Original Research Article **Nanotechnology**

International Journal of Pharma and Bio Sciences

ISSN 0975-6299

CYTOTOXICITY, GENOTOXICITY AND CELL ADHESION STUDY OF ELECTROSPUN SILK/SURFACE MODIFIED NANOSILICA COMPOSITE FOR BONE REGENERATION APPLICATIONS

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ABSTRACT

Natural osseous tissues comprises of a nanocomposite structure which imparts structural and biological properties to it. Hence for successful and efficient regeneration of these tissues, the biomaterial used should essentially mimic the living bone tissue. Since such a biomaterial is currently unavailable, nanocomposites are promising candidates as they can provide the appropriate matrix environment as well as integrate desirable biological properties. The current paper explains an organic-inorganic nanocomposite scaffold that can be utilized for bone tissue engineering which constitutes nanosilica extracted from an agricultural waste such as rice husk and silk extracted from *Bombyx Mori* silkworm. One major advantage is that the material is cost-effective since the source for synthesis is natural. The scaffold is fabricated using electrospinning technique and characterized using XRD, FTIR, FESEM and TG/DTA. The *in vitro* study shows that this scaffold is biocompatible with L929 cells and also promotes cell adhesion with lower genotoxic activity.

KEYWORDS: ***Osseous tissue; Nanocomposite; Silica; Tissue Engineering; Electrospinning; genotoxicity***

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Received on: 23-03-2017
Revised and Accepted on : 22-07-2017
DOI: http://dx.doi.org/10.22376/ijpbs.2017.8.3.p366-372

INTRODUCTION

Tissue engineering is used as a technology to abolish the boundaries of tissue grafts. This area includes a wide strategy by employing *in vivo* method of using cells, scaffolds, cytokines and genetical manipulation for the regeneration of tissue *in vitro*[1]. In bone tissue regeneration, scaffolds are biodegradable and act as a temporary matrix for cellular proliferation and for extracellular matrix deposition. Scaffolds are the extracellular matrices that allows adhesion and proliferation of cells and provides mechanical support until a new tissue is formed[2]. One major approach to design a scaffold for tissue engineering is to mimic the functions of the extracellular matrix components that are naturally found in tissues. None of the biomaterials individually achieve all the targeted properties for a scaffold. Therefore, research is being focused on combining two or more of these biomaterials to improve their properties. This composite or hybrid approach opened up wide possibilities for a more efficient and faster regeneration process [3]. The composite involves a matrix phase and a filler phase which acts as the reinforcement. Common combinations include metal-ceramics, metal-polymers, ceramic-polymers, polymer-polymers and ceramic-ceramics [4]. Metal based composites are not used much now due to their non-biodegradability. Hybrid biomaterials have the advantage of combining properties to provide biological functions and control over their compositional, structural and mechanical features[5]. The crosslinking of tissue-derived scaffolds impacts the structural features and consequently the mechanical and biological responses of the extra cellular matrix (ECM) material. A very promising candidate for bone repair is the silk-silica composite. It effectively combines the mechanical properties of silk and bioactivity of silica. The use of nanosilica can facilitate the biodegradability of silica via particle dissolution[6]. This enables the fabrication of scaffolds with precisely controlled remodeling rates. In the present study, the goal was to economically synthesize nano silica from an agricultural waste such as rice husk and to combine the useful biomaterial properties of silk and silica [7] for composite organic/inorganic biomaterials for bone tissue applications and to study the biocompatibility nature of the scaffold.

MATERIAL AND METHODS

The precursor used for the synthesis of surface modified nano silica is rice husk, which is an agricultural waste that is abundantly available in India. It was collected from rice mill at Nagercoil, Tamil Nadu, India, during the milling season. The Coccoons from Bombyx. Mori silkworm were purchased from Sericulture lab near Hosur, Bangalore, India. All chemicals which were required for the extraction and synthesis of nanosilica and silk fibroin solution was purchased from Sigma Aldrich chemicals private limited, Bangalore.

Extraction of Nanosilica from Rice Husk

Nanosilica was extracted from agricultural waste, Rice Husk, using Sol-gel technique. Briefly, Rice Husk was treated with HCl and EDTA disodium salt for purification and to remove metallic impurities. It was then washed several times with distilled water to remove the traces of the salt followed by the burning of Rice Husk at 650-670 ºC to get Rice Husk Ash. This ash is then treated with NaOH solution for several hours and filtered to get sodium silicate solution which is then treated with HCl solution to form silica gel. The silica gel is then dried at 110 ºC to form nanosilica [8].

Extraction of silk fibroin solution from Bombyx Mori. Silkworm

Silk Fibroin solution was extracted from *Bombyx Mori*. silkworm using Kaplan's Method. Briefly, the silk from *Bombyx Mori* was immersed in sodium hydroxide solution to dissolve the sericin base. The fibers were then washed several times with distilled water. After air-drying overnight, the fibers were dissolved in Lithium Bromide solution to obtain honey like viscous 8% silk fibroin solution [9]. This was then centrifuged to remove the traces of clogged impurities.

Synthesis of Silk/Surface modified silica and Electrospinning of the silk/surface modified silica composite

10 mL of the purified silk solution (8%) was stirred alongwith 1 mL of Aminopropyl tri ethoxy silane(APTES) and 1g of silica. It was stirred continuously for overnight. To this solution, 20 mL of PVA solution was added and continuously stirred for 4-5 hrs to obtain a homogeneous solution of the compound [10]. For electrospinning of silk/surface modified nanosilica fibers, 15 kV voltage was given initially thereby increasing to 20 kV which was used as a source of the electric field. The hybrid solution was contained in a plastic tube connected with a capillary tip. The copper wire connected to a positive electrode (anode) was inserted at the capillary tip and a negative electrode (cathode) was attached to a metallic drum collector [11]. The solution volume was controlled at 0.5 mL/hr to keep proper flow rate for spinning and the distance from the tip to the collector is maintained to be 12 cm. The electrospun scaffold was then characterized using XRD, FTIR, FESEM, TG/DTA. The compatibility of the biomaterial scaffold were further used to determine the cell adhesive property, genotoxicity as well as to determine its cell adhesion activity.

RESULTS AND DISCUSSION

XRD of silk/surface modified silica nanocomposite

The crystallisation phase of the hybrid scaffold is shown in Figure 1. From the figure, 2θ values are obtained at 22.6 º, 28.7 º and 40.6º.

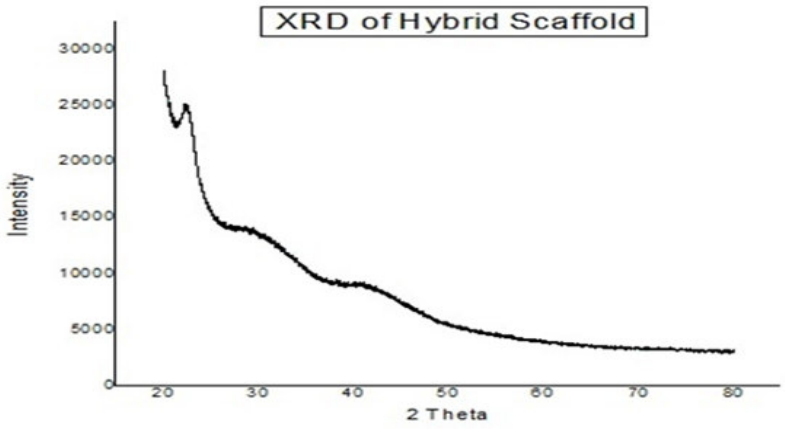

Figure 1
XRD of silk/surface modified silica nanocomposite

The peak at 22.6 º corresponds to the silica group which is correlated with the result obtained by the XRD graph of silica obtained in previous reports [10]. Peak at 28.7 º (d spacing ~ 38Å) corresponds to the silk II structure. Peak at 40.6 º corresponds to the winding of secondary silk structure by APTES. The graph thus obtained can be confirmed that the material used is APTES modified silk/silica nanocomposite.

FTIR of Silk/surface modified silica nanocomposite

Figure 2 shows the FTIR of APTES modified silk/silica nanocomposite. The peak near 3000-3500 cm^{-1} attributes to the -OH stretching due to the attached PVA molecules.

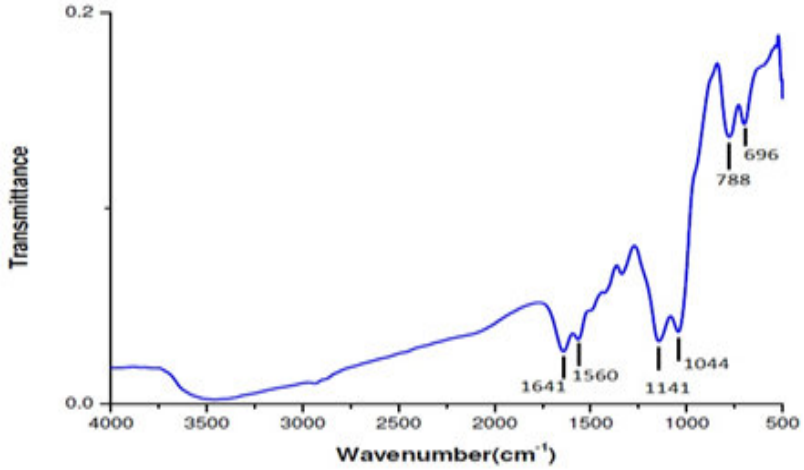

Figure 2
FTIR of silk/surface modified silica nanocomposite

The absorption band representing the Amide I is related to the C-O stretching and it occurs in the range of 1700–1600 cm^{-1}. Amide II, which falls in 1540–1520 cm^{-1} range, is related with the N-H bending and C-H stretching vibration. Amide III occurs in the range of 1220–1300 cm^{-1}, and it results from in phase combination of C-N stretching and C-O bending vibration. Similar structural vibrations were noted by previous reports[12]. This signifies that Amide (-CN) groups can be seen near the 1600-1500 cm^{-1} which explains the presence of silk compounds in the composite. Presence of silica can be detected in 1044, 788 and 696 cm^{-1} which is correlated with the FTIR graph of nanosilica which is significant to previous reports[13].

TG/DTA of surface modified silk/silica nanocomposite

TG/DTA of APTES modified silk/silica nanocomposite which was done in oxygen atmosphere is shown in Figure 3. The onset initial decomposition temperature is 100º and maximum rate of decomposition temperature is set to 850º. The d half of the material decomposition is at 450 °C where the half of the material was decomposed.

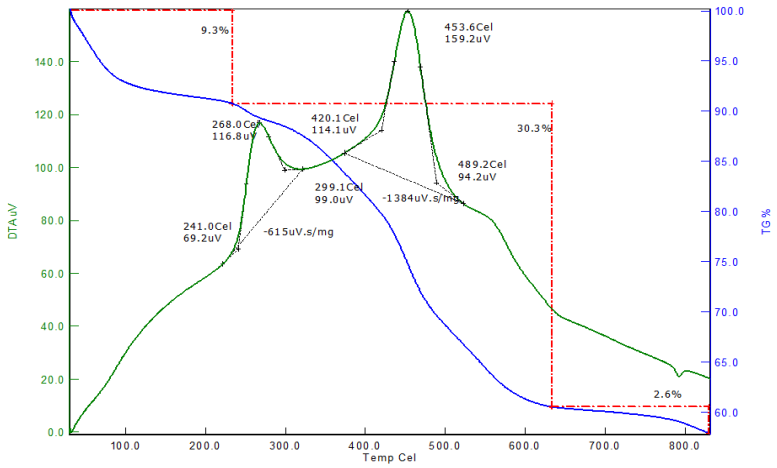

Figure 3
TG/DTA of silk/surface modified silica nanocomposite

Interestingly, the adjacent fibers in silk/APTES modified silica hybrid electrospun nanofibers caused to 'weld' at fiber contact points, as confirmed by SEM images (Figure. 4). Additionally, the fiber diameters showed almost the same size of 2,290 nm (SD = 470 nm). The observed 'weld' at contact points is due to the equilibrium water content, as was verified by thermogravimetric analysis (TGA)(Blue line) analysis shown in the above figure. Moreover, as the silica concentration increased to 10%, the fibers became belts and the juncture extended like a sheet which could not be identified as previously reported nanofiber mats. So in this study, the hybrid nanofibers with the silica concentration of 5%. The Weight loss located between 0 and 200 °C (of about 9.3%) is due to the elimination of water content. The second loss occurring between 250 and 630 °C (of about 30.3%) is due to the decomposition reaction of the silk fibroin content. The third weight loss occurring between 630 and 850 °C (of about 2.6%) is due to the decomposition of other organic compounds present in the rice husk. There are two exothermic peaks in DTA(Green line) curve at 263 °C, 458 °C and one endothermic peak at 800 °C. The area under the two exothermic peaks will give the change in enthalpy values which are -615 µV/mg and -1384 µV/mg. Similar reviews were found in previous reports [10].

FESEM of Silk/surface modified silica nanocomposite

Figure-. 4 shows the FESEM image of APTES modified silk/silica nanocomposite at various magnification. For lower magnification size, dense networks can be seen which decreases with an increase in the magnification. Presence of large consistent pores is apparent from the FESEM image of electrospun membrane and this result in the high pore volume which is needed for a good membrane.

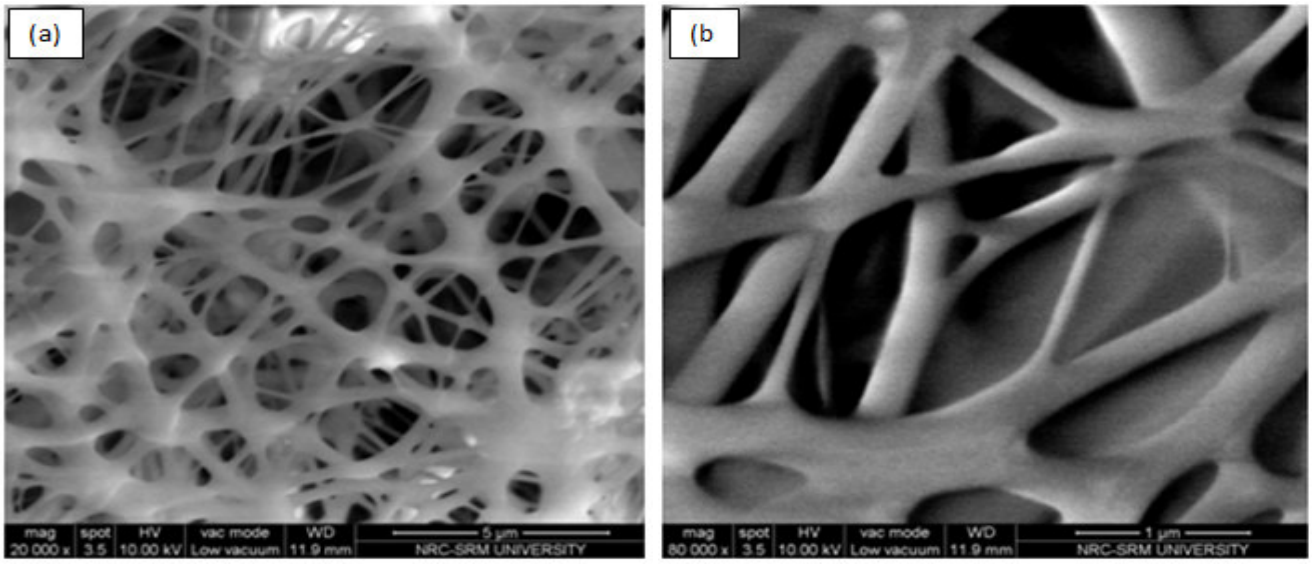

Figure 4
FESEM of silk/surface modified silica nanocomposite under a) 5 µM and magnification and (b) 1 µM magnification

In the process of electrospinning, the addition of SiO_2 particles has significant influence on the fibers morphological characteristics such as fiber diameter, surface roughness and fiber orientation. Hou Et.al [10] reported similar observations for the morphological characterization of silk/silica nanocomposite

In- vitro Study

Cytotoxicity Analysis

Figure 5 shows the cytotoxicity analysis of surface modified nanosilica incorporated silk nanocomposite against L929 cells. From the figure, it is observed that within 24 hrs, the percentage of viability is higher in lower concentrations.

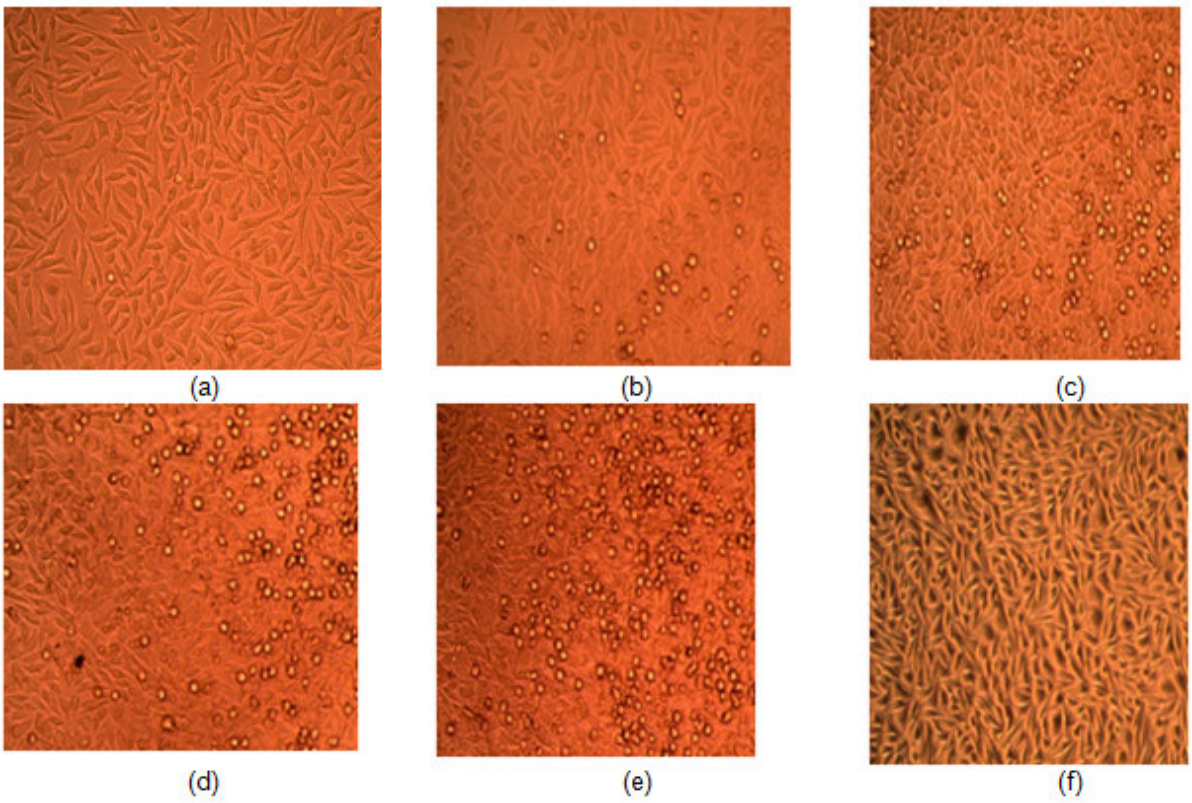

Figure 5
Cytotoxicity analysis for various concentrations of silk/surface modified silica nanocomposite solution: (a) 6.25µl, (b) 12.5 µl,(c) 25 µl,(d) 50 µl, (e) 100 µl and (f) control

The optical density and percentage viability of the fibroblastic L929 cells after incubation with the hybrid concentrations at different concentrations are presented in Table 1. The data showed that the hybrid scaffold were non-toxic to normal cells.

Table 1
Percentage Viabilty for cytotoxicity analysis of silk/surface modified silica nanocomposite

Samples	Average OD at 540nm	Percentage Viability
Control	0.8231	
6.25	0.8058	97.89819
12.5	0.7789	94.63006
25	0.7692	93.45159
50	0.7301	88.70125
100	0.6843	83.13692

At concentrations of 6.25, 12.5 and 25 µg/ mL of the scaffold solution, the percentage viability of cells was 97.89 %, 94.63 %, and 93.45 % respectively whereas for a concentration of 50 and 100 µg/ mL, the percentage of viability is 88.70 % and 83.13 %. In the above case, the percentage viability of the normal cells was greater than 50%, which means that this scaffold is non-toxic to normal L929 fibroblastic cells which is in agreement with the literatures obtained previously[14]. Therefore, surface modified silica incorporated silk can be safely used for bone tissue regenerations.

Genotoxicity Analysis

The Comet assay or single cell electrophoresis is a sensitive and rapid technique for quantifying and analyzing DNA damage in individual cells for the evaluation of genotoxicity. This assay can be used to detect DNA damage caused by double strand breaks, single strand breaks, alkali labile sites, oxidative base damage, and DNA cross-linking with DNA or protein. The Comet assay gain special significance in the field of biomaterials research due to the long latent period between exposure to genotoxic agent and genetic effect becoming apparent.The determination of DNA damage of cells grown on medium extracted with hybrid biomaterial gives a clear idea about the genotoxic potential of the biomaterial scaffolds. The comets resulting from exposure to the extracts of the hybrid scaffold did not differ from that of the control. With control and test scaffold the relatively undamaged cells give comets consisting of a compact head without any prominent tail, indicating double-stranded intact DNA (Figure-. 6(b)).

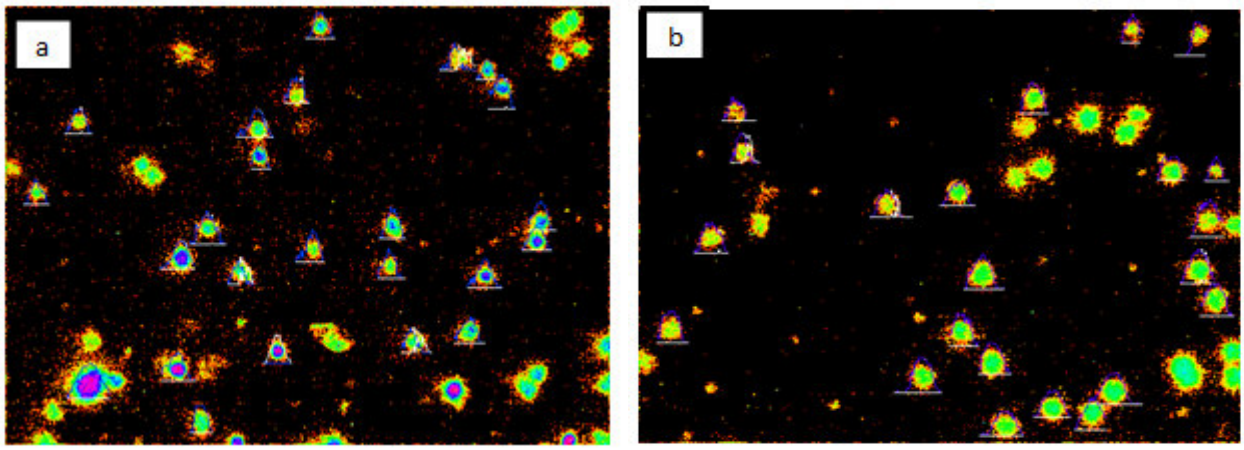

Figure 6
Genotoxicity analysis of control (a) and silk/surface modified silica nanocomposite scaffold(b)

The mean comet lengths for the L929 exposed to the hybrid scaffold as compared with control are presented in Table 2. For the hybrid scaffold, DNA in the head region is more than 99% and in the tail region, it is less than 1%. The diameter of the head region is also maximum.

Table 2
Determination of genotoxic effects of silk/surface modified silica nanocomposite

Samples	Head diameter(px)	% DNA in head	% DNA in tail
Control	21	99.34 ± 0.01	0.84 ± 0.02
silk/surface modified nanosilica composite	27	99.87 ± 0.33	0.16 ± 0.21

In the present investigation, the time (24 h) elapsed between exposures and measuring of the DNA damage is adequate for the repair of the possible lesions and enables to detect whether or not the chemical is able to produce the DNA damage. As per the assay the hybrid scaffold (degrades faster rate in DMEM) did not evoke a significant effect on DNA migration on the electric field at the applied concentrations and found to be non-genotoxic. From the assay it is very clear that the neither the scaffold nor the degradation products or the particles leaching from them affect the genomic integrity of the genetic materials. These non-mutagenic effects suggest their long-term bone tissue engineering applications. Apart from the chemical nature, the physical properties like surface topography, hardness, porosity and surface energy also impart genotoxic effects to the biomaterials. The relative absence of DNA in the tail region of comets of all the samples again reveals that all the characteristics of this hybrid scaffold are well suited for tissue engineering applications. These results are comparable to the reports by Mieszawska et.al [15].

Cell Adhesion study of composite by direct contact Assay

Extraction tests give valuable results on the cytotoxicity of extractable agents. However, low amounts of toxic agents could be undetectable because of the effects of dilution, while toxicity could still occur in the micro environment between cells and material. To analyze such activity, cell adhesion study using direct contact test tests were employed. From Figure. 6, it can be

observed that there are no mechanism of cytotoxicity found in both the control and the scaffolds. The direct contact assay was performed by adding the material to an established cell monolayer covering one-seventh of the monolayer surface. As can be observed in Table 3, both the negative control and the hybrid scaffold showed no reduction in cell metabolic activity which is in consistent with the previous reports[16].

Table 3
Percentage viability for Cell Adhesion property of silk/surface modified silica nanocomposite

SAMPLES	AVERAGE OD (540nm)	Percentage Viability
Control	0.4192	100
Sample	0.3923	93.58302

CONCLUSION

Silk/surface-modified nanosilica scaffold was successfully synthesized using sol-gel technique and further electrospun into fibers. The presence of amorphous nanosilica was confirmed using XRD analysis where a broad peak was obtained in the range of 15°- 40°. The decomposition rates and temperature of the composite were analyzed using TG/DTA technique. FTIR results revealed the functional groups present in the scaffold and its surface morphology was determined using FESEM/EDX analysis which showed a cross network structure that could impart the proper cell adhesion. The porosity was determined using MATLAB software and was found to be in consistent with the results obtained by the Hg Porosimetry results. Cytotoxicity and biocompatibility analysis by cell adhesion revealed that the percentage of viability is 97.89 % (100µg/ mL). The genotoxicity study proved that this scaffold is a suitable biomaterial that has potential applications in treating osteoporosis and bone regeneration.

ACKNOWLEDGEMENT

The author is very thankful to the Chancellor, Noorul Islam University, Kumaracoil for their valuable guidance, constant encouragement and providing basic facilities to carry out the present research work.

CONFLICT OF INTEREST

Conflict of interest declared none.

REFERENCES

1. Sammartino G, Ehrenfest DMD, Shibli JA, Galindo-Moreno P. Tissue Engineering and Dental Implantology: Biomaterials, New Technologies, and Stem Cells. BioMed Research International. 2016;2016:3.
2. Spector M. Biomaterials-based tissue engineering and regenerative medicine solutions to musculoskeletal problems. Swiss Med Wkly. 2006;136(19-20):293-301.
3. Salgado AJ, Coutinho OP, Reis RL. Bone tissue engineering: state of the art and future trends. Macromolecular bioscience. 2004;4(8):743-65.
4. Venugopal J, Low S, Choon AT, Ramakrishna S. Interaction of cells and nanofiber scaffolds in tissue engineering. Journal of Biomedical Materials Research Part B: Applied Biomaterials. 2008;84(1):34-48.
5. Rosso F, Giordano A, Barbarisi M, Barbarisi A. From cell–ECM interactions to tissue engineering. Journal of cellular physiology. 2004;199(2):174-80.
6. Hutmacher D, Goh J, Teoh S. An introduction to biodegradable materials for tissue engineering applications. annals-academy of medicine singapore. 2001;30(2):183-91.
7. Zou H, Wu S, Shen J. Polymer/silica nanocomposites: preparation, characterization, properties, and applications. Chem Rev. 2008;108(9):3893-957.
8. Thuadaij N, Nuntiya A. Synthesis and characterization of nanosilica from rice husk ash prepared by precipitation method. J Nat Sci Special Issue on Nanotechnology. 2008;7(1):59-65.
9. Rockwood DN, Preda RC, Yücel T, Wang X, Lovett ML, Kaplan DL. Materials fabrication from Bombyx mori silk fibroin. Nature protocols. 2011;6(10):1612-31.
10. Hou A, Chen H. Preparation and characterization of silk/silica hybrid biomaterials by sol–gel crosslinking process. Materials Science and Engineering: B. 2010;167(2):124-8.
11. Zhang X, Reagan MR, Kaplan DL. Electrospun silk biomaterial scaffolds for regenerative medicine. Advanced drug delivery reviews. 2009;61(12):988-1006.
12. Song L, He Q, Hu Y, Chen H, Liu L. Study on thermal degradation and combustion behaviors of PC/POSS hybrids. Polymer Degradation and Stability. 2008;93(3):627-39.
13. Liu Y, Huang Y, Liu L. Thermal stability of POSS/methylsilicone nanocomposites. Composites Science and Technology. 2007;67(13):2864-76.
14. Wu C-J, Gaharwar AK, Schexnailder PJ, Schmidt G. Development of biomedical polymer-silicate nanocomposites: a materials science perspective. Materials. 2010;3(5):2986-3005.
15. Mieszawska AJ, Fourligas N, Georgakoudi I, Ouhib NM, Belton DJ, Perry CC. Osteoinductive silk–silica composite biomaterials for bone regeneration. Biomaterials. 2010;31(34):8902-10.
16. Kharlampieva E, Kozlovskaya V, Wallet B, Shevchenko VV, Naik RR, Vaia R. Co-cross-linking silk matrices with silica nanostructures for robust ultrathin nanocomposites. ACS nano. 2010;4(12):7053-63.

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We sincerely thank the above reviewers for peer reviewing the manuscript