Bioceramics for Healthcare Applications

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Wet Chemical Synthesis of Hydroxyapatite Powder and Estimation of Curcumin and Berberine Drug Loading Efficacy

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Schematic representation of HAP powder synthesized by precipitation method

ABSTRACT

Hydroxyapatite (HAP) mineral constitutes ~70% of human bone. Therefore, synthetic HAP holds a prominent place in the context of orthopaedics. Synthetic HAP can act as a carrier to overcome the limitations of hydrophobicity and poor absorption associated with some common anticancer drugs. In the present work, HAP powder was synthesized by facile chemical precipitation method followed by structural characterizations. Furthermore, the potential of HAP powder as a carrier of antitumor drugs such as curcumin and berberine through physical adsorption using absorption measurements has been studied. The synthesised HAP powder has been found to have a higher loading efficacy of ~77% towards curcumin drug as compared to berberine (27%).

KEYWORDS: *Hydroxyapatite (HAP), Raman spectrum*

Introduction

Bioceramics such as hydroxyapatite (HAP), β-tricalcium phosphate and biphasic calcium phosphates possess high biocompatibility, chemical stability, and mechanical strength *in-vivo* [1]. Among these, HAP ($Ca_{10}(PO_4)_6(OH)_2$), is an osteoconductive bioactive ceramic possessing a structural and chemical similarity with bones. The constituent elements of HAP are primarily calcium and phosphorus, with a stoichiometric Ca/P ratio of ~1.667. Synthetic HAP can be made by various chemical methods such as, precipitation, hydrothermal, hydrolysis, mechanochemical and sol-gel method [2]. Among these, the wet chemical precipitation technique is more popular due to its inherent simplicity and cost effectiveness. With hexagonal crystal structure, HAP has negatively charged phosphate anions (PO_4^{3-}) at both the ends and positively charged calcium (Ca^{2+}) cation on the sides along with surface ions such as OH^-. All these act as potential binding sites for biomolecules [3]. The similarity in composition with bone is the reason for the usage of HAP in many interesting applications such as bioimplants due to its superior osseointegration and as carrier to deliver several anti-inflammatory drugs, tumour drugs, proteins, antibiotics, and growth factors [4].

Curcumin, a yellow pigment that is derived from the rhizomes of *Curcuma longa* (turmeric) acts as a bioactive agent and is used to treat diabetes, cancer, arthritis and neurological diseases. Researchers are also examining its therapeutic effect towards inhibition of cancer cells and notably it has been used successfully for the treatment of breast cancer [5]. However, it has a limited use in biomedical applications due to its hydrophobicity and poor absorption. Berberine, another drug, found in the rhizome of the barberry plant is also known for its anti-fungal, anti-viral and anti-bacterial properties. It also works against cancer, inflammation, hypertension and diabetes [6]. The poor water solubility, strong bitter taste and low absorption in the body of this drug restricts its therapeutic applications. To overcome such situations, engineering a suitable carrier to load these drugs and ensure their release can be beneficial.

The purpose of the present study is to synthesize in-house hydroxyapatite powder to load anticancer drugs such as curcumin and berberine and compare their loading efficacy. The powder was synthesized via wet chemical precipitation method using calcium nitrate tetra hydrate, orthophosphoric acid as a precursor. The powder so obtained was characterized by Scanning Electron Microscope (SEM), Energy dispersed X-ray spectroscopy (EDS), micro-Raman and X-ray diffraction (XRD) techniques. The drug loading efficacy was estimated over regular intervals up to 72 h by monitoring the absorbance using a spectrophotometer. A systematic decrease in the absorbance of the HAP-curcumin and HAP- berberine in the solution with increasing time indicated continuous loading of drug onto HAP power. A loading efficiency of 77 % and 27% was estimated for curcumin and berberine, respectively. In previous such studies [7, 8], the surface modification of synthesised HAP rendered it suitable to be used as carrier which also increased the drug loading efficacy. But in the present work, our studies have revealed that HAP powder without any surface modification is also capable to enhance the drug uptake.

Experimental Section

Chemicals and reagents

Calcium nitrate tetrahydrate [$Ca(NO_3)_2.4H_2O$, 99%], orthophosphoric acid (H_3PO_4), ammonium hydroxide [NH_4OH, 28%], berberine and cucurmin were procured from Sigma Aldrich. During synthesis, deionized water (DI) from a Millipore system was used as solvent.

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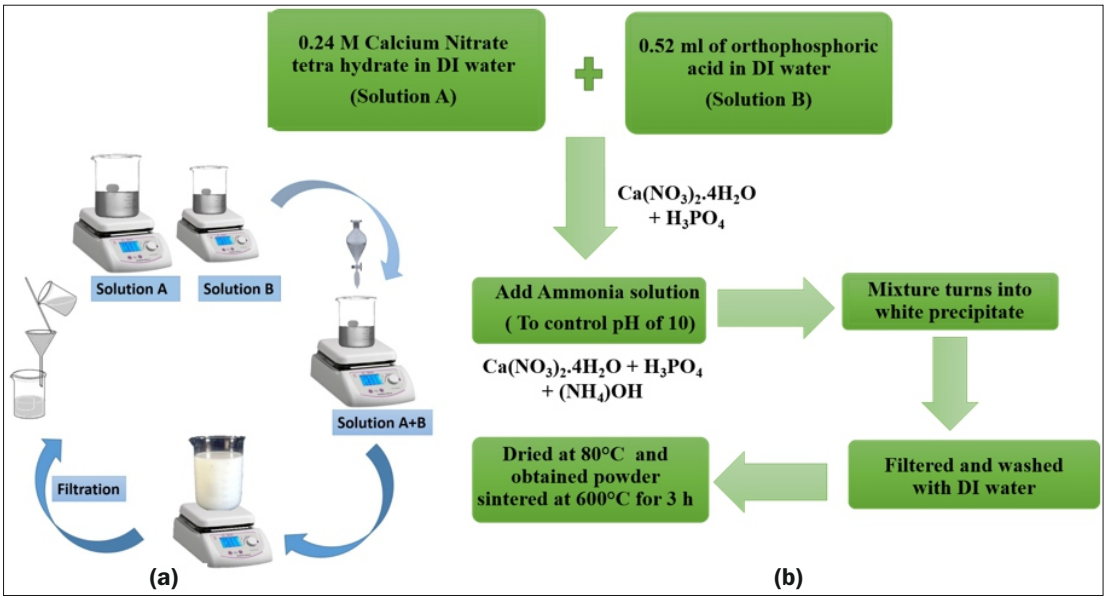

Fig.1: (a) Schematic representation of HAP powder synthesized by precipitation method and (b) flow chart of method of synthesis.

Preparation of HAP powder

HAP powder was synthesized by wet chemical precipitation method [9]. For this, about 0.24 M of $Ca(NO_3)_2.4H_2O$ and 0.5 mL of H_3PO_4 were mixed in 25 mL of deionized water at room temperature in two separate beakers (Solution A and Solution B in Fig.1a). Both solutions were mixed and stirred for 30 minutes followed by dropwise addition of ammonia to maintain pH of the mixture at 10. The reaction was allowed to carry on along with continuous stirring for 2h. After the reaction, the precipitate was removed from the reaction solution by filtration and washed thrice with DI-water, as shown in Fig.1a. Then, it was dried at 80°C in an oven overnight and sintered at 600°C for 3 h to form a powder of HAP. A systematic flowchart of this reaction is shown in Fig.1b.

Characterization techniques

To identify the existence of functional groups in HAP powder, Raman spectrum was recorded (micro-Raman spectrophotometer, M/s. AIRIX Corp., Japan). The presence of crystallographic phases in the sintered HAP was estimated from XRD pattern using Cu K_α radiation (λ=1.5406 Å). The XRD spectrum of the sample was compared with standard diffraction data (JCPDS # 09-0432) of pure HAP. The morphology and Ca/P ratio were studied by a scanning electron microscope (SEM, M/s. S&C, Korea)) and Energy dispersive spectroscopy (EDS) respectively. For SEM analysis, the powder sample was dispersed in ethanol and spread over a carbon tape and dried at room temperature followed by gold sputtering. The drug loading efficacy was studied using UV–VIS spectrophotometer (Model No- UV 2700, Shimadzu Corporation, Japan) in the range of 200-600 nm at absorption wavelengths of 264 nm and 347 nm for berberine and curcumin, respectively.

Encapsulation Efficiency (EE)

Fig.2a-c shows the absorption spectrum of HAP, curcumin and berberine at 1mM concentration dispersed in phosphate buffer saline (PBS, pH=7.4, 10X), respectively. PBS is a non-toxic solution used in many biological laboratories to prevent cell-rupturing or shrivelling up due to osmosis. Curcumin exhibits two distinct absorption peaks at 264 nm and 350 nm, the 264 nm being more prominent of the two. In the case of berberine, absorption occurred at 225 nm, 262 nm and 347 nm and prominent absorption was at 347 nm. Curcumin and berberine were added to HAP (dispersed in PBS) in the ratio of 1:1 separately and ultrasonicated for 30 min to ensure uniform dispersion of the drugs. Both solutions were kept undisturbed and drug loading was studied at regular intervals of 3h, 6h, 24h, 48h and 72h. For this, the solution was centrifuged at 12000 RPM for 15 min and supernatant solution was collected to record UV–VIS spectrophotometer absorption spectrum in the range of 200 to 600 nm. Centrifuging enabled settling down of the drug loaded HAP and unloaded HAP, leaving only the free drug in the solvent absorbance of which was measured.

The encapsulation efficiency (EE) was calculated using the following equation 1 [10].

$$\% \text{ EE} = \frac{C_T - C_F}{C_T} \times 100 \qquad (1)$$

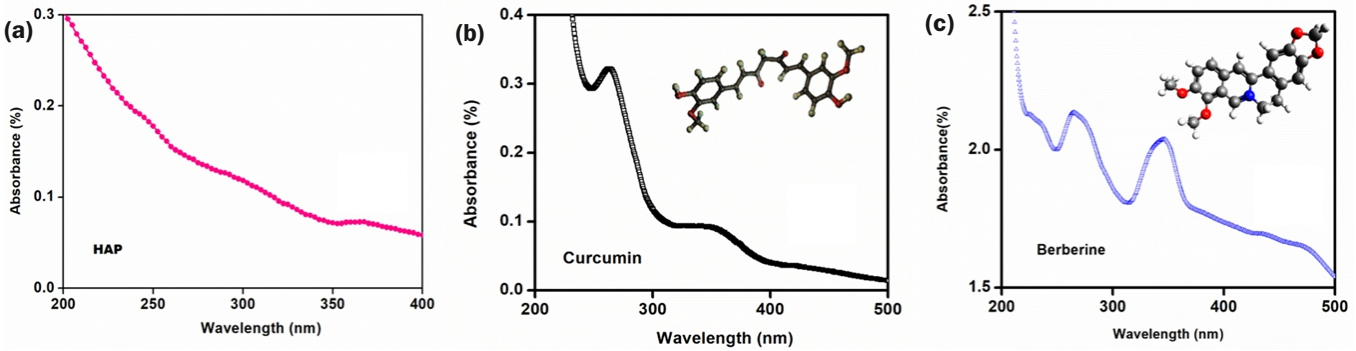

Fig.2: Absorbance spectru of (a) HAP, (b) Curcumin and (c) Berberine.

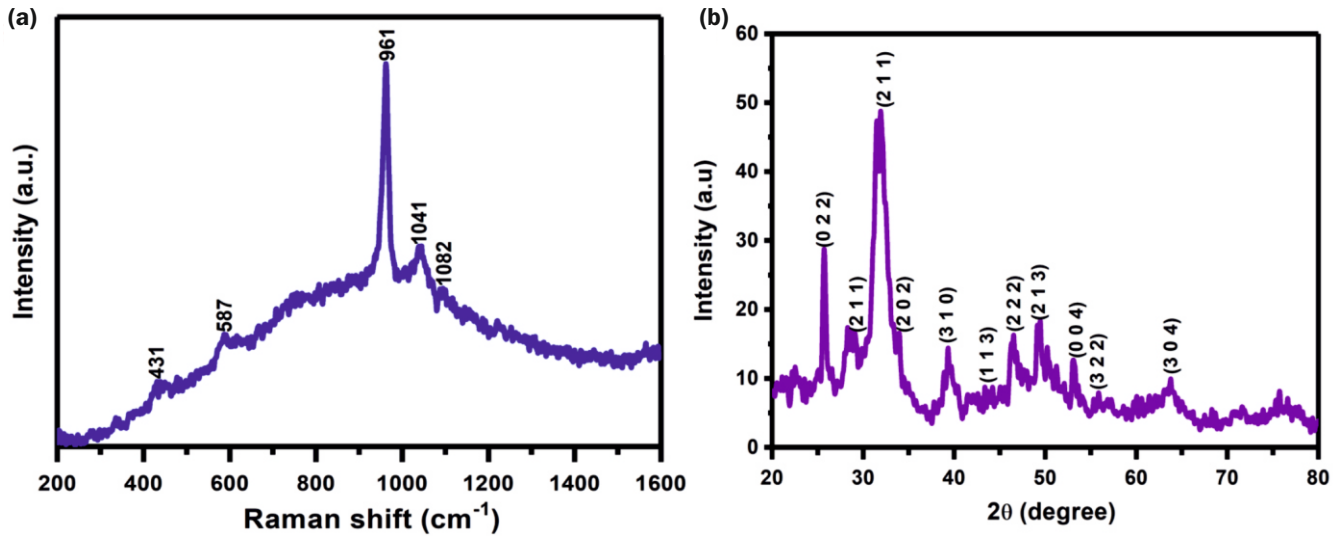

Fig.3: (a) Raman spectrum and (b) XRD pattern of synthesize HAP powder.

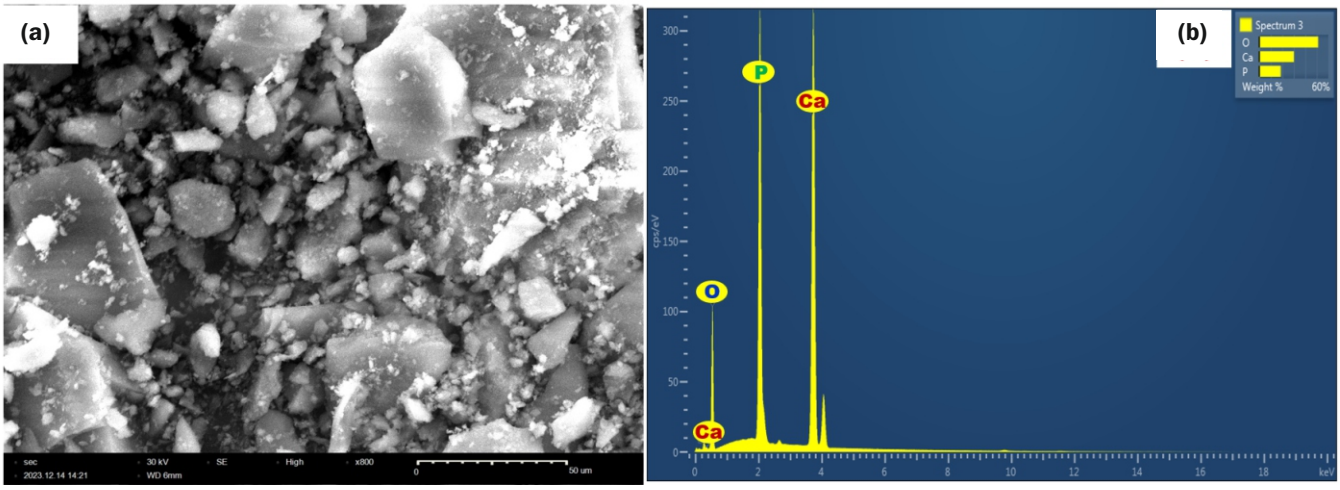

Fig.4: (a) SEM image (b) EDS spectrum of HAP powder.

Where C_T is the measured optical density corresponding to total concentration of curcumin/berberine (1 mM) added and C_F is the measured optical density corresponding to free curcumin/berberine present as supernatant after centrifugation at 12000 RPM.

Results and Discussion

Fig.3a shows the Raman spectrum of as synthesised HAP powder. The characteristic sharp Raman peak at 961 cm^{-1} is attributed to the symmetric stretching mode of $PO_4^{3-}(v_1)$ of HAP. The peaks at 431, 587, 1041 and 1082 cm^{-1} correspond to asymmetric bending mode of $Po_4^{3-}(v_2)$, asymmetric bending mode of $Po_4^{3-}(v_4)$, asymmetric stretching of $Po_4^{3-}(v_3)$ and asymmetric stretching of $Po_4^{3-}(v_3)$ respectively [11].

As seen in Fig.3b, the characteristic peak with highest intensity obtained at $2\theta=31.87^0$ corresponds to the (211) plane of HAP. Other peaks at 2θ = 25.691, 28.284, 33.977, 39.383, 43.891, 46.544, 49.454, 52.941, 55.775, and 63.764 are due to diffraction from (002), (210), (202), (310), (113), (222), (213), (004), (322) and (304) planes of HAP respectively (JCPDS No: 09-0432) [12]. Presence of sharp, narrow and well-defined peaks in the diffraction pattern indicate highly crystalline nature of the synthesised HAP powder. All the diffraction peaks matched well with the standard diffraction data of apatite HAP and the planes of HAP powder reveal the expected hexagonal structure. Absence of impurity peaks such as calcium phosphate and calcium hydroxide confirm the monophase of the prepared HAP.

Fig.4a shows the SEM micrographs of HAP powder with agglomerated morphology. A large variation in particle size (10 to 100 µm) was observed. EDS showed peaks corresponding to only Ca, P, and O. This too confirmed the absence of impurities in the synthesized powder. The average Ca/P ratio was measured as 1.60, which is very close to the theoretical Ca/P value of HAP (1.67) [2].

In Fig.5a, the dispersed colour of curcumin in PBS solution reduced with the increasing time as it loaded onto HAP. However, the colour of the liquid remains pale yellow at the end of 72h for berberine, indicating relatively lesser loading of this drug in the given time. The encapsulation efficiency (% EE) of curcumin and berberine conjugates by HAP powder was calculated using equation (1) on the basis of their optical densities at 264 nm and 347 nm respectively measured at 3h, 6h, 24h, 48h and 72h and plotted in Figure 5b and 5c. It can be noticed that from initial hours, the EE (%) of HAP was more for curcumin which remained high though out the measurement. At the end of 72 hr the maximum EE (%) against curcumin and berberine were estimated as 77% and 27% respectively. In earlier reports, requirement of surface modifications of HAP before loading of curcumin (EE ~85%) [7] and berberine [12] was essential. However, here without any surface modification, we could observe a comparable value of 77 % for curcumin

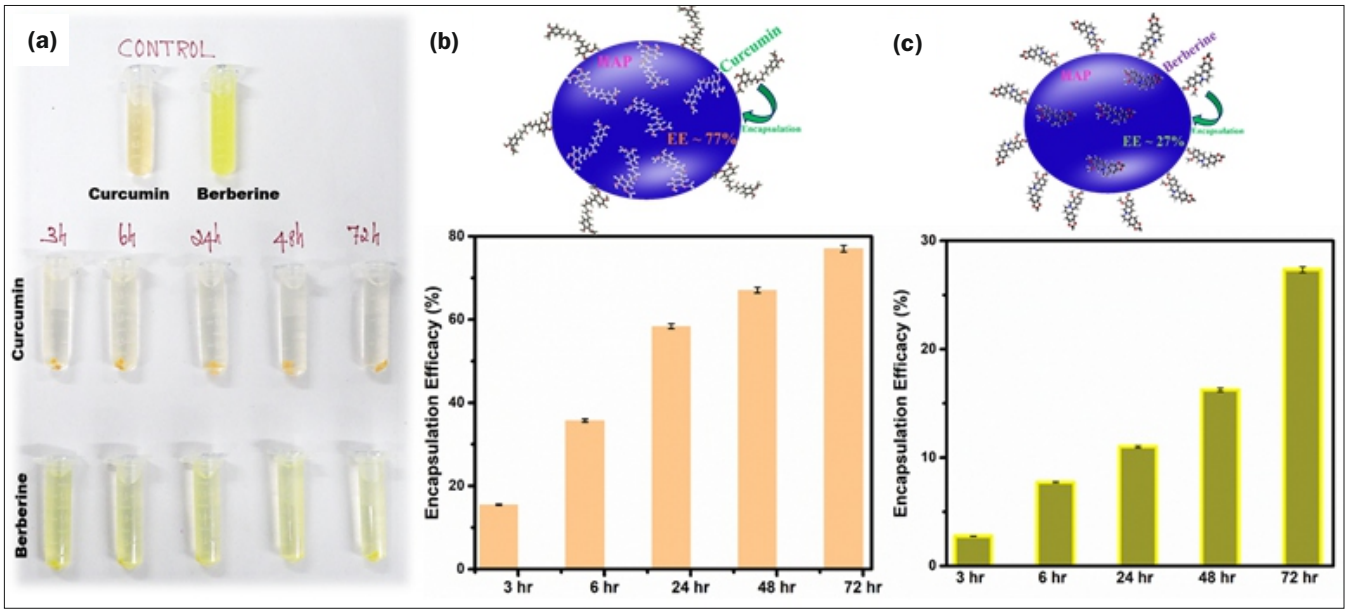

Fig.5: (a) Time dependent photograph of solution of HAP powder loaded with curcumin and berberine taken in Eppendorf tubes, Percentage encapsulation efficiency of HAP powder loaded with (b) curcumin, and (c) berberine.

loading. Hence, this approach of loading HAP with drug e.g., curcumin may be an effective method of its efficient transport and release in the intended region.

Conclusion

Quality of HAP powder synthesized by wet chemical technique was confirmed from the characteristics Raman and XRD peaks and from Ca/P ratio and was found to crystalline and pure with the required Ca/P ratio. Time dependent loading of curcumin and berberine drugs into HAP powder was monitored up to 72 hrs in PBS. The results showed that curcumin has a higher affinity towards the synthetic HAP prepared in this work with encapsulation efficiency about 77% as against 27 % for berberine. This study brings forth the possibility of using synthetic HAP under optimised conditions as an efficient carrier for drug loading applications.

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