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Determination of cytocompatibility and osteogenesis properties of in situ forming collagen-based scaffolds loaded with bone synthesizing drug for bone tissue engineering

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\textbf{ABSTRACT}

Bone tissue engineering using in situ forming 3D scaffolds can be an alternative to surgically treated scaffolds. This work aimed to develop in situ forming scaffolds using poly (lactic-co-glycolic acid) and a bone synthesizing drug (risedronate) with or without the porogenic agent (collagen). Hybrid scaffolds were formed through solvent-induced phase inversion technique and were morphologically evaluated using scanning electron microscopy (SEM). The effect of scaffolds on Saos-2 cell line viability using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide test besides their effect on cell growth using fluorescence microscope was assessed. Furthermore, alkaline phosphatase (ALP) activity as well as Ca\textsuperscript{2+} deposition on the scaffolds was evaluated. SEM images revealed the porous structure for collagen-based scaffolds. Saos-2 cell proliferation was significantly enhanced with risedronate-loaded scaffolds compared to those lacking the drug. Porous collagen-based scaffolds were more favorable for both the cell growth and the promotion of ALP activity. Furthermore, collagen-based scaffolds promoted the Ca\textsuperscript{2+} deposition compared to their counterparts without collagen. Such results suggest that collagen-based scaffolds offer excellent biocompatibility for bone regeneration, where this biocompatible nature of scaffold leads to the proliferation of cells that lead to the deposition of mineral on the scaffold. Such in situ forming 3D scaffolds provide a promising noninvasive approach for bone tissue engineering.

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1. Introduction

Bone tissue engineering is a promising expanding research area for solving bone problems. Bone tissue engineering is a dynamic process that aids in the migration of osteoprogenitor cells to the traumatized bone area, which is then proliferated, differentiated, allowing the matrix formation. Bone tissue engineering uses artificial extracellular matrix (scaffolds), osteoblasts or cells that differentiate into osteoblasts after applying it to the traumatized bone [1]. These approaches aid in the promotion of cell proliferation and hence, the formation of mineralized bone tissues. Among these approaches, three-dimensional (3D) scaffolds play a significant role in the promotion of cell proliferation [2].

An ideal bone tissue engineering 3D scaffold is the one that simulates the extracellular matrix. Bone scaffolds consist of porous, biocompatible, and biodegradable materials that afford both the mechanical support and regeneration of bones. Being porous with interconnected pores, bone scaffolds will assist in the efficient diffusion of oxygen and nutrients which is essential for cell survivability and growth [3]. Metals, ceramics, and polymers are widely used for the fabrication of scaffolds. But polymers have been proven to be attractive candidates compared to metals and ceramics, due to their biodegradability and ease of fabrication [4].

In situ forming scaffolds have been proven to be a striking alternative to those preformed and applied by surgical treatment. In situ forming scaffolds can be formulated in simple steps with low need of equipment and processes [5].

Risedronate sodium, a bisphosphonate drug, belongs to a group of drugs applied in the treatment of bone disorders. Normally, bone undergoes regular turnover which is kept constant by balancing the action of both osteoblasts (bone building cells) and osteoclasts (bone destroying cells). Bisphosphonates prevent bone loss by inhibiting the action of osteoclasts [6]. Therefore, a biodegradable in situ forming scaffold with a drug inhibiting bone resorption like risedronate maximizes the efficacy of implant in the treatment of bone defects.

In our previous study, a type of polymeric in situ forming scaffold to be applied as bone tissue engineering scaffolds was developed [5]. These scaffolds were designed as a hybrid system which contains poly (lactic-co-glycolic acid) (PLGA), collagen, and risedronate. These hybrid in situ forming scaffolds were formed by dissolving all the scaffold components into a biocompatible organic solvent and injecting it into the application site, due to phase separation mechanism [7]. Such in situ forming biodegradable scaffolds were designed to provide a noninvasive approach for implanting the scaffolds in the patients’ body.

Each component used in the preparation of the in situ forming biodegradable scaffolds has its own importance in the bone tissue engineering. PLGA is an example of a biodegradable polymer which possesses the advantage of being self-eliminated, hence avoiding the invasive removal of the polymer from the implantation site [8]. Collagen is the major native extracellular matrix of bone tissue and can promote bone regeneration [9]. Furthermore, collagen was added to the formulation to be a porogenic agent for the formation of porous scaffolds. Risedronate is a potent bisphosphate for the treatment of osteoporosis. Meanwhile, it may also promote the activities of osteoblasts toward bone formation [10]. In our previous study, these risedronate-loaded hybrid in situ forming scaffolds were evaluated for their rheological, injectable, and morphological properties. In addition, in vivo histological study for the treatment of bone defect in jaw bones was evaluated in dogs and confirmed the bone regeneration ability of the prepared scaffold. In this study, we aim to investigate and address the cytocompatibility of the prepared hybrid in situ forming scaffold against Saos-2 cell line using MTT test. The biocompatibility of the scaffold was determined by growing the cells on the surface of the scaffold and then viewed them by microscopic imaging. Finally, mineralization capacity of the cells on the tissue engineering scaffold was assessed.

2. Materials

Risedronate sodium was generously gifted by Marcyrl for Pharmaceutical Industries, Egypt. PLGA copolymer of D,L-lactide and glycolide in a 50/50 molar ratio with an inherited viscosity midpoint of 0.4 dl/g (Purasorb® PDLC 5004; PLGA) was gently donated by Purac Biomaterials Co., Gorinchem, Netherland. Hydrolyzed collagen from fish with molecular weight of 50,000 Da was purchased from Shaanxi Pioneer Biotech Co., Ltd, China. N-methyl-2-pyrrolidone (NMP) was bought from Sigma-Aldrich, St. Louis, Missouri, USA.

3. Methods

3.1. Preparation of in situ forming scaffolds

In situ forming hybrid scaffolds were prepared through solvent-induced phase inversion technique [11]. PLGA was used as an insoluble polymer (30%, w/v) and NMP as an organic solvent. In brief, 10 mg of risedronate and 300 mg of PLGA were added to 1 mL NMP in a screw-capped glass vial and homogenized for 1 min using a probe sonicator (Hielscher, Teltow, Germany) till dissolution. After that, the preparation was poured in phosphate-buffered saline solution (pH 7.4) to formulate the scaffold (IS).

Collagen-based scaffold (IS-C) was formulated as mentioned previously, but with the addition of collagen (5%, w/v) to NMP after dissolving both the PLGA and drug. Finally, all the mixtures were vortexed (KS 4000, IKA, Germany) for 30 s to disperse the collagen. For comparison purposes, drug-free scaffold made only of PLGA was also prepared (Plain IS).

3.2. Characterization of morphological structure

Scanning electron microscope (Jeol JSM-6400; JEOL Ltd., Tokyo, Japan) was used for the investigation of surface and cross-sectional morphology of the in situ forming scaffold preparations at an excitation voltage of 20 kV after coating with a thin layer of gold. The preformed scaffolds in phosphate-buffered saline solution (pH 7.4) were air-dried for 2 days before imaging.

3.3. Sterilization by gamma radiation

The prepared scaffolds were sterilized using gamma radiation (γ-iradiator, Gammacell 1000; BEST theratronics, Ontario,
Canada) at a dose of 10 kGy (1.88 kGy/hr). They were first packed in dry ice inside a polyurethane container and then sterilized to avoid aggregation or melting, which might occur due to the temperature elevation, by \( \gamma \)-irradiation.

### 3.4. In vitro evaluation of scaffold cytocompatibility

#### 3.4.1. Saos-2 cell culture

Human bone osteosarcoma cells (Saos-2) were cultured in McCoy’s 5a Medium supplemented with 2 mM L-glutamine, 100 units/mL penicillin G sodium, 250 ng/mL amphotericin B, 100 units/mL streptomycin sulfate, and 10% fetal bovine serum (FBS).

#### 3.4.2. Effect of scaffolds on saos-2 cell viability

Saos-2 cells (1 \( \times \) 10^4 cells/well) were cocultured with 25 mg of each scaffold in McCoy’s 5a Medium supplemented with 15% FBS for 1 week. The metabolic cell viability was determined by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay [12]. The results were expressed as the percentage of viable cells compared to the untreated cells (control). In this study, paclitaxel was used as a positive control cytotoxic drug.

#### 3.4.3. Cell growth pattern and interaction with scaffolds

Saos-2 cells were incubated for 2 weeks with the scaffolds in 8-chamber cell culture slides (5 \( \times \) 10^4 cells/chamber, SPL Life Sciences, Korea). The cells were stained with acridine orange (AO, 100 µg/mL in phosphate buffered saline pH 7.4 (PBS)), a nucleic acid-binding dye, and were examined by fluorescence microscopy [13]. The slides were investigated under fluorescence microscope with 62HE BFP/GFP/HcRed filter (Axioimager Z2, Zeiss, Goettingen, Germany). Photos were captured by digital camera (Axio Cam MRC3 S/N 4299).

#### 3.4.4. Colorimetric determination of calcium and alkaline phosphatase

To investigate the effects of the prepared scaffolds on calcium deposition and the alkaline phosphatase (ALP) activity, Saos-2 cells were plated (5 \( \times \) 10^4) cells/well in serum-free medium with 0.1% bovine serum albumin (BSA). After incubation, cells were washed with Hanks’ balanced salt solution. Calcium deposition was assayed by a colorimetric kit; calcium detection kit (ab102505, Abcam). Cells were lysed by Triton X-100 (0.5% in PBS) in each well. A volume of 40 µL of lysate was used to estimate ALP activity. ALP activity was measured as a function of p-nitrophenol hydrolysis from p-nitrophenyl phosphate [3]. ALP activities were measured in Saos-2 cells lysate by colorimetric assay after 1 week of treatment, as indicated previously by Schiller et al. [14]. Each cell lysate was incubated with 1 mg/mL 4-nitrophenyl phosphate (substrate) for 30 min before monitoring the absorbance readings at 405 nm. Substrate-free conditioned medium and conditioned medium coincubated with ALP inhibitor, Levamisole, were used for enzyme reaction specificity. The concentration of ALP was normalized by protein concentration that was estimated by Bradford assay. The activity of one unit of enzyme is defined as the activity of hydrolyzing 1 nm of substrate/min/mg of protein. Results were expressed as µg alkaline phosphatase/mg cellular protein.

### 4. Results and discussion

#### 4.1. Characterization of morphological structure

From the presented figure, it was obvious that scanning electron microscopy micrographs of both formulations (IS and IS-C) differed greatly. It was manifested that the formulation (IS-C) showed a porous matrix with well-defined continuous pores as shown in Figure 1a, on the other hand, the formulation (IS) lacking the presence of collagen showed a compact matrix (Figure 1b).

#### 4.2. Effect of scaffolds on saos-2 cell viability

Due to the expected role of scaffolds in bone tissue engineering therapy, their effect on the viability of osteoblast-like Saos-2 cells was investigated. The proliferation of Saos-2 cells on the scaffold was evaluated by MTT assay protocol. This assay depends on determining the activity of mitochondrial dehydrogenases which is assumed to be correlated with cell number and cell viability. The effect of the prepared scaffolds on the metabolic cell viability was investigated for 2 weeks of the coculturing of scaffolds with prepared monolayer Saos-2 cells. The positive control, treated with paclitaxel, behaved in the expected way, as it provided dose-dependent toxic effect on osteoblast-like Saos-2 cells. On the other hand, it was revealed that formulations containing risedronate (IS-C and IS) resulted in a significant elevation in the cell number and cell growth during the first week and this finding was further enhanced in the second week \((p < 0.05)\), where the cell proliferation proportionately increased until the

![Figure 1](image-url)  
**Figure 1.** SEM micrographs for (a): IS-C and (b): IS.
15th day. This confirms the positive impact of the bisphosphonate drug, risedronate, on promoting osteoblastic growth and proliferation. Comparing the two formulae, results showed that the proliferation level of the Saos-2 cells on the IS-C scaffold (containing risedronate and collagen) was significantly higher than that of IS (containing only risedronate) \( (p < 0.05) \), confirming that this scaffold was more favorable for the growth of the cells due to its porous structure. Hence, this confirms the significance of the presence of collagen in the structure of IS-C scaffolds in the osteogenesis process \([15,16]\). On the other hand, a dramatic significant cell death \( (p < 0.05) \) could be observed with the plain scaffold (Plain IS), lacking both risedronate and collagen. That is why, it could be confirmed that the presence of both collagen and risedronate played an important role in the cellular proliferation, as it is clear from Figure 2.

4.3. Cell growth pattern and interaction with scaffolds

Cell adhesion is a complex sequence of physicochemical reactions, affected by different factors including the cell behavior, material surface properties (chemical composition, hydrophobicity, softness, and roughness). Cell adhesion to any scaffold surface is essential in controlling the overall cell behavior with regard to their growth, spread, migration, signaling, extracellular matrix deposition, and differentiation \([17]\). The adhesion of cell layers to the material’s surface provides

![Figure 2](image)

**Figure 2.** The effect of tested scaffolds on metabolic viability of Saos-2 cells during one and two weeks of co-culturing, as estimated by MTT assay, mean SE, \( n = 4 \).

![Figure 3](image)

**Figure 3.** Fluorescence microscope images for Saos-2 cells treated with IS-C with magnification \( \times 100 \).
evidence of the material’s affinity to the cells [18]. The cell/scaffold interaction was investigated by applying a fixed cell count (suspended in medium) onto the surface of each scaffold, the coculture was incubated for 2 weeks, and then the scaffolds were stained with acridine orange (a DNA dye that stains the nuclei for both live and dead cells into bright green). Results presented in Figure 3 show that the presence of risedronate and collagen (IS-C scaffold) promoted cell growth, migration toward, and aggregation around the scaffold fragments in an organized manner. Such cell behavior was induced during the first week of treatment and was further propagated in the second week of treatment. The high attachment and spreading of the cells in the IS-C scaffolds was clearly attributed to the porous biomimetic nature of this hybrid scaffold which contained collagen—the major component of native bone tissue—in addition to the presence of bisphosphonate drug [19]. Furthermore, IS-C with its porous structure would provide greater sites (surface area) for initial cellular attachment on them. On the other hand, the images of the fluorescence microscope (Figure 4) demonstrated that the cells treated with IS scaffold had started to grow and migrate toward the scaffold, although in a chaotic manner, during the second week of treatment. This result is in good correlation with the previous results obtained from the cell viability, where this scaffold (IS) showed lower values of cell viability, compared to the scaffold IS-C. On the contrary, plain scaffold (Plain IS containing PLGA alone) did not show an enhancement in cell growth and migration toward the scaffold (Figure 5).

4.4. Colorimetric determination of calcium and alkaline phosphatase

ALP is an important marker of the cell osteogenic differentiation at the early stages of bone formation, where it is elevated in the case of active bone formation [20]. ALP is a key enzyme whose function is important in the early process of mineralization, as increased ALP activity levels are associated with increased Ca$^{2+}$ uptake and bone formation. ALP is essential in the osteogenic lineages, as it is a key player in the mineralization, regulation of bone cell division and Ca$^{2+}$

Figure 4. Fluorescence microscope images for Saos-2 cells treated with IS with magnification x 100.
binding [21]. Therefore, its expression level was measured in the Saos-2 cells to evaluate the bioactivity of the cells cultured with the tested scaffolds as shown in Figure 6. The estimation of ALP activity in Saos-2 cells revealed that only the scaffold containing risedronate and collagen (IS-C) induced cell differentiation, as revealed by its successes in significant \((p < 0.05)\) enhancement of ALP activity (Figure 6). This demonstrates that this scaffold has the capacity to support the main functional activity of osteoblasts. This enhancement suggested that the presence of risedronate, together with collagen in the scaffold had a synergistic effect, and caused the osteoblastic phenotype to be expressed more effectively. On the other hand, other formulae (Plain IS, and IS-4) did not induce significant change in the ALP activity, compared to the control \((p > 0.05)\), as shown in (Figure 6).

Bone mineralization is a phenotypic landmark that determines the activity of osteoblasts for bone repair and should be supported by any scaffolds aimed to be used for bone tissue

![Fluorescence microscope images for Saos-2 cells treated with plain IS with magnification × 100.](image)

**Figure 5.** Fluorescence microscope images for Saos-2 cells treated with plain IS with magnification × 100.

![Graph showing ALP activity after the co-incubation of formulations with Saos-2 cells.](image)

**Figure 6.** The ALP activity after the co-incubation of formulations with Saos-2 cells (mean ± SE, n = 4).
engineering. Moreover, the results obtained when measuring the Ca\(^{2+}\) binding to the cells, correlated well with the results of ALP activity, where the scaffold containing both risedronate and collagen exhibited the highest Ca\(^{2+}\) deposition on the scaffold (Figure 7). These data prove that the scaffold can clearly promote Ca\(^{2+}\) mineralization by osteoblast cells, and that the cells exhibit full functionality after adaptation to this scaffold after only week, indicating the superior cytocompatibility of the risedronate and collagen-containing scaffolds (IS-C).

The results of ALP activity and Ca\(^{2+}\) deposition are in good correlation with that of the cell viability study, as the formulation containing both risedronate and collagen (IS-C), showed the highest cell viability. Again this is due to the effect of collagen, and risedronate, bisphosphonate group, to promote osteoblastic differentiation [22].

This result suggests that the IS-C scaffolds offer excellent biocompatibility for bone regeneration. The biocompatible nature of the scaffold leads to the proliferation of cells that will lead to the development of finely scaled mineral deposits on the scaffold. Therefore, the presence of essential minerals such as Ca\(^{2+}\) is essential for the cell proliferation as well as for tissue regeneration. Such a mineralized scaffold can improve specific biological functions such as adhesion, differentiation, and proliferation of osteoblastic cells.

5. Conclusion

In conclusion, a three-dimensional hybrid in situ scaffold was successfully fabricated and characterized in terms of cell proliferation, cell attachment, and mineralization characterization. The presence of collagen remarkably improved the Saos-2 cell attachment and cell growth on the scaffold surface. Therefore, this hybrid risedronate/collagen scaffold could be applied as a suitable matrix for the bone tissue engineering applications. It should be noted that this scaffold exhibited the desired biological in vitro properties without incorporation of any growth factor, mineralizing agent, or osteogenic media such as bone morphogenetic protein, dexamethasone, and ascorbic acid. This feature adds to the main advantage of the scaffold for being a noninvasive treatment that the scaffold fabrication becomes an economical process.

References