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Effect of Different Crosslinking Technologies on Hyaluronic Acid Behavior: A Visual and Microscopic Study of Seven Hyaluronic Acid Gels

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ABSTRACT

Background: The mechanical, rheological, and pharmacological properties of hyaluronic acid (HA) gels differ by their proprietary crosslinking technologies.

Objective: To examine the different properties of a range of HA gels using simple and easily reproducible laboratory tests to better understand their suitability for particular indications.

Methods and materials: Hyaluronic acid gels produced by one of 7 different crosslinking technologies were subjected to tests for cohesivity, resistance to stretch, and microscopic examination. These 7 gels were: non-animal stabilized HA (NASHA® [Restylane®]), 3D Matrix (Surgiderm® 24 XP), cohesive polydensified matrix (CPM® [Belotero® Balance]), interpenetrating network-like (IPN-like [Stylage® M]), Vycross® (Juvéderm Volbella®), optimal balance technology (OBT® [Emervel Classic]), and resilient HA (RHA® [Teosyal Global Action]).

Results: Cohesivity varied for the 7 gels, with NASHA being the least cohesive and CPM the most cohesive. The remaining gels could be described as partially cohesive. The resistance to stretch test confirmed the cohesivity findings, with CPM having the greatest resistance. Light microscopy of the 7 gels revealed HA particles of varying size and distribution. CPM was the only gel to have no particles visible at a microscopic level.

Conclusion: Hyaluronic acid gels are produced with a range of different crosslinking technologies. Simple laboratory tests show how these can influence a gel's behavior, and can help physicians select the optimal product for a specific treatment indication.

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INTRODUCTION

Since their introduction in Europe in 1996, crosslinked hyaluronic acid (HA) gels have progressively replaced bovine collagen as the preferred treatment for filling lines and folds,[1] and account for the vast majority of non-invasive aesthetic procedures used in daily practice.

In its native form, the chemical structure of HA is identical across different species. This feature, along with its unique viscoelastic and physicochemical properties, has led to the development of numerous HA-based medical devices. However, due to the short half-life of endogenous HA, chemical modifications are required to obtain long-lasting gels.[2] This is achieved by a crosslinking process, which changes the 3-dimensional structure of the HA chains and results in the formation of either HA microspheres "pearls" or a jelly. While the risk of immunogenicity to HA-derived products is generally low, the altered structure of the 3-dimensional HA gels may result in them being recognized as foreign by the dermis.[3,4]

The raw material in the production of HA gels for aesthetic use consists of pharmacological grade HA chains or HA powder of the same purity, but with different molecular weights, which may vary from 600 kDa to more than 2,500 kDa. The final products differ in terms of their HA concentration and method of crosslinking. Crosslinking methods may be either chemical or physical, but in the field of aesthetic medicine the crosslinking agent that is used to stabilize the majority of HA-based dermal fillers currently on the market is 1,4-butanediol diglycidyl ether (BDDE). The stability, biodegradability, and toxicity profile of BDDE put it ahead of other crosslinking agents such as divinyl sulfone.[5] It should be noted that "natural" crosslinks in the form of Van der Waals forces are also found in all HA preparations developed for aesthetic use.

The basic crosslinking process takes place in 2 steps and is the same for many currently used HA products that use BDDE as the crosslinking agent: (1) dissolution in an alkaline medium and

linearization of the HA, and (2) addition of crosslinking agent under temperature control. However, crosslinking techniques differ from one manufacturer to another, and gels vary in the final amount of crosslinked HA they contain. These differences modify the behavior of the gels so that injection techniques and depths have to be adapted for the HA gel used. The terms used to describe the properties of the different gels are defined in Table 1.

At least 7 different types of crosslinking technology are used in the production of current HA gels. All of these gels are available with lidocaine, which is introduced during the crosslinking process by the manufacturers.

1. Non-Animal Stabilized Hyaluronic Acid (NASHA®)

In this technique developed by Bengt Agerup MD, the addition of a small amount of BDDE introduces minute amounts of crosslinks between the polysaccharide chains, resulting in the formation of an entangled matrix.[6] The degree of crosslinking in the original matrix is estimated to be around 10% to 15% and between 1% to 2% in the final product.[7] It is hypothesized that the slightly viscous matrix thus obtained is dried and then sieved or passed through cleaver filters of different diameters to produce gel particle sizes adapted to the clinical indications of the final product. This process creates solid HA "pearls," which are then suspended in a non-crosslinked vector such as NaCl 0.9% in phosphate buffer (phosphate buffered saline) or a non-crosslinked HA gel. The number and size of the pearls varies depending on the gel indication. The current study used Restylane® (Q-Med, Uppsala, Sweden), a gel with an average pearl diameter of 250 µm (100,000 pearls/mL).[8]

2. 3D Matrix

3D Matrix represents an advancement of Hylacross® technology, but unlike Hylacross is not yet US Food and Drug Administration (FDA) approved (personal communication, Dr. P. Lebreton, Allergan). Surgiderm® products (Allergan-Corneal Industry, Pringy, France) are formulated with 3D Matrix and contain a high ratio of high molecular weight HA to lower molecular weight molecules. In a single-step crosslinking process, the high and low molecular weight molecules are mixed. A greater number of BDDE molecules are attached by both ends or extremities, resulting in more efficient crosslinking.

3. Vycross®

This uses the same crosslinking technique as 3D Matrix, but the proportion of high to low molecular weight HA is reversed, with Vycross® containing a higher proportion of low molecular weight HA. It therefore contains less HA (lower HA concentration) compared with 3D Matrix. Juvéderm Voluma® is so far the only product using this technology to have received FDA approval.

TABLE 1.

Definitions Used to Describe the Properties of Gels Produced With Different Crosslinking Technologies	
(Hydro)gel	Water-soluble polymer crosslinked via chemical or physical bonds.
Monophasic	A monophasic gel consists of a single phase and is usually used to describe a gel looking non-particulate (cohesive).
Biphasic	A biphasic gel traditionally describes a particulate gel, which consists of a phase of semi-solid crosslinked hyaluronic acid particles suspended in a liquid phase.
Cohesivity/ cohesion	Cohesion represents the internal forces that unite a solid or liquid particles. A gel is said to be **cohesive** if it conserves its unity, its cohesivity or cohesion, when placed into an aqueous solution (characteristic of monophasic gels) at a low dilution, for instance 1:3, without agitation. In contrast, a gel is said to be **non-cohesive** if it is unable to conserve its unity, its cohesion, once placed into an aqueous solution (characteristic of biphasic gels).
Monodensified	A gel is described as monodensified if it consists of a single homogeneous crosslinking grade/density zone inside the gel itself.
Polydensified	A gel is described as polydensified if it consists of several crosslinking grades/density zones inside the gel itself.

4. Optimal Balance Technology (OBT®)

This technology is used to produce the Emervel® range of HA gels (Q-Med, Uppsala, Sweden). These have the same HA concentration (20 mg/mL) but, unlike the Restylane products that differ only in their particle sizes, Emervel products differ in their degrees of crosslinking as well as gel calibration, depending on their indication. Thicker or thinner fillers are obtained by varying gel calibration, and firmer or softer fillers by varying crosslinking.

5. Cohesive Polydensified Matrix (CPM®)

Cohesive polydensified matrix (CPM®) technology is used for the Belotero® range of products (Anteis S.A., Geneva, Switzerland, a wholly owned subsidiary of Merz Pharmaceuticals GmbH) and is based on a dynamic double crosslinking. In addition to the classic crosslinking process, 2 additional steps are added: the addition of a new amount of HA followed by a continuation of the crosslinking process. This produces a monophasic polydensified gel that combines high levels of crosslinked HA with lighter levels of crosslinked HA in a cohesive matrix.[9]

6. Resilient Hyaluronic Acid (RHA®)

This is the crosslinking technology used in the Teosyal® (Teoxane Laboratories, Geneva, Switzerland) range of gels. The

technology produces gels with long HA chains stabilized by natural and chemical crosslinks. Only a small amount of BDDE is used to create the gels, which differ in their degree of crosslinking (1.9%-4.0%) as well as their HA concentration.

7. Interpenetrating Network-Like (IPN-Like®)

The Stylage® range of gels (Laboratoires VIVACY®, Archamps, France) use several individual crosslinked matrices, which undergo an interpenetrating network-like (IPN-like®) process to achieve a monophasic gel, resulting in an increased density of crosslinking. The product also contains mannitol, which claims to protect the gel to a certain extent from the effects of free radicals.

In this paper we report on simple and easily reproducible tests that can be conducted in the laboratory to allow us to better understand the properties of HA gels produced by the 7 different types of crosslinking technology.

"Hyaluronic acid gels produced by one of 7 different crosslinking technologies were subjected to tests for cohesivity, resistance to stretch, and microscopic examination."

METHODS

Tested Gels

Between 2006 and 2014, we tested HA gels available on the Swiss market manufactured by one of the 7 different crosslinking technologies: NASHA (Restylane), 3D Matrix (Surgiderm 24 XP), CPM (Belotero Balance), IPN-like (Stylage M), Vycross (Juvéderm Volbella), OBT (Emervel Classic), and RHA (Teosyal Global Action). All of the gels were available with lidocaine, introduced during the crosslinking process by the manufacturers. The tests were conducted on the gels as they became available, with the last tests conducted in 2014 on Vycross, OBT, and RHA. All gels were marketed for aesthetic indications (filling lines or creating volume). The tests were conducted in private practice as well as in private and university laboratories

Microscopic Examination

For microscopic examination, 0.1 mL of each gel was placed on a glass slide and spread as for a hematological examination. The gel's resistance to spreading was noted as a simple estimate of their viscosity. The gels were then colored with toluidine blue at 1 of 2 concentrations (depending on the laboratory where the tests were realized): 0.1% and 0.069% for 30 seconds to 60 seconds before being rinsed twice with double distilled water. Adhesion to the slide during rinsing was examined. The slide was then covered and placed under the microscope for examination of the gel's structure.

Cohesivity Test

When conducted in private practice, 0.6 mL of saline solution (NaCl 0.9%) was combined with 2 drops of a coloring agent (Ecoline® no.548 Talens® blue violine from the Royal Talens Society). To this was added 0.2 mL of the HA gel to be tested by simple pressure on the syringe plunger to avoid any change in the viscoelastic properties of each gel. No other distortion or stress was applied. Finally, 2 drops of ethanol 70% were added and the recipient gently rotated. Photos were taken before and after the addition of the ethanol. Products were measured precisely using Omnican® syringes (Braun, Switzerland). The same test was conducted in a private laboratory by coloring 40 mL of saline serum with Ecoline 548. The investigators then placed 0.9 mL of this colored saline solution in a Petri dish and added 0.3 mL HA gel. Tests were performed a minimum of 3 times for each gel. The different gels were observed visually and under a microscope between slides to see if they remained as long, cohesive strands or disintegrated into multiple stands or smaller particles.

Resistance to Stretch Test

We placed 0.2 mL of each gel on a Petri dish. The gels were then pinched with an Adson's plier to draw them out. A photo was taken of the gel at maximum stretch and the length noted using a measuring tape. The test was performed a minimum of 3 times for each gel.

Equipment

Each laboratory had its own camera, and photographic images were taken with the following cameras: Nikon(R) digital camera D 40 X, lens AF Micro Nikkor 60 mm; Sony® Cyber-shot; Nikon DXM1200F; and Olympus SC100. Microscopic examinations were performed with a Leica® MS5 and a Zeiss Axiokop 40.

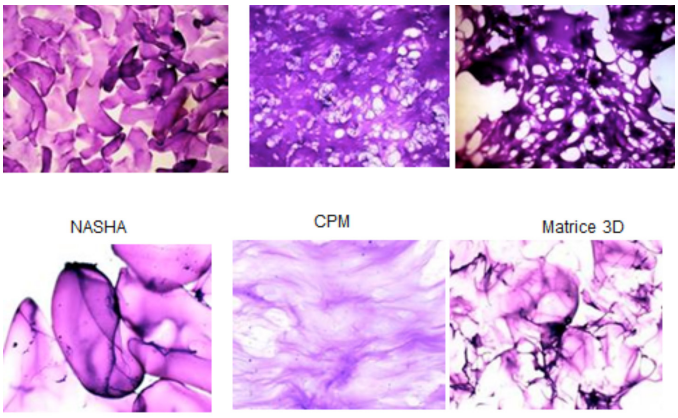
RESULTS

Microscopic Examination

For the 4 HA gels available for testing in 2011-- NASHA, CPM, 3D Matrix, IPN-like -- a difference in viscosity was noted when preparing the slides for examination, particularly when spreading the gels, with NASHA being remarkable for having the least viscosity. In addition, when rinsing with double distilled water, a large amount of the NASHA gel was washed away. This was not observed with the other gels. The most viscous gel was the IPN-like and the most adherent was CPM. 3D Matrix had an adherence between NASHA and IPN-like. Gels produced with the most recent crosslinking technologies (Vycross, OBT, and RHA) were tested in 2014. Of these, RHA had the greatest viscosity and resistance to spreading, but was poorly adherent to the glass slide. Vycross and OBT were similar in having an important viscosity and resistance to spreading, but less so than RHA. During rinsing, the adherence of Vycross and OBT was also similar and greater than that of OBT.

Observation of the gels, with or without added lidocaine, under a light microscope revealed some significant differences in structure (Figures 1 and 2).

FIGURE 1. Appearance of hyaluronic acid gels (NASHA®, CPM® and 3D-Matrix) under the light microscope. The top row images were taken at HCU, Geneva (toluidine blue, original magnification x12.5). The bottom row images were taken at the Laboratory of Histopathology, Viollier, Geneva (toluidine blue, original magnification x25).



NASHA

Hyaluronic acid particles were clearly emphasized and balloon-shaped rather than a round pearl. The structure of the gel was clearly non-cohesive and biphasic.

CPM

The gel had a very specific structure appearing as a continuous network complex, with some areas of the gel having greater staining and appearing more dense than others.

3D Matrix and IPN-Like

These gels had similar structures that were totally different from NASHA or CPM. Compared with CPM, they appeared lighter, less dense, and with less continuous networks.

RHA

The gel appeared as large grains of compressed particles with a nice spreading. The gel resembled Vycross, but with larger particles. There was no real complex continuous network and the gel could be described as non-cohesive or partially cohesive.

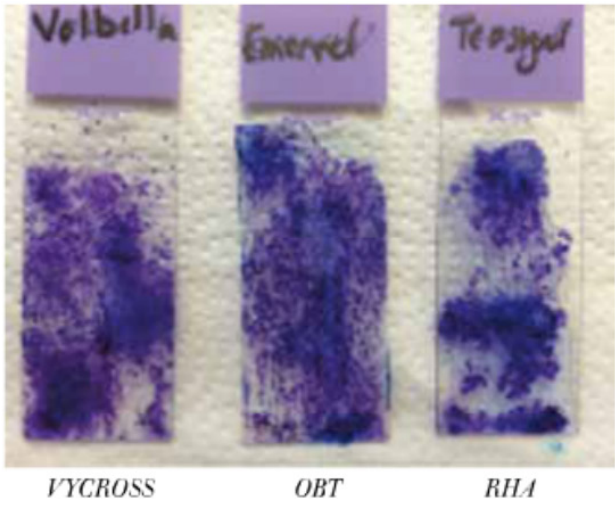
Vycross

When spread on the microscope slide, the gel appeared as fine grains, finer than RHA and OBT. With magnification, the gel appeared as many particles compressed closely together and could be described as particulated, similar to NASHA. Vycross could be described as a non-cohesive or partially cohesive gel.

OBT

On spreading, the gel appeared as fine grains, but not as fine as Vycross. On magnification, the gel appeared as a more or less continuous network comprising particles of different sizes with an appearance similar to IPN-Like. OBT was also classed as a non-cohesive or partially cohesive gel.

FIGURE 2. Appearance of hyaluronic acid (HA) gels (Vycross®, OBT®, and RHA®) under the light microscope. The top row images show a macroscopic view of the HA gels Vycross, OBT®, and RHA colored with toluidine blue. The images below show the appearance of the same gels under the light microscope (original magnification x25).



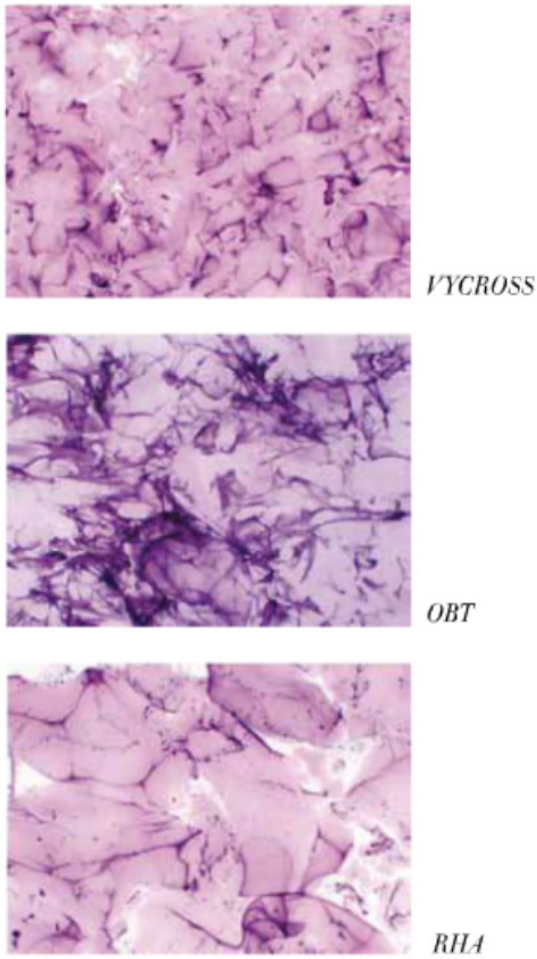
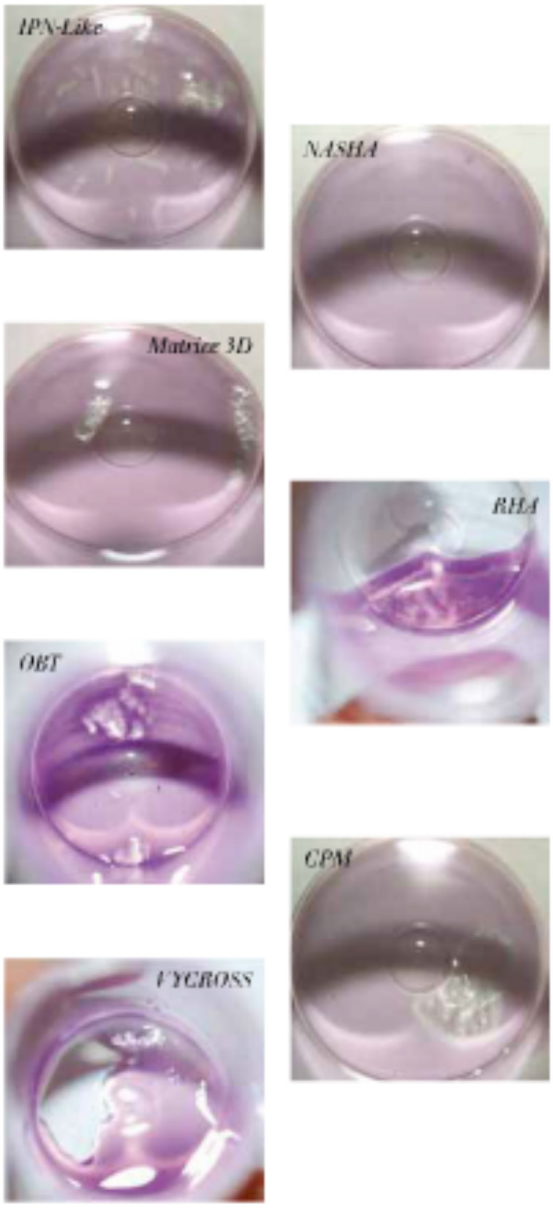


FIGURE 3. Cohesivity test. Investigators placed 0.9 mL of colored saline solution in a Petri dish and added 0.3 mL hyaluronic acid gel. Once placed in the solution, the NASHA® gel disintegrated into multiple very small particles, indicating it was non-cohesive. Only CPM® was truly cohesive, remaining as a continuous long strand. The other gels split into multiple strands, indicating that they were partially cohesive.



Cohesivity Test

Tests performed in private practice showed that the NASHA gel dispersed immediately after contact with saline solution (Figure 3, Table 2). The addition of ethanol increased the dispersion. CPM gel remained completely intact followed in descending order by Vycross, OBT, RHA, 3D Matrix, IPN-like, and finally NASHA. The same results were observed in tests performed at the University of Geneva, Department of Dermatology, in 2008 on the 3 FDA-approved gels (Figure 3).[10] The results illustrate the cohesivity of the different gels, with NASHA being the least cohesive and CPM the most cohesive. The results were the same for all gels, whether or not they contained added lidocaine.

TABLE 2.

Behavior of Hyaluronic Acid Gels Produced by Different Crosslinking Technologies After Contact With Saline Solution

Crosslinking Technology	Cohesivity Test Observation
NASHA®	Disintegration once in contact with saline solution. Microscopic particles, "pearls," of gel visible. Particles are even palpable when the pure gel is massaged between thumb and forefinger.
3D Matrix	Gel disintegrates into multiple strands or "sausages" after several seconds. Addition of ethanol increases the process.
IPN-like®	Gel disintegrates like 3D Matrix®.
CPM®	Gel remains perfectly cohesive with or without the addition of lidocaine. It remains as a single, long strand "continuous sausage," even after the addition of ethanol.
Vycross®	Gel disintegrates as for 3D Matrix®.
RHA®	Gel disintegrates as for 3D Matrix®.
OBT®	Gel disintegrates as for 3D Matrix®.

Resistance to Stretch Test

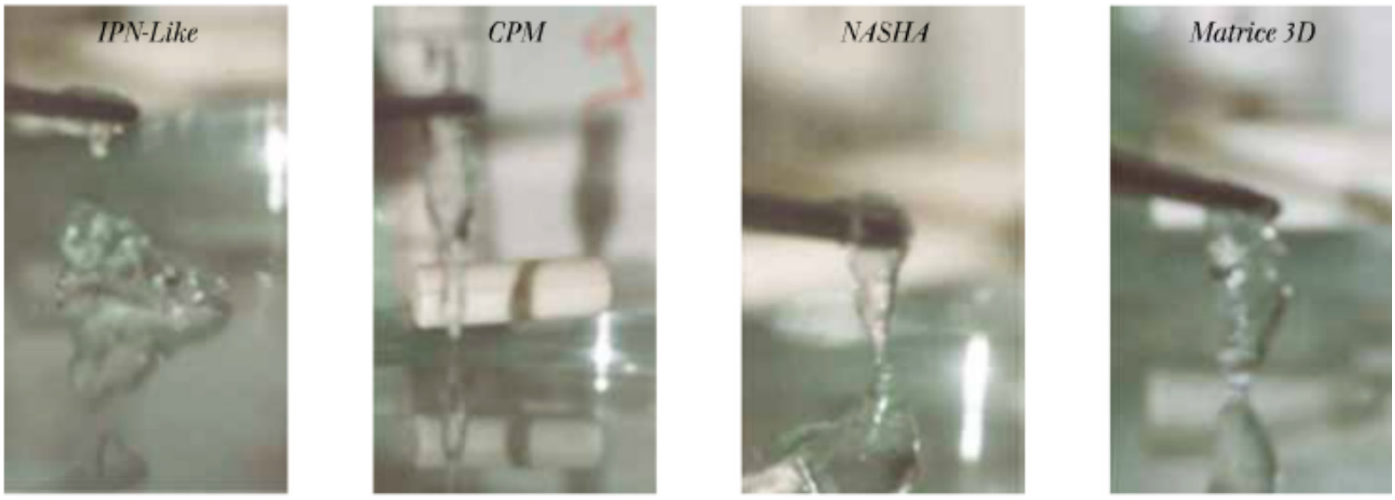
For all the HA gels with the exception of CPM, it was not possible to draw out the gel to a distance greater than 1 cm to 2 cm without the gel breaking (Figure 4, Table 3). This was the case whether lidocaine had been added by the manufacturer or not; addition of liquid lidocaine to an HA gel may modify a product's cohesivity and change its viscoelastic properties. The CPM gel could be drawn to a distance of 3.5 cm to 5 cm without breaking.

DISCUSSION

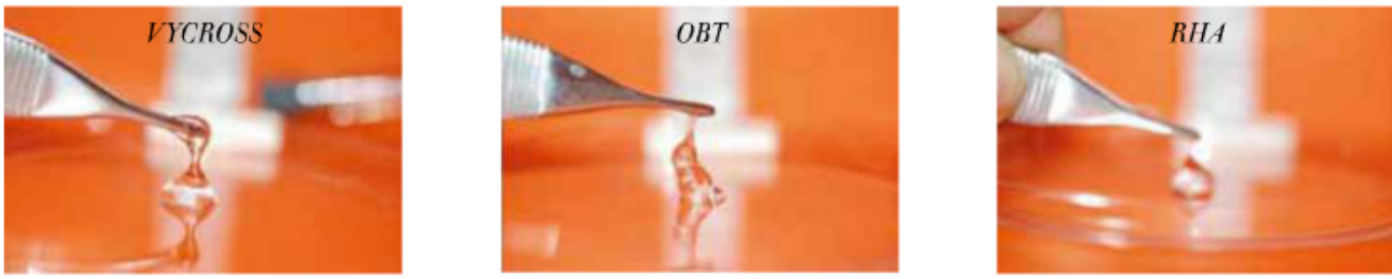
A simple set of tests that can be performed in private practice or in a laboratory reveal large differences in the behavior of currently available HA gels manufactured using different crosslinking technologies. Crosslinking is required to slow down the degradation of endogenous HA, but is also harnessed to change the rheological properties of HA gels with consequences on the effectiveness of a product for a particular indication.

Cohesivity is used to assess the ability of a filler to resist deformation and maintain product integrity and, along with the elastic modulus (G prime) of a gel, is an important determinant of the lift capability of a filler. Cohesivity of gels can be measured quantitatively by the amount of pressure required to compress them between 2 plates. In a qualitative measure of

FIGURE 4. Resistance to stretch test results. The length of stretch was measured against a metric scale (visible in the background of the lower images).



Photos 2 : Tests de résistance à la traction en cabinet.



cohesivity, we observed the dispersion of the gels after mixing with a classic colored saline solution. Some of the gels dispersed completely, others partially, and one remained totally cohesive. Products with high cohesivity such as CPM remain as long continuous strands when mixed. In contrast, the non-cohesive gels are dispersed. A further measure of cohesivity was provided by the resistance to stretch test. The results supported the findings above, with the CPM gel demonstrating the greatest resistance to stretching (3.5 cm-5 cm), while the remaining gels could not be stretched for distances greater than 2 cm.

Although simple, these easily reproducible laboratory tests can help us understand how the different HA gels integrate with the collagen and elastin fibers of the dermis. Biopsies of human skin after injection have shown that the different HA gels have a predictable histologic behavior, which differs by their type of crosslinking.[8,11] CPM, the only monophasic polydensified gel, demonstrates homogenous staining and penetrates all the dermis in a diffuse and evenly distributed manner. Biphasic products such as NASHA appear as large pools of HA distributed as clumps or beads of material in the lower portion of the dermis, with the upper and mid reticular dermis being free of material. Monophasic monodensified products such as 3D Matrix show HA material throughout the dermis, but in large pools. These patterns are consistent between patients and therefore predictable.[8]

The ability of CPM to distribute homogenously across the targeted area and into the surrounding tissues is due to the fact that it contains variable zones of crosslinking density, with areas of higher crosslinking density (harder) interspersed with areas of lower crosslinking density (softer).[12] This creates a gel that retains its integrity on injection and has high resistance to

TABLE 3.

Behavior of Hyaluronic Acid Gels Produced by Different Crosslinking Technologies in Resistance to Stress Test

Crosslinking Technology	Maximum Distance Gel can be Drawn (cms) (Minimum of 3 Tests)
NASHA®	≤ 1.0
3D Matrix	≤ 1.5
IPN-like®	≤ 2.0
CPM®	3.5–5.0
Vycross®	≤ 1.0
RHA®	≤ 0.5
OBT®	≤ 1.5

deformation, for example in areas of high facial movement. The product's low viscosity also means that it is easily injected, with little pressure, through small diameter needles. As a result of its very homogenous tissue distribution, the CPM gel can be injected over a range of tissue depths, including very superficially, for the correction of fine to deep lines. In contrast, Vycross technology creates a gel with a crosslinked mixture of high (>1 MDa) and low molecular weight (short chain) HA with a higher proportion of the latter. This provides the gel with a high G prime (gel hardness) and medium cohesivity, making it suitable for volumizing and subcutaneous or supraperiosteal injection.

Light microscopy confirmed the particulate nature of each product and revealed HA particles of varying size and distribution. CPM was the only gel to have no particles visible at a microscopic level. Among particulate fillers, the shape of the microspheres has previously been shown to be a factor in foreign-body reactions, with granulomatous reactions occurring less frequently after implantation of microspheres with smooth surfaces.[13] Irregular and sharp-edged particles may also induce more severe granulomatous reactions.

CONCLUSION

With the wide choice of HA gels available on the market, it is not always easy to select the best filler for a specific purpose. Despite beginning with the same starting material, HA fillers are produced with a range of different crosslinking technologies. With a few simple and easily reproducible tests, we have shown how these can influence a gel's behavior and consequently require an adaptation of injection technique and probably depth of injection.[10]

There is no single HA gel for all indications, each treatment indication requiring a targeted product. Knowledge of the rheological properties of a gel, combined with proper selection of injection technique and patients individual anatomy, (eg, skin thickness and restrictions regarding nerves and blood vessels), will help physicians select the right product to achieve optimal cosmetic outcomes.

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DISCLOSURES

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