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Material Properties of a Novel Bio Ceramic Scaffold for the Bone Construction and in Vitro Evaluated Tissue Engineering

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ABSTRACT

In this study, a novel three-dimensional porous scaffold was fabricated from nano particles ($CaCO_3$) with the micro-macro architecture for the purpose of bone repair, and their material properties were evaluated *in vitro*. Ideally, scaffold should have the following characteristics: biocompatible and biodegradable, suitable surface chemistry and highly porosity, with an interconnected pore net work. The method may not only accomplish the bone formation on the base of template (scaffold), but also optimize the mechanical properties of new formation. For the *in vitro* the cells were subculture for 5 weeks on the scaffold. The ability of cells to proliferate on this scaffold was assessed by a osteoblasts cells presented a significant increase in alkaline phosphatase activity and calcium deposits were observed at 21 days. Light and scanning electron microscopy revealed the presence of many osteoblast-like cells with development of calcification of the dense collagenous fibril network and bone matrix-like tissue were observed in many area of scaffold, resulting in the formation of bone-like tissue containing osteocyte-like cells. The scaffold properties was characterised by x-ray diffraction (XRD), Fourier-Transform infrared spectroscopy (FT-IR), Scanning Electron Microscopy (SEM), and Compression mechanical tests.

الخواص الجوهرية للسقالةِ الخزفيةِ الحيويةِ المبتكرةِ بهندسة النسيجِ للبناءِ العظمي والمُقَيَّمةِ خارج الجسمِ

الخلاصة

تضمنت الدراسة أبتكار سقالة مثقبة ثلاثية الأبعاد تم انشائها من جزيئات نانوية لمادة كاربونات الصوديوم بشكل هندسي معماري دقيق وكبير لغرض بناء العظام وأعادتها الى الحالة الطبيعية الأولى, وقد تم تقيم هذه التركيبة خارج الجسم. وتمتاز السقالة المثالية المبتكرة والمستعملة في هذه الدراسة بعدة خصائص منها أن تكون متوافقة وقابلة للتفسخ بايولوجيا وذات اسطح مناسبة كيميائيا كما وتضم عدد كبير من المسامات المترابطة والمتداخلة مع بعضها بصورة جيدة وعلى شكل شبكة, كما أن الصفات الميكانيكية لها يجب ان تكون بأحسن المميزات في البناء العظمي للسقالة المبتكرة.

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أن تقيم السقالة المبتكرة خارج الجسم قد تم بأستعمال خلايا بانيات العظم المعزولة من نخاع العظم وعلى مدى 5 اسابيع من زرعها على السقالة فقد لوحظ هناك زيادة كبير في انتشار الخلايا بالأضافة الى الزيادة الهامة في نشاط مادة الفوسفات القاعدية وزيادة ترسيب مادة الكالسيوم خلال 21 يوم من التجربة. أظهر المجهر الضوئي والماسح الألكتروني وجود العديد من خلايا بانيات العظم مع تطور وزيادة المادة الكلسية ووجود كثافة لألياف الكولاجين والمادة العظمية في مناطق عديدة من السقالة والذي ادى الى تشكيل نسيج شبه عظمي يحتوي على خلايا شبه عظمية. لقد تم تميز خواص السقالة المبتكرة بأستعمال الأشعة السينية المنحرفة, المجهر الماسح الألكتروني, أختبارات الضغط الميكانيكية وتحويل الفورييز السبكتروسكوبي تحت الحمراء.

INTRODUCTION

Tissue engineering is an interdisciplinary field that relies on cooperation between physicians, scientists and engineers. It can be defined as the application of scientific principles to the design and construction of living tissue. Bone is a dynamic organ, continuously undergoing remodeling to maintain tissue homeostasis and repair microcracks. Yet, for defects caused by severe trauma, congenital malformations, tumors, infections and nonunion fractures, repairs by bone remodeling are limited and thus surgical interventions are required (1). An ideal scaffold must be biocompatible, possess sufficient transient mechanical properties, highly porous with interconnected pore net work, and surface chemistry that promotes an appropriate regulation of cell behavior such as cell adhesion, proliferation, migration and differentiation. It is favorable that degradation and resorption kinetics match tissue formation *in vitro* / *in vivo* to maintain its structural integrity (2, 3). The preliminary study by Zuki *et al.* (2004) revealed that the mineral composition of cockle shell (Anadara granosa) is almost $CaCO_3$ similar to coral composition.

In vitro cell cultures have the advantage of studying the scaffold under specific conditions, which can be easily reproduced and compared, as opposed to the highly complex environment present in living organisms. Cell cultures (*in vitro*) in two dimensions, plane or flat geometry (PG), are often used to evaluate the cytotoxicity material or its influence on the proliferation and differentiation. In PG configurations, however, it has been shown that direct cell behavior is totally different from the ones displayed in three-dimensional solid geometry (SG) (5). Although, the PG cell cultures proved a first useful step for assessing the suitability of materials for tissue engineering, the SG configuration is necessary in order to observe the behavior of cells under practical conditions.

Therefore, the aims of this study were:

i. to evaluate the morphological and biomechanical properties of newly developed porous 3D-scaffolds
ii. to evaluate the porous 3D-scaffolds seeded with osteoblast cells *in vitro*

MATERIALS AND METHODS

Materials and inspections

Three different materials namely the dextrin, dextran and gelatine were blend with the nanoparticle bio mineral powder of cockle shell ($CaCO_3$). The gelatine was added to the mixture and followed by the heating. The macro porosity was achieved by the macrospores created in the design. The micro porosity was created by gas bubbles CO_2 that released between the individual granules of powder. The compression test of the scaffold was done under the dry condition using universal mechanical testing machine (Instron 4302). The scaffold was also subjected to a 10-day water-soaking test, and examined under high vacuum conditions at 15 KV SEM. FT-IR spectra of calcium carbonate bioceramics scaffold was used to analyses a different materials wave numbers indicated for $CaCO_3$, gelatin, dextran and dextrin, XRD analysis was done to characterize the crystalline/ amorphous nature of the $CaCO_3$ and identify any crystalline phases present.

Isolation and Proliferation of Osteoblast Cells

Isolation and proliferation of osteoblast cells from rat bone marrow (RBM) was conducted using the following method. Sprague-Dawley (SD) rats at 6 weeks of age, with an average body weight of100-200 gram were euthanized using Dolethal, 1ml/kg and the long bones (femur, tibia and humerus) were removed. The diaphyses of the bone were flushed with 10 ml of complete medium after the epiphyses were cut off. The mixture (bone marrow + medium) was resuspended and then 5 ml was transferred into a 75 cm^2 culture flask filled previously with 10 ml of complete medium. The culture medium used was DMEM (Dulbecco's modified Eagles medium), supplemented by 10% fetal bovine serum (FBS) and 10U/ml penicillin G, 10 µg/ml streptomycin and 25mg/ml anphotericin B. After 48 hours, the medium was replaced. The medium was changed every 3 days until the cells completely confluenced in the flask. The cells achieved confluence after 10 to 20 days. The MSCs differentiation to the osteoblast cells was achieved by using the differentiation medium. The differentiation medium was used in this experiment (DMEM high glucose). This was supplemented with 10% fetal bovine serum (DNA Company), 50 mg/ mL gentamicin (DNA Company), 0.3 mg/mL anphotericin B (Gibco), 10^{-7} M dexamethasone (Merck), 5 mg/l ascorbic acid (Merck) and 7 mM β-glycerophosphate (Sigma). The cells were cultured in a humidified atmosphere of 5 % CO_2 at 37°C.

Cells Seeded onto the Scaffolds

Osteoblast cells were detached using trypsin / EDTA (0.25% w/v trypsin / 0.02% EDTA, Sigma). Sample of the heated scaffold (1cm diameter x 1cm length) were prepared and sterilized. They were then inserted into a 25 cm^2 flask after pre-wetting with culture medium. Osteoblast cells were seeded with at a cell density of 1 x 10^8 cells/mL (1 x 10^7/mL for each scaffold was seeded). The scaffolds were placed in an incubator with 5% CO_2, at 37 ˚C, and 90% humidity. After 3 hours of attachment, 10 ml of complete medium was added to flask. Seeded scaffolds were incubated for further attachment overnight. The whole volume of medium was changed every 3 days.

IN VITRO EVALUATIONS

Biochemical Analysis (ALP and Calcium)

The alkaline phosphatise activity (ALP) of seeded scaffold and non-seeded scaffold (controls) was assayed as a measure of the osteoblastic expression at 5, 7, 14, 21 and 25 days post-seeding to evaluate the capability of the biosynthetic scaffolds to generate bone *in vitro*. The alkaline phosphatase activity was measured using a 902 Hitachi automated clinical chemistry analyzer. The calcium (Ca^{2+}) content of each scaffold was assayed in order to quantify the amount of mineralized matrix present. The calcium (Ca^{2+}) was measured using a 902 Hitachi automated clinical chemistry analyzer.

Scanning Electron Microscopy (SEM)

For SEM analysis, sample of scaffold was cut into blocks of ½ cm diameter x ½ cm length. The sample was then fixed in a solution of 4% glutaraldehyde buffer solution at pH 7.3 and 4°C. The coated specimens were observed and analysed using SEM (Tescan VEGA).

Histological Examination

For light microscopy, sample was taken from scaffold and fixed in 10% formaldehyde in phosphate-buffered saline (PBS) at pH 7.4 for 24 hours at room temperature. In the standard of histological techniques, immediately after embedding, the sample was blocked with paraffin wax and then sectioned at 5 µm thick. Finally serial sections were stained with:

- Routine Hematoxylin and Eosin for general histology
- Massons trichrome for demonstration of the collagen fiber and new bone tissue.

The slides were examined under a light microscope.

RESULT and DISCUSSION

SEM analysis revealed that the scaffold contained of macro-micropores with different sizes. These pores were between 30-380 µm (Figure 1) and showed a uniform interior. The intra-architectural scaffold geometry has a major impact on new bone tissue formation, the physical interpretation of microporosity formed in the scaffold depend on the voids that containing the trapped air can make way to provide space for the swelling effect when the scaffold get wet, and these voids formed between the granules or particles in the all mass material (6). In this study the chemical cross linked for the scaffolds that produced by the heating method, will lead to decrease in the degradation rate. This was demonstrated by the water absorption test that revealed the scaffold lasted for 10 days without much visible surface degradation and water absorption. The scaffold obtained by this method, provide the best compression properties. The scaffold was observed to be uniformly sturdy and strong throughout the test. The degradation rates should be adjustable to match the rate of tissue regeneration (7). The strong and clear peaks of a typical XRD reveal for good crystallinity of obtained product. No characteristic peaks for other impurities could be observed only the major $CaCO_3$ reflection peak (8) Figure (2). In addition to, the FT-IR of the scaffold revealed no changes could be observed in the spectra of bioceramic harvested at the different steps in the process simulation Figure (3).

Notwithstanding, the *in vitro* histological examination was considered as one of the most important examinations. The new products of scaffold constructs, which had an initial cell seeding density of 1×10^8 cells/cm^3 were sectioned, stained with haematoxylin and eosin (H&E), and counter stained with Massons trichrome for stem cells. The analysis showed the presence of cells and distributed throughout the interior of the 3D- scaffold and also the formation of a thick surface layer of cells Figure (4). Thus, the histological study in this trial was assessed by evaluating qualitatively of the new scaffolds by the interaction between the osteoblasts cells and the scaffold product material. The differences observed in cells proliferation and continuous to 60 and 90 days after seeding culture, which indicated that multilayer of the cells were observed already accumulated earlier in the outer edges rather than in the middle area of the scaffolds Figure (5). These observations were good in according to Ross and Pawlina (2006) finding. Thus, cell proliferation occurred at different rates throughout the scaffolds. This is likely due to an uneven distribution of cells within the scaffolds during the initial cell-seeding procedure. Initially, more cells anchored to the outer edges than in the center of the scaffolds. The cells were mainly on the surface of the scaffold and few cells inside the scaffold material.

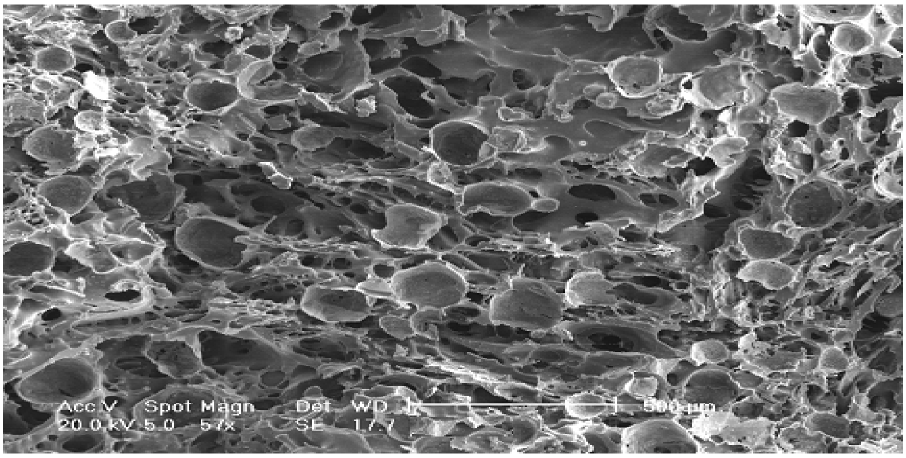


Figure (1) SEM microphotograph of scaffold uninfiltrated show the macro and micro porosity (the magnification bars correspond to 1 mm).

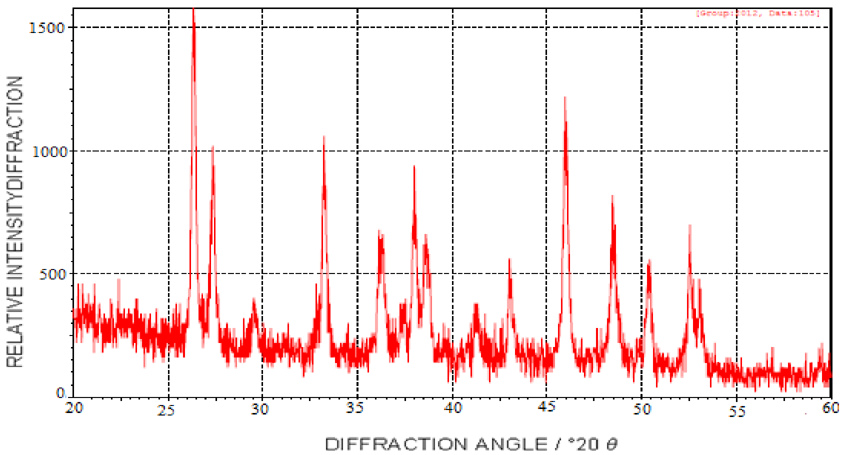


Figure (2) the graphs show the XRD pattern of the scaffold products. The peak at 26$^\circ\theta$ and 28$^\circ\theta$ are characteristic of calcium like graphite, and other peaks are characterized of Calcium. This corresponds well to the specification (Graphite, Ca).

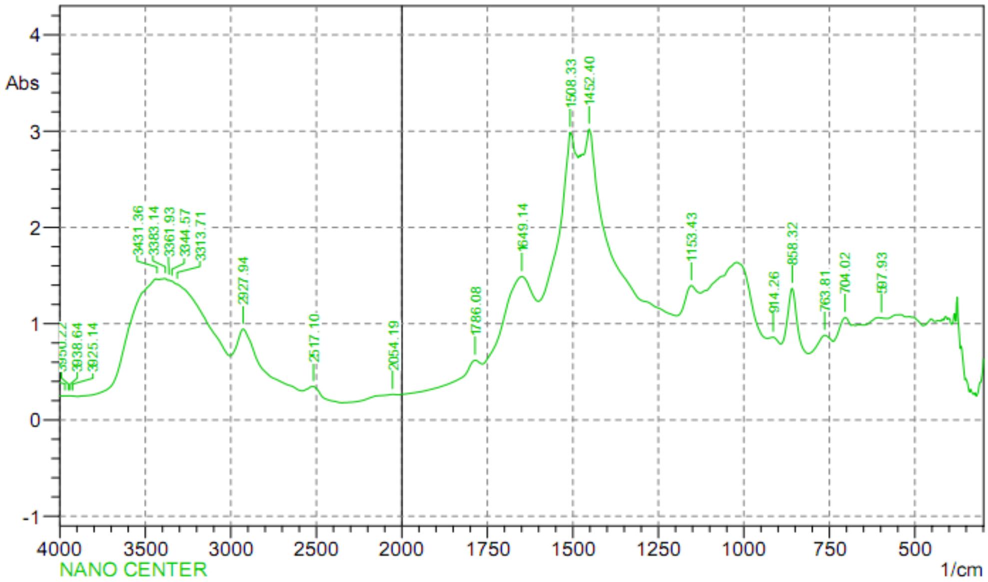


Figure (3) The graphs of FT-IR spectra of calcium carbonate bioceramics; a different materials wave numbers indicated for $CaCO_3$, gelatin, dextran and dextrin.

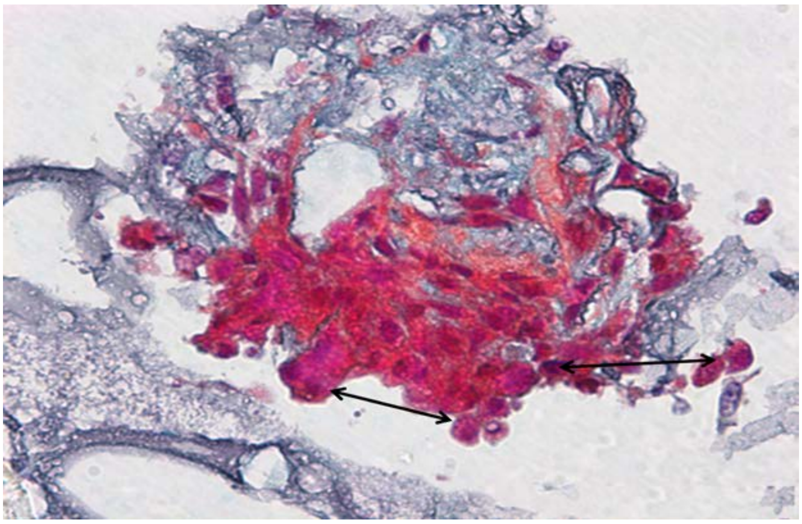
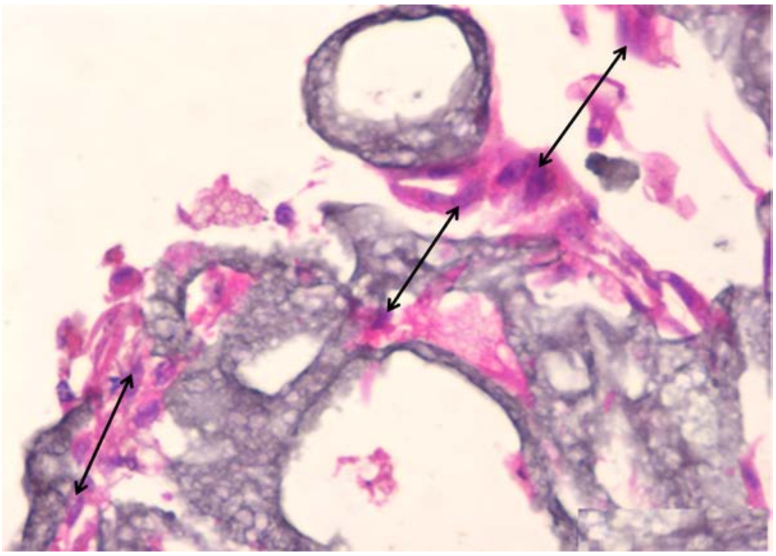


Figure (4) Microphotographs of the scaffold at 90 days post-seeded stain with Masson's trichrome show the osteoblasts cells (black double arrows) on the surface of the scaffold material more than the bottom (x 400).



Figures (5) Microphotographs of the scaffold at 60 days post-seeded stain with H&E show the stem cells (double black arrows) on the surface of the scaffold material more than the bottom (x 400).

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