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USING HYDROXYAPATITE FROM FISH SCALES TO PREPARE CHITOSAN/ GELATIN/HYDROXYAPATITE MEMBRANE: EXPLORING POTENTIAL FOR BONE TISSUE ENGINEERING

Yi-Cheng Huang
Department of Food Science, National Taiwan Ocean University, Keelung, Taiwan, R.O.C., ychuang@mail.ntou.edu.tw

Hao-Wen Chu
Department of Food Science, National Taiwan Ocean University, Keelung, Taiwan, R.O.C.

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Yi-Cheng Huang and Hao-Wen Chu

Key words: hydroxyapatite, fish scale, chitosan, gelatin, bone tissue engineering.

ABSTRACT

Concerns have recently been raised about the treatment and use of hydroxyapatite, an important component of fish scale. The goal of this investigation is to evaluate the feasibility of using hydroxyapatite extracted from fish scale (FHAP) for bone tissue engineering. FHAP or sintered FHAP (sFHAP) was added to chitosan/gelatin (CS/GEL) mixture to prepare membrane. The effect of FHAP and sFHAP on the physical and chemical properties of the membrane and their affinities towards MG63 cells were studied. Accordingly, the prepared CS/GEL/FHAP membrane was characterized by FTIR (ATR-FTIR) and SEM. Following sintering at 800°C for 4 h, the CS/GEL/sFHAP membrane had a rougher surface than the CS/GEL/FHAP membrane. Hydroxyapatite from Sigma (SHAP) was used as a standard and the biological response of MG63 cells on the CS/GEL/sFHAP membrane was determined to exceed that of hydroxyapatite, with higher proliferation and differentiation, as verified by MTT assay and alkaline phosphate assay. Briefly, the biocompatible CS/GEL/FHAP membrane may potentially be useful as a biomaterial for engineering bone tissue.

I. INTRODUCTION

Nowadays, the integrated and sustainable exploitation of fisheries resources is necessary because only 50% of a catch is consumed by humans. Global annual discards are estimated to be around 20 million tons, representing 25% of the total catch [27] and include "non-target" species, processing waste and byproducts. However, many highly valuable compounds, such as chitosan (CS), collagen and hydroxyapaptie, are found in marine by-products. The highest concentration of these bioactive compounds is generally found in the parts of those marine organisms that are discarded [6]. Serious efforts have been made to deal efficiently with the aforementioned by-products and waste, including by recycling. Among the various by-products from marine sources, this study is most concerned with hydroxyapatite.

In recent years, hydroxyapatite has attracted interest for use in bone grafting because of its osteoconductive and bioactive properties [11, 20]. Other medical applications of hydroxyapatite have also been examined because hydroxyapatite is biocompatible, bioactive, non-toxic, non-inflammatory and non-immunogenic [2, 28]. Natural hydroxyapatite biocreamics have recently been extracted from various bio-wastes, including corals [4, 18], cuttlefish shells [26], fish [3, 22], porcine teeth and bones, and bovine bones [1, 2]. Chemical analysis reveals that these products are abundant sources of calcium. Unlike the preparation of hydroxyapatite by such synthetic methods as expeditious microwave irradiation [29], chemical precipitation [23] and radio frequency thermal plasma [32], the extraction of hydroxyapatite from bio-waste is biologically safe (requiring no chemicals) and a potentially lucrative process, especially given the growing global demand for hydroxyapatite bioceramics [2].

In the authors' recent investigation, hydroxyapatite was extracted from fish scale by enzymatic hydrolysis [9]. The experimental results demonstrated that hydroxyapatite that was extracted from fish scale (FHAP) comprised nano-sized particles with a Ca/P ratio of 1.76. In *in vitro* experiments, FHAP significantly promoted the proliferation, osteogenic differentiation and mineralization of MG63 cells. This study develops a chitosan/gelatin/FHAP (CS/GEL/FHAP) membrane to evaluate its potential for use in bone tissue engineering.

Paper submitted 09/09/11; revised 09/26/12; accepted 03/20/13. Author for correspondence: Yi-Cheng Huang (e-mail: ychuang@mail.ntou.edu.tw).
Department of Food Science, National Taiwan Ocean University, Keelung, Taiwan, R.O.C.

Table 1. The composition of CS/GEL/hydroxyapatite membranes.

Sample	CS/GEL	CS/GEL/FHAP	CS/GEL/sFHAP	CS/GEL/SHAP
CS (g)	0.8	0.8	0.8	0.8
GEL (g)	0.8	0.8	0.8	0.8
FHAP (g)	-	0.05	-	-
sFHAP(g)	-	-	0.05	-
SHAP (g)	-	-	-	0.05

Chitosan (CS), a biopolymer that is composed of glucosamine and N-acetylglucosamine, is an N-deacetylated product of chitin. It follows cellulose as the second most abundant natural polymer and has great potential for use as a biomaterial owing to its intrinsic antibacterial activity and low immunogenicity [31]. Structurally, chitosan has some of the characteristics of various glycosaminoglycans (GAGs) and hyaluronic acid present in articular cartilage [30]. Chitosan is a good candidate for cartilage tissue scaffold [7, 21]. However, its bioactivity must first be improved by adding biologically active materials, such as hydroxyapatite or gelatin [15]. Gelatin (GEL), a biocompatible protein, is a partially degraded product of collagen. It reportedly promotes cell adhesion, differentiation and proliferation because it contains the sequence Arg-Gly-Asp (RGD) [14]. GEL has previously been blended into CS for tissue engineering [24, 25].

The goal of this study was to evaluate the potential of hydroxyapatite from fish scale (FHAP) for use in bone tissue engineering. To the best of the authors' knowledge, this is the first report on the use of FHAP as a biomaterial for regenerating bone. This investigation clearly analyzed the properties of CS/GEL/FHAP membranes, including swelling and degradation behavior. The effect of sintered FHAP (sFHAP) on the prepared membrane was discussed. The interaction between MG63 cells and CS/GEL/FHAP membrane was studied by MTT and alkaline phosphate assay.

II. MATERIALS AND METHODS

1. Materials

Chitosan (degree of deacetylation: 93%, Mw: 560 kDa) was purchased from Charming & Beauty Co., Ltd. Gelatin and hydroxyapatite were obtained from Sigma. Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12), fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% L-glutamine, 1% NEAA (non-essential amino acids) and trypsin were obtained from GIBCO (Grand Island, NY). All other chemicals used herein were of reagent grade and were also purchased from Sigma, except where otherwise stated.

2. Extraction of Hydroxyapatite from Fish Scales

Fish scale hydroxyapatite was isolated from tilapia (*Oreochromis* sp.) scales (Ko-Fwu Fishes Co., Taiwan) by enzymatic hydrolysis. In short, after washing to remove impurities, the fish scales were crushed with a disperser (Kinematica, NY, USA), then hydrolyzed under 1% Protease N for 2.5 hours, and 0.5% Flavourzyme (Novozymes, Chiba-shi, Japan) for another 0.5 hour at an optimal pH and temperature. Hydrolysates were stirred and heated in a boiling water bath for 10 min to deactivate enzymes. The hydrolysates were then centrifuged at 12,000 g for 20 min, the residues were dried by hot air and stored at -20°C until use.

3. Preparation and Characterization of CS/GEL/FHAP Membrane

The CS/GEL/FHAP membrane was prepared as described elsewhere [24]. Briefly, GEL was added to chitosan/acetic acid solution (2% w/v). After the CS/GEL solution was mixed to homogeneity, powdered FHAP, sintered FHAP (sFHAP, with FHAP particles sintered at 800°C for 4 hrs) or hydroxyapitite from Sigma (SHAP) was added and stirred for 24 hrs for dispersion. The resulting solution was transferred to 24 well culture plates and left to dry at room temperature for one day. Then, the membranes were neutralized using 10% NaOH-CH_3OH solution (V_{NaOH}: V_{CH3OH} = 1:1) and ddH_2O-CH_3OH solution (V_{ddH2O}: V_{CH3OH} = 1:1) to a final pH 7. Finally, the membranes were dried and stored for further use. Table 1 presents the composition of the prepared membranes.

Chemical structures of CS, GEL, FHAP and the prepared membrane were determined using Attenuated total reflectance-Fourier transform infrared (ATR-FTIR; Perkin-Elmer, FT-IR System, USA). The structural morphology of the membrane was determined by scanning electron microscopy (SEM, Jeol JSM-7000F FE-SEM, Japan). The sections of the samples were sputter-coated with gold (Au) before examination. All of the spectra were obtained and analyzed using the standard software package that was provided by the manufacturer.

4. Swelling Studies

The swelling studies of the CS/GEL/FHAP membranes were performed using the following method [19]. The dry weight of the membranes was denoted w_o. After membranes were placed in a PBS buffer solution at pH 7.4 for 24 hrs, they were removed and the water that was adsorbed on the surface was removed using filter paper. The wet weight of the membranes was recorded as w_w. The swelling ratio was determined using the equation,

$$\textit{Swelling ration}\ (\%) = \frac{w_w - w_o}{w_o} \times 100\ .$$

The swelling ratio was expressed as mean ± S.D. (n = 3).

5. Degradation Test

The degradation of the membranes was examined in a lysozyme-containing medium at 37°C [24, 15]. The membranes were immersed in the medium and incubated at 37°C for seven days. The initial weight of the membrane was denoted w_o. Seven days later, the membrane was washed in de-ionized water to remove ions that had been adsorbed on its surface, and dried. The dry weight was denoted w_t. The degradation of the membrane was calculated using the formula,

$$\textit{Degradation ratio}\ (\%) = \frac{w_o - w_t}{w_o} \times 100\ .$$

The degradation ratio was expressed as mean ± S.D. (n = 3).

6. Cell Culture

A human osteosarcoma cell line (MG-63 cells) was cultured in DMEM/F12 medium that was supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 1% L-glutamine, 1% NEAA and 1% pyruvate at 37°C in a humidified atmosphere that contained 5% carbon dioxide. The media were changed three times weekly.

The prepared CS/GEL/FHAP membrane was used in a cell study. Before the cell culture work, the surfaces of the samples were sterilized by immersing them overnight in 70% alcohol under ultraviolet illumination. The samples were then pre-treated by washing in phosphate buffer saline to remove alcohol.

1) Cell Proliferation

Until 80-90% confluence was reached in tissue culture flasks, the cells were trypsinized with a buffered saline solution that contained 1% trypsin. The cells were then placed in a 24-well cultural plate that contained membranes at a density of 1×10^5 cells/well and allowed to attach for 24 hr. After a prespecified period of incubation, the cells were analyzed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay to determine their viability. Absorbance was measured at 570 nm and 630 nm (SpectraMax 340PC^{384} Microplate Spectrophotometers). Cells that were grown without the membranes were used as a negative control. Cell viability was expressed as a percentage of that of the control.

2) Cell Differentiation

To induce the spontaneous differentiation of MG-63 cells into osteoblasts, a differentiation medium was prepared by adding 56 ug/ml ascorbic acid, 2.42 mg/ml β-glycerophosphate and 50 uM dexamethasone to the growth medium that was described above. Cells (1×10^5 cells/well) were seeded onto the membranes in a 24-well tissue plate and cultured for ten days. The differentiation of MG-63 cells into osteoblasts was evaluated from alkaline phosphatase (ALP) activity.

3) Alkaline Phosphatase Activity Assay

Alkaline phosphatase activity was assayed using the method described by Miyajima, K. [16]. Briefly, after it was cultured for ten days, the sample was washed twice in PBS before the addition of 150 μl SIGMA FAST pNPP substrate solution (Sigma, N2770), which comprised cell lysis buffer and p-nitrophenyl phosphate. After 30 min of incubation at 37°C, enzyme activity was terminated by adding 152 μl 3 M NaOH. The liberated p-nitrophenol was then measured by measuring the absorbance of light at a wavelength of 405 nm. The amount of p-nitrophenol corresponded to alkaline phosphatase activity.

7. Statistical Analysis

All quantitative data are expressed as mean ± S.D. Statistical analysis was performed by two-way ANOVA and the post-test was conducted using GraphPad Prism 5. A p value of < 0.05 was considered to indicate statistically significance.

III. RESULTS AND DISCUSSION

1. Characterization of CS/GEL/FHAP Membrane

Fig. 1 displays the characteristic ATR-FTIR peaks of CS, GEL, FHAP, sFHAP and their composite membranes. The FT-IR spectrum of CS reveals the carbonyl (C=O) stretching of the secondary amide at 1645 cm^{-1}. The peaks at 1020 cm^{-1} and 1069 cm^{-1} corresponded to the skeletal vibration of C-O [8]. The absorption bands of the GEL at 1645 cm^{-1} and 1554 cm^{-1} were associated with the stretching of C=O in amide I and the bending of N-H in amide II, respectively. The peak around 1454 cm^{-1} was attributed to the stretching of C-O in amide III. The peaks in the range 2850-2920 cm^{-1} correspond to symmetric and asymmetric stretching of $-CH_2$ [17]. The spectrum of the CS/GEL membrane includes all of the characteristic peaks of CS and GEL, revealing that the CS/GEL membrane had been effectively formed.

The ATR-FTIR spectrum of FHAP included a peak at 1028 cm^{-1} that corresponded to stretching of a phosphate group (PO_4^{3-}). The small peak at 960 cm^{-1} is attributable to symmetric P-O stretching vibration. The peaks at 1645 cm^{-1}, 1536 cm^{-1} and 1412 cm^{-1} were associated with an out-of-plane bending mode and asymmetric stretching of the carbonate group (CO_3^{2-}) [9]. The authors' previous research has established that hydroxyapatite that is extracted from fish scale by enzymatic hydrolysis contains a carbonate group. In the spectrum of sFHAP, the peaks associated with PO_4^{3-} stretching were narrower and sharper. Furthermore, the peaks at 1645 cm^{-1}, 1536 cm^{-1} and 1412 cm^{-1} were significantly diminished, revealed the decomposition of the carbonate group. The

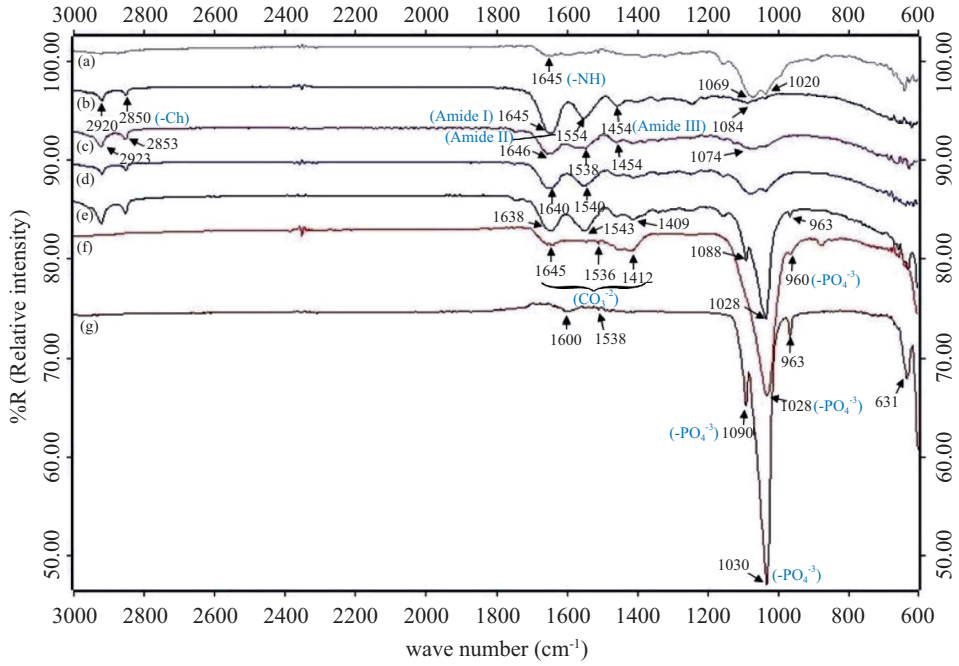
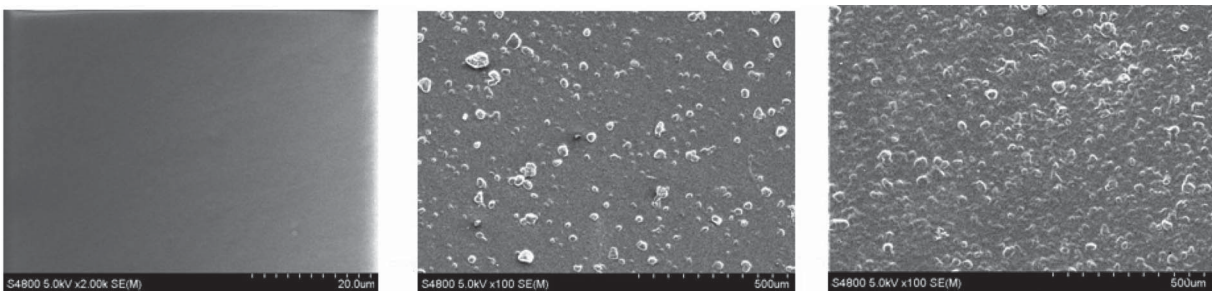


Fig. 1. ATR-FTIR spectra of (a) Chitosan, (b) Gelatin, (c) CS/GEL, (d) CS/GEL/FHAP, (e) CS/GEL/sFHAP, (f) FHAP, and (g) sFHAP.



(a) (b) (c)

Fig. 2. SEM micrographs of CS/GEL/FHAP membrane before (b) and after (c) sintering. The CS/GEL membrane (as a control) is shown in the image (a).

spectrum of CS/GEL/FHAP exhibits a superposition of the bands of the PO_4^{3-} group on FHAP and the amine/amide groups on CS and GEL. The ATR-FTIR spectrum of CS/GEL/sFHAP includes the characteristic absorption peak of the PO_4^{3-} group in FHAP at 950-1050 cm^{-1} and that of the amide group of CS/GEL membrane at 1550-1650 cm^{-1}.

Fig. 2 shows the SEM micrographs of CS/GEL/FHAP membranes. The surfaces of CS/GEL/FHAP and especially CS/Gel/sFHAP membranes were rougher than the CS/Gel membrane. According to the authors' earlier work, sintering FHAP powder formed porous particles; roughened their surfaces, and increased the total surface area [9]. Hence, the homogeneous dispersion of porous sintered FHAP powder in the CS/GEL mixture made the surface of the membrane rougher than those of the CS/GEL and CS/GEL/FHAP membranes.

Fig. 3(a) shows the results of the swelling studies of FHAP composite membranes. The experimental results demonstrate very high swelling capacity and the ability to retain water. The swelling ratios of the CS/GEL/FHAP, CS/GEL/sFHAP and CS/GEL/SHAP membranes varied from 62.5 to 68.4%. Although the addition of sFHAP slightly reduced the swelling ratio of the membranes, the results were not statistically different. More swollen samples have a larger surface area/volume ratio, and therefore exhibit a higher probability of cell growth by attachment to the membrane surfaces. Increased swelling also enables the samples to take up nutrients from the culture media more effectively. However, while the swelling of materials promotes cell adhesion, it worsens their mechanical properties. Research by Prof. Isikli *et al.* has shown that adding non-sintered and sintered hydroxyapatite to chitosan-gelatin scaffold yields a higher Young's modulus and compressive strength than those of CS/GEL scaffold [10]. Accordingly, the CS/GEL/FHAP membrane

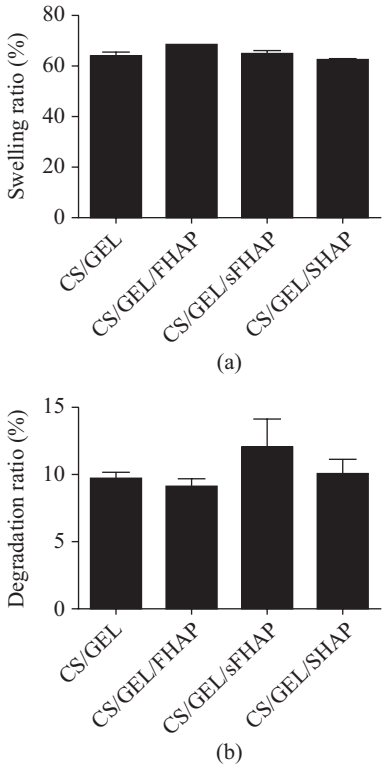


Fig. 3. (a) Swelling behavior and (b) degradation behavior of CS/GEL/ hydroxyapatite membranes.

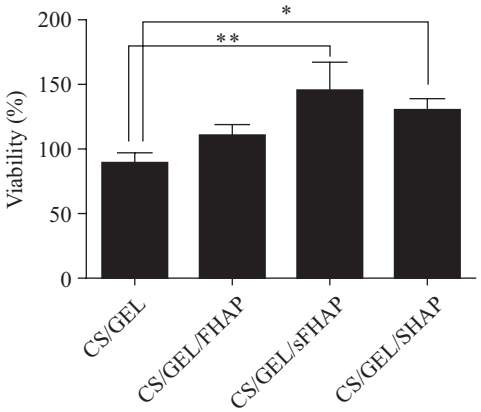


Fig. 4. Effects of CS/GEL/hydroxyapatite composite membranes on MG-63 cell viability after 24 hours, as assessed by a MTT method. Error bars show the standard deviation for n = 6. *P < 0.05 versus CS/GEL; **P < 0.01 versus CS/GEL.

prepared herein exhibited both high swelling and mechanical strength.

Fig. 3(b) plots the degradation rate of the composite membranes. Seven days after placing membranes in lysozyme containg medium, the degradation ratio was 10-12%. No statistical difference between groups was measured. Hydroxyapatite from Sigma (SHAP) was used as a standard for comparison, and the CS/GEL/SHAP membrane had similar properties to those of CS/GEL/FHAP and CS/GEL/sFHAP.

2. Cell Proliferation Test

An MTT assay was utilized to examine the biocompatibility and safety of CS/GEL/FHAP membranes (Fig. 4). According to the experimental results, after 24 hours, FHAP composite membranes had significantly higher cell viability than the CS/GEL membrane, especially in the CS/GEL/sFHAP membrane, indicating that sintered FHAP had the greatest effect on cell viability. We argue that surface roughness is probably responsible for the high cell viability on FHAP composite membranes. Previous studies have established that cell proliferation is correlated with the roughness of the substrate surface [5, 12]. Electrical charge may be responsible for the fact that the cell viability in CS/GEL/sFHAP exceeds that in CS/GEL/FHAP. The high effective negative charge of the hydroxyapatite particles may repel cells and prevent attachment [10]. Moreover, interactions occur between Ca^{2+}, PO_4^{3-} and CO_3^{2-} groups in hydroxyapatite and COO^- and NH_4^+ groups in GEL and CS, respectively [13]. The porous sFHAP particles lost the carbonate group (Fig. 1), reducing the negative charge on the membrane surface. The fact that the sFHAP particles increased the cell viability significantly over that with CS/GEL only agrees with earlier research [10]. The findings demonstrate that the CS/GEL/FHAP membranes were cytocompatible, and have great potential as biomaterials for use in tissue engineering.

3. Cell Differentiation Test

An effective osteo-induction model requires that the cells be able to differentiate into bone-forming cells. Alkaline phosphate (ALP), which is a marker of the osteoblastic phenotype, is expressed when progenitor cells differentiate into osteoblasts. Therefore, as the number of osteoblasts increase, ALP activity increases. The ALP assay is commonly adopted to evaluate cell differentiation capacity. In this study, dispersing sFHAP powder into CS/GEL membrane significantly promoted cell differentiation (Fig. 5), indicating that CS/GEL/sFHAP positively influenced the differentiation of MG-63 cells.

IV. CONCLUSIONS

Hydroxyapatite was extracted from fish scale bio-waste in this study. Both FHAP and sintered FHAP (sFHAP) were used to fabricate CS/GEL/FHAP and CS/GEL/sFHAP membranes to evaluate their potential for use in bone tissue engineering. ATR-FTIR results and SEM images verified that CS/GEL/FHAP membranes were successfully formed. Adding FHAP, and especially sintered FHAP, to a CS/GEL membrane made its surface rougher. The MTT test and ALP activity confirmed that CS/GEL/sFHAP membrane significantly promoted the proliferation and differentiation of cells. Briefly, FHAP has potential medical applications, and fish scale is a cost-effective and environmentally friendly source of hydroyxapatite.

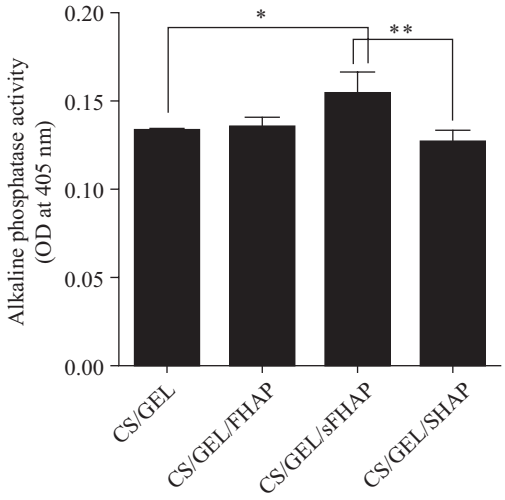


Fig. 5. The alkaline phosphatase activity of MG-63 cells on CS/GEL/ hydroxyapatite composite membranes after culturing for ten days. Error bars show the standard deviation for n = 6. * *P* < 0.05 versus CS/GEL; ** *P* < 0.01 versus CS/GEL.

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