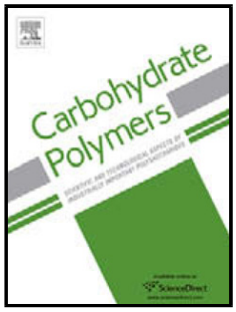

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Cell culture and characterization of cross-linked Poly(vinyl alcohol)-g-starch 3D scaffold for tissue engineering

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Highlights

► PVA and starch can be chemically cross-linked to form a PVA-g-starch 3D scaffold-grafted polymer. ►The absorbency of PVA-g-starch 3D scaffold is up to 800%. ► the strength of the 3D scaffold strength reaches 4 $\times 10^{-2}$ MPa. ►The 3D scaffold was degraded by various enzymes at a rate of up to approximately 30-60% in 28 days. ► *In vitro* experiments revealed that cells proliferate and grow in the 3D scaffold material.

Abstract

The research goal of this experiment is chemically to cross-link poly(vinyl alcohol) (PVA) and starch to form a 3D scaffold that is effective water absorbent, has a stable structure, and supports cell growth. PVA and starch can be chemically cross-linked to form a PVA-g-starch 3D scaffold polymer, as observed by Fourier transform infrared spectroscopy (FTIR), with an absorbency of up to 800%. Tensile testing reveals that, as the amount of starch increases, the strength of the 3D scaffold strength reaches 4 $\times 10^{-2}$ MPa. Scanning electron microscope (SEM) observations of the material reveal that the 3D scaffold is highly porous formed using a homogenizer at 500 rpm. In an enzymatic degradation, the 3D scaffold was degraded by various enzymes at a rate of up to approximately 30-60% in 28 days. *In vitro* tests revealed that cells proliferate and grow in

the 3D scaffold material. Energy dispersive spectrometer (EDS) analysis further verified that the bio-compatibility of this scaffold.

Keywords: Cross-linking, PVA-g-starch, 3D scaffold, Biodegradation, Cell culture

1. Introduction

Biodegradable polymers have been extensively utilized in medicine for a long time. Biodegradable polymer are bio-compatible, bio-absorbent and do not induce an immune reaction or inflammation can be used in medical materials in, for example, sutures, cover coatings, fracture fixing materials and other applications. (Wang, Wu, & Chen, 2004; Jiang et al., 2004; Li et al., 2005, Hsieh et al., 2011; Kuo et al., 2012). In recent years, the use of biodegradable polymer in combination with the cultivation of live cells to form new cartilage tissue has opened up the new research field of tissue engineering (Lanza, Langer, & Vacanti, 1999; Chen, Ushida, & Tateshi, 2000; Lahiji et al., 2000; Yoshimoto et al., 2003). The aim of using these special biopolymers to construct 3D scaffolds in which implanted cells can proliferate and grow, is to combine the advantages of the transplantation of human tissue with those of synthetic repair materials, and so establish principles for tissue engineering. Therefore, the use of biodegradable polymers in applications of biomaterials seems more important than ever.

A 3D scaffold can be used to grow and cultivate cells only under the following conditions, 1. Biocompatible scaffold must be non-toxic to implanted cells or tissues and to promote their growth and adhesion. 2. Bio-absorbable or bio-decomposable a 3D scaffold is an auxiliary tool, whose ultimate purpose is to be able to degradation and then be absorbed or excreted by the body after the cells or tissues have grown in a period suitable for cultivation. 3. Exhibit highly linked pores - when cells are being cultivated using a 3D scaffold, they must be able to adopt the designed shape in the scaffold. As the cells grow in the culture liquid, nutrients must be freely input to, and the waste material

should be excreted from, the structure. Accordingly, porosity is a key factor in cell cultivation. 4. Exhibit excellent mechanical strength and flexibility - the cells will not be unable to maintain their original shapes during the period of cultivation because of changes in the culture liquid or degradation. Hence, 3D scaffold must have a mechanical strength to support the attachment of cells.

This investigation concerns an inexpensive polymer-polyvinyl alcohol (PVA) which is an extensively used water-soluble polymer. PVA contains numerous polar alcohol groups and may form hydrogen bonds with water. It therefore dissolves easily in water. PVA is also a biodegradable material has that has been used in tissue engineering, and production methods include electrospining to form PVA nanofibrous scaffolds (Asran, Henning, & Michler, 2010; Liao et al., 2011); chemical synthesis (Mansur & Costa, 2008; Lynda et al., 2009), freeze-drying (Mohan & Nair, 2008; Mohan, Nair, & Tabata, 2010; Poursamar, Azami, & Mozafari, 2011; Jiang, Liu, & Feng, 2011), and melt-molding particulate-leaching (Oh et al., 2003). Starch, a natural polymer, is a material that is also commonly used in tissue engineering. In most relevant studies starch is blended with other matter to form a porous scaffold (Gomes et al., 2002; Ghosh et al., 2008; Santos et al., 2010; Duarte, Mano, & Reis, 2010; Castillejo et al., 2012). Other methods of producing a porous scaffold, such as chemical synthesis (Xiao & Yang, 2006; Sundaram, Durance, & Wang, 2008), wet spinning (Pashkuleva et al., 2010; Rodringues et al., 2012), and use of a blowing agent (Salgado et al., 2002) have also been reported upon in these literature. The cross-linking method that is proposed in this investigation does not seem to have been described before is utilized herein for the first time.

The goal is to exploit an existing, inexpensive, biodegradable material to form a 3D scaffold material for use in regenerative medicine. Since PVA is inexpensive, easy to obtain, highly biodegradable and biocompatibility, PVA and natural polymer-starch are considered herein. No complex or expensive equipment is required: simple chemical cross-linking method, to form 3D scaffold material and to improve its mechanical

properties and formation. Not only are the structural changes of the material examined: its gel and swelling, formation, mechanical properties, biodegradability, porosity and other characteristics are elucidated. Finally, NIH3T3 cells are transplanted and cultivated to 3D scaffold, and observe the effectiveness of the material for tissue engineering.

2. Experiment

2.1. Formation of 3D scaffold

Poly(vinyl alcohol) (PVA) and soluble starch were obtained from the Nitto Chemical Pharmaceutical Companies. Formaldehyde was obtained from Aldrich Chemical Company. Sulfuric acid (H_2SO_4) was purchased from The First Chemical Company. All materials and chemicals were used as acquired without any processing.

The starch powder (0.5, 0.75, & 1 g) was separately dissolved in RO water (10 mL) at room temperature. The 1 g of PVA was dissolved in RO water (10 mL) at 90 °C. The starch solution was slowly added into the PVA solution and mixed uniformly by a homogenizer at 500 rpm with heating to 90 °C for 30 minutes, until the mixture was viscous. The sample was then cooled to room temperature, before H_2SO_4 (3 mL) and formaldehyde (1.25 mL) were added with stirring. Finally, the sample was poured into the tube and placed in the oven at 60 °C for 50 minutes to form a circle shape.

2.2. SEM analysis

The morphology of 3D scaffold was observed under a field emission scanning electron microscope (FE-SEM Hitachi-4700, Japan). The completely dried blank sample was placed on a copper base, to which it was fastened with conductive tape. It was then plated with platinum in a vacuum deposition machine, before being placed on the sample base of FE-SEM for observation under the desired conditions of an accelerating voltage of 10 kV at a working distance (WD) of 15 mm. Photographs of the enzyme-degraded samples were also taken after the treatment processes. 3D scaffold sample with the

cultured cells was put in paraformaldehyde solution to dehydrate for 12 hours. It was then freeze-dried. The substances on the surface of the sample were then analyzed by EDS (HORIBA, Japan).

2.3. FT-IR analysis

The structure of the graft scaffolds were analyzed with FT-IR (Perkin Elmer FT-IR Spectrum one, USA). The material with the cross-linked PVA-g-starch 3D scaffold was placed in an oven to dehydrate. It was then placed in an FT-IR device for analysis. Scanning was carried out and the spectrum at 400 cm^{-1}-4000 cm^{-1} was recorded to identify the changes in the functional groups in the PVA-g-starch 3D scaffold that were caused by the above process.

2.4. Swelling of PVA-g-starch scaffold

The cross-linked PVA-g-starch 3D scaffold was dried in an oven at 50°C. The dried PVA-g-starch 3D scaffold was immersed in distilled water at room temperature for 48 hours. Excess water was wiped from the swelled PVA-g-starch 3D scaffold and the swelling ratio was calculated using the following below formula.

$$\text{Swelling (\%)} = [\ (Gs - Gi) / Gi\ ] \times 100$$

Gi represents the initial weight of the freeze-dried PVA-g-starch 3D scaffold, and Gs is the weight of the colloid in the swelling state.

2.5. Porosity

The porosity of the PVA-g-starch 3D scaffold is calculated using the liquid displacement method (Nie et al., 2012). The scaffold is submerged in water (V1) exactly for 30 minutes to ensure that enough liquid enters the pores of the scaffold. The new total volume of the liquid and the liquid impregnated scaffold is V2. When the liquid impregnated scaffold is removed, the remaining liquid volume is V3 and the porosity is

given by,

$$P\ (\%) = [(V1-V3) / (V2-V3)] \times 100$$

2.6. Tensile test

The mechanical properties of PVA-g-starch 3D scaffolds were examined by tensile test (Shimadzu EZ tensile, Japan). The sample was placed in the tensile strength tester under a load of 500 N. The tensile strength test was conducted at a rate of 5 mm/min, and the variations in strain and stress of the scaffold were investigated as the maximum breaking strength was approached.

2.7. Biodegradability

The biodegradability test was carried out to observe the degradation of PVA-g-starch 3D scaffold with over time. To simulate the environment of a human body, three enzymes were used in this investigation. They were lipase (20 μg/ml), α-amylase (150.5 U) and lysozyme (20 μg/ml). A thickness of approximately 0.5 cm of each processed 3D scaffold was placed in a 15 mL centrifuge tube with 1mL buffer solution that could be used with the various enzymes. Different concentrations of an enzyme were added. The centrifuge tube was placed in a 37 °C 100 rpm vibrating thermostatic bath for 28 days to perform an *in vitro* test. Every seven days, the 3D scaffold and placed in an environment at 25 °C to dry in completely. The weight lost by the sample was measured each time. The biodegradability is given by the following formula.

$$\text{Biodegradability}\ (\%) = (W_0-W_t)/W_0 \times 100\%$$

W_0 and W_t are the scaffold weights before and after the degradation.

2.8. *In vitro* test

Fibroblasts (NIH3T3 fibroblasts) were cultured in a petri dish with a diameter of 10 cm. The culture liquid comprised DMEM (Dulbecco's Modified Eagle's Medium), 10%

fortified bovine calf serum (FBS) and 1% penicillin streptomycin solution. Its pH value was maintained at 7.4. The incubator settings were 37 °C with 5% CO_2 and 95% relative humidity. The cell culture of PVA-g-starch scaffold which cycles lasted for three days. The numbers of cells were counted every 24 hours. The data thus obtained were used to plot the growth curve.

The cells that well detached from the trypsin were placed in a 15 mL centrifuge; the medium was added in an amount equal to that of trypsin. The system was mixed by centrifugation at 300 rcf and 4 °C for five minutes. Then, the supernatant was removed and 2 mL of medium was subsequently added to form the cell solution. Approximately 1 × 1 × 0.1 cm of scaffold was placed in each of 24-wells plate, and 500 λ cell solutions was added to the surface of the 3D scaffold in each 24-wells plate. The sample was placed in an incubator with 5% CO_2 at 95% relative humidity and 37 °C for 30 minutes. The scaffold was then removed from the incubator, and placed into another well with no additional agents; 2 mL medium was added to initiate the cell attachment experiment. After three days of culturing, the scaffold that contained the cells was immersed in 5 mL 10% formaldehyde overnight so that the cell was fixed to the scaffold, to prepare the test sample for EDS analysis.

3. Results and Discussion

3.1. Morphology of 3D scaffold

SEM was utilized to observe the morphological changes of the chemical cross-linked PVA-g-starch 3D scaffold. Fig. 1 presents SEM image of the PVA-g-starch 3D scaffold. The pores of the 3D material were formed by a homogenizer at 300 rpm. The photographs display the distribution of the pores of the 3D material that was cross-linked by stirring within the material. The physical properties of these materials will be investigated, and the material will be used for cell cultivation.

3.2. FT-IR analysis of cross-linked PVA-g-starch

To confirm whether cross-linking had occurred in the PVA-g-starch 3D scaffold, FT-IR was utilized to examine its structure. Fig. 2 displays the FT-IR spectra of PVA, starch and the cross-linked PVA-g-starch 3D scaffold. Absorption peaks associated with the methylene group (CH_2) of PVA are observed close to 3000 cm^{-1}, 1000 cm^{-1}, and 800 cm^{-1}; a peak associated with the C=O group are observed near 1700 cm^{-1}, and peaks associated with the OH group are observed close to 1150 cm^{-1} and 3400-3640 cm^{-1}. The FT-IR absorption peak of functional group of PVA is also observed in the spectrum of the cross-linked PVA-g-starch material. The CH_2 group of starch and the cross-linked 3D scaffold appeared to yield a weaker FT-IR peak near 3000 cm^{-1} than did the CH_2 group of PVA. Furthermore, the peak of the OH group in PVA close 1150 cm^{-1} gradually became weaker upon cross-linking. Similar result has been reported by Lee and Xiao (Lee, Kung, & Lee, 2005; Xiao & Yang, 2006), the cross-linking agent of Formaldehyde (aldehyde group) can react with PVA and starch (hydroxyl groups) under the presence of an acid catalyst, and forming rings structure of 1, 3-dioxane. The FT-IR absorption peaks of the cross-linked PVA-g-starch demonstrate, significant changes in the major absorption peaks upon cross-linking, implying that the PVA-g-starch had been successfully prepared.

3.3. Swelling ratio of 3D scaffold

A swelling ratio test was performed to determine the extent of swelling of the PVA-g-starch 3D scaffold. It is very important high water content that support tissue engineering, such as tissue-like elasticity, good exchange of nutrients for growth of cells. Fig. 3 displays the rates of absorption of water by the PVA-g-starch 3D scaffold after the cross-linking of PVA using various starch concentrations. The above histogram is the actual forming photographs of the PVA-g-starch 3D scaffold. Fig. 3 demonstrates that, the rate of water absorption increases significantly with the starch concentration. At a PVA to starch concentration ratio of 1:0.5 (ST1), the swelling ratio was 400%; as the

starch concentration increased, the absorption of water considerably, yielding a swelling ratio of 800%. Because of cross-linking with hydrophilic PVA and starch, forming 1, 3-dioxane rings structure, keep a number of water in the scaffold that causes PVA-g-starch have a high swelling. However, absorption of water at ST2 (1:0.75) did not differ much from that at ST3 (1:1), suggesting that PVA-g-starch 3D scaffold that was cross-linked in ST2 (1:0.75) and ST3 (1:1) may have reached saturation level, reducing the degree of water absorption.

3.4. Tensile test

The most important characteristic of a 3D scaffold that is to be used in tissue engineering is that it has a particular strength, which enables it to support the cell while maintaining its shape. The scaffolds with different mechanical properties should be depending on the desired tissue engineering application. PVA and starch have much poor mechanical properties. The addition of starch into PVA could reduce its solubility and increase strength. Table 1 presents the mechanical properties of the cross-linked PVA-g-starch 3D scaffold under wet state. Table 1 indicates that, as the starch concentration increases, the tensile strength falls from 4.3×10^{-2} MPa for the ST1 sample to 3.6×10^{-2} MPa for the ST3 sample, perhaps because more highly cross-linked starch has a lower elasticity. However, regardless of concentration, the elongation ratio at breakage of all cross-linked samples reaches 300%, indicating that the material is an excellent elastomer. The young's modulus of the material is approximately $1.5\text{-}2.9 \times 10^{-2}$ GPa. In the porosity test, the porosities obtained when three starch concentration in the formation of the 3D scaffold were all above 75%, above this value did not seem to influence the strength of the 3D scaffold. PVA and starch are a hydrophilic polymer, and not suitable for using as cell culture. Therefore, PVA need cross-linking another substance to decrease solubility and giving strength for cell culture. These results demonstrate that the cross-linked PVA-g-starch 3D scaffold had strength and porosity for transplanting

cells and the material will be used for cell cultivation in this study.

3.5. Biodegradability of 3D scaffold

As well as maintaining a minimum the required strength during cell culture, tissue engineering implant materials must support the growth of cells, and the scaffold material can slowly degrade into small molecules and excrete. Hence, to simulate the environment of the human body, the enzymes lipase, α-amylase, and lysozyme are utilized in the evaluation of the biodegradability of the scaffold. These three enzymes are all hydrolases. Lipase is a lipid-digesting enzyme, which mainly affects which carbonyl group of material. The α-amylase and lysozyme enzyme are carbohydrate-digesting enzymes, but α-amylase degrades the α-1,4 bonds and lysozyme degrades the glycosidic bonds of β-1,4 bonds.

Fig. 4 plots the biodegradability of 3D scaffold in various enzymes. The extents of enzymatic degradation rates with a PVA to starch ratio of 1:0.5 (ST1) after 28 days are lipase 56%, α-amylase 46% and lysozyme 43%. However, the extent of enzyme degradation falls as the starch concentration increases; the degree of cross-linking therefore increases. With a PVA to starch ratio of 1:1 (ST3), after 28 days, the extents of enzymatic degradation are lipase 27%, α-amylase 35% and lysozyme 31%. The high concentration of starch is presumed to make the structure of scaffold denser and more complete, making it more difficult for the enzymes to degrade. In the control group, the material was placed in aqueous PBS water without enzymes. Although the weight loss increased with the reaction time, the weight loss was insignificant, perhaps because immersing the material in water and caused it to swell, collapsing the weaker bonds in the material. However, the biodegradability of these three concentrations of 3D scaffolds after 28 days was approximately 30% - 60%, suggesting that the 3D scaffold material may be used as long-term implant material.

Fig. 5 presents SEM image of the degradation of the 3D scaffold by various

enzymes. By comparison with the SEM image of the not-degraded PVA-g-starch 3D scaffold in Fig. 1, the SEM image in the Fig. 5 indicates signs of degradation after six days of enzyme treatment. Enzymatic degradation of the pores causes them to become deformed or to be eliminated as presented in square frame (Fig. 5). This result demonstrates that the 3D scaffold after cross-linking can be degraded by enzymes *in vivo*, and can be utilized in tissue engineering applications.

3.6. *In vitro* test

To elucidate the cell culturing conditions in a 3D scaffold, mouse NIH3T3 fibroblasts were implanted into a 3D scaffold and the consequent cell growth. Fig. 6 plots the growth curves of mouse NIH3T3 fibroblasts cells that were implanted in the 3D scaffold. As the culture time increased, the number of cells also increased, reaching 10-15 $\times 10^4$ in 144 hours, indicating that the material supported cell proliferation. Fig. 7 presents the electron microscope SEM and EDS analysis diagrams of cells that were implanted in the 3D scaffold compared with original 3D scaffold. The photograph significant reveals cell growth; EDS analysis confirmed the presence of phosphorus and calcium, proving that cells indeed grew on the cross-linked PVA-g-starch 3D scaffold.

4. Conclusions

The cross-linked PVA-g-starch 3D scaffold reveals that is highly porous and easily formed using a homogenizer at 500 rpm. Formaldehyde (aldehyde group) can react with PVA and starch (hydroxyl groups) under the presence of an acid catalyst, and forming rings structure of 1, 3-dioxane. The FT-IR analysis demonstrates that the PVA-g-starch had been successfully prepared. The produced 3D scaffolds have highly porosity; can keep a large number of water. The absorption of water of 3D scaffold increased significantly with starch concentration, absorbency of up to 800%. The wet state tensile strengths of the 3D scaffolds all exceeded 3.6×10^{-2} MPa, and of these 3D scaffolds

exhibited excellent elongation at breakage and excellent elasticity. A PVA and starch all have biodegradability. The high concentration of starch is presumed to make the structure of scaffold denser and more complete, making it more difficult for the enzymes to degrade. However, the biodegradability of PVA-g-starch 3D scaffolds after 28 days was approximately 30% - 60%, suggesting that the 3D scaffold material may be used as long-term implant material. *In vitro* test, the number of cells gradually increased with the cultivation time in the 3D scaffold, comparison with original 3D scaffold. The EDS analysis of the surface of the scaffold revealed the presence of phosphorus and calcium and those normal cells can attach to, and grow on it. It is suggesting that the 3D scaffold is biocompatible, allowing the proliferation, adhesion and growth of cells. The PVA-g-starch 3D scaffolds have potential use for tissue engineering.

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Table caption

Table 1. Mechanical properties of various cross-linked PVA-g-starch 3D scaffold under wet state.

Table 1. Mechanical properties of various cross-linked PVA-g-starch 3D scaffold under

425 wet state.

Sample	Tensile strength (10^{-2} x MPa)	Elongation to break (%)	Young's modulus (10^{-2} x Gpa)	Porosity (%)
ST1	4.27	336	2.5	75
ST2	3.78	289	1.5	77
ST3	3.59	363	1.9	86

426

427 7-2082.

Figure captions

Fig. 1. SEM image of various cross-linked PVA-g-starch 3D scaffolds. (Concentrations ratio of PVA and starch are ST1=1:0.5, ST2=1:0.75, and ST3=1:1)

Fig. 2. FTIR spectrum of PVA, starch and cross-linked PVA-g-starch 3D scaffold.

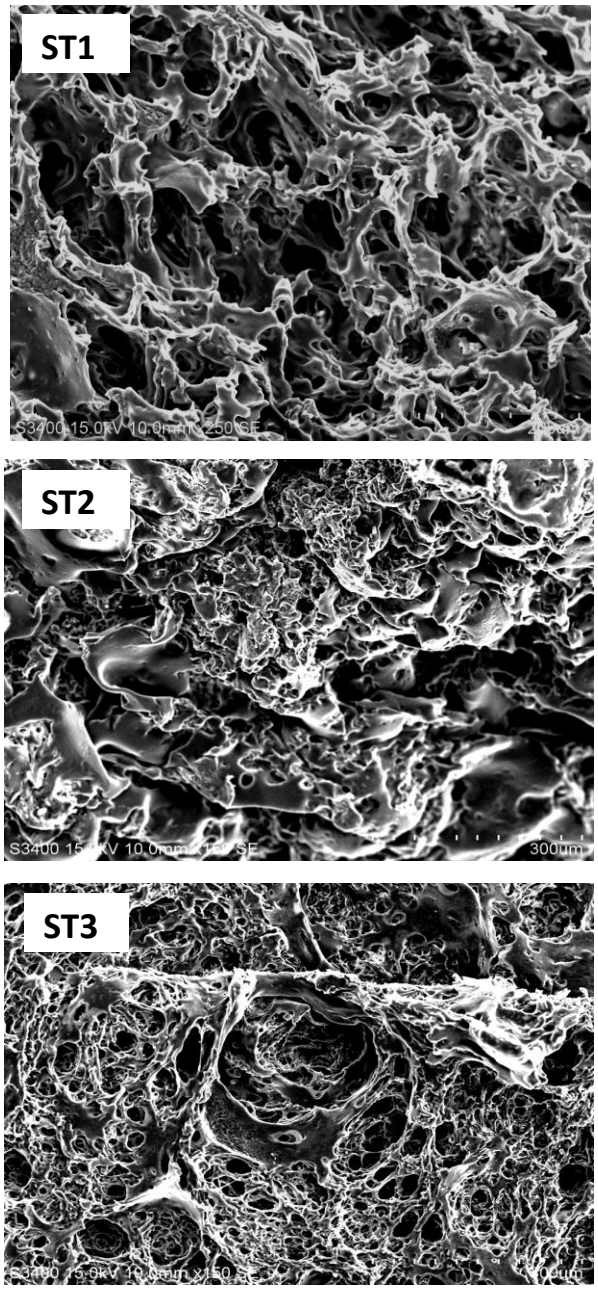
Fig. 3. Swelling ratio of various cross-linked PVA-g-starch 3D scaffolds. The above histogram is the actual forming figure of the cross-linked PVA-g-starch 3D scaffold. (Concentrations ratio of PVA and starch are ST1=1:0.5, ST2=1:0.75, and ST3=1:1)

Fig. 4. Weight loss of various cross-linked PVA-g-starch 3D scaffolds in the various enzymatic degradation at 28 days. (■: lipase, ◆:α-amylase, ▲:lysozyme, ●:control)

Fig. 5. SEM image of degraded PVA-g-starch 3D scaffolds by enzyme. (a : lipase, b ： lysozyme)

Fig. 6. Cell proliferation of NIH 3T3 cells on various cross-linked PVA-g-starch 3D scaffolds as a function of time. (▲:ST1、◆:ST2、■: ST3)

Fig. 7. SEM images of NIH3T3 cells on cross-linked PVA-g-starch 3D scaffold after 3 days of culture. (A: Original material, B & C : materials with Cells, X370 & X2000)



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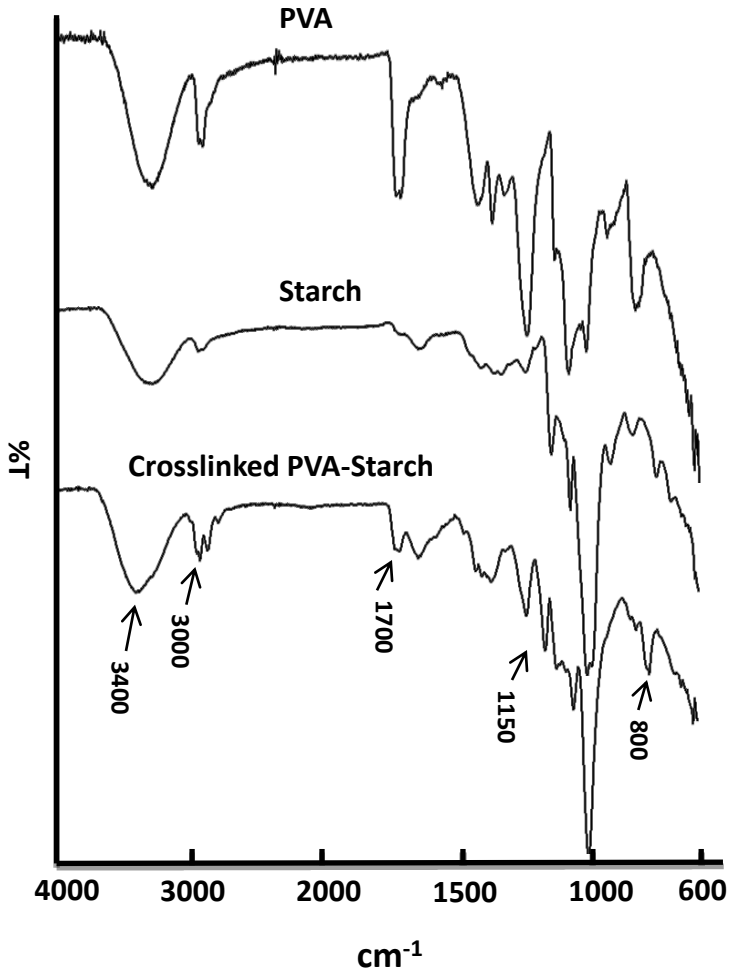

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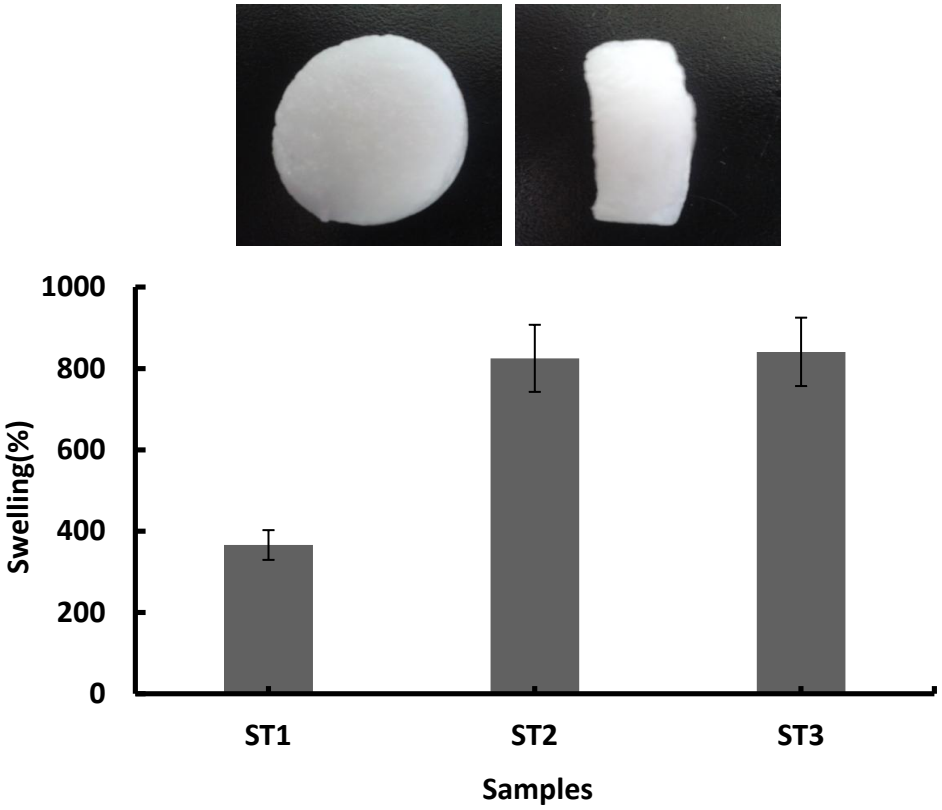

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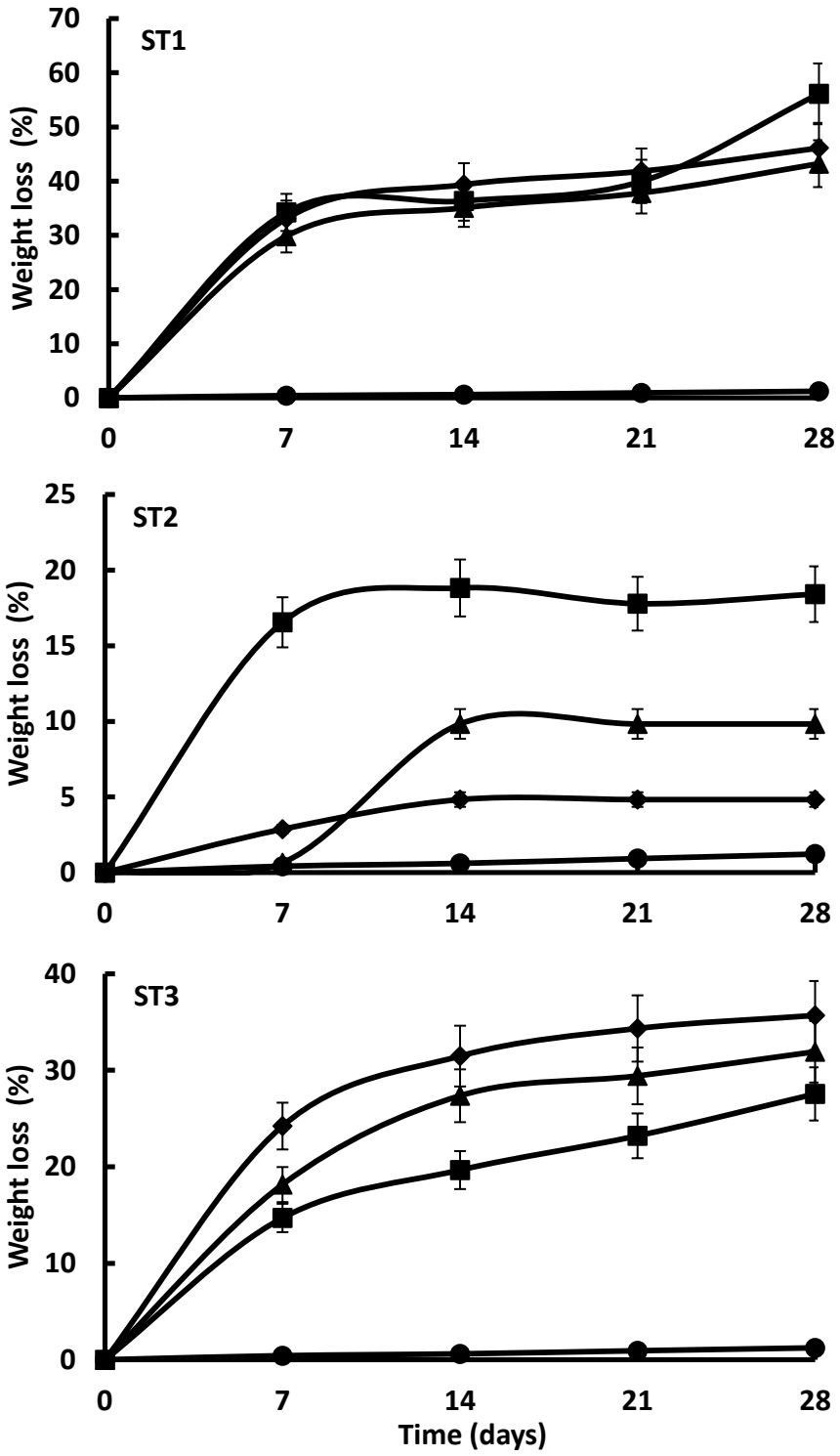

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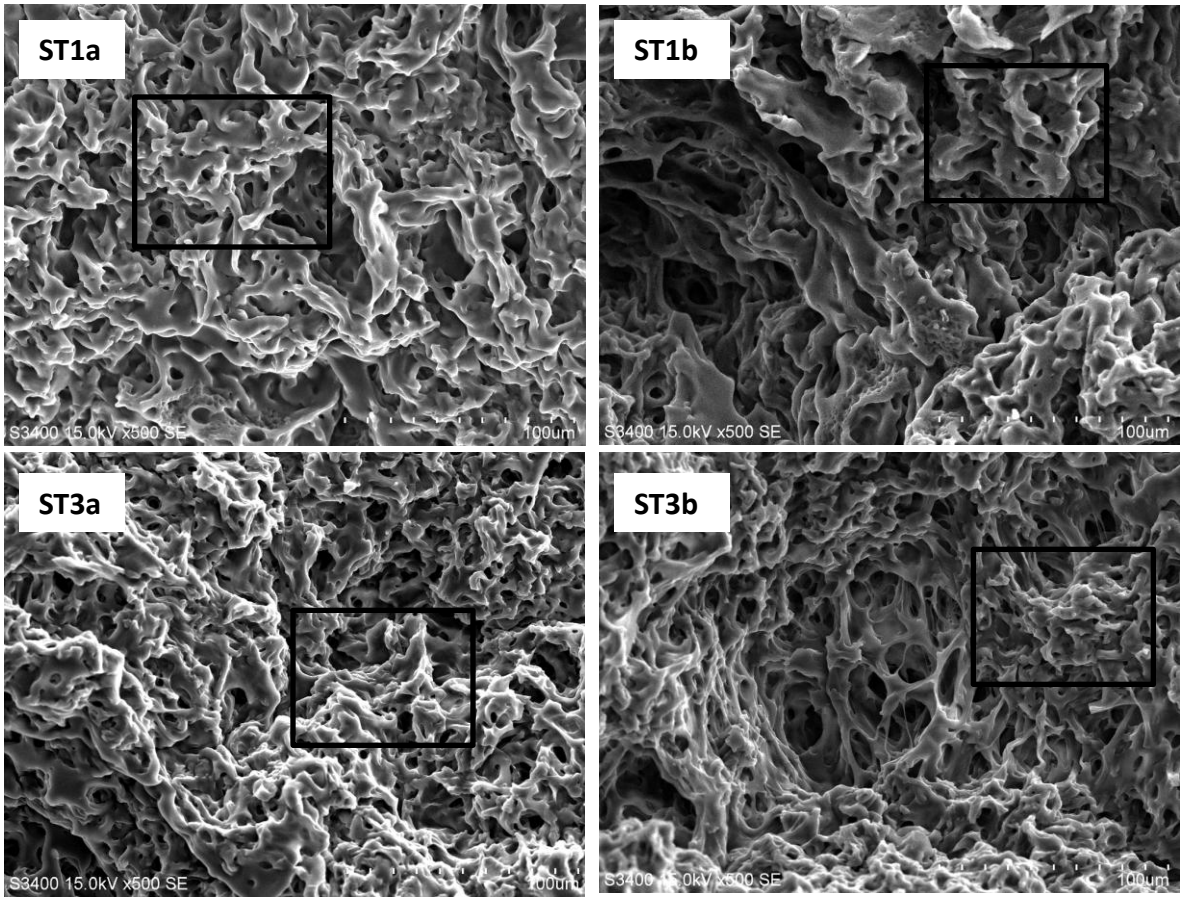

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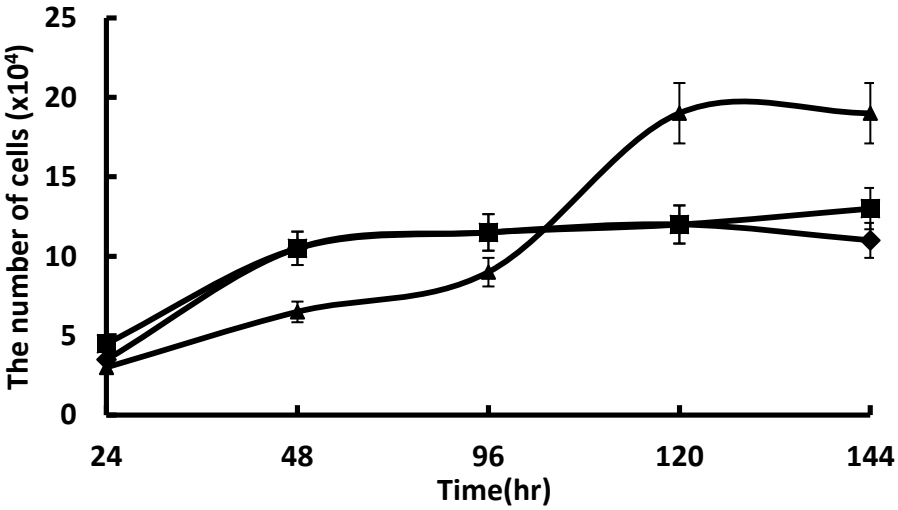

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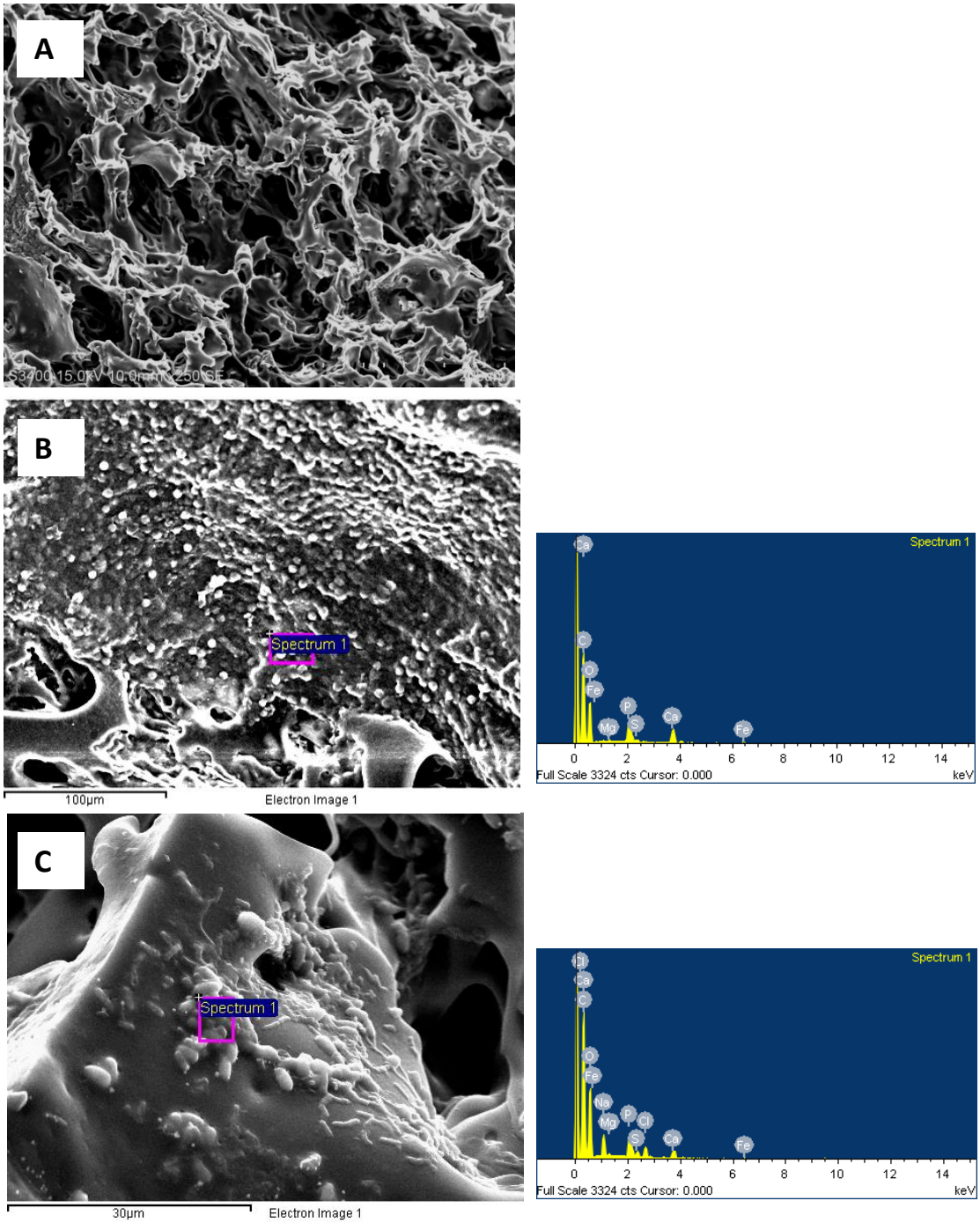

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