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FABRICATION AND CHARACTERIZATION OF POLYCAPROLACTONE/ ZEIN AND POLYCAPROLACTONE/ZEIN/BIOGLASS COMPOSITE SCAFFOLDS FOR BONE TISSUE ENGINEERING

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ABSTRACT

Introduction: Tissue engineering has emerged as an effective alternative treatment modality that aims to regenerate damaged tissues instead of replacing them, taking advantage of the body's self-regenerative capacity. **Aim of the study:** This study aimed to prepare polycaprolactone (PCL)/zein/bioglass composite scaffolds with high porosity and high interconnectivity for bone tissue engineering applications. **Methodology:** PCL, as a synthetic polymer was blended with corn protein (Zein) to fabricate PCL/zein scaffolds. Ananosized bioglass (BG), was also, added to PCL/zein mixture to prepare PCL/zein/BG composite scaffolds using the solvent casting method. Pore size and morphology were investigated using scanning electron microscopy (SEM). The mechanical properties, the porosity and the biodegradation rate of the scaffolds were assessed. Also, the viability and osteogenic differentiation of the mesenchymal stem cells (MSCs) cultured on different PCL scaffolds were evaluated using MTT assay and Alkaline phosphatase activity (ALP) assay, respectively. **Results:** SEM showed that the fabricated scaffolds had highly porous with a highly interconnected structure. High mechanical properties of about 3.41 MPa and higher degradations rate (42%) were obtained by PCL/Zein/BG composite scaffold compared to the pure PCL scaffold and PCL/Zein scaffold. PCL/Zein/BG composite scaffold was shown to be noncytotoxic and supported mesenchymal stem cell (MSC) attachment and differentiation as indicated by viability assay (MTT) and alkaline phosphatase activity (ALP) assay. **Conclusion:** The addition of BG nanoparticles into the scaffold improved the mechanical and degradation rate of the hydrophobic polymers. In addition, the BG promoted better cell adhesion, proliferation, and differentiation. Thus, the study showed that the PCL/Zein/BG composite scaffolds are potential candidates for regenerating damaged bone tissues.

INTRODUCTION

Tissue engineering has emerged as an effective alternative treatment modality that aims to regenerate damaged tissues instead of replacing them, taking advantage of the body's self-regenerative capacity. This approach overcomes the limitations of conventional replacement therapies such as disease transmission, organ rejection and the difficulty to find suitable donors. It is a combinatorial approach of materials science, cell biology, engineering technology, and transplantation that restore, maintain, or improve tissue function by combining scaffolds, cells, and growth factors [(1)].

The concept of tissue engineering is based on inducing the body's regeneration ability by using an artificial three-dimensional (3-D) scaffold. The scaffold provides rigid support for the cell to grow, attach and proliferate in vitro and act as a delivery vehicle that releases therapeutic regulatory components in vivo [2]. Thus, an ideal scaffold should fulfill certain requirements to be successfully used in bone tissue engineering. It should be biocompatible, bond to the tissues without fibrous tissue formation. It also should be highly porous with a proper pore size that allows the cells to adhere, proliferate, and maintain their specific functions as well as the pore interconnectivity act as a pathway for nutrition supply and waste removal. The scaffold's materials should be biodegradable, with a degradation rate matching the new tissue formation rate. Also, the scaffolding materials should possess good mechanical properties and can be fabricated in an irregular shape and architecture and, of course, to be commercially available [3].

Biomaterials used in tissue engineering scaffold construction are broadly classified into polymers and ceramics materials. Each of these individual groups has specific advantages and disadvantages, so the use of composite scaffolds comprised of different material classes is becoming increasingly common.

Poly(ε-caprolactone) (PCL) is a linear hydrophobic semicrystalline aliphatic polyester with a crystallinity degree that reaches 69% and decreases with increasing its molecular weight[4]. PCL exhibits excellent chemical and solvent resistance and good toughness. It has a low glass transition temperature (–60 °C), making PCL rubbery and flexible at room and body temperature [5]. It has a low melting temperature (60 °C) which enables easy processing [6]. PCL has high thermal stability; its decomposition temperature (Td) is about 350 °C, whereas other aliphatic polyesters are between 235 °C and 255 °C [7].

PCL biocompatibility depends primarily on the leaching of low-molecular-mass compounds, either through the presence of leachable impurities or because of the degradation. The reduction in the molecular mass by degradation causes an adverse tissue reaction, denaturation of the loaded agents and worsening the healing, which is considered the main inflammation trigger[8].

The degradation of PCL polymer is prolonged requires 2-3 years for complete removal from the host body. The degradation rate *in-vitro* is influenced by several factors related to the polymer itself, including hydrophilicity, crystallinity, molecular weight, molecular architecture, copolymerization, surface treatment and sample geometry [9]. Other factors related to the environmental condition, such as temperature and pH. PCL degradation is faster in an alkaline environment and a high temperature than in an acidic environment and a low temperature [10].

Zein or maize (*Zea mays* L.) is the main storage protein of corn and comprises about 45-50% of the total protein in corn. It is rich with prolamin and known for its solubility in an aqueous alcohol solution[11]. Zein is an amphiphilic protein possessing both hydrophobic and hydrophilic properties. A little more than 50% of zein's amino acid residues are nonpolar amino acids, including high percentages of leucine, proline, and alanine provide it with a hydrophobic nature which makes it with good resistibility. Zein also has a relatively high content (21%–26%) of polar glutamine, which gives it the hydrophilic property [12].

Zein is non-toxic, biologically active, biodegradable, and antioxidant. Also, it has resistance to microbial attacks and has a high thermal resistance. Zein is soluble in aqueous alcohols, glycols, ethyl

ether, and aqueous alkaline solutions of pH 11.5 or greater. It can form a tough, glossy coating for food products and fibers with antibacterial activity. It is also used to encapsulate essential oils, aroma, and flavors and controlled the release of active additives or drugs [13].

The classical 45S5 bioactive glass is commonly referred to by its commercial name bioglass (BG). This bioglass is a silicate glass based on the 3-D glass-forming SiO_2 network in which Si is fourfold coordinated to O. The quaternary SiO_2 network is composed of SiO_2, CaO, Na_2O, and P_2O_5. The high amounts of Na_2O and CaO, as well as the relatively high CaO/P_2O_5 ratio and the low SiO_2 content <60 mol%, make the glass surface highly reactive in physiological environments and promote apatite formation crystals [14]. In the last years, other former oxides than silica have been proven to be suitable for producing glasses with bioactive properties, particularly phosphate-based glasses and borate-based glasses [15].

Bioglass used as scaffolds for tissue engineering has several excellent properties such as biocompatibility, bioactivity, degradability over time, and interconnected porosity suitable for bone ingrowth. It can also be produced to have similar compressive strength to that of cancellous bone [15]. However, for clinical use, the permeability and mechanical properties of 3-D scaffolds made from bioactive glass prepared by many conventional methods are often conflicting. They cannot satisfy the practical application of repairing load-bearing bones. Also, the difficulty of processing bioactive glass into porous 3-D scaffolds limits its applications [16].

From the above-stated review, PCL has been proved to be a valuable polymer for tissue engineering scaffold applications. However, its slow degradation rate and intrinsic hydrophobicity limit its use as a bone tissue engineering scaffold. On the other hand, zein and gelatin are natural polymers with rapid degradation rates and better biocompatibility. Moreover, the bioactive glass showed high mechanical and bioactive properties to be successfully used in bone tissue engineering.

Therefore, this study was performed to fabricate and evaluate the physical, mechanical, and biological properties of polymers (synthetic and natural)/ ceramic composite scaffolds. In which PCL as a synthetic degradable polymer would be blended with naturally-derived polymers of different origins (plant and animal), zein and gelatin. Bioactive glass (BG) as an inorganic bioactive filler was also introduced to the blended polymers matrix.

MATERIAL AND METHODS

The bioactive glass was prepared by sol-gel method [17] in the chemical lab, by hydrolysis and polycondensation reactions of stoichiometric amounts of the following precursors: tetraethyl orthosilicate (TEOS), $(Si(OC_2H_5)_4$ (Merck, Shacharit, triethyl phosphate (TEP) (BDH Lab), $OP(OC_2H_5)_3$, sodium nitrates ($NaNO_3$) (BDH Annular, England) and calcium nitrates tetrahydrate (CNT), $Ca(NO_3)_2\ 4H_2O$). Sigma-Aldrich, Germamy).

Preparation and characterization of the scaffolds:

Samples grouping:

The **three** scaffold groups were prepared as follows:

Group I: Pure polycaprolactone (PCL) scaffolds (Control group).

Group II: PCL/zain scaffolds.

Group III: PCL/zain/BG composite scaffolds.

The study design of the characterization tests of the scaffolds is summarized in **Table (1)**

Table (1) *Study design for the characterization tests of the prepared scaffolds.*

Test		Group I	Group II	Group III	No. of the samples per test
Morphological assessment (SEM)		2	2	2	6
Porosity		5	5	5	15
Mechanical testing		5	5	5	15
Biodegradation test 2 samples/group were examined by SEM after the testing period		5	5	5	15
Viability and cytotoxicity assay (MTT assay) 2 samples/subgroup were examined by SEM after the testing period	Day 1	4	4	4	36
	Day 3	4	4	4	
	Day 7	4	4	4	
Osteogenic differentiation assay (ALP assay)	Day 1	4	4	4	36
	Day 3	4	4	4	
	Day 7	4	4	4	
No. of samples per group		41	41	41	Total number of samples=123

Preparation of different PCL scaffolds:

The scaffolds of different groups were prepared using the solvent casting/particulate leaching technique as described in previous studies [18-20]. In brief, PCL solution (10% (w/v)) was obtained by dissolving 6 gm of the PCL (M.w =70,000- 90,000 Da Aldrich, Germany) in 60 ml of the chloroform solvent (Aldrich, Germany) using a mechanical stirring. Sodium chloride (NaCl), with a particle size of 315-500 μm, was used as a porogen (pores producing particles) at a PCL/ NaCl ratio of 1:5. The porogen was added slowly to the PCL solution with continuous stirring to obtain a highly homogeneous slurry mix.

The zein powder (Aldrich, Germany) and BG powder was added to the PCL/NaCl mixture solution at room temperature (25 °C± 2) and stirred for an additional 20 minutes to prepare the PCL/ zein scaffold (group II) and PCL/zein/BG (group III) composite scaffolds, respectively.

At the end of each direct stirring, the highly viscous mixture was cast directly into two special disc-shaped Teflon molds of different sizes. The first mold size was 8 mm x 3 mm in diameter and length, respectively, for preparing scaffold samples of porosity, morphological assessment (SEM), degradation test and the *in-vitro* cell-based assays, ***Figure (1a)***. The second one was 9 mm x 18 mm in diameter and length, respectively, for preparing scaffold samples of a mechanical test, ***Figure (2b).***

The samples were dried by natural evaporation of the chloroform solvent at room temperature (25°C± 2) for 48 h for complete hardening of the PCL

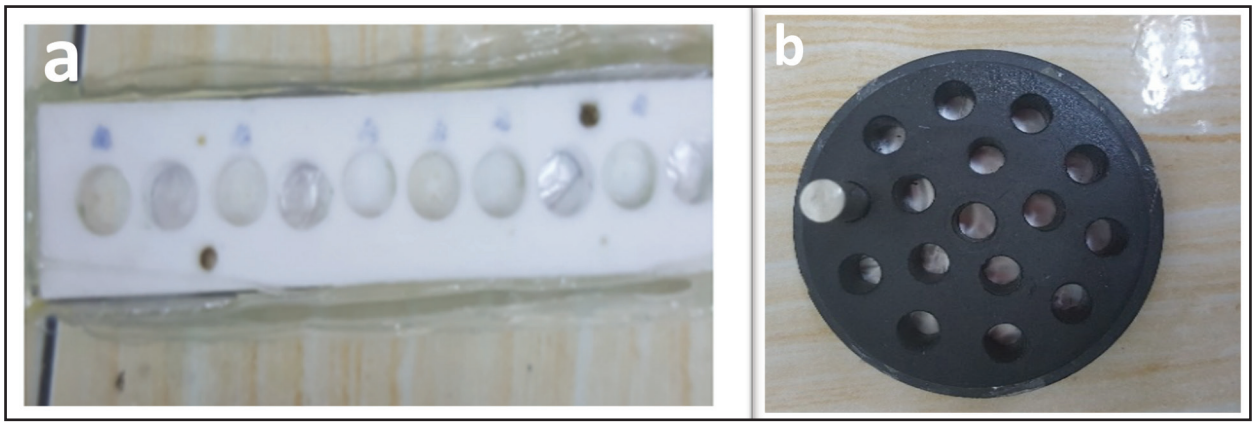

Fig. (1) The Teflon molds and a Petri dish that were used in the preparation of scaffold samples (a) The Teflon molds of 8 mm diameter 3 mm length used to prepare scaffold samples for porosity, SEM and cell-based assay tests, (b) The Teflon mold of 9 mm diameter 18 mm length used to prepare scaffold samples for the mechanical test.

matrix. After complete evaporation of the solvent, the scaffolds were detached from Teflon molds then directly immersed in 200 ml of distilled water for two days to ensure the complete dissolution of the NaCl. The prepared scaffold samples were dried at 40 °C in a dry oven (Fisher isotherm oven model 3600, USA) for 48 hours and stored in clean glass containers before further procedures.

Characterization of the prepared PCL scaffolds:

Porosity measurement:

The porosity of the obtained scaffold samples was measured using a liquid displacement method of Archimedes [21-23]. Absolute ethanol 99.5% was chosen as the displacement liquid. Each sample was immersed in a graduated cylinder of 10 ml capacity containing a known volume of ethanol ($\mathbf{V_1}$). The cylinder was placed in a vacuum and subjected to a series of brief evacuation-repressurization cycles to drive the ethanol into the sample's pores. The level of ethanol containing the infiltrated scaffold sample was recorded as ($\mathbf{V_2}$). The scaffold sample was then removed from the graduated cylinder, and the residual volume of ethanol was recorded as ($\mathbf{V_3}$). On the basis of obtained data, The porosity percentage was then calculated using the following equation[24]:

$$Porosity\% = (V_1-V_3) / (V_2-V_3)\ x100$$

The microstructure, pore morphology, and pore dimensions of the different obtained scaffold samples were examined using Scanning Electron Microscopy (SEM) (Model Philips XL 30 with accelerating voltage 30 K.V magnification x10 up to x 400.000 and resolution 3.5 nm).

In-vitro biodegradation test:

A total number of 15 rounded-shaped samples were prepared; (n=5). Before starting the biodegradation test, each sample was weighed using a 4-digits electric balance (Fisher scientific, USA), with an accuracy of ± 0.0001 mg, to determine its initial dry weight (W_{init}). Each sample was then submerged individually in a plastic vial containing 10 ml of phosphate buffer saline (PBS). The vials were maintained at 37±1°C in an incubator, for six months. The immersion solution was refreshed every four weeks.

At the end of the predetermined immersion period, the samples were rinsed thoroughly by

deionized water dried and placed in an oven at 40 °C for 72 hours. After being entirely dried, the scaffold samples were re-weighed to determine their final weight after degradation (W_{deg}). The weight loss% of each scaffold sample (W_{loss}) was calculated according to the following equation [25]:

$$W_{loss}\% = W_{init} - W_{deg} / W_{init} \times 100$$

Preparation of phosphate buffer saline:

One litre of phosphate-buffer saline (PBS) was prepared according to the method described by ***Foo et al.***[26] where, 8 g of NaCl (Sigma Aldrich, Germany), 200mg of KCl (Sigma Aldrich, Germany), 1.44 g of Na_2HPO_4 (Adwick, El-Naser Pharma Chem Co. Egypt) and 245 mg of KH_2PO_4 (Sigma Aldrich, Germany), were dissolved in 800 ml of distilled water in a suitable container. The pH was adjusted to be ≈ 7.4 with HCl using a pH meter (JANEWAY 3505 pH meter =EU). Distilled water was added until the volume was one liter. The PBS was kept in a refrigerator at about 4 °C until use

Compressive strength test:

To evaluate the compressive strength of the prepared scaffolds, five cylindrical samples from each group with dimensions 9 mm diameter and 18 mm length, were used (n=5). Each sample was individually and vertically mounted on a computer-controlled universal testing machine (model: 3345, with a load cell of 500 N, serial No:2710-113, England). The maximum failure load was recorded in Newton (N). Then, the compressive strength was calculated in MPa, from the recorded peak load divided by sample cross-sectional area.

In-vitro cell-based assays:

Mesenchymal stem cells derived from the bone marrow of rat's femur (RBM-MSC) were used for biological characterization of the prepared scaffolds Cell isolation was performed in a manner as published by ***Smajilagić et al.*** [27] with few modifications. (RBM-MSC was obtained from the central lab of stem cells and biomaterials applied research, Faculty of Dentistry, Ain Shams University).

MTT assay:

An aliquot of MSCs suspension 1X 10^6 cells/ml was cultured onto the surface of the scaffold samples in 24-well tissue culture plates (200 μl/well) with the addition of 10 ml cell culture medium (DMEM) containing 10% FBS, 50 mg /ml penicillin, 50 mg/ml streptomycin and 50 mg/ml antifungal for each well. The Cell culture plates were incubated for 1, 3 and 7 days in a humidified CO_2 incubator, 5% CO_2, at 37°C (Memmert 170 Schwabach, Germany) to allow for cell adhesion and to develop a complete monolayer sheet.

After each time interval, the culture medium was removed from each well, and the (4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-tetrazolium bromide solution (MTT) (bio basic Canada inc) was prepared by dissolving in PBS (5mg/ml). 20 μl of MTT solution were added to each scaffold sample, and incubated at 37 ºC and 5% CO_2 for 5 hours to allow the MTT solution to be metabolized. During the incubation period, the yellow color tetrazolium salt converts into insoluble purple color formazan crystal by reducing enzyme of viable cells. After the incubation period, the precipitated Formosan crystal (MTT metabolic product) was dissolved in 200 μl dimethyl sulfoxide (DMSO) and shaking for five minutes. Using a shaking table at 150 rpm, to thoroughly mix the Formosan into the solvent.

The solutions were taken, centrifuged and the supernatant transferred to a 96-well plate (100 μm in each well) to record the absorbance using an Elisa microplate reader at wavelength 570 nm (Model Mindray MR 96A, china).

The cell viability percent for each group was determined according to the following equation (3):

$$\textit{Cell viability \%} = \frac{\textit{optical density of the test solution}}{\textit{optical density of the cell control}} \textit{ X 100}$$

Alkaline phosphatase ALP activity assay:

MSCs from the third passage were culture on the scaffold samples at a density of 10,000 cells/cm^2 and cultivated for 24 h in the (75 ml DMEM, 15 ml FBS, 5 ml penicillin-streptomycin mixture, 50 mg antifungal). After 24 h, the standard medium was removed and replaced by a differentiating medium containing an osteogenic supplement (10 mm dexamethasone, 0.2 mm ascorbic acid, 10 mm βglycerophosphate) incubated further for another 15 days.

At the end of the incubation period (1, 3 and 7 days), the scaffold samples were washed with PBS and the cells on the scaffolds were lysed with 1 ml cell lysis buffer (20 mm TRIS buffered solution, 0.1wt.% Triton X-100, 1mm $MgCl_{2,}$ and 0.1mm $ZnCl_2$). The lysates were collected from the tested samples, centrifuged at 2500 rpm for 10 minutes. ALP buffer solution was prepared according to the company guidelines (ALP Elisa kit assay), Doxing industry zone, Beijing, China (NOVA protocol)). Then, 100 μl of ALP buffer was added to 250 μl of the clear supernatant and incubated at 37± 0.5 ºC for 5 minutes in the dark place.

NaOH was added to stop the reaction. After that, the cell lysates and the ALP buffer were centrifuged at 16,000 rpm for 10 min. The supernatant was transferred to a 96-well plate with the slandered solutions and their absorbance was measured spectrophotometrically at 405 nm using an Elisa microplate reader.

RESULTS

Physico-mechanical characterization:

SE micrographs of the surface morphology of the scaffolds are represented in **Figure (2a-c)**. All the prepared scaffolds exhibited a highly porous structure with completely interconnected open pores. The pore's morphology of the pure PCL scaffold (group I) and PCL/zein polymers scaffolds (groups II) revealed no change in the pore dimension or interconnectivity. The pore sizes ranged from 200 to 600 μm. On the other hand, group III combined PCL/zein with BG particles showed an apparent decrease in the size and number of the pores. The estimated pore size was in the range of 125 μm to 300 μm. Also, the pore walls of the composite scaffold of group III appeared rougher than the other two groups. The BG particles in groups III appeared as sporadic white distribution.

Porosity measurement:

The results of calculated porosity% measurement were shown in **Figure (3a)** revealed that pure PCL scaffold (group I) showed the highest porosity percent (80.9%). On the other hand, PCL/zein/BG (group II) showed the lowest porosity percent (72.52%).

ANOVA one-way test showed a statistically significant difference between the porosity% of the different groups (*P*-value = 0.001, Effect size = 0.577). However, pair-wise comparisons between the groups revealed no statistically significant difference between groups I (pure PCL) and II (PCL/zein), these two groups showed the statistically significant highest mean porosity%. On the other hand, group III (PCL/zein/BG) showed the statistically significant lowest mean porosity% values.

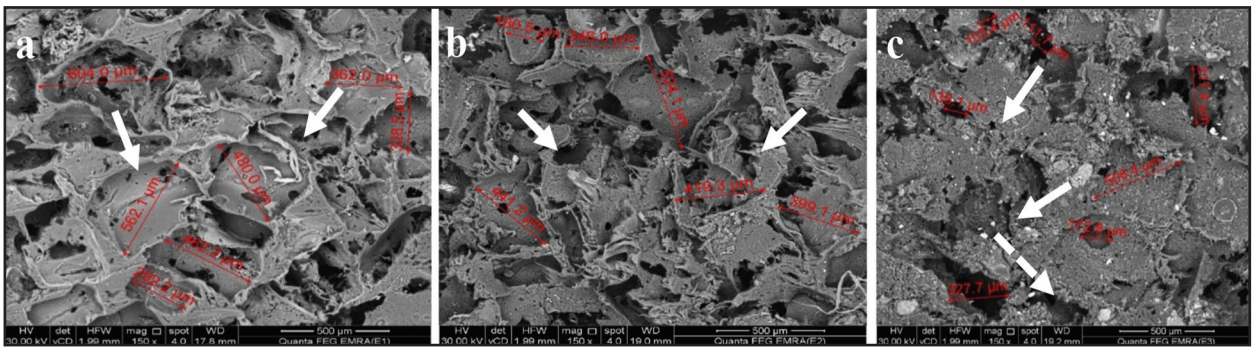

Fig. (2) SE micrographs of the different scaffold groups x 150 **(a)** Group I (pure PCL). **(b)** Group II (PCL/zein). **(c)** Group III (PCL/zein/BG).

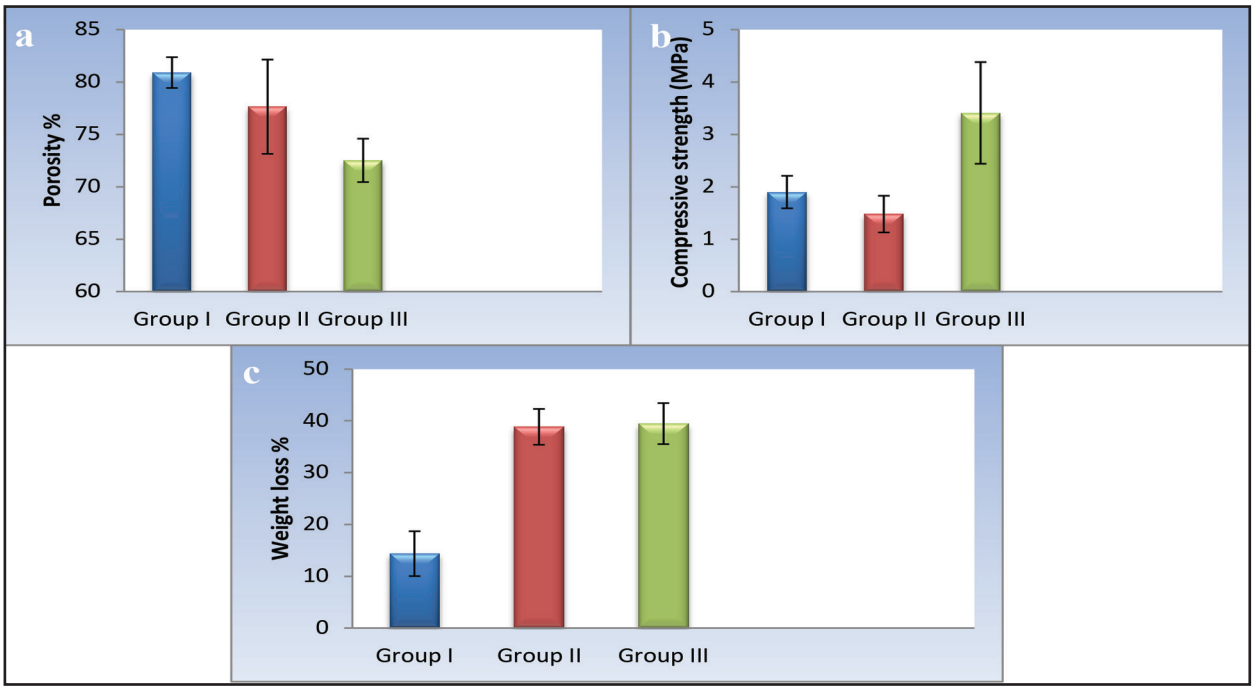

Fig. (3) Physico-mechanical characterization of the three groups (a) Porosity (%) (b) Compressive strength (%). (c) Degradation (%).

The white arrows represent the estimated pore's size, which decreased in group III. Dashed arrows in images **(c)** represent the white spots of BG particles.

In-vitro biodegradation rate:

The results weight loss percentage **Figure (3b)** revealed that pure PCL scaffold (group I) showed the lowest weight loss% (14.37%) among all groups. On the other hand, group III (PCL/zein/BG) showed the highest weight loss% (42.49%) among all groups.

The ANOVA one-way test showed a statistically significant difference between the weight loss% in the different groups (*P*-value <0.001, Effect size = 0.906). Pair-wise comparisons between the groups revealed that group III (PCL/zein/BG) that contains

BG particles showed the statistically significant highest mean weight loss%. Group I (pure PCL) showed the statistically significant lowest mean weight loss% among all groups.

Compressive strength (MPa):

The results of the compressive strength **(Figure 3c)** revealed that group I (pure PCL) showed the lowest compressive strength value (1.9 MPa) among all groups. On the other hand, group III (PCL/gelatin/BG) showed the highest compressive strength value (3.69 MPa). ANOVA one-way test showed a statistically significant difference between the compressive strength of the different groups (*P*-value <0.001, Effect size = 0.755). Pair-wise comparisons between the groups revealed that group III (PCL/zein/ BG) showed the statistically significant highest mean compressive strength values. Likewise, there was no statistically significant difference between group I (pure PCL), group II (PCL/zein) the two groups showed the statistically significant lowest mean compressive strength values.

Biological characterization:

Viability test (MTT assay):

Data of the calculated cell viability percentage of different groups measured at different time intervals (1, 3 and 7 days) are represented in **Figure (4a)**. At all-time intervals of testing, group II (PCL/zein) showed the lowest cell viability percent among all groups at all time intervals (83.8, 96.27 and 91.66%), respectively.

On day one, pair-wise comparisons between the different groups showed that group I (pure PCL) showed the statistically significant highest mean viability%. There was no statistically significant difference between group II (PCL/zein) and group III (PCL/zein/BG); the two groups showed the statistically significant lowest mean cell viability%. On day 7, pair-wise comparisons between the groups revealed that there was no statistically significant difference between groups I (pure PCL) and III (PCL/zein/BG), these two groups showed a statistically significantly higher mean viability% (98.86%, 97.99%) respectively than group II (PCL/zein) which showed a statistically significantly lower mean viability% (91.66%) among all groups.

Osteogenic differentiation (ALP absorbance measured at 405 nm):

The results of ALP activity absorbance measured at 405 nm at all time intervals of testing (1, 3 and 7 days) **Figure (4b)** were found following the trend of MTT assay results, where group V (PCL/zein/BG) showed the highest ALP concentration among all groups. While group II (PCL/zein) showed the lowest ALP concentration among all groups (0.025, 0.0612 and 0.101), respectively.

On day one, one-way ANOVA test showed that there was a statistically significant difference between ALP absorbance values in the different groups (*P*-value <0.001, Effect size = 0.898). Pair-wise comparisons between the groups revealed that there was no statistically significant difference between groups I (pure PCL) and III (PCL/zein/BG); both two groups showed statistically significantly lower mean values. Group II (PCL/zein) showed the statistically significantly lowest mean ALP absorbance among all groups and at all-time intervals.

On day three, one-way ANOVA test showed there was a statistically significant difference between ALP absorbance values in the different groups (*P*-value <0.001, Effect size = 0.999). There was no statistically significant difference between groups I, and III; these two groups showed statistically significantly highest mean ALP absorbance. Group II showed the statistically significantly lowest mean ALP absorbance.

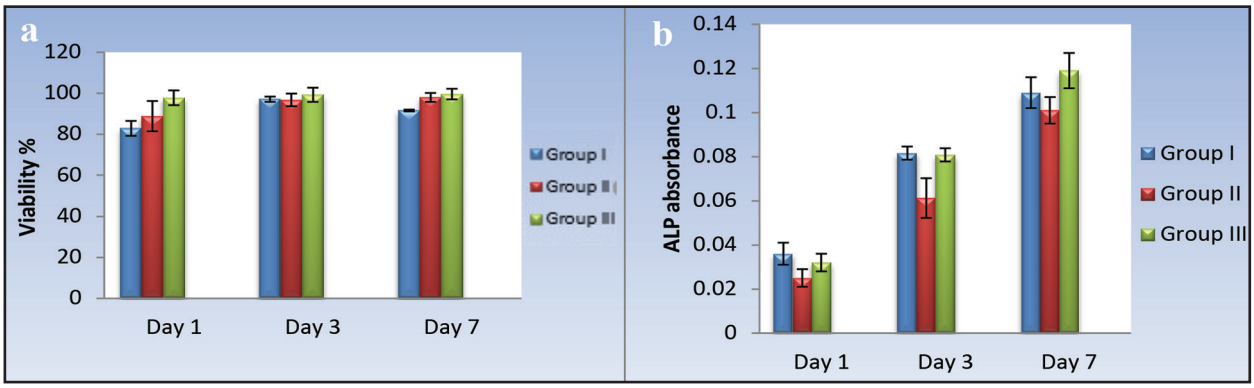

Fig. (4) Biological characterization (a) The mean of the vitality test of the MSCs cultured on the different scaffold groups at different time intervals. (b) The mean of the ALP absorbance of the MSCs cultured on the different scaffold groups at different time intervals.

On day seven, one-way ANOVA test showed there was a statistically significant difference between ALP absorbance values in the different groups (*P*-value = 0.041, Effect size = 0.599). Pair-wise comparisons between the groups revealed that group III showed the highest mean ALP absorbance value. Group I showed statistically significantly higher mean absorbance values. Group II showed the statistically significantly lowest mean ALP absorbance.

DISCUSSION

Tissue engineering has been widely inspected as a promising approach towards the regeneration of critical-sized bone tissue defects (CSDS), overcoming the limitations of the current conventional therapies [28]. Thus, choosing the most appreciated material to produce a scaffold is an indispensable step in constructing a tissue-engineered product. composite scaffolds that combine synthetic/natural biodegradable polymers with bioceramics are considered promising candidates for bone tissue engineering. such materials combination provides the best mechanical and biological performance by combining the advantages of flexibility, easy processing of polymers with the higher strength, stiffness, and bioactivity of inorganic fillers [29].

Among various synthetic polymers, polycaprolactone (PCL) was selected in this current study to prepare different PCL scaffolds for bone tissue engineering applications. PCL is a biodegradable aliphatic polyester with good biocompatibility, acceptable mechanical properties, easy processability, and can be blended and copolymerized with other synthetic and natural polymers [30]. On the other hand, naturally derived polymers from plant origins, (zein) is an amphiphilic polymer with a rapid degradation rate. Thus, blending zein with PCL in this study might overcome the shortcomings present in the PCL and allow a fine-tuning of the wettability and the degradation rate of the PCL scaffolds [31].

Additionally, the bioactive glass powder (BG) was added to the PCL/natural polymer mixture in this current study to produce tissue engineering scaffolds that mimic the structure of the native bone, increase scaffold's bioactivity, improve the mechanical, biodegradation and biological performance of the blended polymer scaffolds. The concentration of the BG used in this study was 20%. The concentration of inorganic filler in the range of 20% to 40% was reported in previous studies [32,33] to be an optimum value that improved the mechanical properties of the composite scaffolds without impacting the porosity.

The calculated porosity% measured by the liquid displacement method **Figure (3a)** revealed that all the prepared scaffolds had satisfactory high porosity% up to 70%, which would be highly beneficial for bone ingrowth. The statistical results of porosity% were confirmed with the SEM results that qualitatively assess the morphology and the pore's size of the prepared scaffolds ***Figure (2a-c).*** The SEM results showed that all the prepared scaffolds of all groups exhibited a highly porous structure with well-interconnected open pores. The pores had a well-defined internal cubical geometry similar to the geometry of the salt particles used in the scaffold's preparation [34].

The statistical analysis of porosity% **Figure (3a)** revealed that pure PCL scaffold (group I) and PCL/zein scaffold (group II) showed a statistically significantly higher porosity% (80.9 and 76), respectively than PCL/natural polymers/BG composite scaffolds (groups III).

This reduction in the pore size of PCL/zein/BG composite (groups III) scaffolds might be attributed to the presence of BG particles leading to a corresponding decrease in the PCL and natural polymers percentage in the total amount of scaffold matrix. Also, the agglomerated cluster formation of the ceramic powder around or inside the pores might occupy the free space available in the pores, leading to reduced pore size dimensions. Additionally, the probability of the adjacent BG particle contact to each other increases and becomes more easily exposed after the extraction of the porogen producing rough walls [335]. The rough pore walls of the composite scaffolds prepared in this study were also reported by ***Maji et al.*** [36], who found that with increasing the bioglass content up to 30% in the chitosan/gelatin composite scaffold, the size of the pores reduced to 100–120 μm and the shape of the pores became irregular, possessing rough pores wall that was beneficial for cell attachment and cell adhesion-promoting faster tissue ingrowth.

On the contrary, the results of the scaffold's porosity in this study are in disagreement with ***Yu et al.*** [37], who found that the presence of ceramic fillers with a varying amount (0 up to 50% wt./wt. of PCL) increased the density but had no significant effect on the porosity values of the scaffolds. Also, ***Hum et al.*** (4) reported that the zein/BG scaffolds had higher mean porosity (89%) than pure zein scaffolds (78%), which might be attributed to the possible dissolution of some of the BG during the leaching of the NaCl particles.

The statistical results of the weight loss% revealed an insignificant weight loss% was observed for pure PCL scaffold (group I), which lost about 14% of its initial weight after immersion in PBS for six months. The slow degradation rate of the PCL scaffold might be attributed to the high molecular weight of the PCL used in this current study (Mw 70,000 Da) which required from two to three years for complete removal from the host body [9] (5). This higher molecular weight increases the chain length necessitating greater numbers of ester bonds to be cleaved to generate water-soluble monomers/oligomers to allow erosion to proceed; consequently, the degradation procedure takes a long time [10].

On the other hand, blending zein with PCL in groups II presented a noticeable weight loss% (36.8%). The increased degradation rate of PCL/zein in groups II might be attributed to the relatively increased hydrophilicity of scaffolds containing natural polymers than that of the pure PCL scaffold due to the presence of polar amino acids in natural polymers that could form a hydrogen bond. In addition, the appearance of functional amide groups on the surface of PCL/zein scaffolds improved the scaffold hydrophilicity and accelerated the degradation rate.

These results are in agreement with several studies [38-40] that reported that blending zein with the PCL accelerates the degradation rate of the PCL scaffolds. ***Wu et al. (*** [39] observed that the zein/PCL scaffolds presented noticeable degradation (32%) during the 28-days of immersion in PBS compared to 1.4% weight loss for the PCL scaffolds.

In addition, the weight loss% of the composite scaffolds in groups III (PCL/zein/BG) that combined natural polymers with BG showed the statistically significant highest mean weight loss% (42.49). Adding inorganic BG fillers to the PCL matrix showed a dramatic effect on the degradation rate of PCL scaffolds, which might be attributed to the addition of the BG could cause an overall increase in the hydrophilicity and water absorption of the PCL matrix, thus increasing the hydrolysis rate. Also, the addition of the BG results in creating a polymer/ceramic interface, which was susceptible to hydrolytic attacks [41].

On the other hand, the results of the scaffold's degradation obtained in this study are in disagreement with ***Tajbakhsh and Hajiali*** [42], who found that no weight loss was observed in the scaffolds containing higher filler concentration during the first two weeks of incubation. However, the polymer molecular weight was found to decrease more quickly and to a large extent in the absence of bioactive glass. The delayed degradation rate in the composite scaffold might be due to the dissolution of ions from bioglass, which leads to a buffering effect of the incubation medium.

The statistical results of the compressive strength in this study revealed that the compressive strength was contrary to the porosity% results. The compressive strength was statistically significantly high in groups III (PCL/zein/BG) that contain BG fillers (3.69 MPa). On the other hand, the compressive strength values of groups I (pure PCL), II (PCL/zein), were decreased to 1.9 MPa, 1.48MPa, respectively.

The results of the compressive strength obtained in this study revealed the positive effects of the BG powder on the mechanical properties of the PCL composite scaffolds. Such improvement in the mechanical properties might be attributed to the lower porosity and the decreased pore sizes of the BG-containing composite scaffolds and the incorporation of stiff BG fillers within the PCL matrix [43,44].

The percentage of the BG (20%) used in this current study was in accordance with the concept of "critical mass fraction" that was reported by ***Cannillo et al.*** [45], who found that introduction of an increasing amount of glass (more than 40%) resulted in inferior mechanical properties of the PCL/bioglass composite scaffolds. Such reduction may be caused by the agglomeration of the glass particles that act as stress concentrators, decreasing the mechanical properties instead of improving them. A similar result was obtained by ***Georgiou et al.*** [46] who manufactured scaffolds of PLA reinforced with $CaO\text{-}Na_2O\text{-}P_2O_5$ glass. Dynamic mechanical analysis (DMA) testing showed that the storage modulus of the solid material was increased from 4.4 GPa to 5.2 GPa by the addition of 20 wt.% glass.

Viability/cytotoxicity was assessed in this study by MTT assay. It is a sensitive and quantitative method for detecting cellular activity, easy to use, and highly reproducible. In the present study, the statistical results of cell viability% results revealed that scaffolds of groups I and IV were more biocompatible than those of group II scaffolds ($P < 0.001$), where their cell viability% at day 7 was 98.86%, and 97.99%, respectively. On the other hand, PCL/zein composite scaffold (group II) showed a lower cell viability% at all time

intervals (1, 3 and 7 days), where the cell viability% was 83.8%, 96.27% and 91.66%, respectively. These results proved that PCL/zein was less biocompatible to MSCs than others groups. On the other hand, group III (PCL/zein/BG) composite scaffold showed low cell viability on day 1 and 3 while on day 7 showed statistically significant high cell viability and decreased cell toxicity, where the mean cell viability% was 91.86%, 96.77% and 97.99%, respectively and the median cell toxicity% was 7.59%, 3.56% and 1.7%, respectively.

The increased viability% of group III that combined zein with BG on day 7 might be attributed to the inclusion of the hydrophilic bioactive glass into the composite structure, which helps reduce the intrinsic hydrophobicity of the PCL and hence increased scaffold's biocompatibility. These results are in agreement with ***Gholamreza et al.*** [47], who found that the PCL scaffolds modified with zein/nBG exhibited a relatively higher rate of MG-63 cell proliferation compared to the unmodified groups, which could be attributed to the increased hydrophilicity of modified scaffolds. Also, the cells on the pure PCL scaffolds had almost spherical morphology with poor spreading tendency, which could be due to the high hydrophobicity of PCL.

Another contributing factor to the increased biocompatibility of the composite scaffolds containing BG particles (groups III) in this study might be attributed to the rough surfaces of the scaffold containing BG particles as was shown in the SEM results **Figures (2)** that enhanced cell adhesion, proliferation and activity. These results are in agreement with ***Sharma et al.*** [48], who showed that after 3 and 5 days, the cells were observed to have a spindle to polygonal morphology with the cell-membrane being quite flattened onto the rough surface of the scaffold created by the nBG particles. The authors proposed that the nanofeatures and rough surface of the nBG particles were believed to be the main boosting factors responsible for the osteoblast's good attachment and well-spread morphology over the scaffold.

Alkaline phosphatase (ALP) is a quantitative polymerase chain reaction present with the plasma membrane of osteoblast in high levels in metrics vehicles seen in the developing bone. Its secretion in the early stage is a marker of osteogenic differentiation [49]. In this study, the MSCs seeded on different scaffold groups were incubated in a culture medium containing osteogenic supplements (10 mM dexamethasone, 0.2 mM ascorbic acid, 10 mM βglycerophosphate) to stimulate cell osteogenic differentiation according to previous studies [50,51]. The ALP activity was evaluated by measuring ALP absorbance and on days 1, 3 and 7. The ALP absorbance was detected spectrophotometrically at 405 nm. The value of the ALP absorbance is proportional to ALP concentration.

ALP absorbance values of MSCs cultured on the different scaffold groups and ALP concentration **Figure (4 b)** revealed that incorporation of BG particles into the PCL/zein matrix in group III causing an increase in the ALP absorbance (0.032, 0.0808 and 0.119) and ALP concentration compared to its values in group II and pure PCL scaffold in group I

The increased ALP activity in groups III (PCL/zein/BG) at all-time intervals may be attributed to the inclusion of BG particles in the composite structure as previously explained the role of BG powder in enhancing the scaffold's biocompatibility. These results are in agreement with several studies [51,52] that reported the role of the BG in enhancing osteogenic cell differentiation. ***Lu et al.*** [51], found that incorporation of 30 wt.% 45S5 bioglass into the synthetic polymer composite scaffold was significantly enhanced human cells ALP activity (300 nmol) compared to the 5 wt.% bioglass

(130 nmol) after 4 weeks *in-vitro* culture as was also confirmed by immunohistochemistry for type I collagen and OCN.

CONCLUSIONS

Blending natural polymers (zein) with the PCL has no effects on both porosity% or the mechanical properties of the PCL scaffolds. On the other hand, such blendings increase the *in-vitro* biodegradation of the PCL scaffolds. Incorporating the BG particles into the PCL/zein matrix dramatically enhances the biodegradation and the mechanical properties of the PCL composite scaffolds and adversely affects the porosity%. Also, adding BG to the PCL/zein scaffold positively enhances the biological properties of the PCL composite scaffolds.

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