Porous spherical hydroxyapatite and fluorhydroxyapatite granules: processing and characterization

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Abstract

This study is aimed at the development of a method to fabricate porous spherical hydroxyapatite (HA)—fluorapatite (FA) granules. The method to produce porous granules is based on liquid immiscibility effect. A suspension of HA–FA powder mixtures in aqueous solution of gelatin and oil as a dispersion media were used. By stirring the mixtures of these immiscible liquids, granules of 50–200 μm diameter can easily be produced. The granules were characterized with respect to their microstructure, phase composition and specific area. In vitro testing of human plasma protein adsorption onto the granules of HA and fluorhydroxyapatite were performed. No kind of difference in the dynamic protein adsorption between pure HA and the HA up to 10 wt% FA materials has been revealed.

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1. Introduction

Hydroxyapatite (HA) is the most stable and biocompatible calcium phosphate. The use of HA in orthopedic applications has become common place, e.g. as a porous block or granule. In dependence of their size, microstructure, pores content and distribution, the granules may find application in dental, periodontal, oral/maxillofacial surgical procedures and skeletal bone surgery [1]. Owing to their physicochemical and biological properties, calcium phosphates have recently been considered as a potential material for a bone drug delivery system. This type of drug delivery system, using a bioactive matrix, can release a therapeutic agent in situ to produce an action associating the osteoconductivity of the material [2–4].

The mineral phase of hard tissues contains significant amount of fluoride. Fluoride ions present in saliva and blood plasma are required for normal dental and skeletal development. The incorporation of fluoride ions into the structure of HA can stimulate bone cell proliferation and increase new mineral deposition in cancellous bone [5]. Besides, the better stability of fluorhydroxyapatite (FHA) in biological environments as compared to HA can also be considered as a positive factor for future prospective application, e.g. for a bone drug delivery system. It has been particularly demonstrated that the FHA showed good integration in the bone tissue and much longer resorption time than classic calcium phosphate [6].

A number of studies have reported the fabrication of HA granulates by various techniques, with irregular or spherical geometry [1,2,7]. The later is much preferable to eliminate non-desirable inflammation reactions from the body soft tissues [2], so the rounded granules with smooth geometry are preferable for medical application. In particular, porous spherical HA granules of 50–200 μm diameter have been produced by the dispersion of well-mixed HA/polymer slurry in a dispersion medium [2,7].
In this context, the present work was aimed at the investigation of the effect of the suspension stirring rate, heat treatment conditions and fluorine substitution for 
OH−
 groups on fabrication and behavior of porous HA—base granules. Also in vitro testing of human plasma protein adsorption onto the granules of HA and FHA was performed.

2. Materials and methods

The HA and FHA powder were supplied by the Institute of General and Inorganic Chemistry RAS. The analytical grade CaCl2, (NH4)2HPO4, NH3OH, Ca3(PO4)2 and CaF2 were used as the starting reagents. The powder of HA was fabricated by precipitation from aqueous solution according to the following reaction:

\[
10\text{CaCl}_2 + 6(\text{NH}_4)_2\text{HPO}_4 + 14\text{NH}_3\text{OH} \Rightarrow \text{Ca}_{10}((\text{PO}_4)_6(\text{OH})_2 + 20\text{NH}_4\text{Cl} + 6\text{H}_2\text{O} \quad (1)
\]

while the FA powder was prepared by solid state interaction as follows

\[
3\text{Ca}_3(\text{PO}_4)_2 + \text{CaF}_2 \Rightarrow \text{Ca}_{10}((\text{PO}_4)_6\text{F}_2 \quad (2)
\]

The synthesis method and route for HA were described in detail elsewhere [8]. Raw product of reaction (1) was calcined at 1000 °C for 8 h in air atmosphere. The reaction (2) was under study in [9,10]. The mixture of tricalcium phosphate, Ca3(PO4)2, and calcium fluoride, CaF2, in equimolar ratio (Ca/P = 1.67) were subjected to the milling in a ball mill at the ratio of 1:3 (powder:balls) with ethanol addition for 6 h. After that the mixture was calcined at 600 °C for 3 h in a furnace with air atmosphere, followed by milling and heat treating at 1100 °C for 2 h in a dry nitrogen atmosphere. The particle size of the powders was estimated from scanning electron microscopy (SEM) measurements (a JEOL JSM-35-CF microscope, Japan). The Ca/P ratio was evaluated by wet chemical analysis. The phase composition of the powders was monitored by comparing acquired spectra with peaks identified in the Joint Committee on Powder Diffraction Standards (JCPDS) database of standards.

The mixtures of HA with FA were prepared in a ball mill using ethanol media and corundum balls at the batch-to-balls ratio of 1:4. After drying at 90 °C, the batches containing 0, 1, 2 and 10 wt% FA were obtained.

For preparation of porous spherical granules, a food grade bovine gelatin P-11 (Russian Standard GOST 11293-89) was used. It was supplied by Gelatin Factory, Moscow, Russia. Vegetable oil, as a dispersion media, was of food grade having the relative density of 0.917 g/cm³ and viscosity of 0.585 Pa s at room temperature. Aqueous solution of 10% gelatin content was prepared by dissolution of 10 g in 100 ml distilled water at 39 °C. Fine powders were added to the above solution in amount of 1 g of powder per 2 ml of gelatin solution. After that, the slurry was dispersed in oil in a flask by stirring with a glass paddle stirrer at 1000 rpm at oil temperature from 39 °C. The stirring results in bead formation due to the surface tension forces. The precipitated beads were washed in acetone followed by ethanol, filtered onto a Buechner cone filter and dried in air. These gelatin-bonded beads were heated initially at 500 °C for 30 min for burning-off gelatin and finally heat-treated at 1200 °C for 1 h in an air furnace to strengthen the granules. A fraction of 50–200 μm diameter was distinguished by standard sieves set.

The specific area of both the powders and granules was measured by BET method (Autosorb 1, Quantachrome, USA), which is based on the measurements of the quantity of nitrogen gas adsorbed onto or desorbed from a solid surface at some equilibrium vapor pressure. The Autosorb 1 has the capability of measuring adsorbed or desorbed volumes of nitrogen at relative pressures in the range 0.01 to slightly under 1.0. The powder samples and granules were examined with a X-ray diffractometer (XRD, a DRON-3 and a Rigaku Denki diffractometers, Cu Kα, radiation, λ = 1.54056 Å). The infrared (IR) spectra were recorded using an IR spectrometer Specord-75. The granules’ size was determined by SEM. The diameter measurement error was equal to about 5%. Optical microscopy observations (a Neophot-32 microscope, Carl Zeiss Jena, Germany) were performed to study the microstructure of sintered granules. Por size, content and size distribution within the granules were analyzed by mercury porometer (an Autoscan porometer, Quantachrome, USA), the method being based on Washburn equation which dictates that as the pressure is increased mercury will intrude progressively into narrow pores. The sintering shrinkage was estimated by the change in granule linear dimensions due to the heat treatment.

The protein adsorption and desorption study was performed on granules of 50–200 μm diameter using FPLC system from Pharmacy Biotech, Uppsala, Sweden. Human plasma pooled from 10 healthy blood donors (male–female) in the age of 18–80 years was provided by the University Hospital, Uppsala, Sweden. Centrifuged (20 800g, 5 min) plasma was diluted with PBC buffer (5.72 ml Na2HPO4·2H2O, 1.36 ml NaH2PO4·H2O and 22 ml Citrate), pH 7.5, to attain a protein concentration of approximately 1 mg/ml. A column (HR 5/5) packed with approximately 0.7 g of respective adsorbent was equilibrated with PBC buffer, pH 7.5, at room temperature. Diluted human plasma was applied to the column at a flow rate of 0.05 ml/min until the protein concentration in the effluent equalled the concentration of the feed solution (approximately 5 ml). At this point all the available protein binding-sites were considered to be occupied and saturation reached. Flow-through fractions from 25, 50 and 75% of saturation were saved and analyzed. The column was then rinsed with PBC buffer, pH 7.5, until a stable baseline was attained. Desorption of adsorbed proteins was accomplished by applying 1% sodium dodecyl sulphate (SDS) in PBC
buffer, pH 7.5 to the column. The protein content in the effluent was measured throughout the study using the BCA protein assay[11] with BSA as standard.

The total binding capacities were calculated as follows

\[
\text{Total binding capacity} = \frac{m_{\text{ads}}}{A_s},
\]

where \(m_{\text{ads}}\) is the amount of plasma proteins adsorbed during the injection phase as obtained by subtracting the proteins that were washed off during the rinsing step. \(A_s\) is the specific surface area of the packed ceramic granules[11].

3. Results and discussion

The characteristics of HA and FA powders are summarized in Table 1. According to the wet chemical analysis data, weight ratio of Ca/P04 is about 0.702 (weight ratio of Ca/P equals to 2.13 and 2.12 for HA and FA, respectively) which is close to the theoretical one[8]. XRD patterns of the calcined powders correspond to JCPDS #9-437 for HA and JCPDS #15-0876 for FA. Estimated unit cell parameters were \(a = 9.432\) and \(c = 6.881\) Å for HA, and \(a = 9.365\) and \(c = 6.884\) Å for FA, respectively. No additional phases were revealed. The IR spectra of the initial powders were typical for HA and FA, respectively. There was only one O–H stretching band at 3573 cm\(^{-1}\) for pure HA spectrum, while the band near 3400–3500 cm\(^{-1}\) was present for both HA and FA, probably due to H\(_2\)O absorptions. Besides, the spectra in the 500–800 cm\(^{-1}\) range contain two strong bands at 601 and 571 cm\(^{-1}\) due to the \(v_4\) mode of the PO\(_4\) tetrahedral in both the compounds, and the band at 631 cm\(^{-1}\) assigned to the Ca\(_{3+}\)–OH librational mode for HA only. The position and intensity of this band were dependent strongly on the introduction of F\(^-\) into the linear [OH]\(^-\) chains. At the substitution of fluorine for OH\(^-\) groups of about 10%, the position of this band corresponds to 637 cm\(^{-1}\), being the characteristic of the FHA spectrum. Shown in Fig. 1 are the IR spectra from the mechanical mixture of 90% HA with 10% FA initial powders (a) and from the sample of the same composition sintered at 1200 °C for 1 h in ambient air atmosphere (b). The difference in the region 600–800 cm\(^{-1}\) is obvious. The solid solution HA–FA formation could probably be concluded from these data. However, after calcination at 1200 °C, the fluoroxyapatite could be expected to form, according to Fujimori et al.[12]. There were no bands at 875, 1418 and 1455 cm\(^{-1}\), which are characteristic of carbonate groups[13], in IR spectra of initial calcined powders of both HA and FA, and in those obtained from HA–10%FA sintered samples.

Fig. 2 shows the X-ray diffraction pattern for the sintered samples containing 0, 2 and 10 wt% FA. The shifting of the main peaks cannot be concluded from these data. A decrease of the peak intensities with an increase of FA content can be seen. It indicates the possibility that the granules are consisted of conjuncted HA and FA particles, because of lower grain size of initial FA powder.

![Fig. 1. IR spectra of initial mechanical mixture HA–10 wt% FA (1) and the sintered at 1200 °C sample of the same composition (2).](image)

![Fig. 2. XRD patterns for sintered HA (A), HA–2 wt% FA (B) and HA–10 wt% FA (C) samples.](image)

| Powder | Ca/P wt. ratio/ at. ratio | BET specific area (sq m/g) | Initial powder particles size (µm) |
|--------|--------------------------|---------------------------|-----------------------------------|
| HA     | 2.13/1.67                | 5.5 ± 0.2                 | 1.0 to 5.0                        |
| FA     | 2.12/1.66                | 13.5 ± 0.2                | Less than 1.0                     |
compared to that of HA powder, according to the data given in Table 1. Therefore, further study is necessary to be performed for understanding the sintering processes and mechanisms in the HA–FA disperse systems.

The two-stage thermal treatment results in the gelatin burning-off followed by shrinkage of the granules due to the sintering process occurring at 1200 °C. Fig. 3 shows the general view of the granules. Geometry of the granules is almost perfect spherical. The shrinkage was equal to 12–15 vol% for all compositions. Open pores content in sintered bodies can be varied by gelatin concentration in starting mixture powder–gelatin and by the heat treatment temperature [7]. The open pores content in the range of 43–62 vol% was obtained for the granules, the mean value being the same for all compositions. The pores size was about 1–6 μm diameter (Fig. 4). According to SEM micrographs there is difference in the microstructure of HA and HA–FA granules: the grain size within the granules becomes reduced with the addition of FA, as it can be seen in Fig. 5.

The total protein binding capacities of the ceramics are summarized in Table 2. The dynamic adsorption of 23 proteins was studied by following the presence of the proteins in the flow-through at three saturation degrees. The chosen degrees were 25, 50 and 75%, i.e. when 25, 50 and 75% of the available protein binding sites have been occupied. The evaluation was purely qualitative.

According to the experimental results there was no difference in the dynamic protein adsorption between pure HA and HA–FA granules: 20 proteins were present in the flow-through fractions corresponding to 25% of saturation and 23 occurred at 50% of saturation (Table 3).

The desorption of proteins was somewhat incomplete with desorption yields of 90%. Table 3 illustrates

| Ceramics   | HA   | HA–1% FA | HA–2% FA | HA–10% FA |
|------------|------|----------|----------|-----------|
| BET specific area of granules (sq.m/g) | 0.728 ± 0.110 | 0.731 ± 0.102 | 0.625 ± 0.211 | 0.593 ± 0.165 |
| Binding capacity (mg/sq m)         | 1.98 | 0.82     | 3.12     | 2.26      |
the qualitative evaluation of the protein contents in the desorption fractions. As can be seen, the protein patterns were identical for the ceramics with one exception: haptoglobin. Traces of haptoglobin could be found in the eluate of granules with the FA content of 1 and 10 wt%, but not in the eluate of the other two materials. The proteins that could be found in the desorption fractions of all materials were albumin, α1 antichymotrypsin, apolipoprotein AI, apolipoprotein D, α2 macroglobulin, α2HS glycoprotein, Zn α glycoprotein, IgA, IgG, IgM, α2 macroglobulin, orosomucoid, serotransferrin and transthyretin. The proteins that did not occur in the eluate from either material were ceruloplasmin, α1 B glycoprotein, hemopexin, LRG, and prothrombin.

Thus, it can be supposed that the minor addition up to 10% of FA to HA does not influence negatively the protein adsorption onto the porous granules, and that the surface morphology is probably more important factor affecting the protein behavior. This is in agreement with the opinion about FHA does not reduce the osteointegration ability of the implanted device [5,14,15]. The addition of fluoride ions can result in a positive consequence due to their influence on the driving force for precipitation of apatite from solution, i.e. for the remineralization of hard tissue, and due to reduction of the solubility of apatite phase by the body fluids [13]. The last effect could be used in designing with ceramic scaffolds intended for bone tissue engineering.

4. Conclusion

The technique to fabricate porous spherical HA and HA–FA (up to 10 wt%) granules was developed; the method is based on a liquid immiscibility effect using the powder–gelatin suspension and oil as the liquid. After the heat treatment at 1200 °C for 1 h in air atmosphere, the granules of diameter range from 50–200 μm with open pores content amount up to 43–62 vol% were obtained. As a result of heat treatment, the solid solution of FA in HA is probably formed, according to the IR data, although this effect needs to be proved by further study.

There were no differences in the dynamic protein adsorption between pure HA and HA–FA granules. The influence of human plasma proteins on cell adhesion can be expected to be the same for both HA and HA–FA (up to 10%) materials with respect to human protein adsorption behavior.

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