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Review

Hyaluronic acid and chondroitin sulfate (meth)acrylate-based hydrogels for tissue engineering: Synthesis, characteristics and pre-clinical evaluation

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ABSTRACT

Hydrogels based on photocrosslinkable Hyaluronic Acid Methacrylate (HAMA) and Chondroitin Sulfate Methacrylate (CSMA) are presently under investigation for tissue engineering applications. HAMA and CSMA gels offer tunable characteristics such as tailorable mechanical properties, swelling characteristics, and enzymatic degradability. This review gives an overview of the scientific literature published regarding the pre-clinical development of covalently crosslinked hydrogels that (partially) are based on HAMA and/or CSMA. Throughout the review, recommendations for the next steps in clinical translation of hydrogels based on HAMA or CSMA are made and potential pitfalls are defined. Specifically, a myriad of different synthetic routes to obtain polymerizable hyaluronic acid and chondroitin sulfate derivatives are described. The effects of important parameters such as degree of (meth)acrylation and molecular weight of the synthesized polymers on the formed hydrogels are discussed and useful analytical techniques for their characterization are summarized. Furthermore, the characteristics of the formed hydrogels including their enzymatic degradability are discussed. Finally, a summary of several recent applications of these hydrogels in applied fields such as cartilage and cardiac regeneration and advanced tissue modelling is presented.

1. Introduction

Hydrogels are polymeric networks that are able to absorb large amounts of water [1–3]. The hydrophilic polymers that make up the networks are crosslinked through covalent and/or physical bonds. Typically, hydrogels are classified based on the source of the polymers used: natural, synthetic or hybrid [4]. Hydrogels were first explored by Wichterle and Lim in 1960 [5]. Since then, they have been successfully developed as materials for contact lenses and other biomedical applications such as (injectable) depot systems for the sustained release of therapeutic proteins [4,6]. Interest in stem cell biology and the emergence of the fields of regenerative medicine and bioprinting have stimulated research regarding the use of hydrogel forming polymers as biomaterials to produce hydrated, cell-instructive and degradable scaffolds to support cellular growth, direct differentiation and to promote native tissue formation *in vivo* [7–10]. Natural hydrogels based on fibrous proteins (e.g. collagen) and polysaccharides that are present in the human extracellular matrix (ECM) are promising for use in tissue engineering due to their inherent bioactivity and high cytocompatibility. Examples of commonly used protein-based materials used for hydrogel fabrication are crosslinked Gelatin Methacryloyl (GelMA [11]) and Matrigel (an extract from mouse sarcoma cells consisting of ECM proteins and polysaccharides) [12]. Glycosaminoglycans (GAGs) are another important group of polymers that can be functionalized to yield hydrogels for use in tissue engineering [13,14]. GAGs are linear anionic polysaccharides that are ubiquitously found in the ECM. The group of GAGs (or mucopolysaccharides) consists of 6 members, namely heparin, heparan sulfate, keratan sulfate, dermatan sulfate, chondroitin sulfate and the only unsulfated GAG, hyaluronic acid [15].

In terms of mass, hyaluronic acid and chondroitin sulfate represent the largest portion of the GAGs found in the human body. All GAGs, except hyaluronic acid are covalently attached to proteins in assemblies termed proteoglycans (Fig. 1A). These proteoglycans are produced in almost all mammalian cells and secreted in the ECM or inserted into

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plasma membranes [16]. Proteoglycans interact with other ECM components to form hydrated gel-like structures that enable tissues to resist compressive forces. Proteoglycans consist of several glycosaminoglycan chains covalently bound to a core protein. Many different types of proteoglycans exist; for example, most chondroitin sulfate proteoglycans can be found in the brain and connective tissues [16]. A well-studied proteoglycan family is the aggrecan-like proteoglycans (Fig. 1A), which are the major sources of chondroitin sulfate in the body. Aggrecan-like proteoglycans consist of a core protein (e.g. aggrecan or neurocan, colored dark blue in Fig. 1A) that has a central region fto which chondroitin sulfate or keratin sulfate chains (depicted in Fig. 1A in yellow and red, respectively) are covalently coupled and a link protein motif (in orange) that is able to bind hyaluronic acid through electrostatic interactions with the carboxylic acid groups and hydrophobic interactions with the *N*-acetyl groups (in light blue, Fig. 1A.). Hyaluronic acid is present as soluble polymer in the ECM, with the highest concentrations in the synovial fluid and the vitreous of the eye. It has been reported to reach molecular weights of millions of Daltons, which is reflected in the physicochemical properties of solutions of HA like high viscosity and viscoelasticity. These properties are in turn important in distributing loads during joint motion and protecting cartilage [16]. Both HA and CS are currently used in clinics. HA is often used as a cosmetic filler (in the form of crosslinked particles) [17] and both HA and CS (co-formulated with glucosamine) are used in the reduction of pain in patients with osteoarthritis of the knee. It should be noted, however, the efficacy of such formulations needs further scientific investigation [18–20]. Because of the ubiquitous nature of both chondroitin sulfate and hyaluronic acid in the human body and their widespread use in clinics, derivatives of these polysaccharides that can form hydrogels have high potential as translatable biomaterials for use as scaffolds in tissue engineering. Currently, several HA-based hydrogels are clinically used as dermal fillers in reconstructive surgery [21].

Structurally, HA and CS are very similar. HA (Fig. 1B) consists of repeating disaccharide units of *D*-glucuronic acid and *N*-acetyl-D-glucosamine, which are linked through alternating β-(1 → 4) and β-(1 → 3) glycosidic bonds. In CS, *N*-acetyl-D-galactosamine combined with D-glucuronic acid constitutes a monomeric unit. The difference between a galactosamine (as present in CS) and a glucosamine (as present in HA) is the position of the OH on the −4 position, in HA this OH is equatorial, for CS it is axial. Furthermore, CS can be sulfated in many different patterns such as unsulfated chondroitin (see Fig. 1C), chondroitin-4-sulfate, chondroitin-6-sulfate, chondroitin-2-6-sulfate and chondroitin-4-6-sulfate (all represented in Fig. 1D) [16].

Among the most frequently studied derivatives of HA and CS are those that have a (meth)acrylate moiety on the HA/CS backbone. Such derivatized polymers are attractive for making hydrogels for tissue engineering due to their one-step synthesis, good biocompatibility and their ability to photopolymerize in the presence of a suitable photoinitiator [22]. Multiple names and acronyms for HA and CS derivatized with methacrylates exist in literature. For HA, Methacrylated Hyaluronic Acid (MHA or MeHA [23,24]), and Hyaluronic Acid MethAcrylate (HAMA [25]) are commonly used. Similarly, MCS (Methacrylated Chondroitin Sulfate [26]) and CSMA (Chondroitin Sulfate MethAcrylate [27,28]) are used. The international union of pure and applied chemistry (IUPAC), in its compendium of polymer terminology and nomenclature, advises that derivatizations of polymers onto the backbone of a polymer are to be mentioned after the name of the backbone polymer. Naming the group installed onto the main polymer before the polymer is meant to imply end-group derivatization of the polymer [29]. In both HA and CS, the methacrylate groups are attached to the backbone (see Refs. [22,23,27,28] for examples) and therefore in this review the terms HAMA and CSMA are used, as these conform to the prescribed nomenclature and can be considered most correct.

In this review, a literature overview is presented discussing hydrogels that contain hyaluronic acid (HA) or chondroitin sulfate (CS) glycosaminoglycans (see Fig. 2). Here specifically, the focus is on methacrylate-derivatized HA and CS, as these polymers have received substantial scientific attention for tissue engineering applications in the last decade [30–33]. An overview of the synthesis and physicochemical characterization of these methacrylate-derivatized polysaccharides is given and research done in elucidating the enzymatic degradability of the covalently crosslinked hydrogels is reviewed. Finally, the combination of methacrylated HA/CS with other polymers and the applications of these hydrogel-forming polymers is discussed.

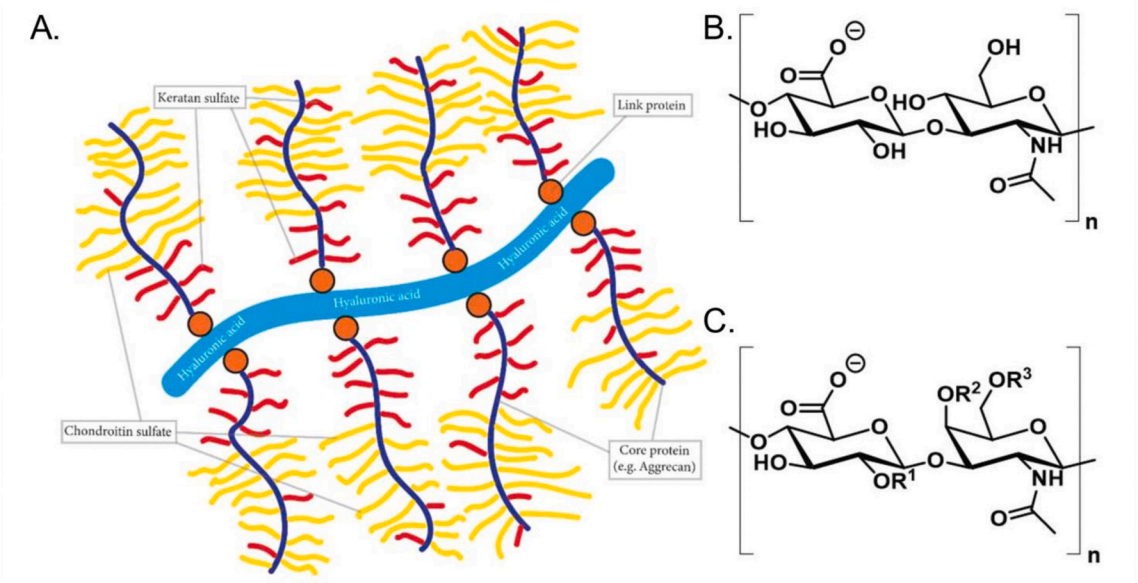

Fig. 1. A: Schematic representation of proteoglycans complexed with a hyaluronic acid molecule; link proteins (in orange) connect the core protein (in dark blue) of the proteoglycan to the HA backbone (in light blue), the core protein contains multiple glycosylation sites where GAGs like keratan sulfate (in red) and chondroitin sulfate (in yellow) are covalently attached. B and C: molecular structures of hyaluronic acid and chondroitin sulfate respectively. For chondroitin sulfate several sites of sulfation are possible: 4-sulfation (where $R^1 = H$, $R^2 = SO_3^-$, $R^3 = H$), 6-sulfation (where $R^1 = H$, $R^2 = H$, $R^3 = SO_3^-$), 2,6-sulfation (where $R^1 = SO_3^-$, $R^2 = H$, $R^3 = SO_3^-$) and 4,6-sulfation (where $R^1 = H$, $R^2 = SO_3^-$, $R^3 = SO_3^-$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

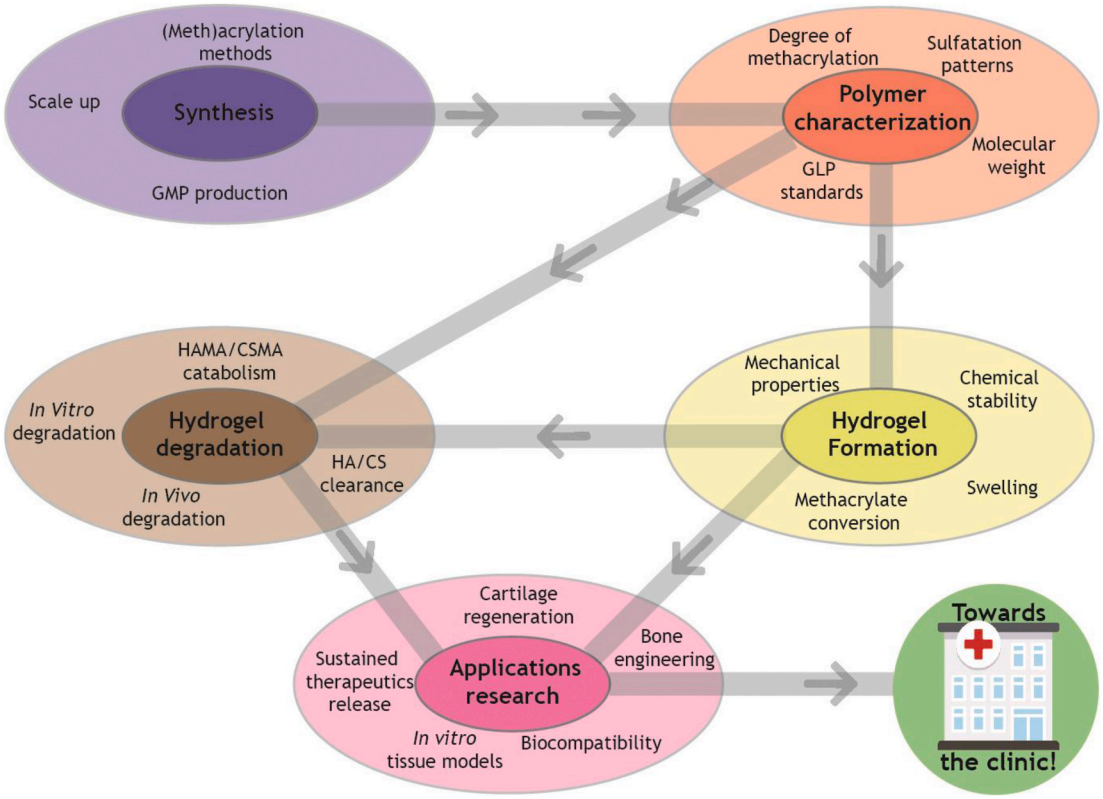

Fig. 2. Schematic overview of the contents of this review and how they affect each other. The goal of this review is to assess the necessary steps for HAMA and CSMA hydrogels to move forward towards the clinic. Therefore, the entire spectrum of preclinical research into these materials is discussed. Specifically, an in-depth overview of the polymer derivatization techniques for both HA and CS is given, followed by a discussion on the methods of characterization in terms of polymer M_w/M_n, DM and sulfation. Furthermore, *in vivo* clearance and enzymatic degradation of HA/CS and HAMA/CSMA in addition to the various hydrogel properties are discussed. Finally, an extensive overview of various applications of HAMA/CSMA containing hydrogels that have been reported in literature is presented.

Throughout this review, recommendations about best practices towards GLP development are given and open challenges that need to be met for the clinical translation of HAMA and CSMA are addressed. In addition, this review gives an overview of the scientific literature published regarding the pre-clinical development of covalently crosslinked hydrogels that (partially) are based on HAMA and/or CSMA.

2. Synthesis of photocrosslinkable derivatives of hyaluronic acid and chondroitin sulfate

Most common groups used for radical polymerization reactions are acrylate and methacrylate functionalities, which are also frequently used for light triggered crosslinking of water-soluble polymers to obtain hydrogels. This section gives an overview of the synthetic methods used to modify HA and CS with either methacrylate or acrylate groups. Additionally, the related challenges, such as scale-up and product reproducibility, that need to be overcome to make methacrylated HA and CS successful clinical products is addressed.

2.1. Synthesis of (meth)acrylated hyaluronic acid derivatives

Introduction of the (meth)acrylate containing moieties on HA chains mainly takes place at one of the two most reactive sites at HA – the carboxylic group of the D-glucuronic acid unit or the primary alcohol group at C-6 of the *N*-acetyl-D-glucosamine unit. As can be seen from the reported examples below, the two main reactions leading towards

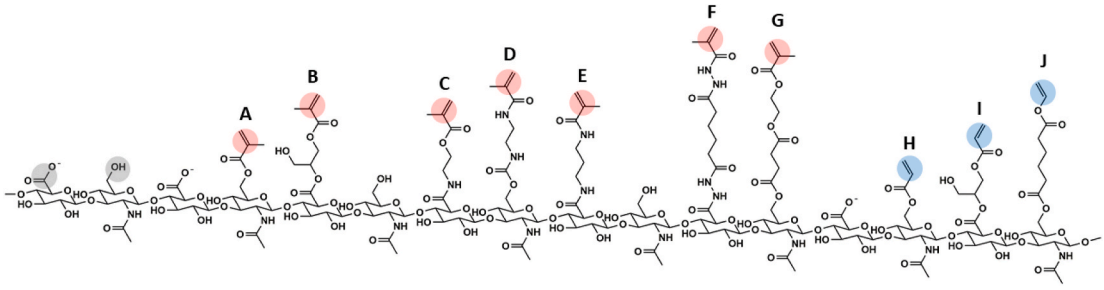

Fig. 3. Schematic representation of HA and corresponding grafted photopolymerizable (meth)acrylate side groups used for hydrogel fabrication. At far left is unmodified HA repeating unit, with grey circles indicating reactive sites on HA – carboxylate of the D-glucuronic acid and C6 primary alcohol of the *N*-acetyl-D-glucosamine monomer. Red circles correspond to reactive methacrylate, whereas blue circles to acrylate groups grafted on HA. Specific side groups result from reactions between HA and methacrylic anhydride (A), glycidyl methacrylate (A,B), 2-aminoethyl methacrylate (C), ethylene diamine and methacrylic anhydride (D), *N*-(3-aminopropyl)-methacrylamide (E), adipic acid dihydrazide and methacrylic anhydride (F), hydroxyethyl methacrylate (G), acrylic acid (H), glycidyl acrylate (H,I) and divinyl adipate (J). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(meth)acrylated derivatives of HA are esterification and amidation reactions.

The most common modification of HA is the esterification of the primary alcohol group with methacrylic anhydride (Fig. 3A) [34–36]. This reaction is performed in the presence of an excess of methacrylic anhydride in water and requires maintenance of the pH between 8 and 10 to drive the reaction forward and to neutralize methacrylic acid, which is formed from the reactions between methacrylic anhydride and water and HA and methacrylic anhydride. Typically, these reactions are performed at 4 °C overnight. Besides water and PBS [37] also water-DMF mixtures [38,39] have been reported as solvents used for the reaction of HA with methacrylic anhydride. Modified polymer can be purified by dialysis, precipitation into organic solvents (usually ethanol or acetone) or a combination of these two methods [39–44]. Degrees of methacrylation can be tailored depending on the feed ratio of methacrylic anhydride and reaction time, ranging from 10 to 49% [39,45,46].

Next to methacrylic anhydride, glycidyl methacrylate (GMA) is often used to introduce methacrylate pending groups on HA [47]. This reaction can take two different routes and thus lead to different products [48,49]. It proceeds via a reversible, transesterification reaction between GMA and the primary alcohol of HA (as first shown for the reaction of GMA and dextran in DMSO catalyzed by DMAP [50]) (Fig. 3A), or via a ring-opening reaction of the oxirane (also named as epoxide) group of GMA and a carboxylic acid group of HA (Fig. 3B). Transesterification is a rapid, reversible reaction, favored in aprotic solvents, such as DMSO, whereas in aqueous medium, at long reaction times the ring-opening reaction product may be favored [48]. Only few authors report on the possibility of formation of the two products simultaneously [48,51]. There are several variations of the reaction between GMA and HA reported. In regard to the reaction medium, water [52,53], as well as mixtures with organic solvents (acetone and DMF) [54,55] have been reported for this reaction. The reaction is usually done in the presence of a catalyst, such as 4-dimethylaminopyridine (DMAP) [56–58] or triethylamine (TEA) [59,60]. Several authors reported the combination of TEA and tetrabutylammonium (TBA), the latter compound being used to improve compatibility with organic solvents [53,61]. These reactions are usually conducted at room temperature, whereas reported reaction times go from 12 h up to several days. The purification of the product relies on the same methods as discussed above, whereas degree of methacrylation can be tuned by GMA content and reaction times and it can reach as high as 90% [48].

Although less explored, several other photopolymerizable methacrylated HA derivatives have been reported. One of them is an aminoethyl methacrylate (AEMA) derivative of HA (Fig. 3C). The methacrylate moiety is introduced via amide bond formation by reaction of AEMA with the carboxylic acid groups of HA, using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) as coupling agent. In this way, the methacrylate group is linked to HA via an ethylene linker. Two main synthetic routes have been reported for preparing HA-AEMA. When the tetrabutylammonium salt of HA (HA-TBA) is used as starting reagent, the reaction can be done in DMSO and in the presence of benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) as coupling agent [62,63]. Alternatively, the same product can be obtained starting from the sodium salt of HA dissolved in water, using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide/*N*-hydroxysuccinimide (EDC/NHS) coupling between –COOH of HA and $–NH_2$ of the AEMA [64,65]. These reactions are performed for 16 h at room temperature. As alternative route, Pitarresi et al. reported also ethylene diamine methacrylate derivative of HA, where the reaction takes place at the primary alcohol site (Fig. 3D) [66]. The synthetic route leading to this derivative involves the conversion of HA to HA-TBA salt dissolved or dispersed followed by –OH activation with bis (4-nithrophenyl) carbonate, and subsequent reactions with ethylenediamine and methacrylic anhydride, respectively [66].

By using 3-aminopropyl methacrylamide (APMAm) as methacrylating agent, an analogous derivative as HA-AEMA was formed, with the linker containing an additional carbon atom (Fig. 3E) [67,68]. This HA derivative was synthesized exploiting EDC coupling chemistry as well, using the combination of EDC and hydroxybenzotriazole (HOBt).

Hahn et al. used adipic acid dihydrazide (ADH) to synthesize photopolymerizable HA [69,70]. First, HA was modified with ADH via EDC coupling and the obtained polymer was subsequently reacted with an excess of methacrylic anhydride to yield adipic hydrazide methacrylate pending functionality on HA chains (Fig. 3F).

However, when performing HA functionalization by using bifunctional linkers, attention should be given to the product characterization, since there is the possibility of intra- and intermolecular crosslinking under the reaction conditions.

In a number of studies hydroxyethyl methacrylate (HEMA) was used as the side chain polymerizable moiety [71–73]. To enable coupling, HA was first converted into a less polar version, namely its HA-TBA salt. Correspondingly, HEMA was modified with succinic anhydride, in the presence of *N*-methylimidazole (NMI), yielding HEMA-succinate. Finally, HA-TBA and HEMA-succinate were coupled in DMSO, with di-tert-butyl dicarbonate (Boc_2O) as coupling agent and DMAP as catalyst, to yield a HA derivative with methacrylate units coupled via hydrolyzable ester groups (Fig. 3G).

HA derivatives used for hydrogel fabrication containing acrylic moieties have also been reported. E.g. Khetan et al. synthesized acrylated HA starting from HA-TBA salt [74] (Fig. 3H). The reaction was performed in DMSO by reaction of HA-TBA with acrylic acid, in the presence of DMAP as catalyst and Boc_2O as coupling agent. More recently, Sigen et al. reported on the reaction of glycidyl acrylate with HA in a water/DMF mixture to yield acrylated HA [75]. Similar as for glycidyl methacrylate, two different products can be formed, resulting from transesterification (Fig. 3H) and ring-opening reactions (Fig. 3I).

Finally, enzymatic synthesis of a photocrosslinkable derivative of HA has also been reported. Qin et al. described the synthesis of HA vinyl esters for UV-crosslinked hydrogels [76] (Fig. 3J). Specifically, the authors made use of lipase-catalyzed transesterification reaction. In short, HA-TBA was dissolved in anhydrous DMSO and the reaction was done at 50 °C, in the presence of divinyl adipate and lipase (from *Candida Antarctica B*). The degree of substitution could be tailored from 0.13 to 1.25 and depended both on the reaction time and the molar ratio between divinyl adipate and hydroxyl groups of HA.

HA and its methacrylated derivatives reported here could be very useful for fabrication of the hydrogels with translational potential. However, it is crucial that in the pre-clinical phase of the studies, such derivatives are prepared according to Good Laboratory Practices (GLP). Therefore, the reactions used to synthesize HA derivatives should be reproducible. This means that the degree of methacrylation has to be reproducible when following reported methods. However, only few authors reported on the reproducibility of the degree of methacrylation [39,66]. In addition to reproducibility, for successful translational use of the polymers, the production process (comprising synthesis and purification) should also be scalable (tens to hundreds of grams), in terms of obtaining the desired product, safety and costs. Many of the reported methods make use of organic solvents (DMSO, DMF, acetone, ethanol), some of which are not easily removed. Importantly, organic solvents require proper disposal and their use and removal on a large scale can be expensive. Methods that make use of organic solvents for reaction should not be considered as the first choice when making HAMA polymers for translational purposes. Rather, methacrylation methods using water as solvent are preferable. An example is given by methacrylation with methacrylic anhydride in water. Different authors reported on similar degrees of methacrylation (20–25%) under comparable reaction conditions [41,42]. Likewise, other reported reactions making use of TBA salts of HA, and other catalysts/coupling reagents (e.g. DMAP, BOP, TEA, EDC, NHS) would not be recommended, as these reagents would have to be efficiently removed. Additionally, reactions which require

multiple steps or long reaction times (e.g. several days in case of methacrylation by glycidyl methacrylate) should also not be considered as the first choice.

Concerning scalability and costs of the production process, purification procedures of the final polymer are very important. Precipitation in organic solvents (e.g. ethanol) and dialysis/freeze-drying are mostly reported. Precipitation in ethanol could be a more straightforward and faster option, considering that ethanol can easily be removed, but the efficiency of the process should be investigated in terms of molecular weight of the polymers used. On the other hand, dialysis/freeze drying is more time consuming and there is a higher risk of contamination, thus further investigation is necessary to evaluate which option is most suitable for large scale purification of such polymers.

From discussed examples it is evident that by far the most frequently used routes for synthesis of HAMA include either reaction with methacrylic anhydride or reaction with glycidyl methacrylate. The reason for this preference is that both methods are simple, one-step reactions and both reagents are inexpensive and easily available despite being used in large excess (5–20 fold). However, the GMA route leads to two products (transesterification and ring-opening), making reproducibility and control over the degree of methacrylation more difficult. Therefore, methacrylation with methacrylic anhydride in water may be more advantageous in terms of reproducibility and scalability, with a reaction duration of typically 8–24 h.

2.2. Synthesis of (meth)acrylated chondroitin sulfate derivatives

Similarly to HA modifications as discussed in the previous section, CS has mainly been derivatized with methacrylate containing functionalities to yield macromers suitable for light triggered crosslinking for hydrogel fabrication. There are several isomers of CS, based on the position of the sulfate group. The most common one is chondroitin 4-sulfate (CS-4), represented in Fig. 4 (left) that has its sulfate group at position C-4, leaving the primary –OH at position C-6 available for chemical derivatization. Another frequently used isomer is chondroitin 6-sulfate (CS-6), shown in Fig. 4 (right). In this polymer the primary –OH group at C-6 is not available for functionalization, although secondary –OH groups might also be reactive. Therefore, most studies concerning methacrylation of CS make use of CS-4-sulfate. Besides, the carboxylic acid group of the D-glucuronic acid unit is another reactive site available for CS derivatization [77].

In contrast with HA, there are fewer reported methacrylated derivatives of CS. Also here, the most common derivatization is the reaction of this polymer with methacrylic anhydride [37,78–86]. This esterification reaction takes predominantly place at the primary –OH at C6 of the *N*-acetyl-D-galactosamine unit (Fig. 5A). This reaction is usually performed in water or PBS, but water/DMSO mixtures have also been used [87,88]. Methacrylic anhydride is used in large excess and basic conditions are needed to drive the reaction forward and to neutralize methacrylic acid, formed as a result of the (aimed) reaction of CS with methacrylic anhydride, but also from the side reaction between methacrylic anhydride and water. The methacrylated product can be precipitated using a nonsolvent (ethanol, methanol, acetone) and/or dialyzed for purification. The methacrylation degree depends on the molar ratio of the anhydride and hydroxyl groups of CS, the reaction time and temperature used, and values as high as 70–75% have been reported [79,89,90]. Reaction with GMA is another very common method for introducing methacrylate side groups on CS. In the same way as for HA, methacrylated products of CS result from transesterification and ring-opening reactions. Li et al. investigated in detail these products and the conditions leading to their formation [91]. In particular, the transesterification product (Fig. 5A) is favored at short reaction times when water is used as solvent, or in aprotic solvents, such as DMSO, whereas after 3 or more days of reaction in aqueous environment, the epoxide ring-opening product (Fig. 5B) predominates [49,92]. This reaction is performed by adding GMA to CS dissolved in either water or PBS at room temperature, followed by stirring for 1–15 days [49,91,93–96]. By changing the reaction time and the amount of GMA, the degree of methacrylation could be tuned. Li et al. reported that the reaction between GMA and CS resulted in the formation of different products due to transesterification, ring-opening reactions, as well as from reaction with glycidol (a product formed from GMA hydrolysis or ester hydrolysis of the CS ring-opening product) [91] (Fig. 5). The complexity of such reactions and several possible formed products are usually not discussed by the majority of authors working on CSMA. However, Reis et al. report on the formation of both transesterification and ring-opening products [92]. Moreover, Guilherme et al. reported that GMA reacted with both carboxyl and hydroxyl groups of CS via epoxide ring-opening mechanism [97]. Such reaction is favored under acidic conditions (pH 3.5) at 60 °C in water [97–99]. Abbadessa et al. reported an alternative method to obtain the methacrylated product [100]. Here, chondroitin sulfate tetrabutylammonium salt (CS-TBA) was used and the reaction was performed in the presence of DMAP, in a polar aprotic solvent (DMSO) for 2 days at 50 °C. By varying feed molar ratio between GMA and the disaccharide units of CS-TBA, the degree of methacrylation could be tuned from 5 to 40%.

Similarly to HA, 2-aminoethyl methacrylate pending moieties (AEMA) have also been introduced in CS [41,101–107]. The reaction takes place at the carboxylic acid moieties of the D-glucuronic acid units of CS via amide bond formation (Fig. 5C). The reported method for preparing such CS derivative has been used by several authors, applying the same reaction conditions. Generally, the reaction is performed in 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, using EDC/NHS chemistry [108].

CS derivatized with APMAm has been reported as well (Fig. 5D) [109]. Here, EDC/NHS coupling chemistry was used, resulting in amide bond formation due to the reaction between the carboxylic acid group of CS and the primary amine of APMAm.

In contrast to methacrylate derivatizations, acrylate derivatives of CS have been explored to a lesser extent. An example is given by the work of Jo et al. where CS was first functionalized with adipic acid dihydrazide (ADH), by reacting –COOH of CS and primary $–NH_2$ of ADH [110]. Subsequently, by means of EDC coupling chemistry, acrylic acid was grafted onto CS-ADH to yield an acrylated CS derivative (Fig. 5E).

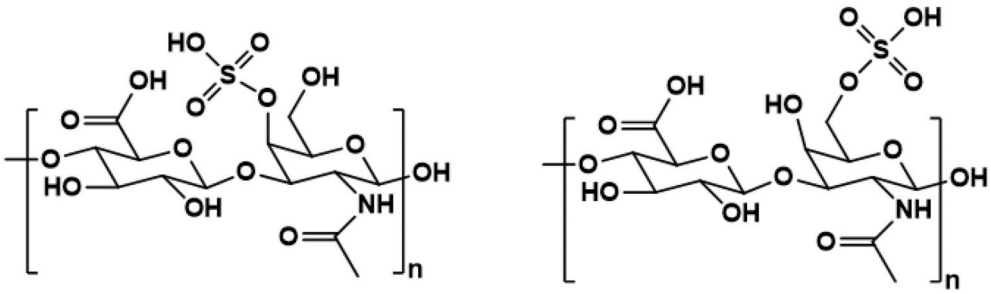

Fig. 4. Molecular structures of the most common isomers of CS, with CS-4 (left) and CS-6 (right).

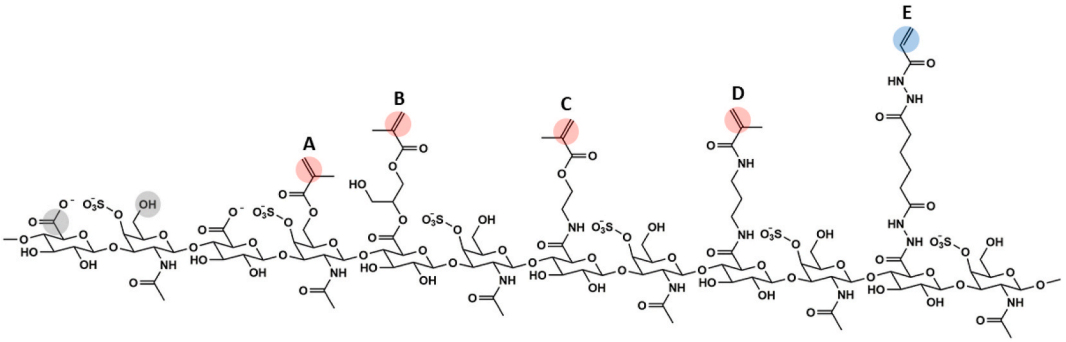

Fig. 5. Schematic representation of CS-4 and corresponding grafted photopolymerizable (meth)acrylate side groups used for hydrogel fabrication. At far left is unmodified CS repeating unit, with grey circles indicating reactive sites on CS – carboxylate of the D-glucuronic acid and C6 primary alcohol of the *N*-acetyl-D-galactosamine monomer. Red circles correspond to reactive methacrylate, whereas blue circle to acrylate groups grafted on CS. Specific side groups result from reactions between CS and methacrylic anhydride (A), glycidyl methacrylate (A,B), 2-aminoethyl methacrylate (C), *N*-(3-aminopropyl)-methacrylamide (D), adipic acid dihydrazide and acrylic acid (E). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Similarly to HAMA, CSMA based hydrogels hold a good potential for translational applications. Therefore, it is necessary to comply to the requirements of Good Laboratory Practice and to pay attention to reproducibility and scalability of the production process, as already discussed for HAMA. Looking at the reported literature examples for preparing CSMA, it seems evident that the two methods mostly used to methacrylate CS are by means of glycidyl methacrylate and methacrylic anhydride. Both of these reactions can be done successfully in water. Hence, both of these methods are attractive in terms of scalability, also considering the affordability of the reagents often used in large excess. However, when using glycidyl methacrylate as methacrylating agent, two products are formed and the reaction times reported are in the range of days (up to 15 days), whereas for the MA method the reaction is usually done in 24 h. The degrees of methacrylation reported for GMA route are in the range from 5 to 40%, whereas for the methacrylic anhydride route the reported values range between 5 and 75% (at different feeding ratios). Reproducibility was not studied in detail by most of the researchers, although some of them used the same method (methacrylic anhydride) to produce polymers with similar degrees of methacrylation (~40%) [79,82]. Based on the given overview, we believe that the methacrylation with methacrylic anhydride in water would be the recommended method for the large scale production, similar as seen for the preparation of HAMA. The purification steps, typically involve precipitation and dialysis/freeze-drying, so in terms of cost-effectiveness the production strategy of the final product has to be further assessed.

2.3. Characterization of HA, CS and their photocrosslinkable derivatives

Proper physico-chemical characterization of HA and CS both before and after (meth)acrylation is crucial to controlling and understanding hydrogel properties like swelling, stiffness, network density, pore size and enzymatic degradability. It has been shown that these properties have their effect on the behavior of cells seeded in these hydrogels as well [111,112]. For CS in particular, the degree of sulfation is also an important parameter that determines amongst other things the hydrogel swelling, pore size and potential protein interactions. Validated characterization methods are required for pre-clinical hydrogel studies under GLP. Validated analysis techniques for important parameters such as molecular weight and Degree of Methacrylation (DM) of the synthesized HAMA and CSMA are therefore crucial to develop. In this paragraph, several methods of determining HAMA/CSMA number and weight average molecular weights (M_n and M_w), molecular weight distributions and sulfation (patterns) are discussed. In addition, several methods to determine the DM are presented and discussed.

2.3.1. Determination of molecular weights of (meth)acrylated HA and CS

The molecular weights and their distributions of HA and CS depend on the source of the materials. For HA, products with molecular weight in the range of 1–2000 kDa are commercially available and are sufficiently reproducible with relatively low polydispersities. For instance, in a recent publication, the absolute PDI of pharmaceutical grade HA of various average chain lengths (from ~0.5 to 287.4 kDa) was reported to be between 1.16 and 1.96 [113]. Pharmaceutical grade HA is mainly produced by biotechnological processes, starting with bacterial culture and subsequent processing through enzymatic cleavage down to the preferred chain length [114]. HA isolated from various animal tissues of similar quality is also available [115]. For CS, the range of available molecular weights is substantially smaller, ranging from 2 to 60 kDa [116,117]. For comparison, the average molecular weight for CS extracted from human articular cartilage is ~16–22 kDa, indicating that the commercially available CS products are similar to the naturally occurring CS [118]. Currently, CS is only available as extracts from various animal tissues, and yield sufficiently monodisperse molecular weight distributions to although research on the biotechnological production of pharmaceutical grade CS is currently being explored [119].

Methods for determining absolute HA(MA)/CS(MA) molecular weight and molecular weight distributions include light scattering techniques such as multiple angle laser light scattering (MALLS) [120]. The average polymer relative molecular weight can also be obtained using aqueous phase gel permeation chromatography (e.g. using dextran-based calibrations). Other methods include: osmometry, viscometry, analytical centrifugation and low-angle laser light scattering [117,120–123]. With regards to the development of a GLP validated method for M_W determinations of HA and CS a good method should yield accurate (i.e. with a standard deviation < 5%) and precise results whilst allowing sufficient range of measurable molecular weights. Considering this information, most molecular weight determination techniques mentioned above should be robust. Of special note is the option of MALLS, as this technique allows for the determination of the absolute molecular weight distribution. Measuring the absolute polymer chain distribution is preferential, as it does not give an estimate that is relative to a calibration curve of reference materials.

2.3.2. Determination of the sulfation patterns of CS

Proper (validated) analysis of pharmaceutical grade CS has been an issue in the past. For instance, in 2015 da Cunha et al. found several contaminations in a study of 16 batches of CS that were labeled 'pharmaceutical grade', with 11 of the samples even containing less than 15% CS [124]. Major contaminants found were maltodextrin, lactose and keratan sulfate. This study showed that further characterization should be required for registry and licensing of CS for pharmaceutical purposes. Any successful clinical translation of CS related products (such as CSMA hydrogels) would be heavily dependent on these stricter regulations.

CS is a heterogeneous polymer due to its different sulfation patterns

that depend on a variety of factors including the type of animal, tissue and the condition of the animal CS is extracted from. For example, Brown et al. studied CS extracted from equine cartilage and found that the ratio of 6-sulfated to 4-sulfated CS disaccharides increased with the age of the animal [125]. Further, degenerative cartilage was also found to consist of significantly less 6-sulfated disaccharides than present in healthy cartilage [125]. In general, the average number of sulfate groups per repeating disaccharide unit on a CSMA chain affects the swelling capacity of formed hydrogels due to charge-charge repulsive forces between these negatively charged moieties leading to attraction of free cations and Donan-Gibbs osmotic swelling, allowing a higher uptake of water into the polymer matrix of CSMA hydrogels over HAMA hydrogels [36].

The ratio of 6- to 4-sulfated CS can be determined via ^{13}C NMR measurements [126]. It is also possible to determine the disaccharide composition of CS through enzymatic degradation using chondroitinase ABC and subsequent quantification of the formed amounts of different disaccharides using e.g. chromatography, mass spectrometry or capillary electrophoresis [127–129]. Although determination of the sulfation patterns of CS is currently not required for clinical usage, it is highly advisable to properly characterize at least the 6 to 4-sulfation ratios of any CS batch that is to be used in a clinical setting, as especially in terms of growth factor binding and hydrogel swelling, the sulfation degree has been found to be highly important [125]. Due to the high error often seen for ^{13}C NMR measurements, it is likely that a GLP method based on depolymerization of the CS polymers and subsequent measurement of the amounts of individual disaccharides would be more suitable [119].

2.3.3. Determination of the degree of (meth)acrylation of synthesized HAMA and CSMA

The DM of HAMA and CSMA is an important parameter because of its effects on the swelling capacities, network densities and mechanical properties of fabricated hydrogels [130]. By far the most used analytical method applied to determine the degree of methacrylation of HAMA and CSMA is by ^{1}H NMR analysis [39]. Usually the intensity of the signals of the vinyl groups (signals can typically be found around ~5.8 and 6.2 ppm) of the methacrylates are quantified and compared with a known peak in the spectrum. Sometimes the DM is also quantified by quantifying the methacrylate methyl peak (at ~1.9 ppm), this quantification typically leads to an overestimation of the DM due to overlap of this peak with other HA/CS related proton peaks [48]. For DM determination via ^{1}H NMR analysis, standard error margins of ~8% are reported which is mostly related to the choice of spectral baseline during analysis [48]. Issues arise with this technique when applied to high molecular weight polymers. In solutions of such polymers (e.g. HAMA of a molecular weight >500 kDa) interactions between the polymer chains, such as hydrogen bonding, occur much more frequently as compared to shorter chain length polymers (at similar concentrations). This increased propensity for interchain interaction causes an increased viscosity and reduced proton mobility of the protons in the vinyl groups and leads to reduced signal intensity, peak broadening and thus less accurate determination of the DM [131]. Standard relative errors of up to ~15% have been reported for higher molecular weight substituted HAs [132]. Several steps can be undertaken to increase the proton mobility and decrease the experimental error of the measurement including increasing the solution temperature, depolymerization with HA degrading enzymes prior to measurement and increasing the ionic strength of the D_2O solution [131,133,134].

Another technique applied to determine the DM of HAMA and CSMA is through quantification of the methacrylic acids formed after hydrolysis of the ester linkages connecting MA to the HA/CS using HPLC analysis. To explain, a accurately weighed dry sample of HAMA or CSMA is dissolved in basic water (typically a 0.1 M NaOH in MilliQ water solution) and is incubated overnight at elevated temperature (~40 °C). All methacrylate-ester moieties connected to the polymers will be hydrolyzed under these conditions and will be released as methacrylic acid. Using a HPLC coupled to a UV detector (measuring at 210 nm) and a suitable calibration curve, the amounts of methacrylic acid in the HAMA/CSMA solutions can then be determined and be used to calculate the DM of the HAMA/CSMA polymer. This HPLC method has been shown to have good reproducibility and accuracy (the typical standard deviation is <1%) and can determine DM regardless of the Mw of the polymer used [36,100,135–137]. For clinical translation of HAMA and CSMA hydrogels, it will be important to develop a validated GLP method of determining DM. The HPLC method is best suited for use in GLP DM determinations, as it has a low error as compared to the ^{1}H NMR method. In addition, HPLC based determination of DM is independent of the molecular weight of the derivatized polymer. Additionally, it will be important to understand how both HAMA and CSMA polymers should be stored. It has recently been shown that ester groups present in the methacrylate groups of HAMA/CSMA polymers are liable to hydrolysis when stored in non-acidic buffers. Due to this hydrolysis the initial DM is reduced over time and potentially toxic side products such as methacrylic acid are introduced [113]. Storage of HAMA/CSMA in the dry state is therefore preferred, although further studies on the shelf life in this state need to be undertaken.

2.4. Formation and characterization of HAMA and CSMA-based hydrogels

Hydrogel formation from HAMA or CSMA is done through covalent crosslinking of these (meth)acrylated polymers. Radical polymerization of the polymer bound (meth)acrylate groups is initiated either thermally or through irradiation with light. In the latter situation, a small amount of initiator is added to the polymer solution to generate sufficient radicals for the crosslinking to proceed upon UV irradiation [1,138]. In most applications, light triggered initiation of crosslinking is used and many photoinitiators have been developed that dissociate into radicals when irradiated with various wavelengths of light (e.g. blue, green and UV). Factors that determine the efficiency of such light initiated reactions are the used light wavelength(s) and intensity, the type and concentration of photoinitiator used and the concentration of oxygen or other radical scavenging molecules present in the sample [1,138]. To use HAMA/CSMA hydrogels for clinical applications, it is important to understand the efficiency of methacrylate conversion. In the following sections, considerations are given regarding methods of quantifying both the crosslinking efficiency and methacrylate conversion after radical polymerization and the effects of the polymer characteristics on the hydrogel properties.

2.4.1. Methacrylate crosslinking efficiency determinations

An important and not always reported parameter of formed HAMA/CSMA hydrogels is methacrylate conversion after crosslinking [36]. Determining the methacrylate conversion is of high importance as an incompletely crosslinked hydrogel can have significantly different properties as compared to a fully crosslinked hydrogel made from a HAMA/CSMA of a similar DM. In addition, unreacted methacrylic moieties (as can be found on partially crosslinked HA/CS derivatized with methacrylic anhydride) are susceptible for ester hydrolysis and will release from the hydrogel as toxic methacrylic acid [139,140]. The typical methacrylate conversion of HAMA or CSMA based hydrogels irradiated with UV light in the presence of a photoinitiator is reported to be between ~90 and 98% [36,48].

Crosslinked hydrogels based on HA as a precursor have already been developed for use in the clinic as dermal fillers. Most HA dermal fillers are crosslinked using 1,4 butanediol diglycidyl ether (BDDE) that received market authorization in 1996 [141]. Dermal fillers based on HA with other crosslinking agents such as bisocarbodiimide, divinylsulfone and 2,7,8-diepoxyoctane are also used in clinics [21]. HA-BDDE gels are crosslinked before administration and are purified to remove unreacted crosslinker from the gels. Free BDDE amounts smaller than 2 ppm are required due to safety regulations regarding potential BDDE

toxicity [141,142]. Considering the reported toxicity of methacrylic acid which is formed upon hydrolysis of unreacted methacrylate groups from HAMA/CSMA-hydrogels, similar requirements for implantation can potentially be expected for these hydrogels. Importantly, this would mean that an *in vivo* crosslinked hydrogel should contain less than 2 ppm of unreacted methacrylate groups. For post-crosslinking implanted hydrogels, a lower crosslinking efficiency could potentially be acceptable for clinical use, if further purification (e.g. dialysis) would be performed. These insights further emphasise the importance of methacrylate conversion measurements after crosslinking.

Determination of the methacrylate conversion can be done in similar ways as the DM. It should be noted however that upon polymer network formation the proton mobility of the vinyl groups of the methacrylates decreases significantly which leads to peak broadening and higher measurement errors when using ^{1}H NMR to quantify the amount of remaining unreacted methacrylates and thus the conversion [136]. Unreacted methacrylates present in the formed hydrogel can also be semi-quantitatively determined via FT-IR analysis [140,143]. The HPLC method described in section 2.3.3. Can easily be validated and used with crosslinked HAMA/CSMA hydrogels to accurately and precisely (standard deviation $< 1\%$) determine the methacrylate conversion kinetics and final conversions under GLP [137].

2.4.2. Effects of polymer characteristics on hydrogel properties

Bencherif et al. reported fully crosslinked GMA derivatized HA hydrogels with a DM ranging from 14 to 90% (please note that the DM calculation used from ^{1}H NMR measurements differs from the indicated norm in this review, causing a overestimation of the DM) [48]. They showed that the swelling ratio (defined as the swollen gel weight divided by initial gel weight) for the 14% DM hydrogel was 4.7, and sharply decreased to 1.8 for 23% DM and subsequently to 1.3 for the 90% DM hydrogel (HA $M_w = 1.6 \times 10^6$ Da, polymer concentration was 5.0 wt%, swelling ratios reported here were recalculated from Ref. [48]). Additionally, the shear modulus of the fully crosslinked swollen HAMA hydrogels increased from 22 to 65 kPa when increasing DM from 14 to 90% [48]. In another contribution, our group showed a similar trend in decrease of swelling ratio from 1.5 to 1 (this means that the hydrogel was dimensionally stable) upon increasing the DM of HAMA from 5 to 30% (DM was determined *via* HPLC as described in 2.3.3., HA $M_w = 1.7 \times 10^6$ Da, polymer concentration of 2 wt%) [25]. Oudshoorn et al. also reported that the HAMA DM affected the compressive modulus, as hydrogels with higher DM became progressively stiffer due to the higher network density. Combining HAMA with other polymers is a frequently investigated method to increase the stiffness of hydrogels and has been reported for a number of polymer combinations [135,144,145]. Interestingly, for high molecular weight HAMA combined with a thermosensitive triblock copolymer, a phenomenon has been observed where phase separation between the HAMA and this synthetic polymer influenced the mechanical properties of the formed hydrogel [146]. For CSMA hydrogels, the swelling ratio was around 2 for a 20 wt%, 15% DM hydrogel (DM determined *via* HPLC [100]). When comparing the swelling of CSMA hydrogels as reported by Abbadessa et al. [100] with the data on HAMA hydrogels in the study of Oudshoorn et al. [25] it is observed that CSMA-based hydrogels have a higher equilibrium swelling than HAMA hydrogels most likely due to the higher charge density present in CSMA over HAMA. Data regarding the mechanical properties of CSMA-only hydrogels are lacking, however, several studies on CSMA hydrogels crosslinked with other methacrylated polymers showed that an increase of the DM of CSMA resulted in an increase of the Youngs modulus of hydrogels [100,135,147].

Insights into the stability of hydrogels based on HAMA and CSMA polymers are essential for their aimed clinical applications. In recent work, the hydrolytic sensitivity of polymer networks based on covalently linked HAMA or CSMA polymers has been investigated. It was found that hydrogels purely based on CS derivatized with GMA or MA degraded in physiological buffer (pH 7.4, 37 °C) after 2–25 days in a crosslinking density dependent manner. HAMA hydrogels were found to be stable in the same conditions over a 50 day timeframe [113]. The observed hydrolysis rate differences were ascribed to the chirality of the amino sugars present in HAMA and CSMA. To explain, CSMA's *N*-acetyl-galactosamine does not form hydrogen bonds with the adjacent oxygen of the glucuronic acid, whereas the *N*-acetyl-glucosamine present in HAMA does. This absence of intra-polymer hydrogen bonds in CS(G)MA allows for higher polysaccharide chain flexibility and hydration in comparison to HAMA. This increase in flexibility and local hydration is likely responsible for the increased hydrolytic sensitivity of CSMA hydrogels [113]. Based on the described results, it is important to consider the hydrolytic liability of the ester bonds in crosslinked CSMA polymers related to the aimed application of the corresponding hydrogel.

The degree and patterning of sulfation found in CSMA also plays a large role in the potential of binding (positively charged) growth factors and other soluble signaling molecules present in the ECM [13]. These factors are typically bound to CS via electrostatic interactions and can greatly affect the response of cells seeded into such hydrogels. An example is given by Lim et al. where CSMA and desulfated chondroitin methacrylate hydrogels were evaluated for their effects on retention of histone (as a model of a positively charged protein) and on cartilaginous differentiation of encapsulated human mesenchymal stem cells. Cells encapsulated in hydrogels based on chondroitin methacrylate showed significant upregulation in gene expression of collagen II and aggrecan after 21 days as compared to those based on CSMA. This difference is explained through the lesser electrostatic interactions of chondroitin based hydrogels with positively charged important growth factors such as TGF-β [148]. Another example of these sulfate based interactions can be found in an investigation of CSMA hydrogels as a material for use in neuronal microenvironment engineering [149]. Here, CSMA hydrogel scaffolds have been shown to promote the maintenance of the undifferentiated state of encapsulated neural stem cells by sequestration of endogenous fibroblast growth factor 2 [149]. Several other publications also report that binding of growth factors by CSMA's sulfate moieties can be useful for growth factor delivery in tissue engineering [36,109].

3. Enzymatic degradation of (crosslinked) HA(MA) and CS(MA)

An important characteristic of HAMA and CSMA based hydrogels for tissue engineering applications is their biodegradability. Here, we will briefly discuss the clearance of free HA/CS from the body, followed by a summary of human HA and CS degrading enzymes and the proposed mechanisms through which HA/CS are degraded. Based on this information some important points of discussion for enzymatic degradation of implanted HAMA and CSMA chemically crosslinked hydrogels are presented.

3.1. Clearance of soluble HA and CS

As a result of the research interest to use of HA for eye, knee and cosmetic applications in the early 1980s, several bio-accumulation studies with radioactively labeled soluble HA have been done [150–154]. When injected directly into the human bloodstream, HA was found to be removed quickly by liver sinusoidal endothelial cells with high endocytosis capacities resulting in a half-life of 2–6 min. Once endocytosed HA was rapidly degraded in the lysosomes of the sinusoidal endothelial cells and most degradation products were found to be excreted renally [152]. HA injected into the knee of rabbits diffuses into the bloodstream where it is subsequently quickly degraded in the liver as described above. The half-life of injected HA in the synovial fluid of healthy rabbits was around 13 h [151,155,156]. The clearance rate of HA thus depends highly on the location of injection and the half-life of HA injected into human skin is one to two days [16,152,156]. Clearance rates by the body of free CS after intravenous injection are very similar to that of HA [16,157].

3.2. Catabolism of HA and CS

As HAMA and CSMA are derivatives of naturally occurring HA and CS, it is important to understand better how the native polysaccharides are degraded *in vivo*. Enzymatic degradation of HA and CS is a complex process which involves many different enzymes and receptors. The full catabolic pathway of enzymatic degradation of HA and CS is not completely understood yet. There are two important HA/CS degrading enzymes that are both part of the same family: hyaluronidase type 1 and 2 (abbreviated as HYAL1 and HYAL2, respectively). HYAL1 and 2 have the highest enzymatic activity in acidic environments and are mainly present in the lysosomes of cells, with HYAL2 is also present on the cell-surface. They are assisted in degrading HA and CS by membrane receptors that mediate HA/CS fragment endocytosis [158,159]. Literature regarding chondroitinases is not reported here as these are bacterial in nature and do not share the specific mechanism of the human CS degrading enzymes [160]. The known human chondroitin sulfate degrading enzymes are part of the family of enzymes termed hyaluronidases (the family name is a misnomer). All known HYALs cleave HA and CS endolytically (i.e. binding to and chain cleavage throughout the backbone).

In Table 1 a comprehensive list of known HA and/or CS degrading enzymes can be found. In addition, known cell-surface receptors that can mediate HA/CS endocytosis are discussed, as well as non-specific lysosomal enzymes capable of exolytically depolymerizing HA/CS. In exolytic action, the enzymes catalyze HA/CS degradation at the end groups of the polymer chains instead of randomly over the polymer chain.

3.2.1. Hyaluronidases (HYALs)

The hyaluronidase family of enzymes all have similar catalytic mechanisms and degrade their substrates via hydrolysis. All human HYALs are endo-β-*N*-acetyl-hexoaminidases, as opposed to HYAL enzymes that are bacterial in origin [164,176]. Most HYALs can cleave both HA and CS, with HYAL4 showing specific activity only towards CS [161,168]. HYAL1 and HYAL2 have long been thought to be the major HA/CS degrading human enzymes as they are the only HYALs present in most somatic cells. HYAL1 is mainly found in the lysosomes of cells, but is also be found in blood plasma and urine [163]. Possibly, HYAL1 is excreted from cells through exosomes or released upon cell death and cleared through the blood stream and glomerular filtration. HYAL1 has no reported enzymatic activity at physiological pH, but has its optimum activity at pH 4–5 [161–163]. HYAL2 is found attached to cell membranes with a Glycosyl Phosphatidyl Inositol (GPI) anchor and this enzyme is also found in the lysosomes of most somatic cells. GPI anchored HYAL2 has been reported to have a pH optimum around 6–7 and has found to be able to shed into the ECM [161,164–167]. Interestingly, HYAL1 and HYAL2 both cleave HA and CS to differently sized products. To mention, HYAL2 degrades HA into 50–60 disaccharides long fragments (~20 kDa), whereas HYAL1 cuts long HA/CS chains into oligomeric tetrasaccharides [165].

HYAL4 only cleaves CS, but is limited in its expression and only being found in the placenta and skeletal muscles [177]. It likely functions similarly to HYAL2 as it has an activity optimum around pH 4–5, but is also found present at the cell surface like HYAL2. HYAL4 might have a role during embryo development, but is likely not involved in systemic catabolism of CS [168,170]. A testicular HYAL was also found and is alternatively named Sperm Adhesion Molecule 1 (SPAM1) or hyaluronidase PH-20. This enzyme is active at both acidic and neutral pH and is found exclusively in the testes. It is present both in the acrosome (a sperm specific organelle) and on the sperm cell surface. Further, it has been reported to shed and have isoforms with different activity profiles regarding pH and can hydrolyze both HA and CS [171–173].

The general proposed mechanism for HA/CS degradation via HYALs is through a 'double-displacement' mechanism, where a single glutamic acid together with several residues that help position the substrate (see Fig. 6) forms the active site [162,178]. One tyrosine responsible for substrate positioning is replaced by a cysteine in HYAL4, likely causing its specificity for CS [158,164]. In the catalysis, the glutamic acid residue acts as an acidic H donor and the carbonyl in the HA/CS *N*-acetylamino group serves as the nucleophile. This mechanism is described in detail by *Stern* et al. [158,164,178]. Practically, the only moiety of HA/CS that is required in the hydrolytic degradation of the glycosidic bond is the *N*-acetylamino group, as this group forms the oxocarbenium ion in the intermediate step (see Fig. 6). Generally, the activity of HYALs is dependent mostly on the protonation of the glutamic acid, which might explain the higher activities observed at lower pH values [164].

3.2.2. Transmembrane protein 2 (TMEM2/CEMIP2)

TMEM2 (also termed Cell Migration Inducing Protein 2 [CEMIP2]) is a recently discovered HA degrading enzyme, which is present in both the lysosomes and GPI-anchored to the cytoplasmic membrane of most mammalian cells. TMEM2 does not degrade CS and is ubiquitously expressed in somatic cells with a reported pH optimum close to physiological pH. Not much is known about the enzymatic mechanism of TMEM2, other than that the enzyme requires Ca^{2+} to exert its catalytic activity. Further, it is not structurally related to the HYALs [174,175]. In a recent publication of *De Angelis* et al. where TMEM2 was studied in a zebrafish model, the enzyme was found to partition into ECM after ectodomain shedding from the cell where it shows catalytic activity [179]. The full role of TMEM2 in HA turnover is still being studied [175].

3.2.3. Lysosomal exoglycosidases

Several lysosomal enzymes exist that can exolytically remove disaccharides sequentially from both HA and CS from their non-reducing ends. These enzymes are also able to cleave other polysaccharides based

Table 1
An overview of degrading enzymes specific for HA and CS. [1]For these enzymes the reported tissue location is based on RT-PCR of mRNA. [2]All cell surface enzymes/receptors are GPI anchored, and can be cleaved by GPIases to release into the ECM.

Enzyme	Enzyme location in the body[1]	Enzyme location in the cell	Enzymatic activity			Substrate specificity	Enzyme molecular weight (kDa)	Reference
			pH range	Optimal pH	At pH 7.4			
HYAL1	Somatic cells, plasma, urine	Lysosome	4–5	4–5	None	HA/CS	48.4	[161–163]
HYAL2	Somatic cells, can shed into ECM	Cell surface, lysosome	4–8	Non bound: 4-5 Membrane bound: 6–7 [159]	Low	HA/CS	53.9	[161, 164–167]
HYAL4	Placenta, skeletal muscle[1]	Cell surface[2], lysosome	4–8	4–5	Low	CS	54.2	[161, 168–170]
SPAM1/PH-20	Testes[1]	Cell surface[2], arcosome	4–9	GPI-Anchored: 7-8 Shedded: 3-4	High	HA/CS	57.8	[171–173]
TMEM2/CEMIP2/ KIAA1412	Somatic cells	Cell surface[2], lysosome	4–8	6–7	High	HA	154.4	[174,175]

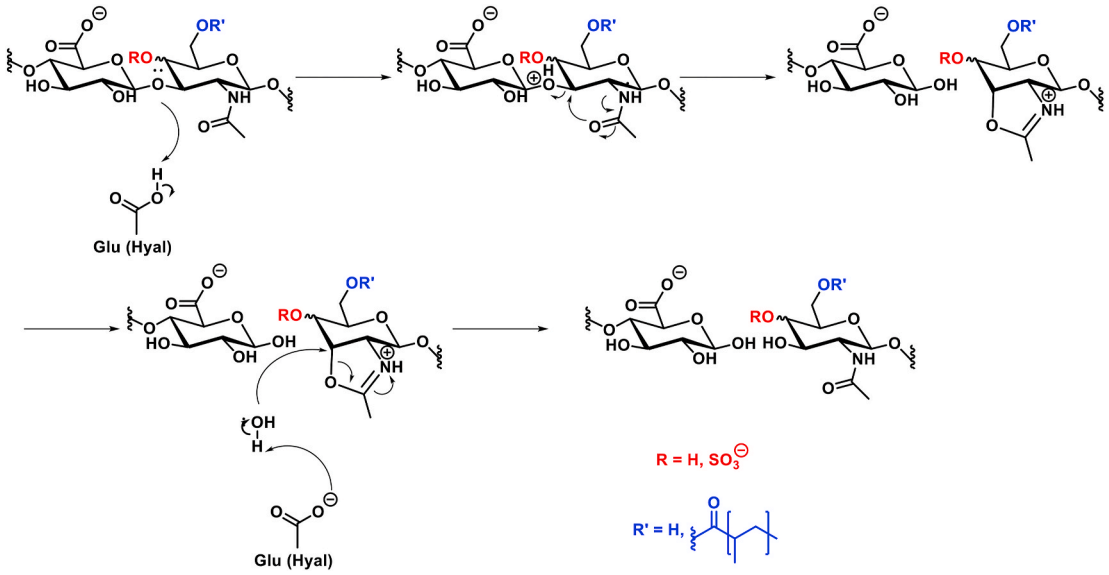

Fig. 6. Proposed mechanism of enzymatic degradation of HAMA and CSMA by HYAL type 1 (reproduced in modified form from Ref. [164]).

on glucosamine, glucuronic acid, and galactosamine [180]. These enzymes (e.g. β-glucoronidase and β-*N*-acetylglucosaminidase) all have optimal activity at acidic pH. Due to their localization in the lysosome, they are of limited interest to the initial enzymatic degradation of implanted/injected HA(MA) and CS(MA) hydrogels and will not be further discussed here.

3.2.4. *HA and CS recognizing receptors for endocytosis*

Receptors present on cell surfaces mediate HA/CS endocytosis into the lysosomal compartments of the cell for further degradation. A well-known example is CD44, which was discovered as cell surface receptor for HA and CS in 1990 by *Aruffo* et al. [181]. It mediates attachment of cells to HA in the ECM and has a role in endocytosis of HA and CS [182]. Upon binding HA, CD44 has also been shown to interact with plasma membrane bound Na^+-H^+ exchangers to acidify the microenvironment outside the cell, increasing the activity of HYAL2 and potentially other cell-surface and ECM present HA/CS degrading enzymes. It is however unclear whether HA can be cleaved when complexed to CD44 receptors [183]. Other HA/CS cell surface receptors include the Hyaluronic acid Receptor for Endocytosis (HARE) [184,185], which is highly expressed in the liver and the lymph-specific Lymphatic Vessel Endothelial hyaluronic acid receptor (LYVE) [186]. A recently discovered cell-surface receptor for HA which is highly homologous with TMEM2 named KIAA1199 (alternatively named CEMIP1) but does not degrade HA is also shown to be involved in endocytosis of HA but not CS [187].

3.2.5. *Locations and routes of enzymatic degradation of HA/CS*

There are three possible locations of degradation of HA/CS by enzymes, namely degradation in the ECM, at the cell surface and in the lysosome of cells (Fig.7ABC). Degradation of HA/CS chains present in the ECM (Fig. 7A) starts with shedding of a degrading enzyme (eg. HYAL2 or TMEM2) from the cell through cleavage of the GPI through

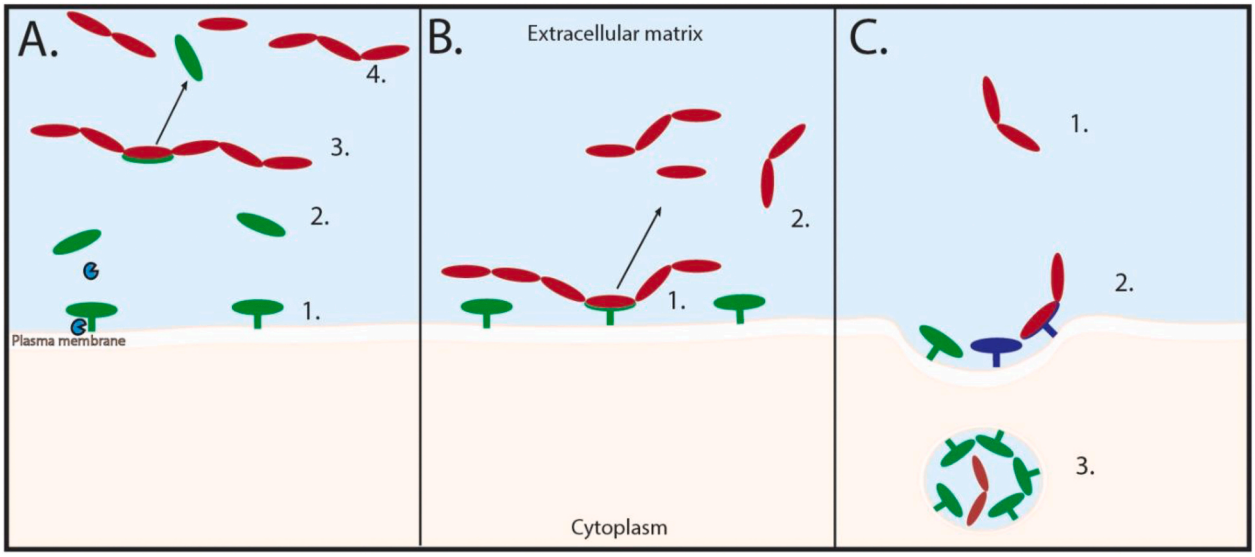

Fig. 7. Three possible routes of HA/CS degradation by matching enzymes. With HA/CS chains in red, HA/CS degrading enzymes in green, endocytosis-mediating receptors for HA/CS are indicated in blue and GPI cleaving enzymes are indicated in light blue. A: Enzymatic degradation of HA/CS chains in the ECM. B: HA/CS cleavage by cell-anchored enzymes and C: (receptor mediated) endocytosis of HA/CS fragments to the lysosome, where complete enzymatic degradation down to single di-saccharides occurs (lysosomal degradation is not pictured). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

which it is anchored to the cell membrane by GPIase (1.) [179,188,189]. When the HA/CS degrading enzyme is subsequently liberated, diffusion into the ECM occurs (2.) followed by binding of the enzyme to HA/CS (3.) and endolytical chain cleavage (4). A second location for enzymatic degradation is the cell surface (Fig. 7B) and some HA/CS degrading enzymes such as HYAL2 are found anchored to the cell, and exhibit activity at physiological pH. Here, soluble HA/CS chains diffuse through the ECM and subsequently bind to the enzyme and are then cleaved (1.) by the cell-surface immobilized enzyme. Subsequently the polymer fragments can then diffuse freely (2.) potentially allowing for their clearance through the liver. The third option (Fig. 7C) is (receptor mediated) uptake of HA/CS chains and fragments into the cell. Where HA/CS (1.) bind to either HYAL2/TMEM2 (serving both as degrading enzyme and cell-surface receptor) or non-degrading HA/CS cell-surface receptors (2.) and are taken up into an endosome (3.) and finally degraded by lysosomal enzymes. Some of the cell-surface present degrading HA/CS enzymes can degrade HA/CS in the lysosome due to their low pH optimum (e.g. HYAL2).

It is probable that all three distinct routes of Fig. 7 are responsible for HA/CS degradation locally by cells. Likely, first enzymatic degradation outside the cell occurs (illustrated in Fig. 7A and B) followed by intracellular uptake and further degradation (Fig. 7C) or diffusion of the smaller polysaccharide fragments into the bloodstream and subsequent clearance from the body as described in paragraph 3.1.

3.3. Enzymatic degradation of methacrylated HA/CS and their hydrogel networks

No human trials have been done so far on hydrogels consisting of either HAMA or CSMA and therefore no information on the degradability of these materials in humans is available. However, much can be learned by studying hydrogels based on covalently crosslinked HA that have already been clinically used as dermal fillers. Most HA dermal fillers are crosslinked using BDDE, this formulation received market authorization in 1996 [141]. It has been reported that HA-BDDE fillers solubilized between 6 and 24 months after implantation which was found to be dependent on molecular weight of the HA used, polymer concentration, hydrogel particle size and crosslinking density. Crosslinking densities of 1–11 BDDE molecules per 100 HA disaccharides are reported for commercialized HA-BDDE gels [142]. HA-BDDE hydrogel particle sizes vary between 200 and 600 μm in spherical diameter [190]. The main pathway of degradation of HA-BDDE gels is through enzymatic action although cleavage of the glycosidic bonds by radicals generated *in vivo* (via e.g. local inflammation) can also be a contributing factor [141]. In general, the enzymatic degradation of HA-BDDE gels injected is substantially slower as compared to degradation of free HA/CS, likely because the hydrogels are immobilized due to their size. In addition, a key factor in enzymatic degradation of crosslinked hydrogels is the crosslinking density. HA-BDDE gels can even be degraded and solubilized by injection of a high dose of HYAL in or in the vicinity of the injected beads, when a patient experiences adverse side-effects of the injected beads [142,191–193].

When regarding HAMA and CSMA based hydrogels, degradation via enzymes present in the ECM and on the cell surface would be most plausible, as bulk hydrogels are immobile at the site of injection and after administration. Enzymatic degradation of HAMA-based hydrogels has been investigated both *in vitro* and *in vivo* in animal models. CSMA-based hydrogels have so far not been tested for HYAL catalyzed degradation *in vitro*. Significant research interest has been devoted to determining the enzymatic degradability of HAMA hydrogels *in vitro* [45,47,136]. Commonly, hydrogel degradation kinetics are investigated via the wet weight method. In this method, a hydrogel is incubated in a buffer with known pH and at 37 °C, and the wet weight of the hydrogel is measured in time and compared to the initial wet weight before incubation (referred to as swelling ratio). Other methods quantify degradation products or the mechanical properties of the gel over time [194]. Typically, there are two distinct mechanisms of degradation of hydrogels, bulk and erosion-based degradation [195]. In enzymatic bulk degradation, the mesh size of the hydrogel is larger than the hydrodynamic diameter of the enzyme, allowing penetration and subsequent enzymatic action throughout the polymeric network. Bulk-degradation is typically observed as an increase in hydrogel wet weight and swelling ratio over time as degradation occurs, eventually resulting in complete liquefaction of the gel. In erosion-based degradation, the enzyme's hydrodynamic diameter is larger than the average mesh size of the hydrogel, only allowing enzymatic action to take place at the outer edges of the hydrogel. Erosion-based degradation typically can be verified by a decreasing hydrogel weight over time [194,195].

The wet weight of HAMA hydrogels when exposed to a fixed concentration of HYALs follows a linearly decreasing trend over time, which is indicative of erosion-based enzymatic degradation where HYALs are cleaving chains at the surface of the hydrogel, reducing the size of the hydrogel in time until fully degraded. As hydrogels based on HAMA completely consist of HYALs matching substrate, it is hypothesized that in addition to limitations related to mesh size, HYALs do not penetrate the polymeric matrix due to the excess of available binding sites already available on the gel surface restricting the mobility of adsorbed HYALs. Baier Leach et al. show that upon increasing the HYAL concentration from 1 U/ml to 100 U/ml for HAGMA hydrogels of several crosslinking densities the degradation rate increased significantly. Additionally, it was found that increasing the crosslinking density from 1.45×10^{-6} to 2.07×10^{-6} mol/cm^3 decreased the degradation rate of HAGMA hydrogels by a factor 10 [47]. Increasing the polymer concentration of the gel both reduces the rate of degradation of HAMA-based crosslinked hydrogels by HYALs. It has also been reported that both increasing the HYAL concentration and a slightly acidic solution pH (i.e. a pH of 4–5) increase the rate of degradation [45,47,136]. Although the enzymatic degradation slows down with increasing DM of HAMA, even at DMs as high as 25% HAMA gels are still fully degraded by HYAL enzymes [196]. This is likely due to the enzymatic mechanism of the HYALs which is mostly dependent on the *N*-acetyl group to cleave the glycosidic bond [158,164]. It is worth noting that this *N*-acetyl group is left unchanged in most (meth)acrylated derivatives of HA and CS. Both HAMA and CSMA are frequently combined with other biopolymers or synthetic polymers for the design of hydrogels for tissue engineering applications. This blending allows for differences in degradation rates of these hybrid hydrogels dependent on the weight fraction and identity of the other hydrogel forming components [145]. Summarizing, the main parameters determining the rate of enzymatic degradation of HAMA-based hydrogels at a fixed pH and temperature are enzyme type and concentration, hydrogel polymer content and crosslinking degree.

The biodegradability of HAMA hydrogels was tested *in vivo* by Baier Leach et al. in a rat model. A 1 wt% HAGMA (obtained by derivatization of HA and GMA in which coupling occurs via ring-opening of the epoxide group) hydrogel with a HA DM of 7% and a molecular weight of $\sim 2 \times 10^6$ Da with 0.03% of *N*-vinyl pyrrolidinone added as a reaction accelerant was photocrosslinked with Irgacure 2959 [47]. The HAGMA hydrogels were implanted subcutaneously in rats and excised two weeks later. Cell infiltrations into the gel were observed and these cells were partially degraded at the edge of the HAGMA gels. Other studies regarding the *in vivo* enzymatic degradation of HAMA hydrogels have shown similar results, with only partial degradation after 42 days of subcutaneous implantation in mouse models [70,197]. These results indicate that HAGMA hydrogels likely share the longevity upon implantation that has been previously been established for HA-BDDE hydrogel dermal fillers. Reports of degradation of CSMA hydrogels *in vivo* are limited. A recent study of PEGDA/CSMA combination hydrogel transplanted in a mouse cranial defect found no degradation of the hydrogel over the course of 8 weeks [94]. In another study done on a CSMA-based cartilage adhesive derivatized with aldehyde functionalities no loss of adhesive properties after 5 weeks of subcutaneous implantation in mice was found, indicating that the enzymatic degradation

of this CSMA hydrogel in this timeframe was limited [198]. It is likely that the observed slow *in vivo* degradation of HAMA and CSMA based hydrogels is due to the relatively low concentrations of the degrading enzymes in the tissue where the gels were implanted.

Any implantation of a biomaterial provokes a systemic or local immune response, known as a foreign body response [199,200]. The severity of the response depends on the level of disruption in the homeostasis of the native tissue. The severity of foreign body responses to implanted materials such as hydrogels are mostly linked to the location of implantation, the type of polymer used to form the networks and the type of crosslinking chemistries utilized and the degradation rate [200]. A foreign body response leading to the formation of a fibrous capsule around the implanted hydrogel can potentially alter the local microenviroment by releasing degrading enzymes, and reactive oxygen species. In addition, the pH of the environment within the capsule can be significantly lowered [200]. The effects of such fibrous capsule formation and of immune responses in general could affect the *in vivo* degradation of HAMA/CSMA hydrogels. Examples of foreign body responses after implantation of HA-based hydrogels are reported, for instance, injection of dermal fillers such as HA-BDDE hydrogels can lead to foreign body granulomas which are treated with corticosteroids [201]. Another example can be found in a report from *Lai* et al. where differences in the foreign body response to HA hydrogels crosslinked using either glutaraldehyde or EDC implanted into the eyes of rabbits were reported [202]. The hydrogels based on EDC crosslinking were found to have better ocular biocompatibility over those crosslinked with glutaraldehyde. Severe tissue responses were seen in the intraocular cavity for the glutaraldehyde crosslinked gels, which was explained by the toxicity of this crosslinking agent. Only a minor immune response was reported for HAGMA hydrogels subcutaneously implanted in rats, with only limited local inflammation observed after two weeks [47].

3.3.1. Considerations regarding enzymatic degradability of HAMA and CSMA based hydrogels for clinical translation

Based on the overview given in this paragraph, some considerations are given regarding the testing of enzymatic degradation of HAMA and CSMA based hydrogels for clinical translation.

A point that applies to all implanted materials that are subject to enzymatic degradation is that enzymatic activity in tissues varies depending on type of tissue, the disease state, inflammation events, and patient [203–205]. As discussed in section 3.2, the different HA/CS degrading enzymes have pH dependent activity profiles [158,164], and the pH at which enzymatic degradation is tested should ideally be similar to the pH of the target tissue, most often this is pH 7.4. When encapsulating cells into HAMA/CSMA hydrogels, the ability of those cells to degrade the hydrogel should also be tested, as most cells will have HA/CS degrading enzymes present on their surfaces [161, 164–167,174,175,179].

The choice of enzyme for *in vitro* hydrogel degradability testing should be based on the intended application and tissue in which the hydrogel will be injected/implanted. For example, when a CSMA-based hydrogel is designed for use in skeletal muscle, testing the enzymatic degradability of the hydrogel is most relevant with HYAL2 and HYAL4, as these enzymes degrade CS in this specific tissue [161,168–170]. As a result of the recent discovery of the HA degrading capabilities of TMEM2, it is recommended to test the degradation of HAMA based hydrogels catalyzed by this enzyme at the pH relevant for the target tissue [175]. Most forms of HYAL and TMEM2 are commercially available as recombinantly made proteins. It should be noted that the hyaluronidase activity unit is defined as the amount of enzyme needed to liberate 1 μmol of *N*-Acetylglucosamine from HA per minute at 37 °C and pH 4.0, indicating that the activity at different pHs might substantially differ [206]. *In vitro* enzymatic degradation tests for CSMA based hydrogels have not been carried out using the relevant HYALs yet, and only the bacterial chondroitinases were used (see e.g. Refs. [207]). HYAL2 has been identified as the most relevant enzyme for CSMA hydrogel degradation due to its presence on cell surfaces and capacity to shed as well as its activity towards CS and its presence in many human somatic cells [161,164–167]. It is worth noting that the activity of HYALs in solution is found to be reduced by ~44% in two days at 37 °C in acidic pH (3–6) [208,209]. It is therefore recommended to refresh the HYAL containing medium at least every second day during *in vitro* degradation testing of HAMA or CSMA based hydrogels.

When assessed *in vivo*, degradation of HAMA or CSMA hydrogels can be further understood through quantification of the HYAL and (HA specific) TMEM2 enzyme concentrations in relevant (excised) tissues. A recent example has been published by Shamskhou and Kratochvil et al. [210]. In this work, intranasally administered gels based on thiolated HA, heparin and gelatin in combination with PEGDA degraded faster in lysates of mice lung fibrotic tissue than in lysates of healthy mice lungs due to the increase in cell expression of HYAL1, HYAL2 and TMEM2. Hydrogels were still present in the fibrotic lung after 21 days; however, it should also be noted that the expression of collagenase was not checked.

The degradation time of HA-BDDE dermal fillers injected into humans is up to 24 months [142]. Since crosslinks are present between primary alcohols of different chains in HAMA/CSMA and HA-BDDE it is likely that HAMA and CSMA-based gels will have similar degradation mechanisms in which the overall crosslink density and polymer concentration determines the degradation rate. Depending on the envisioned application and tissue in which the gel will be implanted/injected, this time period can be potentially be longer than what is typically desired for regenerative medicine applications (i.e. degradation over the span of several months). Therefore, HAMA and CSMA that degrade via ester hydrolysis of the connecting network crosslinks can be interesting alternatives as they do not rely on degradation through enzymatic action only and can degrade in a shorter timeframe [139,140].

4. Composites and hybrids based on HAMA or CSMA for tissue engineering applications

HAMA and CSMA based hydrogels possess different swelling, degradation and mechanical properties depending on the degree of methacrylation and polymer concentration as discussed in section 2.3, which allows to fine tune their properties for the aimed applications. However, HAMA or CSMA have also been combined with different materials, to further tailor hydrogel properties that cannot be obtained by HAMA and CSMA hydrogels alone, yielding hybrid or composite hydrogels. In this review, the term *hybrid* refers to hydrogels formed between two or more polymers (synthetic or biopolymers), whereas the term *composite* refers to hydrogels that also contain organic/inorganic particles. Such blending is usually done to tailor the mechanical and swelling properties of the hydrogels, which could not be achieved by using HAMA and CSMA alone. In the following section an overview of hybrid and composite hydrogels based on HAMA and CSMA is given, with emphasis on the components used to formulate such hydrogels, as well as on their application for tissue engineering. Illustrative examples are summarized in Table 2 (for HAMA) and Table 3 (for CSMA) in the end of sections 4.1 and 4.2 respectively.

4.1. Hybrid hydrogels based on HAMA

4.1.1. Hybrid hydrogels based on HAMA and biopolymers

HAMA has been extensively combined with other biopolymers. Gelatin, being a derivative of collagen, is one of the polymers most frequently combined with photocrosslinkable hyaluronic acid. Both, collagen and HA are constituents of ECM in many tissues, and therefore it is logical to assume that the combination of HA and gelatin can result in ECM mimicking hydrogel materials.

Methacrylated gelatin and hyaluronic acid have been blended at different ratios and photocrosslinked to form hybrid hydrogels, which

Table 2
Examples of hybrid and composite hydrogels based on HAMA.

Hydrogel type	Additional component	Objective	Targeted tissue	Reference
Hybrid	GelMA	Tune mechanical properties and allow cell encapsulation	Cartilage	[40]
Hybrid	GelMA and CSMA	Regulate chondrogenesis and improve mechanical properties	Cartilage	[211]
Hybrid	GelMA	Tune mechanical properties, provide remodeling activity and 3-D printing of heart valve conduits	Heart	[212]
Hybrid	GelMA	Study phenotypic changes of VICs	Heart	[213]
Hybrid	GelMA	Improve wound healing in combination with ADSCs	Soft tissue, skin tissue	[43]
Hybrid	Dextran-HEMA	Tune mechanical and viscoelastic properties for extrusion based 3-D printing	Cartilage	[215]
Hybrid	Collagen I	Tune stiffness to study EMT transition	Heart	[216]
Hybrid	Poly (*N,N*-dimethylacrylamide)	Tune mechanical properties and allow 2 months cell culture	Soft load bearing tissues	[220]
Hybrid	γ-polyglutamic acid	Tune mechanical, swelling and degradation properties and suitable for cell encapsulation	Soft load bearing tissues	[221]
Composite	PLGA microspheres	Tune gelation, degradation and mechanical properties	Heart	[222]
Composite	Calcium phosphate	Increase mechanical properties and degradation resistance	Bone	[224]
Composite	Hydroxyapatite	Promote chondrogenesis	Cartilage	[42]
Composite	GO	Enhance cell expansion	Fluid connective tissue	[225]
Composite	CNTs	Improve antioxidant activity	Fluid connective tissue	[226]

Table 3
Examples of hybrid and composite hydrogels based on CSMA.

Hydrogel type	Additional component	Objective	Targeted tissue	Reference
Hybrid	HAMA, alginate and GelMA	Enhance chondrogenesis	Cartilage	[41]
Hybrid	HAMA and collagen	Tune swelling, mechanical properties and chondrogenesis	Cartilage	[147]
Hybrid	HAMA and collagen	Enhance chondrogenesis	Cartilage	[227]
Hybrid	HAMA and chitosan glycol methacrylate	Improve chondrocyte metabolic activity	Cartilage	[228]
Hybrid	PEG	Model hydrogel for studying the effect of dynamic mechanical loading on chondrogenesis	Cartilage	[230]
Hybrid	PEG	Enhance chondrogenesis	Cartilage	[105]
Hybrid	PEG	Improve biomineralizing ability	Bone	[94]
Hybrid	PEG and agarose	Enhance mechanical properties and chondrogenesis	Cartilage	[232]
Hybrid	PVA	Improve mechanical properties and enzymatic degradability	Cartilage	[235]
Hybrid	PEG and poly(N(2-hydroxypropyl) methacrylamide-mono/dilactate triblock copolymer	Improve printability and chondrocyte cytocompatibility	Cartilage	[100]
Composite	Hydroxyapatite	Promote osteogenesis	Bone	[85]
Composite	Magnetic nanoparticles	Modulate growth factor release	Bone	[236]
Composite	GO and PEG methyl ether-ε-caprolactone acryloyl chloride	Promote cartilage formation	Cartilage	[237]

resulted in improved interaction with cells and improved mechanical properties, compared to gels based on either only crosslinked hyaluronic acid or gelatin [40]. Levett et al. showed that the addition of HAMA to GelMA formulations led to increased compressive modulus after 28- and 56-days culture of encapsulated cells, compared to GelMA only hydrogels. Besides compressive modulus, failure strain and failure strength also improved upon addition of HAMA, most likely due to cell-secreted ECM. The observed good chondrocyte cytocompatibility of these HAMA/GelMA hybrid materials makes this polymer combination very useful for applications in cartilage tissue engineering [211]. The same group also explored the potential of a hybrid hydrogel based on GelMA, HAMA and CSMA [37]. These gels proved to be suitable for long-term culture of human chondrocytes, which produced functional cartilage ECM components. The presence of HAMA and to a lesser extent CSMA were responsible for improved chondrogenesis, better distribution of the ECM components, cellular re-differentiation and improvement of mechanical properties. The authors demonstrated that GelMA/HAMA/CSMA hybrid gel was efficient in mimicking ECM and suitable for cartilage treatment applications [37].

As demonstrated by Duan et al. HAMA/GelMA hybrid hydrogels can also be used for engineering human valve replacement materials [212]. Specifically, they developed a biomaterial loaded with human valvular interstitial cells (VICs) suitable for 3D printing into heart valve conduits. The cells showed high viability and remodeling capacity of the matrix by producing new collagen and glycosaminoglycans. By changing hydrogel composition, material's stiffness could be adjusted, facilitating cell spreading and phenotype maintenance. Additionally, Hjortnaes et al. made use of HAMA/GelMA hybrid hydrogels to study the mechanisms leading to valve disease (Fig. 8B) [213]. These hybrid hydrogels were loaded with VICs and proved to be able to maintain their quiescent phenotype. Only upon their treatment with TGF-β1 VICs differentiated towards myofibroblast-like cells. Therefore, HAMA/GelMA gels represent a good model for controlled phenotypic change of VICs and for studying heart valve disease, which potentially could lead to the development of novel solutions for engineered valves.

Eke et al. developed a GelMA/HAMA hybrid hydrogel loaded with adipose derived stem cells (ADSCs) for stimulating wound healing (Fig. 8A) [43]. The photocrosslinkable polymers dissolved in cell culture medium were supplemented with ADSCs prior to crosslinking and the resulting gels provided a suitable environment for cell proliferation. The authors performed *in vivo* experiments with the cell-laden hybrid hydrogel and showed a 3-fold increase of vascularization compared to the cell-free hydrogel. These findings indicate that ADSCs loaded HAMA/GelMA hydrogels promote rapid wound healing and have potential for skin tissue engineering purposes.

Besides with GelMA, HAMA has also been blended with other biopolymers, such as methacrylated dextran, to design functional hybrid hydrogels. Moller et al. prepared hybrid hydrogels based on HAMA and methacrylated dextran and studied their cytocompatibility and biocompatibility [214]. The reported results suggest good cytocompatibility and biocompatibility of such hyaluronic acid/dextran hybrid hydrogels and encourage their potential use for soft tissue engineering purposes. Moreover, Pescosolido et al. explored formation of semi-interpenetrating networks (semi-IPNs) between HA and

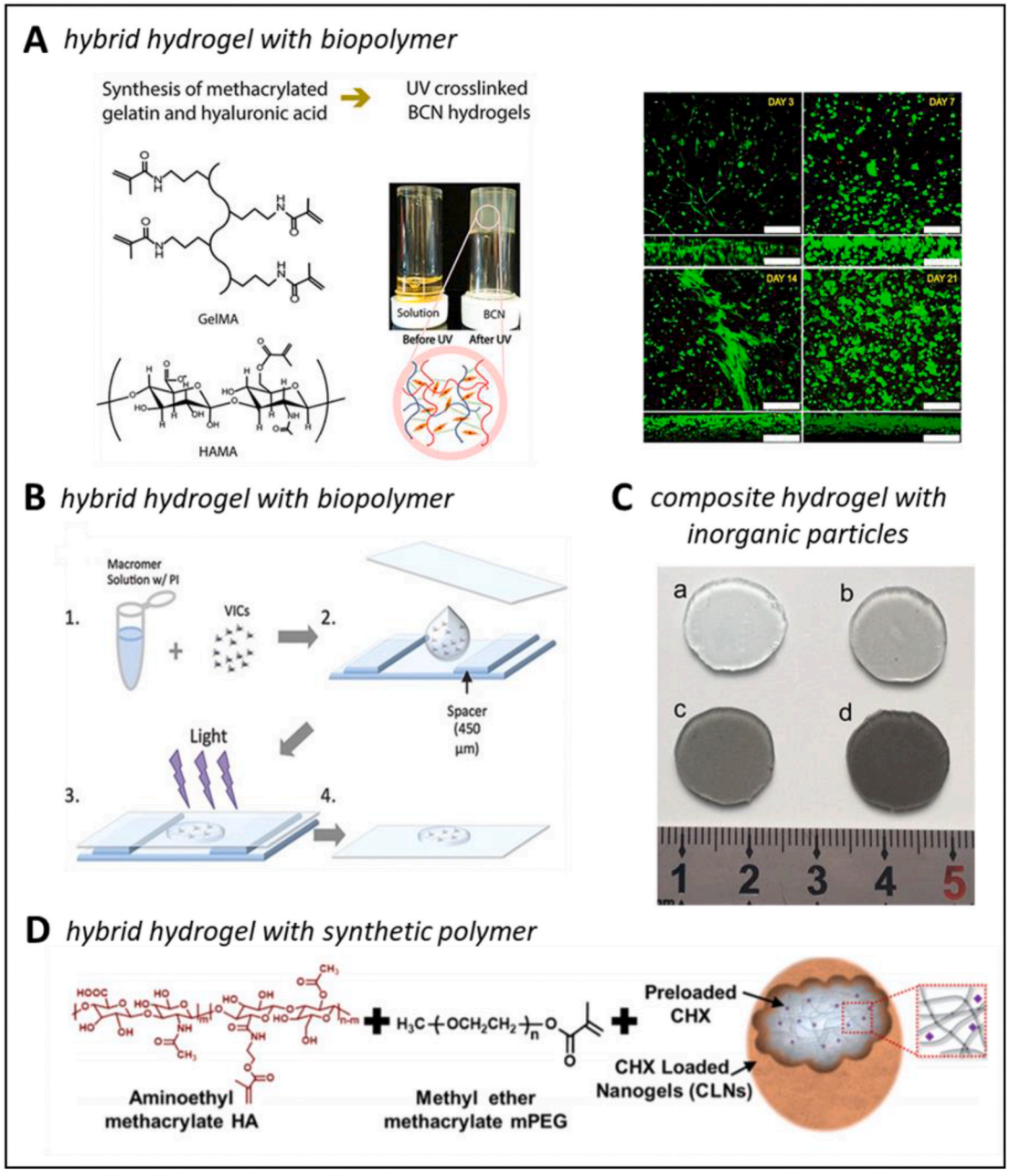

Fig. 8. Examples of hybrid and composite hydrogels based on HAMA. (A, left) Methacrylated hyaluronic acid and gelatin, two main components of the hybrid hydrogel. Both polymers were dissolved in medium containing ADSCs, in which a photoinitiator was dissolved. After UV irradiation, hydrogels were formed, as shown by inversed vials. (A, right) Viability and proliferation of ADSCs are shown. Live-Dead analysis was performed after culturing for 3–21 days, using calcein acetoxymethyl and homodimer-1 for staining live and dead cells respectively (magnification x10). Adapted from Eke et al. [43] with permission from Elsevier. (B) Schematic representation of the fabrication procedure of valve interstitial cells (VICs)-laden HAMA/GelMA hybrid hydrogels. Such hydrogels were used for studying VIC phenotype for heart valve tissue engineering. Adapted from Hjortnaes et al. [213] with permission from Wiley, copyright 2014. (C) Optical images of composite hydrogels based on HAMA and CNTs, at following concentrations: (a) HAMA, (b) HAMA/CNT-0.01, (c) HAMA/CNT-0.05 and (d) HAMA/CNT-0.1 wt%. HAMA/CNT hydrogels promoted HSCs proliferation. Adapted from Zhang et al. [226] with permission from Wiley. (D) Schematic representation of the components of the hybrid hydrogel: HA-AEMA and methacrylated PEG derivative. Such hybrid hydrogel, loaded with chlorohexidine diacetate (CHX) containing nanogels (CLNs), accelerated hemostasis and wound healing *in vivo*. Adapted from Zhu et al. [64] with permission from ACS publications.

hydroxyethyl methacrylate (HEMA) derivative of dextran [215]. Such hybrid hydrogels were proved to be strong, biodegradable and cytocompatible with chondrocytes. The hydrogel's mechanical properties could be tuned by adjusting hydrogel composition. HA/Dex-HEMA solutions displayed suitable viscoelastic properties for extrusion based 3D printing and therefore for fabrication of scaffolds for tissue engineering applications.

Collagen is another biopolymer that has been blended with HAMA to fabricate hybrid hydrogels for biomedical applications. Sewell-Loftin et al. blended collagen type I within a HAMA hydrogel matrix. Collagen type I was mixed with HAMA stock solution prior to UV-crosslinking. The resulting semi-IPN (one component of the network is crosslinked, the other is only entangled) was used to study mechanical cues affecting epithelial to mesenchymal transition (EMT) of endocardial cells, which are essential for heart valve formation [216]. Controllable stiffness and mechanical properties of the hybrid hydrogel

allowed for studying of the EMT phenomenon *in vitro*. The authors demonstrated that mechanical forces indeed play an important role in endocardial EMT and that the prepared hydrogel represents a good platform for studying valve disorders and can act as starting point for developing tissue engineered heart valves. Brigham et al. prepared a semi-IPN from HAMA and collagen type I [144]. The polymers were mixed at the desired concentration in cell culture medium and subsequently exposed to UV, resulting in photocrosslinking of HAMA. The mechanical properties, and in particular compressive moduli, of the HAMA/collagen hydrogels increased as a function of DM of HAMA. Moreover, these gels displayed better fibroblasts viability in comparison to HAMA hydrogel. To explain, fibroblasts adhered to the hybrid hydrogels and proliferated more than on the HAMA gels alone, whereas upon encapsulation, cells maintained high viability. Because of the good mechanical properties and good cytocompatibility, HAMA/collagen semi-IPNs could be of great value in soft tissue engineering applications. A similar system was studied by Suri et al. where a solution of HAMA in PBS and collagen solution (diluted with 0.2% acetic acid) were mixed together and subsequently supplemented with a HEPES solution and DMEM medium. The resulting solution was incubated at 37 °C and allowed to undergo fibrillogenesis process (assembly of collagen fibrils) for 90 min to yield a collagen network [138]. In the following step, the obtained collagen gel was exposed to UV, allowing photocrosslinking of HAMA and resulting in the formation of an IPN. When compared to the corresponding single-component gels, the HAMA/collagen gels exhibit superior mechanical properties and slower enzymatic degradation *in vitro* due to higher crosslink density. Additionally, collagen/HAMA IPNs supported human dermal fibroblast adhesion and proliferation, indicating the suitability of HAMA/collagen IPN for the development of tissue engineering constructs.

4.1.2. Hybrid hydrogels based on HAMA and synthetic polymers

HAMA has been frequently combined with different synthetic polymers to obtain hydrogels with improved mechanical properties or improved release profiles of loaded drugs for pharmaceutical applications.

One of the polymers frequently combined with HAMA is poly (ethylene glycol) (PEG). PEG is a linear, hydrophilic polymer, suitable for hydrogel fabrication. Zhu et al. reported a method for the preparation of hydrogels obtained by photopolymerization of HAMA and methyl ether methacrylate PEG (Fig. 8D) [64]. The hydrogel was intended for wound healing applications, including haemostasis (stopping of bleeding) and antibacterial properties. The hydrogel was used as a carrier for a nanogel loaded with chlorhexidine diacetate (antibacterial agent). The nanogel was prepared by enzymatic degradation (trypsin) of the lysine-based hydrogel (prepared by a direct polycondensation at 80 °C between *p*-nitrophenyl diester monomers and tetra amino lysine-based monomers [217]). The nanogel particles (~50 nm) were dispersed in HAMA/PEG polymer solution prior to crosslinking. The hydrogel loaded with drug-nanogel showed sustained release of the drug (over 240 h), compared to the formulation loaded with drug only. The sustained release was attributed to the drug diffusion from the nanogel, and subsequent erosion of the polymer network of the carrier hydrogel. The antibacterial activity was evaluated by agar diffusion test against *E. coli* and *S. aureus*. The formulation containing drug-nanogel displayed longer (more than 10 days) antibacterial activity than the formulation with drug only (consistent with drug release studies). The individual polymers used for hydrogel preparation, as well as formulations without the drug did not show antibacterial activity. Besides antibacterial activity, in order to achieve the wound healing effect, the hydrogel was tested for its haemostasis properties which was studied in a mouse liver model, with induced liver bleeding. The tested hydrogel formulations, when placed at the site of bleeding, significantly arrested the bleeding process, compared to the gauze control. The stopping of bleeding is most likely caused by the gels being more adhesive than the gauze and acting as a physical barrier. The wound healing ability of this formulation was studied *in vivo*, where the induced wounds on the dorsal area of mice were covered with hydrogels. The wound healing process when using drug-nanogel formulation was accelerated, due to both more sustained antibacterial activity and hemostasis. The control hydrogel without the drug showed limited healing capacity and significant bacterial infection 3 days after the treatment. In another study, HAMA was combined with PEG diacrylate (PEGDA) to form a photocrosslinked hydrogel [218]. A porous hydrogel scaffold was fabricated using these two polymers by means of the leaching method (PMMA beads). Subsequently, a dual compartment-based device was fabricated with an outer porous hydrogel part, and with a central inner compartment filled with a fibrin-based hydrogel and loaded with primary hepatocytes. Such a biomaterial device is intended for a minimally invasive subcutaneous implant, allowing for human primary hepatocytes implantation, where the porous outer compartment has the role to promote vascularization [219].

In the study of Tavsanli et al. dimethylacrylamide (DMA) has also been combined with HAMA to prepare hybrid polymer networks [51]. By changing the degree of methacrylation of HAMA, as well as the amount of DMA, the authors showed that it was possible to obtain gels with tunable viscoelastic properties. DMA was also explored to fabricate hybrid double network (DN) hydrogels with HAMA, as reported by Weng et al. [220]. First, HAMA gel was prepared by photocrosslinking, which was subsequently allowed to swell in a solution of the monomer DMA, supplemented with the crosslinker (*N*, *N*′-methylenebisacrylamide) and a suitable photoinitiator. By exposing this gel to UV irradiation, a second network, based on polydimethylacrylamide (PDMA) was formed. The resulting hybrid hydrogel displayed good mechanical properties, with fracture stress of 5.2 MPa. In addition, HAMA/PDMA hydrogels were cytocompatible and suitable for long-term culturing of dermal fibroblasts (2 months).

Besides PEG, several other synthetic polymers have been combined with HAMA to form hybrid hydrogels. Ma et al. explored the combination of HAMA and a γ-polyglutamic acid methacrylate derivative [221]. The polymers were dissolved in PBS and subsequently photopolymerized to yield a hybrid hydrogel. Several hydrogels were prepared, by changing the ratio between HAMA and methacrylated polyglutamic acid. The resulting hybrid hydrogels could withstand large deformation and stress, making them suitable for potential load-bearing tissue engineering constructs. Moreover, these gels were cytocompatible, whereas swelling and degradation profiles could be tailored by adjusting the ratio between the two components. Degradation was studied *in vitro* in the presence of hyaluronidase, with degradation times ranging from 30 to 70 h. It should be remarked that hyaluronidase only degraded the HAMA component, while methacrylated γ-polyglutamic acid was not degraded. Finally, HAMA/polyglutamic acid hydrogels were also suitable as matrices for the controlled release of pharmaceutical proteins, as BSA as a model protein was released over a period of 5 days.

4.1.3. Composite hydrogels based on HAMA and polymeric microparticles

In several studies, HAMA composites in which poly(lactic-*co*-glycolic acid) (PLGA) microspheres were dispersed have been studied. For example, Tous et al. reported composite hydrogel formation by suspending PLGA microspheres (20–60 μm) in a PBS solution of HAMA, prior to crosslinking [222]. Resulting composite hydrogels displayed tunable physical and mechanical properties and full hydrogel matrix degradation occurred within 2 months, as detected by a colorimetric method based on the reaction between the degradation product uronic acid and carbazole [223]. This composite hydrogel can be useful to stimulate collagen deposition (myocardial bulking) in the process of myocardial infarction repair [222].

Leach et al. also made use of PLGA microspheres embedded in HAMA hydrogels, with the aim to extend the release profile of BSA (model protein) [60]. In particular, HAMA was combined with acrylated 4-arm PEG to form a hybrid hydrogel material, in which BSA loaded PLGA

microspheres (10–25 μm) were encapsulated. Results showed that this hybrid hydrogel released BSA for several weeks, while the protein was released in a few days from the hydrogels without PLGA microspheres. This composite hydrogel was suggested as a suitable scaffold for soft tissue engineering purposes, with the possibility for delivery of therapeutic proteins, in particular growth factors.

4.1.4. Composite hydrogels based HAMA and inorganic particles

Composite gels can also be obtained by incorporation of inorganic particles in the hydrogel network. Jeong et al. prepared HAMA-calcium phosphate (CaP) composite hydrogel by placing HAMA preformed hydrogels in a solution of calcium chloride and phosphoric acid, known as an ion permeation process. Next, the gels containing the ions were immersed in ammonium hydroxide solution, which led to precipitation of calcium phosphate particles (~200 nm) within the hydrogel. Such composite hydrogels had increased mechanical properties, because the CaP nanoparticles act as physical crosslinkers. Also, improved resistance to enzymatic degradation was observed, which was attributed to the nanoparticles sterically restricting the enzyme access. Importantly, this HAMA-CaP composite hydrogel had a good biocompatibility upon intramuscular implantation in rats [224].

Bartnikowski et al. explored a composite HAMA hydrogel loaded with hydroxyapatite particles (needle-like shape; length 50 nm, width 17 nm, 42 wt% loading) for osteochondral tissue engineering applications [42]. In detail, a 3D printed paste based on hydroxyapatite and alginate was used to make a mold, in which HAMA/GelMA solution was poured and subsequently a hydrogel was obtained upon UV polymerization. Moreover, chondrocytes were loaded in the hybrid HAMA/-GelMA hydrogel and evaluated for their viability and extracellular production capability. The authors demonstrated a chondrogenic effect of the hydrogel, which was attributed to the presence of hyaluronic acid. Moreover, the hydroxyapatite containing construct did not have negative effects on chondrogenesis, indicating its potential for treating osteochondral defects.

Although limited, there are some examples in which photocrosslinkable HAMA has been combined with carbon nanocomponents and the resulting hydrogels are usually referred to as *nanocomposite* hydrogels. Whether such composite hydrogels could have clinical translation potential remains to be seen, considering the non-degradability of the carbon nanostructures after enzymatic degradation of the HAMA matrix.

Guo et al. prepared a nanocomposite hydrogel via free-radical polymerization by using HAMA and methacrylated graphene oxide (GO) [225]. By varying the amount of GO from 0.05 to 0.2% w/v the elastic moduli increased, which is attributed to adsorption of polymer chains on GO, leading to stiffening of the matrix. This composite hydrogel was studied for *ex vivo* expansion of $CD34^+$cells and it was found that at 0.05% w/v content of GO, cell expansion was enhanced compared to the GO-free hydrogels, suggesting its possible use for *ex vivo* expansion of cells. The authors hypothesized that maintenance of the cell phenotype and spreading of hematopoietic stem cells could be positively influenced by increased hydrogel stiffness. Considering that in clinical trials of hematology diseases there is a lack of adequate number of cells, authors propose that by using their nanocomposite hydrogels the *ex vivo* expansion can overcome this issue.

Zhang et al. prepared a nanocomposite hydrogel based on HAMA and CNTs (Fig. 8C) [226]. CNTs were first oxidized under acidic conditions, and then dispersed (at a concentration ranging from 0.01 to 0.1 wt%) in HAMA solution prior to photocrosslinking. Based on *in vitro* assays, it was found that these nanocomposite hydrogels displayed antioxidant capacity, neutralizing the effect of superoxide and hydroxyl radicals. Moreover, apoptosis induced by oxidative stress was reduced in cells cultured on such hydrogels, therefore supporting proliferation and phenotype maintenance of hematopoietic stem cells (HSCs) [226].

4.2. Hybrid hydrogels based on CSMA

CSMA has also been exploited for preparation of hybrid hydrogels for biomedical applications. Contrary to HAMA, CSMA is less exploited, but nonetheless there is a considerable amount of work done on such hybrid hydrogels. CSMA has been frequently combined with both biopolymers and synthetic polymers to give rise to functional hybrid hydrogels.

4.2.1. Hybrid hydrogels based on CSMA and biopolymers

CSMA has been frequently combined with multiple biopolymers. For example, Costantini et al. studied novel bioinks for the biofabrication of hydrogel scaffolds for cartilage tissue engineering purposes [41]. A CS-AEMA photocrosslinkable derivative was used, together with HAMA and GelMA. In addition, also alginate was added to this formulation to provide the formation of a physical alginate network (in the presence of Ca^{2+} ions) prior to photocrosslinking of the methacrylated biopolymers. These bioinks were loaded with bone marrow mesenchymal stem cells (BM-MSCs) and these ECM-mimicking bioinks were able to enhance the cell chondrogenic potential. Different combinations of these polymers were explored, and for neocartilage formation CS-AEMA/GelMA/alginate proved to be the most promising combination, as seen from the collagen production data after 3 weeks of culture [41]. In order to produce cartilage-mimicking tissue engineered scaffolds, Guo et al. developed IPN hydrogels based on methacrylated chondroitin sulfate, hyaluronic acid and collagen [147]. Hydrogels were fabricated via 2 steps: self-assembly of collagen into fibers and free-radical polymerization between CSMA and HAMA. Swelling and mechanical properties of such hybrid hydrogels could be adjusted by varying the degree of methacrylation of CSMA or HAMA. Good cytocompatibility of these gels was also demonstrated, using rabbit articular chondrocytes. Moreover, cell production of specific cartilage markers (such as aggrecan and type II collagen) was upregulated. The authors therefore suggested this IPN-based hydrogel as a promising scaffold for cartilage tissue engineering applications. Zhu et al. developed a hydrogel scaffold by combining photocrosslinkable HA and CS with collagen type I to fabricate an ECM-mimicking hydrogel (Fig. 9A) [227]. Such hybrid hydrogels were used to study the chondrogenic effect on encapsulated human mesenchymal stem cells (hMSCs). It was shown that CSMA in combination with HAMA led to promoted deposition of proteoglycans, while compared to HAMA hydrogels calcification of hMSCs was suppressed. This study showed that optimized and carefully selected combinations of biopolymer based components used in cell containing hydrogel formulations could lead to materials with specific maturation and calcification properties of cartilage constructs [227].

Besides gelatin and collagen, chitosan has also been employed in hydrogel fabrication with CSMA. Hayami et al. prepared photocrosslinked hydrogels from blends of CSMA, HAMA and methacrylated glycol chitosan [228]. It was observed that after crosslinking of either chitosan or HAMA together with CSMA, high moduli and high cell viability were achieved. Additionally, compared to any of the 3 single-component hydrogels, long-term metabolic activity of chondrocytes was improved. The obtained results indicate that these hybrid gels have great potential for the development of scaffolds for load-bearing, soft tissue engineering applications. In a study of Costa-Almeida et al. hydrogel fibers were prepared by means of polyelectrolyte complexation and microfluidics techniques [229]. The authors made use of derivatives of negatively charged polysaccharides, such as CSMA, HAMA and alginate, in combination with positively charged chitosan (during the fabrication process, chitosan was dissolved in 1% v/v acetic acid solution, pH 3). Tendon cells were encapsulated in the formed hydrogel fibers, which maintained their phenotype and ECM production capacity.

4.2.2. Hybrid hydrogels based on CSMA and synthetic polymers

The most frequently used synthetic polymer with photocrosslinkable chondroitin sulfate is PEG. This polymer is often used in hydrogel

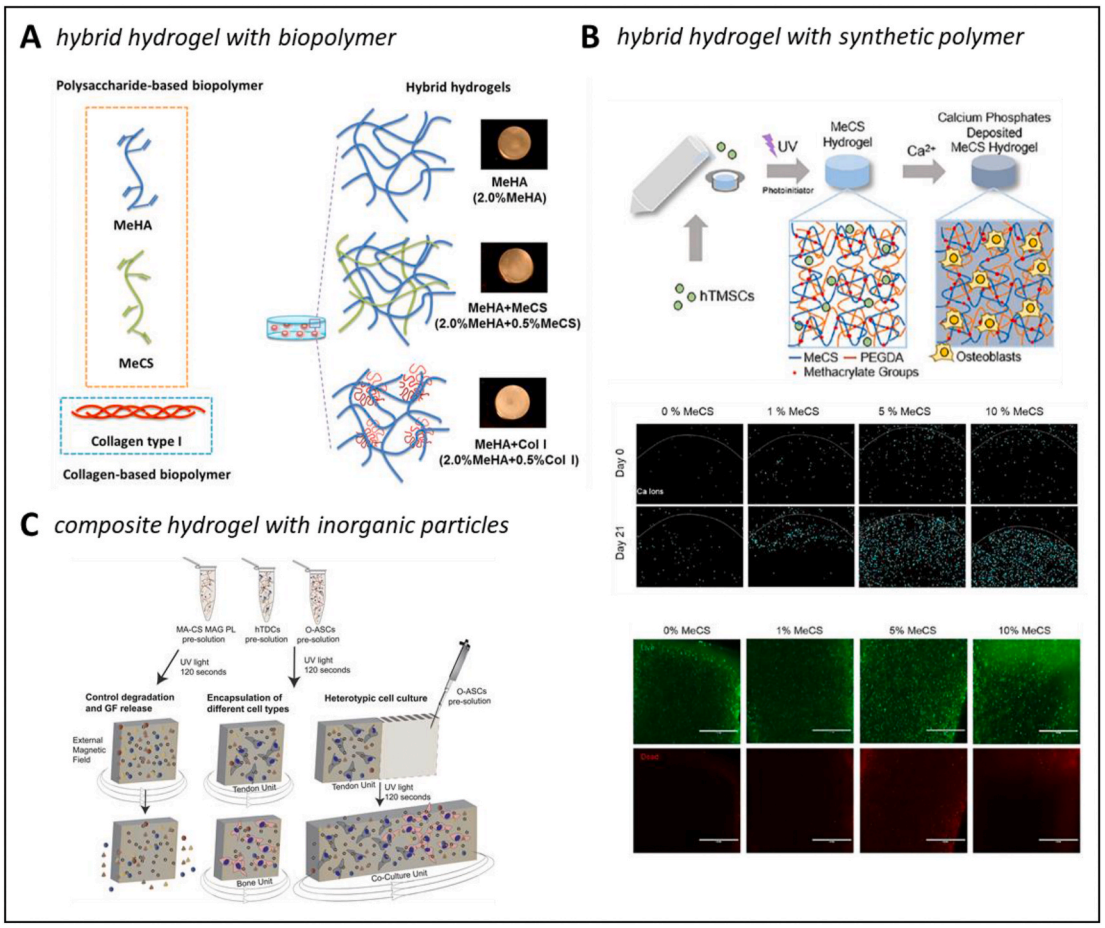

Fig. 9. Examples of hybrid and composite hydrogels based on CSMA. (A) Methacrylated hyaluronic acid and chondroitin sulfate and collagen type I (abbreviated here as MeHA and MeCS). Different combinations of hybrid gels were prepared, with schematic representation of the corresponding networks, alongside hydrogel pictures. Hybrid gels were tested *in vitro* and *in vivo* for chondrogenesis of hMSCs. Adapted from Zhu et al. [227] with permission from Wiley, copyright 2016. (B) Schematic representation of the hydrogel fabrication composed of CSMA and PEGDA (top). The resulting hybrid hydrogel showed biomineralization and calcium ion binding and deposition capacity, due to the presence of the CS component, making it useful for bone tissue engineering applications. Calcium ion deposition in the gel was detected via elemental mapping method, after 21 days (middle). Live-Dead assay indicated good viability of encapsulated hTMSCs after 21 days, (green:live, red: dead), scale bar 1000 μm (bottom). Adapted from Kim et al. [94] with permission from ACS publications. (C) Schematic representation of the preparation of composite CSMA hydrogel, containing iron magnetic nanoparticles. By applying an external magnetic field it is possible to achieve growth factor release from the gel. Also, incorporation of different cell types leads to tendon- or bone-like constructs, which can also exist as a co-culture system. Adapted from Silva et al. [236] with permission from Elsevier. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

formulations, due to its non-degradability and ease of modification. Thus, by controlling the crosslink density of the PEG network, the resulting gels exhibit tunable mechanical, stability and swelling properties. For instance, Khanlari et al. copolymerized CSMA with oligo ethylene glycol acrylates to fabricate hydrogels. The main effect observed in the obtained gels was increased crosslinking density and therefore higher moduli and thus superior mechanical properties compared to CSMA gels [93].

Mostly, CSMA/PEG based hybrid hydrogels have been used to construct model ECM-mimicking hydrogels for studying how external factors, such as chondrogenic factors and mechanical stimuli influence cartilage formation. For example, Villaneuva et al. made use of hybrid gels based on CSMA and PEGDM (poly(ethylene glycol) dimethacrylate) to study chondrogenic effects on cells [230]. Upon loading with chondrocytes, these gels were subjected to dynamic mechanical loading and it was shown that in the presence of CSMA, chondrocytes displayed improved proteoglycan synthesis and collagen deposition. In a related study, Steinmetz et al. also used PEGDM and CSMA to fabricate multi-layer photocrosslinked hydrogels, with adjustable stiffness properties [231]. The authors studied chondrogenic differentiation of the hMSCs embedded in the hydrogel. Upon dynamic mechanical loading, increased collagen expression was achieved, confirming that external mechanical stimuli are indeed regulators of hMSCs differentiation towards osteochondral cell types. In another study, Wang et al. investigated the importance of the mechanical stiffness of CSMA/PEGDMA hydrogels on cartilage formation *in vivo* using a mouse model [105]. Adipose-derived stem cells (ADSCs) and neonatal chondrocytes were encapsulated in the gels fabricated via photocrosslinking of PEGDM and either CSMA or HAMA. The results showed that both hydrogel stiffness and CS/HA are essential for the hyaline cartilage phenotype formation.

CS-based hybrid gels can also be used for bone tissue engineering. In the work of Kim et al. CSMA and PEGDA were employed to develop hybrid hydrogels and tested as biomineralizing scaffolds for bone tissue engineering (Fig. 9B) [94]. This hydrogel was negatively charged, due to the sulfate groups of CS and the biomineralizing ability of the gels was investigated *in vitro*. The prepared hydrogels were incubated in a buffer containing calcium and phosphate ions. Calcium ions were attracted by the CS negative charges on the gel, followed by accumulation of the phosphate ions. A white particulate was formed on the gel surface, corresponding to calcium phosphate. Moreover, it was shown that encapsulated human tonsil-derived mesenchymal stem cells were able to differentiate into osteogenic cellular phenotypes. Upon implantation of

the gel in a cranial defect site, bone formation was induced at the defect site, showing the potential suitability of this hydrogel type for bone tissue engineering [94]. Ingavle et al. made hybrid hydrogels composed of agarose, photocrosslinkable CSMA and PEGDA [232]. First, upon cooling down heated agarose solution, an agarose gel was formed via physical crosslinking, which was then incubated in buffer solution of CSMA and PEGDA for 2.5 h to achieve equilibrium. Next, CSMA/PEGDA were photopolymerized to form the second network. Porcine chondrocytes were embedded in the agarose network during the cooling process (when the temperature reached 39 °C). It was shown that cell viability was markedly improved, along with higher production of GAGs compared to control hydrogel formulation lacking CS. Also, the authors observed an increase in compressive modulus, compared to PEGDA or agarose only hydrogels [232].

Suekama et al. developed a hybrid gel based on CSMA and synthetic polymers [233]. The first network was made by photocrosslinking CSMA and PEGDA, whereas the second network was formed by swelling the gel in the solution of acrylamide, *N*,*N*′-methylene bisacrylamide and a suitable photoinitiator followed by UV irradiation. A variety of gels with different mechanical properties could be obtained by adjusting the concentration and crosslinking density of the networks. This study confirms that formulation of double network gels, also including CSMA, could lead to engineered scaffolds with tunable moduli and failure stresses. The same group also reported a hydrogel based on CSMA and polyacrylamide, without PEGDA [234] and gels with superior mechanical properties were obtained with failure stresses of up to 20 times greater than those of the single component hydrogels.

Poly(vinyl alcohol) (PVA) has been combined with CSMA to fabricate hybrid hydrogels. Bryant et al. prepared methacrylated PVA and then photocrosslinked it with CSMA, when both dissolved in deionized water. The resulting hydrogels exhibited superior compressive moduli compared to individual component hydrogels. Importantly, encapsulated cells underwent chondrogenesis [235].

CSMA has also been combined with a thermo-sensitive triblock copolymer based on PEG and partially methacrylated poly(*N*-(2-hydroxypropyl) methacrylamide-mono/dilactate) (pHPMAlac) [100]. The thermo-sensitive polymer was responsible for physical network formation at temperature above the LCST (27 °C) and also allowed covalent photopolymerization with CSMA. This work showed the suitability of such systems for 3D printing applications of hydrogel scaffolds. Furthermore, encapsulated chondrocytes were viable and proliferating, indicating suitability of the scaffold for cartilage tissue engineering purposes.

4.2.3. Composite CSMA hydrogels containing inorganic components

Derakhshan et al. fabricated a composite CSMA-based hydrogel in combination with hydroxyapatite particles (400 nm) to obtain a scaffold aimed for bone tissue engineering [85]. ADSCs that were incorporated in the hydrogel, displayed calcium deposition ability and upregulation of early osteogenic markers, suggesting the suitability of this composite hydrogel as filler for bone deficiencies.

Silva et al. developed a multifunctional composite gel prepared by incorporating iron-based magnetic nanoparticles (6.9 ± 1.9 nm) in CSMA hydrogel (Fig. 9C). This hydrogel was fabricated in the following way: CSMA was dissolved in platelet lysate (obtained by the lysis of blood platelet concentrates), and supplemented with photoinitiator and magnetic nanoparticles. Gels were obtained by UV irradiation, resulting in a CSMA hydrogel network in which magnetic iron nanoparticles were dispersed. Platelet lysate was used because it is enriched with structural and functional biochemical cues, such as growth factors (among which platelet derived growth factor-BB). Subsequently, by applying an external magnetic field, it was shown that it was possible to modulate growth factor release from the hydrogel. This is attributed to the hypothesis that the external magnetic field (EMF) could cause a temporary destabilization of the matrix, increased permeability and thus triggered release of the loaded growth factors. The release of the growth factor was faster when EMF was applied, as opposed to the control group (without EMF). Osteogenically differentiated ADSCs (adipose derived stem cells), as well as tendon-derived cells were encapsulated in hydrogels without growth factors and able to proliferate and produce bone and tendon related markers. The multifunctional character of the described system, in addition to the possibility of responsive release of the growth factors, indicates its potential suitability as multifunctional biomaterial. The authors proposed that this composite multifunctional and responsive CSMA-based hydrogel is an attractive material for construction of tissue engineered scaffolds for tendon-to-bone interface applications [236].

Carbon nanostructures have also been combined with CSMA to fabricate a nanocomposite hydrogel suitable for cartilage tissue engineering applications [237]. A combination of CSMA, poly(ethylene glycol) methyl ether-ε-caprolactone acryloyl (PECA) and graphene oxide (GO) was used in this study [237]. Heat-initiated free radical polymerization was employed using APS (ammonium persulfate) as initiator to fabricate hydrogels. CSMA, PECA, GO and APS were all dissolved in water and the solution was subsequently brought to 60 °C for 2 h. The prepared hydrogels were cytocompatible (leaching assay) and rabbit chondrocytes cultured on the hydrogels were able to proliferate.

From the reported examples it is evident that the potential of CSMA is not fully exploited in the field of (nano)composite hydrogels, considering that inorganic nanoparticles are known to improve mechanical properties, while providing specific, new functionalities to the hydrogel and still maintaining the biocompatible character of the hydrogel.

The reviewed hydrogels based on HAMA and CSMA, together with different naturally occurring or synthetic polymeric counterparts, result particularly useful for tissue engineering applications. Specifically, in the fields of cartilage and heart tissue engineering several authors reported suitability of the materials to improve physiologically relevant functions, such as chondrogenesis and heart remodeling [41,42,105,147,211,212,227,230,232,237]. In addition, within the field of bone tissue engineering HAMA and CSMA-based hydrogel systems are indicated for promoting osteogenesis [85] and improving biomineralization [94]. Moreover, multiple hydrogel systems were demonstrated to be efficient for cell encapsulation, facilitating studies *in vitro* [40,220,221]. More details on these applications are reported above in the present section and are succinctly summarized in Tables 2 and 3

The hybrid hydrogels reviewed in this section involve the use of other bio- or synthetic polymers in combination with HAMA or CSMA. For use of such materials in clinics, it is necessary that the other components also comply with GLP and GMP requirements, as already discussed for HAMA and CSMA in section 2. Regardless of the type of other component (biopolymer, synthetic polymer, particles etc.), it is important that these are produced according to Good Laboratory and Good Manufacturing Practices. Such materials have to be produced on a large scale and the process has to be reproducible. In addition, suitable analytical methods for assessing the presence of residues of chemical reagents and solvents used during the synthesis process should be defined. The majority of the cited examples do not investigate reproducibility and upscaling of the reported procedures. There are some studies that indicate the feasibility for clinical translation, such as for GelMA, one of the most frequently used polymers in combination with HAMA and CSMA. GelMA is gelatin chemically modified through a methacrylation process, similar as seen for HAMA and CSMA in section 2. GelMA shows some very desirable properties, such as biocompatibility, enzymatic degradability and cell adhesion [238,239]. Most of the examples of the GelMA counterpart used in hybrid gels with HAMA or CSMA report on a methacrylation process with methacrylic anhydride, where the degree of methacrylation is adjustable via the feed ratio of gelatin and methacrylic anhydride. This means that its production, reproducibility of degree of methacrylation and upscaling are required so that the resulting hybrid material can be used for applications in clinics. However, most of the authors of the hybrid hydrogels reviewed

here do not investigate the reproducibility and batch-to-batch variations of the produced polymers and relative hybrid hydrogels. Recently, Zhu et al. looked into reproducibility and controllability over composition and physico-chemical properties of GelMA [240]. Specifically, the authors showed high batch-to-batch consistency and reproducibility of the degree of methacrylation of GelMA. In addition, the reported synthetic procedure can likely be scaled-up for industrial preparation, considering that the authors performed the study on multigram scale of the starting material. Among hybrid gels of HAMA/CSMA with biopolymers, the choice of materials is highly dependent on the actual application and material design, but in terms of GLP/GMP requirements, GelMA would seem to be a very suitable polymer for clinical applications, providing that the HAMA/CSMA component also complies with the requirements. Amongst synthetic polymers, PEG derivatives are frequently used in combination with HAMA and CSMA. These polymers are non-toxic and frequently used in biomedical applications. Therefore, we believe that from synthetic counterparts to prepare hybrid hydrogels, PEG seems a rational choice, provided that the polymer preparation is reproducible, and that upscaling is feasible.

5. Conclusions, recommendations and perspectives

This review gives an overview on the key aspects concerning the synthesis, physico-chemical characterization and applications of photocrosslinkable HA and CS derivatives, intended for biomedical applications, in particular tissue engineering. Such polymers are characterized by high tunability and therefore versatility.

According to reviewed literature, more efforts have been given on the synthesis and application of HAMA derivatives, whereas although explored significantly, CSMA deserves more in-depth and extensive exploitation in constructing photocrosslinkable hydrogels. Although HA and CS are very promising materials for clinical translation, the assessment of their molecular weight distribution and the modification site (exact chemical structure) can often be technically challenging. However, such specifications are necessary for their use in clinics, because a completely defined system with reproducibility for scale-up is pivotal. More attention should be directed to reporting the exact molecular composition of the HAMA or CSMA synthesized. For the production on a large scale of both HAMA and CSMA, the method using methacrylic anhydride in water seems to be the most suitable option as discussed in section 2. However, regarding the large-scale production of such polymers, there are several pitfalls that still need further investigation. Particularly, more systematic studies are necessary for assessing the reproducibility of the degree of methacrylation, also in relation with the molecular weight of the polymers used. Furthermore, a careful assessment of the suitable purification method needs more investigation, as discussed in section 2.

In terms of polymer characterization, the use of a robust (validated) method for M_w determinations (for instance GPC-MALLS) is recommended for investigations towards clinical translation. Evaluation of DM is usually done by ^{1}H NMR measurements, which is prone to large errors. A facile HPLC method that can quantify the amount of methacrylic acid formed after hydrolysis of the methacrylate esters attached to HAMA/CSMA is recommended as a superior, more accurate alternative to ^{1}H NMR based DM determinations. Moreover, monitoring the degradation of the (hybrid) hydrogels and the analytical detection of leachable methacrylic acid and other residues is of great importance before moving to clinics and should also not be overlooked. Here also, the HPLC method mentioned in paragraph 2.3.3. is recommended as it would be excellently suited to be developed as a validated GLP method. Detailed information on the material degradation and related analysis of the degradation products should receive considerate attention during preclinical studies. Determinations of the purity and sulfation patterns of CS are of paramount importance for successful translation to the clinic and also need to be standardized and improved.

An extensive review of the possible enzymatic degradation mechanisms of both HAMA and CSMA polymers and hydrogels are also reported here. Although recently new insights in the cellular degradation of HA have been reported, specifically the discovery of a new ubiquitous HA degrading enzyme (TMEM2), further investigations into the *in vivo* enzymatic degradation of HAMA hydrogels is highly recommended. For CSMA, there are currently no reports in literature that show enzymatic degradability of the hydrogel using the relevant human enzymes (HYALs1, 2 and 3). An important step towards clinical reality for CSMA hydrogels should thus be to establish that human-present enzymes can indeed degrade CSMA-based hydrogels as they can native CS.

We also discuss the use of photocrosslinkable HA and CS derivatives in combination with other polymers, either synthetic or biopolymers with the aim to improve physical, mechanical and biocompatibility properties. As seen from the extensive existing literature, their application, although not limited to, is highly focused on cartilage, bone and cardiac tissue engineering applications. Several of such hybrid hydrogels are attractive candidates for future clinical applications, based on promising *in vivo* results. However, despite a lot of research being done on HAMA/CSMA hybrid gels, there are still several aspects which require more attention to bring these materials closer to patients. First, there is a significant lack in studying the processes necessary for quality control, assessing thoroughly reproducible production of the polymers and hydrogels thereof (and their characterization), as well as developing related upscaling protocols. Furthermore, studies concerning the safety of the polymers and hydrogels and their degradation and clearance from the body have to be properly addressed. Therefore, more studies on the polymers, as well as on the resulting hydrogel materials are necessary for further translational development and use in clinics. It is of paramount importance to successfully characterize such polymers and hydrogels and to produce them in a reproducible, safe manner.

To conclude, both HAMA and CSMA have been demonstrated to be suitable and versatile biomaterials for biomimetic hydrogel scaffolds. In this work, a systematic overview and discussion of the challenges regarding synthesis, physico-chemical characterization, enzymatic degradation and applications of HAMA and CSMA-based hydrogels is given.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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List of abbreviations

ADH	adipic acid dihydrazide
ADSCs	adipose derived stem cells
AEMA	aminoethyl methacrylate
APMAm	3-aminopropyl methacrylamide
APS	ammonium persulfate
BDDE	1,4 butanediol diglycidyl ether
BM MSCs	bone marrow mesenchymal stem cells
Boc_2O	di-tert-butyl dicarbonate
BOP	benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate
BSA	bovine serum albumin
CEMIP1	Cell Migration Inducing Protein 1, Newly found Hyaluronic

	acid cell receptor (see also KIAA1199)
CEMIP2	Cell Migration Inducing Protein 2 (see also TMEM2)
CNTs	carbon nanotubes
CS	chondroitin sulfate
CSMA	chondroitin sulfate methacrylate
Dex	dextran
DM	degree of methacrylation
DMA	dimethylacrylamide
DMAP	4-Dimethylaminopyridine
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DN	double network
ECM	extracellular matrix
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
EMT	epithelial to mesenchymal transition
GAG	glycosaminoglycan
GelMA	gelatin methacryloyl
GMA	glycidyl methacrylate
GO	graphene oxide
GPI	glycophoshatidylinositol
HA	hyaluronic acid
HA-BDDE	hyaluronic acid hydrogel crosslinked with 1,4 butanediol diglycidyl ether
HAMA	hyaluronic acid methacrylate
HAGMA	Hyaluronic acid glycerol methacrylate
HARE	hyaluronic acid receptor for endocytosis
HEMA	hydroxyethyl methacrylate
hMSCs	human mesenchymal stem cells
HOBt	hydroxybenzotriazole
HSCs	hematopoietic stem cells
HYAL	hyaluronidase
IPN	interpenetrating network
LYVE	lymphatic vessel endothelial hyaluronic acid receptor
MES	2-(*N*-morpholino)ethanesulfonic acid
NHS	*N*-hydroxysuccinimide
NMI	*N*-methylimidazole

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