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Biocompatibility evaluation of a novel hydroxyapatite-polymer coating for medical implants (in vitro tests)

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Abstract Nanocomposites consisting of hydroxyapatite (HA) and a sodium maleate copolymer (maleic polyelectrolyte), synthesized by hydrothermal method and deposited on titanium substrates by Matrix Assisted Pulsed Laser Evaporation (MAPLE) technique were tested for the biological properties. Coating bioanalysis was carried out by triple staining of actin, microtubules and nuclei followed by immunofluorescence microscopy. Within 24 h cells that occupied the biomaterial surface displayed the morphology and cytoskeleton pattern similar to the controls. Cells grown on nanocomposite coated surfaces had a higher proliferation rate than their counterparts grown on Ti coated with HA alone, indicating that maleic polyelectrolyte improved surface bio-adhesive characteristics. The capacity to induce cell attachment, spreading and proliferation demonstrated the potential of Ti coated with HA-polymer nanocomposites to be used as scaffolds in dental or orthopedic implantology.

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1 Introduction

Despite the reputation of favorite metal materials in orthopedics and dentistry, Ti-based alloys proved to cause inflammatory responses on long term tissue remodeling due to the unwanted leakage of toxic metal ions in the surrounding areas [1–4]. This major drawback has been overcome by the coating of the metallic surfaces with ceramic-like calcium phosphate containing materials that mimic hydroxyapatite (HA), the natural mineral phase of bone and teeth [5]. HA obtained by synthesis is biocompatible and bioactive in the body and has been shown to improve the rate of osseo-integration of the implant with ordered, living bone [6]. However, the mechanical properties of HA-specifically brittleness, poor tensile strength and impact resistance in many load-bearing applications as well as the HA solubility observed to occur in body fluids in long-term contacts [7, 8] restricted its application. Therefore, in attempt to enhance both biological and mechanical performances of the HA coatings, combinations with various compounds, are continuously created. The composite materials including polymers and hydroxyapatite were largely investigated regarding their preparation, properties, and application. Among these the natural polymers were frequently used due to their excellent availability, biocompatibility, and lack of toxicity. Many natural polymers based on proteins or polysaccharides were examined in this regard: gelatin, collagen, atelocolagen, fibrin, chitosan, hyaluronic acid, and phosphorylated cellulose, were the preferred [9–15]. Some synthetic water soluble polymers were also used as partners in composites with HA [16]. In some cases the polymers acted as crystallization promoters, their effect being attributed to the partial or total interfacial adsorption of

the polymer on the surface of growing crystals. Most polymers associated with HA are polyelectrolytes (polymers bearing charged groups), such as dermatan sulfate, chondroitin sulfate, heparin, hyaluronan, and phosphorylated cellulose [14, 15]. Their effect as crystallization regulators was already described [17]. They demonstrated the ability to control the crystal growth of various inorganic or organic sparingly soluble salts: calcium carbonate, barium sulfate, calcium phosphates. Most polyelectrolytes acting in the crystallization process are anionic, especially bearing carboxylic groups, for instance poly(acrylic acid). In our previous contributions we evidenced the effect of some polyelectrolytes based on maleic anhydride copolymers as inhibitors of crystal growth by precipitation of calcium carbonate [18] or calcium oxalate monohydrate [19]. Added in very low amounts these polyelectrolytes decreased the rate of crystal growth and influenced the size and the shape of crystals. Based on these results we investigated their effect on the phase separation of hydroxyapatite at room temperature, as well as their performance as additives by the preparation of hydroxyapatite by hydrothermal method, when nanosized particles of polymer-HA composite have been obtained [20, 21].

In order to be functional, hybrid coatings with complex chemical composition have to be deposited in such a way that their components to be preserved. Matrix Assisted Pulsed Laser Evaporation (MAPLE) and Pulsed Laser Deposition (PLD), are deposition techniques that differ from each other in target preparation and laser-material interaction mechanisms. MAPLE was derived from PLD to overcome the difficulties it faced including some irreversible decomposition damage, or alteration of the organic or very complex materials that were processed [22]. Specifically in MAPLE an organic/inorganic compound is dissolved in a volatile solvent (matrix) to form a solution. As a consequence, the organic molecule or biomaterial to be deposited is surrounded by a large amount of matrix (frozen solvent). Both thermal and photonic damages during laser irradiation-induced evaporation are therefore reduced or even altogether avoided.

The ongoing development of hybrid coatings requests careful investigation of their biocompatible properties in order to avoid cellular adverse reactions. In vitro tests using established cell lines or primary culture systems provide valuable information to predict biological reactions to materials when placed into or on tissues in the body [23–26].

This study presents the data obtained from the in vitro biocompatibility tests of the novel organo–inorganic nanocomposite based on HA and sodium maleate copolymer synthesized by the hydrothermal method and deposited on Ti substrates by MAPLE technique.

2 Materials and methods

2.1 Ti coated with hybrid nanocomposites of HA–sodium maleate copolymer

The copolymer used as organic partner in the fabrication of hybrid composites was the 1:1 alternating copolymer sodium maleate (NaM)–vinyl acetate (VA) which was obtained from the corresponding maleic anhydride (MA)–VA copolymer. The parent copolymer was synthesized and characterized as previously described [27, 28]. The copolymer NaM–VA was prepared by mild hydrolysis with diluted aqueous NaOH solution at room temperature for 24 h and purified by diafiltration until the filtrate conductivity was lower than 20 μS/cm. The copolymer NaM–VA was recovered by freeze-drying and further referred as MP copolymer. Hybrid nanocomposites, HA–MP were obtained by in situ hydrothermal method starting from soluble salts of calcium, phosphorous and NaM–VA copolymer [20]. Briefly, soluble salts of calcium and ammonium phosphate aqueous solutions were mixed with an appropriate amount of mineralizing reagent (ammonia solution) and MP. The hybrid nanocomposite powders were hydrothermally synthesized at 100 °C, for 3 h at a pressure between 20 and 100 MPa and the resulted precipitate was filtered, washed and oven dried at 100 °C for several hours. 0.2% or 1% HA–MP powder containing 20% NaM–VA copolymer, referred further in the paper as HA–MP1 and HA–MP2, respectively, were used as frozen targets to obtain coatings (thin films) on Ti surfaces (substrates) by MAPLE technique. The main parameters of the depositions were: substrate temperature 30 °C, pressure 13 Pa N_2, distance 3 cm, energy 65 mJ, spot surface 9.2 mm^2. Alternatively HA was deposited on Ti by PLD. The following materials were investigated for the biocompatibility: (a) Ti coated with HA (Ti–HA); (b) Ti coated with HA–MP hybrid structures (Ti–HA–MP1 and Ti–HA–MP2); (c) metallic ceramic composites core-shell structure, stainless steel–Al_2O_3 prepared by a sol–gel method starting from stainless steel powders and soluble salts of aluminum with the following ratios 1:1 (MA1); 5:1 (MA2); 10:1 (MA3) in the National R&D Institute for Non-ferrous and Rare Metals, Romania. Surface coating analysis was done using a FT-IR Spectometer with ATR device (model 2004). All materials were obtained as circular samples with diameter of 12 mm, in order to fit into the 24-well tissue culture plates and were sterilized prior use two times for 90 min at 110 °C each.

2.2 Cell culture

Human Embryonic Kidney cells (HEK293 line) was a generous gift from Dr. E. Condac, Biochemistry Department, Faculty of Biology, University of Bucharest. HEK

cells are very suitable for screening large number of samples for cytotoxic compounds, due to the fact that their viability is affected by slight changes of tissue culture medium parameters (pH, ion concentrations), easy and inexpensive to be cultured. HEK cells were also used in the rapid evaluation of the biomaterial surface qualities to induce cell proliferation; HEK cells are highly proliferative, in a reasonable time period (they almost double the number within 24 h), and in suspension are round shaped, easily detected with Trypan Blue and therefore can be accurately counted. Human Dermal fibroblast culture (HDF) was from Dr. L. Moldovan, National Institute of Research and Development for Biological Sciences, Bucharest. Adult Human Mesenchymal Stem Cells (MSC) were isolated by density gradient centrifugation [29] from whole bone marrow of a 65-year-old patient receiving a bone implant. At second passage MSC observed by phase contrast microscopy displayed a fibloblast-like morphology reported by other studies too [30]. In order to demonstrate that during obtaining and manipulation these cells did not delineate to another phentotype, the MSC phenotype was validated by flow cytometry when cells were found negative for CD14, CD34, CD45 and positive for CD13, CD29, CD90 (data not shown). HEK cells and HDF were cultured in RPMI 1640 medium containing 10% (v/v) FCS (Sigma), 50 U/mL penicillin and 50 mg/mL streptomycin (Life Technologies). All cells were grown in an atmosphere of air /CO_2 (19:1) at 37 °C.

2.3 Reagents and antibodies

Trypan Blue 0.4% solution (Sigma), Cell Titer 96 Aqueous One solution Cell Proliferation Assay (Promega) for MTS assay, 0.05% trypsin–EDTA (Gibco), mouse anti-α tubulin, Alexa Fluor 594 phalloidin, goat anti-mouse Alexa 488 were from Invitrogen Molecular Probes, Vectashield with DAPI (Vector Laboratories, Burlingame, CA).

2.3.1 Cell viability

The standard MTS -3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium inner salt solution assay was performed as follows: 8.0×10^3 HEK cells were seeded in 96-well microtiter plates (Nunc) and incubated over night at 37 °C. Next day the medium was removed and adherent cells were further cultured for additional 24 h into a culture medium resulted from 48 h period incubation with biomaterials. Following the incubation time 20 μL of MTS reagent was added in each well and samples were incubated for 2 h at 37 °C and the absorbance was read 450 nm in a microplate reader (Anthos htIII Lab Tech Instruments). The control was represented by cells grown in standard conditions for 48 h. The absorbance values were directly proportional to number of metabolically active cells (live cells). Alternatively cell viability was determined by Trypan Blue exclusion test. Cells grown in direct contact with the biomaterial surfaces for the indicated time periods were detached by short incubation with trypsin. Cell suspension was diluted with Trypan Blue solution and the mixture was incubated for 5 min at RT. Dead cells were blue whereas viable cells were impermeable for the dye and were counted in a hemocytometer.

2.3.2 Cell proliferation

Cell suspension was layered on the nanocomposite surface and cultured for different time periods. The attached cells were trypsinized and the number of viable cells was determined with Trypan Blue exclusion test. The difference between cell number at the end and the beginning of culture period represented cell proliferation and was expressed as % of the control sample.

2.3.3 Fluorescence microscopy

Cells were cultured on the nanomaterial surface or in standard conditions (borosilicate cover glass), fixed and permeabilized following a previously described protocol [31] and labeled for polymerized actin with phalloidin-Alexa fluor 594 (1:50), microtubules with mouse anti-tubulin antibody (1:200) followed by goat anti-mouse Alexa 488 (1:400). Finally samples were mounted in Vectashield with DAPI (stain for nuclei) and analyzed with a Nikon Eclipse E600 fluorescent microscope. Images were processed using Adobe Photoshop 7.0 software.

3 Results

3.1 Brief characterization of Ti coated with hybrid nanocomposites of HA–sodium maleate copolymer

Fourier transformed infrared spectrum of HA–MP composite revealed that a chemical bond is formed between inorganic phase (calcium phosphate) and copolymer with the participation of phosphate or/and carboxyl group (research project no 46/2005, unpublished results). In order to demonstrate the presence of the HA–MP coating on Ti surface, the comparative FT-IR spectra of titanium substrate and nanocomposite hybrid coating on titanium were investigated. As shown in Fig. 1 most significant are the

band at 1,238 cm^{-1} corresponding to C=O stretching from ester groups from vinyl acetate moieties, the band at 1,563 cm^{-1} attributed to symmetric stretching of carboxylate groups from the maleic moieties and the band at 1,709 cm^{-1} corresponding to C=O stretching mode from vinyl acetate groups. The last band is shifted to lower values probably due to intermolecular hydrogen bonding between inorganic and organic phase. The three characteristic bands corresponding to phosphate groups stretching (960, 1,092, and 1,048 cm^{-1}) from hydroxyl apatite are shifted to 974, 1,017, and 1,087 cm^{-1}, respectively. A possible explanation could be the interaction between phosphate groups and organic phase. The band at 3,222 cm^{-1} corresponds to OH polymer (intermolecular hydrogen bonding).

3.1.1 *Evaluation of soluble toxic compounds in Ti coated with HA and HA–MP nanocomposites*

The purpose of this test was to determine whether soluble toxic compounds were released by different biomaterials following incubation with the culture medium. The culture media after 48 h incubation period with different biomaterials were added onto HEK cells (previously plated), and further incubated with cells for additional 24 h. The cytotoxicity was estimated by determining cell viability by MTS assay and observing cell morphology by phase contrast microscopy. As Fig. 2 showed the viability of HEK cells grown in medium resulted from incubation with Ti–HA nanocomposites was similar with the control and cells had a normal polygonal phenotype. Lower numbers of viable cells, round-shaped, have grown in medium resulted from incubation with stainless steel–Al_2O_3. These data demonstrated that following incubation with the culture medium, toxic soluble compounds were probably released from stainless steel–Al_2O_3 samples whereas Ti coated nanocomposites were not cytotoxic and were eligible for additional further tests.

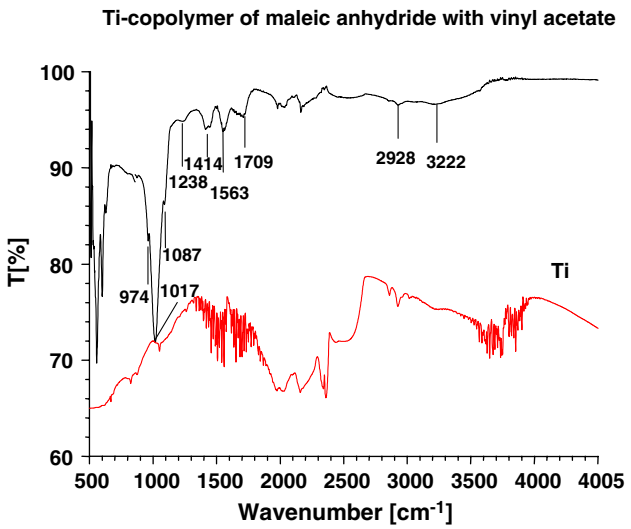

Fig. 1 The comparative FT-IR spectra of titanium substrate and nanocomposite hybrid coating on titanium

3.1.2 *Cell response to the HA–MP nanocomposite surface*

Cell response to the HA–MP nanocomposite surface was evaluated by studies of cytoskeleton dynamics and nuclei morphology. A suspension of HDF, MSC or HEK cells was layered onto the surface of different biomaterials and cultured together with control samples. Cells adhered to biomaterial surfaces were fixed permeabilized and simultaneously stained for actin, microtubules and nuclei and the data were presented in Figs. 3, 4. Within 24 h the polymerized actin long fibers (red fluorescence) that extended throughout the entire cell body forming a network were clearly visible, in both HDF and MSC (Fig. 3), or concentrated in the cell extremities in HEK cells (Fig. 4), similar to what was observed in cells grown in standard conditions. There were no visible differences between actin patterns of HDF and MSC grown on Ti–HA versus Ti–HA–MP naonocomposites (Fig. 3); however the actin staining of MSC appeared more intense in cells grown on Ti–HA–MP2 than on cells grown on Ti–HA–MP1 and less intense in HEK cells grown on Ti–HA than in cells grown on Ti–HA–MP (Fig. 4). Interestingly, on some surface areas of Ti–HA–MP nanocomposites HDF were positive for nuclear staining and poorly stained for polymerized actin, indicating they were superficially attached. In other areas clusters of HDF with strong actin staining were visible demonstrating a good spreading. This suggested that nanocomposite surfaces were organized in discrete areas to which cells preferentially attached. The actin-microtubule images analyzed for MSC cultured on Ti–HA or Ti–HA–MP showed that fine cortical microtubule network (green fluorescence) was aligned with the cell axis, indicating they were in interphase and in most cells did not co-localize with actin. All cell types grown on different Ti–HA or Ti–HA–MP biomaterials had uniformly stained nuclei with normal round shape morphology (Figs. 3, 4), unlike HEK cells which displayed small or fragmented nuclei when were cultured on the surface of metallic ceramic composites stainless steel–Al_2O_3 (MA1) (Fig. 5), demonstrated to contain cytotoxic soluble compounds (Fig. 2). The poor actin staining and round morphology of these cells indicated their superficial adherence to the surface of these biomaterials.

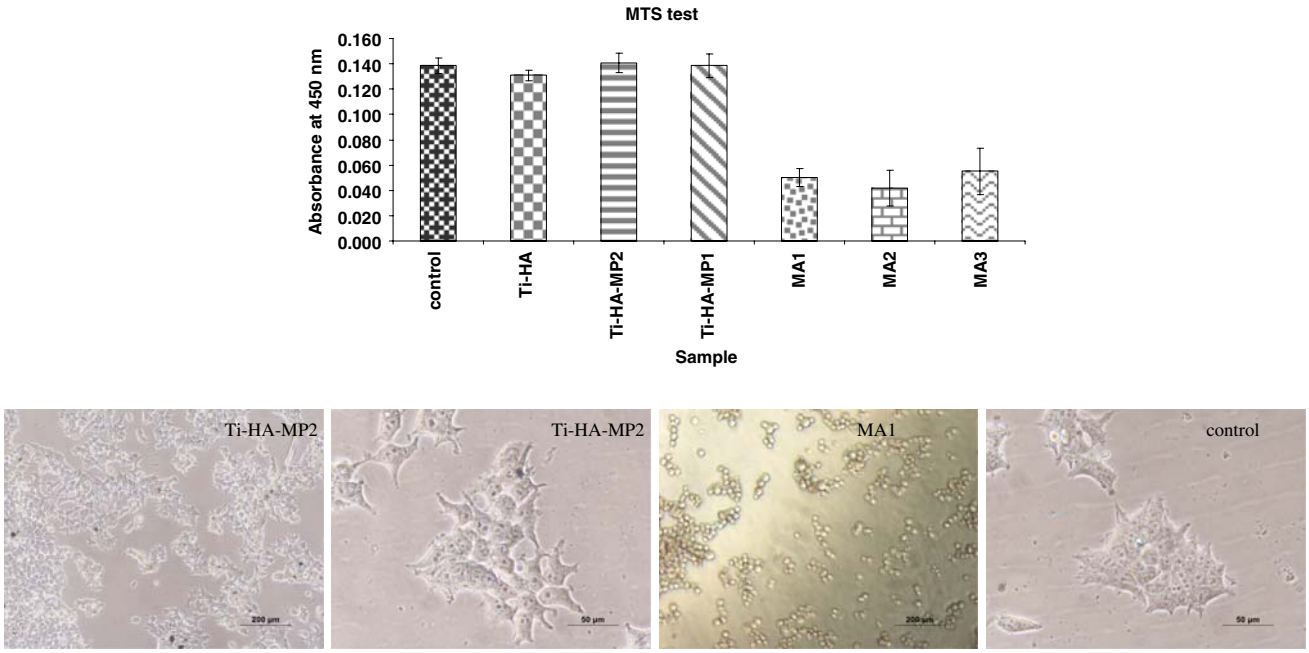

Fig. 2 Cytotoxicity of Ti coated with HA, HA–MP nanohybrids and of stainless steel–Al_2O_3 biomaterials. HEK cells grown in medium resulted from 48 h incubation with different biomaterials were assessed for viability by MTS assay and for morphology by phase contrast microscopy. Values are mean ± SD; $n = 4$

3.1.3 Cell proliferation on Ti–HA and Ti–HA–MP nanocomposites

Cell proliferation on Ti–HA and Ti–HA–MP nanocomposites was studied using HEK cells due to the fast proliferation rate of this cell line. About 5×10^4 cells were layered onto the Ti–HA, Ti–HA–MP1, Ti–HA–MP2 discs in a 24-well plate and cultured for 72 h. Cells were detached with trypsin and the number of viable cells was determined by Trypan Blue exclusion test. The proliferation of cells grown on the Ti surface coated with HA only, was approximately 50% whereas in samples coated with hybrid Ti–HA–MP1and Ti–HA–MP2 was approximately 65% and 77%, respectively, compared to 100% which was the control (Fig. 5).

4 Discussion

The modulation of cell activities in response to extracellular milieu represents the cell biology concept taken into account when new coating strategies are designed in implantology. It is well acknowledged that cellular processes as adhesion, biosynthetic activity, proliferation, differentiation, and inflammation are regulated by biomaterial surface in implant devices.

The hybrid structure consisting of HA and NaM–AV copolymer represents a novel coating expected to induce positive cellular responses at the interface with Ti implants. The innovative elements compared to the existing literature data are represented by both polymer and the synthesis technique [21]. The performances of the copolymers of maleic acid described in the introductive section prompted us to investigate their effect on the phase separation of hydroxyapatite at room temperature, as well as their performance as additives by the preparation of HA by hydrothermal method, when nanosized particles of polymer-HA composite have been obtained. In addition, the deposition of the high molecular weight compound at very low temperatures (–176 to 0 °C) avoiding the decomposition of organic phase was possible by MAPLE technique.

The investigation of Ti–HA and Ti–HA–MP biomaterials in parallel with metallic ceramic composites stainless steel–Al_2O_3, demonstrating the cytotoxicity of the latter, validated the in vitro system used in this study to investigate cytotoxicity. The culturing of cells with medium resulted from previous incubation with different biomaterials represented an useful test to discriminate between the effect on cell viability due to toxic components released into culture medium or to surface properties. In addition, samples screened with this rapid protocol and detected as cytotoxic could be discarded from further tests.

The assembly, length and stability of actin and microtubule filaments control cell shape, attachment, movements and ultimately the proliferation. Microtubules are also critical for cell growth and division. The nuclei morphology represents an indicative for biosynthetic and proliferative activity. All tested cell types grown in

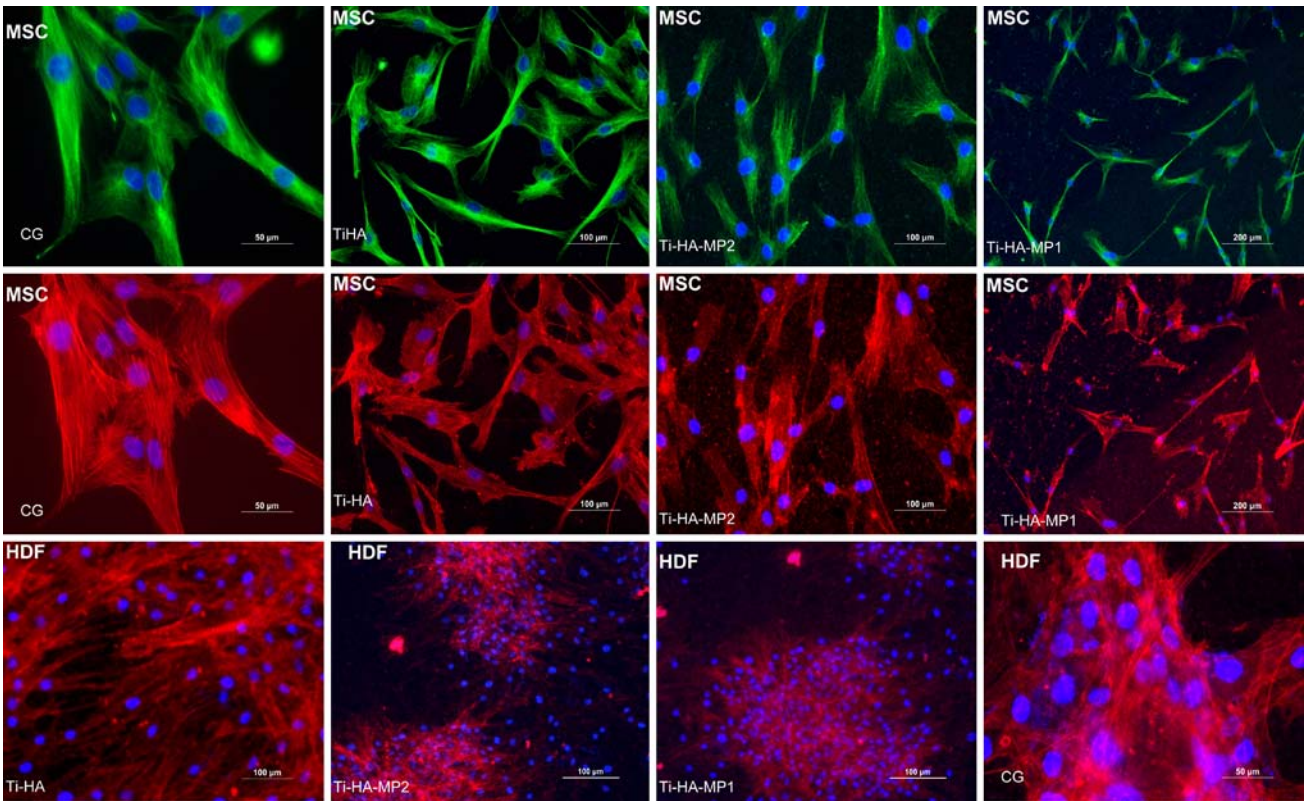

Fig. 3 Cytoskeleton organization and nuclei morphology of MSC and HDF cultured on different biomaterial surfaces. Cells were cultured for 24 h in direct contact with the following surfaces: (**a**) Ti–HA, Ti–HA–MP1, or Ti–HA–MP2 nanocomposites; (**b**) standard borosilicate cover glass (CG). Fixed cells were stained for actin (red), microtubules (green) and nuclei (blue) (see Experimental Section 2.3.3) and analyzed by fluorescence microscopy

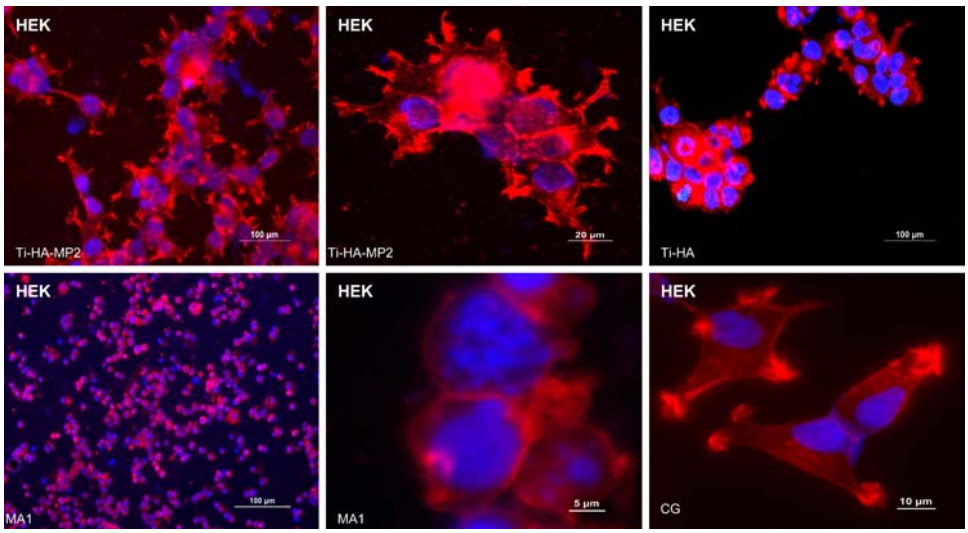

Fig. 4 Cytoskeleton organization and nuclei morphology of HEK cells cultured on different biomaterial surfaces. Cells were cultured for 24 h in direct contact with the following surfaces: (**a**) Ti–HA, Ti–HA–MP1 or Ti–HA–MP2 nanocomposites; (**b**) metallic ceramic composites core-shell structure stainless steel–Al_2O_3 (MA1); (c) standard borosilicate cover glass (CG). Fixed cells were stained for actin (red), and nuclei (blue) (see Experimental Section 2.3.3) and analyzed by fluorescence microscopy

standard conditions, within 4 h were round shaped with low red fluorescence indicating actin filaments partially polymerized at cell periphery or under the plasma membrane (data not shown); within 24 h each cell type acquired the specific morphology, the actin and microtubule networks were clearly visible (Figs. 3, 4). The cell morphology and actin-tubulin patterns in MSC and HDF grown on HA and HA–MP coatings were similar to controls and thus we could assume these surfaces were well tolerated by both cell types. HEK cells preferred HA–MP coatings to HA as demonstrated by cell morphology and actin pattern on these two surfaces. This finding was in

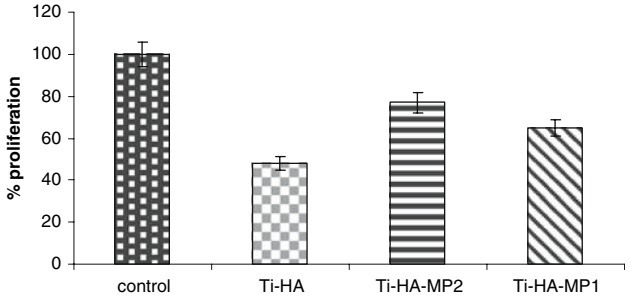

Fig. 5 Proliferation of HEK cells on Ti with HA and HA–MP coatings. Cells plated directly on the biomaterial surface, were cultured for 72 h, detached and counted. Viable cell number was determined by Trypan Blue exclusion test. Values are mean ± SD; $n = 4$

agreement with the proliferation data which showed that the number of HEK cells grown on Ti–HA–MP2 was higher comparatively to Ti–HA–MP1 or Ti–HA. As proliferation is an event that follows cell spreading this result suggested that the polymer substantially enhanced cell adhesion to the biomaterial coating surface. The differences in cytoskeleton and nuclei fluorescence observed in HDF grown on HA–MP1 and HA–MP2 coatings indicate that cells adhered better in some areas than in others and suggested that simultaneous actin-nuclei staining may work as a sensor of the biomaterial surface coating quality. MSC adhered on Ti–HA–MP biomaterials and displayed an undistinguishable cell and cytoskeleton morphology with controls which represent an indicative that these biomaterials are eligible to be further tested for MSC differentiation to osteblasts in vitro. It is interesting to note that surface properties could affect not only cell morphology and cytoskeleton but also nuclei as was observed for HEK cells grown on ceramic composites core-shell structure, stainless steel–Al_2O_3. The fragmented nuclei indicate that following cell interaction with these surfaces severe perturbations of nuclear processes may occur. Moreover, at this time it can be only speculated that cells grown on partially cytotoxic surfaces may still proliferate and generate progenies with altered genome.

5 Conclusion

A novel hybrid coating consisting of hydroxyapatite and a sodium maleate–vinyl acetate copolymer was obtained and deposited on Ti by MAPLE technique. The analysis of cytoskeleton, nuclei and cell viability tests demonstrated that cells adhered and proliferated on HA–NaM copolymer surfaces and that polymer enhances the coating biocompatibility properties. Albeit Ti coated with HA–maleic copolymer represents promising biomaterials in implantology, further research and in vivo tests are needed to fully validate their utilization.

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