A Novel Membrane Diffusion Process for the Preparation of Chitosan/Hydroxyapatite Composite

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ABSTRACT: The aim of this study was to create a suitable method to hybridize hydroxyapatite into chitosan solution with a good dispersion of inorganic matter in the organic phase. The preparation of chitosan/hydroxyapatite composites via a membrane diffusion process was evaluated and optimized. A diffusion time of 3 days, and chitosan, calcium chloride, and disodium hydrogen phosphate with the concentrations of 1.75 % w/v, $0.20M$, and $0.12M$, respectively, were found to be the optimal conditions. Scanning electron microscope observations showed a cluster of needle-shaped hydroxyapatite across the composite film when synthesized with $0.2M$ of calcium chloride solution and $0.12M$ of disodium hydrogen phosphate solution, whereas hydroxyapatite was observed in larger diffuse aggregates when synthesized at a higher concentration of both solutions. Attenuated total reflection-Fourier transform infrared spectrophotometer and X-ray diffraction analyses displayed the specific peaks for the phosphate group and $2\text{-}\theta$ peaks of hydroxyapatite crystals, respectively. These results indicated that hydroxyapatite can be hybridized into a chitosan solution with good dispersion through a membrane diffusion process. © 2013 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 130: 1632–1638, 2013

INTRODUCTION

The restructuring and especially the regeneration of bone is one of the major difficulties in clinical treatments, largely owing to the extremely slow and spatially heterogeneous rate of self-healing, and is often and locally incomplete. Recently, three-dimensional porous scaffolds loaded with specific living cells have been researched. To regenerate tissue in a natural way but quicker and or in desired places, there is an urgent need for easily made and applied biocompatible and biomodifiable scaffold structures that can provide immediate support and structure while encouraging and facilitating natural bone and cellular replacement. Of these three-dimensional porous scaffolds that can be loaded with specific living cells, which have been investigated, hydroxyapatite (HA) is the key choice. This is owing to the fact that it is one of the major inorganic components in natural bone and teeth, and unsurprisingly is osteoconductive,[1] nontoxic, noninflammatory, nonimmunogenic, and can form direct chemical bonds with surrounding tissue.[2] However, on the contrary, HA alone is rough and brittle with a low mechanical strength and high fatigue failure which limits its ability to be surgically manipulated. The resultant search for organic-HA composites to overcome these problems is supported and driven by the observation that natural bone itself is a composite between organic (e.g., collagen) and inorganic (HA) matters. Moreover, the application of HA powders *in situ* is problematic owing to the migration of places other than the implanted area, making sterile perforation of the scaffold matrix prior to surgical implantation desirable.

Chitin, which has inherent wound-healing characteristics,[3,4] is a biocompatible, biodegradable, nontoxic, and non- or weakly immunogenic naturally occurring polymer. Along with its derivative, chitosan, it has been investigated as a potential supporting matrix to solve these problems as well as that of migration of the HA powder when implanted. Chitosan is the partially deacetylated form of chitin, consisting of glucosamine and *N*-acetylglucosamine units linked with β-1,4-glycosidic linkage, and thus shares some bioactivities with various glycosaminoglycans and hyaluronic acid naturally present in articular cartilage.[5] Owing to its own positive charges, chitosan can induce cell adhesion by electrostatic interaction with the negative charges on the cell membrane surface.[6] Therefore, it is of interest to successfully combine the biocompatible positive chitosan with the osteoconductive HA to form a suitable less brittle composite that would be expected to be a good biomaterial for bone tissue engineering.

Several techniques have been investigated for suitability to prepare HA deposited on a substrate. The biomimetic process was first developed by Kokubo and coworkers.[7,8] This process was quite slow, whereas an alternate soaking process revealed a great potential to form calcium phosphate on/in the substrate over a short period of time.[9–11] Dual membrane diffusion system was also reported to form HA on/in chitosan membrane.[12] However, the HA that was deposited covered the substrate surface and hence reduced the original substrate's pore size and porosity. As these macropores facilitate bony ingrowth and implant fixation,[13,14] it is undesirable to reduce the substrate porosity, which can seriously limit the efficiency and applicability of this technique. To attain high-porosity scaffolds, HA should be hybridized into chitosan solution before forming the particular porous structure to avoid clogging the macropore structure. Some techniques such as the chemical wet method,[15] and *in situ* hybridization,[16] have been investigated to synthesize HA in chitosan solution under acidic conditions, and coprecipitate the chitosan–HA mixture under alkaline conditions. The alkaline coagulates chitosan and HA inconsequently forms chitosan–HA with layer-by-layer structure, which is controlled by the rate of alkaline diffusion. This architecture, thus, influences to severe shrinkage of the sample by self-reinforced shrinkage from outside to inside layer after drying.[16]

In this research, we developed an alternative novel method of hybridizing HA into chitosan solution, pH of 7.4, by counter-dialyzing the phosphate ions into the calcium sorption and this method is called "membrane diffusion process." In this process, the membrane barrier is used to slow down the diffusion rate of PO_4^{3-} ions into the Ca^{2+} ions media and hence simultaneously forms HA in the chitosan solution. The obtained chitosan-/HA-hybridized solution is shapeable in various forms such as microparticles, beads, films, as well as three-dimensional porous scaffolds and is expected to have well-dispersed HA crystals throughout the remaining porous chitosan matrix. As a first stage in the development of this process, the optimal conditions to hybridize HA in chitosan solution by the membrane diffusion process were investigated using diffusion time, and the concentrations of chitosan, calcium, and phosphate ions are the key variables of interest. Despite the potentially diverse array of matrix structures, we report on the characteristics of cast sheets, in terms of HA content and dispersion homogeneity, because of the ease of preliminary characterization.

EXPERIMENTAL

Materials
High-viscous chitosan (DD = ~85, determined by FTIR) was purchased from Fluka, Japan. Calcium chloride and disodium hydrogen phosphate were obtained from Carlo Erba Reagent, Italy. Acetic acid was purchased from BDH Laboratory Supplied, England. Sodium acetate and *tris*(hydroxymethyl)aminomethane were bought from Scharlau Chemie S. A., Spain. Hydrochloric acid was obtained from Zen point, Thailand. All chemicals were of analytical grade and used without further purification.

Preparation of Chitosan/HA Composite via the Membrane Diffusion Process
Chitosan and calcium chloride were mixed in 5 mL (0.1M) of sodium acetate buffer, with stirring until homogeneously

Table I. Sample Code and Calcium Chloride/Disodium Hydrogen Phosphate Concentrations

Sample code	CaCl$_2$ (M)	Na$_2$HPO$_4$ (M)
HA1	0.20	0.12
HA2	0.40	0.24
HA3	0.60	0.36
HA4	0.80	0.48
HA5	1.00	0.60
HA6	2.00	1.20

dissolved, and then poured into a dialysis tube (molecular weight cutoff = 6000–8000), sealed, and immersed into 20 mL of disodium hydrogen phosphate solution (pH 7.4 was adjusted with *tris*(hydroxymethyl)aminomethane) at given concentrations. After the indicated dialysis time, the obtained mixture solution within dialysis tube was dialyzed against distilled water for an hour before cast as a film (3 × 3 cm^2) and dried to constant dry weight in oven at 60°C for 24 h.

Effect of Diffusion Time, Chitosan Concentrations, and Calcium Chloride/Disodium Hydrogen Phosphate Concentrations upon HA Formation in Chitosan Solution via Membrane Diffusion Process
Optimization was attempted by varying each of the three parameters individually, and thus assuming that each is independent of the other two.

First, the chitosan and calcium chloride/disodium hydrogen phosphate solutions were kept constant at 2% w/v (0.4/0.24M), whereas the diffusion (dialysis) time was varied from 1 to 5 days.

Second, using the optimal diffusion time from the abovementioned experiment, the calcium chloride/disodium hydrogen phosphate solutions were kept constant at 0.4/0.24M, whereas the concentration of chitosan was varied from 1.0 to 2.0% w/v.

Finally, the optimal diffusion time and concentration of chitosan selected from the abovementioned experiments were utilized, whereas the concentrations of calcium chloride/disodium hydrogen phosphate were covaried, maintaining a fixed calcium : phosphate ratio of 5 : 3 as summarized in Table I. The rational for this ratio is derived from the stoichiometric relationship of the reaction as follows.[17]

$$10CaCl_2 + 6Na_2HPO_4 + 2H_2O \rightarrow Ca_{10}(PO_4)_6(OH)_2 + 12NaCL + 8HCl$$

For all three experiments, the resultant composite mixture solutions were dialyzed against distilled water to remove by-product, cast as a film, and oven dried as above, and all experiments were carried out as three independent repeats.

Analysis of Chitosan and HA Ratio
The chitosan to HA ratio and HA dispersion homogeneity of chitosan/HA composite film were determined in triplicate for each sample by thermogravimetic analysis (TGA), using Model TGA/SDTA851^e, METTLER TOLEDO. A 5–10 mg portion of each sample was placed in the aluminum pan and the TGA run

Materials
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from 50 to 700°C at a heating rate of 20°C/min under oxygen gas flow rate of 20 mL/min, and then held constant at 700°C for 10 min to completely combust the chitosan. The residual weight was defined as that of HA. To determine the distribution of HA in chitosan matrix, three areas of each sample were sampled and the amount of HA in each was measured and compared.

Characterization of Synthesized HA

Attenuated Total Reflection–Fourier Transform Infrared Spectrophotometer

Chitosan/HA composite films were placed on the sample holder. IR spectra were obtained on Nicolet 6700 and scanned from 4000 to 650 cm^{-1} with a resolution of 4 cm^{-1}, and 128 scans were taken using a DLATGS detector.

X-Ray Diffraction

X-ray diffraction (XRD) was measured by a Bruker AXS Model D8. The X-ray source was originated from CuK∞ ($\lambda = 0.154$ nm). Chitosan/HA composite films were scanned at 2θ in the range of 15–55° with a scan speed of 0.02 s^{-1}.

Morphology of Chitosan/HA Composite

Morphology of chitosan/HA composites was observed by scanning electron microscope (SEM). The samples were placed on SEM stuffs and coated with gold at 15 Amp for 3 min before SEM observation. SEM images were observed on a JEOL JSM-5800LV SEM operating at an acceleration voltage of 15 KV and a magnification of 1500×.

RESULTS AND DISCUSSION

### Preparation of Chitosan/HA Composite via Membrane Diffusion Process

The potential use of a novel method, "the so-called "membrane diffusion process," to attain well-dispersed HA crystals in a chitosan solution through hybridization, pH of 7.4, by counterdialyzing the phosphate ions into the calcium ion sorption was evaluated and optimized. The membrane diffusion process is defined here as the process to mineralize inorganic matter into organic solution using a membrane barrier to slow down the diffusion rate of ions from one side of the membrane to the other side where they will mineralize with the other ions trapped in organic solution. As the concentrations of calcium and phosphate used in these assays were set to match the stoichiometric relationship of HA mineralization, namely a 5 : 3 calcium : phosphate ratio,[17] the calcium chloride solution was at a higher molar concentration than disodium hydrogen phosphate. Thus, theoretically more Ca^{2+} ions will typically pass though the membrane to react with PO$_4^{3-}$ ions on the other side. The stoichiometric ratio was an important factor in deciding that disodium hydrogen phosphate should be dissolved in chitosan solution, dialyzed against Ca^{2+} ions to form HA in the chitosan solution. However, we cannot rely on this hybridization as chitosan precipitates with the addition of disodium hydrogen phosphate. However, in this process, the hydroxyl and amino groups of chitosan are considered as important factors as they are likely to interact with Ca^{2+} ions and thus potentially, in addition, direct the production of HA formed within the

chitosan molecule. Therefore, in this method the calcium chloride was dissolved homogeneously in chitosan solution in 0.1M of acetate buffer, pH 5.6, allowing entrapment of Ca^{2+} ions (sorption) within chitosan. As amino groups would be protonated under this condition, thus, hydroxyl groups should be the main functional groups for sorption of Ca^{2+} ions. The chitosan–calcium chloride solution (as opposed to insoluble chitosan–disodium hydrogen phosphate precipitate) was then dialyzed against the disodium hydrogen phosphate solution, pH 7.4. In this process, the membrane was used to separate these two solutions and slow down the diffusion rate of PO$_4^{3-}$ ions to react with Ca^{2+} ions trapped by chitosan to form HA in the chitosan solution. Simple mixing of HA powder in chitosan solution caused phase separation between the organic and the inorganic matter if stirring was removed, whereas hybridization of HA into chitosan solution via membrane diffusion process resulted in the white particles of HA, remaining homogeneously dispersed in the chitosan solution even after 12 h (Figure 1). This phenomenon indicated that the membrane diffusion process removes the otherwise serious problem of phase separation between organic and inorganic substances.

The Effect of Diffusion Time, Chitosan Concentrations, and Calcium Chloride/Disodium Hydrogen Phosphate Concentrations upon HA Formation in Chitosan Solution via the Membrane Diffusion Process

Diffusion Time. To determine the appropriate diffusion time for HA formation in this membrane diffusion method, chitosan, calcium chloride, and disodium hydrogen phosphate were maintained at the concentrations of 2% w/v, 0.4M, and 0.24M, respectively, whereas the diffusion (dialysis) time was varied from 1 to 5 days and the product was assayed on dried cast films. For each sample, essentially the same amount of HA found in all three regions was assayed (bars, Figure 2), suggesting a high degree of homogeneity in HA distribution in the chitosan matrix for each time point assay. However, the average amount of HA in three independent samples increased with increasing diffusion (dialysis) time until reaching the maximum amount of HA (black line, Figure 2). Owing to high error bar, the maximum amount was considered from raw data of each individual sample. It was found that most amount of HA for each individual samples was closed to a value at around 50% for diffusion time of days 3, 4, and 5. Therefore, a diffusion time of 3 days was deemed to be optimal (as no further improvement was noted after this time point) and used in all subsequent experiments.

Chitosan Concentrations. To determine the appropriate concentration of chitosan for HA formation in this membrane diffusion process, the concentrations of chitosan were maintained at 1, 1.5, 1.75, and 2% w/v, with calcium chloride and disodium hydrogen phosphate solutions at constant concentrations of 0.4 and 0.24M, respectively, and allowed to diffuse for the determined optimal time of 3 days, and assayed on dried cast films in triplicate. Figure 3 shows the data for the relationship between concentration of chitosan and percentage of HA in the dried cast composite films. The percentage of HA increased slightly, but not statistically significantly, between 1 and 1.5% w/v and did not increase with further increases in the
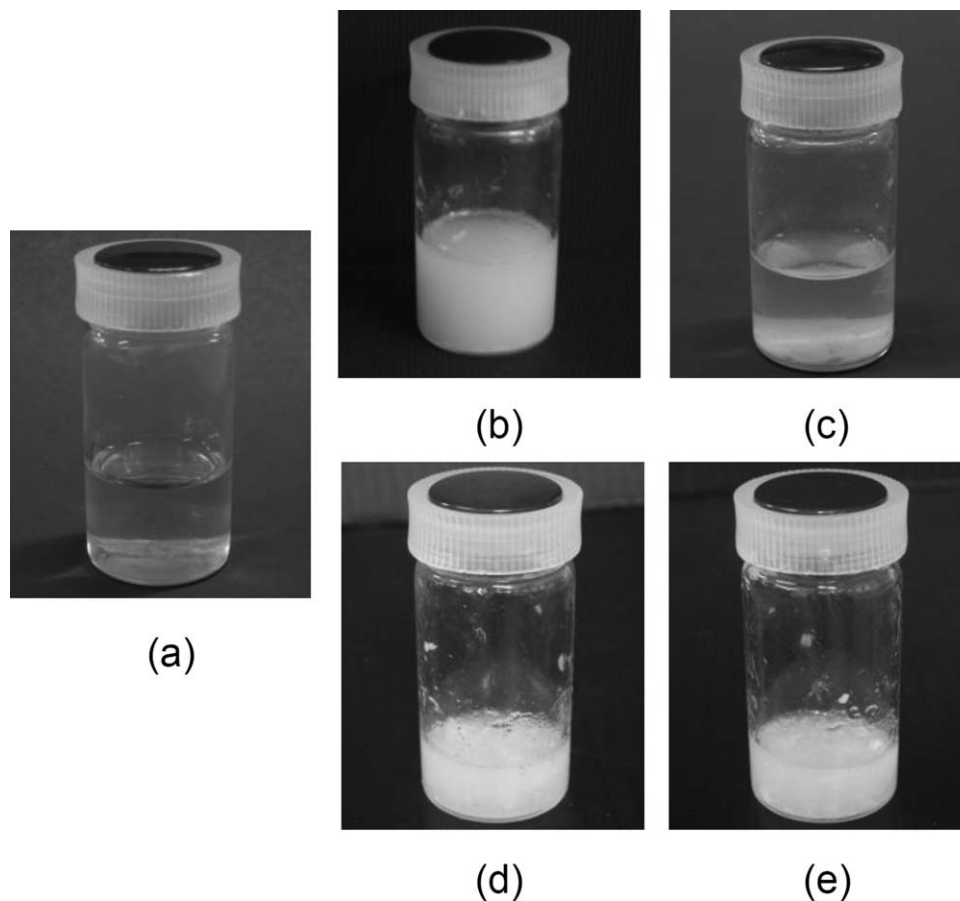
Figure 1. Appearance of (a) chitosan solution, (b,c) simple mixing HA powder in chitosan solution, (b) at the beginning, and (c) after stirring removal for 12 h, (d,e) HA formed in chitosan solution via membrane diffusion process, (d) at the beginning, and (e) after storage for 12 h.

concentration of chitosan up to 2% w/v. We had expected that as high chitosan concentrations would have a higher capacity to encapsulate calcium ions than with a higher quantity of calcium ions sorption, it would form a higher amount of HA. However,

chitosan can be dissolved only to around 1.75% w/v with evidence of insoluble chitosan flakes still remaining in the chitosan solution (2% w/v). Therefore, a chitosan concentration of 1.75% w/v was selected as the appropriate concentration in subsequent experiments.
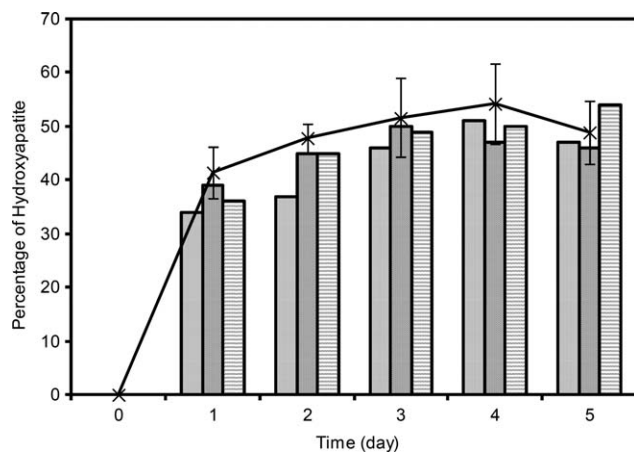

Figure 2. Mean (±SD) percentage of HA dispersed in the chitosan/HA composite films prepared after different diffusion times. Bars represent the %HA from different regions of the same sample, whereas the line represents the average %HA within a sample.
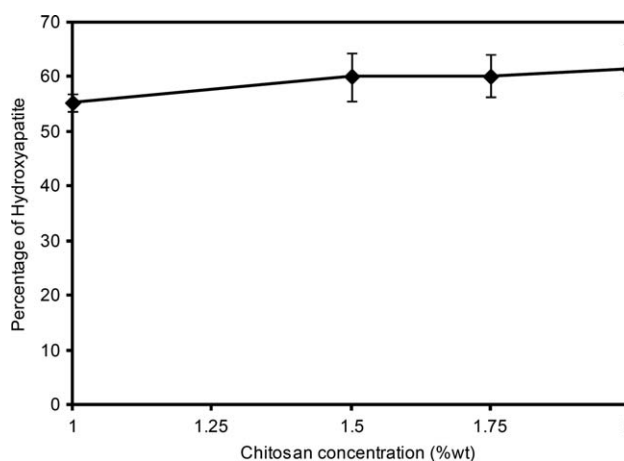
Figure 3. Mean (±SD) percentage of HA in the chitosan/HA composite films prepared from different concentrations of chitosan.
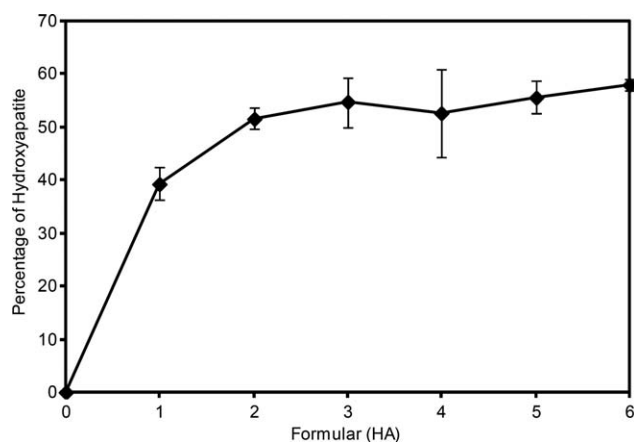
Figure 4. Mean ($\pm$SD) percentage of HA in the chitosan/HA composite films prepared from different concentrations of calcium chloride/disodium hydrogen phosphate.

Calcium Chloride/Disodium Hydrogen Phosphate Concentrations

Finally, to determine how the concentrations of calcium chloride/disodium hydrogen phosphate affected the amount of HA formed in the chitosan complex, they were coaltered in the range of 0.20–2.00 and 0.12–1.20M, respectively, maintaining the 5 : 3 stoichiometry (HA1–HA6, Table I). Chitosan was used at 1.75% w/v and diffusion proceeded for 3 days prior to analysis of the formed complex as dried films. As expected, as the concentration of calcium chloride and disodium hydrogen phosphate increased and hence the amount of HA in the chitosan also increased but nonlinearly, reaching a plateau maxima at around 0.7M calcium chloride/0.42M disodium hydrogen phosphate solution, that is between HA3 and HA4 (Figure 4). This maximal output presumably reflects the total level of calcium ion sorption within chitosan solution. At calcium chloride concentrations lower than 0.6M, calcium ions are encapsulated inside the chitosan molecule and subsequently form HA in contact with diffused phosphate ions. Owing to the ability of
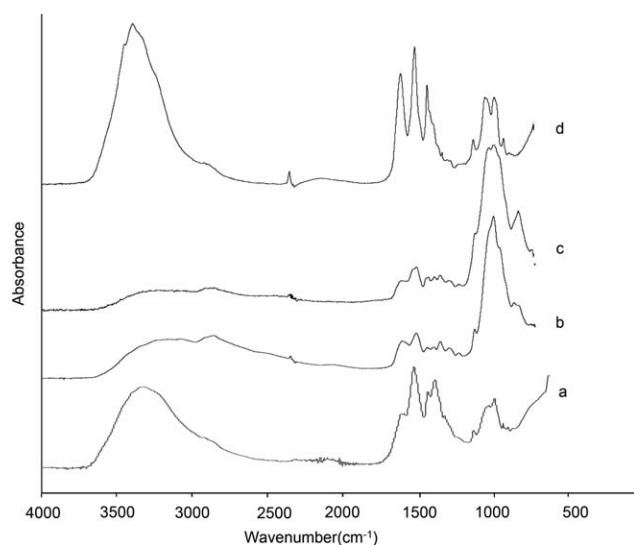
calcium chloride to act as a coagulating agent,[18] when concentrations of calcium chloride are increased, on the other hand, calcium ions are not only encapsulated inside the chitosan molecule, but also surrounded the chitosan molecules forming coagulates. Therefore, the mixture of chitosan and calcium chloride at higher concentrations than 0.6M turned white and opaque before the membrane diffusion process. During the process, the excess-free calcium ions surrounding the chitosan molecules are free to diffuse out and form colloidal HA. Therefore, the quantity of HA was limited by the amount of encapsulated calcium ions.

Characterization of Synthesized HA. Chitosan/HA composite films of HA1, HA3, and HA5 were sampled to determine the functional groups compared with chitosan film by attenuated total reflection–Fourier transform infrared spectrophotometer (ATR–FTIR) analyses with representative spectra shown in Figure 5. Chitosan showed specific peak of OH groups at around 3100–3600 cm^{-1}, CH stretching at 2996–2882 cm^{-1}, amide I at around 1600 cm^{-1}, and —NH$_2$ groups at around 1400 cm^{-1}. HA1, HA3, and HA5 composites also showed a specific peak of OH groups of HA at around 3100–3600 cm^{-1}. HA5 showed a larger and broader peak of OH groups than the other samples, and this may be because of moisture adsorption, but also HA1, HA3, and HA5 composites revealed spectra owing to P—O stretching of HA around 850–990 cm^{-1}. Furthermore, to evaluate the HA formation within the chitosan film, chitosan/HA composite films of HA1, HA3, and HA5 were also examined for the specific 2θ peaks by XRD. Figure 6 shows representative XRD diffractograms of chitosan/HA composites of HA1, HA3, and HA5 compared with chitosan powder. Chitosan powder was used in this assay instead of chitosan film as XRD failed to detect any peak of transparent chitosan thin film (data not shown). Chitosan powder showed a broad 2θ peak around 20°,

Figure 5. Representative FTIR spectra of (a) chitosan films, (b) HA1, (c) HA3, and (d) HA5 composite films.
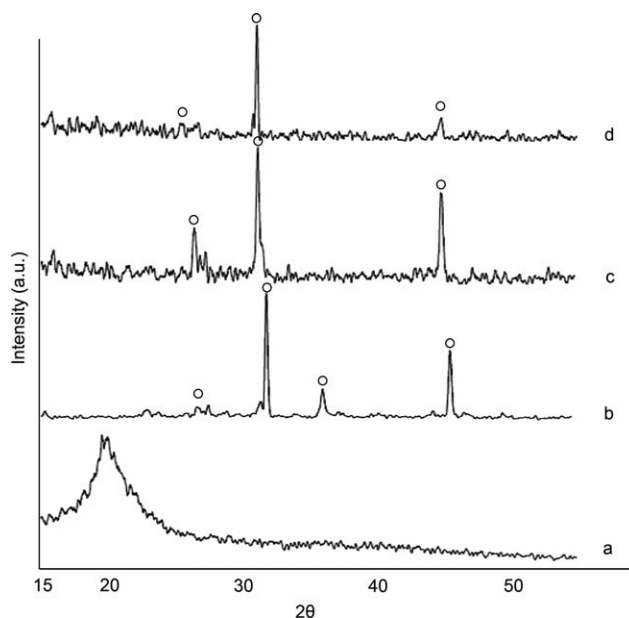
Figure 6. Representative X-ray diffractograms of (a) chitosan powder, (b) HA1, (c) HA3, and (d) HA5 composite films. The specific peaks of (○)HA are shown.
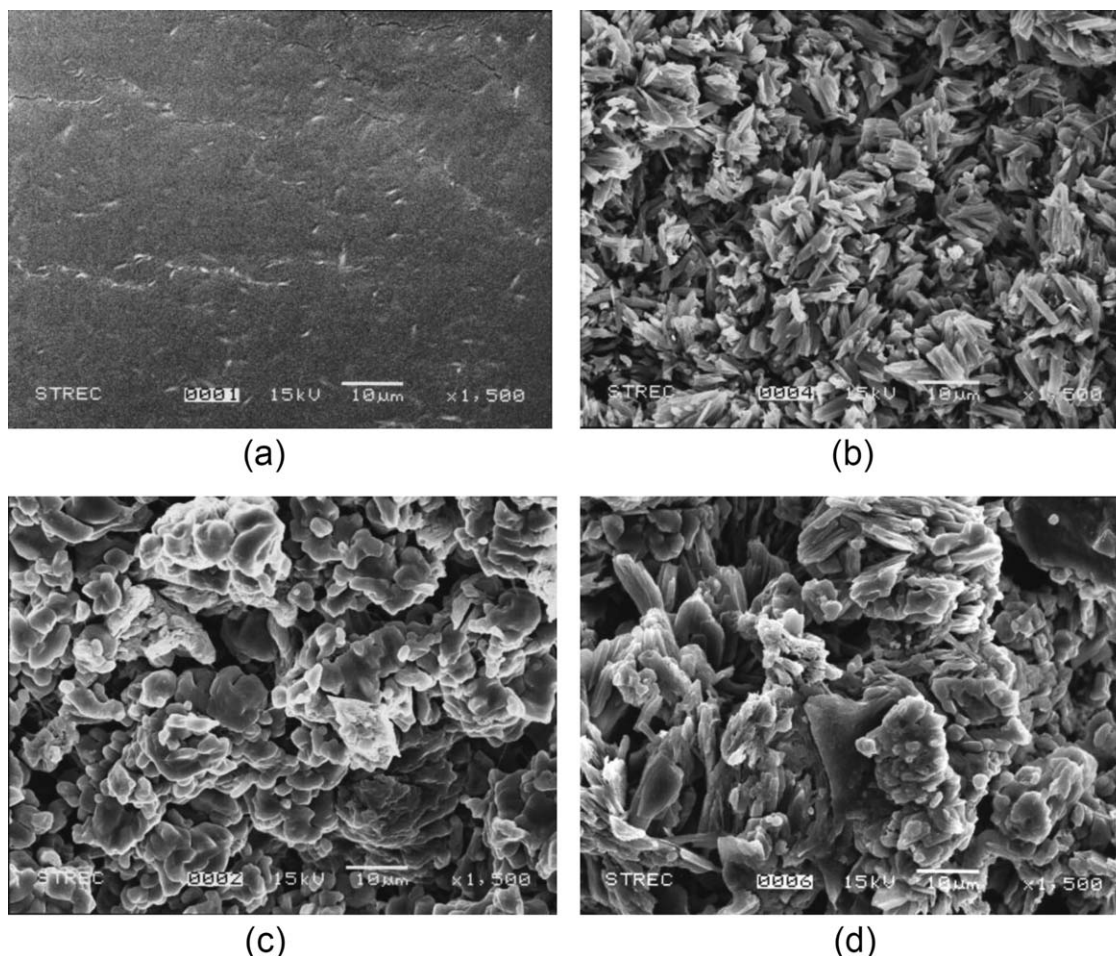
Figure 7. SEM images of (a) chitosan films, (b) HA1, (c) HA3, and (d) HA5 composite films.

whereas chitosan/HA composite films (HA1, HA3, and HA5) showed 2θ of 26, 32, 36, and 45° owing to the specific peaks of HA (JCPDS No. 09-0432). No specific peak of chitosan was observed in chitosan/HA composite films, most likely owing to the HA being dispersed over the entire surface of the composite films. Together, the data from ATR–FTIR and XRD analyses indicated that HA was successfully hybridized into the chitosan matrix via this membrane diffusion process.

The Surface Morphology of Chitosan/HA Composites. The surface morphology of chitosan and the chitosan/HA composite films of HA1, HA3, and HA5 was observed by SEM with representative images shown in Figure 7. At this resolution (magnification, 1500×), the SEM images of chitosan film showed a rather smooth surface. HA1 (prepared by low concentration of calcium ions/phosphate ions) showed a dense cluster of needle-like crystals of HA. HA3 and HA5, which were prepared at higher concentrations of calcium and phosphate ions, showed larger aggregates and significantly more aggregation of HA across all the surfaces and this was more marked in HA5 than HA3. With the crystal size of HA1, also being much smaller than that of HA3 and HA5, at low concentrations of calcium and phosphate ions the orientation of HA crystals occurred during hybridization owing to the slow diffusion rate of ions.

On the other hand, at higher concentrations of calcium and phosphate ions, they diffused to form HA very fast and hence the synthesized HA aggregate into large crystal size.

CONCLUSIONS

HA was successfully hybridized into chitosan solution with good dispersion via a novel membrane diffusion process. The appropriate conditions to prepare chitosan/HA composite were evaluated and found to be maintained at concentrations of 1.75% w/v, $0.2M$, and $0.12M$ for chitosan, calcium chloride, and disodium hydrogen phosphate, respectively, with a diffusion time of 3 days through the membrane . The chitosan/HA composite film showed an even coverage of needle-like HA crystal clusters when using low concentrations of calcium chloride/disodium hydrogen phosphate, whereas aggregation of HA was observed when hybridized at higher concentrations of calcium chloride and disodium hydrogen phosphate.

Chitosan/HA composites prepared via this membrane diffusion process were still in the fluid phase and hence can be shaped or molded into various forms such as bead, film as well as porous scaffold, making it potentially suitable for diverse applications. With the similar chemical reaction, membrane diffusion process

was a trial not only to produce HA, but also to produce calcium phosphates within chitosan solution. Dicalcium phosphate dihydrate was also produced in our laboratory via this process.[19] With the membrane diffusion process suitable for scaling up, the preparation of chitosan/HA composites in the form of a porous scaffold and its subsequent evaluation in terms of its ability to support bone tissue regeneration will now be studied further.

ACKNOWLEDGMENTS

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