Gel Properties of Hyaluronic Acid Dermal Fillers

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BACKGROUND Most of the hyaluronic acid (HA)-based dermal fillers currently on the market are chemically modified with cross-linkers to improve the mechanical properties and duration in vivo.

OBJECTIVE To investigate differences in the properties of dermal fillers that can be related to the respective cross-linking and manufacturing methods used.

METHODS AND MATERIALS Thirteen commercially available HA fillers were analyzed. Two different measures of gel strength were used: the elastic modulus (G′) determined by rheology and a measure of the swelling capacity of the gel (c_{min}). The degree of modification was determined using nuclear magnetic resonance spectroscopy, and the cross-linking ratio was determined using size exclusion chromatography coupled with mass spectrometry.

RESULTS There was a wide variation in gel strength, and the degree of modification varied between 1% and 8% for the HA fillers investigated.

CONCLUSIONS Both measures of gel strength, G* and c_{min}, can be used because the results from the two methods are well correlated. No differentiation in filler properties could be seen as a result of manufacturing process used, except that the nonanimal stabilized HA stabilization process resulted in products with high gel strength and a low degree of modification.

All of the authors are employees of Q-Med.

Most of the hyaluronic acid (HA)-based dermal fillers currently on the market are chemically modified with cross-linkers to improve the mechanical properties and duration in vivo. An HA gel is formed by introducing cross-links between the HA polymer chains so that a three- dimensional network is obtained. The strength of the gel depends on the cross-linking density; a stronger gel has more cross-links than a weaker gel and hence a more dense network. The formation of a cross-link is usually made by chemically binding two HA polymer chains together using a cross-linker molecule. There can be other types of interactions in the network that contribute to the gel strength. These interactions could consist of weaker chemical bonds, such as hydrogen bonds, or entanglements (mechanical interlocking) that could be referred to as natural cross-links (Figure 1). The number of cross-links and the ratio between the different types of cross-links may vary depending on the manufacturing technology chosen.

When a polymer is cross-linked, the resulting gel will have the shape of the container in which it was formed, unless it is a weak gel. A large monolith gel (one large continuous gel piece) cannot be used as a dermal filler; the gels are therefore fragmented into smaller gel particles, which allows the gel implant to form any shape. The properties of these small gel particles will determine the properties of the gel to a great extent. The geometry of the particles may also influence the behavior of the gel. Particles of irregular shape may be more closely packed than spherical particles and interlock with each other, resulting in a gel that behaves as a continuous gel.

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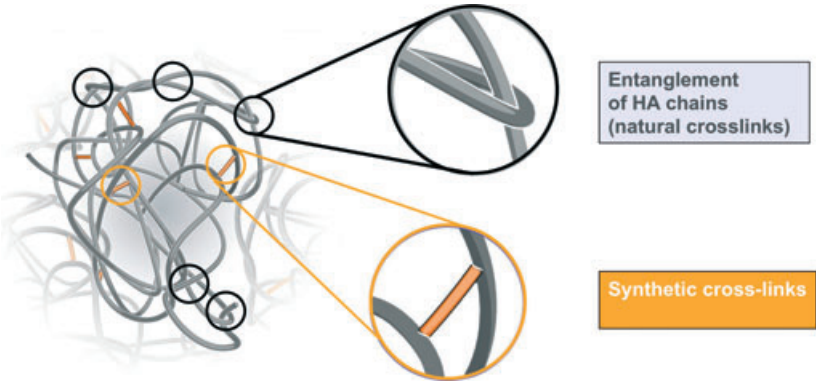

Figure 1. Illustration of synthetic and natural cross-links.

There are several studies on characterization of dermal fillers in which chemical and physicochemical properties have been measured,[1–5] but few studies have tried to find an explanation for the differences in properties. The different manufacturers therefore try to differentiate their dermal fillers by using different names such as "monophasic," "biphasic," "cohesive," and "granular" that, in some cases, have been adopted and frequently used to categorize fillers. Even though there are scientific definitions for some of the terms, there are not many publications in which attempts have been made to correlate the different categories to measurable properties or to describe the scientific rationale for the terms. Another way to differentiate dermal fillers is the different manufacturing technologies. The aim of this study was to investigate whether there are any differences in properties of the dermal fillers that can be related to the cross-linking and manufacturing methods. We limited the study to dermal fillers cross-linked using 1,4-butanediol diglycidyl ether (BDDE), which is the most commonly used cross-linker for HA-based dermal fillers today.

Gel Strength

Gel strength is an important property for dermal fillers. It has been suggested that the lifting capacity of the filler depends on the gel strength,[6,7] which can be determined in different ways. By measuring the rheological properties, the overall resistance to deformation (G*) can be determined and used as a measure of gel strength. An alternative measure of gel strength is the concentration of the gel at maximum swelling. The two measures are described in more detail below.

Rheological Properties as a Measure of the Gel Strength

The mechanical properties of the HA polymer are changed during the cross-linking process. High-molecular-weight HA is a viscoelastic and highly viscous material. When a force acts on a viscoelastic material, it will be deformed in two ways: a viscous, irreversible deformation and an elastic, reversible deformation from which the deformation can recover after the force is removed. The viscoelastic properties of a material can be measured using rheometry. When the material undergoes an oscillatory small-amplitude deformation, the overall resistance to deformation (G*); the elastic modulus, also called the storage modulus (G′); the viscous modulus, also called the loss modulus (G″); and the phase angle (δ) can be determined. The elastic and viscous response of a polymer solution depend on the concentration and molecular weight of the polymer and on the frequency used during the measurements (Figure 2). At low frequencies, which correspond to forces with long duration acting on the material, the viscous behavior dominates. At high frequencies, corresponding to forces of short duration, the elastic behavior dominates. This means that long-acting forces make the solution deform permanently, and short-acting forces give rise to deformations that can recover partially. Visually, a

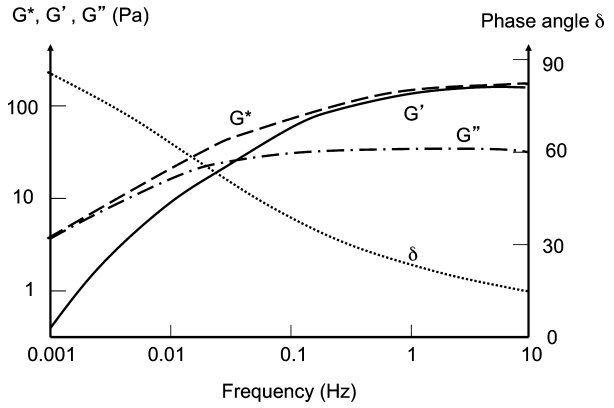

Figure 2. Frequency dependency of the viscoelastic properties of a polymer solution.

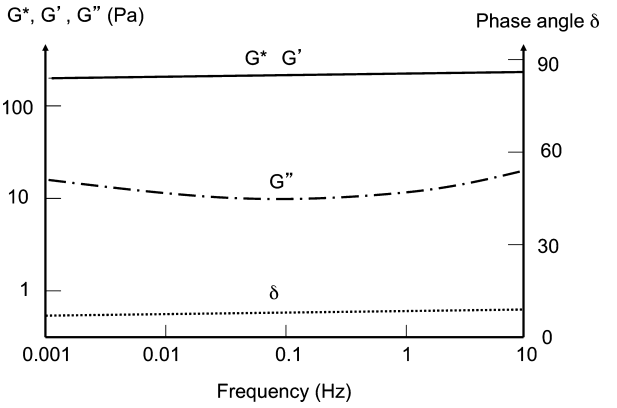

Figure 3. Frequency dependency of the viscoelastic properties of a polymer gel.

concentrated solution of high-molecular-weight HA can be mistaken for a gel because it has such a high resistance to flow. When a syringe with such a solution is emptied, a heap can easily be formed, and for a short time, it seems like it does not deform, but because of its low resistance to deformation because it is a solution, it will be permanently deformed when exposed to long-term forces, such as gravity, and after a while, the heap will become a pool. So even if the preparation looks like a gel, it is and it behaves as a solution.

When cross-links between the polymer chains are introduced, a three-dimensional network of the gel is formed. Just like solutions, gels will have viscous and elastic properties, but the elastic properties dominate over the whole frequency range as a result of the network (Figure 3), which means that the deformation that occurs because of forces of long and short duration will be regained when the force is removed. A strong gel has a high elasticity, meaning that the response to deformation is mainly elastic. Weak gels have a lower elastic modulus, and the ratio of elastic to viscous behavior is usually lower than for a strong gel. Factors that affect the rheological properties of gels are, for example, the polymer concentration and the cross-linking density (including chemical cross-links and entanglements). A higher concentration and a higher cross-linking density render a higher elastic modulus of the gel.

The complex modulus, G*, which for strong gels is almost equal to the elastic modulus G′, is also a measure of how the gel withstands deformation; the higher the complex modulus, the less easily deformed is the gel.

Gel Strength Measured as c_{min}

As a contrast to solutions that can be diluted infinitely, gels can be diluted only to a limited degree before phase separation occurs (Figure 4). The gel

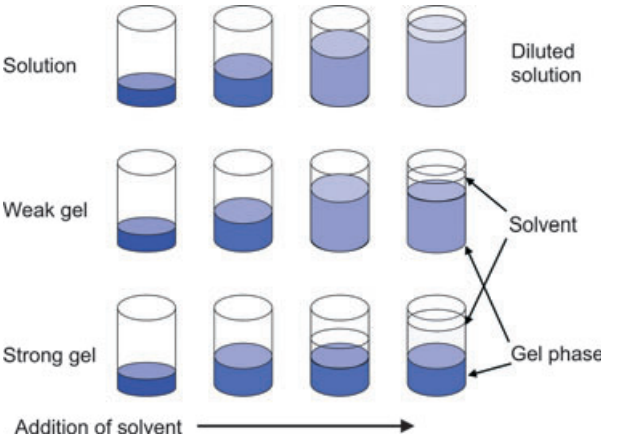

Figure 4. The different behaviors of a solution, a weak gel, and a strong gel when adding solvent. Starting from the left, with an equal amount of preparation with the same hyaluronic acid (HA) concentration, addition of solvent will dilute the solution, whereas gels will swell until they are fully swollen. Addition of more solvent to the gels results in a two-phase system consisting of fully swollen gel and solvent. The HA concentration in the gel phase of the fully swollen gel, c_{min}, is highest for the strong gel.

will absorb added solvent and swell, but it can swell only to a certain extent that is restricted by the polymer network. Addition of more solvent than the gel can absorb will result in a two-phase system. For dermal fillers, this two-phase system consists of a slurry of gel particles in a solution. The maximum swelling of a gel without a two-phase system being formed depends on the cross-linking density of the polymer network. The cross-links (entanglements and chemical cross-links) keep the polymer chains together and limit the possibilities of the chains to move apart. The more cross-links, the tighter the chains are kept together and the more limited the flexibility of the chains is, which reduces the swelling capacity. A stronger gel will thus have less swelling and hence a higher polymer concentration in the gel at maximal swelling.

The HA dermal fillers also contain polymer chains and small gel fragments, formed during the manufacturing process, that are not connected to the gel particles. When an excess of solvent is added, the unbound polymer chains and gel fragments will diffuse out of the gel easily, even though the molecular weight of the polymer is high, because the mesh size of the polymer network in the gel is large. Polymer chains also have the possibility to move end-on (like a snake) through the polymer network of the gel, which allows even large polymer chains to move readily through a gel.

Because the amount of solvent that the gel can absorb depends on the cross-linking density, the HA concentration of the fully swollen gel without any unbound HA molecules (c_{min}) can be used as a measure of gel strength. A weak gel with low cross-linking density will have a low c_{min} because of a high uptake of solvent, whereas a strong gel that absorbs less solvent will have a higher c_{min} (Figure 4).

Degree of Modification, Cross-Linking Ratio, and Degree of Cross-Linking

During the cross-linking process, cross-linker molecules, such as BDDE, become bound to the HA. The BDDE binds mainly through strong covalent bonds in an irreversible reaction; that is, once the cross-linker is bound, it cannot detach. The cross-linker molecules can bind at both ends, creating a cross-link, or at only one end, modifying the HA without creating a cross-link (Figure 5). Adding water to the epoxide during the cross-linking process inactivates the BDDE molecules at the nonbinding end. The formed diol is stable and does not react further with the HA to form more cross-links and will therefore not contribute to gel strength.

The degree of modification (MoD) is defined as the ratio of moles (number of molecules) of linked cross-linker to moles of HA disaccharides (Figure 5). In the example in Figure 5, MoD is 5/36 = 0.14 or 14%. All cross-linker molecules linked to HA, whether they are creating a cross-link or not, are included in the calculation of MoD.

The cross-linking ratio (CrR) is the ratio of cross-linked BDDE to the total number of BDDE molecules bound to HA. The example in Figure 5, illustrates a CrR of 3/5 = 0.6.

The degree of cross-linking (CrD) is the ratio of BDDE molecules that form cross-links to the number of HA disaccharides. In Figure 5, CrD is 3/36 = 0.083 or 8.3%. CrD can be calculated from CrR and MoD: CrD = CrR × MoD.

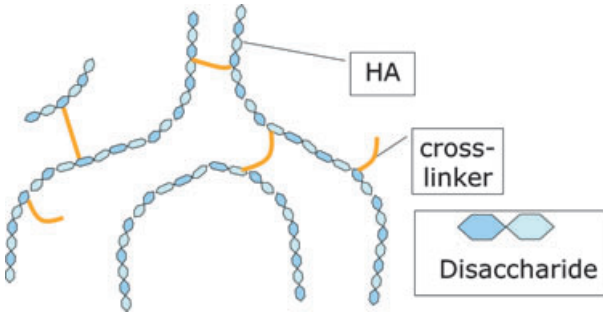

Figure 5. Illustration of cross-linkers bound to HA chains. The cross-linker can bind to both ends, creating a cross-link, or bind to only one end, modifying the HA without contributing to gel strength.

Modification Efficiency

The efficiency in the cross-linking process in terms of gel strength per introduced modification will depend on the manufacturing process chosen. Modification efficiency (MoE) can be calculated to give a measure of the efficiency of the cross-linking process. MoE is calculated from the gel strength (c_{min}) and the MoD.

$$\text{MoE} = \frac{c_{\text{min}}}{\text{MoD}}$$

MoE is a measure of the gel strength achieved for each cross-linker molecule introduced; a higher MoE value indicates a more efficient process.

Materials and Methods

Materials

The HA dermal fillers investigated are given in Table 1. All analyses were performed before the expiry dates of the products. Saline (0.9% sodium chloride (NaCl)) was from Fresenius Kabi AB (Sweden), and Chondroitinase AC was from Sigma Aldrich (St Louis, MO).

*Determination of G**

The viscoelastic properties were measured on an Anton Paar MCR 301 (Anton Paar, Graz, Austria) rheometer equipped with a parallel plate measuring system using a gap of 1 mm. The complex modulus, G*, was measured in a frequency sweep within the linear viscoelastic range determined by a strain sweep. Evaluation of G* was done at 0.1 Hz. Typical precision of the method is approximately 5% relative standard deviation (RSD).

Determination of c_{min}

The concentration of HA in the fully swollen gel (c_{min}) is determined by combining three measurements: HA concentration, swelling factor, and gel content.

The HA content was determined using the carbazole method.[8] Typical precision of the method is 2% RSD.

The swelling factor was determined by adding a known amount of the preparation to a measuring cylinder, adding an excess of saline, dispersing the gel thoroughly, and reading the volume of the swollen gel after sedimentation of the gel particles. The swelling factor was calculated as V/V_0, where V_0 is the initial volume of the gel, and V is the volume of the fully swollen gel in 0.9% NaCl. Typical precision of the method is 2% RSD.

The gel content was determined by adding an excess of saline to a known amount of the preparation and dispersing the gel thoroughly to form a dilute

TABLE 1. Dermal Fillers Used in the Study

Product	*Batch*	*Manufacturer*
Teosyal deep lines	TS27-083502B	Teoxane
Teosyal ultra deep	TSU-082903B	Teoxane
Esthelis basic	311103/3	Anteis
Fortelis extra	510063/2	Anteis
Juvéderm Ultra 2*	X24L506559	Allergan
Juvéderm Ultra 3*	X30L509376, X30L504649	Allergan
Juvederm Ultra 4*	S30L482804, S30L478286, S30L478762	Allergan
Stylage M	EMB090061	Laboratoires Vivacy
Princess Filler	903015/2, 903006/2, 903008/1	Croma
Renofill perfectly volume	410065/2	Laboratoires Renophase
Restylane SubQ TS	9968	Q-Med
Restylane Perlane Lido*	10373	Q-Med
Restylane	10368	Q-Med

*Contains lidocaine 3 mg/mL.

suspension. The diluted suspension of the gel was filtered through a 0.22 μm filter, and the concentration of HA in the filtrate, the extractable part, was determined using the carbazole method. The gel content was calculated as the fraction of HA in the filler that could not pass through the 0.22 μm filter when filtering the diluted suspension of the product. Typical precision of the method is 2% RSD.

c_{min} was calculated as the concentration of HA in the product multiplied by the gel content and divided by the swelling factor.

Determination of MoD

The HA gel preparations were washed using 0.9% NaCl and thereafter completely enzyme digested using chondroitinase AC in deuterated water. Four hundred-MHz ^{1}H nuclear magnetic resonance spectroscopy (NMR) spectra were recorded from the resulting digests. MoD was determined by integrating the signal from the N-acetyl group in HA and a specific signal from the cross-linker. The ratio between the integrals for these two signals (cross-linker/HA N-acetyl) gives MoD after correction for the number of protons responsible for each signal. Details of the method can be found elsewhere.[9] Typical precision of the method is approximately 3% RSD.

Determination of CrR and CrD

HA gel preparations were washed using 0.9% NaCl and completely enzyme digested with chondroitinase AC, yielding a large fraction of plain HA fragments and a minor fraction of HA fragments with cross-linker attached (HA oligosaccharides linked to BDDE residues). In the latter fraction, some of the fragments were cross-linked, and some were mono-linked with the BDDE residues. The different fragments of HA linked to BDDE residues were detected using size exclusion chromatography electrospray ionization mass spectrometry (MS) in the mixtures obtained. The MS was set to detect each fragment in the selected ion monitoring mode. CrR was determined by dividing the sum of the peak areas from cross-linked fragments with the sum of the peak areas from all detected mono- and cross-linked fragments. CrD was then calculated from CrR and MoD. Details of the method can be found elsewhere.[10] Typical precision of the method is approximately 5% RSD.

Results and Discussion

Gel Strength

The dermal fillers chosen in this study are all HA fillers that are cross-linked with BDDE using different manufacturing techniques (Table 2). The products chosen are used for slightly different indications, such as correcting lines and wrinkles, cheek augmentation, and volumizing.

The rheological properties of the different fillers are shown in Figure 6. Because the rheological properties depend highly on concentration, the products have been arranged in order of increasing HA concentration. The declared HA concentration of the products varies from 20 to 25.5 mg/mL (Table 2). Several articles have compared the rheological properties of products manufactured using the Hylacross technology with the stabilized non-animal HA (NASHA) products.[1–5,7] Even though the reported levels differ to some extent, the relationship between the two filler families are the same as in this study; the NASHA family has a higher resistance to deformation (G* or G′) than the Hylacross family.

Not only the cross-linking density, but also the HA concentration of the product and the presence of free or modified HA fragments not connected to the network will affect the gel strength measured by G*. Another measure of the gel strength is c_{min}, the concentration of HA in the fully swollen gel, because a stronger gel having more cross-links (natural and chemical) will swell less than a weaker gel with fewer cross-links and thereby have a higher c_{min}. Unlike G*, any unbound HA or the HA concentration of the product will not affect c_{min} because it depends only on the cross-linking density,

TABLE 2. Product Information

Product	*Hyaluronic Acid Concentration, mg/mL**	*Cross-Linking and Manufacturing Technology*	*Denotation in Text and Figures*
Esthelis basic	22.5	Cohesive and polydensified matrix, CPM technology†	CPM
Fortelis extra	25.5	‡	Fort
Teosyal ultra deep	25	‡	Teo1
Teosyal deep lines	25	‡	Teo2
Stylage M	20	IPN-like technology Interpenetration of cross-linked networks§	IPN
Renofill perfectly volume	24	‡	Reno
Princess filler	23	S.m.a.r.t technology¶	Smart
Juvederm Ultra 2	24**	Hylacross technology††	Hylacross1
Juvederm Ultra 3	24**	Hylacross technology††	Hylacross2
Juvederm Ultra 4	24**	Hylacross technology††	Hylacross3
Restylane	20	Stabilization and NASHA technology‡‡	NASHA1
Restylane Perlane Lido	20**	Stabilization and NASHA technology‡‡	NASHA2
Restylane SubQ	20	Stabilization and NASHA technology‡‡	NASHA3

Product information provided by manufacturers.
*From package insert.
†www.esthelis.com/en/pro/esthelisrange.php Accessed September 28, 2011.
‡No information on cross-linking or manufacturing technology found.
§http://www.vivacy.fr/products.php?id_cat=121 Accessed September 28, 2011.
¶http://www.princessbycroma.com/information_for_doctors/purification_process.php Accessed September 29, 2011.
**Contains 3 mg/mL lidocaine.
††www.allergan.com/products/medical_aesthetics/juvederm.htm Accessed September 28, 2011.
‡‡http://www.restylane.com/en/About-Restylane/The-technology-behind-Restylane/ Accessed September 28, 2011.

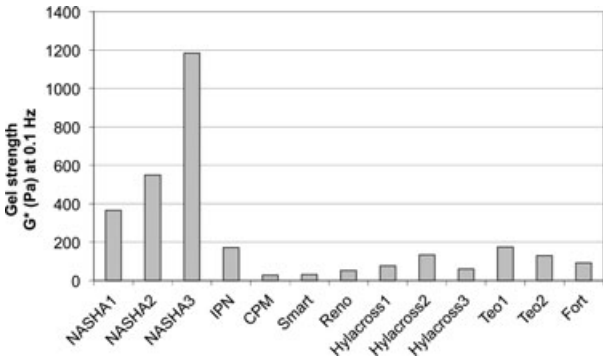

Figure 6. Gel strength measured as G*, the total resistance to deformation, for the investigated hyaluronic acid (HA) filler products. The products are ordered according to the declared HA concentration in the products.

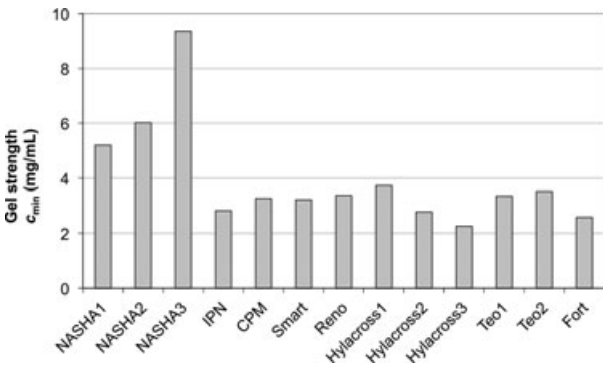

Figure 7. Gel strength measured as c_{min} for the different hyaluronic acid dermal filler products.

which includes natural entanglements and chemical cross-links. The gel strength as measured by c_{min} is shown in Figure 7 for the investigated products. As described in the methods section, c_{min} is calculated from the swelling factor, the gel content (nonextractable HA fraction), and the HA concentration of the product. In this study, the resulting c_{min} value was found to vary mainly because of the differences in the swelling factor of the products. When adding saline, the products swell to 2 to 9 times the original volume. The other factors that influence c_{min} varied

only a small amount between the products; the gel content varied between 74% and 97%, and the measured HA concentration varied between 19.7 and 25.7 mg/mL and was within ±5% of the declared concentrations.

The two different measures of gel strength, c_{min} and G*, show a similar pattern even though a ranking between the products would be somewhat different for the two measures of gel strength. Differences between the two measures of the gel strength is not unexpected because several parameters affect G* (the cross-linking density, the HA concentration of the product, and the presence of unbound HA), whereas c_{min} depends only on the cross-linking density. Even though the two measures of gel strength differ to some extent, it can be seen from Figure 8 that a correlation between c_{min} and G* exists.

It has been suggested that the lifting capacity of a dermal filler depends on the gel strength.[1,6,7] Lifting capacity can be defined as the desired effect of the gel implant in the body (the capacity to lift tissue and resist deformation after the injection). Gel strength is a quantifiable property of the gel, describing its ability to resist deformation. To achieve correction of lines and wrinkles and restore volume, the gel implant must lift the tissue. A strong gel can provide the force required to lift the tissue and resist subsequent deformation, resulting in the desired correction. A high lifting capacity therefore requires high gel strength. A liquid, or a weak gel, will not resist deformation and will therefore displace in the direction of least resistance, and the desired correction will be achieved to less extent. Therefore, the results shown in Figures 6 and 7 for gel strength also show the lifting capacity of the different fillers according to the definition above.

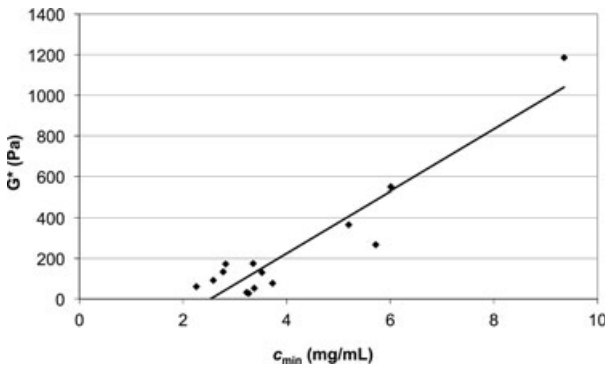

Figure 8. Relationship between the two measures of gel strength: overall resistance to deformation, G*, and hyaluronic acid concentration in the fully swollen gel, c_{min}.

It has also been suggested that the cohesivity of the formulation affects the lifting capacity[7] (or lifting capability, the term used in the reference). The term "cohesive" is often encountered in the literature regarding fillers, although it is seldom defined, and cohesivity is a property much more difficult to measure than gel strength. Lifting capacity is a property of the filler after injection into the body. Because it has been suggested that the presence of free HA decreases cohesivity,[7] it is not relevant for the in vivo situation to measure cohesion of the product before injection because the free HA will leave the implant rapidly, transforming gels with free HA into more cohesive gels. If there is a large difference in cohesion as a result of the presence of unbound HA, products containing more extractable HA may have an advantage during implantation because cohesion will increase after injection. A product containing a larger amount of extractable HA is less cohesive and may be easier to form to the desired shape directly after implantation than a more cohesive product (with less free or extractable HA), and then the product becomes more cohesive after it has been formed into the desired shape as a result of the extractable HA leaving the implant.

Modification Efficiency

The major advantage of HA dermal fillers is the raw material, HA, which is a natural component of human tissues. Even if the HA is produced using bacteria, as is the case for most fillers, the HA molecule is identical and independent of the species and will not be recognized as a foreign material when implanted in the body. In the cross-linking process, it is important not to modify the HA

molecule to such a large extent that it will not be recognized as HA, which may lead to foreign body reactions. The extent of modification of the HA molecules will vary depending on the chosen cross-linking process. The MoD is shown in Figure 9 for the investigated products and was found to vary from 1% to 8%. Some values of the modification can be found in the literature,[2,7] but their values deviate from those found in this study to some extent, especially for the NASHA products. Kablik and colleagues[2] used a method based on enzymatic degradation of the HA fillers followed by high-performance liquid chromatography and ultraviolet (UV) detection of the resulting modified and unmodified fragments. This method has several large uncertainties, for example, chromatographic overlap in combination with low specificity in UV detection. The method used here, based on NMR spectroscopy, is a precise method[9] yielding reliable results because NMR is inherently an accurate technique that is ideal for this type of determination. As mentioned above, one of the main advantages of HA is that it exists in human tissue and therefore will not be recognized as a foreign material. Another advantage of HA fillers is that, if overcorrection is performed, or an adverse event occurs, even though they are rare, the implant can be eliminated from the site of implantation by injecting hyaluronidase.[11] There have been several studies on the degradation of HA fillers with hyaluronidase.[12–14] The degradation rate of the HA fillers varies, and in some of these cases, it seems to be impossible to degrade the filler completely with the enzyme. One explanation for this may be that the MoD is so high that the enzyme does not recognize the HA. This may be an indication that the filler could be perceived as a foreign material. If there is a foreign body reaction to a material, there will be a greater inflammatory reaction that, apart from pain, swelling, and bruising, also results in a greater amount of free radicals,[15] which increases the rate of degradation and decreases the longevity of the material.

The efficiency in the cross-linking process (i.e., the gel strength achieved by each introduced cross-linker molecule) can be calculated and referred to as MoE. Higher MoE means that a stronger gel is achieved with less modification of the HA. When calculating MoE, c_{min} is used as a measure of gel strength. Because both measures of gel strength have a good correlation (Figure 8), any of them could have been used, but c_{min} has the advantage of depending only on the cross-linking density of the gel. The MoE of the different products is shown in Figure 10. All HA fillers have a MoE that is well below 1 except for the products produced using the NASHA stabilization process, for which MoE is >5. The difference in MoE may depend on the efficiency of the cross-linking process as measured by CrR (the ratio of cross-linked BDDE to the total amount of linked BDDE), although the difference in CrR for the different products can only partly explain the difference in

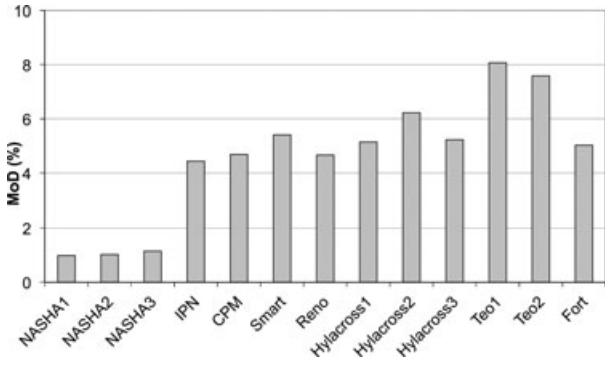

Figure 9. Degree of modification, MoD, for the different hyaluronic acid filler products.

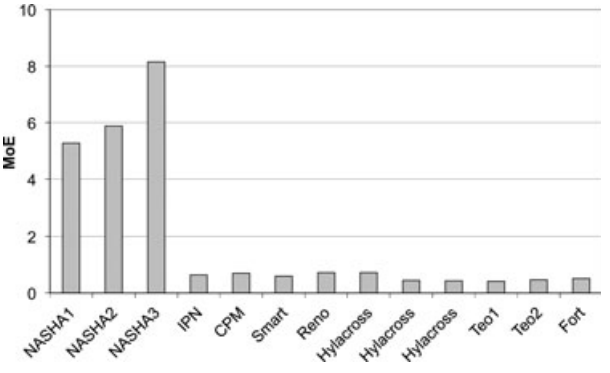

Figure 10. Modification efficiency (MoE) for the different hyaluronic acid fillers.

MoE because CrR only varies between 8% and 17% for the investigated products.

To compensate for the difference in CrR, MoE could be divided by CrR, constructing what may be referred to as the cross-linking efficiency (CrE = MoE/CrR = c_{min}/CrD). Because MoE spans more than a factor of 5 whereas CrR differs only by a factor of 2, CrE will span a factor larger than 2 among the different products. The calculation illustrates that the NASHA process results in a higher gel strength for each linked cross-linker than other HA fillers even when taking CrR into consideration. The factors that can explain the differences are a function of the manufacturing process and the raw materials used. The molecular weight of the HA would affect the MoE; a higher molecular weight would require less cross-linking to achieve the same gel strength. Another explanation for the higher MoE found for the NASHA products would be that more of the natural cross-links, the entanglements, are preserved in the NASHA stabilization process, contributing to the gel strength without changing the MoD of the gel.

Conclusions

Two different measures of gel strength (G* and c_{min}) can be used because the results from the two methods are well correlated. MoD varied between 1% and 8% for the investigated HA dermal fillers. No effects on the filler properties could be seen as a result of different manufacturing processes except for the NASHA stabilization process.

The products manufactured using the NASHA process had the highest gel strength and the lowest MoD of the HA molecules, which resulted in the highest MoE of the investigated products, indicating that the NASHA process is different from the processes used for the manufacturing of the other products.

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