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HIDROGÉIS INJETÁVEIS DE ÁCIDO HIALURÔNICO: ASPECTOS REOLÓGICOS & APLICAÇÕES COMO PREENCHEDORES FACIAIS

THE STATE OF THE ART OF INJECTABLE HYALURONIC ACID HYDROGELS: RHEOLOGICAL ASPECTS & APLICATION AS FACIAL FILLERS

Florianópolis, 2022.

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Trabalho de Conclusão de Curso apresentado como um dos requisitos para a obtenção do grau de Bacharel em Farmácia.

Orientador: Profº. Dr. Thiago Caon
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RESUMO EXPANDIDO

O ácido hialurônico (AH), também conhecido como “hialuronana”, é um dos principais constituintes da pele. Trata-se de um polímero natural do tipo glicosaminoglicano composto por unidades repetidas de ácido D-glucurônico e N-acetil-D-glucosamina. Fisiologicamente, encontra-se presente no tecido conjuntivo, pele e líquido sinovial. Contribui para a integridade mecânica destes tecidos e lubrificação de estruturas intracelulares. No campo da estética facial, o AH é utilizado para recuperar o volume perdido por alguns tecidos, remodelar o contorno e a topografia facial ou ainda suavizar rugas e sulcos. O AH é padrão-ouro em preenchedores faciais por ser um material biodegradável, biocompatível, não-imunogênico, possui alta capacidade absortiva e um comportamento viscoelástico após o contato com meios aquosos. Ainda, procedimentos estéticos com AH podem ser revertidos pela ação de hialuronidades, uma vantagem em relação a outros preenchedores dérmicos. Por outro lado, quando utilizado na sua forma natural, este polímero sofre rápida degradação devido a ação destas enzimas ou a danos oxidativos, apresentando curta duração no organismo (2-3 dias). Assim, modificações químicas do polímero tem sido consideradas a fim de viabilizar e popularizar seu uso como preenchedor facial. O processo de reticulação conecta as cadeias lineares de AH, transformando-as em uma rede tridimensional. Com isto, suas propriedades biofísicas são melhoradas, resultando em estruturas poliméricas mais rígidas e resistentes à degradação. Hidrogéis de AH reticulado não exigem testes de alerginicidade, apresentam alta capacidade de absorção de fluidos e as propriedades biofísicas podem ser ajustadas até a obtenção de um comportamento parecido com cada sítio/local a ser tratado. O BDDE (1,4-butanodiol diglicidil éter), DVS (divinilsulfona) e o PEGDE (polietilenoglicol diglicil éter) tem sido os agentes de reticulação mais utilizados. O tipo e concentração destes agentes afetam o grau de reticulação, viscoelasticidade, coesividade, tamanho de partícula e intumescimento, o que tem relação com o desempenho do produto. Como o AH pode ser obtido a partir de diferentes fontes animais e bacterianas e apresenta um peso molecular variável, uma caracterização periódica deste polímero deve ser realizada para eventuais ajustes da proporção AH: reticulante. Parâmetros reológicos dos hidrogeis também devem ser reavaliados com frequência para garantir a eficácia do produto. Nestes últimos ensaios, é investigado o comportamento destas formulações após serem submetidas a diferentes forças de deformação (torção/cisalhamento lateral e estiramento/compressão). Este comportamento pode ser do tipo viscoso (G") e/ou elástico (G'). O G' fornece informações a respeito da dureza/rigidez do material enquanto o G" tem relação com a mobilidade molecular.

Altos valores de G' indicam hidrogéis com efeito tensor e capacidade de volumização significativos. Por esta razão, são recomendados para preenchimento de rugas profundas da face. Por outro lado, hidrogeis com baixos valores de G' tem uma capacidade de preenchimento (volumização) limitada e então são recomendados para tratar linhas finas de regiões superficiais da face e para preenchimento labial. Ainda que a alta dureza do material resulte em um maior tempo de permanência do hidrogel na pele, limitações durante a aplicação do produto (no momento da saída da seringa) são convencionalmente encontradas devido a dificuldades de escoamento do material. Uma estratégia que tem sido considerada para resolver este problema é a utilização de uma mistura de AH reticulado e não reticulado. Apesar da rápida degradação, o AH não reticulado facilita o escoamento do hidrogel no momento da aplicação. A coesividade, por sua vez, tem a ver com a capacidade de um material não se dissociar devido à afinidade mútua entre os seus componentes. Baixos valores de coesividade indicam que o hidrogel se dissocia e se distribui facilmente na pele. Regiões de alta mobilidade ou mais superficiais exigem sistemas com essas características justamente para evitar o acúmulo indesejável em sítios específicos da pele. Apesar dos avanços no desenvolvimento de preenchedores dérmicos de AH e o conhecimento de que existe um produto adequado para cada região facial, o consumidor ainda exige produtos com uma maior duração. A indústria cosmética entende esta necessidade, mas ainda caminha na direção de entender o efeito de variáveis de formulação no resultado clínico, fato que também foi priorizado nesta revisão de literatura. Por fim, destaca-se a necessidade de investigação de novos candidatos a agentes reticulantes ou modificações químicas capazes de gerar produtos inovadores com ainda mais benefícios ao consumidor.

Palavras-chaves: hidrogéis, ácido hialurônico, agente reticulante, propriedades reológicas.

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THE STATE OF THE ART OF INJECTABLE HYALURONIC ACID HYDROGELS: RHEOLOGICAL ASPECTS & APLICATION AS FACIAL FILLERS

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Abstract

Injectable hyaluronic acid (HA) hydrogels have been popularized in the facial aesthetics as they provide a long-lasting effect, low risk of complications, no require allergenicity tests prior to application and can be removed by hyaluronidases. On the other hand, the development of these systems requires in-depth studies of chemical mechanisms involved in hydrogel formation. Ideal dermal fillers should temporarily fluidize during extrusion through the needle and quickly recover its original shape after application. Hydrogels with more elastic properties, for example, are difficult to inject while viscous materials are too liquid. A balance between both properties should be achieved. Each region of the face also requires products with distinct rheological properties. High G' dermal fillers are preferable for deeper wrinkles whereas the counterpart with lower values of G' are more indicated in superficial wrinkles or lip augmentation. Several factors such as HA molecular weight and concentration, pH, type and concentration of crosslinking agent, particle size, crosslinking reaction time and crosslinking agent/polysaccharide ratio should be considered to modulate the rheological properties desirable for the hydrogel. In this review, the effect of each variable is discussed in detail to guide the development of new dermal fillers in a more rational way.

*Keywords***:** hydrogels, hyaluronic acid, crosslinking agent, rheological properties.

1. Introduction

The skin tissues and skeletal support undergo various changes throughout life, particularly on the face region, as a part of the natural aging process, leading to volume loss. These changes occur mainly due to loss of subcutaneous fat and degradation of collagen fibers (Fakhari & Berkland, 2013; Reuther & Watson, 2016; Rhee, You, & Han, 2014; Wongprasert, Dreiss & Murray, 2022).

The aging process involves intrinsic and extrinsic factors. Intrisic aging is genetically determined by hormonal and biochemical factors, leading to a decrease in the skin elasticity and hydration as well as increase in bone resorption. Extrinsic aging, in turn, is affected by environmental factors such as solar exposure, ultraviolet radiation, repetitive facial expressions and gravity (Macierzyńska, Pierzchala & Placek, 2014; Sadick, Karcher & Palmisiano, 2009; Tezel & Fredrickson, 2008). All together, these changes lead to reduction in collagen levels and other non-collagenous extracellular matrix constiuents, which affect the mechanical properties of skin and lead to the appearance of expression wrinkles.

Dermal fillers emerge as an alternative to reverse the ageing signs given that they are minimally invasive procedures (Tezel & Fredrickson, 2008; Trinh & Gupta, 2021). These formulations are applied in skin to restore the volume loss caused by the agin process (Faivre et al., 2021). Different dermal fillers have been developed over the years to attend a growing demand. According to the origin of the formulation polymer, they can be classified as “synthetic dermal fillers" or “natural dermal fillers”. Calcium hydroxyapatite, polymethylmethacrylate and poly-L-lactic acid are often used in synthetic dermal fillers whereas collagen (bovine and human) and hyaluronic acid in natural dermal fillers (Chang, Yu & Percec, 2018).

The hydrogels have been promising systems as dermal filler due to a capacity for swelling and retaining water in their structure, which makes them flexible and similar to physiological tissues (Ahmed, 2015; Al-sibani, Al-harrasi & Neubert, 2016). Hyaluronic acid (HA) hydrogels have been widely used due to the high biocompatibility and low immunogenicity. In fact, HA hydrogels do not require allergy testing before application, low rate of complications is found and provide a long-lasting effect (6-9 months). Another advantage of this system is the possibility of reversing the procedure with the use of hyaluronidases, which are enzymes responsible for its biodegradation (Breithaupt, Custis & Beddingfield, 2012; Faivre et al., 2021; Fundaró, Salti, Malgapo & Innocenti, 2022; Tezel & Fredrickson, 2008). Although HA is an attractive material for dermal filler preparation, it is characterized by poor mechanical properties and high *in vivo* degradation in its natural form.

In this context, polymer crosslinking has been considered to improve both mechanical properties and product duration in the skin. Once the crosslinking agents change rheological properties and swelling degree of the final product, they can affect the clinical performance of dermal fillers (Guardia, Virno, Musumeci, Bernardin & Silberberg, 2022; Zerbinati, Sommatis, Maccario, Capillo, Grimaldi, Alonci, Protasoni, et al., 2021). The main objective of this review is to understand how the rheological properties and the degree of swelling or crosslinking affect the performance of HA hydrogels, allowing a rational and effective selection of dermal fillers.

2. Hyaluronic acid

HA, also known as hyaluronan, is a natural polymer belonging to the class of glucosaminaglycans. It was first isolated in 1934 by Karl Meyer and John Palmer from the vitreous humor of bovine eyes, which is a colorless, transparent, viscoelastic and hydrophilic structure (Abatangelo, Vindigni, Avruscio, Pandis & Brun, 2020; Pigaiani, Bertaso, De Palo, Bortolotti & Tagliaro, 2020). The name hyaluronic acid derives from the greek word “hyalos” meaning “glass” in a reference to its physical properties; and “uronic acid” in reference to its chemical structure, which is composed of two interconnected sugar molecules, the D-glucuronic acid and the *N*-acetyl-D-glucosamine (Salwowska, Bebenek, Zadlo, Wcislo-Dziadecka, 2016; Sudha & Rose, 2014). The name “hyaluronan” was proposed by Balazs in 1986 in reference to its dissociated form in physiological pH, which results in formation of salts such as sodium hyaluronate (Abatangelo et al., 2020). Adults weighing 70 kg have approximately 15 g of HA synthesized and degraded daily, and approximately half of this amount (7 to 8 g) is found in the skin (Sudha & Rose, 2014; Vasvani, Kulkarni & Rawtani, 2020).

2.1 Chemical structure

Glycosaminoglycans are widely used in biomedical, pharmaceutical, and cosmetic field. They are linear and negatively charged polysaccharides. Unlike other glycosaminoglycans, HA is a representative of this class that does not have sulfate in its structure (Sudha & Rose, 2014; Vasvani et al., 2020).

As already mentioned, the HA is composed of several disaccharide units of D-glucuronic acid and *N*-acetyl-D-glucosamine, which are connected by β1→4 glycosidic bonds for the formation of monomers (Fig. 1) (Dicker, Gurski, Pradhan-Bhatt, Witt & Farach-Carson, 2014; Neuman, Nanau, Oruña-Sanchez & Coto et al., 2015; Salwowska et al., 2016). The molecular weight of HA can vary according to the number of monomers in its structure and

thus low or high molecular weight materials can be found (Chistyakov et al., 2019). The chain size that defines low and high molecular weight polymers is not a consensus in the literature. Most authors consider that high molecular weight HA has a chain size greater than 1000 kDa (Agostino et al., 2017; Chistyakov et al., 2019; Maharjan, Pilling & Gomer, 2011).

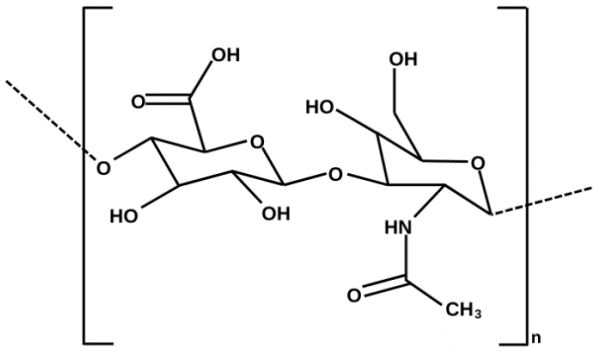

Fig. 1. Chemical structure of hyaluronic acid

2.2 Physiology

HA can be found in various regions of the human body, contributing to different functions. It is the main constituent of the extracellular matrix of connective tissue, skin, synovial fluid and umbilical cord. In the presence of physiological fluids, the formation of a viscous solution is found that hydrate the extracellular matrix, providing volume and resistance to compressive forces. Therefore, HA is crucial for maintaining mechanical integrity of tissues. It also plays a role as lubricating agent on the joints (particulary the high molecular weight HA) (Matarasso, 2004; Pérez, Hernández, Alonso, Pérez-González & Sáez-Martínez, 2021; Sudha & Rose, 2014).

In addition to mechanical protection, HA is an essential macromolecule for the cell membrane composition, acting in cell differentiation processes, morphogenesis and regulation of cell adhesion. It is also involved in the anti-inflammatory (high molecular weight HA) and pro-inflammatory (low molecular weight HA) responses due to its ability to interact with different receptors (Abatangelo et al., 2020; Chistyakov et al., 2019; Fallacara, Baldini, Manfredini & Vertuani, 2018). This polysaccharide can be found intracellularly; however, more studies are still needed to understand its action in this region (Dicker et al., 2014).

2.2.1 Synthesis

Unlike other glycosaminoglycans that are synthesized in the Golgi apparatus of eukaryotic cells, HA is synthesized in the cell membrane (Abatangelo et al., 2020). Three

transmembrane glycosyltransferase isoenzymes kwnon as hyaluron synthases (HAS1, HAS2 and HAS3) are responsible for the synthesis of HA in the human body. The active site of these isoenzymes faces the interior of the cell, where the HA is synthesized, and then it is transported to the exterior through a pore found in the enzyme structure (Fallacara et al., 2018; Salwowska et al., 2016). A down-regulated expression of these enzymes has been observed in older human fibroblasts, which would explain the lower production of HA (Terazawa, Nakajima, Tobita, Imokawa, 2015).

The reaction kinetic and molecular weight of the polymer generated differentiate these isoenzymes. The enzymes HAS1 and HAS2 synthesize HA with higher molecular weight (longer polymer chain) while HAS3 synthesize low molecular weight polymers (shorter polymer chain). Considering the reaction kinetic, HAS1 takes longer to synthesize HA, followed by HAS2 and HAS3 (Vasvani et al., 2020).

2.2.2 Degradation

HA is very susceptible to degradation by the action of hyaluronidase enzymes or oxidative damage caused by free radicals (Fallacara et al., 2018). HA has a very short half-life in the body, lasting from 2.5 to 4.5 min in the bloodstream (Fraser, Laurent, Pertoft, Baxter, 1981), less than one day in the skin and 1 to 3 weeks in the cartilage (Ward, Thibeault & Gray, 2002). The six main hyaluronidases identified in human are Hyal-1, Hyal-2, Hyal-3, Hyal-4, PH-20 and Hyalp 1, which degrade HA in monosaccharides (Salwowska et al., 2016; Stern & Jedrzejas, 2006). These enzymes cleave the internal β-N-acetyl glucosaminidic linkages, resulting in fragments with *N*-acetyl-glucosamine at the reducing terminus and glucuronic acid at the non-reducing end (Zhong et al., 1994). Unlike the Hyal-3, which is only active in acid pH, Hyal-1 and Hyal-2 act in a wide pH range. Hyal-2 degrades HA into fragments up to 20 kDa while Hyal-1 degrades it into tetrasaccharides (Salwowska et al., 2016; Stern & Jedrzejas, 2006). Hyal-4 is more effective in the degradation of chondroitin than in the degradation of HA. PH-20, also known as sperm adhesion molecule 1 (SPAM 1) or testicular hyaluronidase, was first isolated from testicular tissue. It is found in other tissues, but in lower levels than other hyaluronidases. Hyalp 1 is a gene that can be transcribed but not translated in the human body, suggesting that hyaluronidase genes continue to undergo modifications and evolve (Abatangelo et al., 2020; Stern & Jedrzejas, 2006).

3. Hyaluronic acid as facial fillers

The first injectable filling agent was paraffin, but its use was restricted after complications of migration, embolization, and granuloma formation. Silicone was more recently banned by Food and Drug Administration (FDA) for similar complications. Clinical trials with bovine collagen were performed between 1977 and 1978 to improve age-related wrinkles and then this agent was approved by the FDA for cosmetic injection in 1981. On the other hand, allergy tests are required prior to its application, which motivated the search of other filling agents. HA emerged as an alternative due to its high biocompatibility and immunogenicity and long duration of action that can be achieved. Its use was approved in 2003 by the FDA, but it remains the most widely used filler agent to this day (Kontis & Rivkin, 2009).

Hydrogels, which are polymeric networks with great swelling capacity, have been the most traditional cosmetic preparations used for this application as they are able to provide mechanical properties similar to physiological tissues depending on their composition (Ahmed, 2015; Al-sibani et al., 2015). HA has been sleected for the composition of these formulations as it can absorb up to 1,000x its weight in water and it provide viscoelastic properties that may be easily adjusted depending on the application. Therefore, its biological properties and rheological properties have ensured the use as facial fillers (Allemann & Baumann, 2008; Pérez et al., 2021; Rhee et al., 2014).

3.1 Sources of hyaluronic acid

HA can be obtained from animal or bacterial sources and its structure is highly conserved among the different species (Fallacara et al., 2018; Salwowska et al., 2016). The main difference between the animal and bacterial source is the length of the polymer chain. The animal source HA has a longer chain length, ranging from 10,000 to 15,000 monomeric units per chain, presenting an average weight between 4 and 6 MDa. HA obtained from bacteria source, in turn, has a shorter polymer chain, ranging from 4,000 to 6,000 monomeric units per chain and an average weight between 1.5 and 2.5 MDa (Tezel & Fredrickson, 2008).

As in humans, HA is an essential molecule in other mammals (Ucm et al., 2022). HA was first extracted from animal sources such as bovine vitreous humor, umbilical cord, rooster comb, among other animal tissues. Due to the cost issues and complexity of the extraction process, bacterial sources have been prioritized in recent years. The extraction of HA from mammal tissues requires many purification reagents and steps to remove contaminants such as

proteins, nucleic acids and viruses. Therefore, adverse effects are more common in this situation (Fallacara et al., 2018; Liu et al., 2018; Schiraldi, La Gatta & Rosa, 2003).

The first extraction of HA from bacteria sources considered *Streptococcus* spp. As these bacteria are pathogenic to humans, several purification processes have been shown to be necessary to eliminate toxins (Ucm et al., 2022). For this reason, bacteria of the genus Bacillus, particularly *Bacillus subtilis*, have gaining attention since they do not produce toxins, and thus they can be considered a safer option. In addition, no expression of hyaluronidases is found in this microorganism (Widner et al., 2005).

On the other hand, the HA obtained from the natural sources is rapidly degraded, presenting a short duration after application in skin (Chang et al., 2018; Tezel & Fredrickson, 2008). Chemical modifications in its structure have been alternatively considered, resulting in hydrogels with improved mechanical properties (Fallacara et al., 2018).

3.2 Chemical modifications

The chemical modification of HA allows to reduce its degradation rate and thus improve its cilinical performance as facial fillers (Rao, 2020; Tezel & Fredrickson, 2008). Chemical modifications of HA include conjugation and crosslinking methods (Fig. 2) in the following functional groups: carboxylic acid, primary and secondary hydroxyls and *N*-acetyl group (Burdick & Prestwich, 2011; Fallacara et al., 2018).

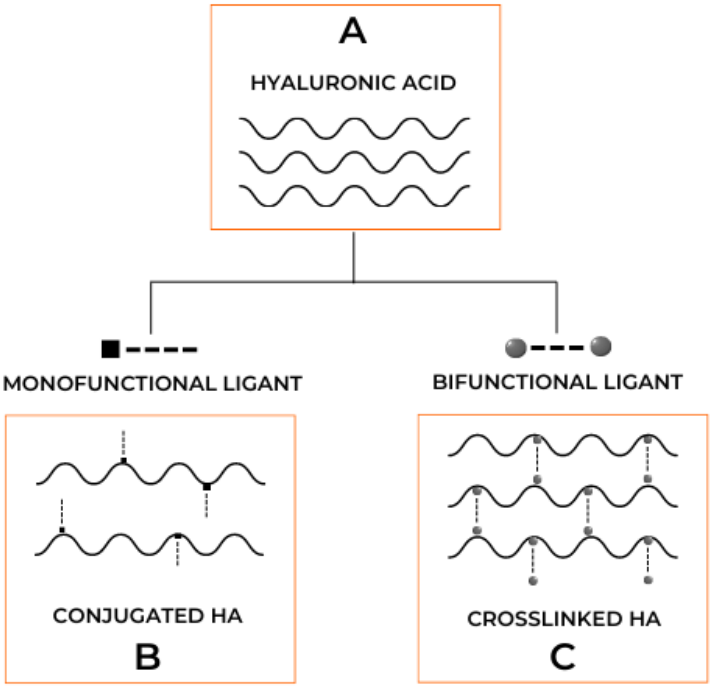

Fig. 2. Main chemical modifications of hyaluronic acid (A): conjugation (B) and crosslinking reaction (C).

In HA conjugation, a compound is grafted onto one HA chain by a single bond. In HA crosslinking, in turn, HA chains are linked together by two or more covalent bonds. In this second method, direct crosslinking, crosslinking of HA derivatives and crosslinking of different HA derivatives can be considered (Schanté, Zuber, Herlin & Vandamme, 2011). Mechanical, rheological and swelling properties are improved depending on the type of crosslinking agent used (Edsman, Nord, Öhrlund, Lärkner c Kenne, 2012; Harrer et al., 2021).

Several methods have been reported for HA crosslinking or conjugation. Although HA is a water-soluble polyssacharide, pH adjustments are required for an effective interaction with the crosslinking agent for the ionization of specific functional groups. On the other hand, this step may lead to HA chain hydrolysis (Maleki, Kjøniksen, & Nystro, 2008). When organic solvents are considered, native HA sodium salt first needs to be converted into its acidic form or a tetrabutylammonium salt for complete solubilization of polymer. In the same way, this additional step could also result in HA degradation (Bergman, Elvingson, Hilborn, Svensk & Bowden, 2007; Pelletier, Hubert, Lapicque, Payan & Dellacherie, 2000).

3.2.1 Hyaluronic acid crosslinking

The crosslinking of HA polymers results in a hydrogel structure with high capacity to retain water without dissolving (Chen, Bolognesi & Vladisavljevi, 2021; Zerbinati et al., 2022). At low concentrations of HA, a Newtonian rheological behavior is observed. In this situation, the viscosity is constant regardless of shear rate. At high concentrations of HA, the applied stress results in an instantaneous elastic strain followed by a viscous, characterizing the viscoelastic behavior (Akhtar, Hanif & Ranjha., 2016; Hennink & Nostrum, 2002).

Different polymer crosslinking agents have been tested to improve the physical properties of the biopolymer and then obtain a more rigid structure (Chen, Garcia & Zimmerman, 2020) as well as resitance to enzymatic and oxidative degradation (Chand, Zhang & Jiang, 2019; Khunmanee, Jeong & Park, 2017; Tezel & Fredrickson, 2008). Crosslinking agents contain two or more reactive ends able to attach certain functional groups, which are separated by a spacer (Fig. 3) (Belsom & Rappsilber, 2020; Bhattacharjee & Ahearne, 2021; Zerbinati, Sommatis, Maccario, Capillo, Grimaldi, Alonci, Protasoni, et al., 2021). The type of crosslinking agent impacts on the characteristics of the final formulation such as degree of crosslinking, rheological properties (viscoelasticity and cohesiveness), particle size and

swelling, which are directly related to the product performance (Belsom & Rappsilber, 2020; Monheit & Coleman, 2006).

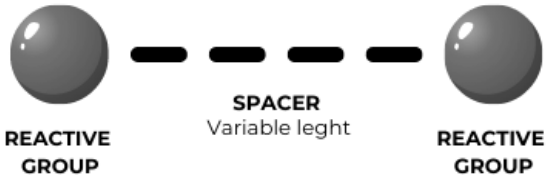

Fig. 3. Crosslinking agent conformation.

Physical and chemical crosslinking methods can be considered for HA (Fig. 4). In physical crosslinking, polymer molecules are hold together through molecular entanglements or interactions such as hydrogen bonds, charge condensation, ionic bonds or hydrophobic interactions (Bustamante-Torres et al., 2021; Caló & Khutoryanskiy, 2014). These polymers undergo sol-gel transitions in response to external stimuli such as pH and temperature. Once these hydrogels are characterized by reversible and variable effects, they have not been considered for application as facial fillers (Chen et al., 2021). Chemical crosslinking, in turn, involves the establishment of irreversible intra- and intermolecular covalent bonds. For this reason, these hydrogels have been prioritized as facial fillers considering that they are more stable to physiological changes (Bhattacharjee & Ahearne, 2021; M. Chen et al., 2021).

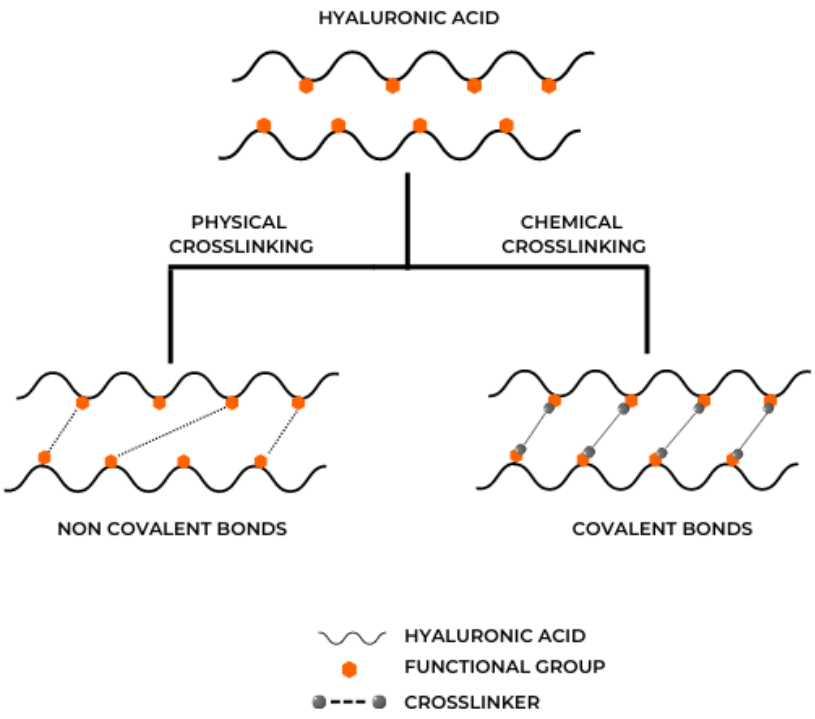

Fig. 4. Physical and chemical crosslinking method.

The HA solution is often alkalized with sodium hydroxide during the crosslinking process and then the crosslinking agent selected is added (Micheels, Sarazin, Tran, Solomon, 2016). Although HA has several sites for interaction with the crosslinking agent, the hydroxyl group represent the main nucleophile. This functional group is deprotonated under alkaline conditions to ensure the reation with the nucleophilic group of the crosslinking agent. The carboxyl group of HA is not preferably considered for the reaction because it can lead to the formation of esters that are more easily hydrolyzed under alkaline conditions (Kenne et al., 2013). After the reaction, part of the unreacted crosslinking agent is found in the medium, which explain some clinical complications. In this context, manufacturers should assure that the techniques used in the production are very effective to reduce the free crosslinker percentage to a safe level (Fundaró et al., 2022; Tezel & Fredrickson, 2008).

Several cross-linked HA manufacturing technologies can be found commercially, but this review will address the impact of crosslinking agents on hydrogel characteristic without considering this aspect. The main products found in the market include Non-Animal Stabilized Hyaluronic Acid (NASHA®), 3D Matrix, Vycross®, Optimal Balance Technology (OBT®), Cohesive Polydensified Matrix (CPM®), Interpenetrating Network-Like (IPN) -Like®) and Resilient Hyaluronic Acid (RHA®) (Micheels et al., 2016).

The main crosslinking agents used for the preparation of HA hydrogels are 1,4-butanediol diglycidyl ether (BDDE), divinyl sulfone (DVS) and polyethylene glycol diglycidyl ether (PEGDE), which will be described in detail in the next sections.

Products with high degree of crosslinking are desirable because they show slower polymer degradation rate, enhanced mechanical properties and greater ability of tissue volume restoration (Herrmann, Hoffmann, Ward, Schulman, 2018). The degree of crosslinking is calculated through the relationship between the number of crosslinker molecules that form double links (reaction with both ends of HA) to the number of HA disaccharides units (Edsman, Nord, Öhrlund, Lärkner, & Kenne, 2012). Therefore, higher crosslink density is the result of more linkages per length of polymer chain (Tolinski, 2009).

BDDE

The crosslinking agent 1,4-butanediol diglycidyl ether (Fig. 5) appears in most of the studies due to its high biocompatibility and lower toxicity (Chand et al., 2019; Fundaró et al., 2022). It was the first crosslinking agent to be used in dermal fillers and it is currently found in market products such as Restylane® and Juvéderm™.

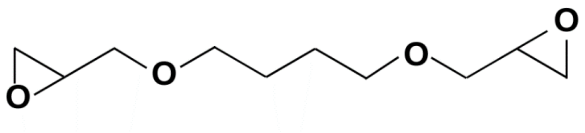

Fig. 5. Chemical structure of 1,4-butanediol diglycidyl ether (BDDE).

The primary hydroxyl groups of HA react with the epoxide group of BDDE under alkaline conditions resulting in ether bonds (Zerbinati, Sommatis, Maccario, Capillo, Grimaldi, Alonci, Rauso, et al., 2021). The reaction is perfomed in strong alkaline conditions to ensure the formation of stable ether bonds (De Boulle et al., 2013).

DVS

Divinyl sulfone (DVS) (Fig. 6) is widely used as crosslinking agent due to its high availability in the market, reproducibility, no organic solvent is required for the reaction with HA (Borzacchiello, Russo, Malle, Schwach-Abdellaoui & Ambrosio 2015; Shimojo, Pires, Lichy & Santana 2015). In this same way, the reaction between HA and DVS occurs under strong alkaline conditions to deprotonate the hydroxyl groups of HA, which react with the vinyl radical of DVS for the formation of stable chemical bonds (Shimojo et al., 2015; Yu & Chau, 2012).

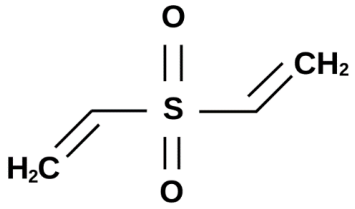

Fig. 6. Chemical structure of divinyl sulfone (DVS).

PEGDE

Polyethylene glycol diglycyl ether (PEGDE) (Fig. 7) is a low toxicity and highly water-soluble crosslinking agent (Zerbinati et al., 2020). The long length of the polymer chain explains the different rheological properties. Unlike other crosslinking agents, PEGDE is a mixture of polymers of different molecular sizes (Monticelli et al., 2019). The chemical reaction between HA and PEGDE also occurs under alkaline conditions involving its epoxide group and the hydroxyl group of HA (Zerbinati, Sommatis, Maccario, Capillo, Grimaldi, Alonci, Rauso, et al., 2021).

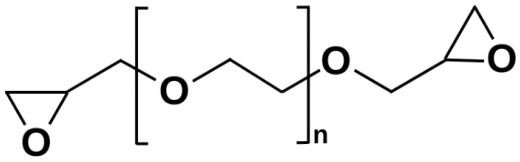

Figure 7. Chemical structure of polyethylene glycol diglycyl ether (PEGDE).

4. Properties of hyaluronic acid hydrogels

The selection of the crosslinking agent impacts on the hydrogel features and performance after application in skin (Monheit & Coleman, 2006; Zerbinati, Sommatis, Maccario, Capillo, Grimaldi, Alonci, Protasoni, et al., 2021). Based on these characteristics, the ideal filler for each site and type of correction required are selected more appropriately (Fundaró et al., 2022). The effect of different parameters (polymer concentration, swelling factor, degree of crosslinking, particle size and rheological properties) on hydrogel characteristics is presented in the Table 1 and will be discussed in detail in the next sections.

Table 1. General and rheological properties of hyaluronic acid dermal fillers and effect on product performance.

PROPERTIES	DEFINITION	EFFECT ON PRODUCT PERFORMANCE
GENERAL		
CONCENTRATION	Total HA in the hydrogel	Hydrogel hardness and duration at the application site
SWELLING FACTOR	Capacity to retain water in hydrogel strucutre and expand	Expansion capacity
DEGREE OF CROSSLINKING	Porcentage of HA monomers linked to a crosslinker molecule	Hydrogel hardness and duration at the application site
PARTICLE SIZE	Size of crosslinked HA fragments	Indication of the most appropriate application site
RHEOLOGICAL		
VISCOELASTICITY	Elastic and viscous behavior	Rheological behavior of the hydrogel after application
G'	Elastic properties and hardness	Ability to resist deformation forces. Increased residence time of dermal fillers in application site
G"	Viscous properties	Capacity to return to its original shape
COHESIVITY	Capacity of adhesion between crosslinked HA molecules	Hydrogel integrity and its distribution in biological tissues (close to application site)

4.1. General properties

4.1.1 Concentration

The concentration of HA in the hydrogel contributes to its duration in the body, hardness as well as degree of crosslinking (Allemann & Baumann, 2008; Borzacchiello et al., 2015). The concentration of HA that appears on the product label (expressed in g/mL) usually refers to the total HA found in the hydrogel, including both crosslinked (insoluble) and free polymer form (soluble). Free HA is added to the hydrogel to facilitate the injection process by increasing lubricity and flow, making it less rigid. On the other hand, this HA form decreases the cohesivity of the products (Borrell, Leslie & Tezel, 2011) and is more susceptible to degradation as already metioned (Kablik et al., 2009), which contribute to a reduction of performance and effectiveness of the product.

4.1.2 Swelling factor

The ability of a hydrogel to absorb water and expand is described as the swelling fator or index, which is calculated by the ratio between de inicial hydrogel volume (V_0) and the hydrogel volume after hydration (V) (swelling factor = V/V_0) (Edsman et al., 2012; Fundaró et al., 2022; Öhrlund & Edsman, 2015).

If the content of water added is greater than the hydrogel's absorption capacity, phase separation can occur, resulting in a hydrogel dispersed in water. This material is not suitable for application as facial filler as it would result in a non-uniform distribution on the skin and application would be more difficult. This fact explains why most HA facial fillers available on the market are “unsaturated” systems, i.e., the water is added at a level below the maximum swelling capacity of the hydrogel to avoid this phase separation (Edsman et al., 2012; Edsman et al., 2015). A high swelling factor describes a hydrogel with high hydration capacity and vice versa (Fundaró et al., 2022).

In physiological conditions, carboxyl and acetyl groups of the HA allow the establishment of hydrogen bonds, retaining water in its structure. This aspect explains how the hydrogel will behave and expand inside the skin after injection. The swelling factor depends on the concentration of HA and the degree of crosslinking (Kablik et al., 2009; Wongprasert et al., 2022).

Maiz-Fernandez et al. (2019), for example, compared the effect of two crosslinking agents (DVS and BDDE) on the swelling factor in HA hydrogels. Different pH conditions and variations in dried to the swollen state of hydrogel were tested. In all tested conditions, BDDE

always provided a greater swelling factor than DVS. The hydrophobic and more rigid structure of DVS would explain these findings, reducing the hydration ability of HA hydrogel (Maiz-Fernández et al., 2019).

Monticelli et al (2019), in turn, compared the swelling degree of HA hydrogels crosslinked with PEGDE and BDDE. *In vitro*, PEGDE showed a higher swelling capacity than BDDE. Authors justified these results due to structural differences of crosslinking agents (Monticelli et al., 2019; Xue et al., 2020). The larger molecular size of PEGDE would result in greater spacing between the HA chains, making the hydrogel structure more flexible. In this way, a greater amount of water accesses the formulation, resulting in a greater swelling index.

4.1.3. Degree of crosslinking and total modification

HA molecules can interact with the crosslinking agent through two main ways, pendant modification or crosslinking. In the first case, one linkage between the crosslinker molecule and the HA disaccharide unit is established. In crosslinking, in turn, two linkages between these agents are established (Fig. 8) (Yang, Guo, Zang & Liu, 2015).

The pendant modification results in a more flexible structure (Kablik et al., 2009; Yang et al., 2015). Crosslinking is more effective in the reduction of degradation rate and to prolong the aestehetic treatment modification and thus it has been prioritized. On the other hand, the application into the skin may be more difficult in this last situation. Both types of interaction contribute to determine the degree of total modification, but only the double bond is required for the determination of the degree of crosslinking (Kablik et al., 2009; Salti & Fundaró, 2020; Yang et al., 2015).

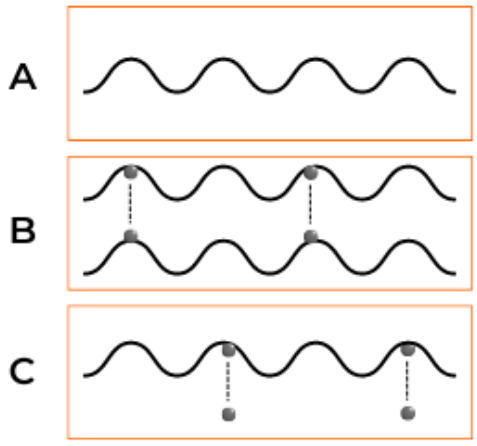

Fig. 8. Free HA molecules (A), formation of two ether bonds between BDDE and two HA molecules generating a more rigid structure (B) and formation of one ether bond between BDDE and only one HA molecule resulting in a more flexible structure (C).

Although pendant modifications are ineffective for the long duration of the hydrogel after application into the skin, it makes the hydrogel less soluble, increasing the degree of swelling (Kablik et al., 2009). The lower the crosslinking degree, the higher the swelling degree, and vice versa. The lower the degree of crosslinking, the more flexible the polymeric network of the hydrogel, which explains the greater degree of swelling (Xue et al., 2020).

The degree of crosslinking describes the percentage of HA monomers that are bound to crosslinking agent. If a hydrogel has a degree of crosslinking of 4%, for example, are present four molecules of the crosslinking agent for every 100 HA monomers (Tezel & Fredrickson, 2008). The degree of crosslinking increases with the concentration of crosslinking agent in the formulation. The greater the degree of crosslinking, the greater the hardness of the hydrogel as well as its ability to resist degradation (Borzacchiello et al., 2015; Herrmann et al., 2018; Kaya & Oytun, 2021; Tezel & Fredrickson, 2008).

On the other hand, high concentrations of non-reactive crosslinking agent can make the application more difficult and affect biocompatibility, leading to rejection of the hydrogel by the body. Several methods to reduce the crosslinking agent remaining in crosslinked hyaluronic acid hydrogels have been studied. In the patent WO2014206701 (Karlssom & Edsman, 2014), for example, the inventors suggest the precipitation of HA using a solvent followed by elimination of residual crosslinking agent. In the patent WHO 2010/015901 (Lebreton, 2010), a process of washing the HA hydrogel by using a dialysis membrane is purposed. Although effective, both methods are not suitable for large-scale production. Moreover, expensive equipments are needed for a precise precipitation in the first case and the process of filling and washing the hydrogel on the dialysis membrane is very difficult. The reduction of crosslinking agent concentration and washing the HA hydrogel with aqueous solutions has been alternatively considered due to the simplicity of operation and lower cost (Jung-Ju, Seong-Won, Wanjin, Kwang-Rok, & Moo-Hyun 2018).

4.1.4 Particle size

A hydrogel block is formed as a result of the crosslinking process (unless it is a weak hydrogel). In other words, hydrogels take the shape of the flask in which they were prepared and then need to be broken into small pieces to pass through the hole of the needle (Allemann & Baumann, 2008; Edsman et al., 2012; Fagien et al., 2019). The compact hydrogel mass may be disintegrated through sieving method or homogenization (Tezel & Fredrickson, 2008). In the first method, hydrogel mass passes through several sieves until they are the desired size,

resulting in average equal size particles. The homogenization method creates a smoother hydrogel with a more regular surface (Fundaró et al., 2022).

Hydrogels with larger particle size have been indicated to treat deeper wrinkles while formulations with smaller particles for superficial wrinkles. Interestingly, larger fragments do not necessarily last longer in the skin than smaller fragments (Kablik et al., 2009). On the other hand, the particle size is related to the hardness of the hydrogel. In general, the larger the particle size, the more is the hardness of the hydrogel (Fundaró et al., 2022).

4.2. Rheological properties

Rheological characterization is a crucial step in the development of hydrogels as it provides information on the clinical performance of the dermal filler in the body (Kaya & Oytun, 2021). These attributes determine the hydrogel behavior after application of different deformation forces (Choi, 2020; Zerbinati, Sommatis, Maccario, Capillo, Grimaldi, Alonci, Protasoni, et al., 2021).

Once the skin tends to tense and stretch, deformation forces act on hydrogel after its application (Borrell et al., 2011). Two main forces causing deformation of hydrogel are (1) lateral torsional or shear force and (2) stretching or compression force (Fig. 9). In the first situation, force is applied across the surface of the hydrogel in a sliding way. The compression force, in turn, is applied vertically (Choi, 2020; Michaud, 2018).

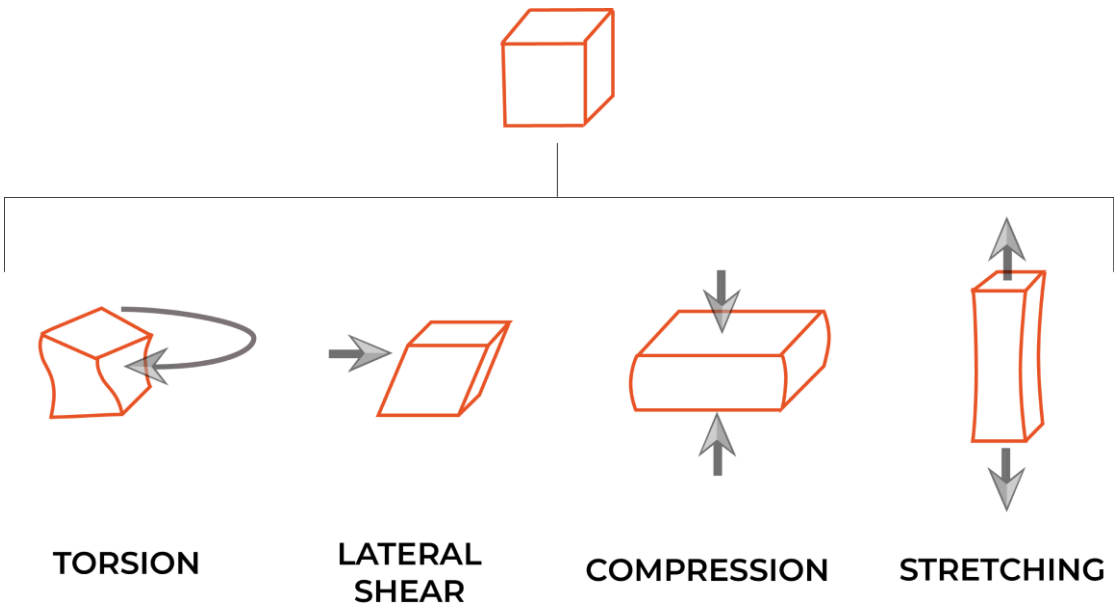

Fig. 9. Deformation forces in hyaluronic acid hydrogels: lateral torsion or shear force and the stretching or compression force.

4.2.1. Viscoelasticity

Viscoelasticity is a parameter used to explain the viscous and elastic behavior after deformation of a material. A material with “elastic” properties is able to recover its original shape after the deformation (up to a certain limit). Rubber-like hydrogels remain elastic up to very high strain and thus they can be considered as a classical example of finite strain elasticity. The material with “viscous” characteristics, in turn, suffer a deformation while the force is applied. In this second case, the material is not able to recover its original shape (Pierre et al., 2015).

A material with elastic properties is difficult to inject while viscous materials are too liquid (Fundaró et al., 2022). Therefore, a balance between both properties is desirable for the hydrogel, before and after its application, as various types of deformation forces may act on the formulation (Borrell et al., 2011; Pierre et al., 2015).

Viscoelastic properties are expressed by four different parameters, the elastic or storage modulus (G'), which evaluates elastic properties; the viscous or loss modulus (G''), which evaluates viscous properties; the shear modulus (G*), which represents the ratio of shear stress and shear strain, and finally, the tan δ, which represents the ratio between viscous and elastic properties (Choi, 2020). All these parameters will be described in detail in the next sections.

-Elastic/storage modulus (G')

The elastic or storage modulus (G') describes the elastic properties of a material, its behavior when a deforming force is applied and its ability to resist this force. This parameter also helps to predict the filler lift capacity and it is associated with hydrogel hardness. A hydrogel with a high G' value is more rigid, has a greater ability to resist deformation. When its shape is changed, the material returns to its original shape more easily. On the other hand, hydrogels with lower G' values result in less rigid formulations, present lower capacity to resist deformation and greater difficulty to return to the original form (Fig. 10) (Borrell et al., 2011; M. S. Choi, 2020; Fagien et al., 2019; Kablik et al., 2009).

Both G' and E' represent elastic modulus. G' is the elasticy when a shear force is applied while E' is the elasticity when a compression stretching force is applied (Fundaró et al., 2022). Crosslinking agents generate structural limits, allowing to storage more energy after application of a deformation force, which explain the higher G' value in this situation. The weakening of covalent cross-links (e.g., increase in temperature), in turn, has an opposite effect.

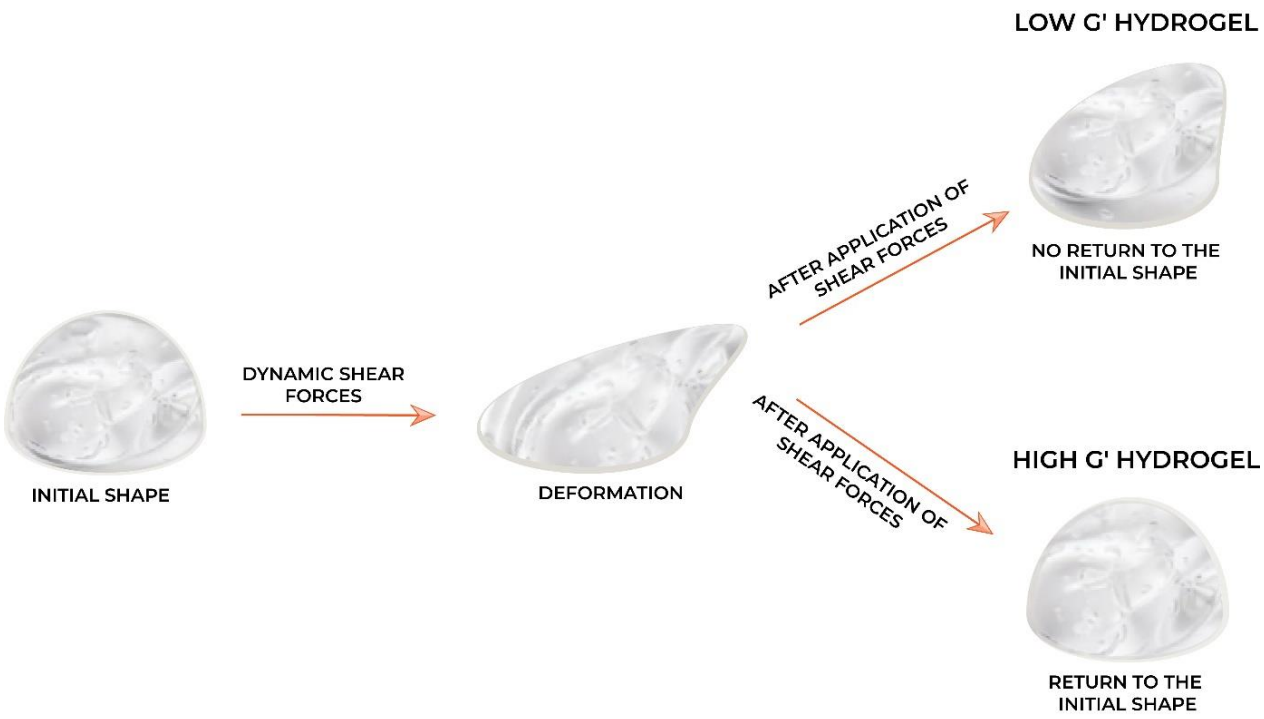

Figure 10. Effect of deformation forces on the shape of high and low G' hydrogels.

As mentioned, the elastic modulus is helpful to predict the lifting capacity of the hydrogel (Sundaram & Cassuto, 2013). Lifting capacity is described as the volumizing capacity of the dermal filler. The higher the lifting capacity the greater the volumizing effect (Borrell et al., 2011). Overall, high lifting capacity or high G' dermal fillers are used for deeper wrinkles, and lower lifting capacity or lower G' dermal fillers are used for more superficial wrinkles or lip augmentation (Kleine-Börger, Meyer, Kalies & Kerscher, 2022; Sundaram & Fagien, 2015).

-*Viscous/loss modulus (G")*

The viscous or loss modulus (G") is a measure of the energy (heat) dissipated or lost per cycle of sinusoidal deformation (Chun et al., 2016). It describes the viscous properties of a hydrogel. Unlike the previous situation, the hdyrogel does not return to its original shape after deformation. High values of G" indicates the energy imparted to the hydrogel by external forces is lost through heat and friction caused by molecular flow. In other words, they indicate a larger amount of viscous molecular motion (Salti & Fundaró, 2020). From a practical point of view, higher G" hydrogels are thicker and then more force is required to flush out them through the needle during application. Once the hydrogel is inserted into the skin, the elastic properties (G')

are predominant (Fagien et al., 2019; Heitmiller, Ring & Saedi, 2021; Jeong et al., 2021; Michaud, 2018). Without molecular entanglements or crosslinking agents, the polymer chains may move more freely, increasing the G" value. As HA hydrogels intended for facial fillers are crosslinked, a reduction of this parameter is found.

-*Shear modulus (G*)*

The shear modulus (G*) represents the total amount of energy required in the form of shear stress to deform the hydrogel. It evaluates the ability of the hydrogel to resist deformation if the deformation is recoverable (more elastic) or non-recoverable (more viscous). This parameter is directly related to the stiffness of a material. The G* is obtained through a mathematical equation that considers the values of G' and G" obtained experimentally with a rheometer (Equation 1), where the G' is the energy stored in the hydrogel during shear deformation, and G" is the energy loss by the hydrogel during shear deformation (Choi, 2020; Sundaram & Cassuto, 2013).

$$G^* = \sqrt{(G')^2 + (G'')^2}$$

Equation 1. Equation used to calculate the shear modulus.

For a hydrogel with only elastic properties, G" is close to 0 and then G' is similar to G*. If the hydrogel has only viscous properties, G' is close to 0 and G" is similar to G*. Therefore, a hydrogel with both elastic and viscous properties will have a G' smaller than a G* (Fig. 11) (Heitmiller et al., 2021). The greater the degree of crosslinking, the greater the value of G' and G*, as the system will have a higher capacity to resist shear deformation (Michaud, 2018).

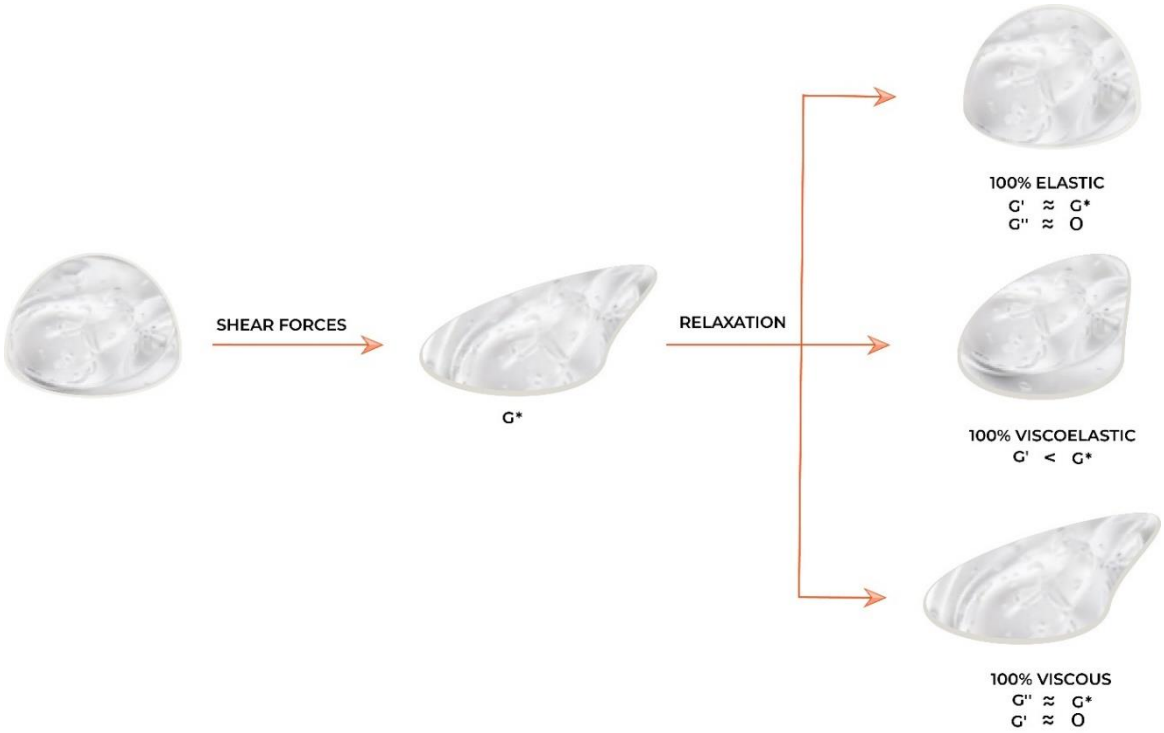

Figure. 11. Effect of shear force on the viscoelastic properties of a hydrogel.

-*Tangential delta (tan δ)*

Tan δ is a parameter used to define if the hydrogel has more elastic or viscous properties. It is calculated by the ratio between G'' and G' (G''/G'). The lower value of δ, the more elastic is the hydrogel as it has a greater elastic modulus (G') than the viscous modulus (G''). If G''>G' (tan δ > 1), the hydrogel behaves more like a viscous liquid. In a opposite situation, where G'> G'' (tan δ < 1), the hydrogel behaves more like an elastic solid material (Choi, 2020; Michaud, 2018; Sundaram & Cassuto, 2013).

HA hydrogels with high tan δ values (softer) are more indicated as dermal fillers of fine lines and superficial wincles. HA hydrogels with lower tan δ values (harder), in turn, are more indicated to volume restoration (Fundaró et al., 2022).

4.2.2. Cohesiveness

The cohesiveness represents the degree of deformation to the hydrogel upon compression (Hurler, Engesland, Kermany & Skalko-Basnet, 2011). This parameter considers the adhesion between the crosslinked HA units and the ability of hydrogel maintain their shape after application of a deforming force (Fig. 12). Cohesiveness is important to preserve hydrogel integrity and tissue distribution (Borrell et al., 2011; Gavard Molliard et al., 2018; Sundaram et al., 2015).

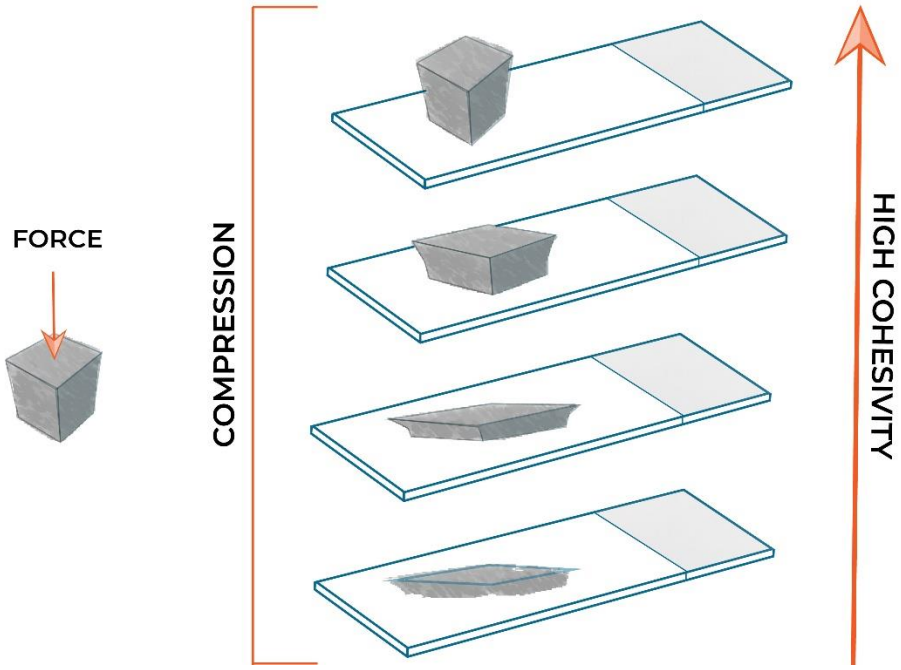

Fig. 12. Effect of compression forces on hydrogel cohesiveness.

Low cohesiveness indicates that polysaccharide molecules that make up the hydrogel have low affinity and attraction among them, dissociating quickly and are more flexible. In an opporsite situation, molecules have high affinity for each other, dissociating more slowly and have a greater ability to be molded (Choi, 2020; Fagien et al., 2019). Hardness (G') and cohesion are inversely related. As the higher the G', the lower the cohesivity and vice versa (Fundaró et al., 2022).

In other words, this parameter is related to the distribution profile or spreadability of the hydrogel after its application. The lower the cohesiveness, the faster the dermal filler is distributed to the local tissues. The higher the cohesivity, more likely that the dermal filler remains in the desired site of application without displacement (Michaud, 2018; Pierre et al., 2015). The first situation is particularly relevant to treat fine lines whereas the second one is desirable during the application of dermal fillers in more deeper areas (Guardia et al., 2022)).

5. Rheological behavior of hydrogels in the facial region

The determination of G' and cohesiveness is a quick and effective way to select dermal fillers for application in specific facial sites. G' represents the physical properties or hardness of hydrogels whereas the cohesiveness is related to the formulation distribution in the application site. Given that different muscle movements are found in each region of the face,

G' and cohesiveness are very useful parameters to guide researchers during the development of new HA hydrogels (Guardia et al., 2022; Lorenc, Öhrlund & Edsman 2017).

Dermal fillers that have low to medium G' (lower lifting capacity and low volumizing effect) and low cohesivity are recommended for the infraorbital area, lips, and to treat fine lines. Low G' dermal filler are less rigid and deforms more easily, adapting to the application region whereas low cohesiveness provides appropriate distribution. As a consequence, no lumps and bumps would be found, mimetizing the natural aspect for the skin (Kapoor et al., 2021; Pierre et al., 2015). These dermal fillers are also characterized by a low swelling degree, which is importat to reduce the edema after injection (Michaud, 2018). Prevelle® and Hylaform®, for example, are not suitable for application in these facial regions as both dermal fillers are crosslinked with DVS, which increase the swelling degree of hydrogels (Kablik et al., 2009).

Medium to high G' dermal fillers with high cohesivity are recommended for application in deeper areas such as the temporal fossa (upper face area), zygomatic and submalar regions (mid face area) as they retain their shape and not spread through the tissue (Guardia et al., 2022; Kapoor et al., 2021). These last characteristics are crucial for formulations applied in chin, jaw, and nose. As these regions are characterized by low shear stress and high compression, hydrogels with lower spreading and appropriate volumizing ability are desirable (Pierre et al., 2015).

High G' dermal fillers and low cohesivity are compatible with applications in the frontal region (forehead) for providing volume, fixation, and spread easily inside the skin. If these events are not observed, an accumulation of formulation may be observed during muscle contraction (Choi, 2020; Michaud, 2018). Nasolabial folds are complex to correct as the perioral zone is characterized by high mobility. In this situation, the dermal filler should have a minimal vertical projection and be easily moldable, which is achieved with a moderate to high G' and low cohesivity (Choi, 2020; Guardia et al., 2022; Pierre et al., 2015).

6. Effect of crosslinking agents on rheological properties

Few rheology studies comparing the performance of HA hydrogels with different crosslinking agents were found in the literature, which can be explained by low availability and high cost of the equipment for this evaluation as well as the requirement of specific technical skills. Advanced studies in this field allow to obtain products with improved clinical performance (and duration) and easier application.

In a study performed by Zerbinati et al. (2021), the rheological behavior of uncrosslinked and crosslinked HA hydrogels was compared at 25 and 37°C. Different HA

concentrations (22, 24, 26 and 28 g mL^{-1}) were tested in hydrogels crosslinked with BDDE whereas a fixed concentration (18 g mL^{-1}) was defined for the uncrosslinked hydrogel. In both temperatures, the elastic modulus (G') increased with higher HA concentrations, which ranged from 56.67 to 120.57 Pa at 25°C and 56.99 to 121.73 Pa at 37°C. The viscous modulus (G"), in turn, demonstrated a similar behavior, varying from 26.03 to 53.19 Pa at 25°C and 21.89 to 52.86 at 37°C. These results confirm that increased concentration of HA tends to make the hydrogel more rigid. This study also evaluated the rheological behavior of the hydrogels at 4, 10, 25, 37, and 45°C to simulate different storage conditions. The elastic modulus (G') was always higher than the viscous modulus (G") in all tempreatures, presenting and slight reduction from 4 to 25°C and similar values between 25 and 45°C. Tan δ values were kept constant at all temperatures. The non-crosslinked hydrogel showed a higher G" than G'. These findings confirm the solid-like behavior of BDDE crosslinked hydrogels and the liquid-like behavior of the uncrosslinked counterpart (Zerbinati, Sommatis, Maccario, Capillo, Grimaldi, Alonci, Rauso, et al., 2021).

In another study performed by the same research team, the rheological properties of HA dermal fillers crosslinked with PEGDE in two temperatures (25 and 37°) and different frequency values (0,1 to 10 Hz) were evaluated. The authors aimed to simulate diffrent storage and injection conditions by changing these parameters. For an increase in applied frequency, the G' values ranged from 149 to 403 Pa at 25°C and from 153 to 378 Pa at 37°C. For the G", the values ranged from 38 to 151 Pa at 25°C and 33 to 135 Pa at 37°C. In all situations, the G' (elastic modulus) was higher than G" (viscous modulus), presenting a tan δ lower than 1, which confirms the solid like behavior of the hydrogel. Under a high shear rate, which was considered to simulate the injection process, the hydrogel viscosity decreased. This finding would result in a rapid extrusion of the gel through the needle. Under a low shear rate, which would simulate a storage conditions, the viscosity increased. This aspect would result in a longer duration of formulation after application in skin. In stability studies performed at extreme temperatures (-20 and 54°C) for 60 days, no significant changes in G' and G" were found, suggesting a high stability (Zerbinati et al., 2022).

Cotofana et al. (2021), in turn, evaluated the effect of changes in angular frequency on different rheological parameters (G', G", G* tan δ) in HA hydrogels crosslinked with BDDE and PEGDE. When angular frequency was increased, G' values varied from 48.5 to 3,116%. The greatest differences in this parameter were found for soft dermal fillers (hydrogels with lower initial G'). If soft dermal fillers are injected into high mobility areas, greater hardness for the hydrogel would be observed, resulting in an undesirable outcome. Although most hydrogels

showed an increase in G" for higher values of applied angular frequency, some formulations had an opposite effect. The decreases in G" indicate that these dermal fillers are less able to resist deformation forces. Based on these results, it is possible to state that the rheological characteristics of hydrogels may change after application in the skin, and they are also related with the region of application in the face (Cotofana et al., 2021).

Borzarichelo et al. (2015) showed that viscoelastic properties vary according to the HA concentration and the HA:crosslinker ratio. In this study, DVS was selected as the crosslinking agent. An increased HA concentration and reduction in the HA:DVS ratio resulted in higher G' values. At 5 g mL^{-1} of HA and the lowest HA:DVS ratio, G' value was 304 Pa (tan δ = 0.058). At the same HA concentration and highest HA:DVS ratio, G' value was 25 Pa (tan δ = 0.12). At 6 g mL^{-1} of HA and lower HA:DVS ratio, G' was 468 Pa (tan δ 0.038). When this HA concentration and the highest HA:DVS ratio were considered, G' was 42 Pa (tan δ = 0.075). In all situations, the G' was higher than the G", confirming the hydrogel solid-like behavior (Borzacchiello et al., 2015). Therefore, harder gels (lower tan δ) could be obtained by increasing HA concentration and reduction in HA:DVS ratio. Similar conclusions were found by Shimojo et al. (2014), who changed the DVS content in HA hydrogels (Shimojo et al., 2014).

The effect of crosslinking agent concentration and reaction time on rheological properties has also been studied. The higher the BDDE concentration in the hydrogel and the longer the reaction time, the higher the G' value. When the reaction time was fixed and the BDDE concentration was increased (0.5 to 10.0 ppm), the G' increased from 1,904 Pa to a value so high that it was not detected by the rheometer. When the BDDE concentration was fixed and the reaction time modified (6 to 24 h), G' increased from 513 to 2,070 Pa (Choi et al., 2014). Moreover, the longer reaction time would increase the degree of crosslinking, contributing to an increased hydrogel hardness (G') (Tezel & Fredrickson, 2008). Overall, these findings demonstrate that not only the addition of higher concentrations of crosslinking agent, but also a longer reaction time can lead to a high G'. The reaction time should be carefully controlled during the production of crosslinked HA hydrogels and free crosslinking agents in solution should be removed at the end to avoid changes in product characteristics during storage.

Two different studies performed by the same research team evaluated the rheological behavior of skinboosters and volumetric dermal fillers crosslinked with BDDE. Skinboosters are used in more superficial injections aiming to improve the skin appearance and texture. The volumetric dermal fillers, in turn, are indicated for deeper injections, improving the lifting effect. According to Garvard-Cohesivity Scale, products both categories varied from “fully disperse” to “fully cohesive”. In G' analyses, volumetric dermal fillers showed values ranging

from 260 to 280 Pa whereas skinboosters ranged from 40 to 120 Pa (compatible with application). As expected, the Restylane family of dermal fillers presented G' values higher than the other commercial products tested, presenting a G' value of 580 Pa for the volumizing filler and 430 Pa for the skinbooster type fillers. The Restylane HA fillers also confirm that lower G' fillers are desirable with more superficial corrections whereas higher G' are recommended for a volumizing effect (La Gatta et al., 2019, 2021).

7. Final considerations

The main challenge during the development of HA-based facial fillers is to find a combination of materials that facilitates the injection process and, at the same time, adapts perfectly to the application region, providing a long-lasting filling effect. Furthermore, each region of the face to be treated requires formulations with specific rheological properties. High G' dermal fillers or high lifting capacity, for instance, are desirable for deeper wrinkles whereas lower G' dermal fillers are recommended for more superficial wrinkles, fine lines or lip augmentation.

The type and concentration of the crosslinking agent; molecular weight and concentration of HA; particle size; pH, crosslinking reaction time as well as crosslinking agent/polysaccharide ratio presented a significant effect on the rheological properties of HA hydrogels. The high degree of crosslinking plays a key role on the volumization or reduction of degradation of HA by hyalunoridases; however, it can make the injection more difficult if a hydrogel with very high hardness is obtained. As already mentioned, the inclusion of uncrosslinked HA with the crosslinked counterpart seems to be crucial in these cases amining to modulate the viscoelastic properties. The optimization of the HA/crosslinking agent ratio in dermal fillers is not only important to achieve distinct viscoelastic properties but also to avoid adverse reactions (e.g., inflammatory effects). BDDE still remains the crosslinking agent most common in commercial HA hydrogels.

Rotational rheology is commonly applied for the characterization of injectable hydrogels, but a clear consensus on ideal conditions of assays is not found. For the same commercial formulation, variable G' and G" values can be found in the literature. Moreover, rotational rheology is not able to mimic the capillary flow and breakup of hydrogels during injection. The tan δ seems to be a simpler and more robust parameter than G' or G" as it is a relative measure (ratio of two main rheological parameters). In fact, this parameter has been found in various recent studies with dermal fillers.

Cohesiveness is another key rheological parameter that is related to the site of application. Regions of the face that suffer frequent deformations require low cohesion of the particles for an effective distribution. On the other hand, reference values for this parameter in each site of application are not yet found in the literature as occur with the elastic modulus. This fact can be explained by the few literature studies evaluating the cohesiveness of injectable HA hydrogels as well as the absence of official methods.

In summary, this review highlights the need for further studies of rheology with facial fillers and standardization of testing condition. The consumers demand products presenting a longer-lasting action (reduction in number of applications) and with few or no adverse effects, motivating the search for new cross-linking agents by the cosmetic industry. In fact, these formulation studies represent an opportunity to explore simpler crosslinking methods and patenting possibilities that add even more value to company.

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Authors' contribution

G.G.G Perera: Conceptualization; Writing - Original Draft.

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