PREPARATION AND PROPERTIES OF NOVEL BIOCOMPATIBLE PECTIN/SILICA CALCIUM PHOSPHATE HYBRIDS

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The development of bioactive polysaccharide-based hybrid materials is necessary for finding new alternatives in the field of biomaterials. As a bioactive water-soluble polysaccharide, pectin was used in this study to prepare reinforced silica gel monoliths through the sol-gel method. *In-situ* mineralization of calcium phosphate was achieved using calcium chloride and phosphate precursors. The properties of the pectin/silica/calcium phosphate hybrid were examined using FTIR, XRD and SEM/EDX techniques. Based on the results of the tests on kidney (Vero) cell lines, the pectin/silica/calcium phosphate hybrid demonstrated very mild cytotoxicity. In addition, the cytotoxicity of different hybrid concentrations was assessed with an MTT test, and the results demonstrated that their non-cytotoxicity for the concentrations investigated.

Keywords: pectin, silica, calcium phosphate, composite

INTRODUCTION

Bone is a multifunctional organ that is essential to life. In recent years, bone regeneration strategies have been developed and consequently, promising alternatives to allografts and autografts have been offered. However, several difficulties, including patient complications, disease transmission, and immune rejection, hampered bone tissue engineering.[1] Biomaterials can be designed with high interconnectivity to create a biocompatible scaffold that can provide a controlled environment for nutrient delivery and waste removal in bone tissue engineering.[2] A porous matrix with pores >300 micrometers and interconnectivity are essential for the attachment and growth of new bone tissue with enough separation to enable nutrient delivery.[3] Recent decades have seen an intensive investigation of biologically active bioresorbable glass scaffolds and calcium phosphate for bone tissue engineering (BTE). Biological responses are similar to those triggered by bones, but they are brittle and weak. Moreover, they suffer rapid strength degradation *in vivo*, which means they are not suitable for load-bearing applications. However, specific natural or synthetic polymers in bone grafting increase physical performance due to their mechanical strength and bioactivity.[4] Polymer and ceramic composite materials have gained interest for BTE applications in recent years, due to their superior mechanical properties and cell-matrix interaction.[5] Bone is an ideal organic/inorganic composite that consists of 65% mineral components and collagen primarily. Due to its high regeneration capacity and osteoconductive properties, hydroxyapatite (HA) is particularly well suited for bone replacement in organic bones.[6] However, HA is intrinsically brittle and slow to degrade, among other negative attributes. In bone regenerative medicine, there has been a lot of interest in polysaccharides and inorganic calcium phosphate.[7] Polysaccharides are beneficial in bone tissue engineering.[5] Polysaccharides, including cellulose,[8-10] chitosan,[11] alginate[12] and starch,[13] were investigated for calcium phosphate mineralization. Researchers have successfully derivatized polysaccharides to modify capsules and hydrogels. Their accessibility and biodegradability make them a good choice for cellular attachment.[4]

Pectin is a brilliant biopolymer for protective coatings applications. Pectin has various physiological functions, such as immunomodulation, antidoting and antioxidants.[14] It has long been utilized in various industries, from foods to pharmaceuticals and cosmetics, owing to its thickening, gel-forming, and stabilizing properties.[15] High amounts of orange peels result as wastes after oranges are squeezed during orange juice production. Peels are considered as wastes by some farmers, while others use them for farm animal feeding or as fertilizer. However, orange peels are also a strategic

source for pectin extraction. This would open the possibility for more effective valorization of this residue.[16]

Silica has numerous functional properties, including biocompatibility, adjustment of surface area, and easy modifiability by various molecules.[17] Furthermore, silicon has been employed as a promising material for bone tissue engineering.[18] It was found that unsupplied silicon leads to abnormal bone formation and reduced bone apatite precipitation in animal trials. In addition, amorphous silica contains polysilicon groups, which promote the role of collagen as a pattern for apatite precipitation.[19] Sol-gel-derived bioactive compounds are fragile and difficult to process during framework design by the 3D method.[20]

Detailed studies for the use of pectin in bone regeneration are scarce. Mostly, pectin is used for the regeneration of other tissues or in drug delivery. To the best of our knowledge, no work has been reported so far on silica gel hybrids prepared from water-soluble pectin extract and tetraethyl orthosilicate. The current study aims to develop such a material and evaluate its ability to initiate calcium phosphate mineralization at pH 7.4. The chemical and internal structure, morphology, and cytotoxicity of the formed hybrid materials were investigated. The results recommend the developed hybrid as a promising alternative biomaterial.

EXPERIMENTAL

Materials

Tetraethyl orthosilicate (TEOS) (≥99%) was purchased from Sigma Aldrich. The other chemicals were of analytical grade and used as received.

Extraction of pectin from orange peel

Pectin is produced primarily through extraction, as described in the literature.[21] The most common method is to extract pectin in a hot diluted robust mineral acid solution. 100 g of dried orange peel was immersed in 500 mL of distilled water, then heated at 80 °C for 2 h at pH 1.5 using 1N HCl. After that, the solution was filtered to obtain the filtrate containing pectin. The pectin solution was adjusted to pH 4-5 by using 1N NaOH, the solution was concentrated by an evaporation system, then the double amount of 70% ethanol was added. The mixture was left overnight to complete precipitation of pectin, and then filtered using a sintered glass funnel or cloth bag.

The titration method is used to determine the degree of methylation (DM). Using the titration method, the DM of pectin was determined. To remove organic acid impurities, pectin (500 mg) was refined in an HCl–ethanol solution. The amount consumed was recorded as V1 after titration with 0.1 M NaOH and phenolphthalein as indicator. After that, pectin saponification was started by adding 20 mL of 0.5 M NaOH. 20 mL of 0.5 M HCl was added when the reaction was completed, and HCl was neutralized with 0.1 M NaOH; the amount consumed was noted as V2. The volume of V1 corresponded to the content of pectin without esterification in the pectin sample, while V2 corresponded to the content of esterified pectin. The calculation of the DM content performed as follows:

$$DM = V2/(V1+V2) \times 100 \quad (1)$$

Preparation of pectin-silica

An organic-inorganic hybrid of pectin/silica was synthesized by the sol-gel method.[22,23] A given amount of pectin (0.5 g) was dissolved in 16 mL of doubly distilled water at room temperature under continuous stirring for 24 h. Then, 4 mL from each of TEOS and acetic acid solutions were added to form a mixture of pH 4 with a total volume of 32 mL. The former was used as a silica precursor and the latter as a catalyst. Subsequently, the mixture was stirred at 60 °C for 2 h to ensure complete hydrolysis of TEOS molecules. The resulting homogenous solution was casted in a 15 mL Falcon tube in a water bath at 40 °C for 48 h to form a homogenous gel. The unreacted residue was extracted from the hybrid materials via washing for three days with distilled water and subsequently dried at 40 °C for 24 h in a vacuum oven.

Preparation of pectin-silica/calcium phosphate preparation

The starting solutions were prepared before the mineralization reaction by dissolving the relevant amounts of calcium chloride $CaCl_2$ (2.22 g) and dipotassium hydrogen phosphate K_2HPO_4 (3.48 g) separately in 100 mL of water, to produce 200 mM solutions of both precursors. Then, the pectin/silica hybrid (0.6 g) was suspended in 100 mL of calcium chloride solution and stirred in double-walled glass vessels, then100 mL of dipotassium hydrogen phosphate was added drop by drop. The solution pH was adjusted to 7.4 and mixed well for 48 h under continuous magnetic stirring. Samples were isolated by centrifugation at 3000 rpm for 30 min, and the white or off-white residues were freeze-dried (Crest Alpha 1-4 LSC plus Germany).

Characterization

FT-IR spectroscopy

Fourier transform infrared spectra (FT-IR) of the materials were obtained using a Mattson 5000 FTIR spectrometer from 500 to 4000 cm^{-1}.

Thermogravimetric analysis

TGA measurements were done with a Netzsch STA 409 PC instrument. All the materials were heated under nitrogen from 25 to 900 °C, at a heating rate of 5 °C/min.

SEM analysis

The surface morphology of pectin/silica and pectin/silica/calcium phosphate composites was observed using a Quanta 250 FEG (Field Emission Gun), provided with an EDX unit (energy dispersive X-ray analysis).

XRD analysis

X-ray diffraction (XRD) patterns were recorded with an Empyrean Powder Diffractometer (Cu Kα, 0.154 nm) between 5 and 70° 2θ, with a step size of 0.01°/s.

Effect of pectin/silica/calcium phosphate materials on cell viability

Following Mosmann (1983), the *in vitro* cytotoxicity test was performed using a colorimetric MTT procedure to evaluate the effect of the pectin/silica/calcium phosphate hybrid material on a normal cell line (kidney cells (Vero)).[24,25] The MTT assay was carried out using a 96-well tissue culture plate, which was inoculated with 1 x 105 cells/mL (100 µl/well) and incubated at 37 °C for 24 hours to develop a complete monolayer sheet. After shaking at 150 rpm for 5 min, the plates were incubated (37 °C, 5% CO_2) for one day to allow the MTT metabolization. Formazan (MTT metabolic product) was resuspended in 200 µL of DMSO and adjusted on a shaking table, at 150 rpm for 5 minutes, to carefully mix the formazan with the solvent. Optical density was recorded at 560 nm and the subtracted background was recorded at 620 nm. Optical density should be directly correlated with cell quantity.

RESULTS AND DISCUSSION

Characterization of pectin-silica gels

Pectin was used in the present study to apply to the silica gel network matrix to produce a biodegradable, biocompatible and bioactive hybrid material. ATR-FTIR spectra of the pectin and the pectin/silica hybrid are shown in Figure 1. The FTIR spectrum of pectin exhibits peaks at 3433 cm^{-1} and 2938 cm^{-1}, which correspond to stretching vibrations of hydroxyl groups and stretching vibrations of methyl ester groups or C-H bonds of the pyranoid ring, respectively.[26] A vibration at 1748 cm^{-1} corresponds to the stretching vibration of carboxyl groups, and the vibration at 1614 cm^{-1} corresponds to the stretching vibration of carboxyl and methyl ester moieties. The stretching vibrations of the -C-O-C- bonds and the -CH groups are mainly found in the region of 1440-1237 cm^{-1}. The polysaccharide "fingerprint" region at 1210-1001 cm^{-1} corresponds to C-O and C-C vibration bands of glycosidic bonds and pyranoid rings.[27] The absorbance bands observed for the pectin/silica hybrid (Fig. 1B) in the region of 3450-3450 cm^{-1} correspond to bending vibrations of -COOH and -SiOH groups. A vibration of the C-O-Si- group is detected at 1246 cm^{-1}, which is absent in the spectrum of native pectin. The vibrations of -Si-O-Si- and -C-O-Si- groups can be seen in the regions of 1132 cm^{-1} and 802 cm^{-1}.[23]

In the field of calcium phosphate mineralization, polysaccharides displayed promising results as bioactive materials. For example, cellulose nanofibers and sodium alginate have been reported as bioactive polysaccharides for preparing hybrid materials with promising applications.[28] In the current study, the pectin/silica hybrid was investigated as a bioactive material for calcium phosphate mineralization. New characteristic peaks for the phosphate group can be seen in Figure 1C. The distinct bands at 1075 and 1153 cm^{-1} (PO_3 mode), 559 cm^{-1} (PO_4 mode), and 947 cm^{-1} (PO_1 mode) are assigned to different vibration modes of the PO_4^{3-} group.[29] These modes could be related to the crystalline calcium phosphates on the hybrid surface.

Pectin/silica was applied as a bioactive material to initiate calcium phosphate mineralization from solutions containing calcium phosphate ions. The obtained pectin/silica/calcium phosphate hybrid was further characterized. The degree of methylation in the sample examined was found to be 44%, indicating that the orange peel pectin was high-methoxyl, quick-setting pectin.

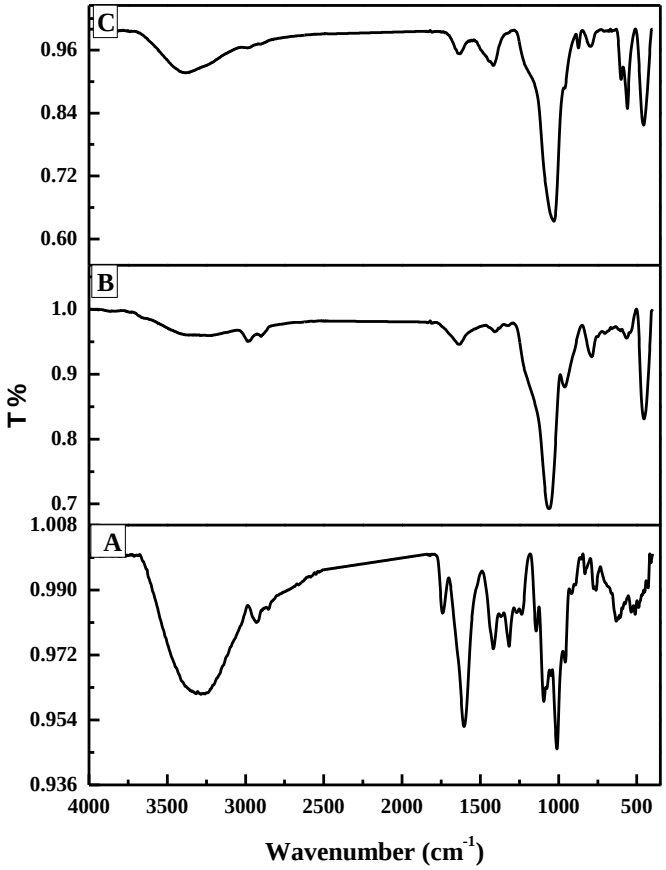

Figure 1: FTIR of (A) pectin, (B) pectin/silica and (C) pectin/silica/calcium phosphate hybrids

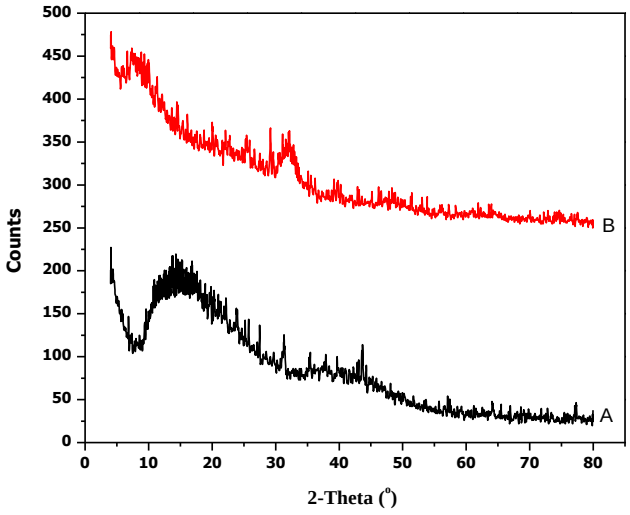

Figure 2: XRD patterns of (A) pectin/silica and (B) pectin/silica/calcium phosphate hybrids

X-ray diffraction patterns of the pectin/silica hybrid before and after calcium phosphate mineralization are presented in Figure 2. The pattern of pectin/silica displays broad reflections at 2θ (°) = 9.8 and 20°, which can be ascribed to the polysaccharide chains. These two bands are broader in the pectin/silica hybrid, which indicates its amorphous glassy structure. However, the XRD pattern of the pectin/silica/calcium phosphate hybrid displayed weak reflections at 2θ (°) = 25.9, 28.4, 31.9 and 38.9, which can be attributed to the partial formation of hydroxyapatite phase.[29] The weak peaks in this pattern indicate that hydroxyapatite, with a low degree of crystallinity, was formed. The precipitation of hydroxyapatite was reported in a study on formation of polysaccharides/calcium

phosphate hybrids.[30] XRD is supported by EDX (Fig. 3D), which shows a distinctive calcium/phosphate ratio of 1.6, nearly equal to that of hydroxyapatite.[31]

The SEM images of pectin, pectin/silica and pectin/silica calcium phosphate hybrids are presented in Figure 3. It is clear from Figure 3B that the pectin/silica hybrid exhibits homogeneous morphology. Moreover, the distribution of pectin fibers in the silica-gel matrix seems homogenous, and there is no phase separation between the inorganic and organic portions. After immersing the pectin/silica hybrid materials into the solution comprising calcium phosphate, the materials shows a calcium phosphate layer comprised of plate-like and spherical globules, as seen in Figure 3C. These spherical particles, which display no separate surface features, are characteristic of amorphous calcium phosphate, and may be transformed, in the hydrolysis-conversion process, into other crystalline phases.

TGA studies were carried out to discover the thermal stability of pectin, pectin/silica, and pectin/silica/calcium phosphate hybrids. The TGA profiles for the pectin/silica and pectin/silica/calcium phosphate hybrids exhibit three weight loss regions, as shown in Figure 4.

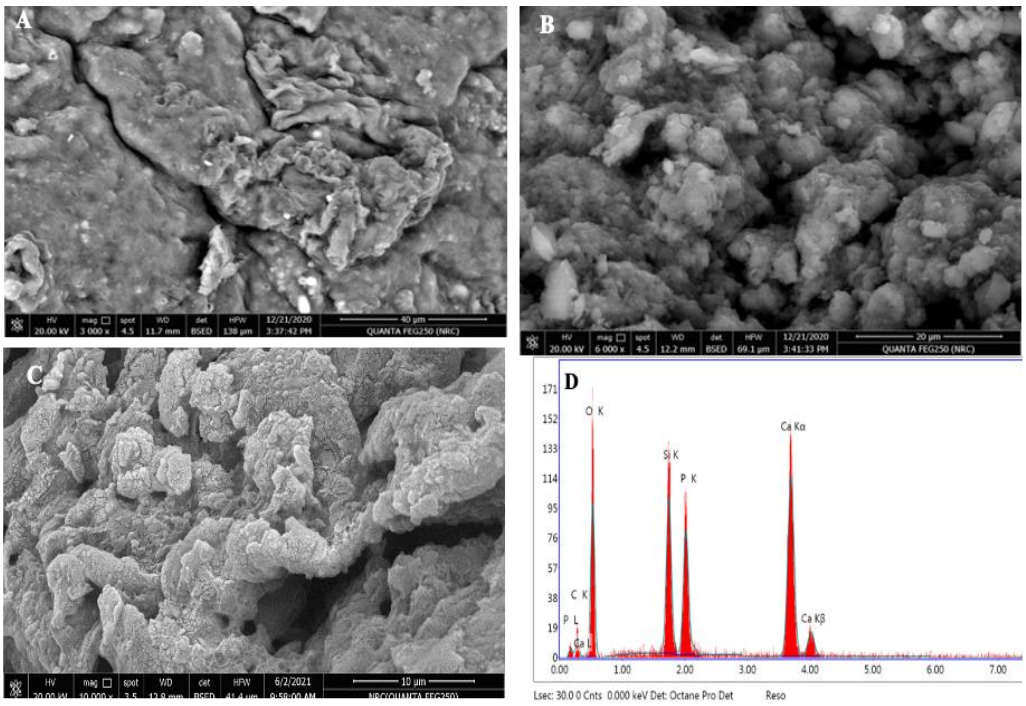

Figure 3: SEM images of pectin, pectin/silica, and pectin/silica/calcium phosphate hybrids

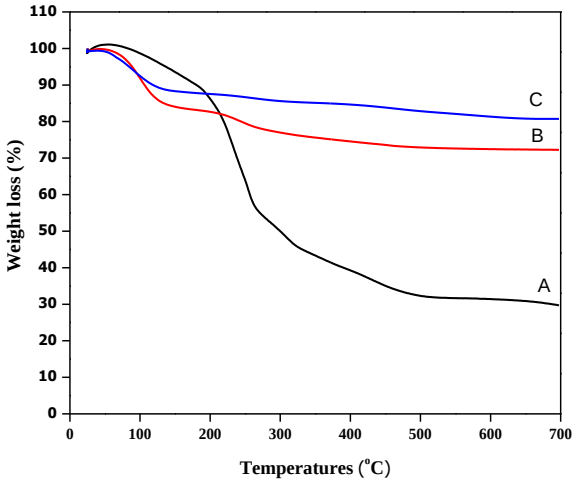

Figure 4: TGA curves of pectin (A), pectin/silica (B) and pectin/silica/calcium phosphate hybrid (C)

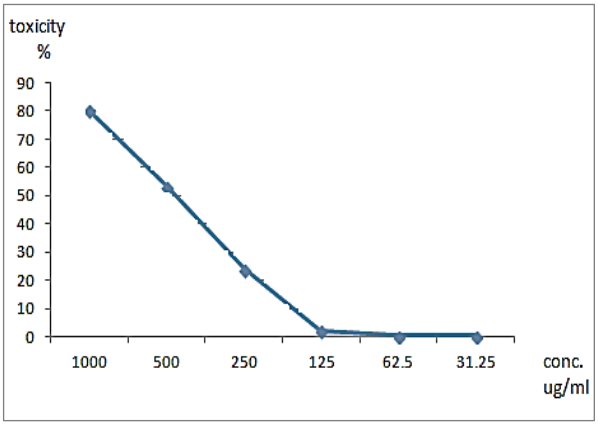

Figure 5. Viability percent of Vero cells treated with pectin/silica/calcium phosphate at indicated concentrations for 24 h

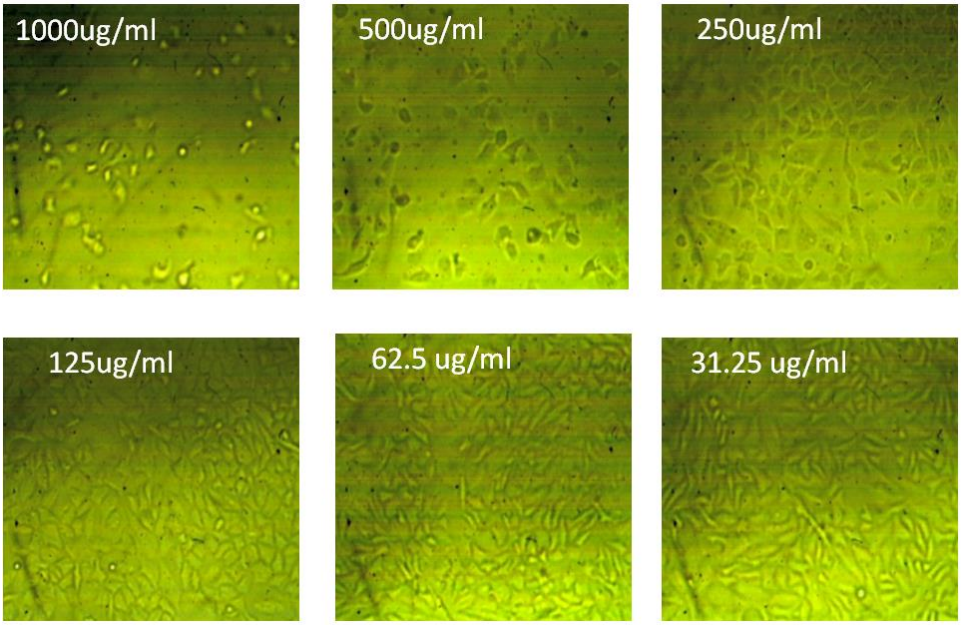

Figure 6: Morphological changes of Vero cells treated with different concentrations of pectin/silica/calcium phosphate hybrids

The first weight loss (nearly 5%) was noticed lower than 120 °C, which is attributed to water evaporation. The second stage in the range between 200 and 350 °C, and is related to the decomposition of the organic portion in the pectin/silica/calcium phosphate material.[32] Thus, the pectin/silica/calcium phosphate hybrid starts degrading earlier than neat pectin, because of the degradation of the organic part in the formed silica gel. The residual weights recorded were 29.7, 72.2, and 80.7% for pectin, pectin/silica, and pectin/silica/calcium phosphate hybrids, respectively, which reflect the thermal stability of the attained hybrids, compared to neat pectin.

Effect of pectin/silica/calcium phosphate materials on cell viability

The effect of the developed pectin/silica/calcium phosphate hybrid on the viability of Vero cells was assessed to define the maximum non-toxic dose (MNTD).[33] Kidney cells were chosen in the present study as an example to investigate the toxicity of the prepared composite since kidney cells are the most vulnerable ones to toxicity. As may be remarked in Figure 5, the viability of Vero cells was greater than 95% when subjected to concentrations of 31.2, 62.5 and 125 µg/mL of the pectin/silica/calcium phosphate hybrid. When the concentrations exceeded 125 µg/mL, it became cytotoxic to the Vero cell line, which means that the concentration of 125 µg/mL can be considered as the maximum viable dose. These results revealed that the cytotoxicity increased with increasing

concentration of the hybrid material, with 50% cytotoxicity (CC50) reached for the concentration of 500 μg/mL.

The morphological changes that occurred in Vero cells when in contact with the hybrid material were dose-dependent (Fig. 6). In general, by decreasing the concentrations of the pectin/silica/calcium phosphate, the cytotoxicity against Vero cells was reduced. The analysis of the images revealed that high hybrid concentrations resulted in Vero cells becoming more floated, shrunken and rounded because of morphological abnormalities. The detailed molecular mechanisms of how bone biomaterials induce osteogenic differentiation of stem cells are mostly unknown. The dissolved phosphorus ions from bone biomaterials may be absorbed by stem cells to procedure ATP metabolites, which send signals to stem cells and induce osteogenic differentiation.[34]

CONCLUSION

A new pectin/silica hybrid was developed via the one-pot sol-gel method, using TEOS as a silica precursor. Calcium phosphate mineralization on the pectin/silica hybrid was explored. It was revealed that the bioactivity of the sol-gel resultant hybrids showed the formation of calcium phosphate with plate-like microparticles. FTIR analysis revealed modes that can be related to the mineralized calcium phosphates on the hybrid surface, while EDX indicated a distinct calcium/phosphate ratio of 1.6 nearly equal to that of hydroxyapatite. SEM confirmed the formation of a homogeneous pectin/silica hybrid and TGA results reflected the thermal stability of the attained hybrids, compared to neat pectin. The viability of Vero cells was greater than 95% for the concentrations of 31.2, 62.5, and 125 μg/mL of the pectin/silica/calcium phosphate hybrid. When the concentrations exceeded 125 μg/mL, it was observed to become cytotoxic to the Vero cell line, which means that this concentration should be considered as the maximum viable dose. These results are promising, suggesting that the developed hybrid material could be considered for application in bone tissue regeneration.

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