VISCOELASTIC PROPERTIES AND THERMODYNAMIC BALANCE IMPROVEMENT OF A HYALURONIC ACID HYDROGEL ENRICHED WITH PROLINE AND GLYCYNE

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Hyaluronic acid (HA), is a linear polysaccharide composed of repeating disaccharide units of d-glucuronic acid and N-acetyl-d-glucosamine linked by β-1-3 and β-1-4 glycosidic bonds that provides viscoelasticity to the dermis, fascia, and most fluid media in humans. HA is a primary component of the extracellular matrix (ECM) of the human connective tissues (1) which regulates and controls several tissue physiological functions *in vivo* (2) and is also present in high concentration in many specialized tissues of the human body.

HA regulates moisture, elasticity, and architecture of tissue, repairing tissue, promoting cell motility, and scavenging free radicals (3-5), thus has a very broad range of applications ranging from orthopedics to aesthetic medicine and surgery (6). The HA hydrogel evaluated in this study has been chemically modified with a crosslinking reaction made with cross-linking agents (PEGDE) based on epoxides, in a strongly basic environment with the formation of ether bonds C – O - C, which is among the most solid and, consequently, the most resistant to degradation (7).

The HA hydrogel evaluated has been enriched with two amino acids non-essential in particular glycine e and proline. Glycine e, proline and hydroxyproline contribute to 57% of total amino acids (AAs) in collagen, the largest and most abundant protein in the body. As proline is a promising and useful ingredient that improves wrinkles due to its ability to increase the elasticity of the stratum corneum, it will also be interesting to examine the additive and/or synergistic effects of proline combined with other anti-aging ingredients which function mainly in the dermis in some effective regimens. Indeed, proline, used with glycine e and leucine, protects skin from UV-damage by restoring the synthesis of HA and reducing the inhibition of elastase, and increases skin moisturization and elasticity (8). In the end, proline under stressful conditions imparts stress tolerance through maintaining cell turgor or osmotic balance, stabilizing cell membranes (9).

Key words: Neauvia, HA PEG crosslinked; proline and glycine e; viscoelastic property; thermodynamic balance

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DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

MATERIALS AND METHODS

The cross-linked HA used is a hyaluronic acid hydrogel 24 mg/ml (MatexLab SPA, Brindisi, IT), which is of probiotic origin and cross-linked using PEGDE (Polyethylene glycol) diglycidyl ether). Rheological characterizations were performed on a Kinexus Instrument Rheometer (Malvern).

Sample preparation

To evaluate the variation of viscoelastic properties of HAP24 PEGde products with and without glycine e and proline (named HAP24-GLYPRO, HAP24, respectively) and only with glycine e (named HAP24-GLY), rheological tests were carried out both on the product immediately after extrusion (time 0) and after 30 min and 4 h of incubation where each sample was weighed (1 gr) and placed in contact with 500 µl of saline solution (0.9% NaCl in deionized water).

Viscoelastic properties

Rheological characterizations were performed on a Kinexus Instrument Rheometer at a temperature of 25°C. A 20-mm diameter plate was used combined with a Peltier lower plate. Before measurement, inertia and oscillatory mapping calibrations were performed. Then, samples were dropped onto the lower plate and then squeezed by the upper tool (plate). Product surplus was removed thanks to a spatula. After the gap was established, sample temperature was set to 25°C with a precision of 0.5°C.

HA hydrogel was subjected to periodic oscillation in a dynamic experiment (small amplitude frequency sweep tests) to evaluate the dependence of the viscoelastic parameters, such as the elastic and viscous moduli, G' and G'', upon the frequency. The mechanical response, expressed as shear stress τ of viscoelastic materials, is intermediate between an ideal pure elastic solid (obeying Hooke's law) and an ideal pure viscous fluid (obeying Newton's law) and, therefore, is out of phase with respect to the imposed deformation as expressed by

$$\tau = G'(\omega)\, Y_0 \sin(\omega t) + G''(\omega)\, Y_0 \cos(\omega t), \quad (1)$$

where $G'(\omega)$ is the shear storage modulus or elastic modulus and $G''(\omega)$ is the shear loss modulus or viscous modulus. G' gives information about the elasticity or the energy stored in the material during deformation, whereas G'' describes the viscous character or the energy dissipated as heat. The phase angle, δ, is equal to 90° for a purely viscous material, 0° for a pure elastic material, and $0° < \delta < 90°$ for viscoelastic materials (1, 9). The frequency range investigated was 0.1Hz–10Hz and fixed strain of 1%. In order to identify the linear viscoelastic response range of the materials, preliminary strain sweep tests were performed on the samples, at the oscillation frequency of 1 Hz and strain of 0.01-2000%. The tests were repeated at least three times on each sample. The tests were carried out at the controlled temperature of 25°C by using a thermostatic bath. In order to avoid water evaporation, the humidity of the chamber containing the samples was controlled by a humidity control accessory.

Statistical analysis

Results were obtained from three individual experiments. The results are expressed as mean ± SD.

RESULTS

The dependence of the elastic and the viscous moduli upon the amplitude strain and the oscillation frequency for the gels obtained by cross-linking of HA with PEGDE are reported. The amplitude strain spectra (Table I) shows a predominance of the elastic modulus (G') compared to the viscous modulus (G"), considered as a solid-like feature. In this way, the hyaluronic acid gel behaves as a three-dimensional network where the deformation feature is the main accommodation mode to the applied stress (Figs. 1, 2).

Moreover, the mechanical spectra (related to the frequency sweep) shows a predominance of the elastic modulus (G') compared to the viscous modulus (G") considered as a solid-like feature; indeed, the elastic modulus is one order of magnitude higher than the viscous modulus (Table II). Since G' is the modulus related to elasticity, when this value exceeds the viscous modulus (G"), which is related to flow, the material can be considered to have an associated structure and hence a yield stress. For a material to have a true yield stress then G' must

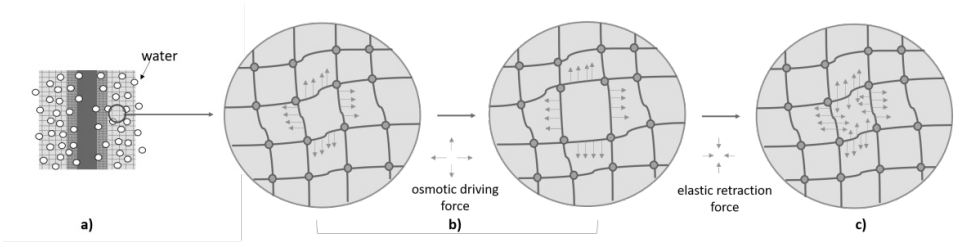

Fig. 1. ***a****) Schematic representation of the gel swelling phases,* ***b****) effect on the hydrogel of the action of osmotic force,* ***c****) effect on the hydrogel of the action of the osmotic force contrasted by the elastic network retraction force.*

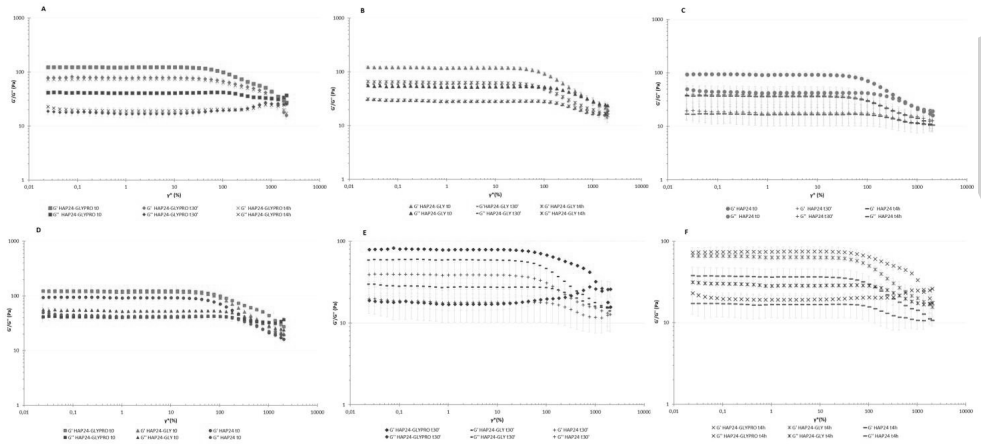

Fig. 2. *The amplitude strain spectra of samples characterized by frequency 1 Hz; stain 0.01-2000%; temperature 25°C. Viscoelastic parameters comparison of HAP24-GLYPRO at time 0, 30 min and 4 h (**A**); viscoelastic parameters comparison of HAP24-GLY at time 0, 30 min and 4 h (**B**); viscoelastic parameters comparison of HAP24 at time 0, 30 min and 4 h (**C**). Viscoelastic parameters comparison at time 0 of HAP24-GLYPRO, HAP24-GLY, HAP24 (**D**); viscoelastic parameters comparison at time 30 min of HAP24-GLYPRO, HAP24-GLY, HAP24 (**E**); viscoelastic parameters comparison at time 4 h of HAP24-GLYPRO, HAP24-GLY, HAP24 (**F**).*

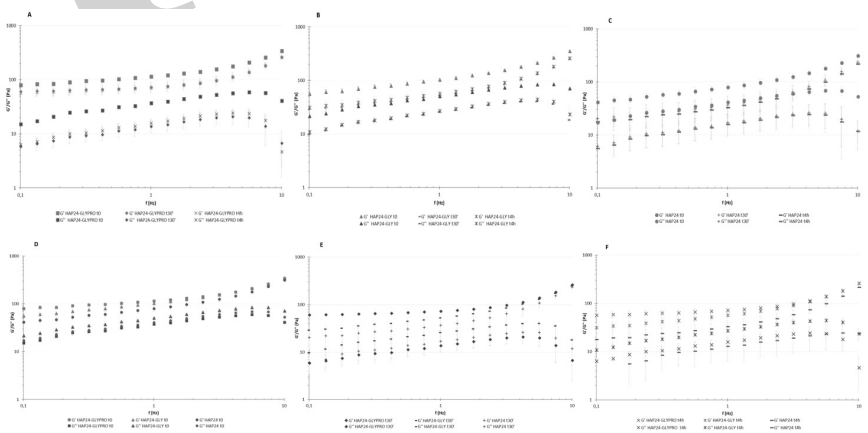

Fig. 3. *Mechanical spectra of samples characterized by frequency 0.1-10 Hz; stain 1%; temperature 25°C. Viscoelastic parameters comparison of HAP24-GLYPRO at time 0, 30 min and 4 h (**A**); viscoelastic parameters comparison of HAP24-GLY at time 0, 30 min and 4 h (**B**); viscoelastic parameters comparison of HAP24 at time 0, 30 min and 4 h (**C**). Viscoelastic parameters comparison at time 0 of HAP24-GLYPRO, HAP24-GLY, HAP24 (**D**); viscoelastic parameters comparison at time 30 min of Intense Lips, HAP24-GLYPRO, HAP24-GLY, HAP24 (**E**); viscoelastic parameters comparison at time 4 h of HAP24-GLYPRO, HAP24-GLY, HAP24 (**F**).*

Table I. *Viscoelastic parameters values of the gels at strain of 10%.*

	G' [Pa]			G" [Pa]			Ƞ* [Pa s]			δ [°]		
	Time 0	Time 30'	Time 4h	Time 0	Time 30'	Time 4h	Time 0	Time 30'	Time 4h	Time 0	Time 30'	Time 4h
HAP24-GLYPRO	125±3	80±9	76±9	42±2	17±3	19±2	21±1	13±2	12±2	18±1	12±1	15±2
HAP24-GLY	121±6	60±6	64±6	54±1	28±2	29±3	21±1	10±1	11±1	24±1	25±1	24±2
HAP24	94±13	39±16	37±9	43±7	18±8	17±6	16±3	7±3	6±2	25±1	25±1	24±3

Table II. *Viscoelastic parameters values of the gels at frequency of 0.5 Hz.*

	G' [Pa]			G" [Pa]			Ƞ* [Pa s]			δ [°]		
	Time 0	Time 30'	Time 4h	Time 0	Time 30'	Time 4h	Time 0	Time 30'	Time 4h	Time 0	Time 30'	Time 4h
HAP24-GLYPRO	105±6	69±15	67±8	32±1	11±3	13±3	31±2	20±4	19±2	17±1	10±1	11±2
HAP24-GLY	89±5	45±6	48±7	43±2	22±2	23±2	28±2	14±2	15±2	26±2	26±1	26±4
HAP24	68±9	31±14	28±8	35±6	14±6	13±5	21±3	10±4	9±3	27±1	24±1	25±6

exceed G" at infinitely low frequencies typical of an ideal gel, as occurred in these samples (Fig. 2).

Viscoelasticity values obtained from the amplitude strain spectra (Fig. 1) of the product containing the amino acids glycine e and proline (HAP24-GLYPRO) and tested at different incubation times showed a gradual reduction of the elastic modulus observed over time (G ‘) and viscous module (G"). This phenomenon is due to the process of diffusion of water within the polymeric network of the gel following the contact of the hydrogel with the physiological solution (Fig 1).

In the product containing only glycine e (HAP24-GLY), the value of the elastic modulus (G ‘) at time zero remained unchanged (121 Pa) compared to that obtained in the product HAP24-GLYPRO (125 Pa), but the values of the viscous module (G “) tended to shrink slightly from a value of 54 to 42 Pa in the sample HAP24-GLY and HAP24-GLYPRO, respectively. This causes a reduction in the distance between the two modules, elastic and viscous, with a consequent liquid-like behavior of the HAP24-GLY gel in respect to the HAP24-GLYPRO gel; in fact, also the values of the δ angle went from a value of 24 to 18 degrees in the sample HAP24-GLY and HAP24-GLYPRO, respectively (Table I).

Moreover, in the HAP24-GLY product, due to the absence of proline, when subjected to swelling, a drastic reduction of the elastic modulus (G ‘) was observed (60 and 64 Pa at 30 min and 4 h of incubation, respectively), compared to the HAP24-GLYPRO product (80 and 76 Pa at 30 min and 4 h of incubation, respectively). These data are also confirmed by the significant increase in the values of the δ angle of the HAP24-GLY sample (25 and 24 Pa at 30 min and 4 h of incubation respectively) compared to the HAP24-GLYPRO sample (12 and 15 Pa at 30 m and 4 h of incubation, respectively) due to the increase in the liquid-like component.

The study of the viscoelastic behavior of the product hyaluronic acid hydrogel 24 mg/ml cross-linked with PEGde in the presence and absence of the amino acid glycine e and proline analyzed for the mechanical spectra (Fig. 2, Table II), related to the frequency sweep, highlighted a similar trend to the one previously described for the amplitude strain spectra.

DISCUSSION

The rheological response is due to the contributions of PEG cross-links for the presence of physical and chemical bonds crosslinks such as electrostatic interactions, hydrogen bonds and also some topological interactions among the HA macromolecules. Moreover, PEG cross-links bring about a reduction of the intrinsic mobility of the

polymer chains with a predominance of the material elastic behavior (G' > G''). The ability of cross-linked HA to absorb water arises from hydrophilic functional groups attached to the polymer backbone while their resistance to dissolution arises from cross-links between network chains. Water inside the hydrogel allows free diffusion of some solute molecules, while the polymer serves as a matrix to hold water together (10) (Fig. 3). Results of previous studies and from this one demonstrate that proline is able to regulate the water diffusion process inside the hydrogel, preventing an excessive swelling of the gel itself (11). Proline is one of the several small molecules classified as an osmolyte or an osmoprotectant. This osmolyte helps mitigate water stress and balance turgor pressure during stress (12). Finally, as our results clearly show, in the absence of proline and glycine (HAP24) in the product, the value of the elastic modulus (G ') already at time zero is drastically reduced (94 Pa) and decreases steadily after 30 min (39 Pa) and 4 h (37 Pa) compared to the G 'values of the HAP24-GLY. This shows that glycine e also plays an important role in the control of cell volume and osmoregulation as it keeps the structure of the hydrogel constant and prevents uncontrolled swelling. The results obtained show that the addition of two amino acids osmolytes, proline and glycine e, in the Neauvia formulation ensures a better control of the hydrogel swelling capacity in the post-implant phase. At the same time, the proline and glycine e guarantee hydration and gives softness to the skin tissue, acting as humectant and emollient, respectively. All these factors lead to their choice for the stabilization of the structure of the hydrogel, especially in terms of viscoelastic properties and thermodynamic balance.

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