

Influence of Particle Size and Fluid Fraction on Rheological and Extrusion Properties of Crosslinked Hyaluronic Acid Hydrogel Dispersions

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ABSTRACT: Crosslinked hyaluronic acid (HA) hydrogels are widely used in gel/HA fluid formulations as a viscosupplement to treat joint diseases; thus, it is important to characterize these hydrogels in terms of their particle size and to investigate the effects of the gel/fluid mixtures on their rheological properties and extrusion force. Hydrogels previously crosslinked with divinyl sulfone were sheared in an Ultra-Turrax unit to produce particles with mean diameters ranging from 20 to 200 μm . Hydrogels with 75–100 μm mean diameters were also evaluated in dispersions containing a 20–40% mass fraction of HA fluid. The mean diameters were measured by laser light scattering and the rheological behavior was determined by oscillatory and steady measurements in parallel plate geometry. The HA hydrogels exhibited the typical behavior of so-called weak gels, as analyzed by the storage and loss moduli G' and G'' , respectively. The viscoelasticity, the viscosity, and the extrusion force increased with the hydrogel particle size. The fluid phase dispersions decreased both moduli. At 40% fluid fraction, the gel characteristics were lost and the dispersion behaved as a fluid. Based on these results, the particle size and HA fluid fraction in hydrogel dispersions may be optimized to develop more efficient viscosupplement formulations. © 2012 Wiley Periodicals, Inc. *J. Appl. Polym. Sci.* 000: 000–000, 2012

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INTRODUCTION

HA is a linear viscoelastic biopolymer composed of a simple repeating disaccharide unit of D-glucuronic acid (GlcUA) and N-acetyl-D-glucosamine (GlcNAc) 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 Native and derivatives from chemical modifications of HA have a broad range of biomedical applications.²

Native HA has a molecular weight between 10^4 and 10^7 Daltons (Da) and is present in joint fluid at a concentration of about 3.5 mg mL⁻¹.³ In joint diseases such as osteoarthritis, the concentration and the molecular weight of HA in the synovial fluid are decreased, resulting in a diminished capacity of the fluid to protect cartilage.⁴

Viscosupplementation is a treatment involving intra-articular injections of high elastoviscous solutions of HA, commercially named hyaluronan or hylans (crosslinked derivatives of hyalur-

onan).⁵ Its purpose is to replace the lost HA, thereby restoring the rheological properties of the synovial fluid. Furthermore, the exogenous HA also potentially stimulates the production of endogenous HA which restores the normal homeostasis of the joint.⁶

In general, commercial viscosupplement formulations are composed of HA hydrogel particles (75–100 μm) crosslinked with divinyl sulfone (DVS) dispersed in HA fluid. The formulations are injected into patients using small-bore needles (27 and 30 G) to reduce undesirable side effects such as pain, bruising, bleeding, and edema. The hydrogel particles provide a large surface area, which enhances their adhesion at the application site. The fluid phase consists of uncrosslinked HA, which facilitates extrusion through the needles, even though it is easily metabolized and does not contribute to the prolonged residence time of the product. Therefore, the balance between the fluid phase fraction and the hydrogel particle size defines the effectiveness of the product.⁷

Although there are wide applications of HA viscosupplements, as far as we know there have been no systematic rheological characterizations of HA hydrogels reported in the literature, nor have their gel/fluid dispersions been associated with the extrusion force.

This study focuses on the effects of particle size and fluid phase fractions on the rheological and extrusion properties of HA dispersions, and aims to contribute to the design of efficient viscosupplement formulations.

EXPERIMENTAL

Materials

Hyaluronic acid (HA) with an average molecular weight of 10^5 Da was obtained by bacterial fermentation using *Streptococcus equi* subsp. *zooepidemicus* ATCC 39920, according to the protocol described by Pires.⁸ Divinyl sulfone and phosphate buffer saline (PBS) solutions were purchased from Sigma–Aldrich. All other reagents were purchased from Merck unless specified otherwise.

Methods

Preparation of Crosslinked HA Hydrogels. To investigate the effect of particle size on the rheological properties, crosslinked hydrogels were prepared in a 1 : 1 ratio of HA/DVS by weight, according to the methodology described by Leshchiner.⁹ The crosslinking reaction was performed by adding divinyl sulfone in a solution 3% (w/w) of HA in NaOH 0.1 mol L^{-1} containing 3% (w/v) NaCl. The pH of the reaction medium was maintained above 9 to facilitate the crosslinking reaction.¹⁰ The reaction was conducted at 25°C for 4 h. The product was washed for 2 days under reciprocal agitation at 200 rpm. After drainage of the wash solutions, the hydrogel was shaken at 200 rpm in a solution of 10 mM PBS for an additional 24 h.

Preparation of Crosslinked HA Hydrogel Particles. The crosslinked hydrogel was sheared in an Ultra-Turrax T25 disperser (IKA, Germany) at 24,000 rpm. The mean diameter of the particles was measured according to shearing time to obtain the required particle sizes. The measurements were performed by laser scattering in a Horiba LA-900 particle analyzer (Horiba Instruments Incorporated, USA). The particle size analysis was performed with the hydrogels dispersed in water. The standard deviation was calculated from ten measurements of the mean diameter.

Morphology of the Hydrogels. The morphology of the hydrogels was analyzed through optical microscopy images using a Reichert Jung—Series 150 Microscope (USA).

Preparation of Gel/Fluid Mixtures. To investigate the effects of the fluid phase on the rheological and extrusion properties of the formulations, the fluid phase was added to the hydrogel particles (75–100 μm mean diameter) to make 20, 25, 30, and 40% (w/w) fractions. The fluid phase consisted of HA (10^5 Da) solution 1% (w/v) in 10 mM PBS. The dispersions were placed in a reciprocal shaker at 200 rpm over 24 h.

Rheological Characterization. The rheological measurements were performed on a Rheometer Haake, model RheoStress 1 (Haake, Germany). The properties of the hydrogels were charac-

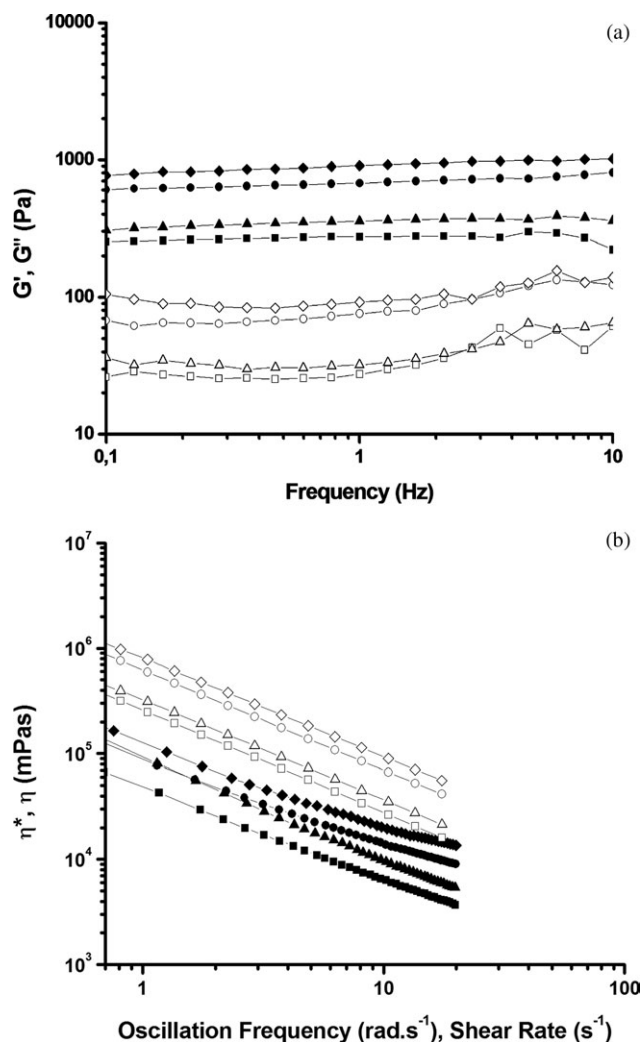


Figure 1. (a) Storage G' (closed symbol) and loss G'' (open symbol) shear moduli of hydrogels as a function of frequency: (■) and (□) $<25 \mu\text{m}$; (▲) and (Δ) $25\text{--}50 \mu\text{m}$; (●) and (○) $75\text{--}100 \mu\text{m}$; (◆) and (◇) $>100 \mu\text{m}$. (b) Complex viscosity (η^*) versus oscillation frequency (open symbol) and apparent viscosity (η) versus shear rate (closed symbol) of hydrogels at 25°C : (■) and (□) $<25 \mu\text{m}$; (▲) and (Δ) $25\text{--}50 \mu\text{m}$; (●) and (○) $75\text{--}100 \mu\text{m}$; (◆) and (◇) $>100 \mu\text{m}$. Temperature at 25°C .

terized in the steady and oscillatory regimes at 25°C , using a parallel plate geometry of 20 mm. Oscillatory measurements were conducted in the linear region, at a stress of 1.188 Pa and in the frequency range of 0.1–10 Hz. Steady shear measurements were obtained at shear rates of $0.1\text{--}20 \text{ s}^{-1}$.

Extrusion Force. The force required to extrude the hydrogels was determined by loading the hydrogels and their gel/fluid dispersions in 1-mL plastic syringes with 30-gauge needles. The measurements were performed in EMIC DL-3000 (Load Cell 10 kg) equipment at 25°C at a 50 mm min^{-1} extrusion rate.

RESULTS AND DISCUSSION

Rheological Characterization of the Hydrogel Particles

Effects of Hydrogel Particle Sizes. Figure 1(a) shows the mechanical spectra of the dynamic moduli: the elastic or storage

Table I. The Power Law Parameters ($G' = A \omega^B$) for Different Particle Size Ranges and Values of $\tan \delta$ (in 4.6 Hz) for HA Hydrogels with Different Particle Sizes

Average particle size range (μm)	A (Pas)	B	Tan δ
<25	357,849	0.03	0.15
25-50	592,925	0.06	0.18
75-100	2,667,473	0.05	0.17
>100	4,837,266	0.07	0.13

Power law relationship between storage modulus and oscillatory frequency ($G' = A \omega^B$).

modulus (G') and the viscous or loss modulus (G'') for the different particle size ranges of the DVS crosslinked HA hydrogels. It is observed that G' is higher than G'' in all studied frequency ranges. The curves are parallel to the frequency axis and both moduli are frequency independent, except at sizes smaller than $50 \mu\text{m}$ (<25 μm and 25–50 μm curves).

According to Ikeda and Nishinari,¹¹ the mechanical spectrum of gels can be related to the gel strength. Weak gels are slightly different than 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 G' higher than G'' ; however, the slope of the G' lines is zero and G'' displays a minimum at intermediate frequencies.^{11,12}

The mean diameter of the hydrogels affects the intensity of the moduli, since the elastic and viscous moduli decrease with the

mean diameter [Figure 1(a)]. This behavior is consistent with the results presented by Sadozai et al.¹³ The authors evaluated the rheological properties of biscarbodiimida crosslinked HA hydrogels which were previously disintegrated in a grinder and fractionated by diameter through cryogenic sieves. G' decreased with the particle size, which was attributed to the rigidity provided by the smaller particles due to the local stiffness imparted by a lower internal water content. Gauthier-Manuel et al.¹⁴ considered the greater contact among smaller particles leading to a higher specific stiffness of the system; this was attributed to the formation of rigid chain-like fibrillar structures. Thus, the lower G' value reflects the lower capacity of smaller particles to store large amounts of energy without breaking their gel structures. However, for the hydrogels prepared in this work, the smaller particles had a higher swelling capability. Therefore, the stiffness may be attributed to the higher number of heterogeneous domains even for the more hydrated smaller particles.

The degree of frequency dependence can also be determined by the well-known power law parameters ($G' = A \omega^B$) described by Ramkumar and Bhattacharya.¹⁵ 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 ω .

The B values define the mechanical strength of the gels. It is known that $B = 0$ for a covalent gel, whereas $B > 0$ for physical gels, according to Khondkar et al.¹⁶

Table I shows the power law parameters and the values of $\tan \delta$ ($=G''/G'$) calculated for the different ranges of hydrogel particle sizes.

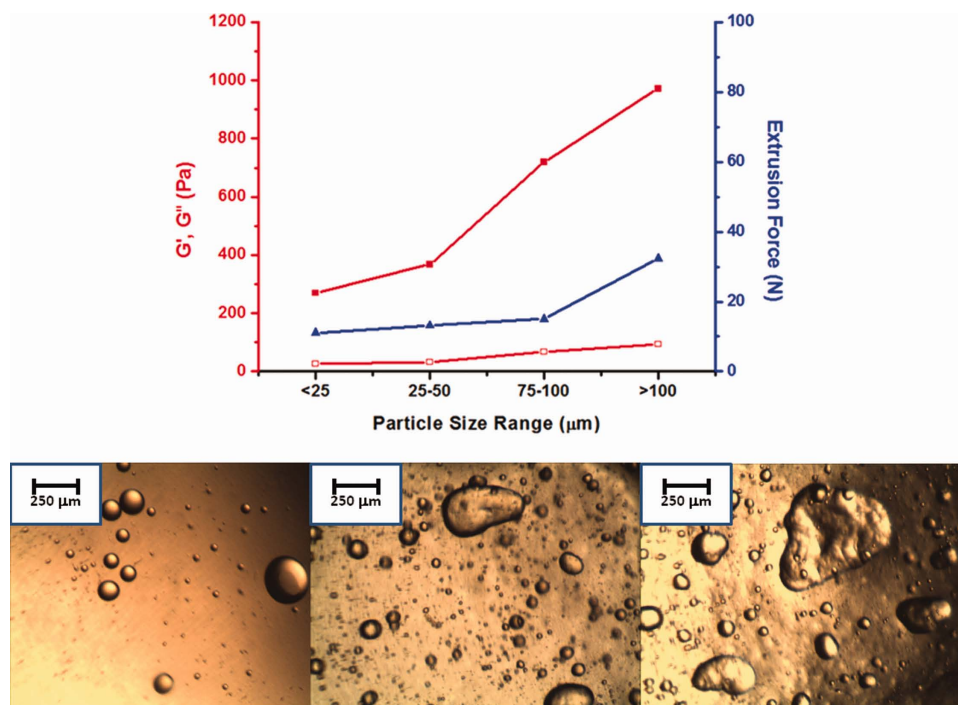


Figure 2. Viscoelastic components (G') and (G'') at 4.6 Hz, morphology and extrusion force through syringes with 30G $\frac{1}{2}$ needles as a function of particle size of the hydrogels at 25°C: (■) G' ; (□) G'' ; and (▲) extrusion force. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Values of $\tan \delta < 0.1$ suggest predominantly elastic gels, while for so-called weak gels, $\tan \delta > 0.1$.¹¹ Based on these criteria, the hydrogels in this work could be classified as weak, irrespective to their particle sizes.

Figure 1(b) shows curves of complex viscosity (η^*) versus oscillation frequency (ω), as well as the apparent viscosity (η) versus shear rate ($\dot{\gamma}$) for the different particle sizes of the HA hydrogels. A strong shear thinning is observed at higher shear rates, with values of flow indexes (n) as low as 0.1–0.32 indicating non-Newtonian pseudoplastic behavior.

Figure 1(b) also indicates that the HA hydrogels tend to depart from the Cox–Merz rule ($\eta^*(\omega) = \eta(\dot{\gamma})$ para $\omega = \dot{\gamma}$), confirming a weak gel structure.¹⁷ As known, the overall Cox–Merz rule is not obeyed by biopolymer dispersions with hyperentanglement (i.e., high-density entanglement), unless the shift factor (α) is considered.¹⁷ The failure of the original Cox–Merz rule suggests that rheological properties of weak gel systems stem from some deformation-susceptible structures. However, it does not necessarily mean that such structures are permanently lost under large deformations.

By multiplying ω in the original Cox–Merz equation by the calculated shift factors (α) in the range of 0.84–0.88, the two $\eta^*(\alpha\omega)$ and $\eta(\dot{\gamma})$ lines from our data were superimposed with a correlation coefficient (R^2) equal to 0.9. From these results, it could be concluded that the prepared HA hydrogels followed the modified Cox–Merz rule, indicating that the hydrogels are weak but able to undergo deformation.

Regarding the viscosity also increased with particle size. It is well known for colloidal and noninteractive particles that the viscosity is proportional to their volumetric fraction. Therefore, higher volumetric fractions are obtained with smaller particles. However, the largest hydrogel particles prepared in this work were swollen less than the smallest ones, probably due to greater entanglement of the polymer chains excluding water. Otherwise, Figure 2 shows the coalescence of larger particles, forming large domains, which leads to the entanglement of the total mass of polymer in the absence of particles. These factors could explain the viscous behavior as affected by the particle size of the hydrogels.

In addition to the viscosity and the viscoelasticity, the larger particle sizes also increase the extrusion force through 30G $\frac{1}{2}$ needles, as expected (Figure 2). Decreasing the particle size below 100 μm reduced the extrusion force $>50\%$. These results point to a compromise in the viscosity and viscoelasticity of the hydrogel formulations for application as viscosupplements.

The rheological behavior reflects the role of interparticle interactions. In general, hard shape models are used to describe the rheology of soft particle suspensions. The approach is successful for low and intermediate volume fractions. At large ones, where the interactions become important, the particular potential through which particles (hard or soft) interact should be determined, because the different nature significantly affects their macroscopic properties. While hard-sphere colloidal suspensions do not exhibit the same diversity of behavior as molecular glasses, deformable colloidal particles, when studied through their concentration dependence at fixed temperature, do exhibit the same variation

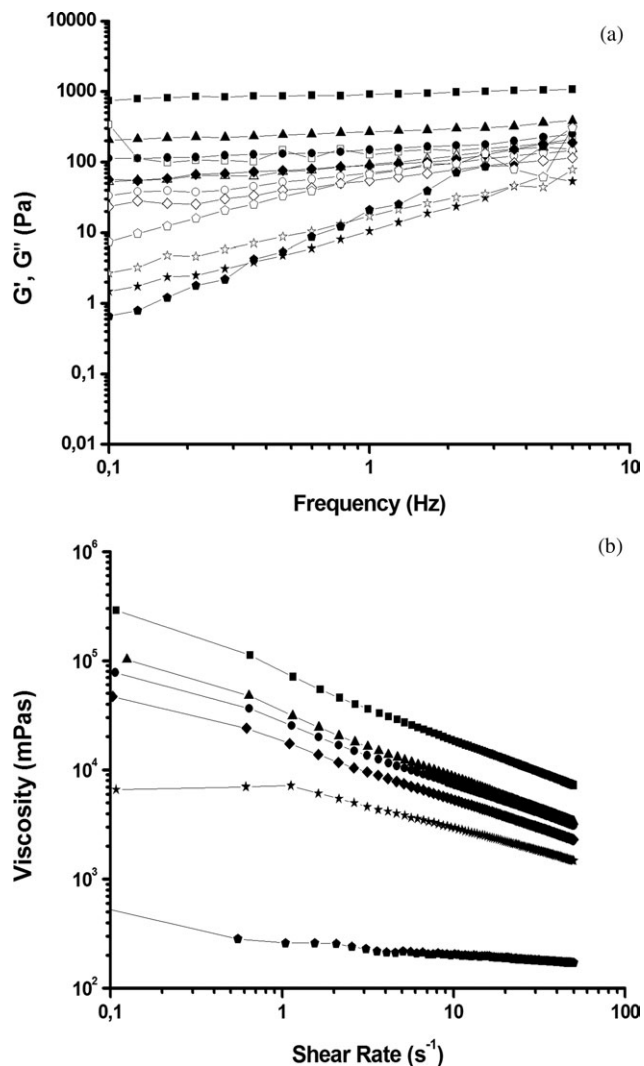


Figure 3. (a) G' (closed symbol) and G'' (open symbol) moduli of hydrogels with different percentages of fluid phase as a function of frequency: (■) and (□) 0%; (▲) and (△) 20%; (●) and (○) 25%; (◆) and (◇) 30%; (closed star) and (open star) 40%; (closed pentagon) and (open pentagon) 100%. The mean diameters of hydrogels in the dispersions were 75–100 μm . (b) Flow curves of hydrogels with different percentages of fluid phase: (■) 0%; (▲) 20%; (●) 25%; (◆) 30%; (closed star) 40%; (closed pentagon) 100%.

in fragility as that observed in the T dependence of molecular liquids at fixed volume. Their fragility is dictated by elastic properties on the scale of individual colloidal particles.¹⁸

Although the presence of small particles reduces the extrusion force, it is still high compared to the forces used in healthy injectable applications. To reduce the viscosity and retain the viscoelastic benefits of the hydrogel particles, commercial viscosupplement formulations contain crosslinked hydrogels dispersed in an uncrosslinked HA fluid.

Rheological Characterization of the Gel/Fluid Mixtures

Effect of the Fluid Phase in the Hydrogel Dispersions. Figure 3(a) shows the oscillation spectra of the hydrogels at different fractions of the fluid phase.

The curves show a decrease of both the G' and G'' moduli with the increment of the fluid phase in the dispersions. At 40% fluid phase, the formulation loses its gel characteristics and displays the typical behavior of a fluid as observed by the crossover of the moduli.

Figure 3(b) shows the flow curves for different fluid phase fractions in the hydrogel dispersions. These curves show a progressively decreasing viscosity with an increased fluid phase, due to the dilution of the hydrogel dispersions. For the pure hydrogel as well as for 10 to 30% fluid fractions, the dispersions show a pseudoplastic behavior with strong shear thinning at all studied shear rate ranges. However, for 40% fluid phase, when the dispersion loses its gel characteristic, the flow curves exhibit Newtonian behavior at low shear rates as observed by the Newtonian plateau; the non-Newtonian pseudoplastic behavior with shear thinning occurs at high shear rates only.

Considering the Ostwald de Waele power law ($\eta = K \cdot \dot{\gamma}^{n-1}$), the values of the K and n parameters^{19,20} are shown in Table II.

The n values indicate that the viscoelastic behavior depends on the fluid phase fraction in the dispersion. The 0.85 n value imparted by the fluid phase only indicates essential Newtonian behavior, as expected.

Figure 4 shows the forces required to extrude these samples through a syringe equipped with 30G $\frac{1}{2}$ needles.

These results show that the addition of a fluid phase reduces the extrusion force by 70%, which facilitates the application of the product and does not compromise the rheological properties of the formulations.

The results of this work indicate a strong tradeoff between the mean diameter of the hydrogels and its viscoelastic and viscous properties, as well as the extrusion force for injection which is related to the fraction of fluid HA in the hydrogel dispersions. For efficient viscosupplementation, the formulations have to contain an optimized amount of crosslinked hydrogel to assure the viscoelastic properties and prolong their *in vivo* residence time. On the other hand, they also need a low extrusion force which is guaranteed by the presence of a greater fluid fraction in the hydrogel dispersion. In this context, the rheological characterization associated with the extrusion force provides insight into the optimization of viscosupplement formulations, consid-

Table II. K and n Values in the Pseudoplastic Domain of η Versus $\dot{\gamma}$ Determined from the Flow Curves, as a Function of the Fluid Phase Fraction in the Hydrogel Dispersions

Fluid phase (%)	K	n
100	300.52	0.85
0	74361.8	0.40
20	32221.1	0.42
25	25440.2	0.46
30	17156.9	0.49
40	6326.9	0.65

The fluid phase (uncrosslinked HA in the fluid) consisted of HA (10^5 Da) solution 1% (w/v) in 10 mM PBS.

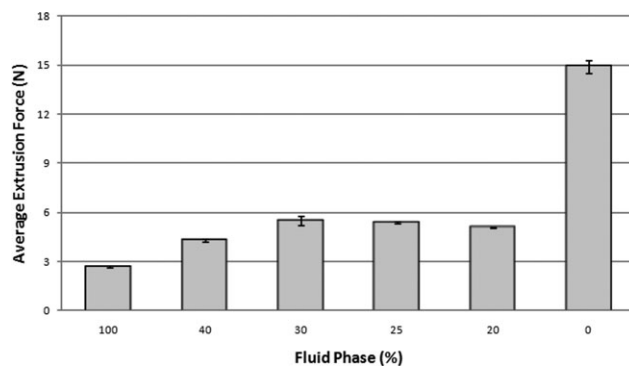


Figure 4. Influence of the fluid phase fraction on the extrusion force of hydrogels. The mean diameters of hydrogels in the dispersions were 75–100 μm .

ering compositions of different sizes of hydrogel particles dispersed in fluid fractions.

A quantitative estimative of the extrusion force, related to the pressure drop inside a tube, can be obtained from the rheological characterization. Similar to a Newtonian fluid, a relationship between flow rate and pressure drop, described by the Hagen–Poiseuille equation, can be obtained for a pseudoplastic fluid considering a particular empirical relation between shear stress and shear rate.²¹

CONCLUSIONS

This study shows that the HA hydrogels crosslinked with divinyl sulfone are weak but able to undergo deformation. There is flexibility of controlling the rheological properties of dispersions of the hydrogels through the particle size and fluid phase fraction. The association of the rheological properties with the extrusion force of the dispersions allows the optimum design of viscosupplement formulations containing different sizes of the HA hydrogels and fluid phase fractions.

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