perforation by topical application of 1% sodium hyaluronate. l. Head Neck Surg. 58, . Indian J. Otolaryngol. Head Neck Surg., 58, 241–244.

Khan, H., Akhtar, N., & Ali, A. (2016). Assessment of Combined Ascorbyl Palmitate (AP) and Sodium Ascorbyl Phosphate (SAP) on Facial Skin Sebum Control in Female Healthy Volunteers. Drug Research, 67(01), 52-58.

Khan, H., Akhtar, N., & Ali, A. (2016). Assessment of Combined Ascorbyl Palmitate (AP) and Sodium Ascorbyl Phosphate (SAP) on Facial Skin Sebum Control in Female Healthy Volunteers. . Drug Research, 67(01), 52-58.

Kim, E., Baba, D., Kimura, M., Yamashita, M., Kashiwabara, S., & Baba, T. (2005). Identification of a hyaluronidase, Hyal5, involved in penetration of mouse sperm through cumulus mass. . 50, 18028–18033.

King, S., Hickerson, W., & Proctor, K. (1991). Beneficial actionsof exogenous hyaluronic acid on wound healing. Surgery , 109, 76–84.

Kleniewska, P., & Pawliczak, R. (2017). The participation of oxidative stress in the pathogenesis of bronchial asthma. Biomed. Pharmacother. , 94, 100-108.

Knopf-Marques, H., Pravda, M., Wolfova, L., Velebny, V., Schaaf, P., Vrana, N., & Lavalle, P. (2016). Hyaluronic Acid and Its Derivatives in Coating and Delivery Systems: Applications in Tissue Engineering, Regenerative Medicine and Immunomodulation. Adv. Healthc. Mater, 5, 2841-2855.

Kogan, G., Soltes, L., & Stern, R. (2007). Hyaluronic acid: A natural biopolymer with a broad range of biomedical and industrial applications. Biotechnology Letters, 29(1), 17–25.

Kulkarni, A., Bari, D., Surana, S., & Pardeshi, C. (2016). In vitro, ex vivo and in vivo performance of chitosan-based spray-dried nasal mucoadhesive microspheres of diltiazem hydrochloride. J Drug Deliv. Sci. Techno. , 31, 108-117.

Kunishima, M., Kawachi, C., Hioki, K., Terao, K., & Tani, S. (2001). Formation of carbox-amides by direct condensation of carboxylic acids and amines in alcohols usinga

new alcohol- and water-soluble condensing agent: DMT-MM. Tetrahedron, 57(8), 1551-1558.

Kunishima, M., Kawachi, C., Monta, J., Terao, K., Iwasaki, F., & Tani, S. (1999). 4-(4,6dimethoxy-1,3,5-triazin-2-yl)-4-methyl-morpholinium chloride: an efficient condensing agent leading to the formation of amides and esters. Tetrahedron , 55, 13159-13170.

La Gatta, A., Schiraldi, C., Papa, A., D'Agostino, A., Cammarota, M., Rosa, A., & Rosa, M. (2013). Hyaluronan scaffolds via diglycidyl ether cross-linking: Toward improvements in composition and performance. Carbohydrate Polymers, 96, 536–544.

Laffleur, F., & Dachs, S. (2015). Development of novel mucoadhesive hyaluronic acid derivate as lubricant for the treatment of dry eye syndrome. Ther. Deliv., 6, 12111219.

Lai, J. (2014). Relationship between structure and cytocompatibility of divinyl sulfone cross-linked hyaluronic acid. Carbohydr. Polym, 101, 203–212.

Langer, K., Mutschler, E., Lambrecht, G., Mayer, D., G., T., & Kreuter, J. (1997). Methylmethacrylate sulfopropylmethacrylate copolymer nanoparticles for drug delivery –Part III. Evaluation as drug delivery system for ophthalmic applications. Int. J. Pharm., 157(2), 219–231.

Larsson, N., Rankin, G., Bicer, E., Roos-Engstrand, E., Pourazar, J., Blomberg, A., . . . Behndig, A. (2015). Identification of vitamin C transporters in the human airways: a crosssectional in vivo study. BMJ Open, 5, e006979.

Laurent, T. C. (1970). Structure of hyaluronic acid. In Chemistry and the Molecular Biology of the Intracellular Matrix, ed. E. A. Balazs,, 703–732.

Laurent, T., & Fraser, J. (1992). Hyaluronan. FASEB J, 6, 2397-2404.

Le Baron, R., Zimmermann, D., & Ruoslahti, E. (1992). Hyaluronate binding properties of versican. J BiolChem, 267, 10003–10010.

Leach, J., & Schmidt, C. (2004). Hyaluronan. Encyclopedia of Biomaterials and Biomedical Engineering. Marcel Dekker, , 779–789.

Lee, H., Ahn, C., & Park, T. (2009). Poly[lactic-co-(glycolic acid)]-grafted Hyaluronic acid copolymer micelle nanoparticles for target-specific delivery of Doxorubicin. Macromol. Biosci., 9(4), 336–342.

Lepperdinger, G., Strobl, B., & Kreil, G. (1998). HYAL2, a human gene expressed in many cells, encodes a lysosomal hyaluronidase with a novel type of specificity. J. Biol. Chem, 273, 22466-22470.

Li, Y., & Li, G. (2016). Is vitamin C beneficial to patients with CAP? . Curr. Infect Dis. Resp., 18, 24.

Lim, S., Martin, G., Berry, D., & Brown, M. (2000). Preparation and evaluation of the in vitro drug release properties and mucoadhesion of novel microspheres of hyaluronic acid and chitosan. J. Control. Release, 66, 281-292.

Liu, L., Liu, J., Li, J., & Du, &. C. (2011). Microbial production of hyaluronic acid: current state, challenges, and perspectives. Microbial Cell Factories, 10(99).

Lokeshwar, V., Rubinowicz, D., Schroeder, G., Forgacs, E., Minna, J., Block, N., . . . Lokeshwar, B. (2001). Stromal and epithelial expression of tumor markers hyaluronic acid and HYAL1 hyaluronidase in prostate cancer. J. Biol. Chem., 276, 11922–11932.

Lokeshwar, V., Schroeder, G., Care, R., Soloway, M., & Iida, N. (2002). Regulation of hyaluronidase activity by alternative mRNA splicing. Journal of Biological Chemistry, 277, 33654–33663.

Luo, Y., & Prestwich, G. (1999). Synthesis and selective cytotoxicity of a hyaluronic acidantitumor bioconjugate. Bioconjugate Chemistry, 10(5), 755–763.

Luo, Y., Kirker, K., & Prestwich, G. (2000.). Cross-linked hyaluronic acid hydrogel films: new biomaterials for drug delivery. J. Control Release, 69, 169-184.

MacNee, W. (2001). Oxidative stress and lung inflammation in airways disease. European Journal of Pharmacology, 429((1-3)), 195–207.

MacNee, W. (2001). Oxidative stress and lung inflammation in airways disease. Eur. J. Pharmacol., 429, 195-207.

Magnani, A., Rappuoli, R., Lamponi, S., & Barbucci, R. (2000). Novel polysaccharide hydrogels:characterization and properties. Polym. Adv. Technol., 11, 488-495.

Maharjan, A., Pilling, D., & Gomer, R. (2011). High and Low Molecular Weight Hyaluronic Acid Differentially Regulate Human Fibrocyte Differentiation. PLoS ONE, 6(10), e26078.

Máiz Carro, L., & Martínez-García, M. A. (2020). Use of Hyaluronic Acid (HA) in Chronic Airway Diseases. Cells, 9(10), 2210.

Maleki, A., Kjoniksen, A., & Nystrom, B. (2007). Characterization of the chemical degradation of hyaluronic acid during chemical gelation in the presence of different crosslinkeragents. Carbohydr. Res., 342(18), 2776-92.

Maleki, A., Kjoniksen, A., & Nystrom, B. (2008). Effect of pH on the behavior of hyaluronic acid in dilute and semidilute aqueous solutions. Macromolecular Symposia, 274(1), 131–140.

Manconi, M., Manca, M., Valenti, D., Escribano, E., Hillaireau, H., Fadda, A., & Fattal, E. (2017). Chitosan and hyaluronan coated liposomes for pulmonary administration of curcum. Int. J. Pharm., 525, 203-210.

Manju, S., & Sreenivasan, K. (2011). Conjugation of curcumin onto hyaluronic acid enhances its aqueous solubility and stability. J. Colloid Interface Sci., 359, 318–325.

Mattheolabakis, G., Milane, L., Singh, A., & Amiji, M. (2015). Hyaluronic acid targeting of CD44 for cancer therapy: from receptor biology to nanomedicine. J. Drug Target, 23, 605-618.

McKee, C. M., Lowenstein, C. J., Horton, M. R., Wu, J., Bao, C., Chin, B. Y., . . . Noble, P. W. (1997). Hyaluronan fragments induce nitric-oxide synthase in murine macrophages through a nuclear factor κB-dependent mechanism . J. Biol. Chem., 8013.

Mendichi, R., Schieroni, A., Grassi, C., & Re, A. (1998). Characterization of ultra-high molar mass hyaluronan. 1. Off-line static methods. Polymer, 39(25), 6611–6620.

Meyer, K., & Palmer, J. (1934). The Polysaccharide of the vitreous humor. .J. Biol. Chem, 107, 629–634.

Mio, K., & R., S. (2002). Inhibitors of the hyaluronidases. Matrix Biology, 21, 31–37.

Mlcochová, P., Bystrický, S., Steiner, B., Machová, E., Koós, M., Velebný, V., & Krcmár, M. (2006). Synthesis and characterization of new biodegradable hyaluronan alkyl derivatives. Biopolymers. , 82, 74-79.

Moldoveanu, B., Otmishi, P., Jani, P., Walker, J., Sarmiento, X., Guardiola, J., & Saad, M. Y. (2009). Inflammatory mechanisms in the lung. J. Inflamm. Res., 2, 1-11.

Moreira, C., Moreira, A., Armstrong, D., Jelliffe, R., Woodford, C., Liggett, P., & Trousdale, M. (1991). In vitro and in vivo studies with sodium hyaluronate as a carrier for intraocular gentamicin. Acta. Ophthalmol., 69(1), 50–56.

Morimoto, K., Yamaguchi, H., Iwakura, Y., Morisaka, K., Ohashi, Y., & Nakai, Y. (1991). Effects of viscous hyaluronate-sodium solutions on the nasal absorption of vasopressin and an analogue. Pharmacol. Res. , 8(4), 471–474.

Muhn, C., Rosen, N., Solish, N., Bertucci, V., Lupin, M., Dansereau, A., . . . Swift, A. (2012). The evolving role of hyaluronic acid fillers for facial volume restoration and contouring: a Canadian overview. Clin. Cosmet. Investig. Dermatol., 5, 147158.

Necas, J., Bartosikova, L., Brauner, P., & Kolar, J. (2008). Hyaluronic acid (hyaluronan): a review. Veterinarni Medicina, 53(8), 374-411.

Nobile, V., Buonocore, D., Michelotti, A., & Marzatico, F. (2014). nti-aging and filling efficacy of six types hyaluronic acid based dermo-cosmetic treatment: double blind, randomized clinical trial of efficacy and safety. J. Cosmet. Dermatol. , 13, 277-287.

Noh, I., Kim, G. C., Kim, M., Park, Y., Lee, K., & al., e. (2006). Effects of cross-linking molecular weights in a hyaluronic acid–poly(ethylene oxide) hydrogel network on its properties. Biomedical Materials,, 1(3), 116–123.

Palumbo, F. S., Pitarresi, G., Mandracchia, D., Tripodo, G., & & Giammona, G. (2006). New graft copolymers of hyaluronic acid and polylactic acid: Synthesis and characterization. Carbohydrate Polymers, 66, 379-385.

Panagopoulou, A., Vázquez Molina, J., Kyritsis, A. M., Vallés Lluch, A., Gallego Ferrer, G., & Pissis, P. (2013). Glass Transition and Water Dynamics in Hyaluronic Acid Hydrogels. Food Biophys, 8, 192-202.

Peer, D., Florentin, A., & Margalit, R. (2003). Hyaluronan is a key component in cryoprotection and formulation of targeted unilamellar liposomes. Biochim. Biophys. Acta-Biomembranes, 1612(1), 76-82.

Pelletier, S., Hubert, P., Payan, E., Marchal, P., Choplin, L., & Dellacherie, E. (2001). Amphiphilic derivatives of sodium alginate and hyaluronate for cartilage repair: rheological properties. J Biomed Mater Res, 54(1), 102-108.

Petrigni, G., & Allegra, L. (2006). Aerosolised hyaluronic acid prevents exercise-induced bronchoconstriction, suggesting novel hypotheses on the correction of matrix defects in asthma. Pulm. Pharmacol. Ther., 19, 166–171.

Platt, V., & Szoka, F. (2008). Anticancer Therapeutics: Targeting Macromolecules and Nanocarriers to Hyaluronan or CD44, a Hyaluronan Receptor. Molecular Pharmaceutics , 54(4), 474-486.

Ponnuraj, K., & Jedrzejas, M. (2000). Mechanism of hyaluronan binding and degradation: Structure of Streptococcus pneumoniaehyaluronatelyase in complex with hyaluronic acid disaccharide at 1.7 Å resolution. Journal of Molecular Biology, 299, 885–895.

Prata, J., Barth, T., Bencherif, S., & Washburn, N. (2010). Complex fluids based on methacrylated hyaluronic acid. Biomacromolecules, 11(3), 769–775. R. Stern, G. K. (s.d.). The many ways to cleave hyaluronan,.

Rahman, I., & Adcock, I. M. (2006). Oxidative stress and redox regulation of lung inflammation in COPD. Eur. Respir. J., 28, 219-242.

Rayle, H. L., & Fellmeth, L. (1999). Development of a process for triazinepromotedamidation of carboxylic acids. Organic Process Research & Development, 3(3), 172–176.

Ré, M., Messias, L., & Schettini, H. (2004). The influence of the liquid properties and the atomizing conditions on the physical characteristics of the spray-dried ferroussulfate microparticles. Drying B , 1174–1181.

Rees, M., Hawkins, C., & Davies, M. (2004). Hypochlorite and superoxidradicals can act synergistically to induce fragmentation ofhyaluronan and chondroitin sulphates. Biochem J.:, 381, 175–184.

Robert, L., Robert, A., & Renard, G. (2010). Biological effects of hyaluronan in connective tissues, eye, skin, venous wall. Pathol. Biol., 58, 187-198.

Robert, L., Robert, A., & Renard, G. (2010). Biological effects of hyaluronan in connective tissues, eye, skin, venous wall. Role in aging. Pathol. Biol. , 58, 187-198.

Romagnoli, M., & Belmontesi, M. (2008). Hyaluronic acid-based fillers: theory and practice. Clinics in Dermatology, 26, 123-159.

Rosiak, J. M., & Yoshii, F. (1999). Hydrogels and their medical applications. Nuclear Instruments and Methods in Physics R. Nuclear Instruments and Methods in Physics Research , B 151, 56-64.

Sakagami, M. (2006). In vivo, in vitro and ex vivo models to assess pulmonary absorption and disposition of inhaled therapeutics for systemic delivery . Advanced Drug Delivery Reviews, 58, 1030-1060.

Sakamoto, A., Matsumaru, T., Yamamura, N., Suzuki, S., Uchida, Y., Tachikawa, M., & Terasaki, T. (2015). Drug transporter protein quantification of immortalized human lung cell lines derived from tracheobronchial epithelial cells (Calu-3 and BEAS2B),

bronchiolar-alveolar cells (NCI-H292 and NCI-H441), and alveolar type II-like cells (A549) by liquid chromat. Journal of Pharmaceutical Sciences,, 104, 30293038.

Sall, I., & Fe´rard, G. (2007). Comparison of the sensitivity of 11 crosslinked hyaluronic acid. Polymer Degr. and Stabil., 92, 915-919.

Schanté, C., Zuber, G., & Herlin, C. V. (2011). Chemical modifications of hyaluronic acid for the synthesis of derivatives for a broad range of biomedical applications. Carbohydr. Polym., 85, 469-489.

Schiraldi, C., La Gatta, A., & De Rosa, M. (2010). Biotechnological Production and Application of Hyaluronan. Elnashar, M. (ed.), Biopolymers.

Schneider, L., Korber, A., Grabbe, S., & Dissemond, J. (2007). Influence of pH on woundhealing: a new perspective for wound-therapy? Arch. Dermatol. Res.

Scott, J., & Heatley, F. (1999). Hyaluronan forms specific stable tertiary structures in aqueous solution: a 13C NMR study. Proc Natl AcadSci U S A, 96, 4850-4855.

Segura, T., Chung, P., & Shea, L. (2005). DNA delivery from hyaluronic acid-collagen hydrogels via a substrate-mediated approach. Biomaterials,, 26(13), 1575–1584.

Seidlits, S., Khaing, Z., Petersen, R., Nickels, J., Vanscoy, J., Shear, J., & Schmidt, C. (2010). The effects of hyaluronic acid hydrogels with tunable mechanical properties on neural progenitor cell differentiation. Biomaterials, 31, 3930–3940.

Shukla, V., Shukla, D., Tiwary, S., Agrawal, S., & Rastogi, A. (2007). Evaluation of pH measurement as a method of wound assessment. Journal of Wound Care, 16.

Silva Bezerra, F., Valença, S., Lanzetti, M., Pimenta, W., Castro, P., V.L., G. K., & Porto, L. (2006). Alpha-tocopherol and ascorbic acid supplementation reduced acute lung inflammatory response by cigarette smoke in mouse. Nutrition, 22, 1192-1201.

Sinhababu, S., & Basu, S. (2013). Modified Ninhydrin Reagents to Detect Colors of Amino Acid Zones on Thin-Layer Chromatographic Plates. Journal of Planar Chromatography , 26(6), 517-520.

Smart, J. (2005). The basics and underlying mechanisms of mucoadhesion. Adv. Drug Deliv. Rev., 57 , 1556-1568.

Sobolewski, K., Bańkowski, E., Chyczewski, L., & Jaworski, S. (1997). Collagen and glycosaminoglycans of Wharton's jelly. Biol. Neonate., 71, 11-21.

Soltés, L., Mendichi, R., Kogan, G., Schiller, J., Stankovska, M., & Arnhold, J. (2006). Degradative action of reactive oxygen species on hyaluronan. Biomacromolecules, 7, 659-668.

Stern, R. (2008). Hyaluronidases in cancer biology. Semin. Cancer. Biol., 18, 275-280.

Stern, R., Kogan, G., Jedrzejas, M., & Šoltés, L. (2007). The many ways to cleave hyaluronan. Biotechnol. Adv., 25(6), 537-557.

Sung, H., Huang, R., Huang, L., Tsai, C., & Chiu, C. (1998). Feasibility study of a natural crosslinking reagent for biological tissue fixation. Journal of Biomedical Materials Research, 42, 560–567.

Tadros, T. (2004). Application of rheology for assessment and prediction of the long-term physical stability of emulsions. Adv. Colloid Interface Sci., 108(109), 227−258.

Tafuro, G., Costantini, A., Baratto, G., Francescato, F., & Semenzato, A. (2020). Evaluating natural alternatives to synthetic acrylic polymers: rheological and texture analyses of polymeric water dispersions. ACS Omega , 5(1), 5280–15289.

Tamer, T. (2013). Hyaluronan and synovial joint: function, distribution and healing. Interdiscip. Toxicol. , 6, 111-125.

Tan, H., Chu, C., Payane., K., & Marra, K. (2009). Injectable in situ forming biodegradable chitosan-hyaluronic acid based hydrogels for cartilage tissue engineering. Biomaterials, 2499-2506.

Toemmeraas, K., & Eenschooten, C. (2007). Aryl/alkyl succinic anhydride hyaluronan derivatives. WO/2007/033677.

Togami, K., Chono, S., & Tada, H. (2016). Alteration in intrapulmonary pharmacokinetics of aerosolized model compounds due to disruption of the alveolar epithelial barriers following bleomycin-induced pulmonary fibrosis in rats. Journal of Pharmaceutical Sciences, 105, 1327-1334.

Tokita, Y., Ohshima, K., & Okamoto, A. (1997). Degradation of hyaluronic acid during freeze drying. Polymer Degradation and Stability, 55(2), 159–164.

Tomihata, K., & Ikada, Y. (1997). Crosslinking of hyaluronic acid with glutaraldehyde. . J. Polym.Sci. Part A: Polym. Chem. , 35, 3553–3559.

Toole, B. (2004). Hyaluronan: from extracellular glue to pericellular cue. Nat. Rev. Cancer, 4, 528-539.

Trommer, H., Wartewig, S., Böttcher, R., Pöppl, A., Hoentsch, J. O., & Neubert, N. (2003). The effects of hyaluronan and its fragments on lipid models exposed to UV irradiation. Int. J. Pharm., 254, 223-234.

Turino, G. M., & Cantor, J. O. (2003). Hyaluronan in Respiratory Injury and Repair. American Journal of Respiratory and Critical Care Medicine, 167(9), 1169–1175.

Turley, E., & Moore, D. (1984). Hyaluronate binding proteins also bind to fibronectin,laminin and collagen. BiochemBiophys. Res. Commun., 121.

Vigetti, D., Karousou, E., Viola, M., Deleonibus, S., De Luca, G., & Passi, A. (2014a). Hyaluronan: biosynthesis and signaling. Biochim.Biophys.Acta, 1840, 2452-2459.

Vigetti, D., Karousou, E., Viola, M., Deleonibus, S., De Luca, G., & Passi, A. (2014b). Hyaluronan: biosynthesis and signaling. BiochimBiophysActa, 1840, 2452-2459.

Volpi, N., Schiller, J., Stern, R., & Soltés, L. (2009). Role, metabolism, chemical modifications and applications of hyaluronan. Curr. Med. Chem., 16, 1718-1745.

Weigel, P., Fuller, G., & LeBoeuf, R. (1986). A model for the role of hyaluronic acid and fibrin in the early events during the inflammatory response and wound healing. Journal of Theoretical Biology, 119(2), 219–234.

Weigel, P., Hascall, V., & Tammi, M. (1997). Hyaluronan synthases. J. Biol. Chem., 272, 13997-14000.

Weiss, C., Abatangelo, G., & Weigei, P. H. (2000). Why viscoelasticity is impornt for the medical usea of hyaluronan and hylans. New frontiers in medical sciences:Redefining hyaluronan, 40, 89-103.

Weissmann, B., & Meyer, K. (1954a). The structure of hyalobiuronic acid and of hyaluronic acid from umbilical cord. J Am Chem Soc, 76, 1753–1757.

Weissmann, B., Meyer, K., Sampson, P., & Linker, A. (1954b). Isolation of oligosaccharides enzymatically produced from hyaluronic acid. J Biol Chem, 208, 417–429.

Weng, J., Tong, H. Y., & Chow, S. F. (2020). In Vitro Release Study of the Polymeric Drug Nanoparticles: Development and Validation of a Novel Method. Pharmaceutics, 12(8), 732.

Witte, M., & Barbul, A. (2003). Arginine physiology and its implication for wound healing. Wound Repair Regen., 11, 419 – 423.

Wouters, E., Groenewegen, K., & Dentener, M. V. (2007). ystemic inflammation in chronic obstructive pulmunary disease: the role of exacerbations. SProc. Am.

Thorac. Soc., 4, 626-634.

Yasuda, K., Armstrong, R., & Cohen, R. (1981). Shear flow properties of concentrated solutions of linear and star branched polystyrenes. Rheol. Acta, 20, 163–178.

Yun, Y., Goetz, D., Yellen, P., & Chen, W. (2004). Hyaluronan microspheres for sustained gene delivery and site specific targeting. Biomaterials, 25(1), 147–157.

Zhang, S., Chang, M. Z., Turley, S., Harrison, R., & EA., T. (1998). The hyaluronan receptor RHAMM regulates extracellular-regulated kinase. J. BiolChem., 273(18), 11342-11348.

7 PUBBLICATION LIST

“Iranian medicinal plants: From Ethnomedicine to Actual Studies.” Piergiacomo Buso, Stefano Manfredini, Hamid Reza Ahmdi-Ashtiani, Sabrina Sciabica, Raissa Buzzi, Silvia Vertuani, Anna Baldisserotto. Medicina 2020, 56 (3), 97.

“Design, synthesis and evaluation of benzothiazole derivatives as multifunctional agents.” Ernestine Nicaise Djuidje, Sabrina Sciabica, Raissa Buzzi, Valeria Dissette, Jan Balzarini, Sandra Liekens, Elena Serra, Elisa Andreotti, Stefano Manfredini, Silvia Vertuani, Anna Baldisserotto. Bioorg Chem. 2020 Aug;101:103960.

“Skin Damages-Structure Activity Relationship of Benzimidazole Derivatives Bearing a 5-Membered Ring System”.
Djuidje EN, Durini E, Sciabica S, Serra E, Balzarini J, Liekens S, Manfredini S, Vertuani S, Baldisserotto A. Molecules. 2020 Sep 21;25(18): 4324.