Synthesis and rheological evaluations of novel injectable sodium alginate/chitosan-nanostructured hydroxyapatite composite bone pastes

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Abstract:
In this paper, nanostructured carbonated hydroxyapatite powder was synthesized using biomimetic method. Physical properties and morphological features of the powder was studied using appropriate techniques such as BET, TEM, XRD and FTIR. The powder was separately mixed with 3 wt. % solutions of either sodium alginate or chitosan to form different pastes. Rheological behavior of the pastes in oscillation mode was measured. Results showed that mean particle diameter and surface area of the powder were 3.9 µm and 154.8 m²/g, respectively. According to dynamic rheological evaluations, all pastes exhibited shear thinning behavior with linear viscoelastic limitations, although paste containing sodium alginate displayed broader linear viscoelastic range than others. Increasing concentration of sodium alginate or chitosan led to increment of viscosity, meanwhile sodium alginate produced a material of higher viscosity than did chitosan. When sodium alginate was employed, lower force was required for injection. Totally, these bioactive pastes potentially can be used for the treatment of hard tissues.

Keywords: Nanostructured carbonated hydroxyapatite, Rheological behavior, Injectability, Biocomposite

1. INTRODUCTION
Until now, numerous bone graft surgeries have been performed for bone injury and disease healing around the world. [1]. Bioactive materials have been extensively considered for bone and dental implants and substitutes [2-5]. Hydroxyapatite (HA) is an osteoconductive and non-toxic material, which has been widely used in dental and orthopedic surgeries. [6]. However there are some clinical limitations when using HA as bone substitute. For example, it is difficult to fit HA blocks in defect site, i.e. it is impossible to completely fill the bone prosthesis gap of an irregular or narrow shape with HA blocks. When HA products are used in the form of granules, it is difficult to prevent migration from the implantation site toward surrounding tissue. [7].

Bone graft in the form of injectable paste gives several advantages over the granule and block forms as it could be molded in defect area even with limited accessibility. HA pastes can be used for the treatment of spinal fragility fracture, cranioplasty, maxillofacial surgery and root canal sealing [8-12].

 Basically, injectable bioactive pastes can be divided into two different categories: cement type and non-cement type. In cement type pastes, microstructure improves by a chemical reaction between the paste ingredients that result in formation of new phase and network as well as an increase in paste viscosity. Therefore, after a certain time, the paste sets and becomes hard. However, in non-cement pastes, there is no chemical reaction between the paste components and no phases are formed. For these pastes, viscosity may just modify by physical interaction of constituents as well as drying process. Calcium phosphate cements (CPCs) made of reactive calcium phosphates and aqueous solution of phosphate salts are the most well known cementitious bioactive materials. In contrast, the pastes made of non-reactive calcium phosphates such as HA and β-TCP and polymeric solutions (for instance, polysaccharides) can be addressed as non-cement pastes, which are adequate, bone fixation and bone-prosthesis gap fillers [21-23].
The rheological properties of cementitious paste such as calcium phosphate cements and polymethyl methacrylate (PMMA) bone cement have been discussed in papers [13–15]. For non-cement pastes, the rheological behavior of aqueous β-TCP suspensions was also investigated by Baroud et al. [16] and Bohnet et al. [17]. For both types of pastes, the role of some parameters such as particle size, powder to liquid ratio and particle size distribution of solid phase has been assessed. Injectability of β-TCP paste with liquid phase of hyaluronic acid and polyethylene glycol or polyvinyl alcohol was measured by Hesaraki et al. [18]. In another work of theirs a non-cement paste composed of bioglass/hyaluronic acid/ sodium alginate was employed and effect of different particle characteristics of powder evaluated on its dynamic and static rheology [19].

In this study, injectable biocomposites are developed based on nanostructured hydroxyapatite and two different polymeric solution: chitosan and sodium alginate. This paper describes an attempt to utilize rheological measurements in the paste as a reasonable indicator of workability.

2. METHODS AND PROCEDURES

2-1- Sample preparation
Calcium carbonate (CaCO_3) [Merck 2076], Dicalcium phosphate anhydrate (Monetite, DCPA) [Merck 2144], Dicalcium phosphate dihydrate (Brushite, DCPD) [Merck 172.06] and Disodium hydrogen phosphate [Merck] were used as starting materials. Powder constituent of the paste, nanostructured hydroxyapatite, was synthesized by using calcium phosphate cement (CPC) production through biomimetic method, as described elsewhere [20]. Three different liquids were considered in this research: distilled water for synthesis of hydroxyapatite as a control sample, 3 wt. % solutions of sodium alginate [Aldrich] and 3 wt. % solutions of chitosan [Chitotech].

2-2- Formulation of pastes
In this research, various hydroxyapatite-based pastes at powder to liquid ratio of 2 g/ml were prepared. This ratio was according to the formation of a consolidated and workable paste. The uniformity test for the paste was accomplished according to the previous work [17].

The chemical formula and the codes of various pastes have been shown in Table 1. In all samples, the biomimetic hydroxyapatite was used as a powder.

<table>
<thead>
<tr>
<th>Samples code</th>
<th>Composition of liquid phase (%Vol.)</th>
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<tbody>
<tr>
<td></td>
<td>Distilled water</td>
</tr>
<tr>
<td>H100</td>
<td>100</td>
</tr>
<tr>
<td>H75-C25</td>
<td>75</td>
</tr>
<tr>
<td>H50-C50</td>
<td>50</td>
</tr>
<tr>
<td>H25-C75</td>
<td>25</td>
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<tr>
<td>H75-N25</td>
<td>75</td>
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<tr>
<td>H50-N50</td>
<td>50</td>
</tr>
<tr>
<td>H25-N75</td>
<td>25</td>
</tr>
</tbody>
</table>

2-3- Characterization and Measurement
Particle size distributions of synthesized carbonated hydroxyapatite (CHA) were examined by laser particle size analyzer (LPSA) via a Fritsch Particle Size ‘analysette 22 instrument in the acetone-alcohol medium. The Barrett–Emmett–Teller method (five point BET) was utilized to calculate the specific surface area of the powders by Micromeritics Gemini 2360 device.

Transmission electron microscopy (TEM; CM200-PEG-Philips, working voltage of 200 kV) was used for characterizing the morphology of nanoapatite. For this purpose, a diluted suspension of CHA in ethanol was deposited onto a carbon film supported by Cu grids.

Phase analysis of CHA powder was performed by a Philips PW3710 diffractometer. This instrument worked with voltage and current intensity of 40 kV and 30 mA, respectively and utilized Cu-Kα radiation (1.542 Å). In addition, the average crystallite size of the synthesized hydroxyapatite was determined by Debye–Scherrer’s formula using (002) atomic plane data situated in 2θ=25.90°:

\[ D = \frac{k\lambda}{\beta\cos\theta} \]  

where D is the crystallite size, k is a constant (shape factor, about 0.9), λ is the X-ray wavelength (1.542Å), β is the full width at half maximum (FWHM) of the diffraction line, and θ is the diffraction angle.

The chemical groups in apatite phase were examined by FTIR analysis. Fine powders of specimens were mixed with KBr powder in the ratio of 10:800 and transparent homogenous discs were formed. In this test, data were collected at room temperature at the wavenumber range of 400–4000 cm⁻¹ and resolution of 2 cm⁻¹ using a spectrometer Bruker Vector 33.
Rheological behavior of the pastes was measured using rheometer instrument (model: physica MCR 301, Anton paar Company, Austria). The device was adjusted on oscillatory mode in which a sinusoidal shear deformation is applied to the sample and the produced stresses are measured. The storage ($G'$) and loss ($G''$) moduli determine viscoelastic characteristic of pastes. The storage modulus represents the elastic part of the paste and the loss modulus shows its viscous portion. In this technique, at first strain sweep is carried out to determine linear viscoelastic region and then in frequency sweep $G'$ and $G''$ are measured as a function of angular frequency ($\omega$).

To assess injectability, the paste was conveyed to a 10 mL standard syringe, and then extruded by a compressive load vertically mounted on top of the plunger. The test was accomplished by a computer controlled testing machine of mechanical properties (ZWICK/ROELL HCR 25/400) at a crosshead speed of 15 mm/min. The applied load was plotted against extension.

3. RESULTS
3.1 Particle size distribution and specific surface area
According to the curve of particle size distribution, the mean particle diameter of the synthesized nanostructured apatite powder is 3.9 µm. In more details, 80% of the particles have a size less than 11.9 µm (Fig. 1). Specific surface area of CHA measured by BET analysis is 154.8 m²/g.

![Particle size distribution of synthesized hydroxyapatite](image)

3.2- Phase composition and chemical groups
Fig. 3 depicts XRD pattern and FTIR absorbance spectrum of the synthesized apatite. In Fig. 3-a, all peaks correspond to carbonated hydroxyapatite phase and no impurity is observed. Peak width broadening represents poor crystallinity of the prepared CHA. Based on Debye–Scherrer equation the calculated size of crystallites is about 10.02 nm, which coincides with TEM results.

In Fig. 3-b, the bands locate at around 570 and 605 cm⁻¹ are attributed to the PO₄ groups. The existence of bands at 870, 1420 and 1470 cm⁻¹ are due to the substitution of carbonate ions (CO₃²⁻) for phosphate ions (PO₄³⁻) in lattice of apatite. The band observed at 870 cm⁻¹ is also related to HPO₄²⁻ which confirms deficiency of apatite phase. Although detecting HPO₄²⁻ peak is really difficult (because of overlapping with carbonate bands), the presence of HPO₄²⁻ instead of PO₄³⁻ indicates a specific disorder in the apatite structure and confirms that the apatite phase is completely amorphous. Two bands are also seen for the sample at around 1650 and 3500 cm⁻¹ due to the adsorbed water and stretching mode of hydrogen bands of OH⁻ in the structure [21].

3.3- Rheometric curves of pastes
Fig. 4 demonstrates the storage/loss moduli vs. strain curves of samples H100, H75-C25, H50-C50, H75-N25 and H50-N50 in strain sweep. According to the figures (4-a) to (4-e), the storage modulus is constant in low values of strain-the linear viscoelastic region (LVE) - and suddenly decreases by increasing the strain. The strain that curve drops is selected for frequency sweep test. There are many scattered points in the curves of pastes synthesized using distilled water which are attributed to inhomogeneity of pastes prepared from distilled water (Fig. 4-a). Based on Fig. 4-b, for H75-C25, the value of $G'$ is higher than $G''$ before 10% strain. This represents gel-like structure of the paste. At strains more than 10%, $G''$ overcomes $G'$ and the paste behaves like fluid. The strain in which $G'$ curve coincides with $G''$ is called flow point. For pastes prepared with chitosan linear viscoelastic region is defined at strains below 1%. The linear viscoelastic region of pastes containing sodium alginate is broader than chitosan-based pastes and the strain considered 10% for these pastes. From Fig. 4, in linear viscoelastic region of polymer containing pastes, storage modulus is higher than loss modulus exhibiting that these pastes have a gel-like structure.
Fig 2. TEM image of n-HA synthesized by biomimetic method.

Fig 3. (a) XRD pattern and (b) FTIR spectrum of synthesized apatite.

Fig 4. $G'/G''$ as a function of strain (a): H100, (b) H75-C25, (c) H50-C50, (d) H75-N25 and (e) H50-N50.
Fig 5. The curves of $\eta^*$-ω and $G'/G''$-ω of samples: (a) H100, (b) H75-C25, (c) H50-C50, (d) H75-N25 and (e) H50-N50.

Fig. 5 shows the rheometric curves of different pastes in frequency sweep mode. The storage modulus indicates ability of paste in energy reservation. The loss modulus specifies the ability of material in energy dispersion. According to Fig. (5-b), viscosity decreases by increasing the frequency that is representative of shear thinning behavior. It should be taken into account that, within linear viscoelastic region, no change is observed in $G'$ and $G''$ values when $\omega$ increases. Moreover, the amount of storage modulus is always higher than loss modulus i.e. the paste has a solid-like behavior. When the amount of chitosan increases, viscosity augments drastically (compare Fig. 5-c to Fig. 5-b). Fig. (5-d) shows that the viscosity decreases by increasing angular frequency. The same behavior is observed for sodium alginate containing pastes. Increasing chitosan content causes $G'$ and $G''$ to become closer that means the behaviour of the paste nears to liquid. In contrast, by increasing sodium alginate the gap between $G'$ and $G''$ enlarges that proves solid nature of the paste.

3-4- Injectability curves of pastes

Fig. 6 illustrates the load against displacement curves of pastes with/without different concentrations of chitosan/ sodium alginate. The injectability of the paste containing 25% chitosan is similar to the control paste. Increasing chitosan to 50% changes injectability considerably as the required force to overcome yield stress is increased by two times. It confirms that when viscosity of the paste increases consequently, injection process is faced with practical difficulties. Moreover, the paste is partially extruded and one-fourth of the paste remains in the syringe. By increasing chitosan up to 75%, the yield stress increases but more volume of the paste is extruded from the syringe. When sodium alginate is added to the paste, a lower force is needed to start injection in compare to the control paste. Sodium alginate enhances injectability so that the paste is completely injected with lower force than the others one.

4. DISCUSSION

In the present study, nano hydroxyapatite was synthesized through biomimetic method. It has been found by other studies that inorganic phase of bone is nanocrystalline carbonated apatite that incorporates into the mineralized collagen matrix during the new bone formation at the material/host bone interface [22]. In other words, HA will be more osteointegrative and osteoconductive, if its chemical composition and structure be more similar to biological apatite in terms of both crystallinity and deviation from stoichiometry [23]. Simulated body fluid (SBF) with ion concentrations similar to those of the inorganic constituents of human blood plasma is able to act as a medium for development of such apatite phase [24]. nano-sized needle-like crystals of pure carbonated hydroxyapatite with high surface area and mean particle diameter of 3.9 μm was obtained.
The elastic and viscous characteristics of material can be determined by applying oscillatory shear to explain its internal structure. In a dynamic test, a sinusoidal oscillation strain is applied to the sample and as a result, the obtained stress compares to the strain. The ratio of stress to strain gives a complex modulus (G*). The phase difference (δ) of two sinusoidal curves of stress (σ) and strain (γ) gives the storage and loss moduli [25]:

\[ G^* = G' + i G'' \]  \hspace{1cm} (2)
\[ G' = G^* \cos \delta \]  \hspace{1cm} (3)
\[ G'' = G^* \sin \delta \]  \hspace{1cm} (4)

Measurement of dynamic rheological properties is performed in three cases: strain sweep, frequency sweep and time sweep. In the case of strain sweep, sequence measurements are done by increasing strain in a constant frequency and temperature. In the case of frequency sweep, the measurements are done at variable frequencies in a constant strain and temperature. The frequency measurement has to be accomplished in linear viscoelastic region to avoid destroying internal structure. For this purpose, at first, the curves are drawn in the strain sweep mode to obtain maximum strain in which material has linear viscoelastic behaviour. This strain can be used for frequency sweep mode.

The shear thinning behavior of paste proves interactions between polymer molecules and HA particles that form internal structures. These structures can be easily destroyed by applying shear stress and then reconstructed by removing it. Since no setting reaction occurred in the pastes and no strong interaction existed between particles, there was no resistance to flow and the paste was easily injected. The shear thinning behavior was observed for all pastes. The frequency-sweep test indicated characteristics of the pastes at variable frequencies and constant strain. This research revealed that all non-cement pastes composed of nano hydroxyapatite and chitosan /sodium alginate had viscoelastic behavior. The linear viscoelastic limit varied by changing strain. The pastes containing sodium alginate displayed linear viscoelastic property in a broader range of strain than the other pastes. By adding polymer, the elastic term was also predominant but distance between storage modulus and loss modulus curves was lower for samples containing chitosan. It seems that increasing concentration caused the sodium alginate paste to behave like solid but chitosan had the opposite effect. Measurement of rheological properties is useful for better understanding of effective parameters on flowability of paste. Although pastes prepared with hydroxyapatite do not set, presence of chitosan in the composition can cause consolidation of pastes due to the gelation phenomenon. Required pressure for injection is divided into two parts. First, the paste requires a pressure to overcome yield stress, second it needs a pressure to flow. The latter is determined by the viscosity of the paste [16]. There are many factors that can influence flowability of pastes such as: P/L ratio [26], chemical composition and reactivity [27], particle size distribution, chemical properties of additives and etc. chitosan and sodium alginate provided a viscous layer between particles which helps particles slip over each other without phase separation [28]. Rheological properties of non-cement pastes can be influenced by using different polymeric solutions. Chitosan and sodium alginate were selected as sources of polymeric solutions. The former is a promising material for bone regeneration because it is biocompatible and biodegradable and the latter is clinically used in the treatment of peptic ulcer disease. Because of its viscosity, sodium alginate could possibly become a new submucosal injectable
material for using in endoscopic resection. [29, 30]. Chitosan has different biological properties that make it attractive to medical applications including: biodegradability, lack of toxicity, anti-fungal effects, acceleration of wound healing and stimulation of immune system [31]. Sodium alginate has been used in food, cosmetics, and pharmaceutical products for 100 years. Because of its viscosity, it has protective effect on mucous membranes and possesses hemostatic effect by acceleration of fibrin formation [32, 33].

5. CONCLUSION
Sodium alginate/chitosan solutions can be mixed with hydroxyapatite nano-particles to prepare injectable biocomposite. Nanostructured carbonated hydroxyapatite is obtained by biomimetic route with mean particle diameter of 3.9 nm and surface area of 154.8 m²/g. Overall, all pastes have non-Newtonian behavior which exhibit shear thinning characteristics. The paste made of sodium alginate has higher viscosity than the one made of chitosan so the former has better injectability than the latter. The elastic term is always exceeded by the viscous one but the distance between storage modulus and loss modulus is smaller in chitosan containing pastes.

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