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# Nanobiocomposites with Enhanced Cell Proliferation and Improved Mechanical Properties Based on Organomodified-Nanoclay and Silicone Rubber

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***Abstract*****—**Bionanotechnology deals with nanoscopic interactions between nanostructured materials and biological systems. Polymer nanocomposites with optimized biological activity have attracted great attention. Nanoclay is considered as reinforcing nanofiller in manufacturing of high performance nanocomposites. In current study, organomodified-nanoclay with negatively charged silicate layers was incorporated into biomedical grade silicone rubber. Nanoparticle loading has been tailored to enhance cell behavior. Addition of nanoparticles led to improved mechanical properties of substrate with enhanced strength and stiffness while no toxic effects was observed. Results indicated improved viability and proliferation of cells by addition of nanofillers. The improved mechanical properties of the matrix result in proper cell response through adjustment and arrangement of cytoskeletal fibers. Results can be applied in tissue engineering when enhanced substrates are required for improvement of cell behavior for *in vivo* applications.

***Keywords*****—**Biocompatibility, Composite, Organomodified-Nanoclay, Proliferation

## I. Introduction

In tissue engineering, a growing body of research deals with improvement of substrates to enhance cell functionality for *in vivo* applications. The cell-substrate interaction necessitates enhanced material properties to influence cell behavior including proliferation, morphology, cytoskeletal structure, and differentiation. Physico-mechanical properties of substrates have been shown to influence cell behavior [1, 2]. Such properties have been manipulated and modified by changing the chemical crosslinking factors [3, 4] or surface topography [5]. Min Lo et al. cultured 3T3 firoblast cells on flexible polyacrylamide sheets and studied cell migration and spreading by change of surface rigidity. The flexibility of substrates was altered by bis-acrylamide as crosslinking agent [6]. Guo et al. similarly created polyacrylamide network by adding bis-acrylamide in order to achieve substrates with different elastic moduli to describe that tissue regeneration trend in biology is markedly influenced by rigidity of matrix surface [7]. Frey et al. prepared substrates made of polystyrene with locally micropillars to investigate how cell migration and morphology are controlled by surface topography [8]. Correspondingly the influence of matrix mechanical properties on glioma cells and neural stem cells were examined by Ulrich et al. [9] and Teixeira et al. [10].

Proteins such as collagen type I [11, 12] or fibronectin [11, 13] have been used to enhance cell adhesion. Surface modifications including plasma [12, 14] and UV [15] treatments have been alternative methods to improve cell attachment. However, such procedures are not economically reliable [16, 17]. In current study PDMS based substrate was modified by inclusion of different mass ratios of organomodified-nanoclay (O-MMT) particles and the resultant mechanical properties were evaluated, and effects of alteration in matrix mechanical properties on cell behavior were studied. For this O-MMT as the extending nanofiller [18] was dispersed in rubbery matrix that improved mechanical properties [19]. Then the response of endothelial cells to altered mechanical properties was analyzed.

## II. Materials and Methods

### A. Preparation of nanocomposites

Medical grade silicone rubber (HTV) was used as the base material and mixed with clay nanoparticles, Closite 15A (Southren Clay Products, USA) with mass ratios of 1%, 2% and 3%. Dicumyl peroxide was employed for cure of samples. Melt mixing method was applied on silicon rubber and O-MMT using a BRABBENDER mixer with the volume of 60 cc at 60 rpm and temperature of 60° C for 20 minutes. Dicumyl peroxide (0.6 % w/w) as crosslinking agent was added to the compound during the mixing on the two roll mill apparatus (Farrel Bridge LTD, UK) at 60 °C [20]. Finally samples were vulcanized at 160 °C by compression moulding at the optimum cure time ($t_{95}$). Curing characteristics was determined in a Monsanto R100S oscillating disc rheometer (ODR) at 3 degrees arc at 160 °C.

### B. Evaluation of Mechanical Properties

The mechanical properties including tensile strength, elastic modulus at tension, and elongation at break were determined using a tensile testing device (Cardano al Campo (VA), Italy). Samples were prepared and tests were performed according to standard protocol of ASTM D412 [21].

### C. Cell Culture and Imaging

Human Umbilical Vein Endothelial Cells (HUVECs) were cultured in DMEM + Ham's F12 (Gibco, USA) containing

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10% FBS (Seromed, Germany) and incubated in5% $CO_2$ at 37°C. Silicone nanocomposite substrates were coated by collage type I (Sigma, USA) of 0.5 mg/mL for proper cell attachment. The confluent cells were transferred to the coated substrate and incubated overnight, after then images of cells were captured and processed.

*D. Image Processing*

The images captured after 24 hours were analyzed using MATLAB-based image analysis code (TheMathWorks, Inc., USA) to approximate adherent cells [22]. To evaluate cell growth the area covered by cells in each image was obtained and the ratio of cell area to the total area was measured through producing binary images and segregation of cells from the background. Then cell density was measured by calculation of number of adherent cells through cell coverage and compared to the number of cells of images of initial culture. Image processing started with conversion of RGB image to the gray scale format. Then an appropriate filter was applied to the resultant image to remove inherent artifacts. After that the binary image was produced, followed by calculation of cell cover parameter. This parameter was computed by the area of adhered cells to the substrate divided by the surface area of the substrate in the image. Variation of cell cover parameter is an indicator of cell growth.

*E. Cytotoxicity Assays*

In order to evaluate biocompatibility and cytotoxicity, MTT assay was performed as follow. First, $10\times10^3$ human umbilical vein endothelial cells (HUVECs) were added to a 96-well plate and were incubated at 37 °C in humidified atmosphere containing 5% $CO_2$. After cell adhesion, the extract of each nanocomposite was directly poured onto the cell monolayer. The plate was then incubated for 24 hours. Subsequently, culture medium of each well was replaced with 100 µL of 3-[4,5-dimethyltriazol-2-y1]-2,5-diphenyl tretrazolium bromide (MTT) with concentration of 0.5 mg/ml and the plates incubated at 37 °C for 4 hours. Then, the MTT solution was removed and plate which contained isopropanol was shaken Cell viability was finally analyzed at 570 nm in an ELISA reader [23]. Wells containing a high number of cells showed higher O.D. value compared to the wells with fewer cells.

## III. Results

*A. Mechanical Properties*

Table I summarizes mechanical properties evaluated for nanobiocomposites. Results indicated stiffening of the matrix as the content of nanoparticles is elevated. An increase of 18% was observed in tensile modulus when the mass ratio of nanoparticles was elevated from 0% to 3%. Simultaneously the tensile strength of the substrate was increased 23% by adding nanofillers up to 3 %. By the same trend, the elongation at break was reduced 19%.

*B. Biocompatibility and Toxicity of Substrates*

MTT results are shown in Fig. 1 indicating high degree of biocompatibility. Percentage of cell viability for samples has been estimated to be 90-100 % in comparison with neat PDMS. In other words, all evaluated samples were found to be nontoxic and biocompatible and cell culture on the O-MMT/silicone rubber nanocomposites showed desirable results.

*C. Cell Growth and Proliferation*

Figure 2 describes typical images of morphology of HUVECs cultured on samples with different mass ration of nanofillers. Cells in all images are well spread and have grown properly. Fig. 4 represents the relative cell density for different groups through measurement of cell cover parameter. Cell density is calculated by normalizing of adhering cells on each sample in respect to the initial cells ($2\times10^3$) transferred to the collage-coated surface (36 $mm^{-3}$) and then dividing dimensionless numbers into the mentioned surface area. Results indicated increase of cell density by elevation of mass ratio of nanofillers, comparable to the increased cell proliferation.

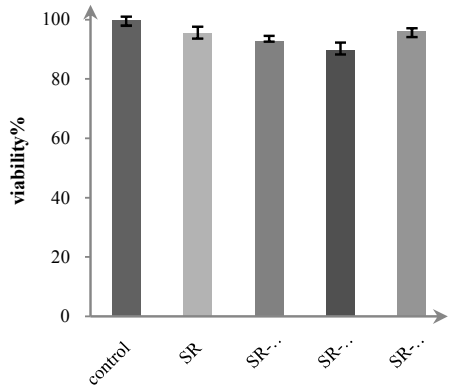


Fig. 1 Cell toxicity results for different samples

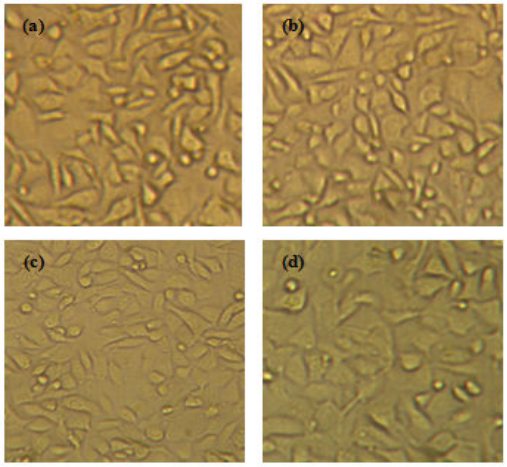


Fig. 2 Comparison of cell morphology on the nanocomposites and control samples; (a) PDMS, (b) PDMS-1% O-MMT, (c) PDMS-2% O-MMT, and (d) PDMS-3% O-MMT, (400x).

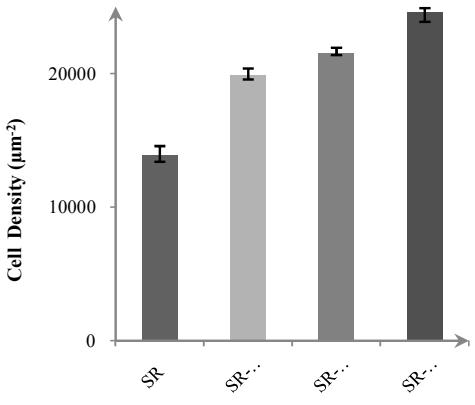


Fig. 3 The relative cell density after 24 hours

TABLE I
MECHANICAL PROPERTIES OF O-MMT/PDMS NANOCOMPOSITES WITH DIFFERENT MASS RATIO PERCENTAGES

| | | Properties | | |
|---|---|---|---|---|
| | | Tensile strength (MPa) | Tensile modulus (MPa) | Elongation at break (%) |
| Nanocomposites | PDMS | 6.72±0.22 | 1.47±0.08 | 356.19±26.72 |
| | PDMS/O-MMT1% | 7.56±0.58 | 1.49±0.19 | 353.55±33.53 |
| | PDMS/O-MMT2% | 7.48±0.66 | 1.57±0.09 | 340.59±25.02 |
| | PDMS/O-MMT3% | 8.26±0.01 | 1.73±0.06 | 289.04±14.30 |

## IV. DISCUSSION

To study effects of substrate elasticity on cell behavior, nanocomposite substrates were used by dispersion of differing mass ratio of nanoparticles of O-MMTs in silicone rubber. Results indicated significant influence of the in polymer matrix on mechanical properties of the substrate. Addition of O-MMT resulted in stronger and stiffer matrix. The decrease of the crosslinking density which was evaluated from cure curves cited that nanofiller act as an inhibitor. In other words, addition of nanoparticles into the polymer matrix would prevent chemical crosslinking, and causes physical integrity [24] with a strong interface between nanofiller and silicone rubber [25]. Such integrity is achieved by uniform distribution of particles into the nanocompostie structure.

In addition to enhancement of mechanical properties, cytotoxicity and biocompatibility analyses revealed improved cell behavior on the modified substrates. Addition of nanoparticles did not result in toxic effects and the resultant substrates found to be biocompatible. Cell growth and alignment was smoothly enhanced in nanocomposite substrates. It has been shown that inorganic nanofiller have higher surface energy than polymer matrix [26]. Silicone rubber is hydrophobic due to the presence of methyl groups, hence PDMS is considered as a low surface energy polymer [27]. Increasing O-MMT leads to the rise of the free surface energy by the mechanism of enhancing surface roughness [28]. Previous studies have shown that silicone rubber polymer reinforced by O-MMT obtained further roughness originated from filler tactoids and agglomerates in reference polymer matrix [29]. Rapid cell growth and proliferation are obtained on the surfaces with higher energy [30], as described by cell cover in this study. By increased surface energy of the substrate, the level of energy differences between cell membrane and matrix surface is elevated and therefore strong contacts are generated which results in desirable cell proliferation and signaling.

Mechanical properties of substrate influences cell functionality. Cytoskeletal arrangement and orientation is highly dependent to mechanical and structural properties of the matrix such as elastic modulus, Poisson's ratio and roughness [31, 32]. When interact with substrate, cellular responses including relaxation time and adaptation by alteration in fibrous structure are defined by local matrix deformability [33, 34]. The adjustment of cell cytoskeleton to the mechanical properties of the substrate roots in the polymerization and depolimerization of actin fibers [35] which act via focal adhesion proteins at the interface of cell-substrate [34].

## V. CONCLUSION

In this research, O-MMT nanoparticles were loaded into the silicone rubber matrix on the basis of melt mixing procedure. Consequently, substrates with higher strength and stiffness were acquired with no toxicity effect. Additionally, enhanced cell behavior was shown by nanobiocomposites. In tissue engineering, reconstruction of damaged tissues and generation of tissue integrity necessitate cell substrates with appropriate mechanical properties with no toxic effects and appropriate cell growth and alignment. Results provide means for improvement of silicone rubber as the cell substrate.

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## REFERENCES

[1] Gentile F, Tirinato L, Battista E, Causa F, Liberale C, Fabrizio EM, Decuzzi P. Cells preferentially grow on rough substrates. *Biomaterials* 2010; 31: 7205-7212.
[2] Cukierman E, Bassi DE. Physico-mechanical aspects of extracellular matrix influences on tumorigenic behaviors. *Semin Cancer Biol* 2010; 20: 139–145.
[3] Wang K, Cai L, Hao F, Xu X, Cui M, Wang S. Distinct cell responses to substrates consisting of poly(ε-caprolactone) and poly(propylenefumarate) in the presence or absence of cross-links. *Biomacromolecules* 2010; 11:2748–2759.
[4] Slaughter BV, Khurshid SS, Fisher OZ, Khademhosseini A, Peppas NA. Hydrogels in Regenerative Medicine. *Adv Mater* 2009; 21: 3307–3329.

[5] Cortese B, Gigli G, Riehle M. Mechanical gradient cues for guided cell motility and control of cell behavior on uniform substrates. *Adv Funct Mater* 2009; 19:2961-2968.
[6] Lo C, Wang H, Dembo M, Wang Y. Cell movement is guided by the rigidity of the substrate. *Biophys J* 2000; 79:144-152.
[7] Guo W, Frey MT, Burnham NA, Wang Y. Substrate rigidity regulates the formation and maintenance of tissues. *Biophys J* 2006; 90:2213-2220.
[8] Frey MT, Tsai IY, Russell TP, Hanks SK, Wang Y. Cellular responses to substrate topography: role of myosin ii and focal adhesion kinase. *Biophys J* 2006; 90:3774-3782.
[9] Ulrich TA, de June Pardo EM, Kumar S. The mechanical rigidity of the extracellular matrix regulates the structure, motility, and proliferation of glioma cells. *Cancer Res* 2009; 69:4167-4174.
[10] Teixeira AI, Ilkhanizadeh S, Wigenius JA, Duckworth JK, Inganas O, Hermanson O. The promotion of neuronal maturation on softsubstrates. *Biomaterials* 2009; 30: 4567-4572.
[11] Yeung T, Georges PC, Flanagan LA, Marg B, Ortiz M, Funaki M, Zahir N, Ming W, Weaver V, Janmey PA. Effects of substrate stiffness on cell morphology, cytoskeletal structure, and adhesion. *Cell Motil Cytoskeleton* 2005; 60:24-34.
[12] Solouk A, Mirzadeh H, Shokrgozar MA, Solati-Hashjin M, Najarian S, Seifalian AM. The study of collagen immobilization on a novel nanocomposite to enhance cell adhesion and growth. *Iranian Biomed J* 2011; 15: 6-14.
[13] Heilshorn SC, Liu JC, Tirrell DA. Cell-binding domain context affects cell behavior on engineered proteins. *Biomacromolecules* 2005; 6: 318-323.
[14] Lopez LC, Belviso MR, Gristina R, Nardulli M, Agostino R, Favia P. Plasma-treated nitrogen-containing surfaces for cell adhesion: the role of the polymeric substrate. *Plasma Process Polym* 2007; 4:S402–S405.
[15] Welle A, Gottwald E. UV-based patterning of polymeric substrates for cell culture applications. *Biomedical Microdevices* 2002; 4:33-41.
[16] Mehta G, Kiel MJ, Lee JW, Kotov K, Linderman JJ, Takayama S. polyelectrolyte-clay-protein layer films on microfluidic PDMS bioreactor surfaces for primary murine bone marrow culture. *Adv Funct Mater* 2007; 17:2701-2709.
[17] Sharma R, RimmerRD, Gunamgari J, Shekhawat RS, Davis BJ, Mazum der MK, Lindquist DA. Plasma-assisted activation of supported Au and Pd catalysts for CO oxidation. *IEEE transactions on industry applications society* 2005; 41: 1373-1376.
[18] Momen G, Farzaneh M. Survey of micro/nano filler use to improve silicone rubber for outdoor insulators. *Rev Adv Mater Sci* 2011; 27:1-13.
[19] Kaneko MLQA, Romero RB, Gonçalves MDC, Yoshida IVP. High molar mass silicone rubber reinforced with montmorillonite clay master batches: Morphology and mechanical properties. *Eur Polym J* 2010; 46:881-890.
[20] Zhang Y, Pang M, Xu Q, Lu H, Zhang J, Feng S. The curing retardation and mechanism of high temperature vulcanizing silicone rubber filled with superconductive carbon blacks. *Polym Eng Sci* 2011; 51: 170–178.
[21] Ma J, Yu Z, Kuan H, Dasari A, Mai Y. A New Strategy to Exfoliate Silicone Rubber/Clay Nanocomposites. *Macromol. Rapid Commun* 2005; 26:830–833.
[22] Haghighipour N, Tafazzoli-Shadpour M, Shokrgozar M, Amini S. Effects of cyclic stretch waveform on endothelial cell morphology using fractal analysis. *Artifcial Organs* 2010; 34:481–490.
[23] Bal BT, Yılmaz H, Aydın C, Karakoca S, Yılmaz S. In vitro cytotoxicity of maxillofacial silicone elastomers: effect of accelerated aging. *J Biomed Mater Res B Appl Biomater* 2009;89:122-1266.
[24] Zhu L, Wool RP. Nanoclay reinforced bio-based elastomers: Synthesis and characterization. *Polymer* 2006; 47:8106-8115.
[25] Li ZH, Zhang J, Chen SJ. Effects of carbon blacks with various structures on vulcanization and reinforcement of filled ethylene-propylene-diene rubber. *eXPRESS Polymer Letters* 2008; 2:695–704.
[26] Tian M, Cheng L, Zhang L. Interface and mechanical properties of peroxide cured silicate nanofiber/rubber composites. *J App Polym Sci* 2008; 110:262-269.
[27] Haji K, Zhu Y, Otsubo M, Honda C. Surface modification of silicone rubber after corona exposure. *Plasma Process Polym* 2007; 4:S1075–S1080.
[28] Ataeefard M, Moradian S. Surface properties of polypropylene/organoclay nanocomposites. *Appl Surf Sci* 2011; 257:2320-2326.
[29] Razzaghi-Kashani M, Gharavi N, Javadi S. The effect of organo-clay on the dielectric properties of silicone rubber. *Smart Mater Struct* 2008; 17:065035(9pp).
[30] Hallab NJ, Bundy KJ, O'connor K, Moses RL, Jacobs JJ. Evaluation of metallic and polymeric biomaterial surface energy and surface roughness characteristics for directed cell adhesion. *Tissue Eng* 2001; 7:55-71.
[31] De R, Zemel A, Safran SA. Dynamics of cell orientation. *Nature Physics* 2007; 3: 655 – 659.
[32] Wei Z, Deshpande VS, McMeeking RM, Evans AG. Analysis and interpretation of stress fiber organization in cells subject to cyclic stretch. *J Biomech Eng* 2008; 130: 031009.
[33] Hsu H, Lee C, Kaunas R. A dynamic stochastic model of frequency-dependent stress fiber alignment induced by cyclic stretch. *PLoS ONE* 2009; 4: e4853.
[34] De R, Safran SA. Dynamical theory of active cellular response to external stress. *Phys Rev E* 2008; 78: 031923.
[35] Nekouzadeh A, Pryse KM, Elson EL, Genin GM. Stretch-activated force-shedding, force recovery, and cytoskeletal remodeling in contractile fibroblasts. *J Biomech* 2008; 41: 2964-2971.

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