The crosslinking degree controls the mechanical, rheological, and swelling properties of hyaluronic acid microparticles

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Abstract: Viscosupplements, used for treating joint and cartilage diseases, restore the rheological properties of synovial fluid, regulate joint homeostasis and act as scaffolds for cell growth and tissue regeneration. Most viscosupplements are hydrogels composed of hyaluronic acid (HA) microparticles suspended in fluid HA. These microparticles are crosslinked with chemicals to assure their stability against enzyme degradation and to prolong the action of the viscosupplement. However, the crosslinking also modifies the mechanical, swelling and rheological properties of the HA microparticle hydrogels, with consequences on the effectiveness of the application. The aim of this study is to correlate the crosslinking degree (CD) with these properties to achieve modulation of HA/DVS microparticles through CD control. Because divinyl sulfone (DVS) is the usual crosslinker of HA in viscosupplements, we examined the effects of CD by preparing HA microparticles at 1:1, 2:1, 3:1, and 5:1 HA/DVS mass ratios. The CD was calculated from inductively coupled plasma spectrometry data. HA microparticles were previously sized to a mean diameter of 87.5 μm. Higher CD increased the viscoelasticity and the extrusion force and reduced the swelling of the HA microparticle hydrogels, which also showed Newtonian pseudoplastic behavior and were classified as covalent weak. The hydrogels were not cytotoxic to fibroblasts according to an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. © 2014 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 00A:000–000, 2014.

Key Words: hyaluronic acid, microparticles, crosslinking, divinyl sulfone, viscosupplements


INTRODUCTION

Hyaluronic acid, or hyaluronan (HA), is a linear biopolymer composed of disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine linked through a β-1,3 glycosidic bond. The disaccharides are in turn linked to each other through a β-1,4 glycosidic bond.1 HA occurs naturally in vitreous humor, synovial fluid, and umbilical cord. HA can be extracted from cockscomb or produced by certain strains of Streptococcus by fermentation. Microbial HA is similar to HA derived from native sources, and its molecular weight varies from $10^4$ to $10^7$ Da.2

Its high capacity for holding water and high viscoelasticity, as well as its inherent biocompatibility and biodegradability, make HA a suitable biomaterial for various medical and pharmaceutical applications, such as in joint and cartilage diseases. However, its rapid degradation by oxidation and elimination by enzymes when implanted or injected into the human body considerably limit the applications of HA. Chemical crosslinking reactions have often been used to create a polymer network with greater stiffness and less susceptibility to enzymatic degradation.3 Crosslinked HA swells in water but does not dissolve,4 and its residence time in the site of application is prolonged.5 According to Milas et al. (2001), crosslinking HA preserves the biocompatibility and physical functionality of unmodified HA. However, the molecular weight, molecular size, mechanical and rheological properties and swelling of the polymer in solution or suspension are substantially affected.5

On the other hand, crosslinked fluid HA generally produces highly viscous solutions that are difficult to inject. Therefore, viscosupplements should contain crosslinked particles, not only fluid HA. Small particles, such as microparticles, increase the surface area available for interactions. Therefore, HA microparticles promote adhesion in the site of action and act as efficient scaffolds for cell growth in tissue regeneration. In addition, HA microparticles also reduce

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the viscosity of fluid HA, allowing it to be injected with appropriate needles, and reducing undesirable side effects such as pain, bruising, bleeding and edema.6

Four functional groups on HA molecules can undergo chemical modification: the carboxylate group, the acetylamo group, the reducing end-group of the polymer, and the hydroxyl groups. Furthermore, the glycosidic bonds can also be broken by oxidation to provide shorter chains or oligosaccharides.4

HA crosslinking reactions typically employ polyfunctional chemicals such as divinyl sulfone (DVS), 1,4-butanediol diglycidyl ether (BDDE), or adipic acid dihydrazide (ADH).4

The crosslinking reaction based on divinyl sulfone (functionality = 2) creates sulfonyl-bis-ethyl crosslinks between hydroxyl groups of the polysaccharide (functionality = 4), giving rise to an infinite network of HA chains (Fig. 1).7,8

Most HA-based viscosupplements are composed of HA microparticles crosslinked with DVS (HA/DVS) dispersed in fluid HA. Despite their importance, systematic studies in the literature about the physicochemical properties of HA/DVS microparticles remain scarce. In general, these studies compare the performance of different crosslinkers or characterize the swelling properties only as a function of DVS concentration.5,9

In previous work, we studied the influence of the particle size and fluid HA fraction on the rheological and extrusion properties of dispersions containing HA microparticles at a 1:1 HA/DVS mass ratio.10 The microparticles exhibited behavior typical of so-called weak hydrogels, as analyzed by the storage and loss moduli. The viscoelasticity, viscosity, and the extrusion force increased with the particle size for HA/DVS microparticles in the range of 25–125 μm.

This work extends the previous findings by investigating the influence of the degree of crosslinking conferred by the HA/DVS mass ratio on the mechanical, rheological and swelling properties of HA microparticles in the size range of 75–100 μm. Unlike the literature data on viscosupplements, this study considers only HA/DVS microparticles, not their mixture with fluid HA. The importance of this focus is to evaluate the ability of the physicochemical properties of HA/DVS microparticles to be modulated through CD control.

**EXPERIMENTAL**

**Materials**

Hyaluronic acid with an average molecular weight of 105 Da was obtained by bacterial fermentation using *Streptococcus equi* subsp *zoopidemius* ATCC 39920, according to the protocol described by Pires et al.11 Divinyl sulfone and phosphohe buffered saline solution (PBS) were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents were purchased from Synth® (Diadema, SP, Brazil) unless specified otherwise.

**Preparation of crosslinked HA hydrogels**

The crosslinked hydrogels were prepared in HA/DVS mass ratios 1:1, 2:1, 3:1, and 5:1, as described by Leshchiner et al.12 The reaction was performed by adding DVS in a solution (3%, w/w) of HA in NaOH (0.1 M) containing 3% (w/v) NaCl. The pH of the reaction medium was kept above 9 because this facilitates the crosslinking reaction. The reaction was carried out at 25°C for 4 h. The product was washed for 2 days under reciprocal agitation at 200 rpm. The wash solution was decanted, and the hydrogel was shaken at 200 rpm in a solution of 10 mmol L⁻¹ PBS for an additional 24 h.

**Preparation of hyaluronic acid microparticles**

The crosslinked hydrogels were sheared in an Ultra-Turrax T-25 homogenizer (IKA Labortechnik, Staufen, Germany) at 24,000 rpm. The mean diameter of the particles was adjusted throughout the shearing time to obtain particles in the range of 75–100 μm as previously described.13 Particle size measurements were performed by laser scattering in a Horiba LA-900 particle analyzer (Horiba Instruments, Irvine, CA). The particle size measurements were performed with the microparticles dispersed in water. The standard deviation was calculated from ten measurements of the mean diameter.

**Swelling determination**

After synthesis, the HA/DVS microparticles were swollen to equilibrium in phosphate buffered saline solution (PBS) at 25°C for 72 h. The microparticles were weighed after swelling, and their dry weight was determined by drying under vacuum (1 mmHg) at 25°C for 3 days, according to a protocol described by Shu et al.13 The swelling ratio (SR) was calculated with Eq. (1):

\[
\text{Swelling ratio (SR)} = \frac{W_s}{W_d}
\]  

where \(W_s\) (in g) is the weight of the sample at 25°C after the wash steps (i.e., \(W_s\) is the swollen weight of the HA/DVS microparticles) and \(W_d\) (in g) is the dry weight.14

The percentage of equilibrium water content (EWC) was calculated according to Eq. (2):

\[
\% \text{ EWC} = \left( \frac{W_s - W_d}{W_s} \right) \times 100
\]  

The EWC was calculated at pH 7 in PBS at 25°C. All measurements were made in triplicate.

**DSC measurements**

The state of the water in the swollen HA/DVS microparticles was determined by the respective fractions of free and
bound water, which were obtained through thermal analysis. The assays were performed by differential scanning calorimetry (DSC) using a Model 2920 differential scanning calorimeter from TA Instruments (TA Instruments, New Castle, DE). To examine the state of the water, the temperature was cooled to −40°C and then heated to 40°C at a heating rate of 5°C min⁻¹ under 60 cm³ min⁻¹ of nitrogen gas flow. Using peak areas normalized for sample mass, the endotherm associated with water loss was obtained and compared with the theoretical value for water as described by Collins and Birkinshaw.  

The fraction of free water in the total amount of water was calculated as the ratio of the endothermic peak area for the water-swollen microparticles to the melting endothermic heat of fusion (334 J g⁻¹) for pure water, as described in the work of Mansor and Malcolm. The bound water was expressed as the difference between the total and free waters.

**Crosslinking degree**

The theoretical crosslinking degree of the microparticles was calculated by the stoichiometry of the reaction. The experimental CD values were obtained through sulfur analysis in the water washes by inductively coupled plasma (ICP) spectrometry using a Perkin-Elmer 3100XL Inductively Coupled Plasma Optical Emission Spectrometer (Perkin Elmer; Norwalk, CT). The experimental CD was calculated as the difference between the added mass of DVS and that obtained by ICP.

**Chemical modifications**

Infrared spectrometry (FT-IR) was used to identify the chemical modifications of HA in the crosslinking reaction. Infrared spectra of dried HA and HA/DVS hydrogels were obtained using a Shimadzu IR Prestige-21 infrared spectrophotometer (Shimadzu, Kyoto, Japan) in the spectral range of 4000–675 cm⁻¹. The spectra represent an average of 64 scans, with a resolution of 4 cm⁻¹ with CO₂ peaks removed.

**HA concentration**

The HA concentration was measured by the modified carbazole method according to a protocol described by Bitter and Muir.

**Rheological measurements**

Rheological measurements were performed on a rheometer Haake RheoStress 1 (Haake, Karlsruhe, Germany). The properties of the HA/DVS microparticles were characterized in steady and oscillatory regimes using parallel plate geometry of 20 mm at 25°C. Oscillatory measurements were conducted in the linear region at a stress of 1.188 Pa under a frequency range of 0.1–10 Hz. Steady shear measurements were carried out at shear rates of 0.1–50 s⁻¹. All rheological measurements were performed using swollen HA/DVS microparticles equilibrated in PBS.

**Extrusion force**

The force required to extrude the HA/DVS microparticles (3% (w/w) HA in starting solution) was determined in 1-mL plastic syringes with 30-gauge needles. The measurements were performed in an EMIC DL-3000 Universal Test Machine (São José dos Pinhais, PA, Brazil) with load cell 10 kg at 25°C at a 50 mm min⁻¹ extrusion rate.

**Cell viability**

The quantification of reduction of MTT 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Molecular Probes) was used according modified MOSMANN (1983) method to evaluate cell viability in the presence of HA and HA/DVS microparticles. We used VERO fibroblast-type cells, a cell line originating from African green monkey kidney (Cercopithecus aethiops) obtained from Adolfo Lutz Institute, São Paulo, Brazil. The cells at the 28 passage were cultivated in Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco, Grand Island, NY) supplemented with 10% Fetal Bovine Serum (FBS, Gibco, Grand Island, NY) and 1% penicillin and streptomycin (PS, Hyclone, Logan, UT) at 37°C in an atmosphere with 5% CO₂ until the time of the cell viability assay. Extracts of the HA and HA/DVS microparticles were obtained by incubating them in DMEM containing 10% FBS at a proportion of 0.2 g mL⁻¹ of medium for 48 h at 5% CO₂ and 37°C. This method is in agreement with the International Standard Organization (ISO 10993 Part 5 1992-E). Vero cell suspensions (2 × 10⁵ cells mL⁻¹) were inoculated into a 96-well cell culture plate (n = 5) and cultured with DMEM with 10% FBS at 37°C for 24 h. After this, the medium was replaced by the extract obtained from the HA and HA/DVS microparticles and the cells were maintained under these conditions for 24 h. DMEM with phenol 0.5% was used as the positive control toxicity (PCT) and DMEM with 10% FBS as the negative control toxicity (NCT). After incubation time, the medium was removed and the wells were washed with 200 µL PBS and 200 µL pure DMEM. Next, 200 µL of MTT solution in culture medium (0.5 mg MTT/mL) were added, and the plate was incubated in the dark for 4 h at 37°C. The medium with MTT was removed and 200 µL of dimethyl sulfoxide (DMSO, Sigma–Aldrich, St. Louis, MO) was added and absorbance was determined at ƛ = 595 nm (FilterMax F5 Multi-Mode Microplate reader, Molecular Devices, Sunnyvale, CA).

**Statistical analysis**

Each experiment was carried out in triplicate unless otherwise specified. All results are presented as mean ± standard deviation (SD). The experimental data from all the studies were analyzed using analysis of variance (ANOVA). Statistical significance was set to P value < 0.05.

**RESULTS**

**Microparticle characterization**

**Size and HA content.** We characterized the hyaluronic acid microparticles in terms of average particle size and HA content as a function of the initial HA/DVS mass ratio (Fig. 2).

**Chemical modifications and crosslinking degree.** The chemical modifications of hyaluronic acid were previously identified in IR spectra of the HA microparticles by the characteristic peaks for DVS appearing between 1384 and 1280 cm⁻¹, which were attributed to the sulfone group (νSO₂ = 1350, 1310 cm⁻¹).
The theoretical value for the degree of chemical modification or CD of the HA microparticles, calculated from the stoichiometry of the HA/DVS reaction, was compared with the experimentally determined values obtained from ICP analysis (Fig. 3).

**Mechanical properties.** Table I shows the extrusion force data for HA/DVS microparticles as a function of experimental CD values.

**Swelling properties.** DSC analysis was used to evaluate the water content of the HA microparticles in terms of the EWC, that is, the fractions of bound and free water. The DSC thermograms of the fully swollen microparticles (Fig. 4) were characterized by endothermic peaks with a maximum between 0.5 and 3.8°C.

Table II summarizes SR and the water content of the HA/DVS microparticles in terms of the EWC, fraction of bound water and free water as a function of CD and HA/DVS.

**Rheological properties.** Figure 5(a) shows the oscillation spectra from which we determined the elastic or storage modulus ($G'$) and the viscous or loss modulus ($G''$) as a function of frequency for HA/DVS microparticles with different CDs obtained from the corresponding HA/DVS mass ratios.

Table III summarizes the values of $\tan \delta (=G''/G')$ and the power law parameters calculated in steady and oscillatory regimes from the spectra of Figure 5 as a function of the CD obtained from different HA/DVS mass ratios.

Figure 6 summarizes the obtained results by correlating $G'$, $G''$, SR, and CD; images of hydrogels composed of HA/DVS microparticles are also shown.

**Cell viability.** Figure 7 shows the cell viability, as assayed by MTT, of fibroblasts cultured in the presence of HA microparticles.
TABLE II. Influence of CD and HA/DVS Mass Ratio on the Water Content of HA Microparticles (in Starting Solution, 25°C, 4 h)

<table>
<thead>
<tr>
<th>CD (%)</th>
<th>HA/DVS Mass Ratio</th>
<th>SR (%)</th>
<th>EWC (%)</th>
<th>Wb (%)</th>
<th>Wf + Wfb (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>90</td>
<td>1:1</td>
<td>23.6 ± 0.9</td>
<td>95.8 ± 0.2</td>
<td>9.37</td>
<td>86.37</td>
</tr>
<tr>
<td>39</td>
<td>2:1</td>
<td>38 ± 2</td>
<td>97.4 ± 0.1</td>
<td>16.96</td>
<td>80.42</td>
</tr>
<tr>
<td>26.4</td>
<td>3:1</td>
<td>52 ± 2</td>
<td>98.07 ± 0.07</td>
<td>18.76</td>
<td>79.31</td>
</tr>
<tr>
<td>14.2</td>
<td>5:1</td>
<td>73 ± 2</td>
<td>98.62 ± 0.04</td>
<td>41.22</td>
<td>57.39</td>
</tr>
</tbody>
</table>

CD = crosslinking degree; SR = swelling ratio; EWC = equilibrium water content; Wb = bound water; Wf = free water; and Wfb = freezing bound water. Mean ± standard deviation n = 3.

DISCUSSION

Figure 2 shows the size of the microparticles ranged from 75–100 μm, values that were expected based on the factors of viscoelasticity and extrusion force demonstrated in our previous work.10

The content of HA in the microparticles decreased with HA/DVS mass ratio, as a consequence of the variations in the crosslinker mass as well as of the total water content in the microparticles due to the differences in the crosslinking degree. As expected, at the lowest values of the HA/DVS mass ratio, the CD was greater, reaching 90% of the binding sites at a HA/DVS mass ratio of 1:1. At a HA/DVS mass ratio of 5:1, only 14% of the binding sites in the material were modified.

The degree of crosslinking predicted by the theoretical calculations always exceeded the experimentally determined crosslinking degree of the microparticles. We observed that only ~50% of the crosslinker effectively modified the polymer, and the remainder was eliminated in the washing steps (Fig. 3).

Table I shows the extrusion force increased as the crosslinking degree increased, as expected. High values, such as 14.9N for the extrusion force, were obtained for a crosslinking degree of 90%, which corresponds to a HA/DVS ratio of 1:1. The extrusion force decreased with the CD, reaching the level of 6.8N for extrusion force at 14.2% CD (HA/DVS ratio 5:1), which is adequate for avoiding undesirable side effects with injection.5

Swelling characterization is very important because the character of the water can determine the overall permeation of nutrients into and the exit of cellular by-products out of the hydrogel.20 According to Zawko et al., the swelling ratio (SR) of the HA hydrogels is the maximum attainable swelling in water.14

The HA/DVS microparticles with the highest CD showed the lowest SR, EWC and bound water content, indicating a more compact structure. The increase in CD decreases the availability of functional groups that interact with water; thereby diminishing the swelling degree. The microparticles were also swollen in phosphate buffered saline solution (PBS), which is important because these fluids approximate in vivo conditions (Fig. 4, Table II).

At physiological pH, the HA is hydrated extensively by water because the water forms hydrogen bonds with the N-acetyl and carboxyl groups. The dipole attraction of the hydrogen bond to the carboxyl group results in HA's affinity for retaining water. The swelling capacity is dependent upon concentration, crosslinking density, and the processes used to hydrate the microparticles.6

Figure 5(a–c) shows that all HA/DVS microparticles exhibited typical gel-type mechanical spectra, with G’ higher than G’’ in all studied ranges and both moduli lying parallel to the frequency axis.

According to Kablik et al., the CD plays an important role in defining the moduli of the hydrogel. A hydrogel with a lower number of crosslinks (covalent bonds) has a greater length of the HA molecule between links; thus, less force is required to deform the hydrogel. As the network is tightened by increasing the number of crosslinks, the hydrogel becomes stiffer. Pendant-type modification of HA hydrogels has little effect on the moduli because they do not form a crosslinked network.6

Xuejun et al. reported that this behavior demonstrates an increased elasticity for the crosslinked HA hydrogel relative to network stabilization through the strong covalent crosslinks that reduce the intrinsic mobility of the chains and increase the relaxation time characteristic of motion. Consequently, the polymer chains cannot release stress during the period of oscillation and show elastic behavior.21

Figure 5(b) shows that the measured values of log G’ at 4.6 Hz for HA decreased linearly with the increase of log HA/DVS mass ratio. This frequency was set due to the range of physiological frequency of the knee when running (3–5 Hz).22

The slope of the straight line was 1.81, similar to the value of 2 often observed for slopes of double-logarithmic plots of G’ versus the concentration of biopolymers with a large number of potential binding sites along each chain (i.e., high functionality; HA = 4 and DVS = 2).23 This behavior also suggests the entanglement of chains, as well as intra- and intermolecular chain interactions due to increases in the degree of crosslinking.24

According to Ikeda and Nishinari, the mechanical spectrum of hydrogels can be related to the gel strength. Weak gels are slightly different from conventional gels in two respects: the moduli have low frequency dependence, and the magnitude of G’ is often 10 times smaller than the magnitude of G’’. In contrast, strong gels exhibit a G’ that is higher than G’’; however, the slope of the G’ line is zero, and G’ displays a minimum at intermediate frequencies.25,26

The degree of frequency dependence can also be determined by the well-known power law parameters (G’ = Aoωp) described by Ramkumar and Bhattacharya.27 In the power law relationship, G’ is the storage modulus, ω is the
oscillation frequency in rad s\(^{-1}\), \(A\) is a constant, and the exponent \(B\) is the slope in a log–log plot of \(G'\) versus \(\omega\). The \(B\) values define the mechanical strength of the gels. The value of \(B = 0\) for covalent gels, whereas \(B > 0\) corresponds to physical gels, according to Khondkar et al.\(^{28}\)

The flow curves of HA/DVS microparticles display non-Newtonian and pseudoplastic behavior [Fig. 5(c)] with a significant viscosity decrease over a narrow range of the shear rate (shear thinning). We also observed an increase in viscosity with decreasing HA/DVS mass ratio, indicating the presence of interchain associations at higher degrees of crosslinking.

Considering the power law described by Ostwald de Waele (\(\eta = K \gamma^n\)), the parameters \(K\) and \(n\) can be determined from these spectra, where \(K\) is the fluid consistency index and \(n\) is the flow behavior index.\(^{29,30}\)

The values of \(\tan \delta\) and the slope of the curve of \(G'\) in Table III show that the strength of the hydrogels increases with increasing CD. Values of \(\tan \delta < 0.1\) suggest predominantly elastic gels, while for so-called weak gels, \(\tan \delta > 0.1\).\(^{25}\) Based on these criteria, the hydrogels in this work

<table>
<thead>
<tr>
<th>Crosslinking Degree (%)</th>
<th>HA/DVS Mass Ratio</th>
<th>(A) (Pa.s)</th>
<th>(B) (Slope)</th>
<th>(\tan \delta)</th>
<th>(K)</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>90</td>
<td>1:1</td>
<td>794</td>
<td>0.08</td>
<td>0.17</td>
<td>72,260</td>
<td>0.417</td>
</tr>
<tr>
<td>39</td>
<td>2:1</td>
<td>192</td>
<td>0.07</td>
<td>0.24</td>
<td>29,478</td>
<td>0.393</td>
</tr>
<tr>
<td>26.4</td>
<td>3:1</td>
<td>99</td>
<td>0.15</td>
<td>0.27</td>
<td>21,179</td>
<td>0.431</td>
</tr>
<tr>
<td>14.2</td>
<td>5:1</td>
<td>24</td>
<td>0.24</td>
<td>0.34</td>
<td>9020</td>
<td>0.402</td>
</tr>
</tbody>
</table>

FIGURE 5. Rheological characterization. (a) Oscillation spectrum of HA microparticles with different HA/DVS mass ratios: (■) 1:1, \(G'\); (□) 1:1, \(G''\); (▲) 2:1, \(G'\); (△) 2:1, \(G''\); (●) 3:1, \(G'\); (○) 3:1, \(G''\); (●) 5:1, \(G'\); (○) 5:1, \(G''\). (b) Storage modulus \((G')\) at 4,642 Hz as a function of HA/DVS mass ratio. (c) Viscosity of HA microparticles with different HA/DVS mass ratios: (■) 1:1; (▲) 2:1; (●) 3:1; and (○) 5:1.

FIGURE 6. \(G'\), \(G''\) (at 5Hz) and SR (in phosphate buffered saline solution at 25°C) as a function of crosslinking degree obtained by ICP. Lower panel: Images of the HA/DVS hydrogels composed of HA/DVS microparticles.
could be classified as weak, irrespective of HA/DVS mass ratio.

The B values indicate an increase in the viscoelastic properties of hydrogels at lower HA/DVS mass ratios. Moreover, the B values were small, suggesting covalent hydrogels. The values of n showed a pseudoplastic behavior independent of the HA/DVS mass ratio.

Table 1 and Figure 6 show the major variations in the swelling and extrusion force occur with CDs below 50%: the extrusion force of the hydrogels doubles, and the swelling decreases by ~50%. Above 50% CD, the decrease in swelling (~30%) does not affect the extrusion force by more than 6%. The viscous component G’ and viscoelastic component G” increase proportionally with CD with the predominance of G’ in the studied frequencies. As the CD increases, the HA/DVS microparticles hydrogels become more viscoelastic. Therefore, there is a compromise among the viscoelasticity, swelling properties and extrusion force, which can be modulated by CD. For the HA/DVS microparticle hydrogels, an adequate extrusion force for injection could be obtained at a lower CD (~20%) at the expense of the viscoelasticity. At higher CDs, the extrusion force could be modulated by mixing the HA/DVS microparticles with fluid HA.

The results in Figure 7 reveal no potential cytotoxicity of the DVS residue according to the standard values. Therefore, the HA/DVS microparticles are potentially useful for in vivo applications.

CONCLUSIONS
The crosslinking degree that occurs in the reaction of HA with DVS has notable effects on the mechanical, swelling, and rheological properties of HA microparticles. Its correlation with these properties represents a novelty in the field and allows the control, modulation and optimization of the required properties of HA/DVS microparticle preparations. Moreover, the microparticles were not cytotoxic, increasing their value for use as viscosupplements or in other in vivo-specific applications.

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