

# *In Vitro* Evaluation of Polyurethane-Chitosan Scaffolds for Tissue Engineering

Imelda Olivas-Armendariz<sup>1</sup>, Perla García-Casillas<sup>1</sup>, Adriana Martel Estrada<sup>1</sup>,  
Alberto Martínez-Villafañe<sup>2</sup>, Laura A. A. de la Rosa<sup>3</sup>, Carlos A. Martínez-Pérez<sup>1\*</sup>

<sup>1</sup>Institute of Engineering and Technology, Autonomous University of the City of Juárez, Ciudad Juárez, México; <sup>2</sup>Department of Physics of Materials, Research Center of Advanced Materials, Chihuahua, México; <sup>3</sup>Institute of Biomedical Science, Autonomous University of the City of Juárez, Ciudad Juárez, México.

Email: \*camartin@uacj.mx

Received July 17<sup>th</sup>, 2012; revised August 16<sup>th</sup>, 2012; accepted September 2<sup>nd</sup>, 2012

## ABSTRACT

In this work the use of Polyurethane (PU)-Chitosan(CH) scaffolds prepared by thermal induced phase separation (TIPS) for osteoblast proliferation and bone mineralization is described. Primary rat calvaria osteoblasts were seeded in the scaffolds and it was shown that supported cell adhesion and growth. The behavior osteoblast cells growing in the scaffold in function of the different ratio of PU and CH is presented. The results showed that TIPS is an appropriate technique for the production of PU-CH scaffolds with high potential for application as cell scaffolds in bone tissue engineering.

**Keywords:** Biomaterials; Composite

## 1. Introduction

Tissue engineering combines cell biology, materials chemistry, and processing to recreate viable tissue that restore and maintain the function of the body. The scaffold, a three dimensional porous material is created to support the cell source and growth factors to facilitate the differentiation of the cells and its proliferation. Much has been done, but the state of the art is far from perfect scaffolds production, and there is much more to do in order to realize the potential of tissue engineering. The requirements are complex and specific to the structure and function of the tissue of interest [1]. Therefore, the scaffold fabrication technique needs to be developed appropriately to manufacture the scaffold with the desired physical-chemical and mechanical properties. Also, factors such as porosity and pore size are very important for vascularization and the tissue in growth but they are difficult to control precisely during the process [2]. In the search to find the material that meet all this characteristics it is common to make blends of two or more polymers [3-6]. Chitosan, a natural polysaccharide, has generated an enormous interest due to its diverse advantages like its low cost, easy access (by deacetylation of chitin, for being the second most abundant natural polymer), positive charge biocompatibility, and antimicrobial activity [7-9]. However, the poor flexibility in the regula-

tion of mechanical properties and the limits of degradation has restricted its use. On the other hand, the PU has been utilized for the development of several kinds of materials and devices for biomedical applications, the preparation process and its velocity of degradation can be controlled and the physical and mechanical properties can be adjusted. In a previous work we present PU-CH blends bonded by chemical interaction with tunable properties [10]. Here in we present the evaluation for application for bone tissue engineering by means of seeding osteoblast rat calvaria cells into the scaffold; the results in cell proliferation and growth as well its mineralization suggest that these scaffolds are potentially appropriate for bone tissue engineering.

## 2. Materials and Methods

### 2.1. Scaffolds Preparation

The preparation of the Polyurethane prepolymer was prepared following a methodology previously described [2]. Briefly, Polycaprolactone diol (Mn 2000) and Polycaprolactone triol (Mn 900) were dissolved in 1.4 dioxane with *c*-hexane as a co-solvent. Once the reagents were dissolved; 1.6 diisocyanatehexane was added and 0.5% in weight of dibutyltin dilaurate was used as catalyst, the ratio PU/solvent was 20% w/v. Afterwards, chitosan of high molecular weight dissolved in a 0.5 M ace-

\*Corresponding author.

tic acid solution with a ration CH/solvent of 5% w/v was added and stirred to obtain different homogeneous mixtures of 90/10, 80/20, 70/30, 60/40, 50/50 (PU/Chitosan wt%). Once homogenized the solution was templated at  $-78^{\circ}\text{C}$ , then the solvent was extracted by a freeze drying system for 48 h afterwards the composite was cured at  $60^{\circ}\text{C}$  under reduced pressure for 48 h.

## 2.2. Osteoblast Cell Culture

The ability of scaffolds to support osteoblast cell adhesion and growth was evaluated for the different PU/CH composites, the scaffolds were sterilized by immersion in 70% ethanol for 5 minutes, followed by washing with sterile DI water and finally exposure under UV light in a laminar flow hood for 12 hours each side of the scaffold. After that, the scaffolds were incubated at  $37^{\circ}\text{C}$  in  $\alpha$ -MEM and 10% v/v FBS overnight in order to degas them. Primary rat calvaria osteoblasts were obtained by collagenase digestion of calvaria bone from 14 days old Wistar rats. In all the experiments, cells at first passage were used. The cells were seeded onto the PU, chitosan, and PU/chitosan scaffolds at a density of 50,000 cells per sample. Cells were cultured in  $\alpha$ -MEM supplemented with 10% FBS, 3% penicillin-streptomycin, 3 mM  $\beta$ -glycerophosphate and 10  $\mu\text{g/ml}$  ascorbic acid, and then placed in an incubator at  $37^{\circ}\text{C}$  with 5%  $\text{CO}_2$  and humidified air for a periods of 3, 7, 14, 21 and 28 days.

At the end of this period the scaffolds were gently rinsed with PBS and then the adherent cells were trypsin-detached and counted using a hemocytometer (blood counting chamber neubauer improved double ruled). The number of adherent cells was expressed as the percentage of those originally seeded on each respective sample surface. Cell proliferation was assessed using colorimetric indicator Alamar Blue assay (Alamar BioSciences, Monterrey, México). After the incubation time the samples were washed with PBS and placed into fresh, sterile 12-well culture plates. 2 ml of  $\alpha$ -MEM containing 1% v/v FBS and 10% v/v Alamar Blue indicator were added to each plate containing samples, and the plates were incubated for 4 h at  $37^{\circ}\text{C}$ . Absorbance of the extracted dye, which is proportional to the number of cells attached to the scaffold, was measured spectrophotometrically with a microplate reader (microplate spectrophotometer Bench mark plus, BIO-RAD) at wavelengths of 570 and 600 nm. In order to quantify the number of cells attached to the scaffolds, a calibration curve from a known number of osteoblast cells reacting with the Alamar Blue indicator was generated.

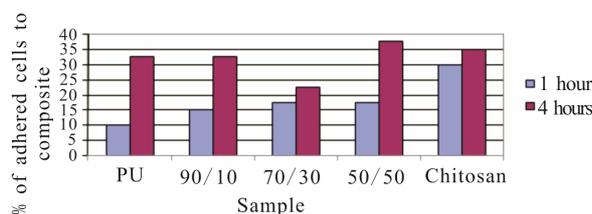
Also, alkaline phosphatase activity of the cells was measured as an early marker of the osteoblastic phenotype using an alkaline phosphatase substrate kit (PIERCE biotechnology, Monterrey, México). Cells were lysed

with 1% Triton X-100 in DEPC-treated water and three freeze-thaw cycles. The lysed cells were collected and stored at  $-70^{\circ}\text{C}$ . A volume of the sample was added to 100  $\mu\text{l}$  of the p-nitrophenyl phosphate solution and incubated at room temperature for 30 minute and then the reaction was stopped by the addition of 50  $\mu\text{l}$  of 2 N NaOH. The production of p-nitrofenol was determined by the absorbance at 405 nm. The results of alkaline phosphatase activity were normalized by the number of cells on the scaffolds. At designated time points, cell attachment and proliferation were visualized qualitatively using Scanning Electron Microscopy. The cells on samples were fixed at room temperature with 1% and 3% of gluteraldehyde for 1 and 24 h, respectively. The samples were then dehydrated sequentially using ethanol series (50%, 70%, 90%, 95%, and 100%) for 10 minutes each. Samples were dried overnight, and analyzed by SEM. In addition, energy-dispersive spectroscopy was performed to assess the elemental composition of the inorganic elements deposited on the scaffolds.

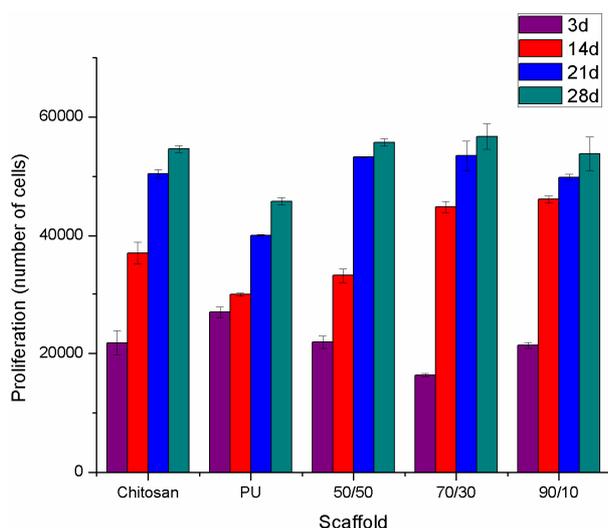
## 3. Results and Discussion

Polyurethane Chitosan composites had a high and interconnected porosity, a necessary requirement for tissue formation *in vitro* and *in vivo*. It is well known the processing parameters like template temperature and polymer concentration have an enormous influence in the morphological and properties of the materials; in this work the temperature was hold at  $-78^{\circ}\text{C}$ . Therein the chitosan scaffolds can have a bigger porosity than PU under the same processing conditions so it can be observed a different morphology and structure since the Chitosan and blends show greater porosity than pure polyurethane, therefore the union of the chitosan and polyurethane has an influence in the morphology of the composite. This can be tuned in a way that the scaffold maintains the form desired and the indispensable space for the formation of new tissue via proliferation and differentiation of cells or secretion of its extracellular matrix. There are compatibility and miscibility between both polymers according with our previous results and can be prepared according to the require conditions [10].

Rat calvaria osteoblast cells were cultured onto the scaffolds to evaluate the cell-surface interactions such as adhesion, proliferation, cell viability and spreading. The results of adhesion and proliferation are shown in **Figures 1** and **2**, respectively. The results of cell adhesion revealed that during the first hour the osteoblast attachment on chitosan is higher than that on PU and the composites; the osteoblast adherence was similar for PU and the composites (10% - 17% approximately), meanwhile for chitosan was around 30%. However, important differences were evident after 4 h. PU increases its adherence



**Figure 1.** Cell adhesion on PU, chitosan, and PU/chitosan composites.



**Figure 2.** Cell proliferation on PU, chitosan, and PU/chitosan composites.

significantly from 10% to 30%. On the other hand, 90/10 and 50/50 composites duplicate their adherence, meanwhile, opposed to the fast increase in cell number observed during the first hour, chitosan increases only from 30% to 35% and the composite 70/30 from 17% to 22%, respectively. These events are evidence that the rat calvaria cell adhesion was not only time dependent, but also a function of the material ratio in the composite. Chitosan content in the composite plays an essential role in osteoblast adhesion; since chitosan has a higher hydrophilicity than polyurethane that deals with a more favorable topography and chemistry or surface energy for pure osteoblast adhesion, chitosan has a higher adherence than the PU and composites. Osteoblast proliferation in the PU, chitosan and composites was evaluated by using the indicator Alamar Blue. The number of cells in PU, chitosan and composites increases over time in the evaluated period. The representative graph for cell proliferation shows similar cell growth trend during cultivation. It was seen that at early time point (day 3), the cell density on composites is lower than PU; the presence of chitosan on the composite surface retards the proliferation of rat calvaria osteoblast cells at early time point that it is in good agreement with the results obtained in a previous work by where they studied the responses of mesenchy-

mal stem cell on Chitosan-coraline composite [11]. After 14 days, the cell density on PU, chitosan, and composites was higher than those at 3 days and the cell number in the composites gradually became comparable to PU and chitosan. Nevertheless, the osteoblast proliferation on all polymeric composites showed a decrease after 14 days of culture compare to the cell seeded initially. An explanation for this behavior could be attributed to the low levels of initial attachment of cells at the surface. On the other hand, the cell density on the PU/chitosan composites was generally higher than PU and chitosan after 21 days; however, there is not a significant difference suggesting that the presence of chitosan in the composite does not have influence in the cell proliferation at the long term. In addition, the osteoblast proliferation was faster during day 3 and 14 of culture but slow down considerably thereafter. The proliferation behavior could be explained for the changes that occurred in the composition and organization of the extracellular matrix in order to be competent for the mineralization process [12]. Another possible explanation for this fact is that cells could reach confluence on the places where they were seeded, the rate of cellular death and proliferation is equal and hence the proliferation is stopped [13].

**Figures 3** and **4** show SEM images of PU, CH, and Composites seeded with osteoblast cells for 7, 14, and 21 days of culture. The appearance and size of the osteoblasts on the polymers is consistent with the morphology of osteoblast cells. After 7 days of culture (**Figures 3(a)-(c)**) cells were attached to the pores and the osteoblasts could be observed in clusters and randomly attached on the material surface. At day 14, more cells can be seen into the samples where it can be appreciated that the cells have proliferated at the adjacent areas through the pores. It can be seen several layers of cells, as well as extracellular matrix (ECM) secreted by the cells, like microvilli forming fibers between them. In **Figure 3(f)**, the morphology of individual osteoblast cell on the 70/30 composite after 14 days of culture can be appreciated where it presents the typical morphology that exhibit osteoblast cells and they are anchored to the surface by filopodia. In **Figure 4** it can be appreciated that after three weeks of culture the osteoblast cells covered the whole composite and filled the pores with uniform distribution.

The SEM images reveal the existence of calcium-phosphate mineralization with morphology in form of spherulites or nodules that it is a biomineralization phase and it was characterized by energy-dispersive spectroscopy (EDS). The EDS spectra from the composite-cell surface showed both calcium and phosphate signals from cells on the PU, chitosan and composites where it was shown higher mineralization on 90/10, 70/30, and 50/50 composites. These results suggest that the proliferation of

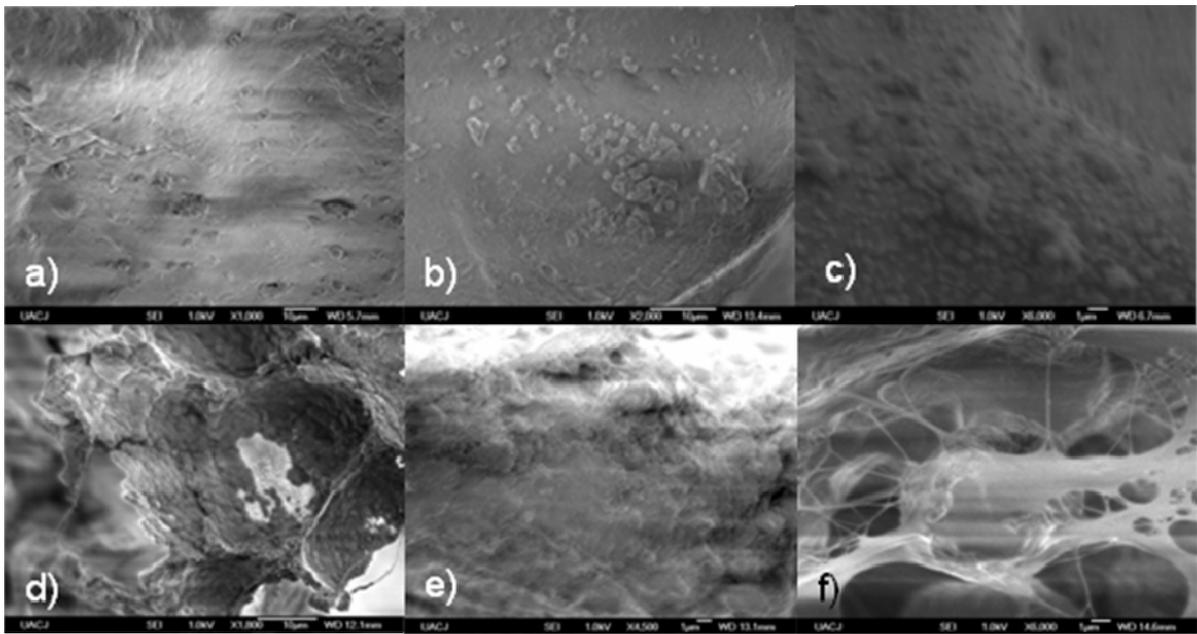


Figure 3. SEM micrographs of osteoblasts cultured on PU, chitosan, and composites. Osteoblasts cells on (a) 90/10 composite, (b) 70/30 composite and (c) Chitosan after 7 days of culture; osteoblasts cells on (d) 70/30 composite and (e) PU after 14 days of culture; (f) Individual osteoblast cell on 70/30 composite after 14 days of culture.

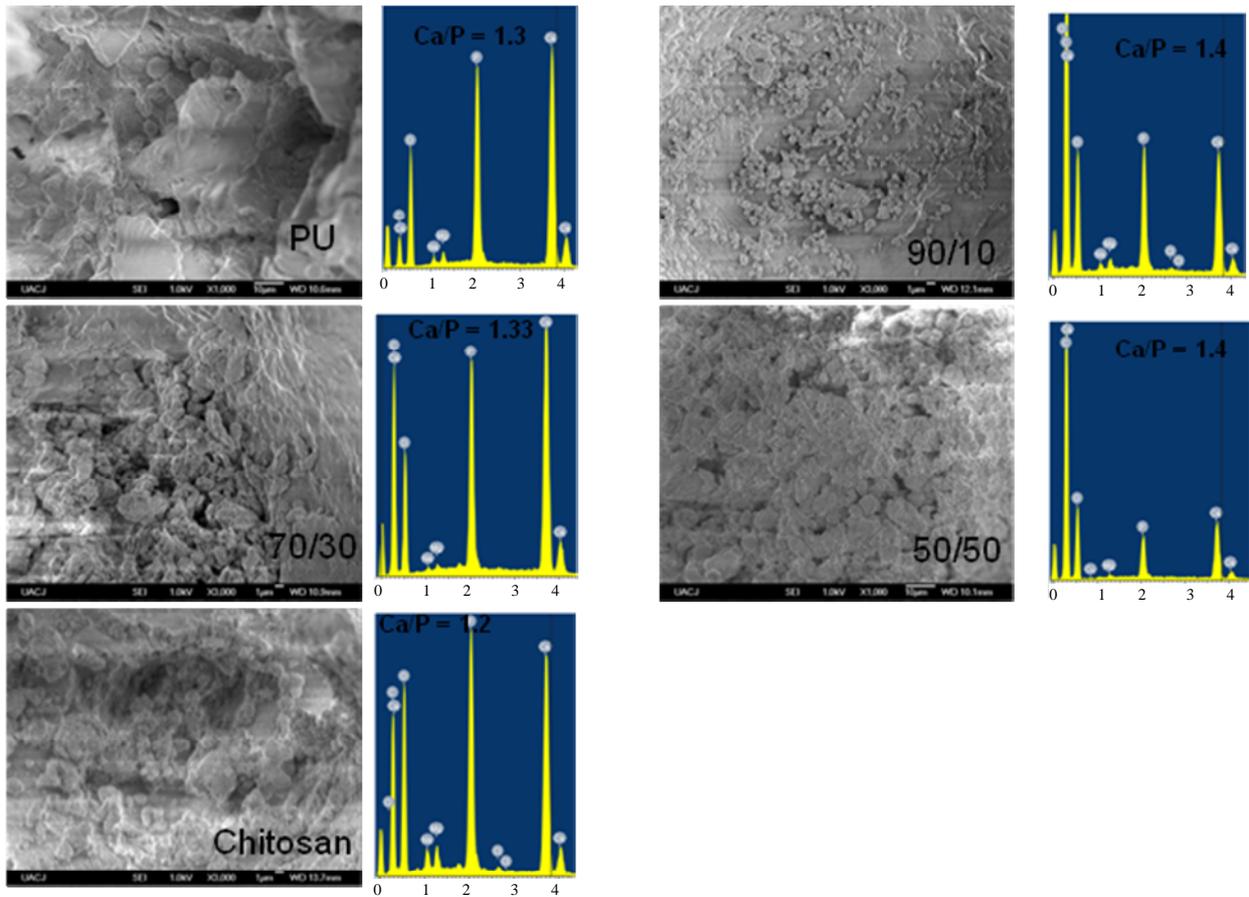


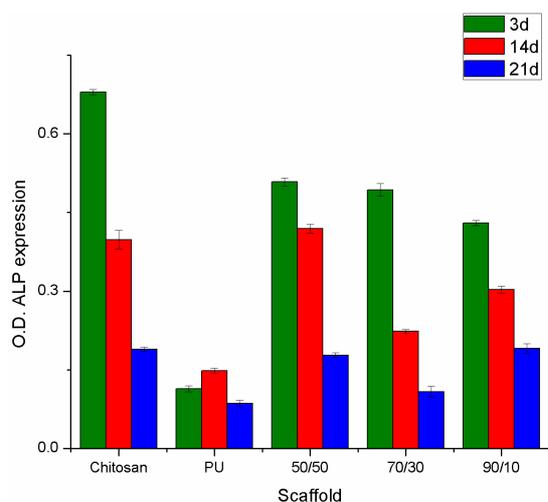
Figure 4. SEM micrographs of osteoblasts cultured on PU, chitosan and composites after 21 days of culture. EDS spectrum of PU, chitosan and composites after 21 days of culture.

rat calvaria osteoblast cells is directly related to the porosity of the material. It is generally accepted that the open and interconnectivity porosity improve cell seeding and proliferation in the scaffolds; moreover, it is crucial for nutrient and waste exchange [14]. On the other hand, the calcium content in the composites shows a similar trend of the results obtained in the cell proliferation. So, the obtained results in the mineralization and proliferation suggest that the composites are good candidates for applications in bone tissue regeneration.

Alkaline phosphatase (ALP) activity was measured as an indicator of osteoblast cell differentiation. **Figure 5** exhibits the general behavior of ALP activity for PU, chitosan, and composites during a 21-day culture period. All samples expressed alkaline phosphatase activity throughout the study period where the highest activity of ALP was presented at seven days of culture in all the samples. The decrease in ALP activity after the second week is typically attributed to its down-regulation in more mature osteoblast phenotypes that is in agreement with others works [15]. The alkaline phosphatase activity of cells increases as the chitosan content in the composite increase. These results suggest that the presence of chitosan improved ALP activity of individual cells, which is commonly used as a signal of normal osteoblast development and phenotypic expression that it is very important for bone mineralization. Through the alkaline phosphates, the osteoblast cells generate phosphates ions, which are secreted into the extracellular medium. These ions bond to calcium and generate nucleation sites for the production of calcium phosphates similar to the mineral phase of bone.

#### 4. Conclusion

The synthesis and processing of polyurethane and chito-



**Figure 5.** Alkaline phosphatase activity for osteoblasts cultured on PU, chitosan and PU/chitosan composites.

san composite as scaffolds shown to have physicochemical properties that are attractive for future application in bone tissue engineering. Future investigations with these materials will be made involving evaluation *in vivo*.

#### 5. Acknowledgements

The authors are very gratefully to National Science Council of México, CONACyT for their financial support through the project sep-conacyt 2007-84339.

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