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Review

Chemical modifications of hyaluronic acid for the synthesis of derivatives for a broad range of biomedical applications

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ABSTRACT

Hyaluronic acid (HA) is widely used for numerous medical applications, such as viscosupplementation, eye surgery and drug delivery. A broad range of HA-based materials have been developed and described for the enhancement, modulation and control of its therapeutic action, based on chemical modification of polysaccharides. The purpose of this paper is to review the various chemical modification methods and synthetic routes to obtain HA derivatives, encompassing all applications.

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1. Introduction

The increasing number of biomedical uses for hyaluronic acid (HA) has encouraged the development of a broad range of HA-based derivatives with enhanced or modulated properties. HA has been the subject of many previous reviews focusing on its biological functions and medical applications (Kogan, Soltés, Stern, & Gemeiner, 2007; Laurent, 1998; Laurent & Fraser, 1992), and, more specifically, on its uses for viscosupplementation (Moreland, 2003), wound healing (Jiang, Liang, & Noble, 2007) and drug delivery (Gaffney, Matou-Nasri, Grau-Olivares, & Slevin, 2010; Oh et al., 2010). The numerous derivatives synthesized for these applications have also been thoroughly reviewed (Larsen & Balazs, 1991; Mori, Yamaguchi, Sumitomo, & Takai, 2004; Vercruysse, Prestwich, & Kuo, 1998). In addition to the extensive literature available, the Glycoforum – Hyaluronan Today website publishes articles on biological aspects of HA, its applications and HA derivatives (http://www.glycoforum.gr.jp/science/hyaluronan/hyaluronanE.html).

The present review focuses on chemical modifications made to HA and their use for the synthesis of HA derivatives. Numerous chemical modifications, aimed at enhancing, modulating or controlling the therapeutic action of HA and developing new products, have been described in the literature. These are performed in different solvents, target different sites on HA and yield different results in terms of modification efficacy and chain length damage. The method should be chosen carefully, based on the starting material and final product characteristics sought.

After a first section presenting the important aspects of HA, the second part of this paper reviews the numerous chemical reactions described for HA modification, indicates the advantages and drawbacks of the methods used and reports recent advances in the field. Subsequently, existing types of HA derivatives are reviewed, focusing on the chemical routes used for their synthesis. The final section describes the different techniques used for the characterization of HA and its derivatives.

2. Hyaluronic acid, its physicochemical properties, biosynthesis, degradation and applications

2.1. Chemical structure and physicochemical properties

HA is a linear polysaccharide made of repeating disaccharide units of D-glucuronic acid and N-acetyl glucosamine linked by β(1,4) and β(1,3) glucosidic bonds (Fig. 1). In physiological conditions, HA is in the form of a sodium salt, therefore negatively charged and referred to as sodium hyaluronate. In these conditions, it is highly hydrophilic, surrounded by a sphere of water molecules linked by hydrogen bonds. The physicochemical properties of HA have been thoroughly investigated and reviewed (Day & Sheehan, 2001; Fouissac, Milas, Rinaudo, & Borsali, 1992; Lapčík, Lapčík, De Smedt, Demeester, & Chabreček, 1998; Laurent & Fraser, 1992).

Its molecular weight can reach 8.10^6 Da, 10^7 Da or 10^8 Da according to different authors and depending on the enzyme that catalyzed its synthesis (Girish & Kemparaju, 2007; Kogan et al., 2007; Stern, Kogan, Jedrzejas, & Šoltés, 2007). Due to such high molecular weight values and strong intermolecular interactions, HA aqueous solutions are highly viscous and shear-thinning. Treatments in alkaline or acidic conditions have been shown to induce hydrolysis of HA chains (Ghosh, Kobal, Zanette, & Reed, 1993; Maleki, Kjøniksen, & Nyström, 2008). Degradation in alkaline con-

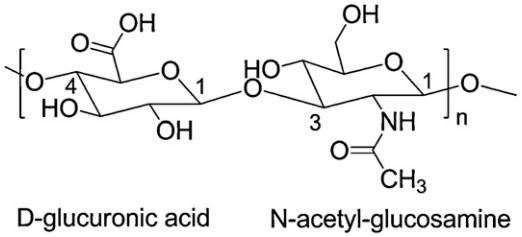

Fig. 1. Chemical structure of hyaluronic acid and target sites for chemical modification.

ditions has been shown to be more marked and faster than in acidic conditions.

2.2. HA occurrence in the organism and physiological functions

HA is naturally present in vertebrate organisms, as well as in bacteria. Its highest occurrence in the human body is in the extracellular matrix (ECM) of connective tissues. HA is especially abundant in the synovial fluid of joints, the dermis of the skin and the vitreous body of the eye (Fraser, Laurent, & Laurent, 1997; Laurent & Fraser, 1992; Robert, Robert, & Renard, 2010).

Owing to its high molecular weight and its capacity to retain a high amount of water, HA's primary role in the body is its structural and hydration role. It maintains an open, hydrated and stable extracellular space in which cells and other ECM components, such as collagen and elastin fibers, are firmly maintained, and acts as a lubricant and shock absorber, especially in joints.

It has been established and confirmed that HA is also involved in cellular activity, including regulation, migration and adhesion (Laurent, Laurent, & Fraser, 1995; Lee & Spicer, 2000). High molecular weight HA exhibits anti-angiogenic and anti-inflammatory properties, whereas low molecular weight fragments (<100 kDa) have the opposite biological activity; they are inflammatory, immuno-stimulatory and angiogenic (Stern, Asari, & Sugahara, 2006). Many authors have reported and reviewed the numerous functions of HA and HA fragments in wound healing (Chen & Abatangelo, 1999; Stern et al., 2006), as well as in tumor growth and cancer proliferation, during which CD44 cell receptors are overexpressed (Stern, 2008; Sugahara et al., 2006; Zhang, Underhill, & Chen, 1995).

2.3. Degradation of HA

It is well known that the half-life of HA after injection into skin and joints is no longer than 24 h (Brown, Laurent, & Fraser, 1991) since HA is naturally degraded in the organism by a complex enzymatic mechanism involving hyaluronidase (Hyal) enzymes and HA cell internalization by CD44 cell surface receptors (Aruffo, Stamenkovic, Melnick, Underhill, & Seed, 1990; Girish & Kemparaju, 2007; Jedrzejas & Stern, 2005). It is assumed that HA degradation is a highly organized and tightly controlled process to generate HA fragments of precisely defined size for the desired biological function. Hyal-1 and Hyal-2 are the most widely expressed hyaluronidases. Molecular modeling has indicated that the recognition sites of the Hyal-2 enzymes and CD44 receptors are the carboxylic groups of HA (Banerji et al., 2007).

HA can also be naturally degraded in the organism by reactive oxygen species (ROS) (Lurie, Offer, Russo, Samuni, & Nitzan, 2003; Stern et al., 2007). The mechanism of HA degradation differs according to the ROS involved and has been previously reviewed in detail (Stern et al., 2007).

2.4. Therapeutic uses

The unique properties of HA have led to its use for an extensive range of medical applications (Kogan et al., 2007). Firstly, HA is administered by injection to compensate for its loss as a result of ageing, disease or surgical procedures. Clinical uses include dermal filling (Athre, 2007; Kablik, Monheit, Yu, Chang, & Gershkovich, 2009), viscosupplementation in deteriorated joints (Moreland, 2003) and ophthalmic surgical aids (Balazs, 2008). For such applications, HA has the advantage of being biocompatible and safe, inducing minimal foreign body reaction.

Owing to its important physiological role in tissue repair as mentioned above, HA is also used for wound healing (King, Hickerson, Proctor, & Newsome, 1991; Lin, Matsumoto, Kuroyanagi, & Kagawa, 2009). Hyalofill®, Hyalogran®, and Ialuset® are examples of wound dressings or bandages impregnated with HA solutions. Both HA and hyaluronidases have also been reported to be used as tumor markers for different cancer types, owing to their role in tumor growth and proliferation (Auvinen et al., 2000; Franzmann et al., 2003).

Today HA is also being investigated for drug delivery purposes. The internalization of HA by cells through CD44 receptors during its enzymatic degradation enables intracellular delivery of drugs via conjugation to HA or entrapment in HA particles (Esposito, Menegatti, & Cortesi, 2005; Peer & Margalit, 2004a; Platt & Szoka, 2008). Such systems have been shown to considerably enhance drug solubility, absorption and/or efficacy (Drobnik, 1991; Larsen & Balazs, 1991; Oh et al., 2010; Vercruysse et al., 1998) and have been specifically studied for targeting anticancer drugs to tumor cells over-expressing CD44 receptors (Hua, Knudson, & Knudson, 1993; Platt & Szoka, 2008).

2.5. Extraction or synthesis of HA

HA was initially isolated from bovine vitreous humor and later from rooster combs (Shiedlin et al., 2004) and human umbilical cord. Advances in biotechnology have led to the development of genetically modified bacteria producing high yields of HA and today, most commercial products are derived from *Streptococcus equi* (Restylane®, Juvederm®, etc.), which avoids the risk of contamination by animal pathogens. Efforts are increasingly focusing on the production of bacterial HA using high-yield and less costly methods with effective purification techniques (Rangaswamy & Jain, 2008).

Research has also been conducted on the preparation of HA fragments with specific uniform sizes by controlling the degradation of high molecular weight HA using various techniques, including acidic, alkaline, ultrasonic and thermal degradation. These techniques are reviewed in detail (Stern et al., 2007). Another approach to obtain HA oligosaccharides of defined length has been their production by chemoenzymatic synthesis (DeAngelis, 2008). This method has led to the commercialization of monodisperse HA oligomers under the name Select-HA™ (Hyalose LLC) with low polydispersity index values.

3. Various chemical methods used for the synthesis of HA derivatives

HA can be chemically modified in two different ways: cross-linking or conjugation. HA conjugation and HA cross-linking are based on the same chemical reactions and only differ in that, in the first case, a compound is grafted onto one HA chain by a single bond only, whereas in the second case, different HA chains are linked together by two bonds or more, as depicted in Fig. 2. In addition, there are different types of crosslinking procedures: direct crosslinking, crosslinking of HA derivatives and crosslinking of different HA derivatives. The chemical modification of HA can be performed on the two available functional sites of HA: the carboxylic acid group and the hydroxyl group (Fig. 1). An amino group can also be recovered by deacetylation of the N-acetyl group. It is not known which of the hydroxyl groups reacts, though it is reasonable to assume that the reaction occurs mainly on the hydroxyl of the C6 of the N-acetylglucosamine moiety of HA because of the better accessibility of reagents to primary alcohols.

Numerous methods have been reported for HA crosslinking or conjugation. Some methods are performed in water while others, since they use reagents sensitive to hydrolysis, need to be performed in organic solvents, such as dimethylformamide (DMF) or dimethylsulfoxide (DMSO). In this case, the native HA sodium salt first needs to be converted into either its acidic form or a tetra-

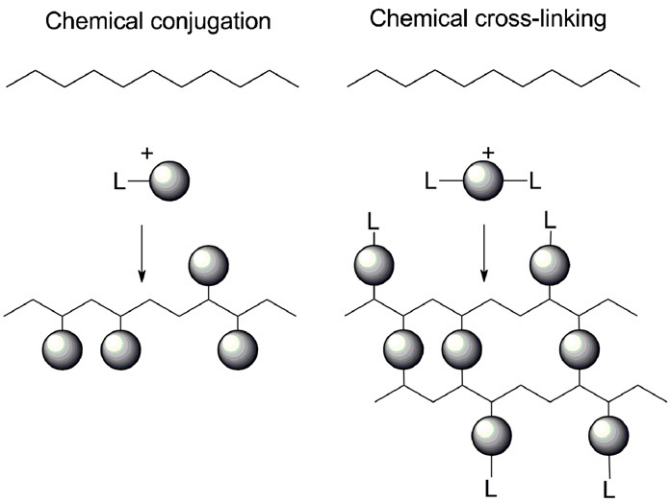

Fig. 2. Chemical conjugation and chemical cross-linking of a polymer.

butylammonium (TBA) salt for solubilization in organic solvents. This requires an additional step, increasing the chances of HA chain fragmentation associated with chemical and physical treatments (Bergman, Elvingson, Hilborn, Svensk, & Bowden, 2007; Pelletier, Hubert, Lapicque, Payan, & Dellacherie, 2000). Since HA is soluble in water, the easiest method is to perform the reaction in water. However, in aqueous conditions, some reactions are pH-dependent and need to be performed in acidic or alkaline conditions, which have been shown to induce significant HA chain hydrolysis (Maleki et al., 2008).

These aspects have encouraged research teams to explore new synthetic routes for the development of HA derivatives with appropriate characteristics according to their specific needs. In some cases, the effectiveness of the reaction is not a criteria, as low substitution or crosslinking degrees are sufficient for the desired effect (Yeom et al., 2010), whereas for other applications, high degrees of substitutions are required (Schneider et al., 2007).

This section reviews the different chemical techniques described in the literature for the modification of the three target sites of HA. Table 1 summarizes all the chemical modification techniques described in this section.

3.1. Modification of the –COOH

3.1.1. Amidation

3.1.1.1. Amidation with carbodiimides. Amidation in water with carbodiimides is one of the most widely used methods for HA modification (Bulpitt & Aeschlimann, 1999; Oh et al., 2010; Prestwich, Marecak, Marecek, Vercruysse, & Ziebell, 1998; Vercruysse et al., 1998). The carbodiimide used is predominantly 1-ethyl-3-[3-(dimethylamino)-propyl]-carbodiimide (EDC) for its water solubility. Danishefsky and Siskovic (1971) were the first to convert the carboxyl groups of polysaccharides including HA into amides. They used EDC at pH 4.75 to activate the carboxylic groups, which then reacted with an amino-acid ester. The presence of amide bonds was detected by infrared, chromatography and electrometric titration, and a substitution degree of 38.6% was calculated.

The reaction mechanism has been studied in detail (Nakajima & Ikada, 1995). The first step of the amidation reaction starts with the activation by EDC of the HA carboxylic acid, which forms an O-acyl isourea intermediate. The second step of the reaction is the nucleophilic attack by the amine on the activated HA, which leads to the formation of the amide bond (Fig. 3a). However, the O-acyl isourea intermediate is highly reactive and also reacts with water, in which case it quickly rearranges into a stable N-acyl urea by-product, thus preventing any further reaction with the amine.

The reaction is very delicate as it is strongly pH-dependent and the optimal pH for both steps is different. Indeed, carboxylic acid activation by EDC is best performed in an acidic environment (pH 3.5–4.5) (Nakajima & Ikada, 1995), whereas amide formation is best done at high pH, when the amine is deprotonated. At such a high pH, EDC is more rapidly hydrolyzed into the N-acyl urea by-product and no amidation can occur. The compromise is therefore not easy to define and amines with high pK_a values are not easily conjugated to HA using this method.

Even though most authors have shown evidence of amidation (Danishefsky & Siskovic, 1971; Follain, Montanari, Jeacomine, Gambarelli, & Vignon, 2008; Nakajima & Ikada, 1995), Kuo, Swann, and Prestwich (1991) contradicted all previous literature by demonstrating that no amide linkage was formed between the carboxylic acid groups of HA and amino groups. Only the N-acylurea by-product was obtained using the same pH of 4.75 as previous authors. Indeed, at this pH value, the protonated amine is not as nucleophilic and does not react easily with the activated HA.

On the basis of this finding, Kuo et al. proposed using carbodiimides not as an activator but as the reagent itself. Thus biscarbodiimides were employed to crosslink HA and form stable bis(N-acylurea) cross-linked gels (Fig. 3b).

Replacing diamines by dihydrazides, which have much lower pK_a values of 2–3, made it possible to obtain higher coupling degrees, of up to 56% (Pouyani & Prestwich, 1994a). By adding a large excess of adipic dihydrazide (ADH), no cross-linking was observed but only single functionalization and dihydrazide-bonds were formed (Fig. 3c).

In order to prevent the formation of the irreversible N-acylurea by-product, Bulpitt and Aeschlimann (1999) reported the use of N-hydroxysuccinimide (NHS) or 1-hydroxybenzotriazole (HOBt) with EDC to form more hydrolysis-resistant and non-rearrangeable intermediates. NHS or HOBt reacts with the O-acyl isourea and the resulting activated HA intermediate undergoes nucleophilic attack by the amine. The mechanisms are shown in Fig. 3d. Esters of HOBt led to higher degrees of substitution than esters of NHS, suggesting greater reactivity towards the amine.

Amidation using EDC has the advantage of being able to be performed in water from the native HA sodium salt used, without previous handling. In addition, this method does not lead to cleavage of the HA chain according to the authors cited above and therefore maintains its high molecular weight responsible for its valuable viscoelastic properties. However, reagents need to be added in large quantities as some hydrolysis of EDC cannot be avoided and the amine is mostly protonated at the required pH reaction range.

Some authors have performed the same amidation reaction but replacing water with DMSO (Bulpitt & Aeschlimann, 1999; Schneider et al., 2007). This way, degrees of substitution of up to 60–80% were obtained, suggesting that EDC hydrolysis was minimized. However, HA first needs to be converted from its native sodium salt form into its acidic form to be soluble in the organic solvent.

No breakthroughs have been published since then concerning amidation using carbodiimides. Authors have used the methods described previously with EDC and NHS or HOBt to synthesize novel derivatives through the introduction of new functional compounds or by adapting the known methods to more complex systems, such as liposomes (detailed in the next section of this review).

3.1.1.2. Amidation with 2-chloro-1-methylpyridinium iodide (CMPI). Magnani, Rappuoli, Lamponi, and Barbucci (2000) described an amidation reaction using 2-chloro-1-methylpyridinium iodide

Table 1
Chemical modifications of hyaluronic acid.

HA target site	Reaction type	Activator	Reagents	Solvent	References
–COOH	Amidation	Carbodiimides	EDC, NHS	Water (pH 4.75–7.5) or DMSO	Danishefsky and Siskovic (1971), Vercruysse et al. (1998), Prestwich et al. (1998), Bulpitt and Aeschlimann (1999), Oh et al. (2010), Pouyani and Prestwich (1994a), Bulpitt and Aeschlimann (1999) and Schneider et al. (2007)
		CMPI	CMPI, triethylamine	DMF or DMSO	Magnani et al. (2000), Della Valle (1994), Young et al. (2004) and Della Valle and Romeo (1989)
		CDMT	CDMT, NMM	Water/acetonitrile	Bergman et al. (2007)
		1,1′-Carbonyl-diimidazole	1,1′-Carbonyldiimidazole	DMSO	Bellini and Topai (2000) and Borzacchiello et al. (2010)
	Ugi condensation		Formaldehyde, diamine, cyclohexyl isocyanide	Water (pH 3)	De Nooy et al. (2000), Crescenzi et al. (2003a, 2003b) and Maleki et al. (2007)
	Esterification	Diazomethane	Trimethylsilyl diazomethane, acetic acid	DMSO	Jeanloz and Forchielli (1950) and Hirano et al. (2005)
		Alkyl halides	Alkyl iodides or bromides	DMSO	Della Valle and Romeo (1986) and Pelletier et al. (2000)
		Tetraethylene glycol tosylate	Tetraethylene glycol tosylate	DMSO	Huin-Amargier et al. (2006)
		Bisepoxides	Butanediol-diglycidyl ether	Water (acetic acid, pH 2–5)	De Belder and Malson (1986) and Tomihata and Ikada (1997a)
	Oxidation	Sodium periodate	Sodium periodate	Water	Jia et al. (2004) and Glass et al. (1996)
–OH	Ether formation	Bisepoxides	1,2,3,4-Diepoxybutane	Water (0.2 M NaOH, pH > 13)	Laurent et al. (1964)
			Butanediol-diglycidyl ether	Water (0.25 M NaOH, pH > 13)	Malson and Lindqvist (1986) and Piron and Tholin (2002)
			Ethyleneglycol diglycidyl ether and polyglycerol polyglycidylether	Water (1 M NaOH, pH 14)	Yui et al. (1992)
			Epichlorohydrin or diepoxyoctane	Water (pH 10 then pH 4)	Zhao (2000)
		Divinyl sulfone	Divinyl sulfone	Water (0.2 M NaOH, pH > 13)	Balazs and Leshchiner (1968), Collins and Birkinshaw (2007), Ramamurthi and Vesely (2002) and Eun et al. (2008)
		Ethylenesulfide	Ethylenesulfide, dithiolthreitol (DTT)	Water (pH 8.5–10)	Serban et al. (2008)
	Hemiacetal formation	Glutaraldehyde	Glutaraldehyde	Water (pH 2)	Tomihata and Ikada (1997b), Crescenzi et al. (2003a, 2003b) and Collins and Birkinshaw (2007)
	Esterification	Alkyl succinic anhydrides	Octenyl succinic anhydride	Water (pH 9)	Toemmeraas and Eenschooten (2007) and Eenschooten et al. (2010)
		Acyl-chloride activated carboxylate		DMSO	Pravata et al. (2008)
		Methacrylic anhydride		Water (pH 8–10)	Seidlits et al. (2010)
	Carbamate formation	Cyanogen bromide (CNBr)		Water (pH 9–10)	Mlčochová et al. (2006) and Chytil and Pekař (2009)
–$NHCOCH_3$	Deacetylation/amidation	Hydrazine sulfate		Water followed by DMSO	Bulpitt and Aeschlimann (1999), Bellini and Topai (2000), Dahl et al. (1988), Crescenzi et al. (2002) and Oerther et al. (2000)

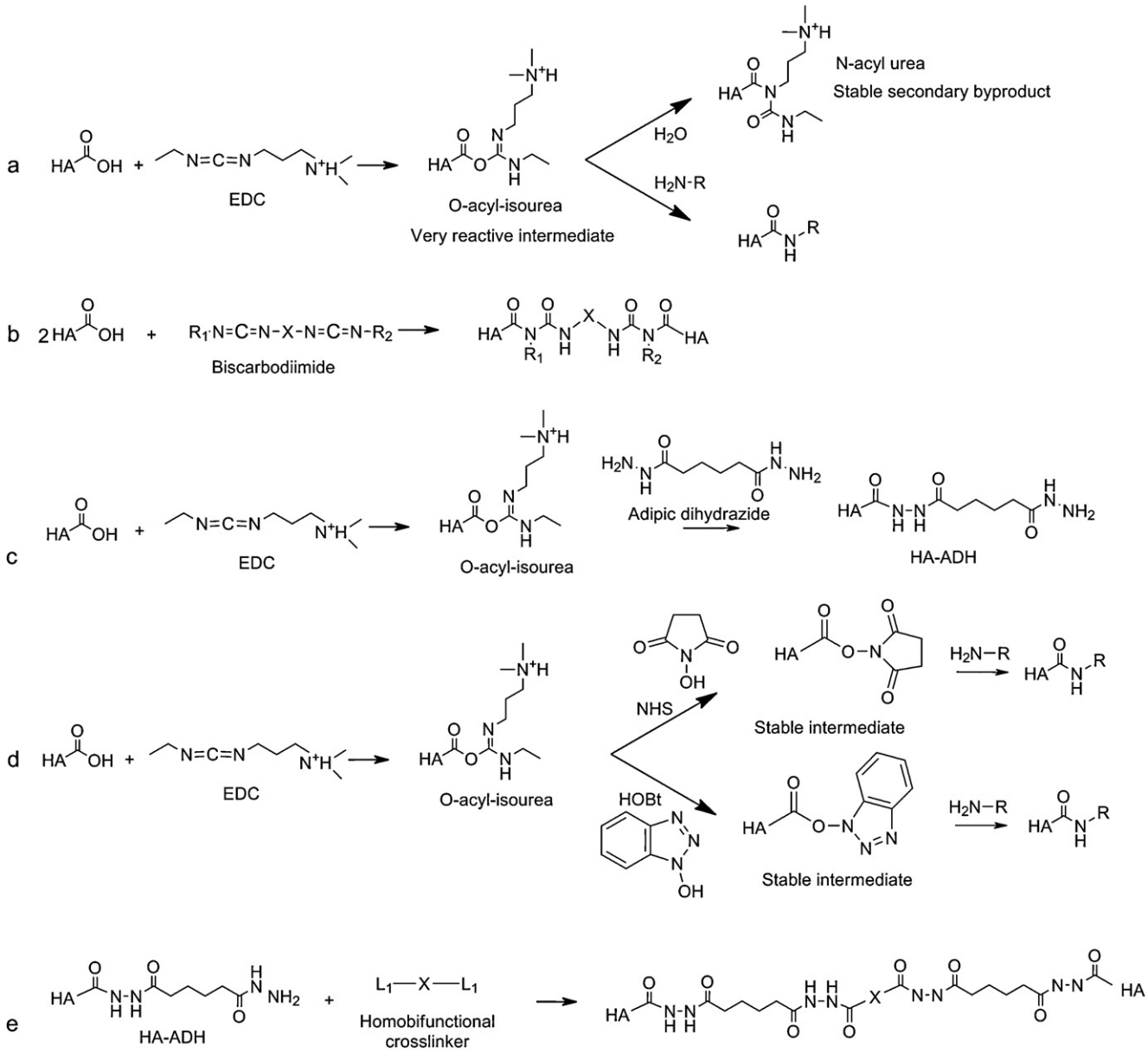

Fig. 3. HA amidation mechanism with EDC (Nakajima & Ikada, 1995) (a), crosslinking of HA with biscarbodiimides (Kuo et al., 1991) (b), amidation of HA with EDC and adipic dihydrazide (Pouyani & Prestwich, 1994a) (c), with EDC/NHS and EDC/HOBt (Bulpitt & Aeschlimann, 1999) (d), HA crosslinking with a homobifunctional crosslinker (Bulpitt & Aeschlimann, 1999) (e).

(CMPI) as the activating agent of the carboxyl groups of HA. This reaction is performed in dimethylformamide (DMF), an anhydrous organic solvent, to minimize CMPI hydrolysis. The HA sodium salt must first be converted into a tetrabutylammonium (TBA) salt to allow its solubilization in the organic solvent. 1,3-Diaminopropane was used to form crosslinks between the HA chains. Firstly, CMPI reacts with a carboxyl group of HA, forms a pyridinium intermediate and releases a chloride ion, which is neutralized by tetrabutylammonium. The nucleophilic diamine then attacks the activated HA carboxyl and forms the amide bond (Fig. 4a). Triethylamine neutralizes the iodide ion released. The drawback to this method is its need to be performed in an organic solvent, which requires a longer purification process and the addition of a preparation step to convert the HA sodium salt in its TBA salt. However, ^{13}C NMR studies showed that the calculated crosslinking degree was similar to the theoretical value, suggesting that 100% of the CMPI reacted with the corresponding carboxylic acid sites. This method is therefore highly effective as it uses low amounts of reagents compared to the previous methods with carbodiimides.

When no amine is added to the reaction medium, esterification occurs as the CMPI-activated HA reacts with its own hydroxyl groups, forming an ester crosslink between the HA chains (Fig. 4b). Such gels are called auto-crosslinked gels. Della Valle patented the procedure performed in dimethyl sulfoxide (DMSO) (Della Valle, 1994; Della Valle & Romeo, 1989). However, this reaction is not as quick as amidation as hydroxyl groups are less nucleophilic than amino groups. The CMPI-activated carboxyl groups of HA can also react with a non-activated carboxyl group but the resulting unstable anhydride subsequently reacts with a hydroxyl group to form the same ester crosslink. The unique feature of auto-crosslinking compared to other crosslinking techniques is that no bridge molecules are present between the crosslinked HA chains. This ensures that only the natural components of HA are released during its degradation in the organism. Young, Cheng, Tsou, Liu, and

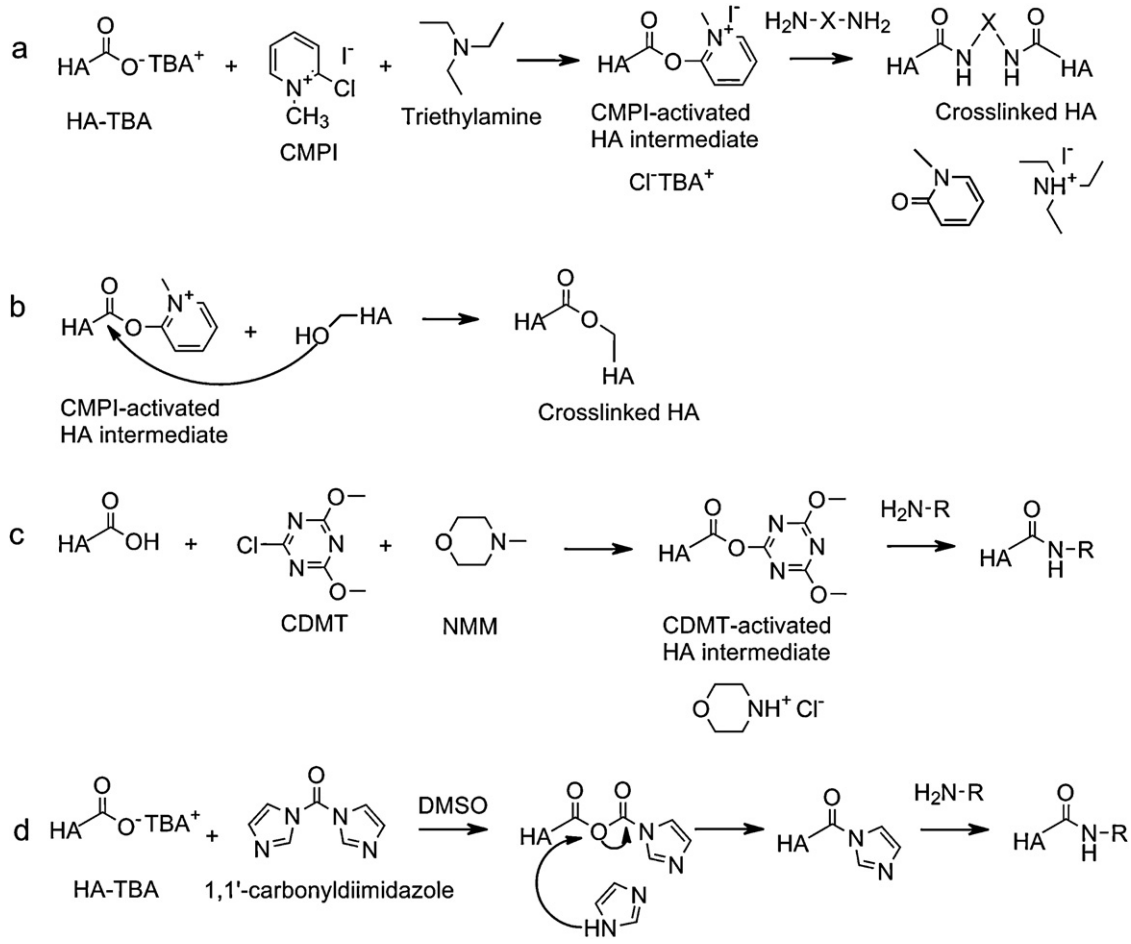

Fig. 4. Crosslinking reaction of HA with amines using CMPI (Magnani et al., 2000) (a), using CMPI alone (Della Valle, 1994) (b), amidation with CDMT (Bergman et al., 2007) (c), with carbonyldiimidazole (Bellini & Topai, 2000) (d).

Wang (2004) showed that crosslinked HA hydrogels of this type were much more rigid and resistant to *in vitro* enzymatic degradation when using CMPI rather than EDC, confirming the higher reactivity of CMPI.

3.1.1.3. Amidation with 2-chloro-dimethoxy-1,3,5-triazine (CDMT). Bergman et al. (2007) recently described a novel method for HA amidation using 2-chloro-dimethoxy-1,3,5-triazine (CDMT) as the activating agent of the carboxylic groups. The reaction is performed in a mixed solvent with water and acetonitrile (3:2) for an optimal solubilization of the reagents. No intermediate is needed for this amidation reaction in a water mixture. Firstly, CDMT reacts with the carboxylic acid to form a CDMT-activated HA intermediate. N-methylmorpholinium (NMM) is added to the mixture to neutralize the chloride ions which are formed. The CDMT-activated HA intermediate then reacts with the amine to form the amide bond (Fig. 4c). Substitution degrees of up to 25% were obtained using only 2:1 of HA:CDMT amounts and the authors suggest that higher degrees can be obtained by increasing the amount of CDMT. This is a promising method to obtain high grafting yields while performing the reaction in an aqueous mixed solvent and in mild conditions.

3.1.1.4. Amidation with carbonyldiimidazole. HA amidation with 1,1′-carbonyldiimidazole as the activating agent of the HA carboxyl groups was patented by Fidia (Bellini & Topai, 2000). The reaction described is performed in DMSO from HA–TBA salt. Carbonyldiimidazole reacts with HA to form a highly reactive intermediate which quickly rearranges into a more stable HA–imidazole intermediate. This last intermediate reacts with an amine to form the amide bond (Fig. 4d). The described procedure is long compared to the previous methods which are usually performed overnight, as the formation of the imidazole intermediate takes 12 h followed by a 48-h amidation reaction. However, the reaction does not release a strong acid but only CO_2 and imidazole, which are non-toxic compounds.

3.1.2. Ugi condensation

Several authors have described Ugi condensation for HA crosslinking (Crescenzi, Francescangeli, Capitani, et al., 2003; Crescenzi, Francescangeli, Taglienti, Capitani, & Mannina, 2003; De Nooy, Capitani, Masci, & Crescenzi, 2000; Maleki, Kjøniksen, & Nyström, 2007). The method uses a diamine as a cross-linker to form diamide linkages between the polysaccharide chains. The reaction is performed in water at pH 3 with formaldehyde, cyclohexyl isocyanide and the diamine. First of all, the diamine condenses with formaldehyde to form a protonated diimine which then reacts with the cyclohexyl isocyanide. The carboxyl group of HA then eliminates the activated cyanide intermediate to form an (acylamino) amide bond (Fig. 5a). The use of formaldehyde, which is known to be carcinogenic, requires specific handling. However, this method leads to the formation of a secondary amide, adding a second pending group, in this case, a cyclohexyl.

3.1.3. Ester formation

3.1.3.1. Ester formation by alkylation using akyl halides. Della Valle and Romeo (1986) patented esterification by alkylation of HA carboxylic groups using alkyl halides, such as alkyl iodides or bromides

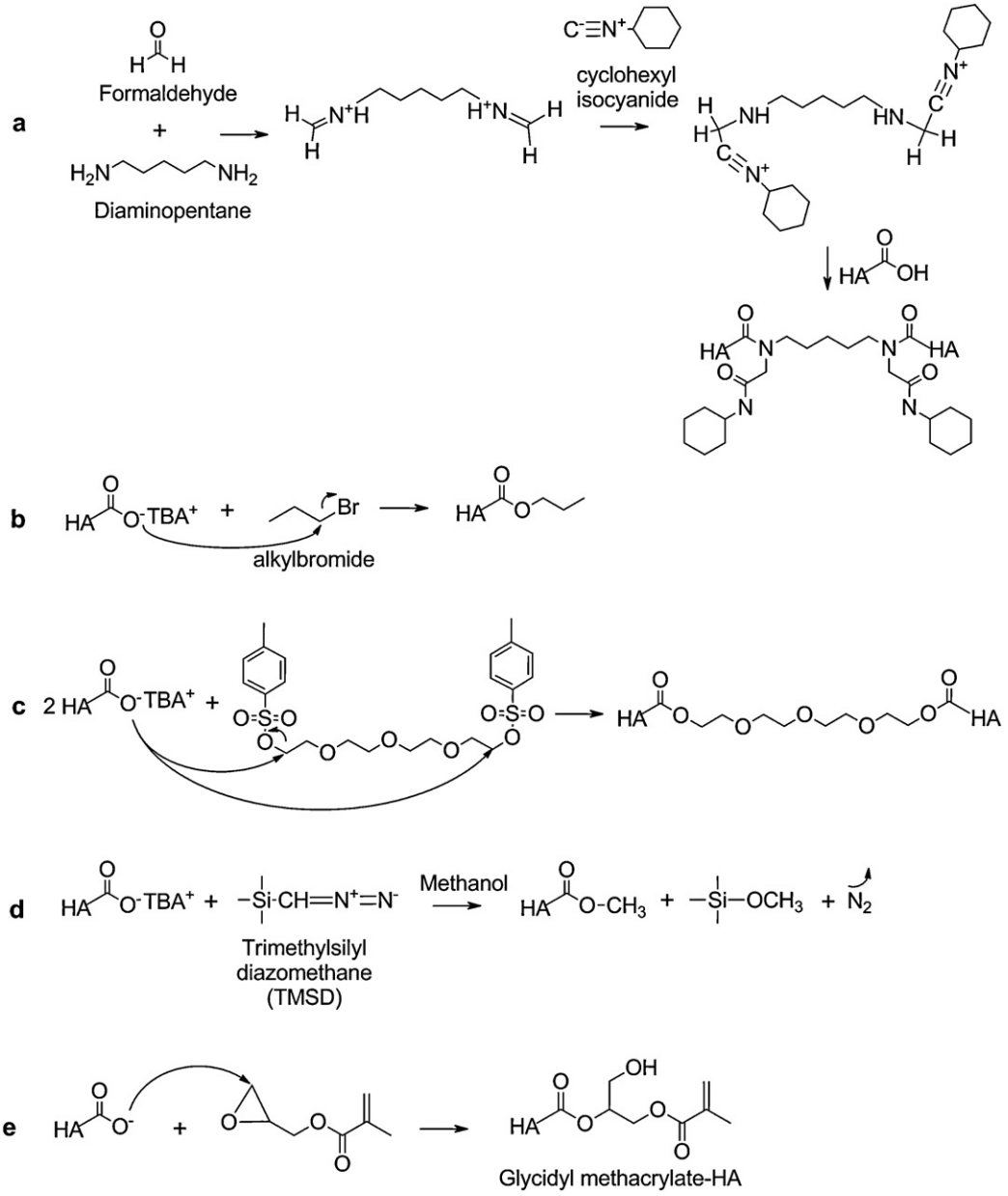

Fig. 5. Ugi condensation reaction (De Nooy et al., 2000) (a), esterification using alkyl halides (Della Valle & Romeo, 1986) (b), esterification with tetraethylene glycol ditosylate (Huin-Amargier et al., 2006) (c), esterification using trimethylsilyl diazomethane (Hirano et al., 2005) (d), esterification with glycidyl methacrylate (Bencherif et al., 2008) (e).

(Fig. 5b). The reaction was performed over a period of 12 h at 30 °C. Pelletier et al. (2000) synthesized amphiphilic HA esters using the same method with bromide alkyls, performing the reaction over 24 h. The reaction has to be performed in DMSO, meaning that the native HA sodium salt has to be first converted into its TBA salt.

3.1.3.2. Ester formation by alkylation using tosylate activation. Another example of the same esterification chemistry is using tosylate as the leaving group, as described by Huin-Amargier, Marchal, Payan, Netter, and Dellacherie (2006). HA crosslinking was performed by esterification using tetraethylene glycol functionalized by two tosylate groups (Fig. 5c). The reaction is performed in DMSO from the TBA salt of HA.

3.1.3.3. Ester formation using diazomethane. Jeanloz and Forchielli (1950) were the first to report the esterification of HA using diazomethane. The reaction is performed in an organic solvent, DMSO, from HA–TBA salt. Hirano et al. (2005) described the preparation of methyl ester of HA using trimethylsilyl diazomethane (TMSD) as the carboxylic group activator. TMSD reacts with HA to form an intermediate which then reacts with acetic acid to recover the methyl ester (Fig. 5d).

3.1.3.4. Ester formation using epoxides. Several authors have described the reaction of HA with glycidyl methacrylate to synthesize methacrylated HA (Bencherif et al., 2008; Leach, Bivens, Patrick, & Schmidt, 2003; Prata, Barth, Bencherif, & Washburn, 2010; Weng, Gouldstone, Wu, & Chen, 2008). The reaction is performed in water in the presence of excess triethylamine as a catalyst. Bencherif et al. (2008) suggest that the reaction occurs mainly on the carboxylic groups of HA and that the secondary trans-esterification on the hydroxyl groups is reversible (Fig. 5e).

3.2. Modifications of the –OH

3.2.1. Ether formation

3.2.1.1. Ether formation using epoxides. Laurent, Hellsing, and Gelotte (1964) were the first to report HA crosslinking. They used 1,2,3,4-diepoxybutane as the crosslinking agent and performed the reaction in strong alkaline conditions at pH 13–14 (0.2 M NaOH and 0.1% sodium borohydride) and at 50 °C for 2 h. Malson and Lindqvist (1986) patented the crosslinking of HA using butanediol-diglycidyl ether (BDDE) in a 0.25 M NaOH solution (Malson & Lindqvist, 1986). Piron later improved the method by mixing BDDE in the 0.25 M NaOH solution before adding it to the HA powder for a more homogeneous hydrogel (Piron & Tholin, 2002). The reaction consists of the epoxide ring opening to form ether bonds with the HA hydroxyl groups (Fig. 6a). Other bisepoxides have been used to prepare crosslinked HA gels, such as ethylene glycol diglycidyl ether and polyglycerol polyglycidyl ether (Yui, Okano, & Sakurai, 1992).

When HA is subjected to such high pH values (pH > 13) above the pK_a value of the hydroxyl groups (approximately 10), the latter are almost all deprotonated and are thus more nucleophilic than the deprotonated carboxyl groups. The epoxides therefore react preferentially with the hydroxyl groups to form ether bonds. However, when the pH is lower than the pK_a value of the hydroxyl group, a smaller quantity of hydroxyl groups is deprotonated and the anionic carboxyl group is predominant, thus promoting ester bond formation. This has been demonstrated by De Belder and Malson (1986), who performed the crosslinking of HA with BDDE in acidic conditions (pH 2–4.5) (Fig. 6b).

Tomihata and Ikada (1997a), however, observed the formation of ether and not ester bonds, even when the crosslinking with bisepoxides was performed in slightly acidic conditions. This was probably due to the pH values used (4.7, 6.1 and 8), at which a higher quantity of hydroxyl groups is deprotonated than at pH values lower than 4.5.

Today, BDDE is used for most crosslinked HA hydrogels currently on the market. In addition to easy synthesis, HA–BDDE degradation products have not demonstrated any cytotoxicity and epoxide compounds are hydrolyzed into simple diols (Nishi, Nakajima, & Ikada, 1995).

Zhao (2000) patented a double cross-linking method based on the consecutive reaction of epoxides in alkaline conditions (pH 10) followed by acidic conditions (pH 4). The epoxides used were epichlorohydrin and 1,2,7,8-diepoxyoctane.

3.2.1.2. Ether formation using divinyl sulfone. HA crosslinking with divinyl sulfone (DVS) was patented by Balazs and Leshchiner (1968). The reaction is performed at high pH values (0.2 M NaOH, pH > 13) and creates sulfonyl bis-ethyl linkages between the hydroxyl groups of HA (Fig. 6c). This crosslinking method has the advantage of occurring at room temperature, which limits the degradation of HA in alkaline solutions compared to higher temperatures. Balazs and Leshchinger showed that the reaction starts shortly after addition of DVS (5–10 min) and 1 h is sufficient for the completion of the reaction. They also found that the presence of salts such as NaCl in the reaction medium increased the crosslinking degree. Studies by other authors confirmed the efficiency of the crosslinking method with DVS (Collins & Birkinshaw, 2007; Ramamurthi & Vesely, 2002). However, Eun et al. (2008) showed that HA–DVS crosslinked gels degraded more rapidly than HA–ADH-BS[3] hydrogels crosslinked by the carboxylic groups of HA. Even though the starting material DVS is highly reactive and toxic, the biocompatibility of the HA–DVS hydrogels was confirmed by histological analysis.

3.2.1.3. Ether formation using ethylene sulfide. Serban, Yang, and Prestwich (2008) used ethylene sulfide (also known as thiirane) to synthesize 2-thioethyl ether HA derivatives (Fig. 6d). The ethylene sulfide ring is opened by a nucleophilic attack of the HA hydroxyl group with the addition of dithiothreitol (DTT). The reaction is performed at pH 10 overnight and at pH 8.5 after addition of DTT over a period of 24 h. The authors state that if the carboxylic group of HA reacts with ethylene sulfide, an unstable intermediate is formed, which rearranges into a carboxylic group and reforms ethylene sulfide. The presence of the grafted thiol groups, which cannot undergo further crosslinking, has been shown to have a radical scavenger action, protecting cells from reactive oxygen species.

3.2.2. Hemiacetal formation using glutaraldehyde

Several authors have used glutaraldehyde (GTA) for HA crosslinking (Crescenzi, Francescangeli, Taglienti, et al., 2003; Tomihata & Ikada, 1997b). Tomihata and Ikada demonstrated the formation of hemiacetal bonds between the hydroxyl groups of HA (Fig. 6e) by IR measurements. By using the same procedure on a polymer which contained only hydroxyl groups, the crosslinked gel was also formed (Tomihata & Ikada, 1997b). The authors observed that the reaction could be performed in an acetone–water medium but not in an ethanol–water medium, suggesting inhibition of the crosslinking reaction caused by the side reaction with the hydroxyl groups of ethanol. Glutaraldehyde crosslinking needs to be initiated in an acidic medium (pH 2) to activate the aldehyde and catalyze the reaction. However, the hemiacetal bond can be hydrolyzed and recover the starting materials in acidic conditions. Indeed, Collins and Birkinshaw (2007) demonstrated GTA crosslinking to be unstable and were able to stabilize the hydrogel by neutralizing it through swelling in buffer. Glutaraldehyde has the disadvantage of being toxic, requiring specific handling during the reaction and thorough purification of the final product.

3.2.3. Ester formation

3.2.3.1. Ester formation using octenyl succinic anhydride (OSA). Toemmeraas and Eenschooten (2007) patented HA modification using alkyl succinic anhydrides, such as octenyl succinic anhydride (OSA), under alkaline conditions (pH 9) in water. The hydroxyl groups of HA react with the anhydride to form ester bonds (Fig. 6f). Eenschooten, Guillaumie, Kontogeorgis, Stenby, and Schwach-Abdellaoui (2010) later optimized the reaction parameters using an experimental design. Values of 43% of substitution could be obtained using 50 times more OSA than HA. However, the reaction was quite fast, with a substitution rate of 18% being obtained after only 6 h.

3.2.3.2. Ester formation with activated compounds. Pravata et al. (2008) described a novel method for grafting an acyl-chloride activated carboxylate compound onto the hydroxyl groups of HA to form ester bonds (Fig. 6g). The carboxyl groups of the compound to be grafted were first activated by chloroacylation with thionyl chloride and reacted at room temperature with HA in an organic solvent. In their study, the authors used this method to graft poly(lactic acid) (PLA) oligomers. As the reaction was performed in an organic solvent (DMSO), HA was previously converted to a cetyltrimethylammonium bromide (CTA) salt. HA–CTA was more hydrophobic and easier to prepare than HA–TBA using a one-step reaction with CTA-bromide.

3.2.3.3. Ester formation with methacrylic anhydride. HA esterification with methacrylic anhydride was performed to obtain methacrylated HA (Fig. 6h) (Burdick, Chung, Jia, Randolph, & Langer, 2005; Seidlits et al., 2010; Smeds & Grinstaff, 2001). The reaction is performed in ice cold water for 12 h at pH 8–10. The presence of methacrylate groups enabled further photo-crosslinking of the HA derivatives, as described in Section 4.1.4.

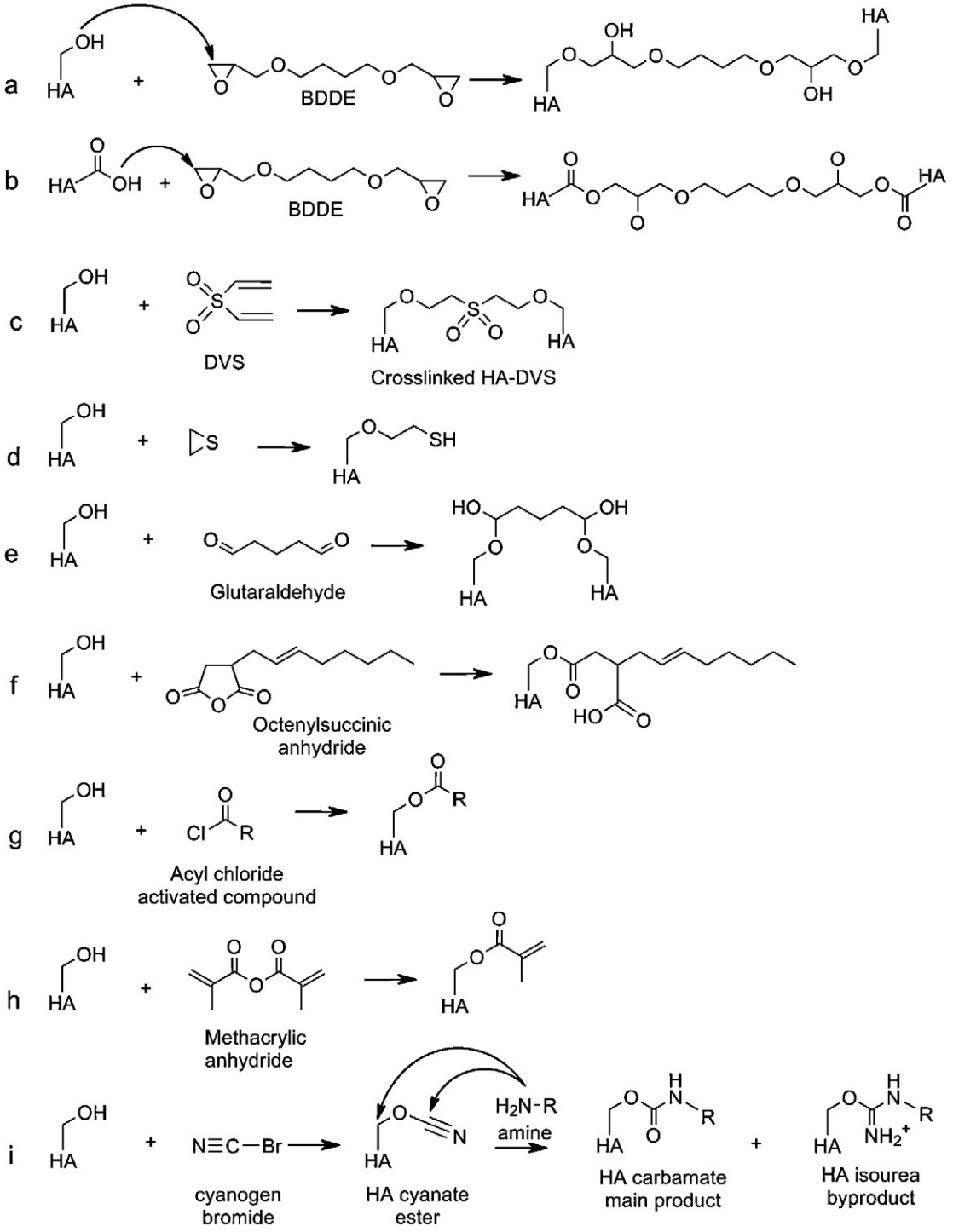

Fig. 6. HA crosslinking with BDDE in alkaline conditions (Malson & Lindqvist, 1986) (a), in acidic conditions (De Belder & Malson, 1986) (b), crosslinking with divinyl sulfone (Balazs & Leshchiner, 1968) (c), HA modification with ethylene sulfide (Serban et al., 2008) (d), crosslinking with glutaraldehyde (Tomihata & Ikada, 1997b) (e), modification with octenyl succinic anhydride (Toemmeraas & Eenschooten, 2007) (f), with an acyl chloride activated compound (Pravata et al., 2008) (g), with methacrylic anhydride (Seidlits et al., 2010) (h), activation with cyanogen bromide and carbamate formation (Mlčochová et al., 2006) (i).

3.2.4. Carbamate formation

Mlčochová et al. (2006) describe the synthesis of HA derivatives using cyanogen bromide (CNBr) to activate the hydroxyl groups of HA. An activated HA cyanate ester is formed, which reacts with the amine to form mainly N-substituted carbamate bonds and an HA–isourea secondary product (Fig. 6i). This reaction has the advantage of being performed in water using the native HA sodium salt. High degrees of substitution of up to 80% were achieved using only a slight excess of reagents and in a reaction time of only one hour. However, a high pH (up to 10) is needed for the coupling to occur, inducing a reduction in the molecular weight of the HA polymer chain. Chytil and Pekař (2009) also used this method to synthesize a wide range of HA derivatives with tunable properties.

3.2.5. Oxidization with sodium periodate

Aldehyde groups were added to HA after reaction with sodium periodate, which oxidizes the hydroxyl groups of the D-glucuronic acid moiety of HA to dialdehydes, thereby opening the sugar ring

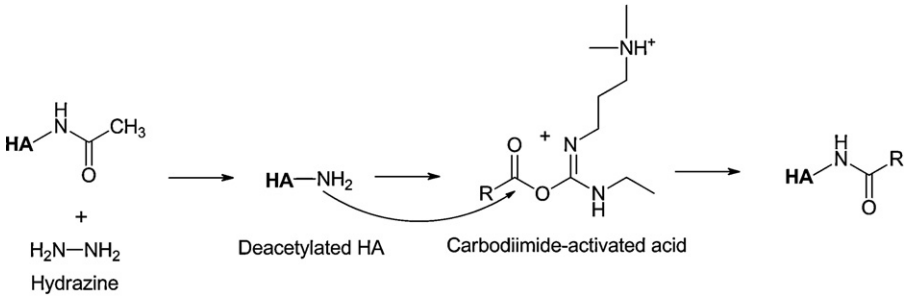

Fig. 7. HA deacetylation and amidation (Bellini & Topai, 2000).

(Jia, Colombo, Padera, Langer, & Kohane, 2004). Measurements indicated a decrease in molecular weight upon reaction from 1.3 MDa for native HA to 260 kDa for the resulting HA–aldehyde. This method was used for grafting peptides onto the aldehyde groups (Glass, Dickerson, Stecker, & Polarek, 1996) or for crosslinking with HA–hydrazide derivatives to form a vehicle for bupivacaine (Jia et al., 2004). However, this reaction led to a significant decrease of HA molecular weight.

3.3. Modification of the $-NHCOCH_3$

Deacetylation of the N-acetyl group of HA recovers an amino group which can then react with an acid using the same amidation methods described in Section 3.1.1. Deacetylation is usually performed using hydrazine sulfate over a period of five days at 55 °C, which induces severe chain fragmentation (Bellini & Topai, 2000). Since then, even milder treatments have been reported to induce HA chain degradation via β-elimination of the glucuronic moiety (Bulpitt & Aeschlimann, 1999; Crescenzi et al., 2002; Dahl, Laurent, & Smedsrod, 1988).

Bellini and Topai (2000) patented the amidation of HA by reaction of an acid with the deacetylated amino-group of HA. The acid was first activated using a carbodiimide. The deacetylated HA amine reacts with the activated acid and forms the amide bond (Fig. 7). Oerther et al. (2000) used this method to crosslink HA with the carboxylic groups of alginic acid. Crescenzi et al. (2002) used deacetylated HA for further crosslinking using Ugi condensation (Fig. 5a). The deacetylated amino groups react with the carboxylic groups of HA to form an auto-crosslinked hydrogel. Platt and Szoka mention the possibility of using enzymes for HA deacetylation, which was previously performed on the N-acetylglucosamine moiety of heparin and heparan sulfate (Duncan, Liu, Fox, & Liu, 2006; Platt & Szoka, 2008).

4. HA derivatives

Numerous HA derivatives have been described in the literature, designed for a range of applications, in particular drug delivery. In this section, we present several interesting examples of HA derivatives and their synthesis methods.

4.1. HA hydrogels for supplementation

4.1.1. Direct chemical crosslinking

Due to the short half-life of HA solutions after injection, most commercial HA products for articular injections (such as Synvisc® by Genzyme) and for dermal filling (Restylane® by Q-Med, Juvederm® by Allergan, Teosyal® by Teoxane, Glytone® by Pierre Fabre, etc.) are first stabilized by crosslinking to obtain non-soluble hydrogels with longer residence times and a mechanical effect. Manufacturers claim dermal filling effect durations of from 6 to 12 months depending on the product. Clinical case studies have reported effect durations of 6–9 months (Bennett & Taher, 2005; Lemperle, Morhenn, & Charrier, 2003; Narins et al., 2003; Piacquadio, Jarcho, & Goltz, 1997). Many commercially available injectable HA hydrogels are crosslinked with butanediol diglycidyl ether (BDDE) (Sall & Férard, 2007) (Fig. 6a and b). Other commonly used crosslinking agents include divinylsulfone (Fig. 6c) or glutaraldehyde (Fig. 6e), as detailed in the previous section of the present review. With the aim of obtaining derivatives with longer residence times after injection, techniques for the synthesis of HA derivatives are continuously being explored. For example, Collins and Birkinshaw (2007, 2008a) compared different crosslinking agents to identify the most effective ones.

Zhao (2000) patented the synthesis of double crosslinked HA, subjected to two consecutive crosslinking reactions with bisepoxides, the first at high pH (10) and the second at low pH (4). The resulting double crosslinked hydrogels demonstrated a lower water absorption capacity and less degradation when subjected to hyaluronidase digestion than the single cross-linked hydrogels.

4.1.2. Chemical crosslinking of functionalized HA

Some authors have described the crosslinking of HA–amine or HA–hydrazide derivatives obtained with commercially available homo- or heterofunctional crosslinkers (Bulpitt & Aeschlimann, 1999; Pouyani & Prestwich, 1994a). These crosslinkers include bis(sulfosuccinimidyl)suberate (BS^3), 3,3′-dithiobis(sulfosuccinimidyl)propionate (DTSSP) or 2-methylsuberimidate (DMS). They react with HA–hydrazide derivatives at pH values above 5 or HA–amine derivatives at pH values above 8 due to the higher pK_a of the amino groups (see Fig. 3e) (Bulpitt & Aeschlimann, 1999). The biocompatibility of the resulting crosslinked gels has to be carefully studied as these will be degraded internally by enzymatic processes (Jedrzejas & Stern, 2005). The *in situ* generation of potentially toxic degradation hydrazide-products may result in side effects which need to be evaluated using *in vivo* methods (Bulpitt & Aeschlimann, 1999).

4.1.3. In situ crosslinking of functionalized HA

Shu, Liu, Luo, Roberts, and Prestwich (2002) formed hydrogels by crosslinking HA–disulfide derivatives. First of all, thiol-modified HA was prepared using a carbodiimide-mediated hydrazide chemical method. Hydrogels were then formed under mild conditions by air oxidation of thiols to disulfides (Fig. 8a). This type of reaction is interesting because it does not rely on synthetic crosslinkers.

Kurisawa, Chung, Yang, Gao, and Uyama (2005) synthesized *in situ* crosslinkable hydrogels from tyramine-grafted HA by treatment with horseradish peroxidase and H_2O_2. HA–tyramine was first synthesized with EDC and HOBt. Formation of the hydrogel was performed *in situ* using two syringes, one containing HA–tyramine and H_2O_2 and the second containing horseradish peroxidase to induce the crosslinking reaction, which proceeds at the C–C and C–O positions between phenols, as shown in Fig. 8b. The *in situ* crosslinked HA hydrogels proved to be biocompatible.

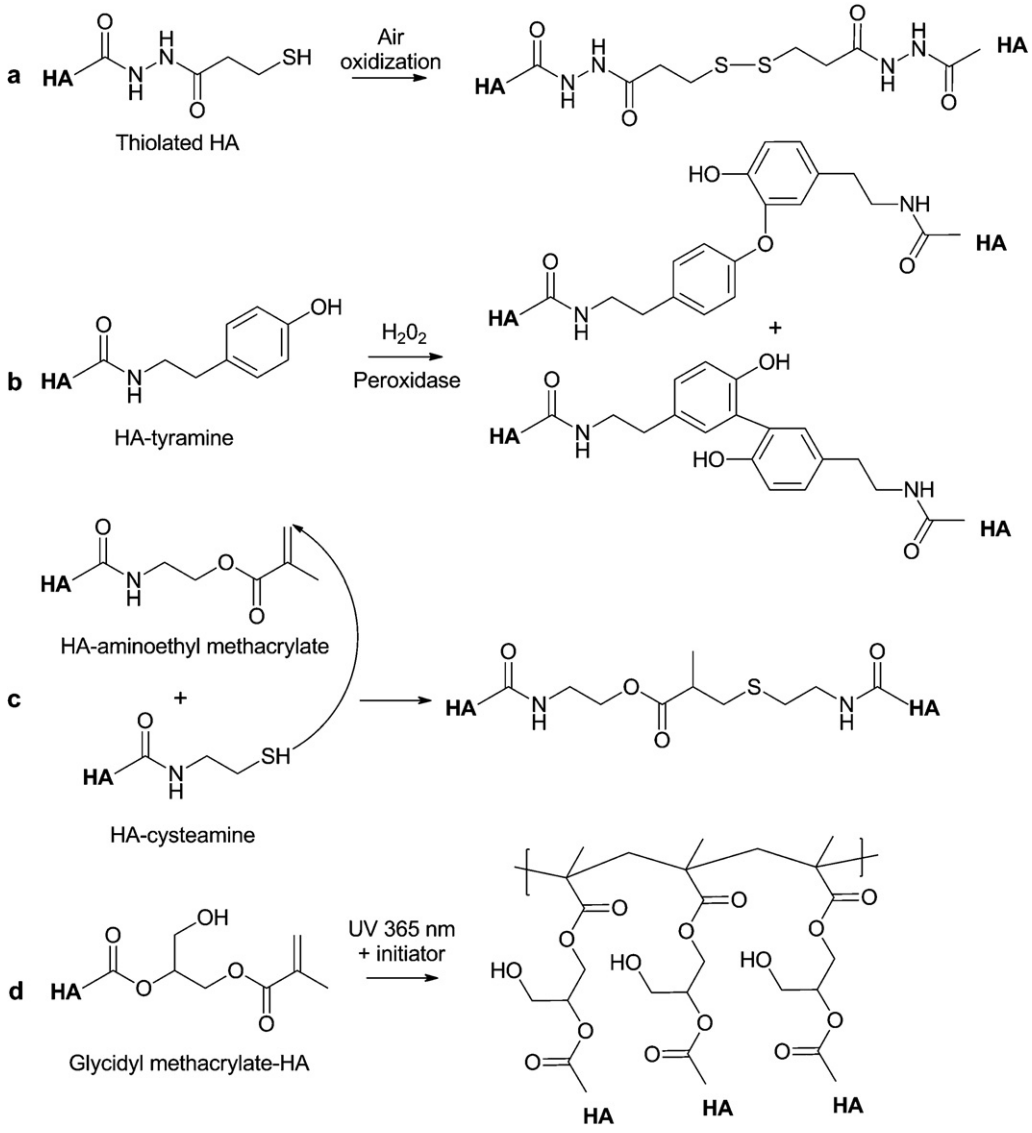

Fig. 8. *In situ* crosslinking of thiolated-HA derivatives (Shu et al., 2002) (a), of HA–tyramine by oxidation (Kurisawa et al., 2005) (b), of two different HA derivatives (Oh et al., 2010) (c). Photocrosslinking of glycidyl methacrylate–HA (Leach et al., 2003) (d).

Oh et al. (2010) recently reported the formation of HA hydrogels from functionalized derivatives containing groups reactive towards one another. For instance, HA–aminoethyl methacrylate or HA–aminopropyl methacrylamide comprising double bonds react with HA containing thiol groups such as HA–cysteamine (Fig. 8c). No additional reagent is required for the reaction and no toxic byproducts are formed, making it a suitable method for *in situ* formation of HA hydrogels.

4.1.4. Photocrosslinking of functionalized HA

Several authors have also described the synthesis of HA hydrogels by photocrosslinking (Bencherif et al., 2008; Leach et al., 2003). The methacrylated-HA products obtained via glycidyl methacrylate (Section 3.1.3.4) (Fig. 5e) or via methacrylate anhydride (Section 3.2.3.3) (Fig. 6h) were further crosslinked by free-radical polymerization when subjected to UV-light (365 nm) and a photo-initiator such as 2-oxo-ketoglutaric acid or 4-(2-hydroxyethoxy)phenyl-(2-hydroxy-2-propyl)ketone (marketed as Irgacure 2959 by Ciba Specialty Chemicals, Switzerland) (Fig. 8d).

4.1.5. Physical hydrogels

As an alternative to chemically crosslinked hydrogels, some authors have synthesized amphiphilic derivatives by grafting hydrophobic chains such as hexadecylamine (Borzacchiello, Mayol, Schiavinato, & Ambrosio, 2010) or polylactic acid (Palumbo, Pitarresi, Mandracchia, Tripodo, & Giammona, 2006) to HA. The resulting associative polymers form strong interactions between the hydrophobic chains, which are disrupted when subjected to a strong force, typical of elastic behavior. The amphiphilic HA derivatives thus obtained behave like gels compared to the initially viscous fluid of native HA.

4.2. HA conjugates for drug delivery

4.2.1. HA–drug conjugates

Conjugation of drugs to HA was reported as early as 1991 for the improvement of drug delivery (Drobnik, 1991). This technique aims to form a pro-drug by covalently binding the drug to HA. The drug is released once the covalent bond is broken down in the organism, ideally at the specific target site. Vercruysse et al. (1998) reviewed

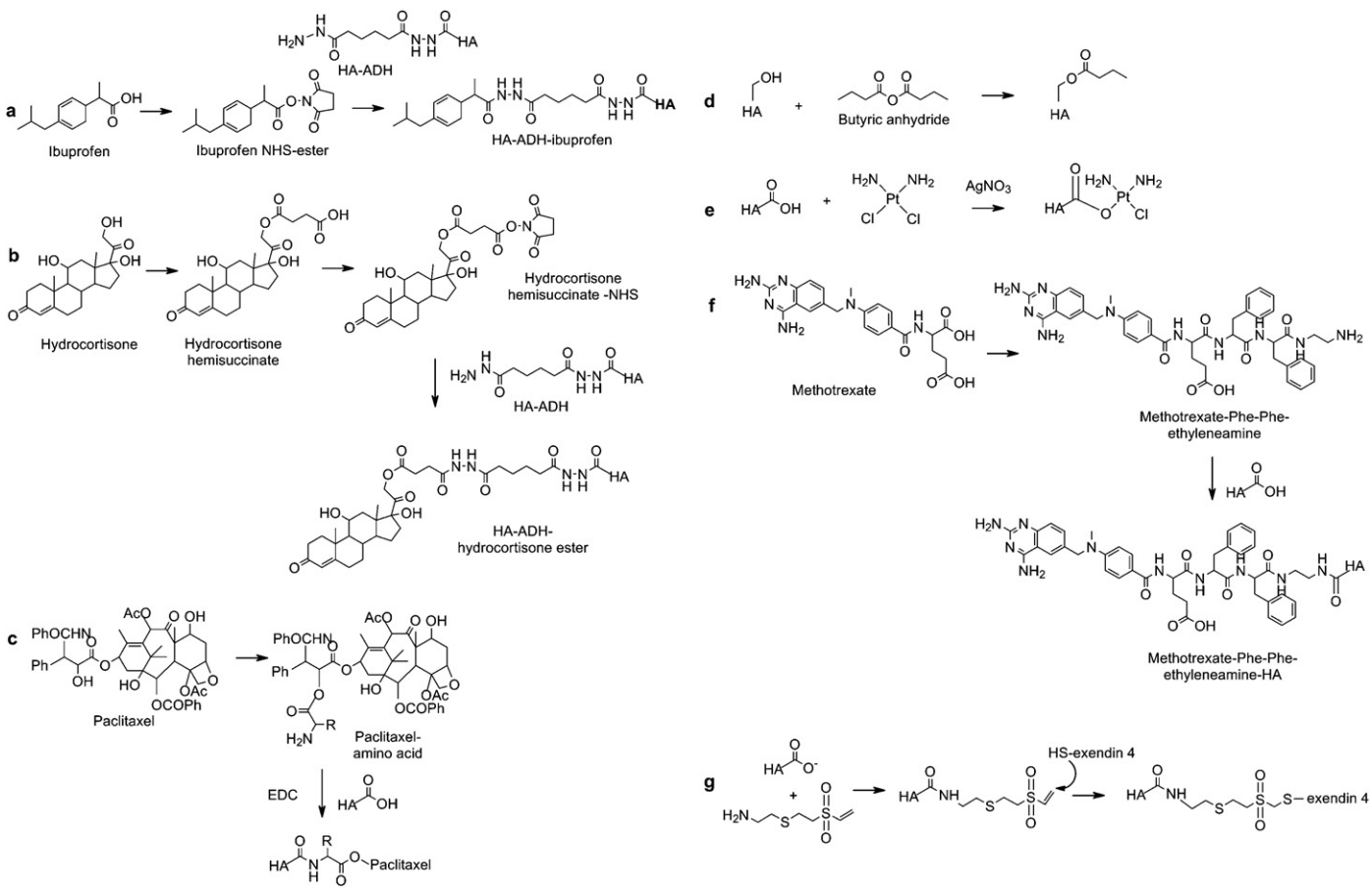

Fig. 9. Conjugation onto HA–ADH of ibuprofen (Pouyani & Prestwich, 1994a) (a), of hydrocortisone hemisuccinate (Pouyani & Prestwich, 1994a) (b), conjugation of paclitaxel via an amino acid linker (Xin et al., 2010) (c), of butyric acid (Coradini et al., 1999) (d), HA–cisplatin conjugation (Cai et al., 2008) (e), HA–methotrexate conjugation via Phe–Phe peptide and ethylenediamine (Homma et al., 2010) (f), HA–exendin 4 conjugation via vinyl sulfone (Kong et al., 2010) (g).

studies showing that by conjugating drugs to HA, their therapeutic effect was enhanced.

Pouyani and Prestwich (1994a) described the grafting of ibuprofen, a nonsteroidal anti-inflammatory drug, or hydrocortisone, a steroidal anti-inflammatory drug intended for local injections into arthritic joints. HA is first conjugated with adipic dihydrazide (ADH) and ibuprofen is converted to an activated ester using carbodiimide and NHS. Both intermediates then react together to form a hydrazide bond (Fig. 9a). Hydrocortisone was grafted to HA–ADH via its hemisuccinate derivative, previously converted into an activated ester in the same way as ibuprofen (Fig. 9b). This way, an ester linkage is formed between HA and hydrocortisone that is more easily hydrolyzed by enzymatic mechanisms in the body to release the starting hydrocortisone.

Paclitaxel has been conjugated to HA in a similar way to hydrocortisone via its hemisuccinate NHS activated ester onto HA–ADH (Luo & Prestwich, 1999). Paclitaxel is a poorly soluble antimitotic chemotherapeutic agent which causes tumor cell death by disrupting mitosis. Its solubility was greatly increased by conjugation to HA. In addition, studies suggest that, using this method, paclitaxel could be released inside tumor cells by intracellular enzymatic hydrolysis of the ester bond to carry out its activity (Luo, Ziebell, & Prestwich, 2000).

Proteins have been conjugated to HA–hydrazide in a similar way (Marecak, 2001). The HA–protein conjugates demonstrated more durable blood concentrations than formulations containing the protein alone.

Most examples of HA–drug conjugates that have been synthesized using carbodiimide chemistry have been prepared from HA–hydrazide intermediate and not from native HA. Indeed, Vercruysse et al. (1998) stated that drugs were not covalently grafted directly onto HA due to the formation of N-acylurea byproducts (also see Section 3.1.1.1).

As an alternative, Xin, Wang, & Xiang (2010) later described the conjugation of paclitaxel onto HA using carbodiimide via amino acid linkers. An amino acid was previously grafted by its carboxylic group onto a hydroxyl group of paclitaxel. The intermediate was then conjugated by the amino group of the amino acid onto the carboxylic group of HA using EDC and NHS activation in DMF (Fig. 9c). Hydrolysis of the carbodiimide was minimized by the use of an anhydrous organic solvent and degrees of substitution of 10–15% were obtained. The authors confirmed the formation of nanoparticles in aqueous solution due to the amphiphilic nature of the HA–amino acid–paclitaxel conjugate. The hydrophobic paclitaxel is likely to be entrapped in the hydrophilic HA outer shell. A higher level of drug release was observed relative to the HA–ADH–paclitaxel conjugate described by Luo and Prestwich (1999). The authors suggest that this difference is due to the electron-withdrawing effect of the protonated amino group, which weakens the ester bond and thus facilitates its hydrolysis and the release of paclitaxel (Xin et al., 2010). Another hypothesis could be the facilitated recognition of the esterase enzymes thanks to the presence of an amino acid. This method offers new perspectives for HA–drug conjugation using different linkers that are more "biological-like" with tunable release rates.

Drugs such as anthracycline antibiotics comprising an amino group can also be conjugated on the hydroxyl groups of HA using cyanogen bromide activation, as described in Section 3.2.4

(see Fig. 6i) (Cera, Terbojevich, Cosani, & Palumbo, 1988). Carbamate bonds and hydrazide bonds are not enzymatically degraded, unlike ester bonds. In addition, by grafting the drug onto the hydroxyl groups, the carboxylic groups of HA are unmodified, thus preserving HA's natural recognition and biodegradability by enzymes, which may be advantageous for cell internalization. Drug release may therefore be faster with respect to drugs conjugated on the carboxyl groups of HA. Indeed, the more quickly the protective HA shell around the conjugated drug is degraded, the more quickly the drug is released.

Other drugs comprising a hydroxyl group, such as cortisone, hydrocortisone and fluorocortisone, were conjugated to HA by esterification and patented by Fidia (Della Valle & Romeo, 1986). HA–methylprednisolone conjugation is another example (Kyyronen et al., 1992). The drugs were grafted from their bromide form as described in Section 3.1.3.1 (Fig. 5b). It would be interesting to compare *in vivo* drug release rates of hydrocortisone grafted via an ester bond and via a hydrazide spacer, but no studies of this type have been reported.

Leonelli et al. (2005) suggested another method for conjugating paclitaxel to HA by esterification via a 4-bromobutanoic acid derivative of paclitaxel. This derivative reacted with HA–TBA in N-methyl-2-pyrrolidone (NMP) over a period of 7 days. However, this solvent is more toxic than DMF and the reaction is very long. Rosato et al. (2006) reported that this HA–paclitaxel conjugate had a much stronger antitumor activity than the free drug.

Recently, Platt and Szoka (2008) reviewed a number of studies examining HA–drug conjugates for selective CD44 receptor-mediated cell internalization by cells. The conjugated drug will be internalized together with HA and released inside the cell once the covalent bond is hydrolyzed by intracellular enzymes (Coradini, Pellizzaro, Miglierini, Daidone, & Perbellini, 1999; Luo, Kirker, & Prestwich, 2000; Luo, Ziebell, et al., 2000). Tumor cells have been shown to over-express CD44 receptors, allowing specific targeting of drugs to tumors (Platt & Szoka, 2008).

Coradini et al. conjugated butyric acid, a tumor-cell growth inhibitor, from butyric anhydride in DMF to HA in the presence of pyridine or dimethyl amino pyridine. The anhydride reacts with the hydroxyl groups of HA and forms ester bonds in the same way as with methacrylic anhydride, as described in Section 3.2.3.3 (Fig. 9d). Covalent HA–butyric acid demonstrated enhanced cellular uptake and thus enhanced antitumor activity (Coradini et al., 1999).

Cai, Xie, Bagby, Cohen, and Forrest (2008) studied the conjugation of cisplatin onto HA with the aim of reducing its renal toxicity. Cisplatin was grafted onto the carboxyl groups of HA with the use of silver nitrate as the activating agent (Fig. 9e), which increased drug concentrations in the targeted tissue and induced sustained-release kinetics.

Recently, Homma et al. (2009) described the synthesis of HA–methotrexate conjugates with reduced side effects to be used in the treatment of osteoarthritis. Methotrexate was linked to HA through a small peptide sequence and a PEG–diamine spacer, with grafting ratios of 1–3%. First of all, a methotrexate–peptide–PEG–amine compound was synthesized by a multi-step reaction using carbodiimide chemistry in DMF. The terminal amino group of the compound then reacted with the carboxyl groups of HA using EDC and HOBt in tetrahydrofuran (THF) and water. The authors observed that the peptide chain was necessary for the drug's effectiveness, suggesting it may promote enzyme recognition of the conjugate, which is essential for the drug to be released and biologically active. The PEG spacer was also added to promote enzyme access to the peptide chain. A further study of this method determined that the optimal peptide chain was phenylalanine–phenylalanine, with ethylenediamine as the linker, and that the bonding ratio should be higher than 1.3%, ideally 3.8% (Homma et al., 2010) (Fig. 9f).

Kong, Oh, Chae, Lee, and Hahn (2010) also recently presented a novel method for conjugation of exendin-4, an antidiabetic peptide, onto HA via a vinyl sulfone cysteamine intermediate. In a first step, vinyl sulfone cysteamine was prepared from divinyl sulfone (DVS) and cysteamine hydrochloride. The amino group then reacted with HA–TBA in DMSO using benzotriazol-1-yloxytris(dimethylamino) phosphonium hexafluorophosphate and diisopropylethylamine to form HA–vinyl sulfone cysteamine by amidation. The vinyl group of the intermediate then reacted with the terminal cysteine group of exendin-4 to form a covalent linkage (Fig. 9g). The resulting conjugate demonstrated a longer half-life and better glucose-lowering capabilities than the free drug.

Kim, Checkla, Dehazya, and Chen (2003) reported the preparation of crosslinked HA–DNA matrix formulations intended for use as gene delivery systems. Crosslinking was performed using adipic hydrazide and EDC. The resulting matrices demonstrated gradual release of DNA, which is advantageous compared to non-protected HA or liposomes systems with faster release profiles.

4.2.2. HA conjugation to carrier systems

4.2.2.1. HA conjugation to liposomes. Yerushalmi, Arad, and Margalit (1994) reported the coating of liposomes by covalent conjugation of HA. The carboxyl groups of HA were linked to the amino groups of phosphatidylethanolamine of liposomes using carbodiimide chemistry (Fig. 10). Peer and Margalit (2004a, 2004b) showed that HA covalently coated liposomes circulated longer than non-coated liposomes. Drugs encapsulated in the liposomes accumulated in tumor cells and reduced tumor growth, with lower systemic toxicity. Taetz et al. (2009) recently adapted HA–liposome conjugates for the delivery of siRNA to lung cancer cells.

4.2.2.2. HA conjugation to nanoparticles. Rivkin et al. (2010) encapsulated paclitaxel into phosphoethanolamine lipids, which were further covalently grafted with HA. The nanoparticles formed proved to be effective as selective tumor-targeted nanovectors. Nanoparticles were also formed by covalently conjugating HA to polylactide-co-glycolide (PLGA) via a polyethylene glycol (PEG) spacer (Hyung et al., 2008; Yadav et al., 2007). Both PLGA and HA were first activated with EDC and NHS, then mixed together with the diamine linker. The particles were loaded with doxorubicin and demonstrated increased effectiveness compared to nanoparticles of PLGA alone.

4.2.3. HA conjugates (crosslinked or modified) as drug carrier systems

As an alternative to HA conjugation onto particles, some authors have described the synthesis of HA derivatives as particles or other drug delivery systems themselves. For example Choi et al. (2009) reported HA modification with hydrophobic 5β-cholanic acid via an aminoethyl-amide linker using EDC and NHS in DMF. The resulting amphiphilic derivative formed nanoparticles in physiological conditions and showed prolonged blood circulation and a high affinity for tumor cells *in vitro*. These nanoparticles are therefore promising drug carriers for tumor-targeted drugs.

HA nanogels for siRNA delivery were described by Lee, Mok, Lee, Oh, and Park (2007). Firstly, thiol–HA was prepared using the carbodiimide method with cystamine followed by dithiothreitol (DTT) treatment to cleave the disulfide bonds. Crosslinked HA-nanogels were then generated by an inverse emulsion method from the thiol–HA solution containing siRNA during which the disulfide bonds were formed. The encapsulation method induced no damage to the siRNA and uptake by CD44-expressing cells.

Microspheres prepared from HA esters, obtained using alkyl halides, have been used as drug delivery systems for drugs such as hydrocortisone (Benedetti, Topp, & Stella, 1990). The authors observed that hydrocortisone was released faster when encapsu-

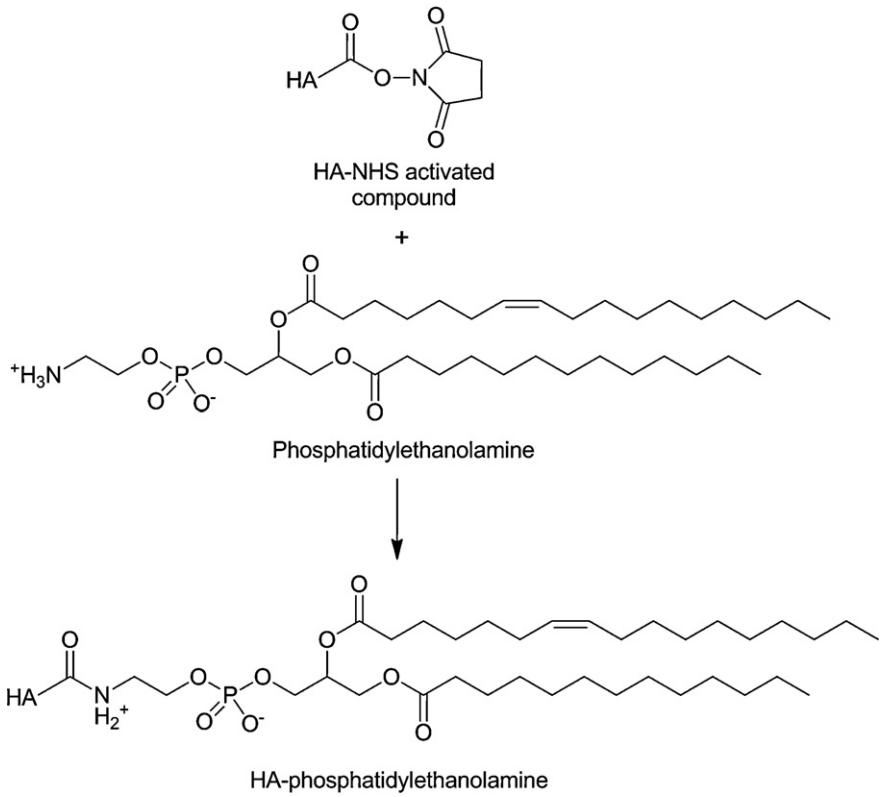

Fig. 10. HA conjugation to liposomes (Yerushalmi et al., 1994).

lated into HA microspheres than when covalently conjugated to HA. Esposito et al. (2005) also formed microspheres from HA esters using either spray-drying or inverse emulsion. In the former case, the drug was dissolved in a solution containing HA and spray dried. In the latter case, a water-in-oil emulsion was formed containing the HA and drug solution in the water droplets emulsified in oil with a surfactant (sorbitan trioleate, Span® 85) and further centrifuged to isolate the HA microspheres. Dehazya and Lu (2002) patented the process of making HA microspheres by inverse emulsification.

Later, Yun, Goetz, Yellen, and Chen (2004) reported the preparation of HA microspheres using carbodiimide and adipic hydrazide combined with the inverse emulsion method. Plasmid DNA was incorporated into the microspheres when mixed into the HA solution prior to the addition of the crosslinking reagents. The microspheres obtained were shown to deliver structurally intact plasmid DNA and transfect cells *in vitro* and *in vivo*.

Recently, Lai et al. (2010) reported carbodiimide crosslinked HA hydrogels for potential use as cell sheet delivery systems. The authors showed a better ocular biocompatibility in comparison with HA discs crosslinked with glutaraldehyde. This appears to be a promising approach since cell sheet engineering is an emerging technique for tissue regeneration (Yang et al., 2006).

Luo, Kirker, et al. (2000) reported the preparation of drug-loaded crosslinked HA films for the sustained release of drugs at wound sites. The drug was dissolved in the HA–ADH solution prior to crosslinking to trap it inside the hydrogel network.

The formation of HA composites has also been reported by covalently linking HA to other polymers, such as carboxymethylcellulose (Burns et al., 1991), poly(vinyl alcohol) or poly(acrylic acid) (Cascone, Sim, & Sandra, 1995). A recent example developed by Antunes et al. (2010) are poly(L-lactic acid)(PLLA) macroporous hybrid scaffolds coated with HA. Preformed PLLA scaffolds were immersed in an HA solution which was then crosslinked in water/acetone with glutaraldehyde. Applications for this type of material generally concern bone tissue engineering.

4.2.4. Non-covalent HA derivatives

Native HA was used to encapsulate ofloxacin inside microspheres obtained by spray-drying a solution containing both HA and ofloxacin (Hwang, Kim, Chung, & Shim, 2008). The microspheres demonstrated increased ofloxacin uptake relative to non-HA microspheres for improved lung delivery. Kim, Hahn, Kim, Kim, and Lee (2005) described the use of HA microspheres for the sustained release of recombinant therapeutic proteins. The microspheres were obtained by spray-drying a native HA solution comprising the protein, in this case a recombinant human growth hormone, and a non-ionic surfactant, polysorbate 80 (Tween® 80). The microparticles obtained were further mixed with a lecithin solution and spray-dried a second time. Even though recombinant proteins are easily denatured during spray-drying, the authors observed almost no denaturing. Their optimized formulation demonstrated prolonged release after injection. These results are very promising for the delivery of therapeutic proteins, especially in terms of reducing injection frequency, one of the main challenges in this area. Other protein delivery systems of this type were reviewed recently by Oh et al. (2010).

The negative charges of the carboxyl groups of HA have also been used to form ion complexes with positively charged compounds. For example, HA was mixed with positively charged tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) to form non-covalent ion complexes. These nanocomplexes showed prolonged blood circulation *in vivo*, therefore prolonging the anticancer therapeutic effect (Na et al., 2008). Nanoparticles formed by ion complexes of HA and cisplatin were described by Jeong et al. (2008). In aqueous solution, cisplatin is positively charged and forms ion complexes with HA when simply allowed to mix in

water for three days. Saettone, Giannaccini, Chetoni, Torracca, and Monti (1991) had previously described the formation of HA ion complexes with pilocarpine, which showed improved bioavailability for ophthalmic delivery. In the same way, liposomes presenting a cationic outline were non-covalently coated by ion complexation of negatively charged HA (Esposito, Geninatti Crich, & Aime, 2008). Jederstrom, Andersson, Gråsjö, and Sjöholm (2004) described the formation of ionic complexes between HA and insulin by successively decreasing ionic strength and increasing pH of the solution by dialysis. The authors observed the formation of stable complexes after optimization of the dialysis parameters with improved biological activity for potential oral administration.

4.3. HA coatings

Coating of biomaterial surfaces by HA has been widely described and reviewed by Morra (2005). The review describes different types of coating methods, including covalent linking to surfaces, ionic coupling or adsorption. For example, HA has been covalently linked to metallic stent surfaces using carbodiimides (Verheye et al., 2000). Pitt et al. (2004) describe another method to attach HA to metals via condensation of HA–hydrazide derivatives onto aldehyde-functionalized metal surfaces.

More recently, multi-layer film systems for biomaterial surface engineering have been reviewed (Crouzier, Boudou, & Picart, 2010). Schneider et al. (2007) synthesized layer-by-layer films comprising native HA and cationic amine-modified HA. Firstly, the native HA was converted to its acidic form and dissolved in DMSO to react with a monoprotected ethylenediamine using EDC and NHS. The second amino group was deprotected to obtain the cationic amine-modified HA. Multilayer films were then formed using the layer-by-layer technique described by Decher, Hong, and Schmitt (1992). Richert et al. (2004) had previously reported the improvement of cell adhesion and resistance of crosslinked HA–poly(L-lysine) films for surface modification of biomaterials. The rigidity of the crosslinked films played a major role in the improved cell adherence. Such multilayer films are promising materials for the coating of prosthetic implants.

5. Physicochemical characterization methods

5.1. Structural characterization

5.1.1. Nuclear magnetic resonance spectroscopy (NMR)

Uni-dimensional ^{1}H NMR is the most common tool to characterize HA derivatives, as shown in early studies by Pouyani and Prestwich (1994b) or Bulpitt and Aeschlimann (1999). The NMR-spectra show the presence of the conjugated compounds and make it possible to calculate the degree of modification of HA. A typical spectrum of native HA solubilized in D_2O is shown in Fig. 11a and presents broad signals due to the high viscosity of the solution. When dissolved in D_2O containing NaOH, the lower viscosity of HA alkaline solutions leads to more clearly defined peaks (Fig. 11b). The broad multiplet between approximately 3.2 and 3.9 ppm corresponds to the signals of the protons in the sugar rings. They are all superimposed which makes it difficult to assign each proton individually. The broad doublet at 4.5 ppm corresponds to the two anomeric protons attached to the carbons adjacent to two oxygen atoms. The $-CH_3$ protons of the N-acetyl group of HA are assigned to the well defined peak at 1.95 ppm. The signal is reduced slightly in alkaline pH to 1.90 ppm. This peak is usually used as the reference to calculate degrees of modification as it is rarely modified when synthesizing new HA derivatives.

NMR spectrometry is a very useful method for quantifying and characterizing chemical modification. For example, L-alanine was grafted onto the carboxylic acid of HA and the spectrum of the purified conjugate is shown in Fig. 11c. The new peaks at 1.33 ppm and 4.14 ppm correspond to the methyl group and the –CH of L-alanine, respectively. The substitution degree (DS) is easily determined by calculating the ratios of peak integrals from the L-alanine methyl group (δ = 1.33 ppm) with the methyl protons of the N-acetyl group of HA (δ = 1.9 ppm). The peak of the –CH group of L-alanine at 4.14 ppm can give an approximation of the DS but the peak is too broad to give a precise value. The methyl peak of alanine has the advantage of not being superimposed with the peaks of the native HA, enabling easy and precise calculation of the DS. When the molecules grafted present signals which are superimposed with the HA peaks, the degree of substitution is more difficult to assess using uni-dimensional ^{1}H NMR.

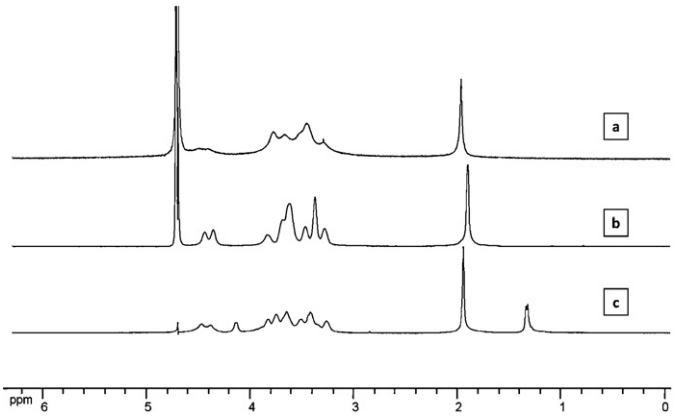

Fig. 11. ^{1}H NMR spectra of hyaluronic acid in D_2O (a) and D_2O with NaOH (b) and ^{1}H NMR spectra in D_2O of HA–alanine (c).

Eenschooten et al. (2010) obtained similar results for amphiphilic HA derivatives using DMSO-D_6 or D_2O, showing that ^{1}H NMR is a reliable technique even for amphiphilic compounds. However, Pravata et al. (2008) found differences on spectra using D_2O or D_2O/DMSO-D_6 solvents.

Two-dimensional (2D) NMR spectra have been presented by several authors (Crescenzi, Francescangeli, Capitani, et al., 2003; Crescenzi, Francescangeli, Taglienti, et al., 2003; Pravata et al., 2008; Sahoo, Chung, Khetan, & Burdick, 2008). Crescenzi et al. used diffusion-ordered 2D NMR (DOSY) which makes it possible to differentiate molecules bound to HA from those which have not been covalently linked (Crescenzi, Francescangeli, Capitani, et al., 2003; Crescenzi, Francescangeli, Taglienti, et al., 2003). This technique enables verification of the effectiveness of the purification step and was used by Sahoo et al. (2008) to confirm the covalent linkage of methacrylate–lactic acid onto HA.

Solid state carbon NMR (^{13}C NMR) measurements were performed on HA using CP-MAS. De Nooy et al. observed that the increase in the theoretical degree of crosslinking led to increased intensity of the crosslinked carbon signal. To determine these types of changes, as well as the degree of crosslinking, extremely high frequencies and a high number of scans are necessary (De Nooy et al., 2000). Several other authors report the use of ^{13}C NMR in solution or solid state as well as two-dimensional ^{1}H NMR and ^{13}C NMR (Follain et al., 2008; Magnani et al., 2000; Mlčochová et al., 2006).

NMR is without a doubt an essential tool for the characterization of HA products. However, common uni-dimensional ^{1}H NMR does not provide information on covalent linking and requires complementary characterization.

5.1.2. Infrared spectroscopy (IR)

Another common and complementary characterization technique used for HA is infrared spectroscopy (IR), particularly Fourier

transform infrared spectroscopy (FTIR) (Schneider et al., 2007; Young et al., 2004; Zhao, 2006). It allows to determine the type of bond which has been formed during the HA modification. For example, Magnani et al. (2000) observed by using FTIR, the appearance of C=O amide bands at about 1630–1640 cm^{-1} which correspond to the new amide bonds formed between the diamines and the carboxylic groups of HA. At the same time, as the amount of crosslinking reagents was increased, the band intensity of the carboxyl groups at about 1720–1740 cm^{-1} decreased. Mlčochová et al. (2006) confirmed the presence of alkyl chains on HA by the additional peaks observed at between 2850 and 2930 cm^{-1} corresponding to –CH and $–CH_2$ vibrations.

However, this method does not enable accurate quantification of the degree of modification. In some cases, no information can be extracted from FTIR spectra, as observed by Tomihata and Ikada, who obtained no difference between the spectra before and after crosslinking, except for a band at 1650 cm^{-1} which can be attributed to the ion exchange of the sodium salt to the acidic form of HA (Tomihata & Ikada, 1997a).

5.1.3. Primary amine quantification by ninhydrin assay

A colorimetric test method using ninhydrin was reported by Kuo et al. (1991) to quantify the free primary amino groups of the amine-functionalized HA. The negative results confirmed that no primary amines were grafted onto HA via carbodiimide-mediated amidation. Therefore, this quick test easily determines whether amine molecules detected by NMR are covalently linked to HA or if they are free in solution.

5.2. Morphology

5.2.1. Microscopy

Scanning electron microscopy (SEM) is often used to characterize the morphology of dry HA derivatives (Esposito et al., 2005; Hwang et al., 2008; Luo, Kirker, et al., 2000; Luo, Ziebell, et al., 2000). Images show that native HA has a fibrous and irregular structure whereas HA–ADH has a highly porous and sheet-like surface (Pouyani, Harbison, & Prestwich, 1994). For HA microspheres or coated liposomes, in particular, SEM is used to characterize shape, surface properties or size distribution. For example, Esposito et al. (2005) observed spherical microparticles or irregularly collapsed microparticles depending on the parameters used for their formation.

Transition electron microscopy (TEM) can be used to determine the surface characteristics of dried nanoparticles (Bodnár et al., 2009; Hyung et al., 2008; Jeong et al., 2008; Yadav et al., 2007). However, the images obtained are not as precise, although they still enable calculation of particle size and shape.

Another microscopy method used for HA derivatives is atomic force microscopy (AFM). Unlike the previous two techniques it can provide information on the morphology of HA in aqueous conditions (Jederstrom et al., 2004; Schneider et al., 2007). Schneider et al. (2007) used it to observe the topography and roughness of HA crosslinked multi-layer films.

5.2.2. Dynamic light scattering

Dynamic light scattering (DLS) measurements provide information on the hydrodynamic behavior of HA microparticles or nanoparticles in solution and enable measurement of their diameter and size distribution (Bodnár et al., 2009; Hyung et al., 2008; Jederstrom et al., 2004; Jeong et al., 2008). For example, Yadav et al. (2007) observed differences in HA–PLGA nanoparticle size and size distribution depending on the processing parameters.

5.3. Physical characterization

5.3.1. Swelling measurements

The capacity of crosslinked HA hydrogels to retain water is measured by their swelling properties (Jeon et al., 2007). Collins and Birkinshaw (2007) used swelling measurements to compare the crosslinking densities of HA hydrogels formed with different crosslinking agents. High swelling ratio values correspond to the lowest crosslinking density; the denser the hydrogel network is, the lower its water uptake capacity and its swelling ratio. It was thus shown that glutaraldehyde and divinyl sulfone were the most effective crosslinkers relative to carbodiimide and bisepoxide. Ghosh et al. (2005) observed that, as expected, the swelling ratio decreases as the amount of crosslinking agent – in this case poly (ethylene glycol) diacrylate – increases.

5.3.2. Compression test

Ibrahim, Kang, and Ramamurthi (2010) used a uniaxial compression test to determine the apparent crosslinking density of HA hydrogels. The gels were compressed with a defined initial force and compression rate. The results were in line with the swelling ratio, showing that increasing the amount of crosslinker decreased the apparent crosslinking density. Collins and Birkinshaw (2008b) also used a compression test to calculate the swollen modulus, G_e, of glutaraldehyde-crosslinked HA.

5.3.3. Thermal analysis

Differential scanning calorimetry (DSC) is often used to characterize the thermal behavior of HA derivatives or HA hydrogels, providing information on their hydration properties (Collins & Birkinshaw, 2007, 2008b; Kafedjiiski et al., 2007; Luo, Kirker, et al., 2000; Luo, Ziebell, et al., 2000). The DSC thermogram of native HA presented by Luo et al. presents a broad endothermic peak at 89.5 °C followed by two sharp exothermic peaks at 243.6 and 258.7 °C. This indicates the crystalline nature of HA (Luo, Kirker, et al., 2000; Luo, Ziebell, et al., 2000). The peaks above 300 °C correspond to HA decomposition. Thermograms of crosslinked HA–ADH no longer show an exothermic peak, the material no longer being crystalline but amorphous. Studies by Barbucci, Rappuoli, Borzacchiello, and Ambrosio (2000) provided information on the hydration water of HA derivatives. The endothermic peak attributed to the dehydration heat was lower for native HA than for sulfated HA, the latter therefore containing more hydration water. Indeed, the OSO_3^- groups are more polar than the hydroxyl groups of HA. Yadav et al. (2007) state that the glass transition temperature has a significant effect on drug release.

Collins and Birkinshaw (2008b) used dynamic mechanical thermal analysis (DMTA) to study the influence of different crosslinkers and water content on the elastic modulus, the storage modulus and the mechanical loss factor ($\tan\delta$) of crosslinked HA gels.

5.3.4. Molecular weight measurements

Size exclusion chromatography coupled to multi-angle light scattering (SEC-MALS) is a conventional method to determine the molecular weight of modified polymers. Preserving the chain length of the HA is a critical point in the synthesis of HA derivatives since its lubricant, shock-absorbent and space-filling properties are closely correlated to its high viscosity and molecular weight. Some degradation is inevitable during chemical modification processes. Indeed, HA is sensitive to various procedures, especially lyophilization and exposure to alkaline conditions (Tokita, Ohshima, & Okamoto, 1997). This type of analysis coupled to light scattering detection has the advantage of measuring the real molecular weight. The values obtained are not dependent on eventual changes in the hydrodynamic sphere following HA functionalization and thus do not lead to false interpretation. The molecular weight is

calculated using the specific refractive increment index (dn/dC) of the polymer. The dn/dC values of native HA are usually within the range of 1.430–1.670 mL/g depending on the solvent and measurement parameters used (Bergman et al., 2007; Hokputsa, Jumel, Alexander, & Harding, 2003; Mendichi, Schieroni, Grassi, & Re, 1998).

Another method reported to measure the molecular weight of HA is asymmetric flow-field-flow fractionation (AFFFF). Maleki et al. (2007) described this method for the study of the hydrolytic degradation of HA during the crosslinking process with EDC. It was used to measure the molecular weight and molecular weight distribution at different time points of the crosslinking reaction. After an initial increase, the molecular weight demonstrated a significant decrease after seven days in the presence of the crosslinking reagents.

5.3.5. Rheological measurements

As described earlier in Section 2.1, HA solutions are highly viscous and shear-thinning due to the high molecular weight of HA and the entangled coil formation in solution. Rheology studies are therefore essential to evaluate the effect of derivatization or crosslinking (Barbucci et al., 2000; Borzacchiello et al., 2010; Huin-Amargier et al., 2006; Ibrahim et al., 2010; Maleki et al., 2007). Measurements can be performed in steady shear mode or in oscillatory mode.

A typical dynamic viscosity curve of an HA solution as a function of shear stress shows a reduction in viscosity as the shear rate increases, demonstrating the shear-thinning property of HA. Modified HAs with lower molecular weights also have a reduced viscosity profile but can still exhibit shear-thinning properties. Huin-Amargier et al. (2006) described the synthesis of associative polymers by grafting long hydrophobic chains onto HA. Amphiphilic polymers of this type form strong physical interactions, which are broken when submitted to high shear forces. The shear-thinning behavior is thus much more marked than that of native HA.

Oscillatory shear experiments on crosslinked HA hydrogels make it possible to determine the shear storage modulus (or elastic modulus), G', and the shear loss modulus, G'', giving information on their viscoelastic characteristics. G' reflects the elasticity of the material whereas G'' represents its viscous behavior. Barbucci et al. (2000) presented the results of oscillatory shear experiments on HA solutions and crosslinked gel. For unmodified HA solutions, G'' is higher than the elastic modulus G' and they both increase in a linear manner as the frequency increases. This behavior is typical for viscous liquids. For HA hydrogels, the elastic modulus G' is higher than G'' and they are both independent of the shear frequency, which is a typical form of behavior for viscoelastic solids. Both forms of behavior were also demonstrated by Borzacchiello et al. (2010). According to Barbucci et al. (2000), these measurements make it possible to distinguish chemical hydrogels from entangled networks. The oscillatory shear mode therefore provides important information on the nature of the resulting hydrogels.

6. Conclusion

Thanks to its valuable physicochemical properties, HA is currently widely used in a number of therapeutic applications. The design and synthesis of innovative HA derivatives for biomedical applications is still of major interest for the improvement of drug efficacy and targeting. The numerous chemical modification methods described above offer a broad spectrum of options for the synthesis of new derivatives with various physicochemical properties. This review may be used as a tool for the design or improvement of HA derivatives for existing or new applications in the future.

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