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Research Article

Characterization of Polymeric Solutions as Injectable Vehicles for Hydroxyapatite Microspheres

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Abstract. A polymeric solution and a reinforcement phase can work as an injectable material to fill up bone defects. However, the properties of the solution should be suitable to enable the transport of that extra phase. Additionally, the use of biocompatible materials is a requirement for tissue regeneration. Thus, we intended to optimize a biocompatible polymeric solution able to carry hydroxyapatite microspheres into bone defects using an orthopedic injectable device. To achieve that goal, polymers usually regarded as biocompatible were selected, namely sodium carboxymethylcellulose, hydroxypropylmethylcellulose, and Na-alginate (ALG). The rheological properties of the polymeric solutions at different concentrations were assessed by viscosimetry before and after moist heat sterilization. In order to correlate rheological properties with injectability, solutions were tested using an orthopedic device applied for minimal invasive surgeries. Among the three polymers, ALG solutions presented the most suitable properties for our goal and a non-sterile ALG 6% solution was successfully used to perform preliminary injection tests of hydroxyapatite microspheres. Sterile ALG 7.25% solution was found to closely match non-sterile ALG 6% properties and it was selected as the optimal vehicle. Finally, sterile ALG 7.25% physical stability was studied at different temperatures over a 3-month period. It was observed that its rheological properties presented minor changes when stored at 25°C or at 4°C.

KEY WORDS: alginate; carboxymethylcellulose; hydroxypropylmethylcellulose; injectability; microspheres.

INTRODUCTION

Minimal invasive surgery has been commonly used over the past two decades since it uses smaller incisions and partial anesthesia, induces fewer complications and less post-operative pain as well as a fast patient recovery. As result, research in this particular area is being pushed forward in order to obtain further improvements (1). Minimal invasive surgery can be used to repair some damaged areas involving the procedure itself alone (endoscopy, thoroscopy and laparoscopy) or the procedure associated with injection of materials to fill defects and/or to deliver drugs. When injectable materials are used, the surgical procedures will depend on factors such as the type of materials, their rheological properties, setting time, etc. If the material is a single liquid solution, the procedure becomes easier. However, when solid particles are mixed with the liquid solutions, other features should be taken into consideration, namely the shape, density, porosity, size, and amount of particles to be used, since the solid phase has to be efficiently carried by the liquid solution (vehicle). Thus, the vehicle must be easy to manipulate (e.g., be injectable) and present a suitable viscosity to enable microparticles transportation. On the other hand, viscosity at different shear rates must be approximately constant since manual injection rate is difficult to control. In order to fulfill all these requirements, several polymers have been tested as injectable materials. Some of them include chitosan (2,3), Na-alginate (4), hyaluronic acid (5,6), hydroxypropylmethylcellulose (HPMC; 7–10), carboxymethylcellulose (NaCMC; 11), poly(propylene fumarate) (12,13), poly(ethylene glycol)-dimethacrylate), poly(β-caprolactone) (14), carboxylmethyl chitin (15), polymethylmetacrylate, and others (16). However, the use of specific solid particles in suspension requires an optimization of the vehicles in terms of their viscosity, injectability, and stability.

In this study, we were interested in selecting a suitable vehicle for injectable hydroxyapatite (HAp) microspheres developed at our laboratory (17). The preparation and characterization of these ceramic microparticles of spherical shape and uniform size (diameter around 500 μm) have been previously described, and *in vitro* studies revealed that they can be used as supports for culturing osteoblastic-like cells,

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suggesting their applicability as cell microcarriers for bone regeneration applications (18). However, in order to display adequate handling properties and to enable minimally invasive implantation, the microspheres must be combined with a vehicle. To accomplish our goal, we tested three polymers (Na-alginate (ALG), HPMC, and NaCMC), in terms of viscosity and injectability. Finally, ALG was selected as the most promising solution and its stability was evaluated over a 3-month period storage at different temperatures.

MATERIAL AND METHODS

Materials

Sodium alginate (Protanal 10/60LS) with a high α-L-guluronic acid content (65–75%, as specified by the manufacturer) was kindly donated by Pronova Biopolymers, Norway and used without further purification.

Sodium carboxymethylcellulose and hydroxypropylmethylcellulose are the cellulose derivatives most used for medical applications and also the most widely used polysaccharide in pharmaceutical industries. Sodium carboxymethylcellulose is prepared by treating cellulose with aqueous sodium hydroxide followed by reaction with sodium chloroacetate and it was purchased from Guinama, Spain. Hydroxypropylmethylcellulose (Methocel E4M Premium) which is prepared from cellulose using methyl halide and propylene oxide as reacting groups was a gift from Colorcon, USA. Hydroxyapatite is a calcium phosphate stable at pH≥ 4.2 with a chemical structure $[Ca_{10}(PO_4)_6(OH)_2]$ similar to the bone mineral phase. It was purchased from Plasma Biotal, Ltd., UK. All products were of pharmaceutical grade.

Preparation of the Polymeric Solutions

Sodium alginate was dissolved in deionized water for 24 h in order to obtain a homogeneous dispersion. The cellulose derivatives were dispersed in deionized water under low-speed mechanical agitation.

Preparation of the Microspheres

Hydroxyapatite microspheres were optimized in our laboratory in order to fill all the requirements for injectability (19,20). Briefly, a suspension of HAp particles in an ALG 3% (*w*/*v*) solution was extruded drop-wise into $CaCl_2$ solution followed by sintering at 1,200°C for 1 h.

Evaluation of the Rheological Behavior

Flow Assay

The rheological behavior was assessed by performing flow measurements at 20°C using a viscometer Viscotester VT550 (ThermoElectron, UK), fitted with concentric cylinder geometry (SV-DIN), between 1 and 1,000 s^{-1} (unless high viscosity did not allowed it) and backwards, with a 60 s delay period between measurements. The experimental results were fitted with Power law model $\tau = k \times \dot{\gamma}^n$ and consistency (*k*), and flow index (*n*) were determined. In power law equation, τ is the shear stress and $\dot{\gamma}$ is the shear rate. The power law model fits the experimental results for many materials over two or three decades of shear rate, and it is used extensively to describe the non-Newtonian flow properties of liquids (21).

Injectability (Extrusion) Assay

The injectability of the mixtures was evaluated using an injection device (LP2 Stainless Steel Delivery System—Biomet, Portugal) commonly used in vertebroplasty surgical procedures, which consisted of a plastic syringe (20 mm internal diameter), a cannula (2.7 mm internal diameter and 173 mm length), and a polymeric connection tube (Fig. 1). The syringe was filled up with the polymeric solution and the whole device was mounted on a Texture Analyzer TA-XT2*i* (Stable Mycro Systems, UK) working in compression mode. During extrusion tests, samples were assayed in triplicate applying the force vertically and using a plunger displacement rate of 1 mm/s. Results were expressed as the force needed to push the solution out from the cannula. Some preliminary tests using non-sterile ALG 6% (*w*/*v*; NS-ALG 6%) mixed with 20% or 40% (*w*/*w*) of microspheres were also performed.

The influence of ALG concentration on the extrusion force of ALG solutions and the effect of sterilization on the extrusion force of ALG 6% (*w*/*v*) solution were statistically evaluated by one-way analysis of variance (ANOVA). Post hoc comparisons of the means of individual groups were performed using Tukey's Honestly Significant Difference test. A value of $p<0.05$ was taken to denote significance. Statistical analysis was performed with SPSS 15.0 for Windows software (SPSS Inc., Chicago, IL, USA).

Influence of Sterilization on the Rheological Behavior

Sterilization of polymeric solutions was carried out in an autoclave (15 min at 121°C) and their mechanical properties were accessed after a storage period of 24 h at 20°C as described previously.

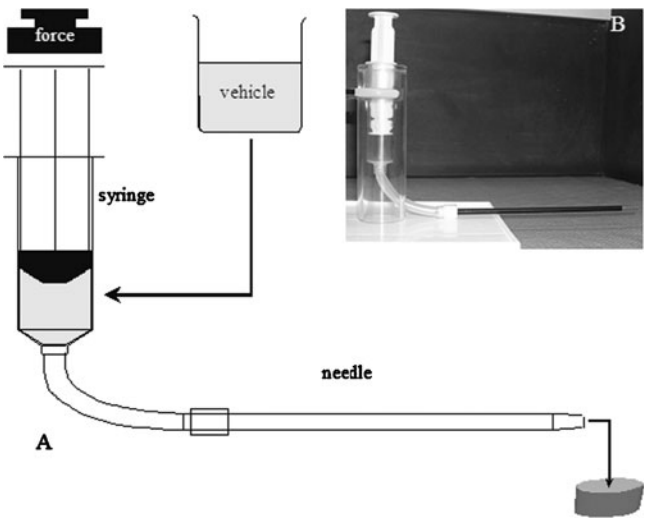

Fig. 1. Injectability device. Schematic representation of the procedure and device used to evaluate the injectability of the polymers. Injectability device scheme (**A**) and image of the injectability device (**B**)

Influence of Sterilization on Alginate Molecular Weight

Molecular weight was characterized at room temperature by High Performance Size Exclusion Chromatography (HP-SEC) using a modular system, composed of an isocratic pump (K-1001 Knaeur), a vacuum degasser (K-5002 Knaeur), a viscometer/right angle laser light scattering (RALLS) dual detector (T60 Viscotek), and a refractive index detector (K5002 Knaeur) operating at the same wavelength as the RALLS detector (670 nm). Separations were performed in a set of PL aquagel-OH mixed columns. The mobile phase consisted of 0.1 M $NaNO_3$ with 0.02% (*w*/*v*) NaN_3 and the flow-rate was maintained at 1.0 ml/min. Samples were dissolved in the mobile phase at 1 mg/ml, sterilized, filtered and injected through a manual injection valve equipped with a 116 μl loop.

Evaluation of the Physical Stability

The physical stability of the sterile ALG 7.25% (*w*/*v*; S-ALG 7.25%) solution was studied after storage for 3 months at 4°C, 25°C, and 40°C. Rheological properties were assessed on three batches performing flow measurements at 20°C using a viscometer (Viscotester VT550, ThermoElectron, UK) fitted with concentric cylinder geometry, in the shear rate range from 1 to 500 s^{-1} and backwards, with 60-s delay period between measurements. The effect of storage time and temperature on the viscosity of ALG solution was statistically evaluated by one-way ANOVA. Post hoc comparisons of the means of individual groups were performed using Tukey's Honestly Significant Difference test. A value of $p<0.05$ was taken to denote significance. Statistical analysis was performed with SPSS 15.0 for Windows software (SPSS Inc., Chicago, IL, USA).

RESULTS

Microspheres Properties

After sintering at 1,200°C, spherical-shaped microparticles with a uniform size were obtained as depicted in Fig. 2.

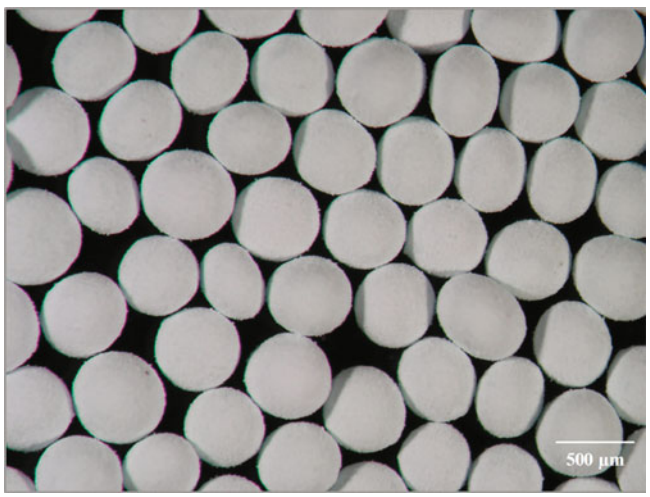

Fig. 2. HAp microspheres sintered at 1,200°C for 1 h. The picture was obtained by optical microscope, showing the homogeneous size and sphericity of microspheres after sintering

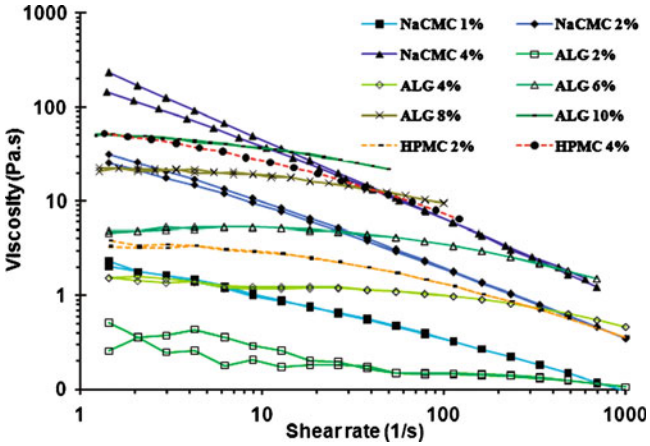

Fig. 3. Rheograms of polymeric solutions at different concentrations (T=20°C). NaCMC solutions (*filled square* NaCMC1%, *filled diamond* NaCMC2%, and *filled triangle* NaCMC4%) showed a decrease of viscosity for the whole range of shear rates tested while most of the ALG solutions (*empty diamond* ALG4%, *empty triangle* ALG6%, and *multiplication symbol* ALG8%) presented a viscosity approximately constant at shear rates below 30 s^{-1}

The microspheres diameter and compression strength were found to be around 500 μm and 0.35 N, respectively, as described in detail elsewhere (17).

Evaluation of the Rheological Behavior

When polymeric solutions are used in injectable systems their rheological behavior becomes one of the most important properties to be studied. In this work, the rheograms obtained showed that all polymeric solutions presented shear thinning behavior characterized by a decrease of viscosity with increase of shear rate (Fig. 3). The parameters of the Power law model are presented in Table I. The flow index, n, varies from 0.140 to 0.834, which confirms the shear thinning behavior. Flow indexes between 0 and 1 are characteristic of shear thinning liquids, while for shear-thickening liquids $n>1$ and for Newtonian liquids $n=1$. The polymeric solution with higher viscosity was NaCMC 4% (*w*/*v*).

An increase in consistency, k, following an increase in concentration was observed for all polymers. Increasing the concentration of NaCMC and HPMC, the rheological behavior showed a trend to increase the shear-thinning (lower n values) behavior whereas ALG solutions behaves closer to

Table I. Parameters of the Power Law Model

Product	Concentration (% *w*/*v*)	*K*	*n*	R^2
NaCMC	1	1.05	0.623	0.9987
	2	51.63	0.279	0.9939
	4	337.83	0.140	0.989
HPMC	2	14.32	0.480	0.9928
	4	126.33	0.391	0.9850
ALG	2	0.34	0.834	0.999
	4	4.69	0.669	0.9964
	6	20.47	0.614	0.995
	8	37.95	0.704	0.9972
	10	74.62	0.695	0.9977

k consistency, n flow index, and R^2 R-squared value

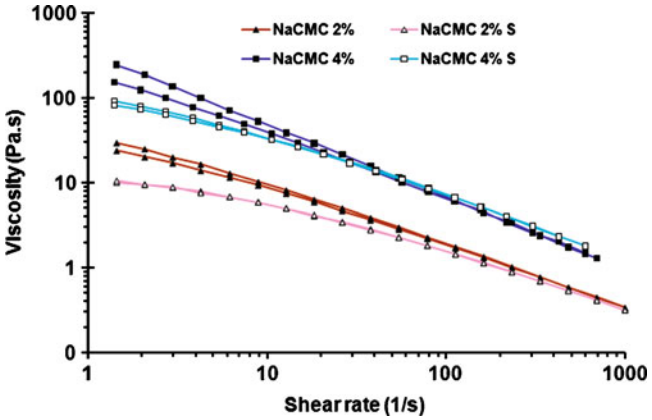

Fig. 4. Effect of sterilization on NaCMC solutions viscosity. After sterilization, a decrease of 50% in NaCMC solutions viscosity was detected for low shear rates, while at high shear rates the decrease was smaller. Sterile solutions are represented by *S*

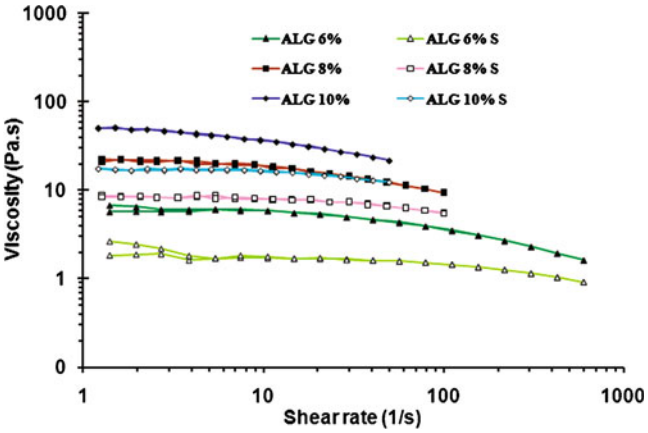

Fig. 6. Effect of sterilization on Na-alginate solutions viscosity. Alginate solutions viscosity dropped sharply after sterilization. However, it is still approximately constant at low shear rates. Sterile solutions are represented by *S*

Newtonian fluid, showing a decrease in viscosity only at medium to high shear rates. This behavior did not change with concentration as flow indexes remained approximately constant.

The polymeric solutions did not present thixotropy (represented by a hysteresis area between up and down curves) with the exception of the NaCMC solutions at higher concentrations.

Low modification of the mechanical properties after sterilization was found for cellulose derivatives solutions (Figs. 4 and 5). In contrast, the ALG solutions showed a marked decrease of viscosity (Fig. 6).

Injectability

In order to evaluate the injectability, polymeric solutions were extruded through a specially designed injection apparatus (Fig. 1) and force versus distance values were recorded and plotted (Fig. 7). The curves show the evolution of applied force during polymeric solution movement through the device.

The evaluation of extrusion force showed that HPMC 2% (*w*/*v*), NaCMC 2% (*w*/*v*), and ALG 2% (*w*/*v*) solutions were able to extrude at low forces, respectively, 33.3±1.4 N, 35.4±3.8 N, and 16.3±0.7 N. Furthermore, ALG 4% (*w*/*v*) and ALG 6% (*w*/*v*) solutions were extruded at similar forces (18.4±1.3 N and 34.3±0.8 N, respectively) but HPMC 4% (*w*/*v*) and NaCMC 4% (*w*/*v*) needed around 100 N to be extruded (123.5±7.4 N and 92.7±5.6 N, respectively).

In Fig. 8, it is observed that the force to extrude sterile ALG 6% (*w*/*v*; S-ALG 6%) decreased to about 20 N (18.7±0.6) as the result of sterilization and, as expected, sterile ALG 8% (*w*/*v*) and sterile ALG 10% (*w*/*v*) needed higher force, 47.5±2.6 N and 82.7±1.3 N, respectively.

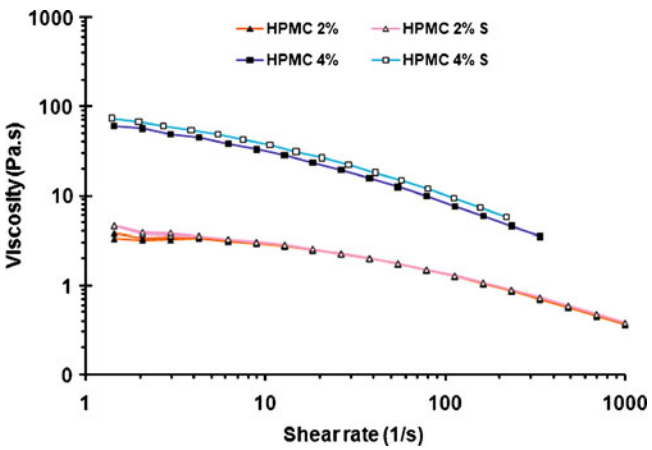

Fig. 5. Effect of sterilization on HPMC solutions viscosity. HPMC solutions viscosity was almost the same before and after sterilization. Sterile solutions are represented by *S*

Evaluation of Physical Stability

ALG solution stability was markedly affect when stored at 40°C, while minor modifications were observed at 25°C or 4°C (Fig. 9). After storage at 40°C for 1 day, the viscosity of ALG solution decreased (data not shown) and it continued to decrease with time. After 1, 2, and 3 months the viscosity was 56%, 36%, and 25%, respectively. In contrast, after 3 months, the ALG viscosity at both 4°C and 25°C did not fall below

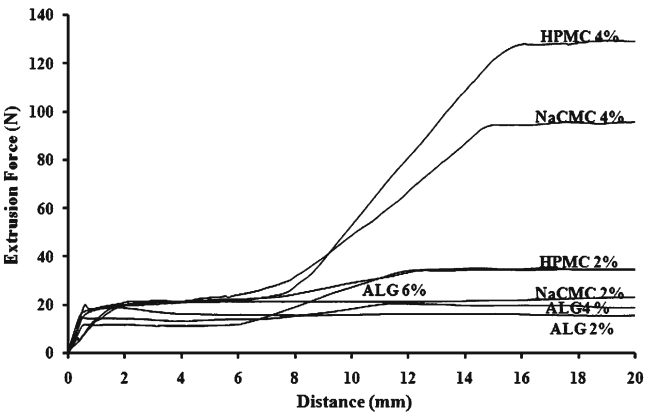

Fig. 7. Curves of injectability of polymeric solutions after extrusion using the device shown in Fig. 1. The curves were drawn using the values recorded during extrusion process and correspond to different polymeric solutions. The curves profile identifies the position of solutions inside of the device

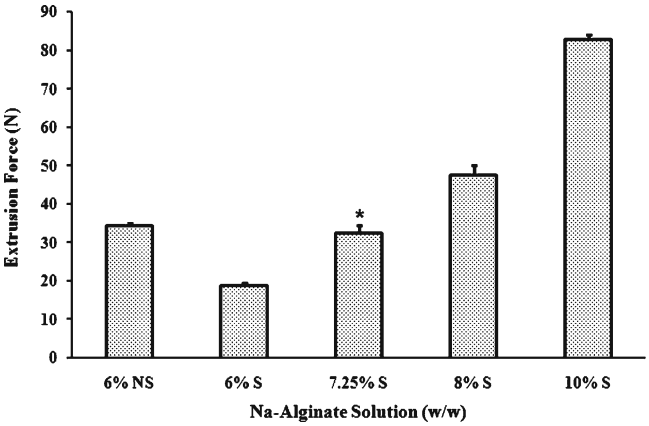

Fig. 8. Extrusion force of Na-alginate solutions. Na-Alginate solutions were autoclaved at 121°C for 15 min. *NS* represents a solution non-autoclaved and *S* represents autoclaved solutions. *Asterisk* not significantly different from 6%NS

85%. No statistically differences could be detected between 1 and 3 months ($p=0.899$) during storage at 4°C.

DISCUSSION

The design of an orthopedic device and the rheological properties of a vehicle play an important role on the injectability of materials. In terms of rheological properties, a shear-thinning behavior affects the injectability since a high shear-thinning leads to a decrease in viscosity when extrusion rate increases. As mentioned above, most of polymers analyzed in this study presented a shear-thinning behavior. Among those, NaCMC and HPMC solutions present the highest shear-thinning making them less suitable to use as vehicles since their ability to push a solid phase (when used) forward will be drastically affected if extrusion rate change occurs.

However, those polymers were less affected by the sterilization process. On opposition, ALG solutions presented a rheological behavior closer to Newtonian fluid ($n=1$) being less dependent of extrusion rate and more suitable to carry a solid phase across an orthopedic device.

On the other hand, as reported in the literature, ALG solutions undergo a decrease of viscosity after treated at temperatures above 80°C due to a degree of depolymeralization (22,23). Accordingly, in our study ALG solutions viscosity dropped considerably after sterilization by autoclaving.

Considering the orthopedic device design, it can be observed (Fig. 7) that the extrusion process is also affected by each component of the device. At the beginning, extrusion force increases in order to extrude the solution out from the syringe due to the high reduction of extrusion area. However, as the connection tube was filled up (first plateau—constant value of extrusion force) the extrusion force became approximately constant as it was more difficult to force solutions out of the syringe than fill up the connection tube. Once solution reached the cannula, extrusion force increased again until the solution started to come out from it. Cannula diameter is smaller than connection tube diameter increasing the extrusion force again. Following this last slope, a second plateau was observed. At high viscosities (HPMC 4% and NaCMC 4%), the influence of the cannula was marked by a high increase in the extrusion force. Therefore, during a surgical procedure, the use of cannulas of smaller length and higher diameter could eventually decrease the extrusion force needed to accomplish the injection procedure.

The extrusion process is difficult when mixtures prepared from solid and liquid phases are used and it becomes worst if the liquid phase presents high or low viscosity. For high viscosity solution a high force has to be applied to extrude a mixture, while for low viscosity solutions a phenomenon known as filtering will occur and solid particles will not be pushed through the cannula (24). Furthermore, low viscosity solutions easily invade blood vessels and may cause embolus in the circulatory system (25–28). On the other hand, solution's viscosity should be approximately constant at different shear rates (close to a Newtonian fluid) since injection is usually performed manually (variable injection rate). Among the studied vehicles, ALG solutions presented

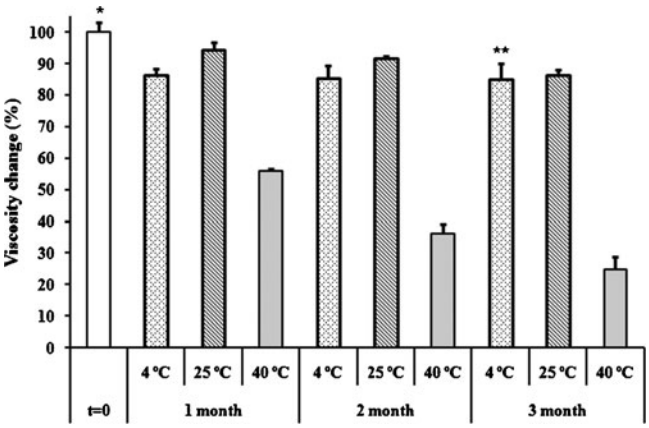

Fig. 9. Influence of storage time on S-ALG 7.25% solutions viscosity at 51 s^{-1}. The viscosity was highly affected after storage at 40°C. After 1 month the viscosity decrease was notorious and continued for the second and third months. At both 4°C and 25°C, only a slight decrease of viscosity was observed. *Asterisk* significantly different from all storage temperatures and times ($p<0.05$)—exception for samples stored at 25°C for 1 month. *Double asterisk* not significantly different from samples stored at 4°C for 1, 2, or 3 months and samples stored at 25°C for 2 months ($p>0.05$)

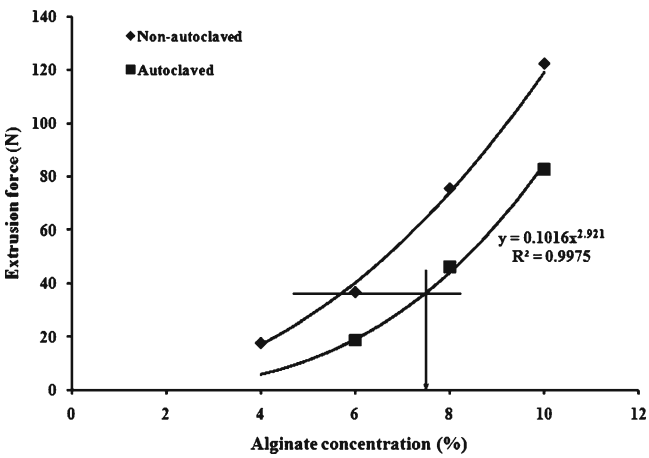

Fig. 10. Extrusion forces of Na-alginate after and before sterilization. Extrusion forces were measured on both autoclaved and non-autoclaved ALG solutions. Sterile ALG concentration that matched NS-ALG 6% extrusion force was computed from the regression equations of autoclaved solutions

the closest behavior to a Newtonian fluid ($n=1$) and they were easily injected using the orthopedic device above described. Thus, NS-ALG 6% was used to perform some preliminary tests, using both 20% and 40% (*w*/*w*) of HAp microspheres (diameter of 535±35 μm). In those assays, both mixtures were able to be extruded; however, the mixtures containing 40% (*w*/*w*) of microspheres were only extruded at forces of 166±40 N while mixtures prepared with 20% (*w*/*w*) of microspheres were extruded at 42±5 N. In the literature, values in the range 40-300 N (most accepted is 100 N) have been found as forces suitable to perform material injections which suggests that, in terms of rheological properties, NS-ALG 6% could be an appropriate solution to work as an injectable vehicle (29–31).

Although viscosity of NS-ALG 6% proved, empirically, to be proper to keep microspheres in suspension and to accomplish several injection assays, the sterilization method decreased its viscosity and the equilibrium of microspheres in suspension was lost. Therefore, new studies had to be performed in order to find a suitable concentration that, after sterilization, presents rheological properties similar to NS-ALG 6%.

Besides injectability, high mechanical strength of the injected mixtures was also a goal in this study. This objective can be accomplished by injecting a mixture combining the maximum possible amount of ceramic microspheres. However, the extrusion of sterile ALG 8% and sterile ALG 10% needed high extrusion forces; consequently a lower percentage of microspheres would have to be used to enable extrusion at forces below 100 N. Therefore, we focused on finding the alginate solution concentration whose rheological properties are similar to those of NS-ALG 6%. A mathematical model relating extrusion forces with ALG concentration was elaborated (Fig. 10). Afterwards, the ALG concentration that presented an extrusion force similar to NS-ALG 6% was calculated. S-ALG 7.25% was found to be the appropriate solution to replace the NS-ALG 6%. To confirm our approach, injectability tests using S-ALG 7.25% were performed and results showed that both S-ALG 7.25% and NS-ALG 6% presented similar extrusion forces (Fig. 8), which was also confirmed by the rheological tests. The statistical tests showed that the ALG concentration influenced the extrusion force, and the extrusion forces found for S-ALG 7.25% and NS-ALG 6% were not significantly different ($p=0.662$).

Upon submission to moist heat sterilization (121°C, 15 min), a decrease in the viscosity of alginate solutions was observed, as discussed before. In order to understand this behavior, the molecular weight of those solutions was measured and a decrease of more than 15% occurred. Before sterilization the molecular weight of alginate solutions was 85 kDa and after sterilization it has dropped to 70 kDa. This behavior was expected since moist heat sterilization promotes polymer breakdown, resulting in the reduction of the average molecular weight and consequently altering the rheological properties of the polymer solution (32).

Since both NS-ALG 6% and S-ALG 7.25% presented similar rheological behaviors and S-ALG 7.25% was stable for a period of at least 3 months when stored either at 25°C or at 4°C (behavior considered acceptable for a pharmaceutical product of extemporaneous preparation), it was chosen as the vehicle to perform future injectability tests using different concentrations of HAp microspheres.

CONCLUSIONS

In order to use a polymeric solution as a vehicle capable of carrying solid particles, suitable rheological and chemical properties should be reached. In this investigation, we were looking for a vehicle suitable to carry HAp microspheres through an orthopedic device during an injection procedure. Among the polymeric solutions studied, ALG presented the closest behavior to a Newtonian fluid, so it was selected as the polymer for future studies.

NS-ALG 6% proved to be able to carry 40% (*w*/*w*) of HAp microspheres through an orthopedic device. Moreover, S-ALG 7.25% presented rheological properties similar to NS-ALG 6%. For injection into a bone defect, sterility is a mandatory feature, and so S-ALG 7.25% was selected as the most promising solution. It proved to be stable over 3 months when stored at 4°C which further reinforces its suitability as injectable solution for ortophedic surgery.

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