

Original Research Article

<https://doi.org/10.20546/ijcmas.2020.910.122>

Biocompatibility Study of Collagen I Modified PCL/Gelatin/Chitosan Composite Nanofibrous Scaffold for Fibroblast and Osteoblast Cells

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ABSTRACT

Keywords

Composite scaffold, L929 mouse fibroblasts, Human osteoblasts, Polycaprolactone, Gelatin, Chitosan, Collagen I

Article Info

Accepted:
10 September 2020
Available Online:
10 October 2020

In present study, a previously fabricated tripolymer composite scaffold of PCL/gelatin/chitosan was modified with collagen I and the modified scaffold were studied for biocompatibility of L929 mouse fibroblasts and human osteoblast cells. Phase contrast microscopy confirmed the viability of L929 mouse fibroblasts and human osteoblasts cultured with the modified composite scaffold which indicated that scaffold did not produce any toxic effect on both cells. Giemsa staining demonstrated the fibroblastic characteristic morphology of L929 and osteoblastic characteristic morphology of human osteoblast cells. FE-SEM study illustrated the adhesion and proliferation of L929 fibroblasts and osteoblasts, and retained their characteristic morphology on the modified PCL/gelatin/chitosan composite scaffold. Therefore, these results indicated that both cells were viable, proliferating, and showing their phenotypic morphology on collagen I modified PCL/gelatin/chitosan composite nanofibrous scaffold. Hence, the collagen I modified PCL/gelatin/chitosan composite nanofibrous scaffold might be an acceptable contender for skin and bone tissue engineering applications.

Introduction

Tissue engineering is an interdisciplinary field, nowadays which is being used for the development of biological substitutes and to restore, maintain, or improve the function of diseased tissue (Ma, 2008; Langer and Vacanti, 1993; Praveen, 2006). The key aspects of tissue engineering are cells, a scaffold and growth factors which play a pivotal role in tissue regeneration and tissue healing, although all the three components are essential, but scaffold plays a critical role for

tissue regeneration (Ott *et al.*, 2008; Ikada *et al.*, 2006; Gautam and Ambwani; 2019). The main role of the scaffold in tissue engineering is to provide a biomimetic environment to the cells so that cells can proliferate and differentiate in a specific tissue (Sharma *et al.*, 2011). Thus, a tissue engineering scaffold should be structurally and chemically similar to the extra-cellular matrix (ECM) of our body. The extra-cellular matrix of our body mainly made up of nanoscale collagen (protein) fibers which are embedded in the gel of polysaccharides (Aumailley and Gayraud,

1998). Therefore, a scaffold which is made up of nanoscale fibers would show architectural similarity to the ECM of our body. Recently, electrospinning is an attractive and simple technique which is used to produce continuous nanofibers for tissue engineering application (Murugan and Ramakrishna, 2007).

The electrospinning process controls the scaffold architecture at the nano and micro scales for flexible tissue design that improves cellular response and biocompatibility because of the morphological similarities to the three-dimensional ECM protein fiber network (Zong *et al.*, 2005). It has also been seen in previous studies that electrospun scaffold possess nanoscale topography which comprises many vital properties similar to the native ECM of our body such as high porosity and very high surface area-to-volume ratio that initiates the cell adhesion, proliferation and migration (Lim and Mao, 2009; Jun *et al.*, 2018). Additionally, in order to fulfill the diverse need of tissue engineering, chemical composition of scaffold is also an important aspect. To mimic the chemical composition of ECM various materials have been exploited.

In previous study, a composite scaffold of PCL/gelatin/chitosan (Gautam *et al.*, 2014) has been fabricated on the basis of excellent properties of PCL, gelatin and chitosan. Since, PCL is a synthetic polymer (Zhang *et al.*, 2005) which possess high mechanical properties (Chen *et al.*, 2011) but low cell adhesive properties due to its hydrophobic nature (Kim *et al.*, 2006; Li *et al.*, 2006; Gautam *et al.*, 2012). On the other hand, gelatin is a natural polymer and it is denature form of collagen protein which shows good cell adhesive properties (Ghasemi-Mobarakeh *et al.*, 2008) but low mechanical strength (Bigi *et al.*, 2001). Further, chitosan is a natural polysaccharide and it is deacetyl form of chitin that consists of N-acetylglucosamine

and glucosamine similar to glycosaminoglycans which is a major component of natural ECM (Chen *et al.*, 2010; Mourya *et al.*, 2010). Chitosan exhibits good cell adhesive and proliferative properties whereas it also shows poor mechanical stability which is almost similar to gelatin (Pok *et al.*, 2013; Albanna *et al.*, 2013). Thus, the combination of PCL/gelatin/chitosan (Gautam *et al.*, 2014) to fabricate an electrospun scaffold was found remarkable that mimicked the chemical composition (protein and polysaccharide) more similar to the extracellular matrix which provoked cell proliferation properties to the scaffold, and also provided the required mechanical strength.

In this study, an attempt has been made to mimic the previously fabricated scaffold chemically more similar with native ECM of skin and bone to increase the cell affinity for skin and bone tissue engineering applications. Hence, the previously fabricated PCL/gelatin/chitosan composite scaffold was modified with collagen I. Since, collagen I is a major component of natural ECM of skin and bone which bears different cell binding sites such as Arg-Gly-Asp (RGD) and Gly-Phe-Hyp-Gly-GluArg (GFOGER) (Khew *et al.*, 2007; Gautam *et al.*, 2013) that influences adhesion mechanism of cells to the scaffold. Further, in the present study, collagen I modified tripolymer composite scaffold of PCL/gelatin/chitosan were exploited for biocompatibility study of L929 mouse fibroblasts and human Osteoblasts cell line.

Materials and Methods

Chitosan (degree of deacetylation $\geq 75\%$) powder, PCL (Mw = 80,000), Gelatin (type B bovine skin) and collagen (type I from calf skin) were purchased from Sigma-Aldrich (St. Louis, MO). Phosphate buffer saline (PBS) and Dulbecco's modified eagle medium

(DMEM) were also procured from Sigma-Aldrich (St. Louis, MO). Giemsa stain, methanol and Chloroform were obtained from Fisher Scientific, Mumbai, India. Glacial acetic acid was acquired Qualigens Fine Chemical, Mumbai, India. Fetal bovine serum (FBS) was purchased from Hyclone, U.S.A. Skin fibroblast-like cells (L929) from mouse connective tissue was received from National Centre for Cell Science (NCCS Pune, India). Human osteoblast cell line was obtained from Jaslok Hospital and Research Centre, Mumbai, India. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) was obtained from Himedia laboratories Pvt. Limited, Mumbai, India.

Fabrication of collagen I modified PCL/gelatin/chitosan composite scaffold

First of all, PCL/gelatin/chitosan composite nanofibrous scaffold was synthesized by electrospinning method and then the scaffold was modified with 1wt% collagen I solution. The electrospinning of PCL/gelatin/chitosan composite nanofibrous scaffold was done according to previously reported parameters (Gautam *et al.*, 2014). Subsequently, the fabricated electrospun PCL/gelatin/chitosan composite nanofibrous scaffold was submerged in EDAC (10 mg/mL in PBS) solution and put at 4°C for 4 h. The treatment of EDAC was given to activate carboxylic group of gelatin present in scaffold. After EDAC treatment, scaffold was treated with 1wt% collagen I concentration at 4°C for 24 h (Ma *et al.*, 2005)

Afterward, the modified scaffold was air-dried overnight and the morphology of this scaffold was investigated by FE-SEM (Quanta 200F Model, FEI, Netherland). Before imaging, the gold coating was done of the modified scaffold using a sputter coater (Biotech SC005, Switzerland).

Cell culturing of fibroblasts and Osteobalsts

L929 mouse fibroblast cells were maintained in DMEM media which was supplemented with 10% FBS, penicillin (100 units/mL) and streptomycin (100µg/mL). Similarly, human osteoblasts were also cultured in DMEM supplemented with penicillin (100 units/mL) and streptomycin (100µg/mL), 10% FBS, 2µL/mL glutamine and 12µL/mL insulin. Confluent mouse fibroblasts and human osteoblasts were detached from culture flask by trypsinization using 0.25% Trypsin-EDTA for 5 minutes and then suspended in complete growth medium to perform next experiments.

Cell viability of fibroblasts and osteoblasts cultured with modified scaffold

Cell viability of L929 mouse fibroblasts and human osteoblasts incubated with collagen I modified PCL/gelatin/chitosan composite nanofibrous scaffolds were demonstrated by phase contrast microscopy (Zeiss, Pvt. Ltd. Bangalore, India) for 1, 3 and 5 days. The modified scaffolds were sterilized by UV treatment from both sides for 30 minutes each.

Before cell seeding, scaffolds were immersed in 100 µL DMEM media in 96 wells plate overnight at 37°C to make scaffolds surface more efficient for cell attachment. After PBS washing, scaffolds were incubated with L929 mouse fibroblast cells and human osteoblast cells at a density of 5×10^3 cells/well (volume 200 µL) and 1×10^3 cells/well (volume 200 µL) respectively for 1, 3 and 5 days in CO₂ incubator (BINDER, Germany). During cell culture, the media was changed every 24 h. After incubation period, cell-scaffolds constructs were observed under the phase contrast microscopy. The experiment was performed in triplicates.

Morphological characteristics of fibroblasts and osteoblasts cultured with modified scaffold

Histological staining (Giemsa staining) was performed to investigate the morphological integrity of L929 mouse fibroblasts and human osteoblasts cultured with collagen I modified PCL/gelatin/chitosan composite scaffold for 1, 3 and 5 days. Scaffolds were placed in 48 well culture plate and incubated with 5×10^3 cells/well fibroblasts and 1×10^3 cells/well osteoblasts at 37°C in CO_2 incubator. After incubation period, media was discarded and cell-scaffold construct in culture plate were fixed with 50% methanol for 1 h at room temperature. Consequently, giemsa stain was added into the wells of plate for 5 min. Afterwards, cell-scaffold construct was washed with distilled water and the stained cells with scaffold were examined under phase contrast microscope.

Adhesion of fibroblasts and osteoblasts cells on modified composite scaffold

Adhesion of L929 mouse fibroblasts and osteoblasts on collagen I modified PCL/gelatin/chitosan composite scaffolds were demonstrated by FE-SEM. Scaffolds were cultured with 100 μL DMEM media overnight in 96 well culture plate in CO_2 incubator at 37°C . After PBS washing, scaffolds were cultured with L929 fibroblasts and human osteoblasts at a density of 5×10^3 cells/well and 1×10^3 cells/well respectively for 1, 3 and 5 days. After incubation period, scaffold were rinsed with PBS three times and fixed with 2.5% glutaraldehyde at 4°C for 6 h.

Consequently, scaffolds were dehydrated with gradient concentration of ethanol (50%, 70%, 95%, and 100%) at 4°C for 30 minutes each for FE-SEM analysis to observe the morphology of attached cell on collagen I

modified PCL/gelatin/chitosan composite nanofibrous scaffold.

Results and Discussion

Morphology of collagen I modified PCL/gelatin/chitosan composite nanofibrous scaffold

The PCL/gelatin/chitosan composite scaffold was modified by immersing in collagen I (1.0 wt %) solution as discussed above. The fiber morphology of PCL/gelatin/chitosan and collagen I modified PCL/gelatin/ chitosan composite scaffold has been analyzed by FE-SEM as shown in (Fig. 1). The PCL/gelatin/chitosan composite nanofibrous scaffold showed random fiber morphology with 350 ± 55 nm fiber diameter and 2.5 ± 0.59 μm pore size [Fig.1 (a-b)]. The collagen I modified PCL/gelatin/chitosan composite scaffold also showed random fiber morphology but fibers were found collapsed to each other [Fig.1(c-d)]. The collapse fiber morphology may be arise from the immobilization of collagen I on the surface of PCL/gelatin/chitosan scaffold. The modified composite scaffold showed fiber diameter in the range 426 ± 114 nm and pore size in the range 1.0 ± 0.36 μm . The modified composite scaffold showed higher fiber diameter and lower pore size as compared to the unmodified scaffold which might be due to the immobilization of collagen I on the surface of PCL/gelatin/chitosan scaffold.

Cell viability of fibroblasts and osteoblasts cultured with modified composite scaffold

Phase contrast microscopy of L929 fibroblasts and human osteoblasts cultured with modified scaffold has been shown in Fig. 2 for 1, 3 and 5 days of incubation period. It is clear from Fig. 2, L929 mouse fibroblast cells cultured with collagen I modified PCL/gelatin/chitosan composite scaffold were fully viable and

increased in density with incubation period. This indicates that any toxic substance was not leach out from modified composite scaffold or scaffold materials did not produce any toxic effect on the cells, and the cells were observed proliferating in nature with

incubation period. Similar results were found with human osteoblasts. These cells were also found to increase in density with incubation period that indicated the proliferating nature of the cells with scaffold.

Fig.1 Morphology of electrospun PCL/gelatin/chitosan and collagen I modified PCL/gelatin/chitosan composite scaffold: (a) and (b) PCL/gelatin/chitosan composite scaffold at 5,000x and 10,000x magnification; (c) and (d) collagen I modified PCL/gelatin/chitosan composite scaffold at 5,000x and 20,000x magnification respectively

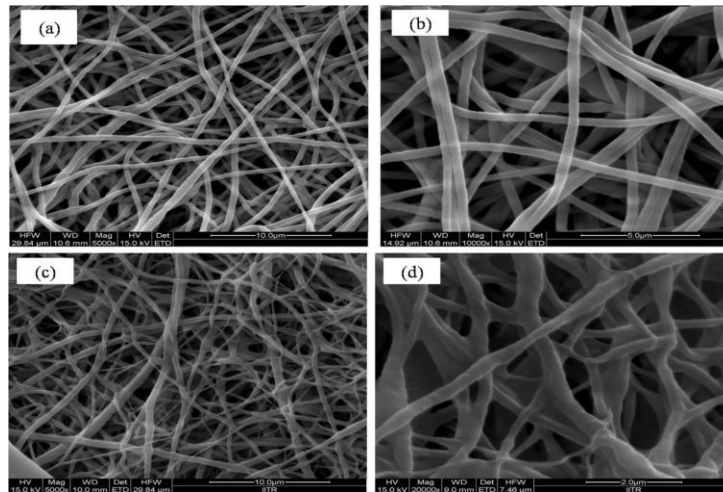


Fig.2 Phase contrast microscopy images of L929 cells and human osteoblasts incubated with collagen I modified PCL/gelatin/chitosan composite scaffold at different magnification. White arrows represent the scaffold in the wells of tissue culture plate

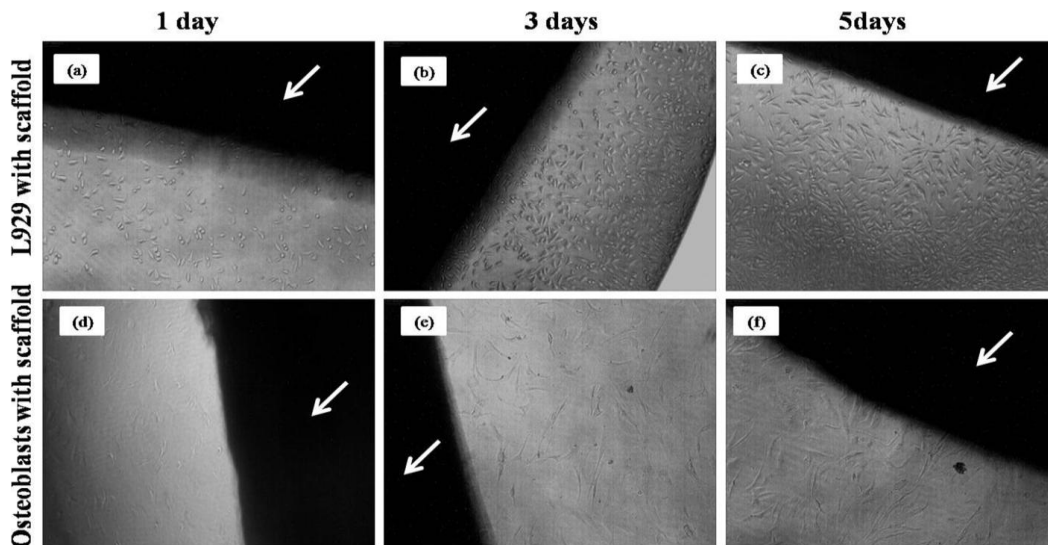


Fig.3 Morphology of osteoblast cells incubated with PCL/gelatin/collagen I composite scaffold at different magnification: arrows represent the scaffold in the wells of tissue culture plate

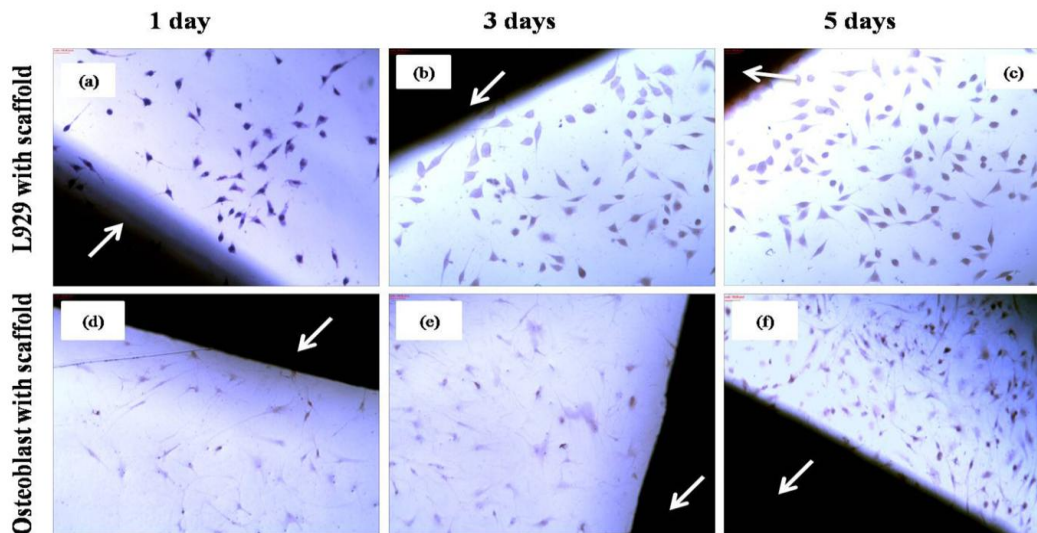
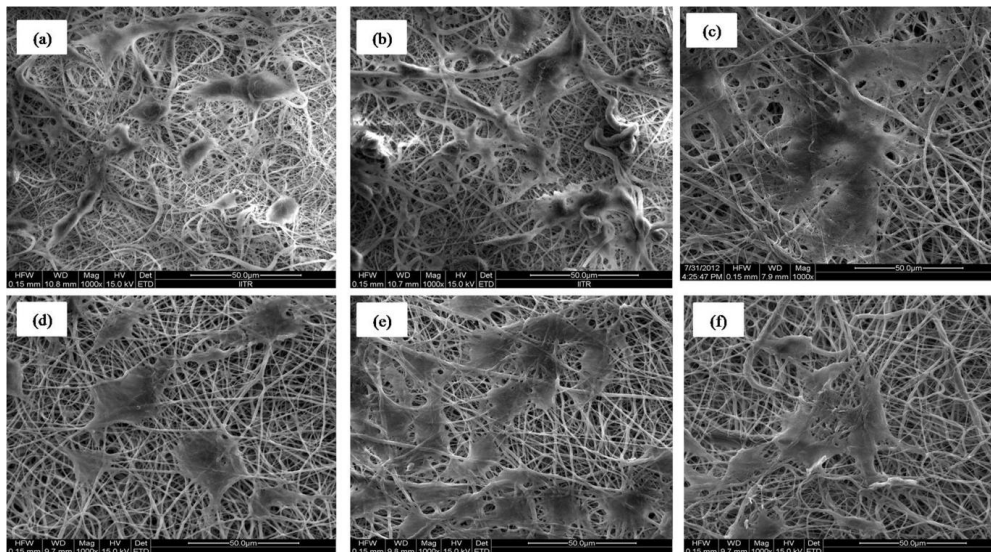


Fig.4 Adhesion of L929 and osteoblast cells on PCL/gelatin/collagen composite scaffold: (a), (b) and (c) represent L929 cell adhesion on composite scaffold after 1, 3 and 5 days respectively at 2000x magnification; (d), (e) and (f) represent L929 cell adhesion on composite scaffold after 1, 3 and 5 days respectively at 1000x magnification



Morphology of fibroblasts and osteoblasts cultured with modified composite scaffold

Giemsa staining of L929 mouse fibroblasts and human osteoblasts was shown in Fig. 3 cultured collagen I modified with

PCL/gelatin/chitosan composite nanofibrous scaffold to study their morphology after 1, 3 and 5 days. After 1 day of cell culture, mostly L929 fibroblasts were found to alter into spindle shape morphology whereas after 3 day of incubation period, mostly cells

converted into spindle shape morphology which is phenotypic characteristic of these cells (Fig. 3). After 5 days of cell culture also, maximum cells were observed in spindle shape morphology with increased density. This characteristic morphology of L929 fibroblasts also discussed in earlier studies (Theerakittayakorn and Bunprasert *et al.*, 2011). Additionally, the morphology of human osteoblasts was found to alter into polygonal shape after 1 day of cell seeding. After 3 days of incubation period, mostly cells converted into polygonal morphology and after 5 days, the cells were converted prominently into polygonal morphology (Fig. 3) which is a phenotypic characteristic of these cells. Besides this, the cells were also observed in high density with incubation period. This type of morphology of osteoblast cells was also reported previously on the titanium based scaffold (Yang *et al.*, 2002). Thus, Giemsa staining confirmed that both cells maintained their characteristic morphology in the presence of composite scaffold.

Adhesion of fibroblasts and osteoblasts on modified composite scaffold

FE-SEM images showed the adhesion of L929 mouse fibroblasts and human osteoblasts on the collagen I modified PCL/gelatin/chitosan composite nanofibrous scaffold (Fig. 4). After 1 day of cell culture, some L929 mouse fibroblasts were found in round morphology and few cells were observed in spindle shape. After 3 days of incubation period, they started spreading on the scaffold. Subsequently, after 5 day of cell culture, the cells were found merge with fiber morphology. Similarly, osteoblast cells were found adhere to the surface of modified composite scaffold as shown in Fig. 4. After 1 day of cell culture, the osteoblast cells started to convert into polygonal morphology over the modified scaffold. After 3 days, mostly cells converted into its characteristic

polygonal morphology and polygonal shape became more prominent. After 5 days of cell culture, the osteoblast cells were found merge with fiber morphology. These results showed that the surface of scaffolds were suitable for the cells and provided a more favorable environment to the cells for adhesion and proliferation because both cells are gaining their characteristic morphology on the scaffold even after 1 day of cell culture. Thus, FE-SEM results also confirmed the L929 fibroblasts and human osteoblasts viability and proliferation, and also supported the results of Phase contrast microscopy and Giemsa staining.

In conclusions a composite nanofibrous scaffold of PCL/gelatin/chitosan was successfully modified with collagen I to enhance the cell affinity of scaffold. L929 mouse fibroblasts and human osteoblast cells were found viable when incubated with the modified composite scaffold that confirmed the biocompatibility of this scaffold for both cells. Further, mouse fibroblasts and human osteoblasts both cells maintained their phenotypic morphological characteristic throughout the culture period. FE-SEM images showed the adhesion and also the characteristic morphology of these cells on the modified composite scaffold with incubation time that confirmed the viability and proliferation of both cells on the scaffold. Thus, the biocompatibility study and FE-SEM results of modified PCL/gelatin/chitosan composite scaffold indicated that the scaffold might be a good candidate for skin and bone tissue engineering applications.

Acknowledgment

The author is grateful to IIT Roorkee for work related to the fabrication of scaffold and Jaslok Hospital and Research Centre, Mumbai for carried out biocompatibility study.

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How to cite this article:

Sneh Gautam. 2020. Biocompatibility Study of Collagen I Modified PCL/Gelatin/Chitosan Composite Nanofibrous Scaffold for Fibroblast and Osteoblast Cells. *Int.J.Curr.Microbiol.App.Sci*. 9(10): 1017-1025. doi: <https://doi.org/10.20546/ijcmas.2020.910.122>