

Evaluation of Physico-Mechanical Properties and *In Vitro* Biocompatibility of Compression Molded HDPE Based Biocomposites with HA/Al₂O₃ Ceramic Fillers and Titanate Coupling Agents

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ABSTRACT: The present article demonstrates how the stiffness, hardness as well as the cellular response of bioinert high-density polyethylene (HDPE) can be significantly improved with combined addition of both bioinert and bioactive ceramic fillers. For this purpose, different amounts of hydroxyapatite and alumina, limited to a total of 40 wt %, have been incorporated in HDPE matrix. An important step in composite fabrication was to select appropriate solvent and optimal addition of coupling agent (CA). In case of chemically coupled composites, 2% Titanium IV, 2-propanolato, tris iso-octadecanoato-O was used as a CA. All the hybrid composites, except monolithic HDPE, were fabri-

cated under optimized compression molding condition (140°C, 0.75 h, 10 MPa pressure). The compression molded composites were characterized, using X-ray diffraction, Fourier transformed infrared spectroscopy, and scanning electron microscopy. Importantly, *in vitro* cell culture and cell viability study (MTT) using L929 fibroblast and SaOS2 osteoblast-like cells confirmed good cytocompatibility properties of the developed hybrid composites. © 2011 Wiley Periodicals, Inc. *J Appl Polym Sci* 124: 3051–3063, 2012

Key words: HDPE; composite; coupling agent; microstructure; cell adhesion

INTRODUCTION

High density polyethylene (HDPE) is an important biocompatible semi-crystalline thermoplastic material with significant ductility. In view of the fact that natural bone is an anisotropic complex composite material, the semicrystallinity in polyethylene has been suggested to provide the direction dependent characteristic properties, when used in a composite with bioactive hydroxyapatite (HA).¹

HA has been well recognized as the foremost biomaterial with orthopedic and dental applications because of its excellent biocompatibility, bioactivity, and osteoconductivity.² The applicability of HA/HDPE composites in orthopedics has been clinically realized as low load bearing implants.^{3,4} The reliability in terms of long term clinical survival is questionable because both the

phases in this composite are only physically interlocked by the shrinkage of polyethylene during cooling from their processing temperature.⁵

To improve the mechanical properties of these composites, especially with the improved interfacial bond strength, the use of coupling agents (CA) appears to be a better option. Since, the polyethylene is nonpolar and hydrophobic, the introduction of polar group can effectively increase the affinity between polyethylene and HA. The interaction between a polymer matrix and filler can be mechanical or chemical or both; however the chemical bonding has important implication in improving the properties.⁵

In the field of polymer-based composites, CA have been shown to have an important contribution in promoting the molecular bonding and consequently, the improved interfacial adhesion.^{6,7} Organo titanates and zirconates are two surface modifiers with similar structures that can be used as modifying filler.⁸ Among all these, titanate is the fillers which can be used frequently, while zirconates are advantageous for certain applications⁹ like grafting etc. Several studies^{10–14} demonstrate the possible application of titanate or zirconate surface modifiers^{11,15,16} or zirconate alone¹³ or in combination with other CA, to a wide range of composite systems.⁸

In the above backdrop, one of the major aims of the present work is to develop chemically coupled

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HDPE-HA- Al_2O_3 biocomposite using a simple low cost processing route, which will exhibit a desired combination of mechanical properties and good biocompatibility. For this purpose, bioactive HA and bioinert alumina particles are used as reinforcing ceramic fillers into the HDPE matrix. Alumina is selected as a second reinforcing phase, because of its excellent properties such as high wear resistance, good biocompatibility, and stability in physiological environments. In our earlier work, the results of compression molding of HDPE-HA- Al_2O_3 system reveal that 40 wt % ceramic filler addition can provide better combination of mechanical properties. In view of the same, it has been decided to reinforce HDPE matrix with 40 wt % HA or 40 wt % Al_2O_3 or 40 wt % HA + Al_2O_3 (1 : 1 ratio) in the presence of CA and the identical compression molding conditions, as reported earlier¹⁷ are used to consolidate such composites. The CA addition is made in the presence of solvent and it has been shown how an aromatic solvent can cause cell death. Overall, the present work possibly reports the first comprehensive physical and biocompatibility evaluation of CA treated HDPE-HA- Al_2O_3 biocomposites.

EXPERIMENTAL PROCEDURE

Starting powders

In this study, HDPE has been used as a matrix of the composite. HDPE (density: 0.95 g/cm³) was procured from Swasan Chemicals, Mumbai, India. HA was synthesized by widely reported suspension-precipitation route, as described in our earlier work.^{6,18} The precursor materials were CaO (M/s S.D Fine-Chem Lit., Product No. 37614), H_3PO_4 (M/s Merck, CAS No. 7664-38-2), and NH_4OH (M/s Qualikems, Product No. A025112).

The particle size distribution was measured using laser particle size analyzer (Analysette 22, Fritsch GMBH, Germany) and the as-synthesized HA powders have average size (d_{50}) of 1.9 μm . Besides HA, commercially available alumina ($\alpha\text{-Al}_2\text{O}_3$, average size: 4.8 μm , 99.4% pure, Carborundum Universal India) is used as another ceramic filler in the composite. Titanium IV, 2-propanolato, tris isoocadecanoato-O was used as CA and was obtained from Kenrich Chemicals, USA.

Composite preparation

As part of this study, a number of polymer-ceramic compositions were formulated (Refer Table I). Unreinforced HDPE is designated as CS1 sample. HA powders of 40 wt % were mixed with HDPE matrix without and with CA to produce CS2 and CS3 composites, respectively. CS4 and CS5 compo-

TABLE I
Composition of the Various Polymer-Ceramic Composites, Developed in the Present Work

S. no	Sample designation	Composition	Coupling agent (wt %)
1	CS1	HDPE	–
2	CS2	HDPE + 40 wt % HA (16.5 vol % HA)	–
3	CS3	HDPE + 40 wt % HA	2
4	CS4	HDPE + 40 wt % Al_2O_3 (13.6 vol %)	–
5	CS5	HDPE + 40 wt % Al_2O_3	2
6	CS6	HDPE + 20 wt % HA (8.4 vol %) + 20 wt % Al_2O_3 (6.7 vol %)	–
7	CS7	HDPE + 20 wt % HA + 20 wt % Al_2O_3	2

sites were composed of 40 wt % Al_2O_3 without and with CA, respectively. CS6 and CS7 are the composite compositions made from simultaneous addition of both HA and Al_2O_3 [40 wt % fillers (1 : 1)] without and with CA to 60 wt % HDPE powder, respectively. It can be mentioned here that the optimized mechanical and biological properties could be achieved in case of 40 wt % HA reinforced HDPE/HA composites.¹⁷ In all the cases, the starting powders of different compositions were mechanically blended in a planetary ball mill in dry condition for 12 h. Both the balls and jars were made of natural agate (silica). The weight of the balls used was 6–7 times more than the total weight of powder mixtures. Compression molding (SCM-30, Ms/- Santec Automation, Delhi, India) was used for the processing of these composites. The polymer-ceramic powder blends were subsequently compression molded into 12 mm diameter cylindrical specimens at a constant pressure of 10 MPa and at a temperature of 130°C for 0.5 h in case of monolithic HDPE and at 140°C for 0.75 h for all the composite samples. The thickness of the compression molded samples was around 3 mm.

Characterization

The density was measured in water for composites and in ethanol for HDPE using Archimedes's principle. Four samples per composition were used for density measurements. The phase identification of both the starting powders and compression molded samples was performed using X-ray diffraction with Cu $K\alpha$ radiation (Rich-Seifert, 2000 D). The obtained patterns were later analyzed using JCPDF database software.

Fourier Transform Infrared Analysis (FTIR, vortex 70, BRUKER) was carried out using powders of ball milled samples in the range of 400–4000 cm^{-1} to obtain information about the various chemical bonds present in the samples.

The polished surface microstructures as well as indentation images were studied using SEM (FEI-SEM,

model JSM-6330F, Philips, The Netherlands). Prior to SEM observation, the samples were sputter-coated with a thin Au-Pd coating in order to obtain sufficient conductivity on the surface. The compositional analysis of the composites was carried out with the help of EDS equipped with SEM.

The hardness of the developed composites was measured by Vickers micro-hardness tester using a number of replicates of each composition. The E-modulus was measured using impulse excitation method and the dynamic elastic properties analysis instrument (Dynamic Elastic Properties Analyzer: Jagdish Electronics, Bangalore, India) was used for this purpose. For the dynamic modulus measurements, the samples of known dimensions were impacted upon by a standard steel rod close to the high frequency sensor of the instrument to induce the vibrations in the sample.¹⁹ For the disc shaped samples, two measures of dynamic Young's modulus, E (E_1 and E_2) are made independently from the two resonant frequency measurements, and then a final value E is determined by averaging the two calculated values E_1 and E_2 , as per the following formulation:

$$E_1 = [37.6991f_1^2 D^2 m(1 - \mu^2)] / (K_1^2 t^3)$$

$$E_2 = [37.6991f_2^2 D^2 m(1 - \mu^2)] / (K_2^2 t^3)$$

$$E = (E_1 + E_2) / 2$$

where, E = Young's modulus; E_1 = First natural calculation of Young's Modulus; E_2 = Second natural calculation of Young's Modulus; f_1 = First natural resonant frequency of the disc; f_2 = Second natural resonant frequency of the disc; D = diameter (mm) of the disc; m = mass (g) of the disc; μ = Poisson's ratio for the specimen as determined; K_1 = First natural geometric Factor; K_2 = Second natural geometric Factor; t = thickness (mm) of the disc.

In vitro cytocompatibility

Cell culture experiments

Mouse fibroblast (L929) and human osteoblast-like (SaOS2) cell lines were procured from ATCC (American Type Culture Collection, Manassas, VA, USA) Prior to seeding cells on biomaterials surfaces, the cells were revived. The *in vitro* cytocompatibility of the as-synthesized polymer ceramic composites were performed using the standard cell culture protocol as per ISO-10993-1 (Guidance on selection of Test) guidelines, as described in our earlier work.^{17,20,21}

MTT assay

For MTT assay, L929 and SaOS2 cells were cultured following the previously described cell culture method. To study the cell adhesion and proliferation

behavior on the developed composites (Refer Table I), the autoclaved samples were placed in the 24 well plate and cells were seeded at a density of 3×10^5 cells/mL. Subsequently, the culture plate was incubated for 3, 5, and 7 days in CO₂ incubator. After the respective incubation periods, the medium was aspirated and the samples were washed twice with Phosphate buffer saline (PBS). Fresh DMEM (without phenol red) and reconstitute MTT (3(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium-bromide: SIGMA, USA) solution in 10 : 1 ratio was added in each well and the plate was incubated for 6–8 h. In the meantime, the culture plate was monitored under the phase contrast microscope (Nikon, Eclipse 80i, Japan) to check for the formation of purple color formazane crystal. After the incubation, samples were removed from the well and 300 μ L of dimethyl sulfoxide (DMSO) (stock solution) was added in each well, including control. The optical density of the solution was measured at 540 nm using ELISA automated microplate reader (Bio-Tek, ELx800).

To analyze the statistical significance of various experimental measurements, the commercially available software SPSS-13 was used. The statistical analysis was carried out by adopting univariate analysis of variance (ANOVA) method. In particular, the *post hoc* multiple comparisons of the mean of independent group were made using Tukey test (for homogeneous data) at statistical significant value, $p < 0.05$.

RESULTS

Processing results

The compression molding conditions, i.e., the combination of temperature of 130/140°C with curing time of 30/45 min and a pressure of 10 MPa were selected based on our earlier study.¹⁷ On the basis of the chemistry and the molecular structure of HDPE, as well as for the purpose of establishing chemical coupling between polymeric chains and ceramic fillers (HA or alumina), the titanates were selected as CA. In terms of processability to cylindrical shaped compact without any visible porosity, 2 wt % of CA along with isopropyl alcohol (IPA) as a solvent (10 wt %) was found comparatively better among all the trial batches. At a higher amount of CA, the thermo-plastic behavior of HDPE was enhanced and therefore, the consolidation becomes difficult.

As far as the consolidation is concerned, pure HDPE shows maximum density of 95% ρ_{th} , whereas other composites possess density, varying in the range of 95–98.5% ρ_{th} (see Fig. 1). It is interesting to note here that, the use of CA consistently provides better densification than that of composites prepared without CA.

Figure 2(a,b), present the XRD patterns of the starting powders and polished compression molded

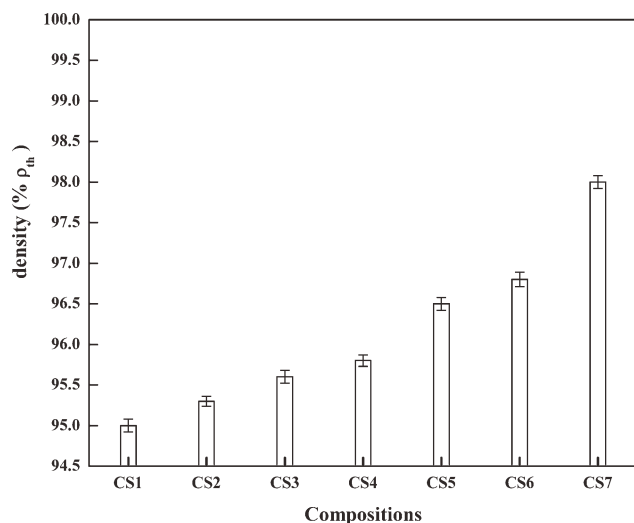


Figure 1 Comparative representation of obtained % densification of the compression molded samples produced by the presence and absence of coupling agent at the time of mixing.

specimens. The analysis of the XRD peaks of starting HA powders [see Fig. 2(a)] reveals the presence of three major characteristic peaks of pure HA, located at 2θ : 31.8° , 32.9° , and 34° . The sharp peak of HDPE powders at 2θ : 21.9° and 24.1° clearly indicates

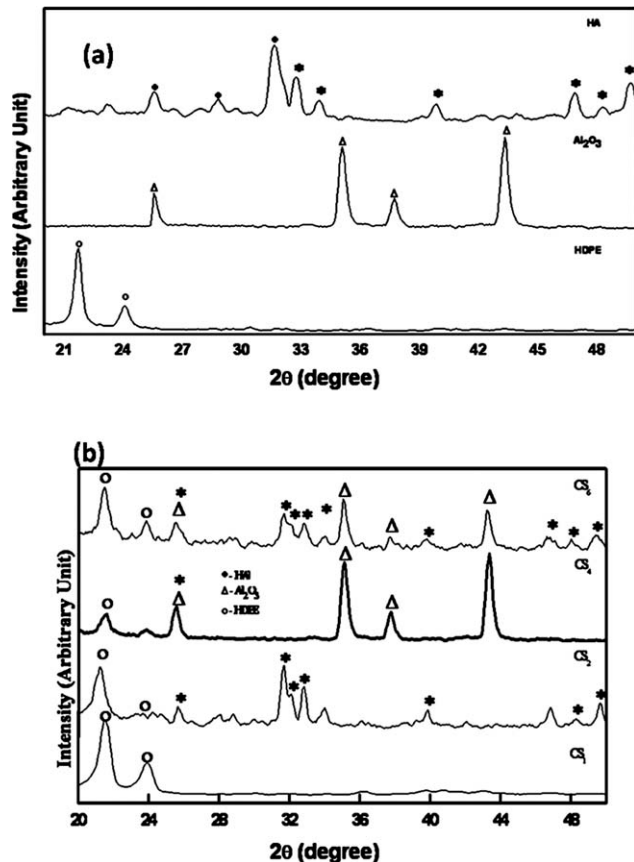


Figure 2 (a) XRD Trace of the Starting phases i.e., HDPE, HA, and Al_2O_3 . (b) XRD Traces of Compression molded samples CS1, CS2, CS4, and CS6.

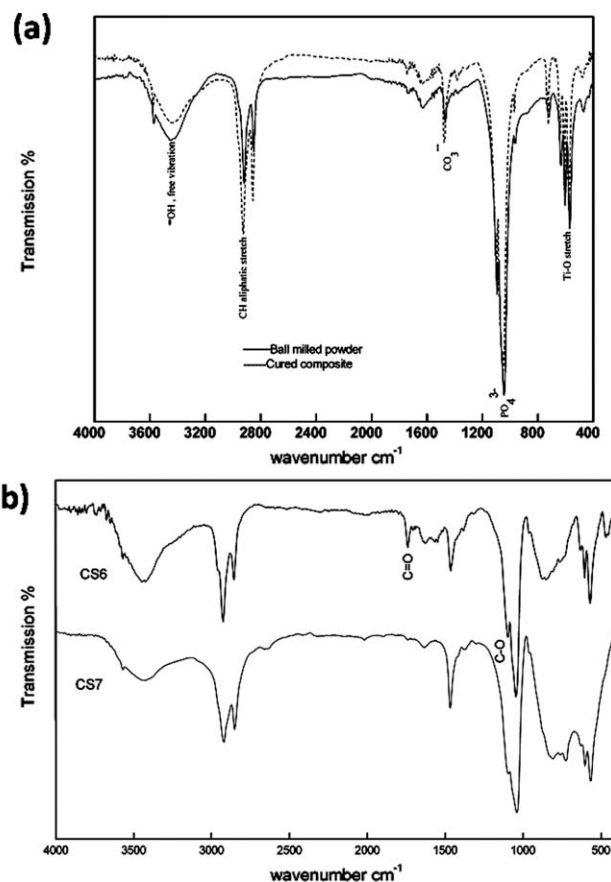


Figure 3 (a) Comparative FTIR trace of the ball-milled powder and compression molded composite. (b) FTIR trace clearly evidenced the alcoholysis reaction taking place in the CS7 composite system.

higher degree of crystallinity. The 'smooth molecular structure' of polyethylene molecular chains and the absence of bulk side groups enhance the crystallinity of HDPE. The presence of α -alumina is confirmed by characteristic peaks at 2θ : 35° , 37.9° , and 43.5° . In case of composites, XRD graph [see Fig. 2(b)] confirms that the microstructure of developed composites predominantly contain the HA and alumina fillers along with semi-crystalline HDPE. Because of the least quantity of CA present in the composite matrix, it was difficult to detect from XRD. However, it is clearly evident using SEM-EDS analysis.

FTIR spectrum, represented in Figure 3(a,b), shows the presence of structural OH^- (632 and 3501 cm^{-1}), $-OH$ from polymer backbone chain (3416 cm^{-1}) and PO_4^{3-} (1093 , 1027 , and 604 cm^{-1}) for HA. The characteristic peak of $C=O$ bond at 1726 cm^{-1} and several peaks for $C-H$ bending (polymer characteristic peak) in the range of $1200\text{--}1250\text{ cm}^{-1}$ were observed. More discussion on FTIR results is provided in next section.

The distribution of HA and Al_2O_3 particles in HDPE matrix can be assessed from SEM images and such representative SEM micrographs of polished samples are presented in Figure 4. In order to

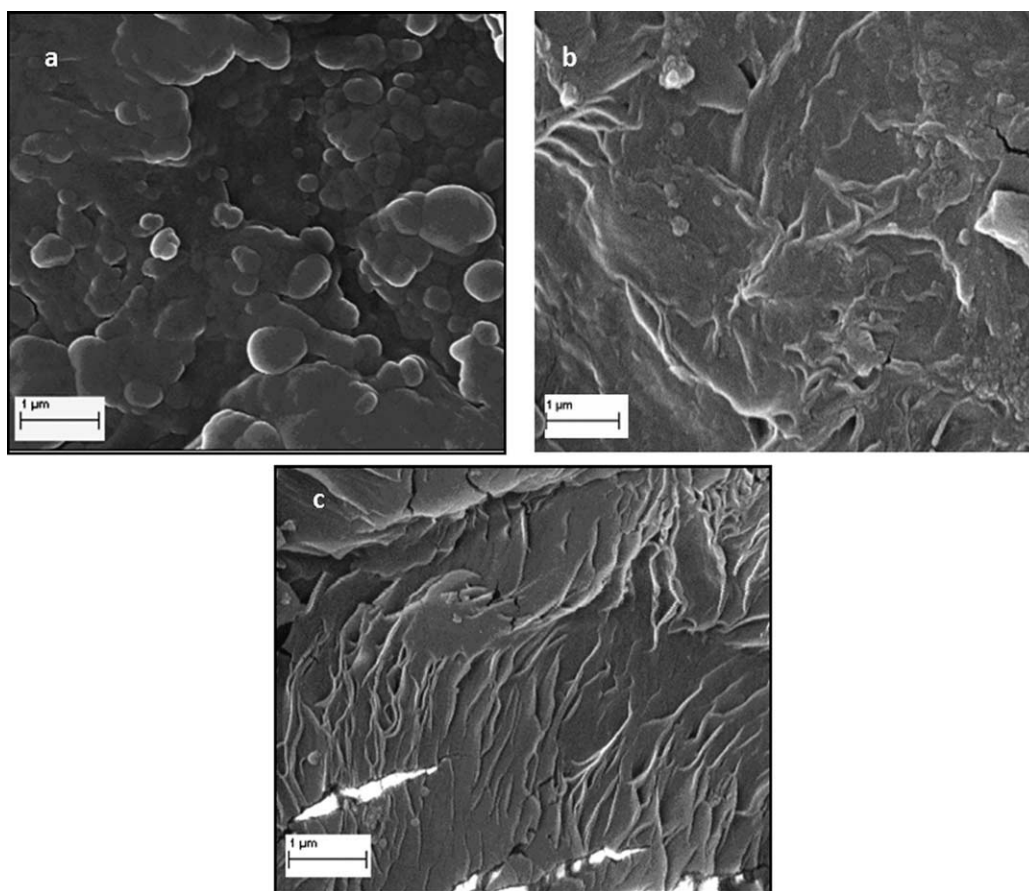


Figure 4 SEM images illustrating the surface morphology of (a) CS3, (b) CS5, and (c) CS7.

provide such evidence, a representative SEM image is shown in Figure 5(a), which reveals the uniform dispersion of ceramic fillers. SEM investigation also reveals that uniform dispersion of HA and alumina particles are preserved in the compression molded composite. The EDS compositional analysis of the polished composite surface reveals strong peak of Ca, P, and Al, which signifies the presence of both types of ceramic fillers in the composite microstructure [Fig. 5(a,b)].

Figure 6 shows the variation of hardness values as obtained for different prepared composites, which were measured using Vickers microhardness tester. The hardness enhancement in the presence of CA for a given composition can be realised from the data presented in Figure 6. In particular, higher hardness values were measured in the range of 190–256 MPa for chemically modified composites and such hardness values are near to the hardness of human cortical bone (234–760 MPa).²² From the experimental results, it is also evident that CS5 composite exhibits similar values, compared to that of composite CS7.

Figure 7 shows SEM images of the Vickers indents for all the samples. It is clearly observed that, in case of

pure HDPE, the indents are not clearly distinct. This indicates the softness of HDPE and a significant amount of viscoelastic recovery even after the removal of the applied load. However, in case of composites, indentation impressions are quite distinct [Fig. 7(b–d)].

From the dynamic elastic modulus values (Fig. 8), better properties in the presence of CA can be again confirmed. The *E*-modulus values presented in Figure 8 should be used for comparison purpose among the investigated composites only and should not be used for comparing with other composites or natural tissue/bone, where different test methods are used. For sample CS1, the dynamic elastic modulus was found as 0.96 GPa and it rises upto 8.5 to 11.3 GPa for the compositions filled with ceramic fillers without and with Titanium IV, 2-propanolato, tris isooctadecanoato-O, i.e., in samples CS6 and CS7, respectively. Likewise mentioned earlier, the statistical analysis of the data was performed using SPSS-13 software.

Cell adhesion behavior

To assess the cytotoxicity property of the developed polymer-ceramic composites, cell culture study by

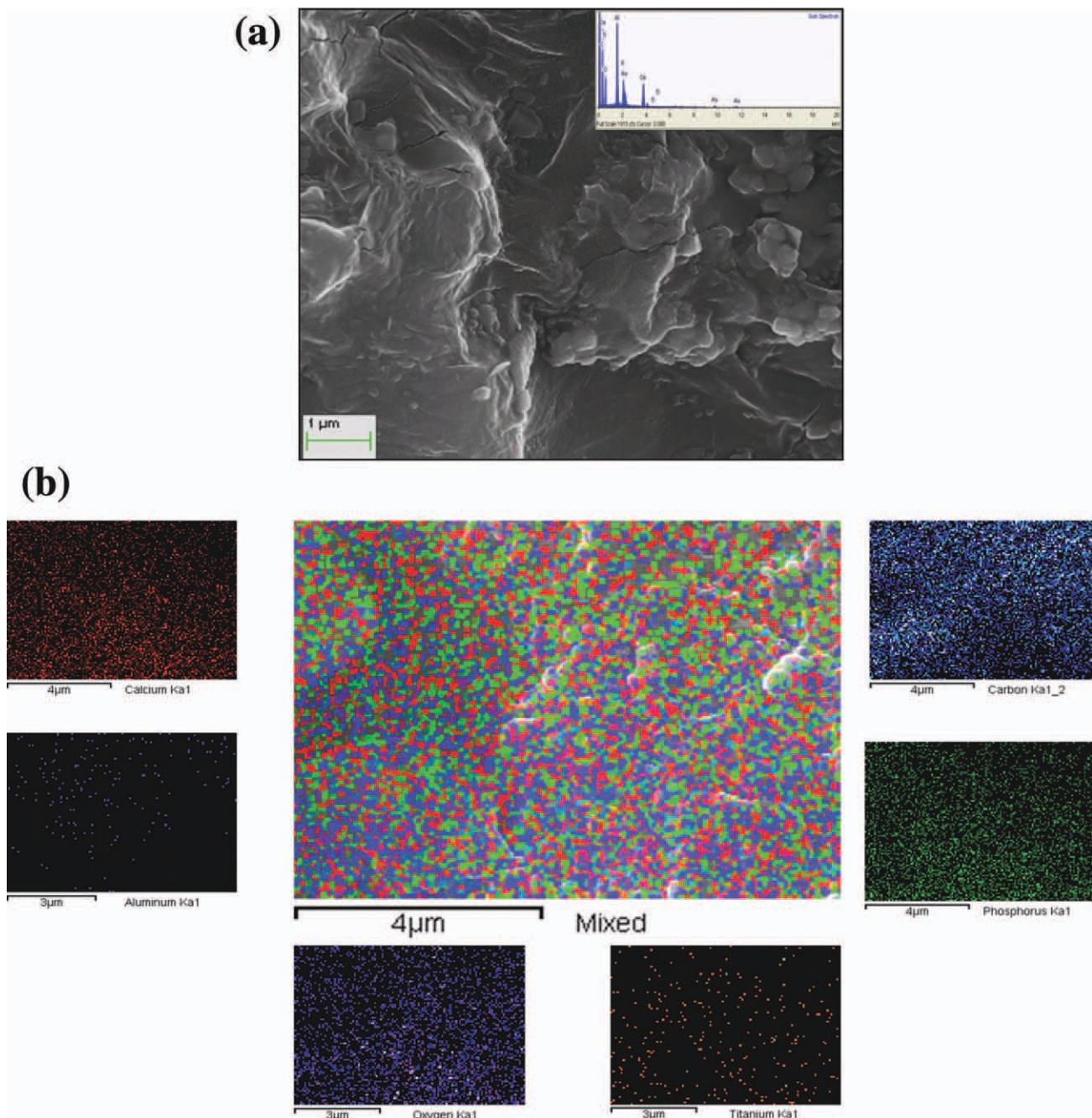


Figure 5 (a) Representative SEM micrograph, revealing the fracture surface morphology of CS₇ composition. (b) Mapping of the different elements on some selected region of the composite using SEM-EDS system. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

direct contact method using mouse fibroblast cells (L929) and human osteosarcoma (SaOS2) cell lines were carried out under scientifically controlled condition as per ISO-10993-1 guidelines (Biological testing of Medical Devices-Part 1: Guidance on selection of Test (ISO 10933-1)). As part of our experiments, L929 and SaOS2 cells were seeded on selected samples and kept in a CO₂ incubator (37°C) for desired incubation periods. Cell attachment on the standard biocompati-

ble disc (poly-L-lysine-coated glass cover slips, negative control sample) is shown as reference behavior. Observing Figure 9, it is clear that fibroblast cells proliferate to a considerable extent on the control sample. At many of the investigated regions, it was observed that the cells were attached with each other with their extended lamellipodium. In case of composites, the cells are flattened with their filapodia in multiple directions to facilitate the increased cell-material

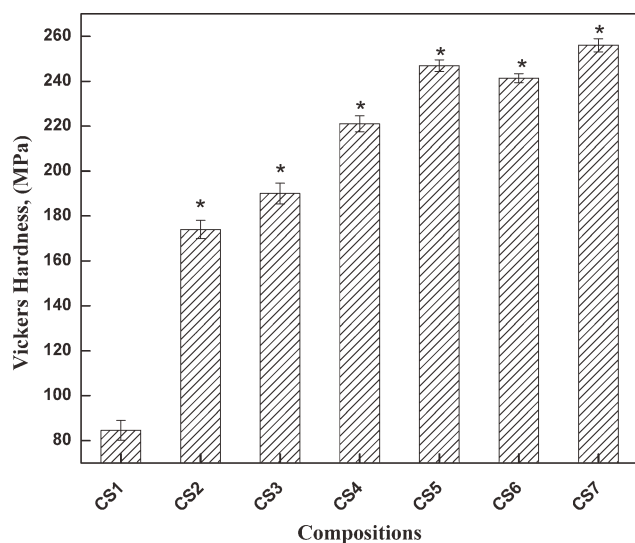


Figure 6 Vickers hardness of the prepared compositions. The asterisk mark (*) represents significant statistical difference at $p < 0.05$.

interaction. In addition, there is a little signature of globular shaped cells. The cell viability results will be discussed in the next section.

Figure 10 clearly shows that the SaOS2 cells were significantly spread with their multidirectional and elongated actin filament (filopodia) extension. Overall morphological features of SaOS2 cells suggest evidences of good cell-to-cell attachment and cell adhesion on composite surfaces. A closer observation of SEM images in Figure 10 also reveals the formation of extracellular matrix (ECM). It is known that ECM provides support to anchorage-dependent cells as well as regulates the inter-cellular communication, and the dynamic behavior of the cell. This qualitatively suggests a higher SaOS2 cell activity than L929 cells on the same substrate.

Figure 11 reveals the observation of nonviable cells on the surface of the composite prepared using aromatic solvent (xylene) at the time of ball milling. Although, other solvents are not used, the present study suggests that xylene as a solvent should be avoided in developing HDPE-HA- Al_2O_3 composites.

MTT assay

To investigate cell viability on the surface of the developed composites, MTT assay was performed. L929 and SaOS2 cells were cultured on the

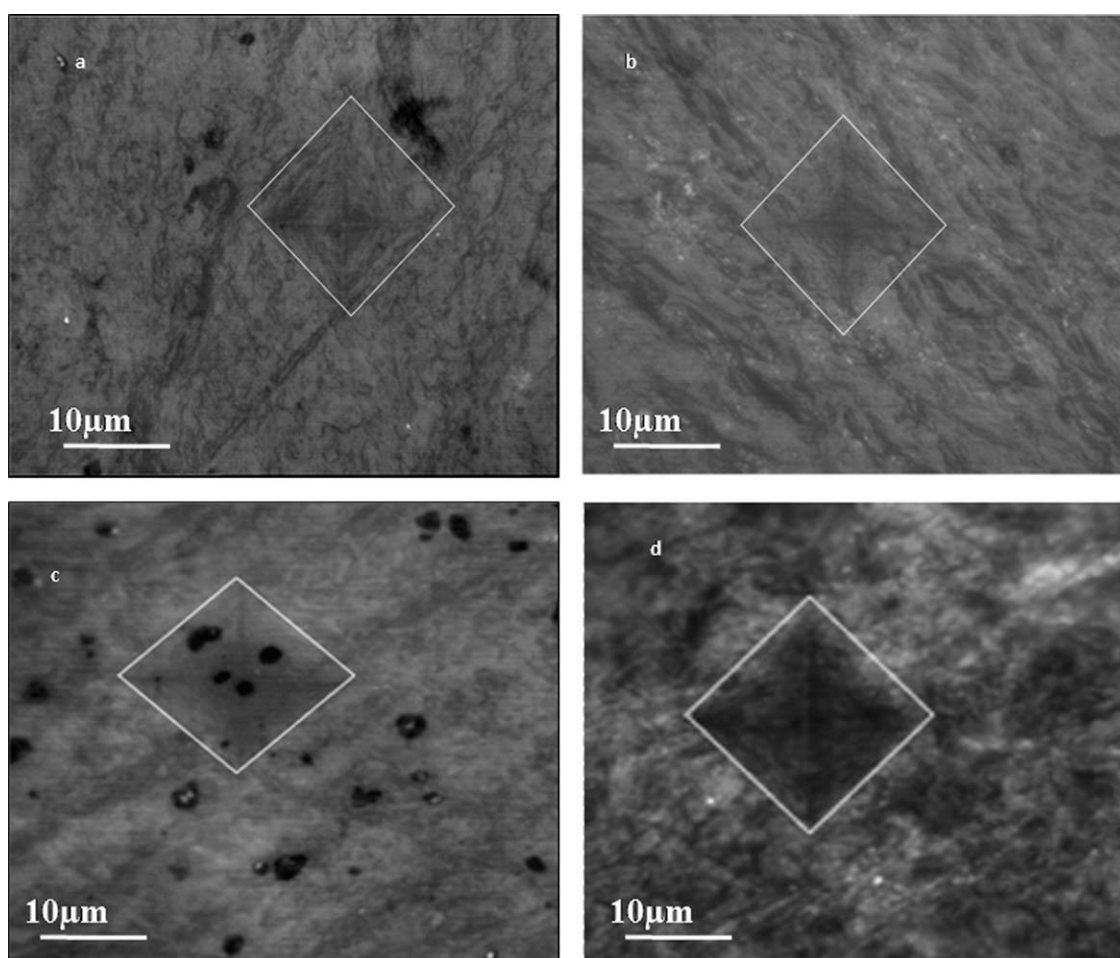


Figure 7 SEM images showing the Vickers indentation on compositions (a) CS1, (b) CS3, (c) CS5, and (d) CS7.

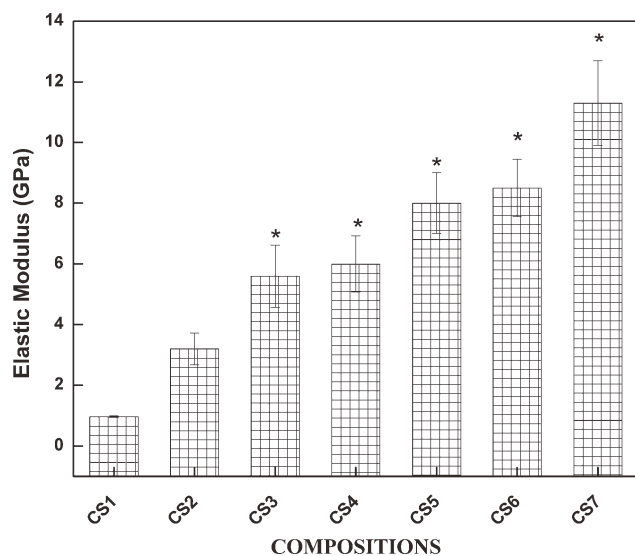


Figure 8 Variation of elastic modulus of the prepared compositions. The asterisk mark (*) represents significant statistical difference at $p < 0.05$.

composites following the previously described cell culture method.

Figure 12 shows the MTT assay results, obtained for the metabolically active mouse fibroblast (L929) cells on control, HA, CS3, CS5, and CS7 composites, after 3, 5, and 7 days of incubation. The SPSS-13 software was used to analyze the statistically significant difference in the obtained biochemical assay results. The univariate analysis of variance (ANOVA) method was adopted to analyze the result statistically. In particular, the *post hoc* multiple comparisons of the mean of independent groups were made using Tukey test at statistically significant value, $p < 0.05$. The asterisk mark (*) represents significant statistical difference at $p < 0.05$ and error bars correspond to ± 1.00 SE (standard error). The mean optical density increases with the incubation period for all the analyzed samples. However, the composite compositions CS3, CS5, and CS7 showed the significant difference in the cell density among the 3, 5, and 7 days of incubation periods. The similar results were also obtained for the composites, while the biochemical assay results were analyzed for SaOS2 cells adhered on prepared composites (Fig. 13). MTT results in Figure 13 confirm that the chemically coupled composites can support the metabolically active osteoblast cells and cell viability increases with incubation time. Therefore, the investigated materials can be used as a good substrate to support adhesion and proliferation of mitochondrially active osteoblast-like cells. MTT values obtained with L929 and SaOS2 cells, in combination, establish good cytocompatibility property of these composites in the context of bone replacement applications.

DISCUSSION

Processing results

Under optimized processing condition, the density data (Fig. 1) indicate that maximum consolidation is achieved in case of chemically coupled ceramic filled HDPE sample (CS7). In case of HDPE-based composites, it is observed that more than 95% theoretical density (ρ_{th}) can be achieved for all the samples at optimized processing condition. It is possible that the higher surface energy of the micron sized HA particles sometimes causes agglomeration and renders lower densification in the composites, compared to alumina reinforced composites. The calculated relative density of CS4 was 95.8% ρ_{th} and with the addition of CA, it increases to 96.5% ρ_{th} in CS5 composite. Similarly, CS6 shows 97.0% relative density, which was increased to 97.7% by the addition of CA (CS7). Therefore, a comparative study of the densities of composites with those of chemically coupled systems, when both fabricated by conventional molding technique establish that the use of CA provides better interfacial bonding by the incorporation of polar bonds and thereby better densification (Fig. 1). The introduction of polar group into the HDPE matrix provides better arrangement to ceramic grains as a resultant of interfacial bonding.

The analysis of FTIR results [Fig. 3(a,b)] provides more information on chemical bonding between ceramic fillers and polymer molecules. A comparison of FTIR spectra obtained with polymer blend and compression molded compact reveals that the intensity of FTIR peak at 3417 cm^{-1} for OH^- band changes during the compression molding of composite mixture. The peak at 3417 cm^{-1} ($-\text{OH}$) from polymer backbone chain, those at $1093, 1027, 604\text{ cm}^{-1}$ for PO_4^{3-} and peaks at $632, 3571\text{ cm}^{-1}$ for $\text{O}-\text{H}$ bending of HA, and peak at 1726 cm^{-1} for $\text{C}=\text{O}$ are clearly visible. This might be due to oxidation, which takes place during the compression molding at ambient atmospheric conditions, producing carbonyl groups in the polymer-ceramic system. It is possible that $(\text{HA})\text{Ca}^{+2}-\text{O}_2\text{P}(\text{O})-(\text{CH}_2)_2-\text{OC}-\text{O}-(\text{Polymer})$ bridges as well as H-bonding are formed at the interface of polymer.²³ It can be said that the formation of monomolecular layer is formed as a result of alcoholysis reaction with the CA. The surface active protons play a good role in this mechanism. Ti nucleus might act as electro-phobic or electro-philic for causing a catalytic rearrangement and redistribution of the molecular structure and molecular weight of polymeric phase. The prime advantage of titanate derived CA is their reaction with free protons at inorganic interface for the formation of monomolecular layer.

Microstructure

Figure 4(a-c) displays the surface morphology of ceramic filled HDPE matrix. SEM images of the

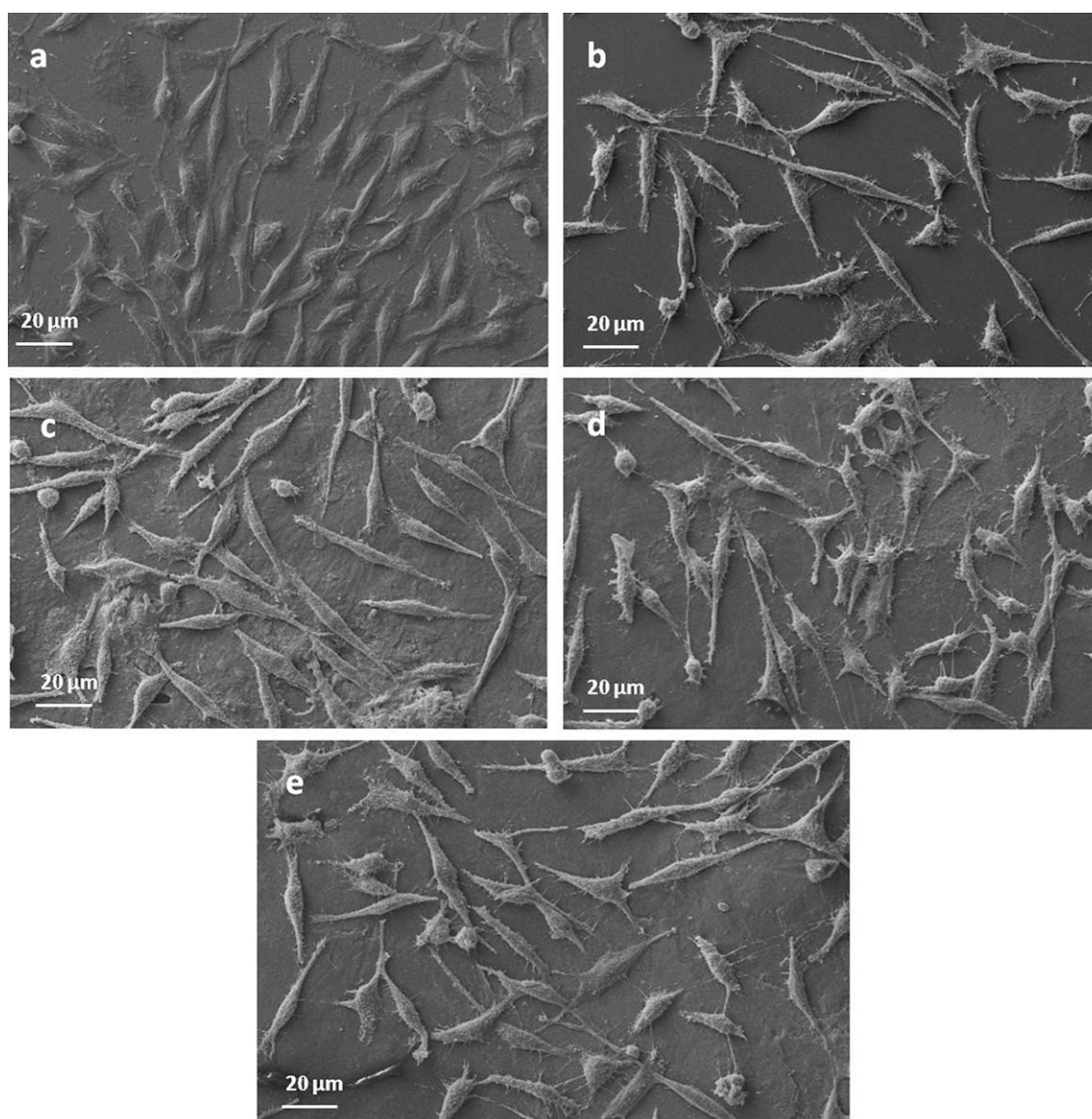


Figure 9 Scanning electron micrographs revealing cellular functionality of L929 mouse fibroblast cells (after incubation of 24 h) on various sample surfaces: (a) control, (b) HA, (c) CS3, (d) CS5, and (e) CS7.

microstructures of 40 wt % HA particles reinforced HDPE composites with CA (CS3) are presented in Figure 4(a). It is clear that the use of CA provides better interfacial bonding between the fillers and polymer molecules. Figure 4(b) shows the polished surface microstructure of CS5 sample and Figure 4(c) presents that of CS7, where alumina and HA particles (1 : 1) are dispersed in polymer matrix with CA. The use of CA at the time of mixing provides better increase in the interfacial bond strength.²⁴ Figure 4(c) shows the homogeneous mixing of the polymer-ceramic surface after curing.

The use of micron sized alumina fillers allows a greater surface area, to be available for polymer/filler interaction, adhesion, and interlocking. SEM-EDS compositional mapping of different elements

essentially reveals good distribution of ceramic fillers in polymer matrix [Fig. 5(a,b)].

Mechanical properties

From the experimental results, it is evident that the use of CA provides better bonding to the molecules. The hardness for pure HDPE (CS1) was found to be 84.6 MPa, which is increased to 256 MPa by the addition of 20 wt % HA and 20 wt % Al_2O_3 in presence of CA. It can be easily observed in Figure 6, that chemically coupled system shows higher values rather than the composites prepared without CA. The detailed univariate analysis using *post hoc* test among different compositions reveals that the hardness of all the developed hybrid polymer-ceramic

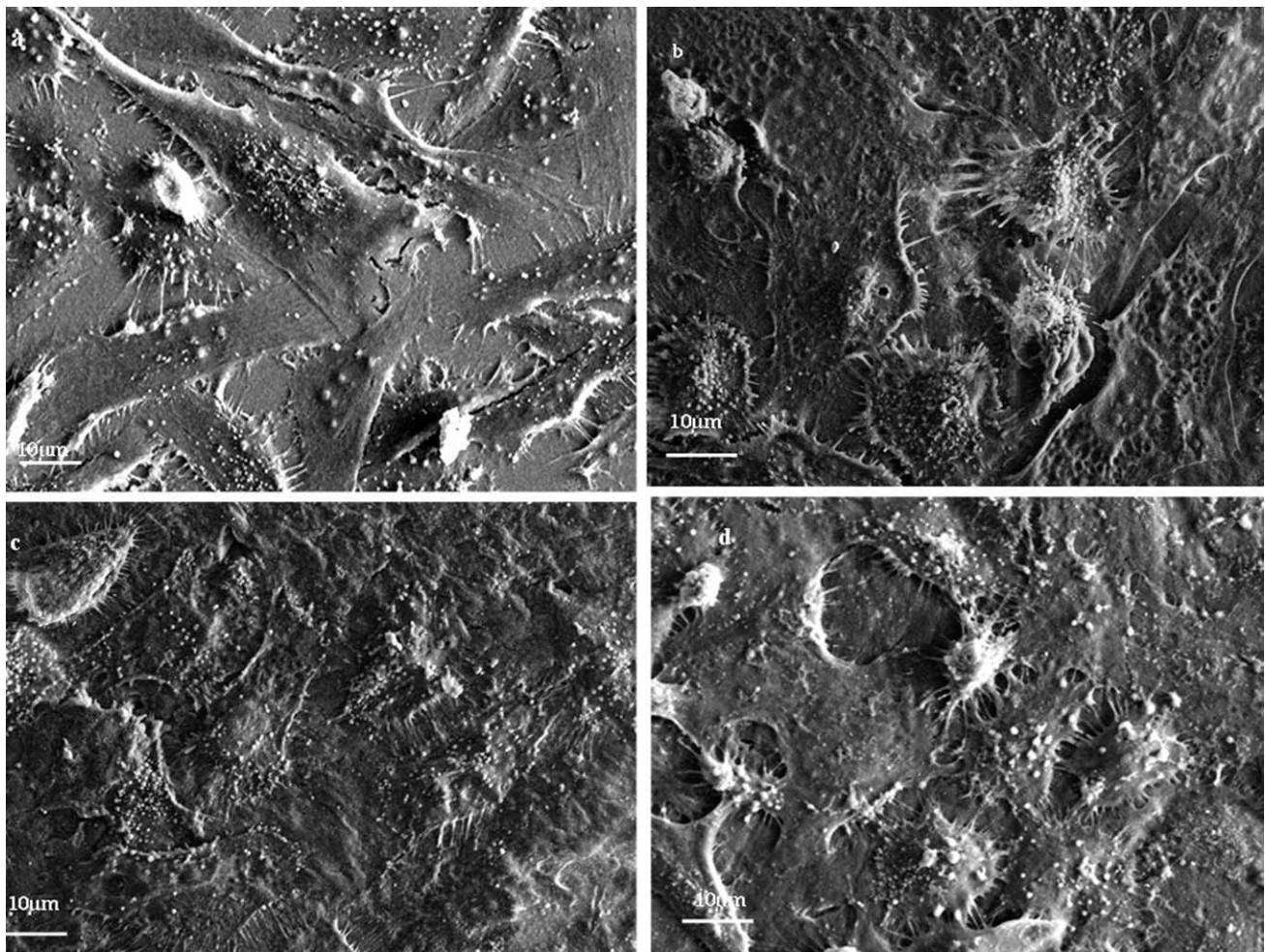


Figure 10 Scanning electron micrographs revealing cellular functionality of SaOS2 human osteoblast cells (after incubation of 72 h) on various sample surfaces: (a) HA, (b) CS3, (c) CS5, and (d) CS7.

composites show statistically significant difference with CS1 composition (Fig. 6).

In general, most of the polymers exhibit viscoelastic behavior, when loaded. This type of deform-

ability is possibly enhanced at indent contacts in presence of complex stress conditions. It is evident that pure HDPE, being softer, can easily distort under indentation loading, which accounts for low

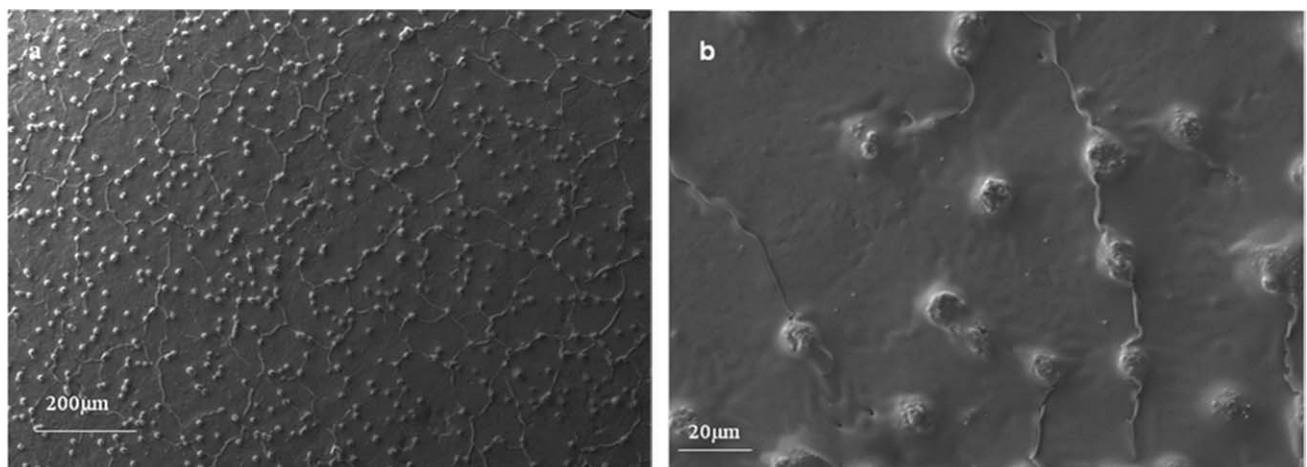


Figure 11 Scanning electron micrographs revealing dead L929 mouse fibroblast cells (after incubation of 24 h) on sample CS7 processed using xylene.

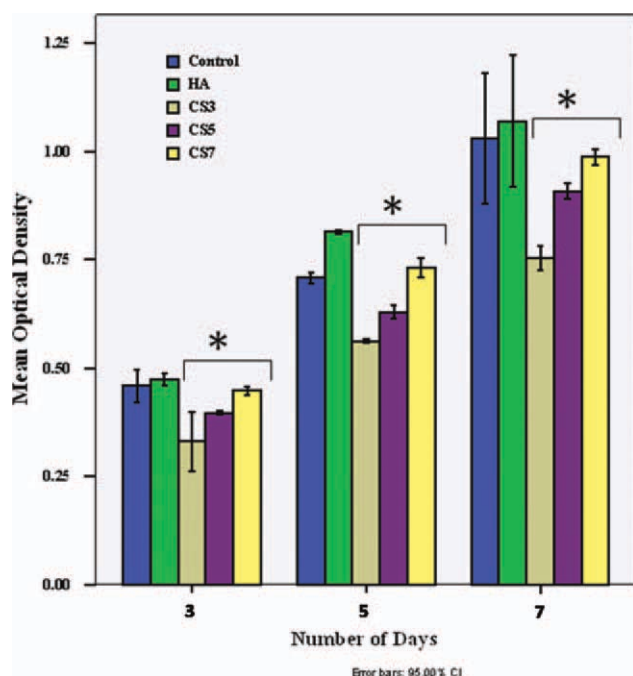


Figure 12 MTT assay results showing the L929 cells on control, HA, and Hybrid composite samples after 3, 5, and 7 days of culture. * represents significant difference at $p < 0.05$ with respect to compositions and Error bars correspond to ± 1.00 SE for number of days of culture. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

hardness of polymer. It is clearly observed in Figure 7 that in case of pure HDPE (CS1), the indent impressions are not clearly distinct, indicating the softness of the thermoplastic phase and a significant amount of recovery of viscoelastic matrix even after the removal of load. Except CS1, all other prepared composites showed a better resistance to deformation. This might be due to the presence of the ceramic phase as well as the presence of CA in the polymer matrix, which provides better hardness in CS3, CS5, and CS7 composite systems.

It can be reiterated here that Young's modulus or Elastic modulus (E), as reported in the present work, are indicative values, as values of E , like many other mechanical properties (hardness, fracture toughness) are dependent on test method employed. Nevertheless, the E -values presented in Figure 8 can be used to compare dynamic elastic modulus values among the investigated composites only.

The *post hoc* multiple comparisons show significantly higher dynamic elastic modulus of all the polymer-ceramic composites with respect to CS1 and CS2 compositions (Fig. 8). From our experimental results, it is clear that the addition of titanate CA along with alumina particles evidently improves the hardness and dynamic elastic modulus properties of the composites with respect to monolithic HDPE (Figs. 6 and 8).

As far as the microstructure of the prepared polymer ceramic composites is concerned, it can be said that the CA molecules are placed in polymer matrix, which results in interfacial bonding between the polymer and ceramics phases. It is also expected that the mechanical interlocking of ceramic fillers with the HDPE matrix allows effective load transfer from polymer matrix to harder ceramic phases, leading to better modulus and hardness with respect to HDPE.

The advantage of titanate treatment lies in the enhancement of hydrophobicity, organophilic, and organofunctionality. The incorporation of titanates in polymeric system often recruits dispersion, adhesion and rheology of the system.²⁴ The CA provided improved interfacial adhesion between polymer and ceramic phase by the incorporation of polar groups. Long chain structure of HDPE matrix is entangled with the ceramic phases by hydrogen bonding, which reflects the formation of comparatively harder surface compared to pure polymer (CS1). On the other hand, it is believed that identical assortment and better mechanical interlocking of micron sized harder alumina fillers leads to better hardness and stiffness.

Biocompatibility evaluation

It needs to be mentioned here that an earlier study¹⁷ from our research group reported good cytocompatibility property of HDPE-HA- Al_2O_3 hybrid composites. However, those composites were fabricated

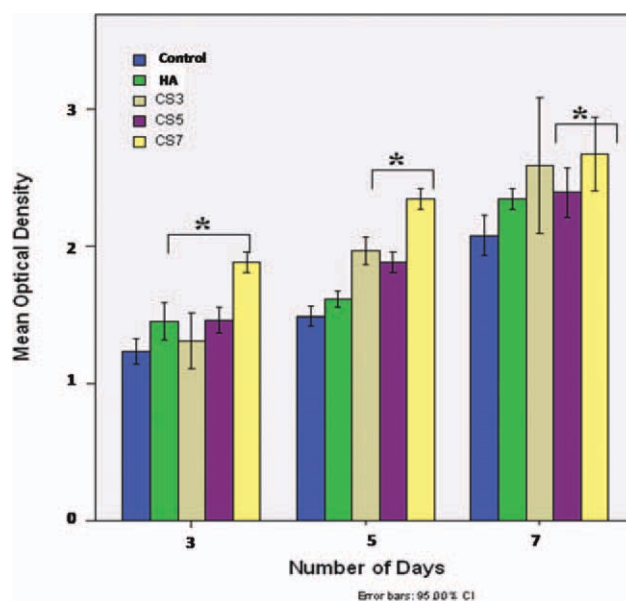


Figure 13 MTT assay results showing the SaOS2 cells on control, HA, and Hybrid composite samples after 3, 5, and 7 days of culture. * represents significant difference at $p < 0.05$ with respect to compositions and Error bars correspond to ± 1.00 SE for number of days of culture. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

without any CA. Therefore, it was of great interest to assess the biocompatibility property of similar composite compositions with the addition of CA.

In the present study, some selected samples CS3, CS5, and CS7 along with HA and control sample were placed in culture medium, containing L929 or SaOS2 cells. After 24 and 72 h incubation of L929 and SaOS2 cell lines, respectively, in CO₂ incubator (37°C), the seeded samples were dehydrated and dried using Hexa methyl di-silazane (HMDS). Subsequently, SEM images were acquired and comparative studies of cell adhesion behavior on different surfaces were made. The comparison among the composites (CS3, CS5, and CS7) suggests that the cells adhered on the composite surfaces has comparable cell density to that of HA (Fig. 9).

A closer observation of SEM images [Figs. 9(a–e)] clearly reveals that apart from lamellipodia extension, the cell adhesion and spreading were facilitated through their filopodium extension on the substrates. In all the cases, cellular functionality, cell-to-cell contact is clearly evident, which form cellular network. As far as the comparative cell behavior is concerned, more flattening with multidirectional extension of filopodium is observed in the CS3, CS5, and CS7 composite. The fine extensions of the growth cone are known as “filopodia”. The filopodia morphology is the evidence of healthy and migrating cell. The composite surface leads to the similar cell behavior because of the fact that composite substrate supports cell adhesion and proliferation. The flattened cells increase the cell-material interface area and hence, the biocompatibility of the developed composites can be realized. In addition, the cell migration is also realized on the developed composites, as evidenced by the presence of extended lamellopodium on biocomposite surfaces. Figure 10 illustrates the expanded morphology of human osteoblast cell line on the prepared polymer-ceramic composites. Human osteoblast, SaOS2 cells, symbolize fully mature osteoblastic cell morphology with wide spreading and good proliferation on the substrate (Fig. 10).

On the basis of the above discussion, it can be safely stated that all the hybrid polymer-ceramic composites with CA exhibit good cellular functionality property. The term “good cellular functionality property” has been used to emphasize that the cells have adhered, flattened (SEM results), and the number of viable cells increases with incubation time (MTT results). This can be explained on the basis of homogeneous distribution of HA in the biocomposites. Among various constituent phases (HDPE, HA, Al₂O₃, and CA), HA is the most biocompatible (bioactive) phase and therefore, the presence of HA is expected to facilitate initial adhesion of the anchorage dependent cells, followed by the cell flattening and cellular bridge formation.

Another point requires brief mention. It is important to use appropriate solvent with CA. From Figures 11(a,b) it is observed that when aromatic solvent-like xylene was used at the time of powder mixing before compression molding, cell toxicity was observed in composites processed with xylene. In particular, the apoptosis of fibroblast cells on composites fabricated using these solvents were recorded under similar culture conditions. However, the use of IPA solvent does not have any such adverse effect. Therefore, the combination of CA and solvent needs to be appropriately selected, while developing polymer-ceramic biocomposites.

MTT assay results (Fig. 12) using mouse fibroblast cells (L929) on control, HA, CS3, CS5, and CS7 after 3, 5, and 7 days of culture, showed that the cell density was increased with the incubation period. The increased cell density showed statistically significant difference among CS3, CS5, and CS7 composites with respect to timescale of incubation. The same analysis has been carried out for SaOS2 cells adhered on prepared composites (control, HA, CS3, CS5, and CS7), which shows that the number of cells adhered on the composite surfaces were increased with an increase in incubation period (Fig. 13). The *post hoc* multiple comparison among the samples revealed that the sample CS7 showed significant difference with rest of the compositions. All other compositions followed the similar trend of cell viability with time. The viability of the SaOS2 cells seeded on the composites was found to be superior in case of prepared polymer-ceramic composites than that of cells cultured on HA. In conclusion, it can be said that the prepared polymer-ceramic hybrid composite shows good cytocompatibility as well as enhanced cell viability property with respect to incubation period.

Our experimental results reveal better cell viability with SaOS2 cells than L929 cells, when cultured on identical samples under similar culture conditions. The enhanced cell adhesion and viability of SaOS2 cells compared to L929 cells on the prepared composites can be explained by giving the reference to earlier reported results. Wang et al.²⁴ reported that different cells prefer different surfaces, e.g., a higher percentage of osteoblast-like cells preferred to attach to a rougher surface,²⁵ while fibroblasts likes to grow more on smoother surfaces.²⁶ In our study, L929 cells and SaOS2 cells also showed the similar trend, as the SaOS2 cells showed higher density compared to L929 cells. It also needs to be mentioned here that the polymer samples were coarse ground using emery paper (No. 1200). Unlike fine diamond polish in case of ceramic/metallic samples, the used polymer samples were rough compared to the diamond finished samples.

It is important to mention here that *in vivo* tests are recently being conducted on CS7 samples in rabbit's femur and after 14 weeks of implantation

histopathology analysis revealed active bone formation with the presence of osteoid and osteoblasts at bone implant interface. As osteoclasts are also present at the implanted sites, such observations indicate the process of bone remodeling. The observation of bone integration as well as the absence of any significant inflammation around the implantation site reveals that the investigated material does not release any toxic agent *in vivo* and such material can be used as an implant material. The results will be reported later. Further clinical trials and other biological test protocols, as prescribed in ISO-10993 will be carried out in future before such materials can be used as implants.

CONCLUSIONS

In the present work, the effect of CA on physico-mechanical and *in vitro* biocompatibility of the compression molded HDPE-based composites has been studied. On the basis of the measurements of physical properties as well as evaluation of the cytocompatibility property, we arrive at the following conclusions;

1. The consolidation of HDPE/HA/Al₂O₃ compositions with 2 wt % CA, using compression molding (140°C, 0.75 h, 10 MPa pressure) leads to the development of chemically coupled hybrid polymer-ceramic composites with around 98% relative density (ρ_{th}).
2. XRD results confirm the presence of HA and Al₂O₃ in semi-crystalline HDPE. FTIR data indicate alcoholysis reaction, which results in better interfacial locking in the investigated polymer-ceramic system. This was facilitated by surface bearing protons.
3. The microstructural analysis confirms homogeneous distribution of ceramic particles in the polymer matrix. The use of micron sized alumina fillers allows a greater surface area, to be available for polymer/filler interaction, adhesion and interlocking. SEM-EDS compositional mapping of different elements essentially reveals good distribution of ceramic fillers in polymer matrix.
4. A comparison in terms of dynamic elastic modulus and hardness reveals better material properties of the chemically coupled HDPE-HA-Al₂O₃ composites *vis-à-vis* uncoupled composites of similar compositions.
5. Biocompatibility test using L929 mouse fibroblast cell line and SaOS2 human osteoblast cells provide clear evidence in support of cell adhesion and cellular functionality of surfaces of chemically coupled composites. The cell-to-cell interaction and multidirectional extension of filopodia has been explained on the basis of homogeneous distribution of HA phase.

6. The results of MTT assay essentially reveals good viability of both L929 and SaOS2 cells in contact with the chemically coupled composites. Also, the number of live cells increases systematically with an increase in incubation time for the developed composites. The combination of good cell adhesion and cell viability was explained on the basis of good cellular functionality of both polymer and ceramic phases i.e., HDPE, HA, and Al₂O₃.

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