Synthesis and character investigation of new collagen Hydrolysate/polyvinyl alcohol/hydroxyapatite Polymer-Nano-Porous Membranes: I. Experimental design optimization in thermal and structural properties

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Abstract Development of bioorganic–inorganic composites has drawn eyes to extensive attention in biomedical fields and tissue engineering. So many attempts to prepare hydroxyapatite (HA), in conjunction with various binders including polyvinyl alcohol (PVA), and collagen has performed for late 20 years. We applied a method based on the phase separation for making of polymer porous membranes. This procedure is induced through the addition of a small quantity of water (polymer-rich phase) to a solution with HA precursors (polymer-poor phase). Thermal and structural composite properties of collagen Hydrolysate (CH)–PVA/HA Polymer-Nano-Porous Membranes were analyzed by Design of experiment that was undertaken using D-optimal approach, to select the optimal combination of nano composites precursor. The resulted composite characters were investigated by Fourier transform infrared, scanning electron microscopy (SEM) and thermal gravimetric analysis. Based on the SEM images, this new method could be clearly concluded to porous CH–PVA/HA hybrid materials. Finally the hemocompatibility of nano-composite membranes were evaluated by the hemolysis study.

Keywords Tissue engineering · Polymer porous membranes · Mixture experimental design · D-optimal approach · Hydroxyapatite collagen biocomposite

Introduction

Some biocomposite materials composed of organic–inorganic components were used in biomedical fields for bone reconstituting and tissue engineering. Hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$), as a well known phosphates in the biologically active phosphate ceramic family, has been widely used in the biomedical field, especially in dental and orthopedic surgery (Kapoor and Batra 2010; Kikuchi et al. 2004; Liu and Chou 1994; Petit 1999) by virtue of its similarity to natural bone mineral. It is known to be a bioactive and biocompatible material with excellent osteoconductive properties (de Bruijn and van Blitterswijk 1998; Hamadouche and Sedel 2000; Layman and Ardoin 1998), as HA shows a strong interaction with bio-molecules such as collagen (COL), glycoprotein, etc. (Girija et al. 2004; Itoh et al. 2003).

Collagen is the major structural protein of connective tissue such as skin, bone, cartilage, tendons and ligaments. Because of its biological properties and availabilities, it is widely used as a biomaterial with multiple physical forms such as sponges, films membranes, wire and fabrics. Among relevant properties of COL are low immune response, low toxicity, the ability to promote cellular growth and attachment, homeostasis and the ability of COL solution to reconstitute in vitro according to the microfibrillar structure found in natural tissues (Sionkowska 2000). Type (I) collagen, the major structural protein of natural bone, has been shown to enhance proliferation and differentiation of osteoblasts (Zamboni and Grano 1995). Several studies in dental medicine have pointed out a possible role for collagen type I in guided bone regeneration around HA implants in the jaw (Brunel et al. 2001; Caiazza et al. 2000).

Many composite materials based on natural and/or synthetic components were prepared and tested as bone

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grafts. Due to the compositional similarity with bone, collagen and hydroxyapatite based composite materials were extensively studied in the literature (Bandyopadhyay-Ghosh 2008; Fikai et al. 2010a, b; Wahl and Czernuszka 2006). The addition of a supplementary component is done in order to improve some properties of the materials or induce new properties. PVA is a scaffold supporting material for tissue engineering applications. It has been frequently utilized for cell culture attachment (Chen et al. 2007; Doyle et al. 2009; Michaels et al. 1995) and as a hydrogel for bioartificial pancreas (Chuang et al. 1999; Qi et al. 2004). PVA, as a safe component, has been cleared by the United States Food and Drug Administration (FDA) (Lindemann 1971). Also, preparation of PVA/HA blends has been reported to exploit the advantages of both materials (Kim et al. 2008). COL-PVA/HA composite materials have been poorly studied in the literature (Asran et al. 2010; Degirmenbasi et al. 2006; Kobayashi et al. 2004; Poursamar et al. 2009). From recently published papers about synthesis of ternary COL-PVA/HA composite materials, a work is the synthesis and the characterization of COL-PVA/HA hybrid materials with 1:2:3 wt compared to COL/PVA hybrid 1:2 wt. They have shown the composite properties of the materials are strongly influenced by their composition and processing. For this purpose, both types of materials were dried by controlled drying (at 30 °C) and freeze drying. Based on SEM images and porosity measurement, they concluded that the stratified morphology of the hybrid materials is due to the presence of PVA. The freeze drying was revealed to induce a porous morphology while the controlled air drying was leading to compact morphology. The stratified presence of HA has resulted in a denser material with higher density and lower porosity (Fikai et al. 2010c). In other work PVA/COL/HA composite materials was prepared by electrospinning technique. Microscopic photographs showed that PVA/COL/HA biocomposite nanofibers had a similar nanostructure to bone in which the nano rod-shape HA crystals were aligned along PVA/COL nanofibers. The most striking properties of electrospun PVA/COL/HA biocomposite nanofibers scaffold not only the composition but also mimicking the hierarchical structure of extracellular matrix and mineral organization in bone at the nanoscale level which might be open up a wide variety of future applications for higher bioactive bone graft materials (Asran et al. 2010). Since the properties of the materials are strongly influenced by their composition and processing, in this research, we used the mixture design in the experimental design (Design-Expert, Version 7.0.0, Trial MFC Application, Stat-Ease, Inc., Minneapolis, MN, USA), to optimize the formulation of the ternary CH-PVA/HA composite materials. After thermo gravimetric analysis, the structural properties of synthesized nano composites were

measured. The relationships between the different combinations and thermal and structural properties of products were analyzed by Design-Expert to select the optimal combination of nano composites precursors from thermal and structural properties point of view. Finally the hemocompatibility of nano composite membranes were evaluated by the hemolysis study.

Experimental

Materials

Collagen was extract from Bovine tendon, (SERVA Co). The average molecular weight of collagen was found to be 350 kDa. We heated the collagen with water in the temperature range between 80 and 90 °C for 3 h to obtain CH (Lowe (1937). Polyvinyl alcohol, (Denki kagaku kogyo Co, Korea, MW = 84,000 g mol⁻¹). Ca(NO₃)₂·4H₂O powder (Merck), P₂O₅ (Merck), ethanol (Merck).

Preparation of CH-PVA blends

Polymer blends were prepared by mixing ingredients (CH, PVA) according to an experimental design. Precursors gradually added into small quantity of water (2 ml) and dissolved in it by ultrasound bath (type: PUL 200, England) for 1 h to obtain a clear dense solution. Then we were mixing the solution by magnetic stirrer (Heidolph, model: D-91126 Schwabach) at 1,400 rpm for 30 min at room temperature.

In situ preparation of CH-PVA/HA Nano-Porous Membranes

In situ preparations of CH-PVA/HA Nano-Porous Membranes were done by starting from dissolving of requiring amount (according to DOE method) of P₂O₅ and Ca(NO₃)₂·4H₂O in 10 ml ethanol separately. This solutions mixing at 1,400 rpm in room temperature, and then added the CH-PVA blends to them in same conditions and covered the backer with parafilm sheet. After 2 h obtained a dense solution that was put in an oven (ShimiFann E.o 155) in 70 °C for 12 h to obtain a Polymer-Nano-Porous Membrane.

Characterization

The samples were characterized by FTIR, SEM and TGA; the density was measured by the Arthur method. IR spectroscopic measurements were performed on a BRUKER FTIR (model: TENSOR 27) spectrophotometer. The spectra were recorded over a 400–4,000 cm⁻¹ wave

number range with a resolution of 1 cm^{-1} . The particles size and size distribution of CH-PVA containing HA nanoparticles Polymer-Nano-Porous Membranes were determined by SEM (XL30 model, Philips Co, Holland). All samples were covered with a gold layer prior to imaging with sputter coater (Bal-Tec Co, model: SCD 005, Swiss). These experiments were performed at room temperature. Thermogravimetric analysis (TGA) (Perkin Elmer, model: pyris Diamond), operating at the following conditions: weight of the sample of nano composite blends, heating rate, $20 \text{ }^{\circ}\text{C min}^{-1}$; maximum limit of heating, $800 \text{ }^{\circ}\text{C}$; Thermal stability of samples was determined as the slope of the TGA curve in the temperature range $489\text{--}759 \text{ }^{\circ}\text{C}$. The density was measured by the Arthur method (ASTM D792-08 1999). The samples were weighed and allowed to stay under vacuum (0.4 at.) for 30 min and thereafter immersed in xylene for further 30 min. After the immersion, the samples were weighed in air and in xylene. Based on the above sets of masses the density of each sample was measured.

Experimental design

Mixture experimental design was used for a statistical and mathematical evaluation of measurements to fit the experimental data to the model for optimization Processes. A response surface method that is usually applied for optimizing of the final products occurred in various proportions (Giesbrecht and Gumpertz 2004). This method gives a set of regression equations, as the output. These equations can be used for optimization of the system.

Independent variables, including biopolymer (CH) (X_1), a synthetic modifier polymer (PVA) (X_2), and biocompatible nanoparticles (HA) (X_3). Satisfy a statistical criterion called D-optimality. D-optimal is a initial design in mixture design methodology. D-optimal concept is an algorithm that can be used for the selection of samples from a larger set.

Mixture formulation was investigated using a D-optimal design approach (DuMouchel and Bradley 1994). D-optimal design was computed by Design-Expert in 16 experiment units. The ranges of levels of the CH, PVA and HA are 0–98.94, 0–98.94 and 1–75 % in polymeric blends respectively.

Thermal stability (from TGA measurements) of polymer blends and density of them were evaluated as responses of experiments. The measured properties are summarized according to the mixture design methodology in Table 1. These properties were evaluated using the Design-Expert software. The mathematical relationship of the responses and the independent variables (X_i) were modelled by a second-order polynomial function as follows:

Table 1 Mixture design in various runs and the correspondent response

Run no.	Independent variables			Dependent variables	
	X_1 CH (%)	X_2 PVA (%)	X_3 HA (%)	Y_1 TGA ($^{\circ}\text{C}$)	Y_2 Density (gr/cm^3)
1	32.286	27.371	40.341	651	0.43
2	98.938	0.056	1.005	582	0.562
3	0.031	98.939	1.029	489	39.56
4	53.343	21.148	25.508	647	1.069
5	0	49.485	50.515	683	1.388
6	25	0	75	757	0.157
7	0	25	75	742	1.101
8	0.031	98.939	1.029	487	38.47
9	61.997	0	38.003	658	0.531
10	48.898	50.101	1	600	15.112
11	0	25	75	745	1.028
12	48.898	50.101	1	602	15.05
13	98.938	0.056	1.005	588	0.556
14	71.384	22.637	5.978	621	11.61
15	25	0	75	759	0.159
16	16.39	65.676	17.933	532	0.408

$$\begin{aligned}
 Y = & \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \\
 & + \beta_{123} X_1 X_2 X_3 + \beta_{12} (\beta_1 - \beta_2) X_1 X_2 (X_1 - X_2) \\
 & + \beta_{13} (\beta_1 - \beta_3) X_1 X_3 (X_1 - X_3) \\
 & + \beta_{23} (\beta_2 - \beta_3) X_2 X_3 (X_2 - X_3)
 \end{aligned} \quad (1)$$

where Y is the predicted response, $\beta_1, \beta_2, \beta_3, \beta_{12}, \beta_{13}, \beta_{23}, \beta_{123}, \beta_{12} (\beta_1 - \beta_2), \beta_{13} (\beta_1 - \beta_3), \beta_{23} (x\beta_2 - \beta_3)$, are regression coefficients and x_i are factors (independent variables). The results from regression analysis are given in Table 1. On the basis of regression equations it was possible to plot the response surfaces of the individual parameters at chosen conditions. For significant sequential models test, analysis of variance (ANOVA) was performed by employing a statistical methodology. ANOVA includes a full analysis of variance, prediction equations, and case statistics and help to determine which model, if any, significant to choose.

Hemolysis assay

First, for red blood cell's (RBCs) preparation, peripheral blood, which is the chief source of mononuclear cells (lymphocytes and monocytes) for immunologic studies of humans (Strober 2001), was collected from healthy human donor by venipuncture and 0.3 % of EDTA was added. RBCs were separated by centrifugation at $2,000 \times g$ for 30 min. RBCs were then washed once in PBS-EDTA 0.075 % and three times in an isotonic buffer (PBS). RBCs

were then suspended in this buffer at a cell density of $5\text{--}108\text{ cells ml}^{-1}$ (Dufour et al. 2005). Then for hemolysis assay, CH-PVA polymeric blend and CH-PVA/HA nano composite membrane dissolved in small portions of distilled water and 1 M HCl (Chen et al. 2004) respectively, and for each sample, prepared solutions in 5, 10, 15 and 20 mg ml^{-1} concentrations. These solutions volume received to 900 and 100 ml of RBCs suspension were added and the reaction was performed at $25\text{ }^{\circ}\text{C}$ during 30 min. Unaltered RBCs were then removed by a $10,000\times g$ centrifugation and the absorbance of the supernatant at 540 nm was compared with two control samples in order to determine the percentage of hemolysis. The positive control prepared by adding 100 ml of RBCs suspension to 900 ml of distilled water. Positive control was totally hemolysed with distilled water. The negative control prepared by adding 100 ml of RBCs suspension to 900 ml of PBS. The RBCs in PBS remained safe (Dufour et al. 2005). Each experiment has been performed at least twice. The hemolysis percent can be obtained through the following equation (Zhang et al. 2010):

$$\text{Hemolysis (\%)} = \frac{OD_t - OD_{nc}}{OD_{pc}} \times 100 \quad (2)$$

where OD_t [the absorbance also called optical density (OD) (McNaught and Wilkinson 1996)], is the absorbance of the test sample. OD_{pc} and OD_{nc} are the absorbance of the positive and negative controls, respectively.

Results and discussion

Nano-Porous Membranes identification

The present authors introduced a novel method for preparation of CH-PVA/HA nano hybrids through in situ synthesis of HA in CH-PVA blending solution. This procedure requires the use of a solvent with a low boiling point that is easy to evaporate. For example ethanol used to dissolve P_2O_5 and $\text{Ca}(\text{NO}_3)_2\cdot 4\text{H}_2\text{O}$ then phase separation is induced through the addition of CH and PVA blending in a small quantity of water as solvent then a polymer-rich and a polymer-poor phase are formed. Following heating out of the ethanol at its boiling point and during night vacuum-drying to evaporate the solvent a porous scaffold is obtained. The COL as a natural polymer plays as a significant component of extracellular matrix for example about 30 % of the dry weight of bone and 90–95 % of its non mineral content (Nimni et al. 1988). It is reported that the use of COL matrices don't change the phenotypic expression of cultured cells (Reid 1990; Reznikoff et al. 1987; Rocha et al. 1985). Combination of COL and water-soluble polymers such as PVA make the blends that can be

use as a films, hydrogels or sponges according to our trial and the other study (Cascone et al. 1995). This bio artificial material might meet our demands in a range of medical applications including dialysis membranes, wound dressing, artificial skin, cardiovascular devices, nerve guide channels and as implantable devices to release biologically active substances in a controlled manner (Cascone et al. 1994; Cascone et al. 1995). Chemically and physically modified biodegradable polymers such as CH-PVA in this study may be used as scaffolds for tissue engineering purposes.

The spatial distribution of the hydroxyapatite particles in the porous structure formed in the CH-PVA/HA samples were analyzed using a SEM (XL30, Philips Co, Holland). The Fig. 1a show the polymer matrix (CH-PVA) without applied the HA nano particles. It can be observed that the polymer matrix have a homogeny morphology. In Fig. 1b image for the used HA nano particles in CH-PVA matrix; it can be observed that the HA particles have size in the range of almost 38–42 nm.

As shown in this figure, HA nano particles were randomly dispersed in the polymer matrix and shown well

Fig. 1 SEM image of CH-PVA polymeric blend (a); and HA nano particles distribution in the CH-PVA matrix (b)

distribution in each part of this matrix which is noticeable to be mentioned.

Infrared spectroscopy

Samples show peaks at $3,400\text{--}3,500\text{ cm}^{-1}$, corresponding to the hydroxyl groups. The characteristic peaks of NH containing $3,342\text{ cm}^{-1}$ (amide A, N–H stretching), $3,082\text{ cm}^{-1}$ (amide B, N–H stretching), $1,662\text{ cm}^{-1}$ (amide I, C=O stretching), $1,556\text{ cm}^{-1}$ (amide II, N–H bending and C–N stretching) and $1,240\text{ cm}^{-1}$ (amide III, CN stretching and N–H bending) (Chang and Tanaka 2002a, b; Friess and Lee 1996; Lai et al. 2007; Sachlos et al. 2006; Sionkowska et al. 2004; Wahl et al. 2007). For the PVA spectrum no obvious C=O, amide A, B and II bands was observed. An absorption band at $2,943\text{ cm}^{-1}$ representing the --CH_2 aliphatic group of PVA comparing with the CH spectrum (Lai et al. 2007; Sionkowska et al. 2004).

The characteristic bands of HA powder containing $3,555$ and 622 cm^{-1} (structural OH^- ions, stretching and vibration mode, respectively), $1,040\text{ cm}^{-1}$ (PO_4 bend ν_3), 603 and 561 cm^{-1} (PO_4 bend ν_4) (Eslami et al. 2008) and $1,600\text{ cm}^{-1}$ (CO_3^{2-} , which an indication of the presence of carbonate apatite. This might have originated through the absorption of carbon dioxide from the atmosphere (Komath and Varma (2003)).

In CH–PVA blend spectrum, the amide A band shifted to higher wavenumber gradually compared to that of the pure COL dispersion. The amide B band disappeared, because of amide B band was observed only for CH/PVA blend weight ratio (99:1) (Komath and Varma (2003).

In CH–HA spectrum, addition to the typical amide bands of COL, it could be seen a broad phosphate band in the range from $1,150$ to 950 cm^{-1} derives from the P–O asymmetric stretching mode of the $(\text{PO}_4)^{-3}$ group. The triple degenerate bending modes of the O–P–O bond were exhibited at 600 and 572 cm^{-1} (ASTM D792-08 1999; Ficai et al. 2010c).

After incorporating HA nanoparticles into the PVA matrix a shift of the predominant broad absorption band occur associated with the OH stretching of PVA from $3,278$ to $3,296\text{ cm}^{-1}$. This result suggests that the hydrogen bonding became stronger in case of PVA–HA than pure PVA due to the increase in the number of OH groups by addition of HA. The major absorption band of PO_4^{3-} stretching in HA, moved from $1,019$ to $1,033\text{ cm}^{-1}$. This shift might be attributed to the interactions between PVA molecules and HA particles (Asran et al. 2010). Also the spectra of PVA adsorbed on HA clearly influence the recovering of the carbonyl-stretching region (Kim et al. 2008). In CH–PVA/HA nano porose membrane (Fig. 2) the intensity of the amide A band has decreased as compared to that of CH–PVA 1:2 materials while the amide B band could not be identified. As we mentioned before (Eslami et al. 2008), the presence of

Fig. 2 FTIR spectra for CH (a), PVA (b), HA (c), CH–PVA (d), CH–HA (e), PVA–HA (f) and CH–PVA/HA (g)

HA has induced a shift of the amide I and amide II bands at the low and high wavenumber, respectively (ASTM D792-08 1999).

As shown in Fig. 2e, g, the FT-IR spectra of CH/HA and CH–PVA/HA composite materials are very similar to the spectra of real bone (Evans et al. 1992; Paschalis et al. 1996). The appearance of an amide I mode at $1,662\text{ cm}^{-1}$ indicates that CH/HA composites adopt a predominantly α -helical configuration and this is confirmed by the appearance of amide II mode at $1,556\text{ cm}^{-1}$ (Doyle et al. 1975; Payne and Veis 1988). The red shift of the $1,339\text{ cm}^{-1}$ band in COL has been effectively used to confirm the chemical bond formation between carboxyl ions in COL and HA phases (Chang et al. 2001; Kikuchi et al. 1999). The $1,339\text{ cm}^{-1}$ band in COL does not simply represent the carboxyl group, but it is one of a number of bands in the range of $1,400\text{--}1,260\text{ cm}^{-1}$ which are attributed to the presence of COL in biological tissue (Liu et al. 1996). The band at $1,339\text{ cm}^{-1}$ in COL is attributed predominantly to the so-called wagging vibration of proline side chains (Chang et al. 2003). It is believed that the red shift in the CH/HA system is encouraged by the wagging vibration through the covalent bond formation with Ca^{2+} ions of HA nanoparticles (Chang et al. 2003). It is known that the carboxylic acid groups of CH at $\text{pH} > 7$ are largely ionic in

form (Jakobsen et al. 1983) and offer binding sites for Ca ions because of the ionic attraction.

Mixture design and response surface methodology

The Response surface methodology followed by D-optimal design was applied to find optimal level of independent variables including biopolymer CH (X_1), a synthetic modifier polymer PVA (X_2), and biocompatible nanoparticles HA (X_3) shown in Table 1.

The analysis of variance by calculating F value was employed to choose the best model matched with the data. The p value less than 0.05, indicate that the data were normally distributed. Also, the similarity between R^2 and adjusted R^2 shows the adequacy of the model to predict the response by optimization process. The non-significant lack-of-fit (more than 0.05) showed that the model is valid for present study. Non-significant lack-of-fit is good for data fitness in the model.

Thermal behavior and experimental design optimization

Degradation of CH-PVA film shows three weight loss stages. The first weight loss takes place at 50–176 °C due to the loss of adsorbed moisture via evaporation of the trapped water; the second stage at 200–400 °C involves the elimination reactions of H_2O and residual acetate groups because of partially hydrolyzed COL and PVA. The degradation step at 400–550 °C is more complex and includes the further degradation of these polymer residues to yield the carbon dioxide and hydrocarbons (Peng and Kong 2007). In CH-PVA/HA nano composites, the surface of HA nanoparticles will contact with the CH and PVA molecules to make a coordination bond between Ca^{2+} and $RCOO^-$ in CH molecules and a strong interact between the hydroxyl groups on the surface of HA and PVA hydrophilic molecules (Chang and Tanaka 2002a, b; Ficaï et al. 2009; Kim et al. 2008; Peng and Kong 2007). These reactions were influenced on tertiary weight loss stage of CH-PVA blend materials. According to experimental design results this weight loss temperature increased with increasing the amount of HA nanoparticles (Table 1). CH-PVA/HA sample shows a huge exothermic peak at higher temperature. It is believed that the huge exothermic peak at ~630 °C is caused by the decomposition of CH and PVA molecules combined with HA nanoparticles. In this sample HA nanoparticles are uniformly dispersed in the CH-PVA matrix as shown by SEM analysis (Fig. 1b). We know from FT-IR (Fig. 2g) that the nanoparticles having the width of several nanometers are chemically bound with the CH and PVA molecules. The organic-inorganic bond energy was released as a huge amount of exothermic heat as shown in Fig. 3b for the CH-PVA/HA sample. When TGA is

Fig. 3 The TG and DTG thermogram of CH-PVA polymer blend film (a) and optimized nano composite of CH-PVA/HA blend (b)

considered as the response, the cubic equation fitted to the data was presented as follows:

$$Y_{TGA} = 5.853X_1 + 4.875X_2 + 6.653X_3 + 0.027X_1X_2 + 0.032X_1X_3 + 0.038X_2X_3 - 1.571X_1X_2X_3 + 3.976X_1X_2(X_1 - X_2) - 5.481X_1X_3(X_1 - X_3) - 5.473X_2X_3(X_2 - X_3) \quad (3)$$

The analysis of variance for TGA response (Table 2), presented as follows: According to this analysis, the Model F value of 201.06 implies the model is significant. There is only a 0.01 % chance that a *Model F Value* this large could occur due to noise. Values of *Prob > F* less than 0.0500 indicate model terms are significant. In this case linear mixture components, X_1X_2 , X_1X_3 , X_2X_3 , $X_1X_2X_3$, $X_1X_2(X_1 - X_2)$, $X_1X_3(X_1 - X_3)$, $X_2X_3(X_2 - X_3)$ are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. Therefore, all of the model terms are significant. The non-significant lack-of-fit (more than 0.05) is good for data fitness in the model.

The good correlation between the measured values and those predicted by the model obtained by R^2 parameter ($R_{TGA}^2 = 0.996$), that confirms the quality of this model. In addition, the *Pred R²* of 0.8312 is in reasonable agreement

Table 2 The analysis of variance table for TGA as the response

Source	Sum of squares	df	Mean square	F value	p value (prob > F)	
Model	117,520.3	9	13,057.81	201.0555	<0.0001	Significant
Linear mixture	108,243.8	2	54,121.91	833.3332	<0.0001	
X_1X_2	5,809.457	1	5,809.457	89.45015	<0.0001	
X_1X_3	461.5786	1	461.5786	7.107079	0.0372	
X_2X_3	744.2024	1	744.2024	11.45873	0.0148	
$X_1X_2X_3$	1,488.964	1	1,488.964	22.92608	0.0030	
$X_1X_2 (X_1 - X_2)$	1,132.128	1	1,132.128	17.43175	0.0058	
$X_1X_3 (X_1 - X_3)$	680.8583	1	680.8583	10.4834	0.0177	
$X_2X_3 (X_2 - X_3)$	580.6854	1	580.6854	8.941007	0.0243	
Residual	389.6779	6	64.94631			
Lack of fit	215.1779	1	215.1779	6.165555	0.0556	Not significant
Pure error	174.5	5	34.9			
Cor total	117,910	15				

Fig. 4 The three dimensional response surface diagram for the thermal behavior (a) and density (b), from the minimum value to 99 % of CH and PVA, and from 1 to 75 % of HA in CH-PVA/HA Polymer-Nano-Porous Membranes

with the $Adj R^2$ of 0.9917. These values confirm that the equation of the model is highly reliable. This indicates also that the model terms are significant at 95 % of probability level.

According to three dimensional response surface diagram (Fig. 4), tertiary weight loss temperature increases with content of HA in the blend, (as seen in Fig. 4a). The effect of both CH and PVA are opposite, (as seen in Fig. 4a).

Density and experimental design optimization

CH-PVA/HA nano composites were successfully synthesized. Density of the CH-PVA/HA can be introduced by ethanol evaporation and it can be controlled using the change in amount of CH, PVA and HA in composition of porous membranes. The characterization of the density (Table 1) was measured by the Arthur method (ASTM

D792-08 1999). The mixture design also was used for evaluation of these characteristic. When density of CH-PVA/HA nano composites is considered as the response, the cubic equation fitted to the data was presented as follows:

$$\begin{aligned}
 Y_{Density} = & 0.010X_1 + 0.416X_2 - 0.278X_3 - 1.860X_1X_2 \\
 & - 1.860X_1X_3 + 6.551X_2X_3 - 2.441X_1X_2X_3 \\
 & + 1.073X_1X_2(X_1 - X_2) - 9.124X_1X_3(X_1 - X_3) \\
 & - 1.657X_2X_3(X_2 - X_3)
 \end{aligned} \quad (4)$$

The analysis of variance for density of CH-PVA/HA nano composites response (Table 3), presented as follows: According to this analysis, the Model F value of 1,116.95 implies the model is significant. There is only a 0.01 % chance that a *Model F Value* this large could occur due to noise. In this case linear mixture components, X_1X_2 , X_1X_3 , $X_1X_2X_3$, $X_1X_2 (X_1 - X_2)$, $X_1X_3 (X_1 - X_3)$, $X_2X_3 (X_2 - X_3)$

are significant model terms and the non-significant lack-of-fit (more than 0.05) is good for data fitness in the model.

The good correlation between the measured values and those predicted by the model obtained by R^2 parameter ($R^2_{Density} = 0.999$), that confirms the quality of this model. In addition, the $Pred R^2$ of 0.9703 is in reasonable agreement with the $Adj R^2$ of 0.9985.

These values confirm that the equation of the model is highly reliable. This indicates also that the model terms are significant at 95 % of probability level. The *Lack of Fit F value* of 2.85 implies the Lack of Fit is not significant relative to the pure error.

According to three dimensional response surface diagram (Fig. 4b), achieved that, density increases with content of PVA in the blend, (as seen in Fig. 4b). The effect of both CH and HA are opposite, (as seen in Fig. 4b).

Optimum sample predicted by Design expert software. Predicted levels of CH, PVA and HA are 13.93, 24.07 and 62 % respectively. The predicted and observed values of TGA and density are 726.42, 746.46 °C and 2.43, 2.30 gr cm⁻³ respectively. We could be observed that the predicted values of responses (TGA and density) are in reasonable agreement with their observed values. Thermal analysis of optimum sample is shown in Fig. 3b.

Hemolysis

Each of CH-PVA polymeric blend and CH-PVA/HA Nano-Porous Membrane has been tested at various concentrations for its hemolytic activity. The results are given in Fig. 5 CH-PVA polymeric blend do not cause any hemolysis in the concentration range used. CH-PVA/HA Nano-Porous Membrane gives rise to a concentration

dependent hemolysis. HC_{50} , which is defined as the concentration of surfactant that bursts 50 % of the RBC, is equal to 18.633 mg ml⁻¹.

Conclusion

We conclude that the stratified morphology of the CH-PVA/HA membranes could be varying by changing experimental conditions. The method of DOE helped to identify the effect of CH, PVA and HA on thermal and structural properties evaluated on the base of this method. The properties of the polymer-nano porous membranes are strongly influenced by their precursors' composition. As a result, based on thermal gravimetric analyses and density.

Structural measurements by the Arthur methods, seems the presence of PVA to improve compression resistance of these CH-PVA/HA nano composite materials so density was decrease with content of PVA in the blends. The highly dense scaffolds have low porosity, high absorption capacity and strength and it is worth to be mentioned in these composite tertiary weight loss temperature was increases with content of HA in the blends. The CH-PVA polymeric blends have excellent biocompatibility, according to the hemolysis tests and the CH-PVA/HA Nano-Porous Membranes gives rise to a concentration dependent hemolysis. We hope these results may be hopeful and by considering suitable porous composite method may result a reasonable composite with acceptable physical properties in near future. We suggest by considering these result attempts by salt leaching and freeze drying or microwave solvent evaporation to synthesize new composite with

Table 3 The analysis of variance table for density of CH-PVA/HA nano composites as the response

Source	Sum of squares	df	Mean square	F value	p value (Prob > F)	
Model	2,623.621	9	291.5135	1,116.949	<0.0001	Significant
Linear mixture	1,897.988	2	948.9939	3,636.119	<0.0001	
X_1X_2	27.77097	1	27.77097	106.4059	<0.0001	
X_1X_3	18.66954	1	18.66954	71.5333	0.0001	
X_2X_3	1.230647	1	1.230647	4.715287	0.0729	
$X_1X_2X_3$	35.95968	1	35.95968	137.7814	<0.0001	
$X_1X_2(X_1 - X_2)$	82.49106	1	82.49106	316.0687	<0.0001	
$X_1X_3(X_1 - X_3)$	18.86838	1	18.86838	72.29517	0.0001	
$X_2X_3(X_2 - X_3)$	53.25228	1	53.25228	204.0389	<0.0001	
Residual	1.565945	6	0.260991			
Lack of fit	0.839869	1	0.839869	5.783611	0.0612	Not significant
Pure error	0.726077	5	0.145215			
Cor total	2,625.187	15

Fig. 5 Hemolytic activity of CH-PVA polymeric blend (filled diamond); and CH-PVA/HA Nano-Porous Membrane (filled square); as a function of their concentration

reasonable porosity and mechanical properties for bioengineering tissue.

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References

- Asran AS, Henning S, Michler GH (2010) Polyvinyl alcohol–collagen–hydroxyapatite biocomposite nanofibrous scaffold: mimicking the key features of natural bone at the nanoscale level. *Polymer* 51:868–876. doi:[10.1016/j.polymer.2009.12.046](https://doi.org/10.1016/j.polymer.2009.12.046)
- ASTM D792-08 (1999) Standard test methods for density and specific gravity (relative density) of plastics by displacement. Annual Book of ASTM Standards, vol 8.01, part A, American Society for Testing and Materials, West Conshohocken
- Bandyopadhyay-Ghosh S (2008) Bone as a collagen–hydroxyapatite composite and its repair. *Trends Biomater Artif Organs* 22: 116–124
- Brunel G, Brocard D, Duffort JF, Jacquet E, Justum P, Simonet T, Benque E (2001) Bioabsorbable materials for guided bone regeneration prior to implant placement and 7-year follow-up: report of 14 cases. *J Periodontol* 72:257–264
- Caiazza S, Colangelo P, Bedini R, Formisano G, De Angelis G, Barrucci S (2000) Evaluation of guided bone regeneration in rabbit femur using collagen membranes. *J Dent Implants* 9: 219–225
- Cascone MG, Silvio L, Sim B, Downes S (1994) Collagen and hyaluronic acid based polymeric blends as drug delivery systems for the release of physiological concentrations of growth hormone. *J Mater Sci Mater Med* 5:770–774. doi:[10.1007/BF00120374](https://doi.org/10.1007/BF00120374)
- Cascone MG, Sim B, Downes S (1995) Blends of synthetic and natural polymers as drug delivery systems for growth hormone. *Biomaterials* 16:569–574
- Chang MC, Tanaka J (2002a) FT-IR study for hydroxyapatite/collagen nanocomposite cross-linked by glutaraldehyde. *Biomaterials* 23:4811–4818
- Chang MC, Tanaka J (2002b) XPS study for the microstructure development of hydroxyapatite–collagen nanocomposites cross-linked using glutaraldehyde. *Biomaterials* 23:3879–3885
- Chang MC, Ikoma T, Kikuchi M, Tanaka J (2001) Preparation of a porous hydroxyapatite/collagen nanocomposite using glutaraldehyde as a crosslinkage agent. *J Mater Sci Lett* 20:1199–1201. doi:[10.1023/A:1010914621089](https://doi.org/10.1023/A:1010914621089)
- Chang MC, Ko CC, Douglas WH (2003) Preparation of hydroxyapatite–gelatin nanocomposite. *Biomaterials* 24:2853–2862
- Chen ZF, Darvell BW, Leung VWH (2004) Hydroxyapatite solubility in simple inorganic solutions. *Arch Oral Biol* 49:359–367
- Chen J, Zhang Y, Du GC, Hua ZZ, Zhu Y (2007) Biodegradation of polyvinyl alcohol by a mixed microbial culture. *Enzyme Microb Technol* 40:1686–1691. doi:[10.1016/j.enzymmicro.2006.09.010](https://doi.org/10.1016/j.enzymmicro.2006.09.010)
- Chuang WY, Young TH, Yao CH, Chiu WY (1999) Properties of the poly(vinyl alcohol)/chitosan blend and its effect on the culture of fibroblast in vitro. *Biomaterials* 20:1479–1487
- de Bruijn JD, van Blitterswijk CA (1998) New developments in implant coatings: Biomimetics and tissue engineering. In: Schnettler G, Markgraf W (eds) *Biomaterials in Surgery*. Stuttgart, Germany, Thieme, pp 77–82
- Degirmenbasi N, Kalyon DM, Birinci E (2006) Biocomposites of nanohydroxyapatite with collagen and poly(vinyl alcohol). *Colloids Surf B Biointerfaces* 48:42–49. doi:[10.1016/j.colsurfb.2006.01.002](https://doi.org/10.1016/j.colsurfb.2006.01.002)
- Doyle BB, Bendit EG, Blout ER (1975) Infrared spectroscopy of collagen and collagen-like polypeptides. *Biopolymers* 14:93957. doi:[10.1002/bip.1975.360140505](https://doi.org/10.1002/bip.1975.360140505)
- Doyle AD, Wang FW, Matsumoto K, Yamada KM (2009) One-dimensional topography underlies three-dimensional fibrillar cell migration. *J Cell Biol* 184:481–490
- Dufour S, Deleu M, Nott K, Wathélet B, Thonart P, Paquot M (2005) Hemolytic activity of new linear surfactin analogs in relation to their physico-chemical properties. *Biochim Biophys Acta* 1726:87–95
- DuMouchel W, Bradley J (1994) A simple Bayesian modification of D-optimal designs to reduce dependence on an assumed model. *Technometrics* 36:37–47. doi:[10.1080/00401706.1994.10485399](https://doi.org/10.1080/00401706.1994.10485399)
- Eslami H, Solati-Hashjin M, Tahriri M (2008) Synthesis and characterization of hydroxyapatite nanocrystals via chemical precipitation technique. *Iran J Pharm Sci* 4:127–134
- Evans LA, Macey DJ, Webb J (1992) Calcium biomineralization in the radular teeth of the chiton, *Acanthopleura hirtosa*. *Calcif Tissue Int* 51:78–82
- Ficai A, Andronescu E, Ghitulica C, Voicu G, Trandafir V, Manzu D, Ficai M, Pall S (2009) Collagen/hydroxyapatite interactions in composite biomaterials. *Mater Plast* 46:11–15
- Ficai A, Andronescu E, Trandafir V, Ghitulica C, Voicu G (2010a) Collagen/hydroxyapatite composite obtained by electric field orientation. *Mater Lett* 64:541–544. doi:[10.1016/j.matlet.2009.11.070](https://doi.org/10.1016/j.matlet.2009.11.070)
- Ficai A, Andronescu E, Voicu G, Ghitulica C, Ficai D (2010b) The influence of collagen support and ionic species on the morphology of collagen/hydroxyapatite composite materials. *Mater Charact* 61:402–407. doi:[10.1016/j.matchar.2010.01.003](https://doi.org/10.1016/j.matchar.2010.01.003)
- Ficai M, Andronescu E, Ficai D, Voicu G, Ficai A (2010c) Synthesis and characterization of COLL–PVA/HA hybrid materials with stratified morphology. *Colloids Surf B Biointerfaces* 81:614–619. doi:[10.1016/j.colsurfb.2010.08.009](https://doi.org/10.1016/j.colsurfb.2010.08.009)
- Friess W, Lee G (1996) Basic thermoanalytical studies of insoluble collagen matrices. *Biomaterials* 17:2289–2294
- Giesbrecht FG, Gumpertz ML (2004) Planning, construction, and statistical analysis of comparative experiments. Wiley, New Jersey, p 693
- Girija EK, Yokogawa Y, Nagata F (2004) Apatite formation on collagen fibrils in the presence of polyacrylic acid. *J Mater Sci Mater Med* 15:593–599
- Hamadouche M, Sedel L (2000) Ceramics in orthopaedics. *J Bone Joint Surg Br* 82:1095–1099
- Itoh S, Yamaguchi I, Suzuki M, Ichinose S, Takakuda K, Kobayashi H, Shinomiya K, Tanaka J (2003) Hydroxyapatite-coated tendon chitosan tubes with adsorbed laminin peptides facilitate nerve regeneration in vivo. *Brain Res* 993:111–123
- Jakobsen RJ, Brown LL, Hutson TB, Fink DJ, Veis A (1983) Intermolecular interactions in collagen self-assembly as revealed

- by Fourier transform infrared spectroscopy. *Science* 220:1288–1290. doi:[10.1126/science.6857249](https://doi.org/10.1126/science.6857249)
- Kapoor S, Batra U (2010) Preparation and bioactivity evaluation of bone like hydroxyapatite: bioglass composite. *Int J Chem Biol Eng* 3:24–28
- Kikuchi M, Suetsugu Y, Tanaka J, Itoh S, Ichinose S, Shiniyama K, Hiraoka Y, Mandai Y, Nakatani S (1999) The biomimetic synthesis and biocompatibility of self-organized hydroxyapatite/collagen composites. *Bioceramics* 12:393–396
- Kikuchi M, Ikoma T, Itoh S, Matsumoto HN, Koyama Y, Takakuda K, Shinomiya K, Tanaka J (2004) Biomimetic synthesis of bone-like nanocomposites using the self-organization mechanism of hydroxyapatite and collagen. *Compos Sci Technol* 64:819–825. doi:[10.1016/j.compscitech.2003.09.002](https://doi.org/10.1016/j.compscitech.2003.09.002)
- Kim GM, Asran AS, Michler GH, Simon P, Kim JS (2008) Electrospun PVA/HAP nanocomposite nanofibers: biomimetics of mineralized hard tissues at a lower level of complexity. *Bioinspir Biomim* 3:1–12
- Kobayashi H, Kato M, Taguchi T, Ikoma T, Miyashita H, Shimmura S, Tsubota K, Tanaka J (2004) Collagen immobilized PVA hydrogel-hydroxyapatite composites prepared by kneading methods as a material for peripheral cuff of artificial cornea. *Mater Sci Eng, C* 24:729–735. doi:[10.1016/j.msec.2004.08.038](https://doi.org/10.1016/j.msec.2004.08.038)
- Komath M, Varma HK (2003) Development of a fully injectable calcium phosphate cement for orthopedic and dental applications. *Bull Mater Sci* 26:415–422
- Lai G, Du Z, Li G (2007) The rheological behavior of collagen dispersion/poly(vinyl alcohol) blends. *Korea Aust Rheol J* 19: 81–88
- Layman DL, Ardoin RC (1998) An in vitro system for studying osteointegration of dental implants utilizing cells grown on dense hydroxyapatite disks. *J Biomed Mater Res* 40:282–290. doi:[10.1002/\(SICI\)1097-4636\(199805\)](https://doi.org/10.1002/(SICI)1097-4636(199805)40:3<282::AID-JBMB10974636199805>3.0.CO;2-1)
- Lindemann MK (1971) Encyclopedia of polymer science and technology. In: Mark HF, Gaylord NG, Bikales NM (eds), *Vinyl Alcohol Polymers*, vol 14. Wiley, New York, pp 149–239
- Liu DM, Chou HM (1994) Formation of a new bioactive glass-ceramic. *J Mater Sci Mater Med* 5:7–10. doi:[10.1007/BF00121146](https://doi.org/10.1007/BF00121146)
- Liu K, Jackson M, Sowa MG, Ju H, Dixon IMC, Mantsch HH (1996) Modification of the extracellular matrix following myocardial infarction monitored by FTIR spectroscopy. *Biochim Biophys Acta* 1315:73–77
- Lowe B (1937) Experimental cookery from the chemical and physical standpoint. In: a laboratory outline (eds), *gelatin*, 2nd edn, Wiley, New York, pp 969–970
- McNaught AD, Wilkinson A (1996) IUPAC (Gold Book). Blackwell Scientific Publications, Oxford, p 2257
- Michaels JD, Nowak JE, Mallik AK, Koczo K, Wasan DT, Papoutsakis ET (1995) Interfacial properties of cell culture media with cell-protecting additives. *Biotechnol Bioeng* 47:420–430
- Nimni ME, Cheung DT, Strates B, Kodama M, Sheikh K (1988) Bioprosthesis derived from cross-linked and chemically modified collagenous tissues. In: Nimni ME (ed) *Collagen*, vol 3. CRC Press, Boca Raton, FL, pp 1–38
- Paschalis EP, DiCarlo E, Betts F, Sherman P, Mendelsohn R, Boskey AL (1996) FTIR microspectroscopic analysis of human osteonal bone. *Calcif Tissue Int* 59:480–487. doi:[10.1007/BF00369214](https://doi.org/10.1007/BF00369214)
- Payne KJ, Veis A (1988) Fourier transform ir spectroscopy of collagen and gelatin solutions: deconvolution of the amide I band for conformational studies. *Biopolymers* 27:1749–1760. doi:[10.1002/bip.360271105](https://doi.org/10.1002/bip.360271105)
- Peng Z, Kong LX (2007) A thermal degradation mechanism of polyvinyl alcohol/silica nanocomposites. *Polym Degrad Stab* 92:1061–1071. doi:[10.1016/j.polyimdegradstab.2007.02.012](https://doi.org/10.1016/j.polyimdegradstab.2007.02.012)
- Petit R (1999) The use of hydroxyapatite in orthopaedic surgery: a ten-year review. *Eur J Orthop Surg Tr* 9:71–74. doi:[10.1007/BF01695730](https://doi.org/10.1007/BF01695730)
- Poursamar SA, Rabiee M, Samadikuchaksaraei A, Tahriri M, Karimi M, Azami M (2009) Influence of the value of the pH on the preparation of nano hydroxyapatite: poly vinyl alcohol composites. *J Ceram Process Res* 10:679–682
- Qi M, Gu Y, Sakata N, Kim D, Shirouzu Y, Yamamoto C, Hiura A, Sumi S, Inoue K (2004) PVA hydrogel sheet macroencapsulation for the bioartificial pancreas. *Biomaterials* 25:5885–5892
- Reid LM (1990) Stem cell biology, hormone/matrix synergies and liver differentiation. *Curr Opin Cell Biol* 2:121–130
- Reznickoff CA, Loretz LJ, Pesciotta DM, Oberley TD, Ignjatovic MM (1987) Growth kinetics and differentiation in vitro of normal human uroepithelial cells on collagen gel substrates in defined medium. *J Cell Physiol* 131:285–301. doi:[10.1002/jcp.1041310302](https://doi.org/10.1002/jcp.1041310302)
- Rocha V, Ringo DL, Read DB (1985) Casein production during differentiation of mammary epithelial cells in collagen gel culture. *Exp Cell Res* 159:201–210
- Sachlos E, Gotora D, Czernuszka JT (2006) Collagen scaffolds reinforced with biomimetic composite nano-sized carbonate-substituted hydroxyapatite crystals and shaped by rapid prototyping to contain internal microchannels. *Tissue Eng* 12:2479–2487
- Sionkowska A (2000) Modification of collagen films by ultraviolet irradiation. *Polym Degrad Stabil* 68:147–151. doi:[10.1016/S0141-3910\(99\)00176-7](https://doi.org/10.1016/S0141-3910(99)00176-7)
- Sionkowska A, Skopińska J, Wisniewski M (2004) Photochemical stability of collagen/poly (vinyl alcohol) blends. *Polym Degrad Stab* 83:117–125. doi:[10.1016/S0141-3910\(03\)00232-5](https://doi.org/10.1016/S0141-3910(03)00232-5)
- Strober W (2001) Current protocols in immunology. In: Coligan JE (ed) *Current protocols in immunology*, vol Appendix 3F. Wiley, London
- Wahl DA, Czernuszka JT (2006) Collagen-hydroxyapatite composites for hard tissue repair. *Eur Cells Mater* 11:43–56
- Wahl DA, Sachlos E, Liu CZ, Czernuszka JT (2007) Controlling the processing of collagen-hydroxyapatite scaffolds for bone tissue engineering. *J Mater Sci Mater Med* 18:201–209
- Zamboni G, Grano M (1995) Biomaterials in orthopaedic surgery: effects of different hydroxyapatites and demineralized bone matrix on proliferation rate and bone matrix synthesis by human osteoblasts. *Biomaterials* 16:397–402
- Zhang K, Zhao M, Cai L, Wang ZK, Sun YF, Hu QL (2010) Preparation of chitosan/hydroxyapatite guided membrane used for periodontal tissue regeneration. *Chin J Polym Sci* 28:555–561