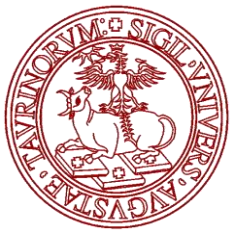

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Innovative ceramic surfaces for prosthetic application

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Introduction

Biomaterials

The interaction between living and non-living matter has always been a fascinating topic for men. For centuries, humans have been trying to implement the body's function by using inorganic materials. Around 300 BC, Phoenicians used ivory sculpted teeth in a creative way, then stabilized with gold thread to create a fixed bridge. Around 500 BC, Etruscans have customized the gold bands for soldering animals to restore oral function in humans (Abraham 2014). Still, about 600 b.C. the Mayans were distinguished by using pieces of shells as prostheses as substitutes for the mandible teeth. The X-rays images taken in the 70s by Maya's jaws show the compact formation of bone around the bone structure that looks surprisingly similar to the presence of blade (Abraham 2014).

A material that interacts with biological systems to evaluate, treat, sustain or replace any tissue, organ or function of the body is called biomaterial.

The terms "Biomaterial" was coined in the United States in the late 1960s at Clemson University, although the definition was formally formed in 1991 during the "2nd International Biomedical Consensus Conference". For the past 50 years, these materials have played a key role in medicine and biology, contributing considerably to increasing life expectancy and improving quality of life (Franchin & Nicola 2003). Although what constitutes the very first biomaterial may remain somewhat ill - defined, it is clear that modern biomaterials are evolving at an intense pace. From simple implants like intraocular lenses, which restore sight to millions of cataract patients every year and were first used more than half a century ago, to more complex materials that not only perform mechanical tasks in the body, but can even direct body's response for maximum restorative power. Indeed, researchers are becoming adept at creating grafts and implants that not only mimic the body but actually encourage it to 'colonize' the foreign material itself, growing new skin, cartilage or blood vessels. Think for example at vascular grafts that could stimulate the growth of new vessels (Tiwari et al. 2002) or retinal prosthesis that can restore some vision lost because of retinitis pigmentosa.

This biomaterial boom is partly due to the advances in molecular, cellular and tissue biology. A better understanding of body tissue and its interaction with materials, coupled with increased collaboration of scientists from different disciplines to help spread this knowledge and enable its use in engineering, has allowed the rapid development we are seeing (Burns 2009).

Each implanted biomaterial causes the biological response of an organism; the organism interacts and modifies the biomaterial itself. The human organism can activate many complex biological mechanisms that have defense functions with malicious events. These processes are essential defense mechanisms, but are also the main obstacle to the application of medical devices. To overcome this barrier, biomaterials must be biocompatible.

Biocompatibility is a key feature of interaction between organism and non-living matter. It has been defined as the ability of a host to be well-supported by the host, by providing an adequate response to its specific application (Williams 1987).

In fact, this definition provides little information on how to evaluate biocompatibility or how to improve biocompatibility. We consider the procedural definition: it derives from ISO standards on biocompatibility, a series of detailed tests that, if approved, can be a biocompatible material. Many ISO tests provide cytotoxicity, sensitivity, irritation, genotoxicity, carcinogenicity, reproductive toxicity, and sterilizing agent residue tests (Brunette et al. 2001). 2009 updated tests are visible in table 1.

Device categorization by			Biologic effect									
nature of body contact		contact duration	CYTOTOXICITY	SENSITIZATION	IRRITATION OR INTRACUTANEOUS REACTIVITY	SYSTEMIC TOXICITY (ACUTE)	SUBCHRONIC TOXICITY (SUBACUTE TOXICITY)	GENOTOXICITY	IMPLANTATION	HAEMOCOMPATIBILITY	CHRONIC TOXICITY	CARCINOGENICITY
Category	Contact	**A_limited** (<= 24h) **B_prolonged** (>24h to 30d) **C_permanent** (> 30d)										
Surface device	Intact skin	A	X	X	X							
		B	X	X	X							
		C	X	X	X							
	Mucosal membrane	A	X	X	X							
		B	X	X	X	O	O		O			
		C	X	X	X	O	X	X	O		O	
	Breached or compromised surface	A	X	X	X	O						
		B	X	X	X	O	O		O			
		C	X	X	X	O	X	X	O		O	
External communicating device	Blood path, indirect	A	X	X	X	X				X		
		B	X	X	X	X	O			X		
		C	X	X	O	X	X	X	O	X	O	O
	Tissue/Bone/Dentin	A	X	X	X	O						
		B	X	X	X	X	X	X	X			
		C	X	X	X	X	X	X	X		O	O
	Circulating blood	A	X	X	X	X		O^		X		
		B	X	X	X	X	X	X	X	X		
		C	X	X	X	X	X	X	X	X	O	O
Implant device	Tissue/Bone	A	X	X	X	O						
		B	X	X	X	X	X	X	X			
		C	X	X	X	X	X	X	X		O	O
	Blood	A	X	X	X	X	X		X	X		
		B	X	X	X	X	X	X	X	X		
		C	X	X	X	X	X	X	X	X	O	O

Table 1. *Guideline for biocompatibility testing. ISO 10993-1 Evaluation and testing, 2009 edition. X = test for ISO 10993-1 O = additional tests*

To gain an insight into how biocompatibility is, we can focus on what some of these tests consist on. Cytotoxicity refers to cell damage caused by materials, either by direct contact or by leachable substances. Sensitization refers to a materials ability to induce specific delayed-type hypersensitivity in the body upon initial exposure. Irritation refers to a non-specific inflammatory response to a single, repeated or continuous application of the material. Systemic toxicity can be acute within the 24 hours, subacute when you give a single dose or multiple doses of a test sample during a period from 14 to 28 days, subchronic at 90 days but not exceeding 10% of the life cycle of the device, chronic if you give single or multiple exposures to medical devices, materials, extracts during at least 10% of their lifespan of the test animal. Genotoxicity refers to gene or point mutations, small deletions, mitotic recombination or microscopically visible chromosome changes. Test on implantation permit to assess the local effects of implant material on living tissue: comparison is made with

reactions observed to medical devices whose clinic acceptability has already been established. Hemocompability tests evaluate the effects of medical devices or materials that are in contact (or indirect contact) with blood, or blood components (Saliterman 2006). Clearly, *in vitro* biocompatibility tests are the preface of all *in vivo* tests because they permit to study the cell-material interaction and look at the preparatory cell behavior on the surfaces.

Bone tissue

Bone tissue is a highly organized, dynamic and vascular connective tissue, highly specialized and able to regenerate and remodel without permanent scars. It has a great ability to mobilize and release the physiological source of mineral quantity based on metabolic demand. These characteristics make the bone an interesting tissue, in fact it is constantly subject to intensive research on the world in relation to histological aspects and systemic functions. In general, the vertebrate skeleton has the main role to provide support for soft tissues and the protection of spinal components (ie, undifferentiated hematopoietic cells). In addition, the skeleton is represented as a mineral reservoir and supports muscle contraction, facilitating movements. For these reasons, any change in this tissue can alter body balance and quality of life. In fact, the ability to self-heal bone tissue allows small lesions to heal spontaneously without any treatment (Gemini-Piperni et al. 2014). Nevertheless, when lesions are extensive (e.g. exposed bone fracture) or further pathology such as insufficient blood supply (Oryan et al. 2014), osteomyelitis (Calori et al. 2011), tumors and systemic diseases (e.g., diabetes) (Wada et al. 2013) are present, bone healing can be compromised. In these cases there is the need of certain clinical adjustments because large amounts of bone need to be applied in the treatment of the injured tissue. The first choice for filling these gaps along the fractures is the autogenous bone, which is obtained from specific areas of the same patient, for example iliac crest, parietal bone and mandible, respecting the chosen treatment and extension of the lesion (Gulan et al. 2012; Gemini-Piperni et al. 2014).

Bone interface prosthesis

A prosthesis (from Ancient Greek prósthesis, "addition, application, attachment") is an artificial device that replaces a missing body part, which may be lost through trauma, disease, or congenital conditions or that makes a part of the body work better.

A biomaterial can be used as prosthesis if it has some features: *biocompatibility*, *high specific strength*, excellent *corrosion and wear resistance* (Albrektsson et al. 1981; Affatato et al. 2001; Sun et al. 2008).

Make an attempt to understand better how this means. Biocompatibility has already been defined as the ability of a biomaterial to be well-supported by the host and to provide the appropriate response. The strength of a material is its resistance to failure with permanent deformation. For metals, polymers, woods and composites, the "force" in the selection tables refers to the voltage load (as failure is for yield). For fragile (ceramic) materials, stress insufficiency is fracture and "traction resistance" is very variable. The "force" in the selection tables is therefore the "compression force" (which requires a much larger load). A strong material requires high loads to deform (or break) permanently. The specific force is the strength divided by density.

Corrosion resistance refers to the strength and material that can corrode the material. Various materials possess this property inherently depending on their corrosion resistance. Some other methods can also be used to withstand corrosion, such as painting, hot galvanizing, and combining these methods with the coating. In essence, corrosion is the process where a material is oxidized from the environment and loses the electrons in its result. Therefore, corrosion resistance is the ability to retain this binding metallic energy and resist the deterioration and chemical degradation that occurs during surface exposure to this environment. Wear resistance is the ability of the material to withstand wear processes.

MATERIAL	WEAR RESISTENCE
METALS	High toughness allows to adapt to each particle or impact angle. Susceptible to corrosive substances and to softening effects at high temperatures.
CERAMICS	High toughness. Resistant to high temperatures and corrosive substances. Resistant to low erosive wear.

Wear resistance of metals and ceramics.

More than these properties, biomaterial should support osteoinduction, osteoconduction and osteointegration.

Osteoinduction means that primitive, undifferentiated and pluripotent cells are somehow stimulated to develop into the bone-forming cell lineage (Albrektsson & Johansson 2001). It is also defined as the process by which osteogenesis is induced (Wilson-Hench 1987). Consequently, osteoinductive materials stimulate host mesenchymal stem cells from surrounding tissues to differentiate into bone forming cells (Giannoudis et al. 2005).

Osteoconduction defines the ease with which materials can be colonized by host bone cells and blood vessels (Giannoudis et al. 2005). An osteoconductive material permits vascularization and infiltration of precursors on its surface or down into pores, channels or pipes (Albrektsson & Johansson 2001).

Osteointegration is histologically defined as the direct anchorage of an implant by the formation of bone tissue around the implant without the growth of fibrous tissue at the bone – implant interface (definition was coined by Per-Ingvar Branemark in 60s). Therefore, osteointegration implies strong interactions between the host bone tissue and the grafted materials (Giannoudis et al. 2005).

Titanium and its alloys are widely and successfully used in medicine as implant materials. The longevity of implants (dental and orthopedic prostheses) depends on the integration of the implant with the surrounding bone.

Titanium

Titanium is a chemical element with the Ti symbol and the atomic number 22. It is a transition metal with a silver color, a low density and a high resistance. The element appears within a series of mineral deposits, mostly rutile and ilmenite, widely distributed in the earth's crust and in the lithosphere and are found in almost all living beings, rocks, waterfalls and solids (Tengvall & Lundström 1992).

Like aluminium and magnesium surfaces, titanium and its alloys oxidize immediately upon exposure to air. It readily reacts with oxygen at very high temperature forming titanium dioxide. It is, however, slow to react with water and air, as it forms a passive and oxide coating that protects the bulk metal from further oxidation. Due to the spontaneous emerging superficial oxide coating, titanium and its alloys show a *high biocompatibility* and a *low potential of corrosion* (Parr et al. 1985). Titanium can also be alloyed with iron, aluminium, vanadium, and molybdenum, among other elements, to produce strong, lightweight alloys for aerospace (jet engines, missiles, and spacecraft); military, industrial process (chemicals and petro-chemicals, desalination plants, pulp, and paper), automotive, agri-food, medical prostheses, orthopedic implants, dental and endodontic instruments and files, dental implants, sporting goods, jewelry, mobile phones, and other applications (Tengvall & Lundström 1992). Pure titanium is ductile, about half as dense as iron and less than twice as dense as aluminum; it can be polished to a high luster. It exhibits *high specific strength* and *good chemical inertia* in the human body (Hu et al. 2013; Albrektsson et al. 1981; Sun et al. 2008). It is almost as *resistant* as platinum, capable of withstanding attack by dilute sulfuric and hydrochloric acids as well as chloride solutions, and most organic acids (Tengvall & Lundström 1992).

For their features, commercially pure titanium is the current dental implant material of choice (Belser et al. 2004; Pjetursson et al. 2007; Jung et al. 2012). Nevertheless, there are some limits on the use of this material.

Firstly, its ability to make precipitate HA on its surface is not as good as that of some ceramics. To improve the osseointegration, the implants are often coated with hydroxyapatite layers, for example surface is modified by plasma electrolytic oxidation (PEO) (Wirtz et al. 1991; Yerokhin et al. 1999; Walsh et al. 2009). Other problems are that titanium, in few cases, might be an allergen (Evrard et al. 2010; Sicilia et al. 2008) and may diffuse not only within the

adjacent tissues, as it's proven by the elevated concentrations found in the vicinity of oral implants (Koutayas et al. 2009) and in regional lymph nodes (Onodera et al. 1993), but also systemically (Jacobs et al. 1998). Moreover, we can also consider the esthetic problem of dental implants : the metal implant that becomes visible in mouth when gingival recession takes place. Also, there is an ongoing discussion on sensitivities associated with failure of dental implants made of this material (Hedenborg 1988; Evans 1994; Pigatto & Guzzi 2009; Depprich & Naujoks 2014; Sicilia et al. 2008). Under certain circumstances such as a low surrounding pH value or mechanical stress, titanium might show corrosive behaviors (Reclaru & Meyer 1994; Toumelin-Chemla et al. 1996; Tschernitschek et al. 2005). Currently, there is a lack of evidence for a clinical relevance of this hypothesis (Wenz et al. 2007), but the rising popularity of metal-free reconstructions motivates clinicians to offer an implant made of another material than titanium.

Oxide ceramics

Oxide ceramics could be a possible alternative to titanium: *white-colored* ceramic implants can feature esthetic benefits in both supra- and sub-crestal areas (Borgonovo, Censi, et al. 2013; Borgonovo, Corrocher, et al. 2013). In particular in the presence of a gingival recession, the white color better mimics dental hard tissues (Bengazi et al. 1996; Heydecke et al. 1999). Owing to their *high biocompatibility*, ceramic materials are perfectly suitable as implant material in medicine (Christel et al. 1989; Akagawa et al. 1998; Degidi et al. 2006).

Alumina exhibits a high wear resistance and excellent mechanical properties. Therefore, it is a desirable material for dental and orthopedic prostheses. However, it has high fracture rates (Piconi & Maccauro 1999) and due to its bioinert behavior it is not favorable for applications such as bone substitutes with direct contact to bone, because of its disability of bonding with surrounding tissues (Wittenbrink et al. 2015).

Zirconia has been successfully used in dentistry for restorations such as root posts (Kakehashi et al. 1998; Bateli et al. 2014), crowns and bridges (Heintze & Rousson 2010; Komine et al. 2010), and implant abutments (Glauser et al. 2004; Zembic et al. 2013). The use of zirconia in oral implantology and fixed implant prosthodontics (Guess et al. 2012) is still in its developmental stages and little research has been conducted so far for oral implants regarding the mechanical stability and ceramic aging (Chevalier 2006; Sanon et al. 2013). The aging of

zirconia can result in a decrease in the initially high flexural strength which may lead to fatigue fractures under “normal” masticatory loading (Piconi & Maccauro 1999). Zirconia implants may undergo slow degradation during long term implantation in the human body (Swain 2014) also because they are sensible to hydrothermal degradation: phase transformation is accelerated in aqueous environments (Kim et al. 2000; Chevalier et al. 2004). Alumina and yttria stabilized zirconia (Y-TZP) are suitable for biomedical applications, due to their good mechanical and tribological properties and proved biocompatibility (Kohal et al. 2013; Larsson & Vult 2009; Schliephake & Hefti 2010; Zinelis et al. 2010; Zhou et al. 2007; Wenz et al. 2007). Y-TZP and other newly developed high-performance ceramics of zirconium dioxide (ZrO_2) or zirconia possess good initial mechanical strength (Christel et al. 1989), exhibit favorable tissue compatibility (Degidi et al. 2006), and show osseointegration comparable to that of titanium (Depprich et al. 2008; Lee et al. 2009; Kohal et al. 2009; Kohal et al. 2013).

The development of mixed oxides ceramic materials may be able to combine the properties of both alumina and zirconia, emphasizing specific properties, and could be the key for good alternative bearing surfaces (table 2). The addition of a fraction of zirconia to alumina or vice versa results in a “composite” material of increased toughness. The word “composite” refers to the combination, on a macroscopic scale, of two or more materials different in composition, morphology, and general physical properties (Salernitano & Migliaresi 2003). Two kinds of composites can be prepared in the alumina – zirconia system: a zirconia matrix reinforced with alumina particles (alumina-toughened zirconia, ATZ) or an alumina matrix reinforced with zirconia particles (zirconia-toughened alumina, ZTA).

Material property	Alumina	Zirconia	ATZ (Zirconia Matrix)	ZTA (Alumina Matrix)
Toughness (MPa$\sqrt{m}$)	4	7	5–6	7–8
Fatigue limit (K_{IO}) (MPa$\sqrt{m}$)	2.5	3.5	2.5–3	5–6
Hardness (Hv)	1800	1200	1300	1700
Hydrothermal stability	High	Low	Medium	High

All values given here are quoted by the specified manufacturer, CSIC Institute (Madrid, Spain).

Table 2. *Material Properties of Alumina, Zirconia, Alumina-Toughened Zirconia (ATZ), Zirconia-Toughened Alumina (ZTA)* (Affatato et al. 2006).

However, in the case of ATZ, the problem related with hydrothermal stability still remains with the need to stabilize zirconia with yttria (Green et al. 1989). Moreover, the hardness of ZTA composites is greater, leading to higher wear resistance (Affatato et al. 2006).

The advantages and disadvantages of titanium and the characteristics of ceramics are summarized in table 3 and 4.

Titanium	
Advantages	**Disadvantages**
High biocompatibility	HA coating needed
Low potential of corrosion	Allergen (some cases)
High specific strength	May diffuse (some cases)
Good acid resistance	Some failures
Good chemical inertia	Metal coloured

Table 3. *Titanium properties.*

	ALUMINA	*ZIRCONIA*
White-couloured	✓	✓
High biocompatibility	✓	✓
High wear resistance	✓	
Good mechanical and tribological properties	✓	✓
High fracture rates	✗	
Osseointegration	✗✗	✓
Reduced plaque accumulation		✓
Slow degradation		✗✗

Table 4. *Advantages (✓) and disadvantages (✗) of alumina and zirconia. The great problem of alumina is the lack of direct bonding with bone, that is, insufficient osseointegration. The principal problem of the zirconia is the slow degradation.*

Bioactivation of ATZ and ZTA

Bioactivity, in case of bone bonding materials, can be described as the ability to grow bonelike apatite on the materials surfaces. It has been shown that apatite formation is induced by the presence of particular hydroxyl sites on the surface. The introduction of (OH) functional groups on the materials surfaces by acid and base treatments seems to represent a promising solution to induce bioactivity. Such processes have allowed to grow apatite on the materials surfaces when they were immersed in the simulated body fluid (SBF).

It is also known that the Al-OH functional group has no affinity for calcium and phosphate and do not induce apatite formation. By contrast, zirconia gels with tetragonal or monoclinic structure were proved to effectively induce apatite nucleation by the specific arrangement of the ZrOH functional group which are a nucleation supply for apatite crystal formation. The work of Kokubo et al. has shown that a zirconia composite (70 vol.% zirconia, 30 vol.% alumina)

was able to form apatite layer on the surface after acidic or basic treatments. (Gianmario Schierano et al. 2015)

Cell models to study the biocompatibility of bone interface materials

Preclinical models, although not perfect, offer a prescreening method for testing novel biomaterial (Pearce et al. 2007). However, to ethically rationalize the use of animals and to further our detailed knowledge, *in vitro* testing is required. In terms of bone research, a plethora of osteoblast cell models have emerged to tackle the surge in research in this area and to compensate to some degree for the ever present problem relating to sustainable supply of primary cells from either healthy or diseased donors. Primary mesenchymal stem cells mainly deriving from the bone marrow are also very important when dealing with bone regenerative purposes. The first isolation method relied on the ability these cells have to adhere to tissue culture plastic (Friedenstein et al. 1966; Friedenstein et al. 1970; Friedenstein et al. 1968). To reduce the high heterogeneity of whole bone marrow cultures, Haynesworth et al. introduced the separation of the MSCs through gradient centrifugation (Haynesworth et al. 1992). In later studies, these cells were able to properly differentiate into bone cells (Pittenger et al. 1999). More recently, adipose tissue was also proposed as an alternative source of mesenchymal cells, which have been employed in osteogenic models with contrasting results (Zuk et al. 2001; Chou et al. 2011; Zuk et al. 2011; Lin et al. 2013).

The most used human osteoblast cell line, SaOs-2cells were originally isolated from an 11-year old Caucasian female in 1975. SaOs-2 cells display a mature osteoblast phenotype and tend to form a calcified matrix typical of woven bone (Rodan et al. 1987). SaOs-2 cells share with primary human osteoblasts a similar expression profile of cytokines, growth factors and receptors for parathyroid hormone (Bilbe et al. 1996). The structure of the collagen synthesized by SaOs-2 turned out to be similar to that of collagen produced by primary human osteoblast cells. (Fernandes et al. 2007).

MG-63 cell line derives from a juxtacortical osteosarcoma diagnosed in the distal diaphysis of the left femur of a 14-year-old male. (Billiau et al. 1977) MG-63 cells represent an immature osteoblast phenotype. Although inconsistent data are available in literature as regards their

mineralization capabilities (Czekanska et al. 2012), MG-63 cells have been used in spite of their limitations in long-term studies concerning cell behavior on biomaterials (Lincks et al. 1998).

As osteocytes are terminally differentiated cells embedded within a mineralized matrix, they are quite difficult to obtain in culture for in vitro study (Kartsogiannis & Ng 2004). So far, research has been limited to chick, rat, and mouse (Nijweide et al. 2003; Semeins et al. 2012; Gu et al. 2006; Halleux et al. 2012). Among others, Bonewald's group deserves to be mentioned for developing immortalized cell lines such as: MLO-Y4, MLO-A5 and IDG-SW3. Kato established the clonal line MLO-Y4 (murine long bone osteocyte Y4) with osteocyte-like characteristics (Kartsogiannis & Ng 2004; Kato et al. 1997). These cells are endowed with extensive, complex dendritic processes, produce large amounts of osteocalcin and are positive for osteopontin and connexin 43. The MLO-Y4 cells also support osteoclast formation and activation (Zhao et al. 2002). The mouse derived MLO-A5 cell line representing late osteoblast/early osteocytes was shown to mineralize spontaneously in culture even in the absence of beta-glycerophosphate and ascorbic acid (Kato et al. 2001). Both MLO-Y4 and MLO-A5 are usually cultured in monolayer on collagen-coated plastic.

Among the osteoblast cell lines, MC3T3-E1 cells are endowed with a pre-osteoblastic phenotype. Notably, several sub-clones of these widely diffused murine cells have been established. Some of them undergo mineralization with the addition of ascorbic acid and inorganic phosphate. (Quarles et al. 1992; Wang et al. 1999). Interestingly, in sub-clone 4, specific temporal changes from proliferation to nodule formation and mineralization have been described, resembling the intramembranous osteogenesis in vivo. (Sudo et al. 1983). MC3T3-E1 cells represent a reliable alternative to primary human osteoblasts as in vitro cell model for various research areas (Czekanska et al. 2012).

Aim of the work

The aim of this work was to investigate the biological *in vitro* response of osteoblasts to two ceramic composites. In particular, a zirconia matrix reinforced with alumina particles (alumina-toughened zirconia, ATZ) or an alumina matrix reinforced with zirconia particles (zirconia-toughened alumina, ZTA) with or without a specific hydrothermal treatment, were used in the study. The biological response has been evaluated in terms of viability, protein adsorption, cell adhesion, cell morphology, cell differentiation, and bone matrix production using MC3T3-E1 cells, a widely used cell model of pre-osteoblast. The present study was performed in order to assess the biocompatibility, the early cell response and the osteoinduction of the considered ceramic composites, upon specific surface treatments.

Materials and Methods

Sample preparation

High purity powders were used to produce the oxidic disks: Taimei Al_2O_3–16 wt%ZrO_2 (ZTA, Taimicron, Taimei, Japan) and ZrO_2–20 wt%Al_2O_3, (ATZ, TZ-3Y20AB, Tosoh, Japan), in form of "ready to press" powders, so that no additional mixing was required before pressing. Green samples were obtained by linear pressuring at 80 MPa followed by Cold Isostatic Pressing at 200 MPa. The best conditions for the sintering process were: heating at a rate of 50°C/h up to 700°C, followed by a 2 h dwell; heating at a rate of 100°C/h up to temperature sintering of 1500°C, followed by a 2 h dwell (Vallée et al. 2014). Fully dense materials were obtained by this procedure, as reported elsewhere (Faga et al. 2012). The resulting samples were 12 mm planar disks with thickness ranging between 4 and 5 mm.

Surface treatment

Samples were mirror polished with diamond suspension in ethanol with decreasing granulometry to the final surface roughness of less than 1 μm (Vallée et al. 2014). The surface treatment was obtained by hydrothermal cycles (patent number: TO2012A000029 and PCT/IB2013/050425). Implants were then treated with phosphoric acid under hydrothermal conditions with the purpose of inducing bioactivity (G. Schierano et al. 2015).

Washing and sterilization of samples

Washing was done sonicating samples in 5% SDS solution for 14 minutes, deeply rinsing them with ultra high purity water and keeping them in dH_20. Sterilization was done dipping samples in two 70% ethanol and 95% ethanol solutions. Samples were placed in 24-well culture plates.

Cell culture

MC3T3-E1 cells (ECACC, Salisbury, UK) were used in this work. These cells, derived from mouse calvaria, constitute a well-known in vitro model for studying osteoblastic proliferation and differentiation (Peterson et al. 2004; Quarles et al. 1992). MC3T3 cells were maintained in α-modified essential medium (α-MEM, Life Technologies, Milan, Italy) supplemented with 10 % fetal calf serum (Life Technologies, Milan, Italy), 1% L-glutamine (Life Technologies, Milan, Italy), 1% penicillin/streptomycin (Life Technologies, Milan, Italy) at 37°C in humidified air with 5% CO_2. The cell culture medium was changed every 2-3 days. Confluent plates were subcultured using trypsin/EDTA solution (Life Technologies, Milan, Italy). Cells were cultured on samples placed in 24 multi-well plates (BD, Milan Italy) and on glass, used as control (CTRL).

Cell viability

MC3T3-E1 cells were plated at density of $4*10^4$ cells/well in 24-well culture dishes. The cell viability was assessed by using CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Promega) according to the manufacturer's protocol at 1, 3 and 5 days of culture. This test is based on the bioreduction of a tetrazolium compound, [3 – (4,5-dimethylthiazol-2-yl) – 5 – (3-carboxymethoxyphenyl) – 2 – (4-sulfophenyl) – 2H – tetrazolium, inner salt; MTS], into a brown formazan product by dehydrogenase enzymes. This reaction takes place in metabolically active cells, thus in viable cells. For this assay, culture medium was removed, samples were washed with Phosphate Buffer Saline with Ca/Mg (PBS) before the addition of DMEM without phenol red. The samples were then incubated for two-three hours at 37°C in a humidified atmosphere containing 5% of CO_2. A 100µl of incubated medium was transferred to 96-well plate and the optical density (OD) was read at 490 nm in a micro-plate reader ("FilterMax F15" molecular devices, Orleans, USA). Test was done three times for each surface. To compare the viability results a non parametric test (Mann-Whitney test) was used as a scoring method.

Protein adsortion

According to literature (Canullo et al., 2016), to quantify the amount of protein adsorbed, the titanium disks were incubated in presence of Fetal Bovine Serum (FBS) at 2% concentration in Phosphate Buffered Saline (PBS), at 37°C for 30 minutes. After that, the samples were rinsed twice with PBS and the adsorbed protein was eluted from the disks using Tris Triton buffer (10mM Tris (pH 7.4), 100 mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 10% Glycerol and 0.1% SDS) for 10 minutes. Total protein amount was quantified through SERVA BCA Protein Assay Micro Kit (SERVA Electrophoresis GmbH, Heidelberg, Germany) following the manufacturer's instructions.

Cell adhesion

Cell adhesion assay was performed on titanium samples using a 12-well plate as support. Cells were detached through a treatment with trypsin for 3 minutes, after that, detached cells were carefully counted and seeded at 3 x 10^3 cells/disk in 500 µl of growth medium on the disks with different roughness. The 12-well plates were kept at 37°C, 0.5% CO_2 for 10 min. After fixation in in PBS 4% Paraformaldehyde for 15 min at room temperature, cells were washed two times with PBS and then stained with 1µM DAPI (Molecular Probes, Eugene, California, USA) for 15 min at 37°C to observe cell nuclei. Images were acquired using a Nikon Eclipse T-E microscope with a 4× objective. The cell nuclei were counted using the 'Analyze particles' tool of ImageJ software (ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/).

Cell morphology

Paxillin is multi-domain protein that localizes in cultured cells primarily to sites of cell adhesion to the extracellular matrix (ECM) called focal adhesions. It is a 68-kDa protein containing multiple tyrosine and serine/threonine phosphorylation sites within its two SH2- and one SH3-protein binding domains, as well as multiple LIM-motifs (Salgia et al. 1995). Paxillin functions

as an adaptor protein between FAK and the actin cytoskeleton, recruiting several proteins to the focal adhesion site during attachment, and it is involved in cytoskeletal reorganization in many cell types (Turner 2000).

To test focal adhesions, expression of paxillin was visualized by immunocytochemical double staining. MC3T3-E1 cells were seeded on the samples at a concentration of $2*10^4$ cells/well in 24-well plates and incubated for 24 hours. All following steps were performed at room temperature. After incubation, cells were rinsed once in cold PBS with Ca/Mg and fixed with 4% paraformaldehyde for 10 min. The fixed cells were washed twice with PBS with Ca/Mg washing solution. Blocking and membrane permeabilization was carried out simultaneously with 1% BSA+0.1% Triton (Sigma-Aldrich, Milan, Italy) in PBS solution for 10 min.

The permeabilized cells were washed three times with PBS with Ca/Mg washing solution. Paxillin staining was performed by incubations with anti-Paxillin N-Term 04-581 antibody from Millipore (Merk, Darmstadt, Germany) (1:200 in BSA 1%) for 1 hour followed by three washing steps. Subsequently, the samples were incubated for 40 min with anti-rabbit Alexa Fluor 488 secondary antibody (1:800 in BSA 1%) and rinsed with PBS three times. Cell nuclei were stained for 20 min with DAPI (1:500 in BSA 1%) and with Rhodamine-Phalloidine (1:100 in BSA 1%) (Life Technologies, Milan, Italy). The cells were washed again with PBS. The localization and distribution of focal adhesions were evaluated with a Nikon Eclipse Ti-E microscope using a Nikon Plan 10X/0,10; Nikon Plan Fluor 40X/0,75; Nikon Plan Apo VC 60X/1,40 (Nikon Instruments, Amsterdam, Netherlands). Images were processed with MetaMorph® Microscopy Automation & Image Analysis Software and focal adhesions were counted by ImageJ 1.48v (Wayne Rasband National Institutes of Health, USA) software.

Osteoblastic differentiation

At 80% confluence, cells were induced to differentiate by incubation in α-MEM supplemented with 10% fetal bovine serum (Life Technologies, Milan, Italy), 10 mM β-glycerophosphate (Life Technologies, Milan, Italy), 50 μg/ml ascorbic acid (Life Technologies, Milan, Italy), 1% penicillin/streptomycin, 1% L-glutamine for an additional culture period. The differentiation medium was changed every 3 days.

Real time PCR

MC3T3 were cultured in 24-well plates (three replicates for each experimental point). In each well we seeded $4*10^4$ cells/well. At days 0, 7, 14 and 21 of differentiation induction, RNA was extracted from cells by Ambion RNA extraction kit (Thermo Fisher Scientific) by means of additional mechanical lysis with a tip. RNA samples were preserved in eppendorf by freezing at -80°C before markers expression analysis.

Real-time polymerase chain reaction (qRT-PCR) was performed using specific Taqman probes (Roche). For gene expression analysis 1 μg of total RNA was retro transcribed in cDNA after DNAse treatment to avoid genomic amplification. For each target gene, specific primers were designed using UPL Roche software choosing intron-spanning assays. Primers sequences are listed in Table 5. All cDNA samples were analyzed in triplicate. For each cDNA sample, the C_t value of the reference gene was subtracted from the C_t value of the target gene to obtain the ΔC_t. The level of expression was expressed as the mean $\pm$SD of triplicate samples. For statistical analysis ANOVA was applied followed by Bonferroni test.

Forward and Reverse Primer Sequences Used for RT-PCR				
Gene	***Accession Number***	***Forward***	***Reverse***	***Species***
ALP	MGI:87983	aatgaggtcacatccatcctg	cacccgagtggtagtcacaa	Mus musculus
BSP	MGI:96389	gaaaatggagacggcgatag	cattgttttcctcttcgtttga	Mus musculus
OC	MGI:88156	tgaggaccatctttctgctca	tggacatgaaggctttgtca	Mus musculus

Table 5. *Primer sequences used for RT-PCR are showed: Alkaline Phosphatase (ALP), Bone Sialoprotein (BSP) and Osteocalcin (OC).*

ALP Activity

Intracellular ALP activity was determined with the Alkaline Phosphatase Assay kit (Abcam, ab83369, Cambridge, UK). The kit uses p-nitrophenyl phosphate (pNPP) as a phosphatase substrate which turns yellow when dephosphorylated by ALP. Osteoblasts were cultured for up to 21 days in osteogenic media containing 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate. Cells were rinsed twice with PBS, lysed with 0.05% Triton X-100 and incubated with the reagent solution containing phosphatase substrate (Sigma-Aldrich, Milan, Italy) at 37°C for 15 min. Optical density (OD) was measured at a wavelength of 405 nm with a microplate reader ("FilterMax F15" molecular devices, Orleans, USA). To adjust the final alkaline phosphatase concentration per total protein content, part of the cell lysates obtained for ALP quantification was incubated with BCA™ (Thermo Fisher Scientific, Waltham, MA, USA) Protein Assay, as per the manufacturer's instructions. OD was measured at a wavelength of 562 nm and data were normalized, as described previously (Mussano et al. 2010). Three replicates were done for ALP staining and standard samples.

Alizarin Red Staining Quantification

After 21 days of culture (four replicates), mineralized bone-like nodules were estimated following incubation of wells with an Alizarin Red S solution (40 mM ARS in a 10% (w/v) cetylpyridinium chloride (CPC) buffer dissolved in 10 mM Na_2PO_4). Monolayers in 24-well plates ($4*10^4$ cells/well) were washed with PBS and fixed in 10% (v/v) formaldehyde at room temperature for 10min. The monolayers were then washed twice with excess dH_2O. The plates were then left at an angle for 2min to facilitate removal of excess water. Then, cells were stained with 800 μL of ARS (pH 4.2) per well. The plates were incubated at room temperature for 30min with shaking. After aspiration of unincorporated dye, 2mL of 20% methanol and 10% acetic acid solution was added to each well for quantification of staining. The plate was incubated at room temperature overnight with rough shaking. The monolayer, now loosely attached to the plate, was then scraped from the plate with a cell scraper and aliquots (150μL) were transferred in 96-well format using opaque-walled, transparent-bottomed plates. Plates were read at 450 nm.

Calcium Assay Kit Quantification

After 21 days of culture, mineralized bone-like nodules were estimated following incubation of the calcium deposed within the extracellular matrix was quantified colorimetrically through the Calcium Assay Kit (Cayman Chemical, Michigan, USA). Absorbance of the lysates was measured at 570 nm.

Statistical analysis

Statistical analysis were done by GraphPad Prism 6 (GraphPad Soſtware Inc, La Jolla, CA, USA), using the non parametric test Mann-Whitney or the ANOVA analysis followed by Bonferroni test. A *p*-value of less than 0.05 was considered significant.

Results

MC3T3-E1 show a proper grow on all surfaces

The effect of composite ceramics on cell viability and proliferation of MC3T3 cells by MTS assay is shown in fig. 2. We can see MTS results of cell culture studies on treated or untreated ATZ and ZTA (ATZ +, ZTA+, ATZ -, ZTA-) and on control (CTRL). Globally, the graph shows that ceramics, on average, seem to increase proliferation of osteoblasts. Actually, after 24, 48 and 72 hours of culturing, OD values were not significantly different neither between the various types of surfaces nor between ceramics and control. However, these results indicate that composite ceramics were not cytotoxic and that cell proliferation was similar to the control. Three replicates for each experimental point were employed.

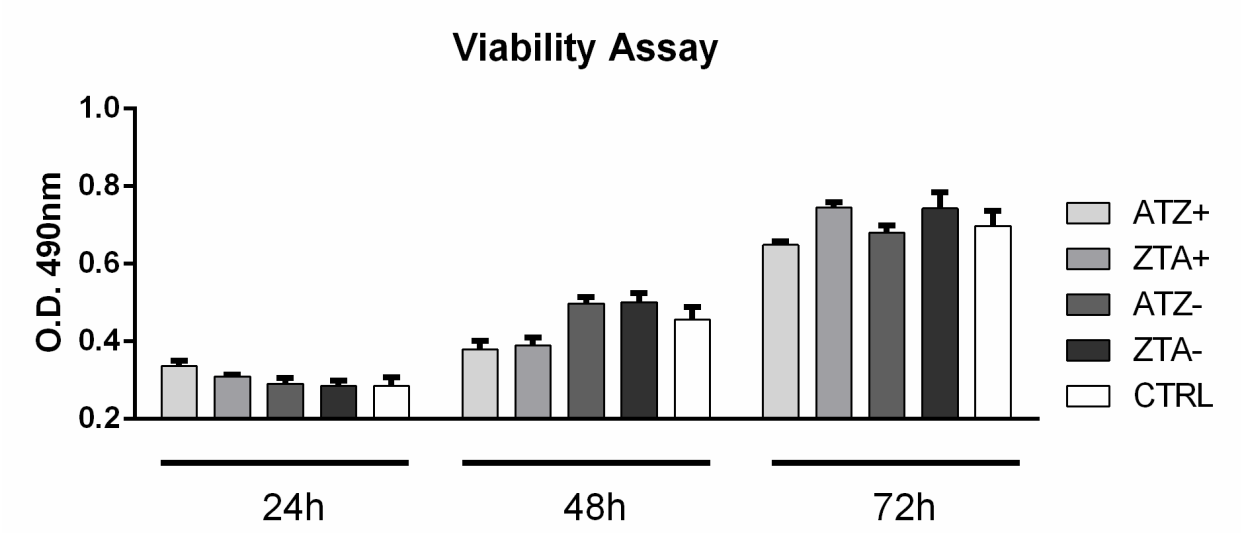


Fig. 1. *Viability assay to assess the cellular viability. Composite ceramics seem not to damage the viability of MC3T3. Ceramics don't significantly improve the cell viability. A p-value <0.05 was considered as significant using a Mann-Whitney test. No significant differences were observed between the different surfaces.*

Hydrotermal treatment enhance cell adhesion and protein adsorption

To further investigate the ability of the considered surfaces in the influence of early cellular response, we evaluated the protein adsorption and the cell adhesion.

As showed in figure 2 Hydrothermal treatment of both ATZ and ZTA significantly increase the protein adsorption level. No difference in protein adsorption were detected in untreated ATZ and ZTA compared to control condition.

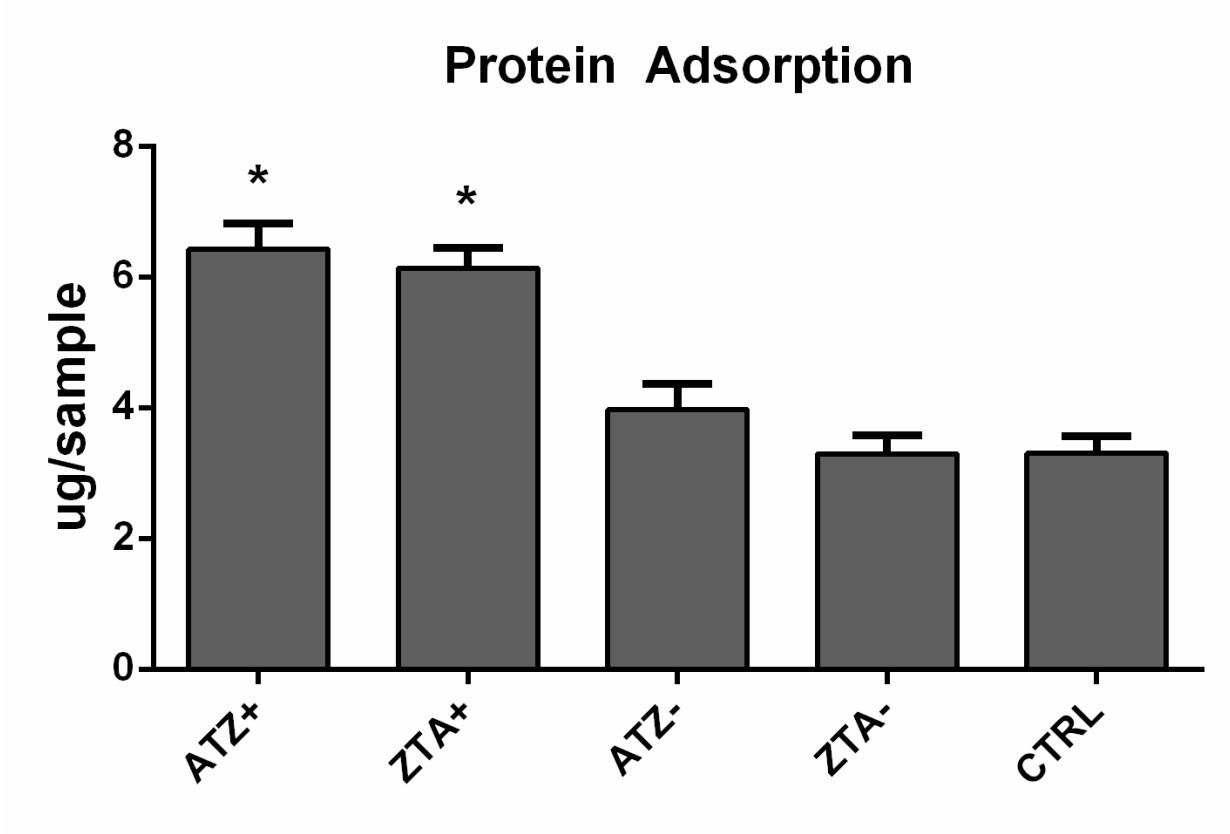


Fig. 2. *Protein adsorption quantification after 30 min of treatment. The symbol (*) indicates the statistical significance vs. CTRL condition using Mann Whitney test considering a p value < 0.05.*

In order to study how cell adhesion is influenced by the considered surfaces, cell adhesion assay was performed. As shown in figure 3 all composite ceramics significantly increase the level of cell adhesion compared to control condition. Interestingly, both treated ATZ and ZTA significantly increase the cell adhesion than untreated ATZ and ZTA.

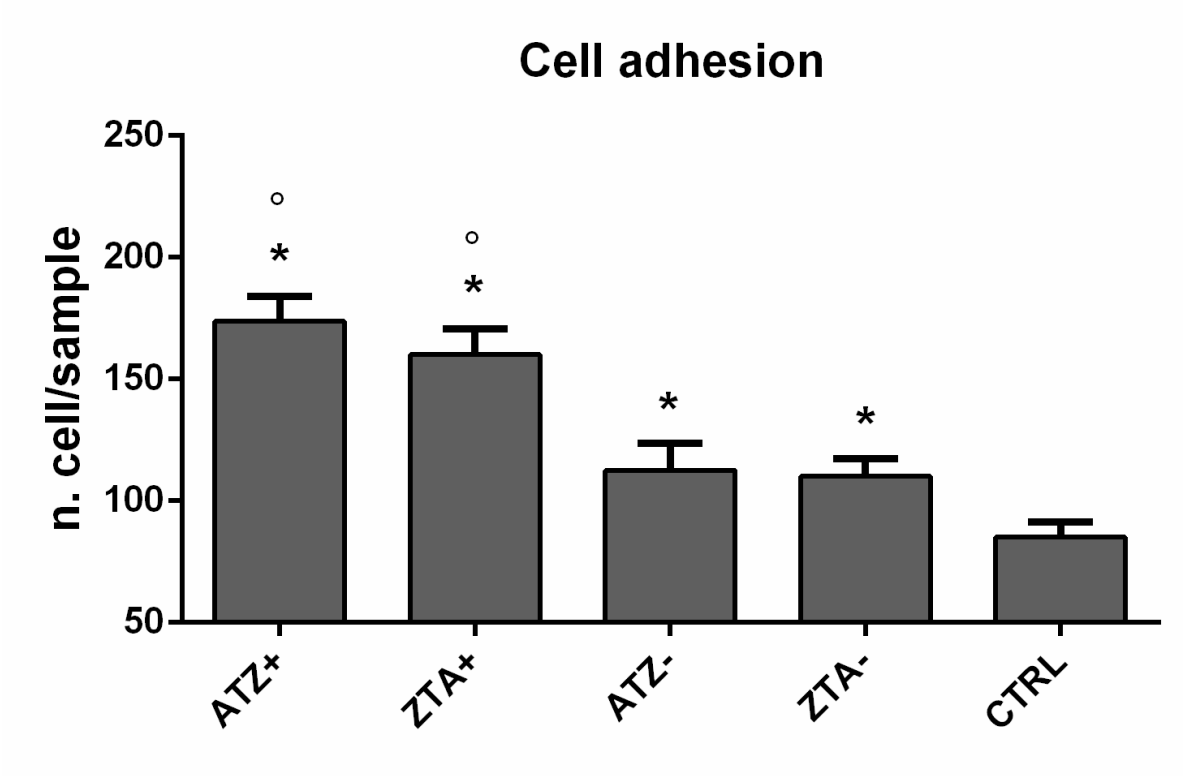


Fig. 3. Cell adhesion evaluation after 10 minutes from seeding. The symbol (*) indicates the statistical significance vs. CTRL condition, while the symbol (°) indicates the statistical significance vs. ATZ- and ZTA- using Mann Whitney test considering a p value < 0.05.

MC3T3 cells' morphology is not affected by different ceramics

The evaluation of cell morphology at 24h, showed no particular differences in cell shapes among the different surfaces as shown in figure 4-7. Cells were stained with DAPI to observe nuclei and with Rhodamine Phalloidine to visualize cytoskeletons. Images of MC3T3 plated on different surfaces were acquired at different magnifications. However, cells seeded on ATZ+ and ZTA+ seem to show a more elongated and oriented morphology and a proper level of spreading compared to the other surfaces.

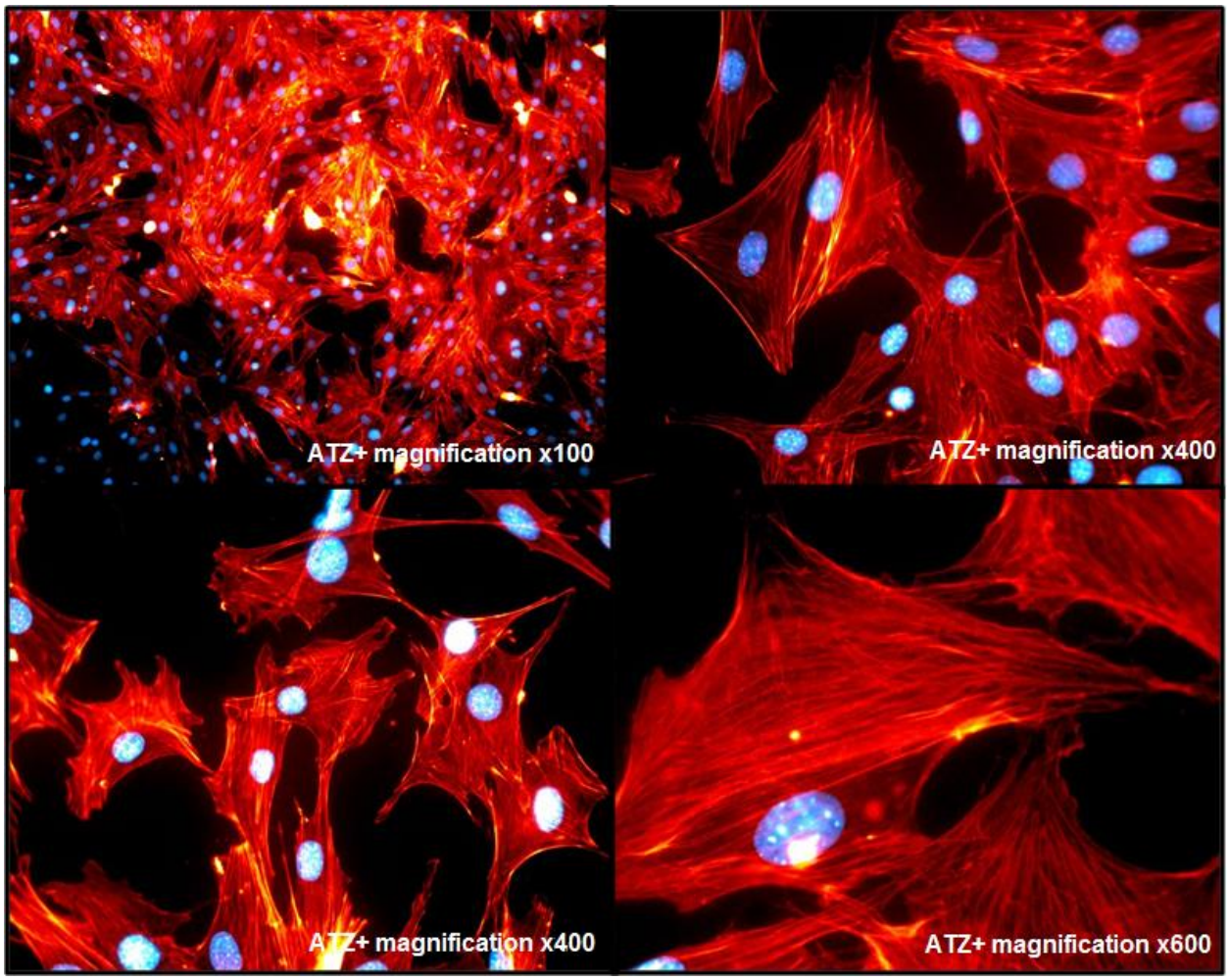


Fig. 1. *Fluorescence microscopy of MC3T3 cultured on* ***ATZ+*** *at different magnifications: 100X, 400X and 600X. Cells were stained with DAPI (blue) to visualize the nucleus and with Rhodamine-Phalloidine (red) to visualize actin.*

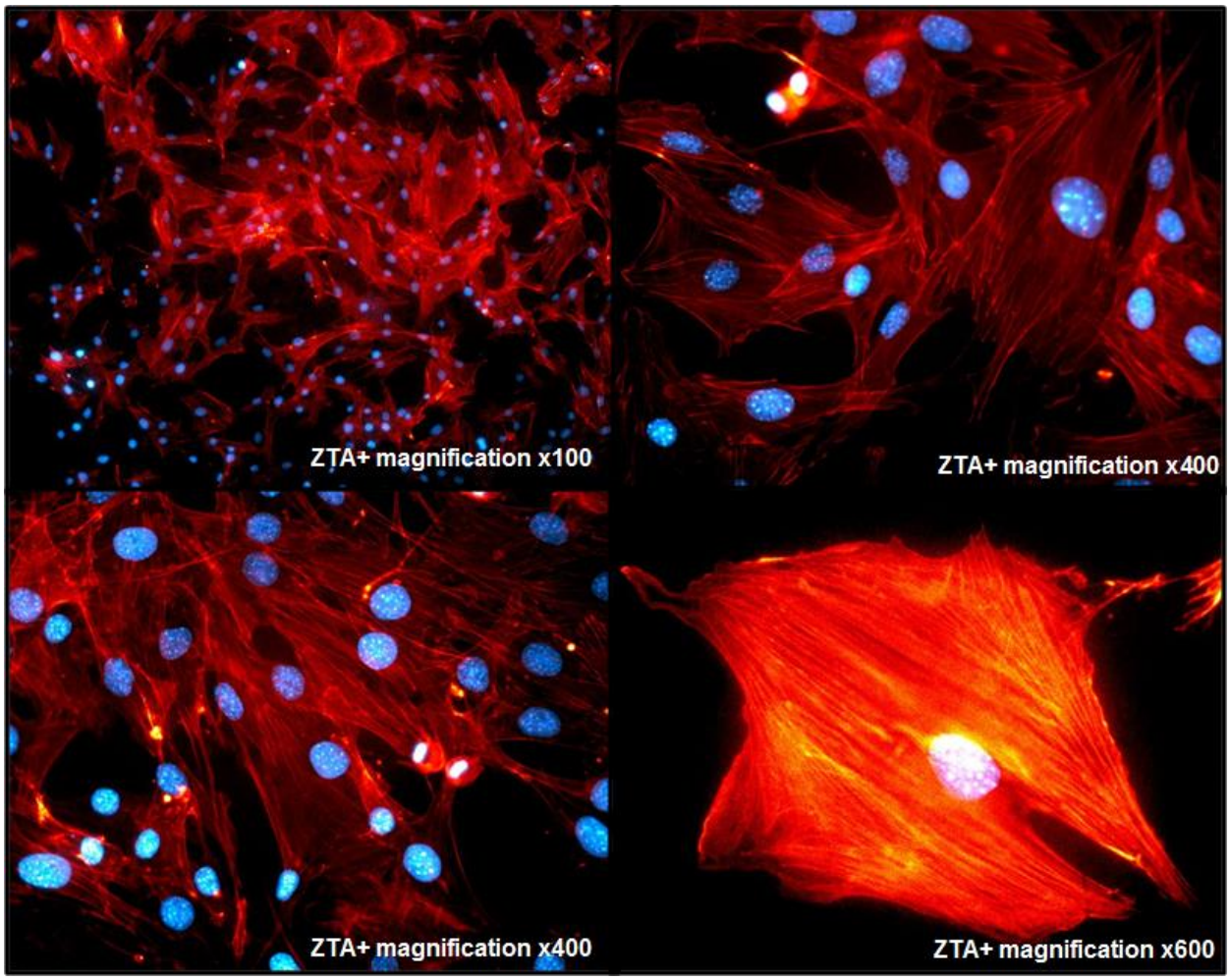


Fig. 2. *Fluorescence microscopy of MC3T3 cultured on* ***ZTA+*** *at different magnifications: 100X, 400X and 600X. Cells were stained with DAPI (blue) to visualize the nucleus and with Rhodamine-Phalloidine (red) to visualize actin.*

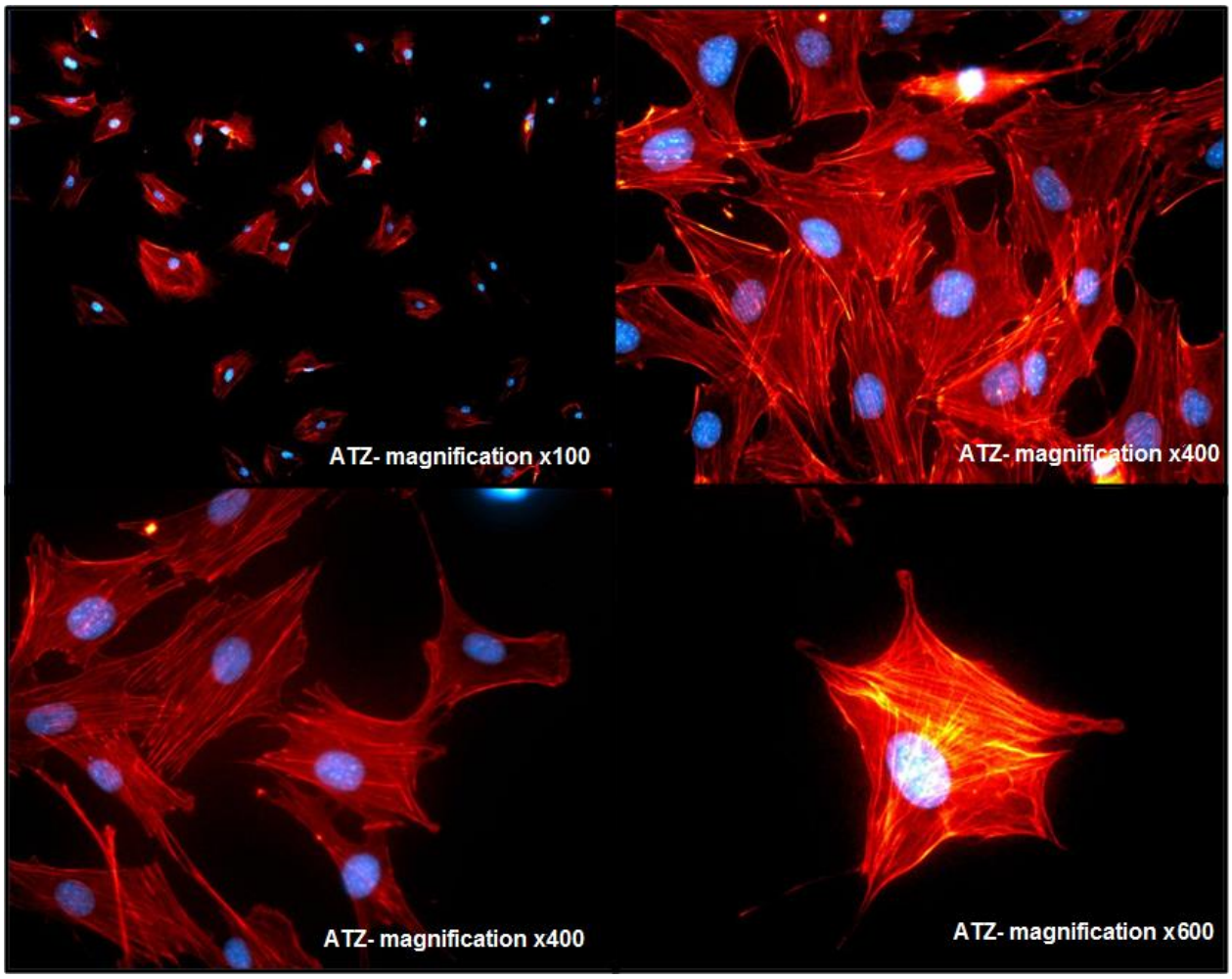


Fig. 3. *Fluorescence microscopy of MC3T3 cultured on* ***ATZ-*** *at different magnifications: 100X, 400X and 600X. Cells were stained with DAPI (blue) to visualize the nucleus and with Rhodamine-Phalloidine (red) to visualize actin.*

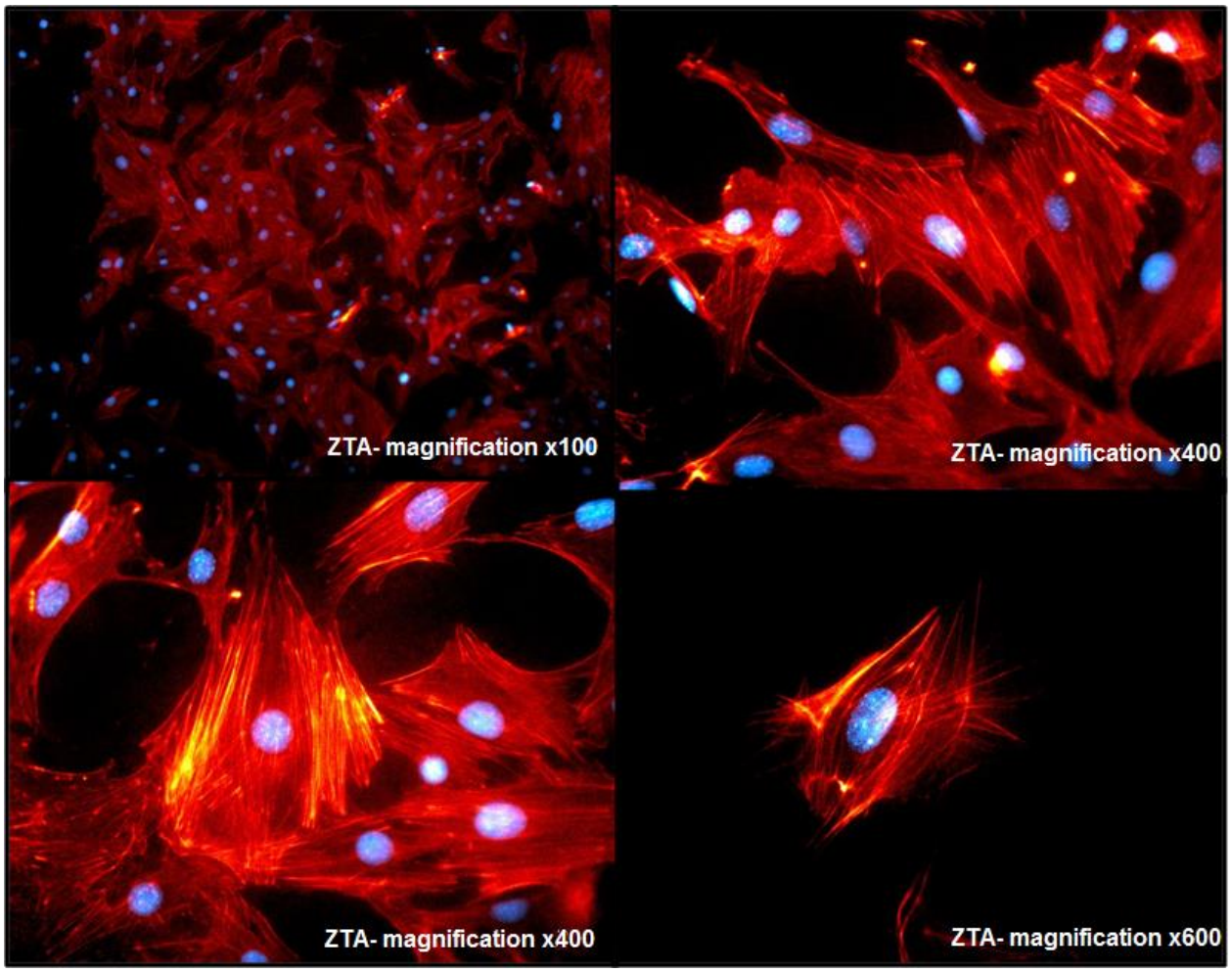


***Fig. 4**. Fluorescence microscopy of MC3T3 cultured on **ATZ-** at different magnifications: 100X, 400X and 600X. Cells were stained with DAPI (blue) to visualize the nucleus and with Rhodamine-Phalloidine (red) to visualize actin.*

In figure 8 is showed the quantification of cell area performed by ImageJ software. This analysis showed no significative difference among ceramic materials and CTRL condition. Interestingly it is possible to appreciate a bigger cell area mean of MC3T3 seeded on treated ATZ and ZTA even if there is no significance.

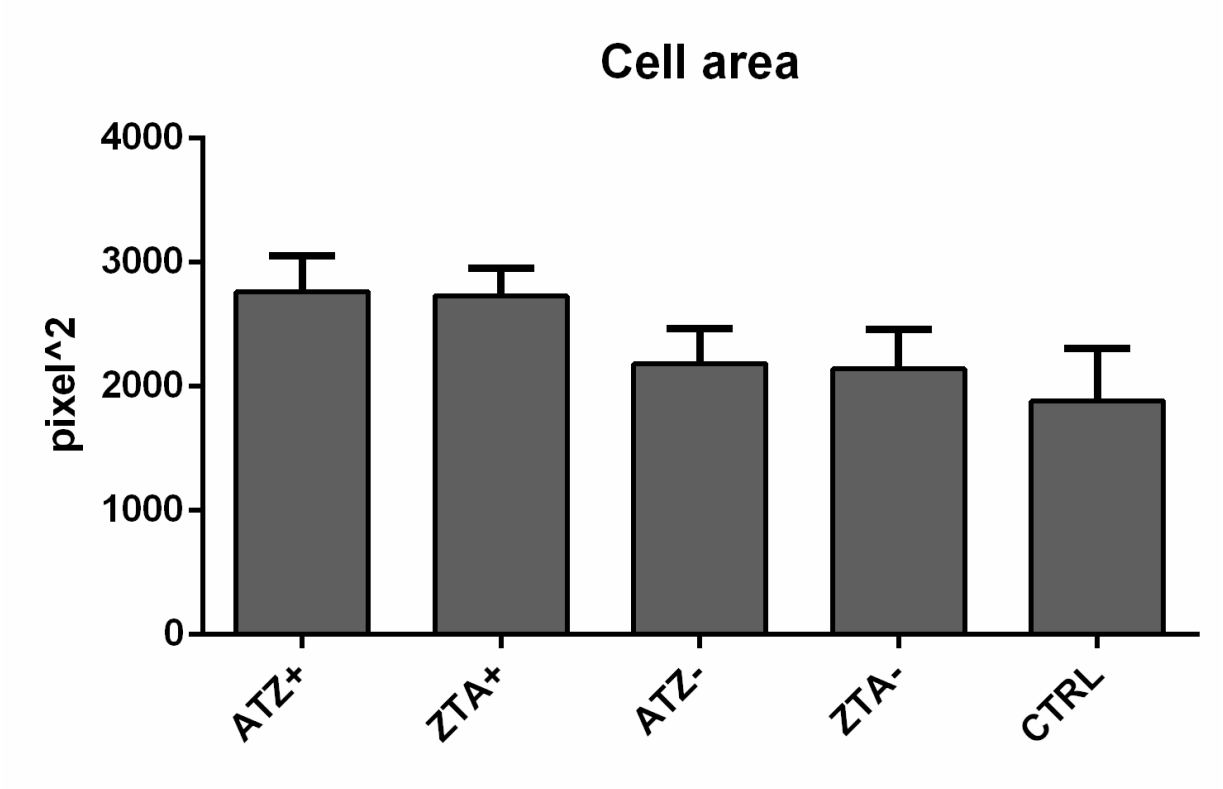


Fig. 8. Cell area analysis of MC3T3-E1 plated on ATZ+, ZTA+, ATZ-, ZTA- and CTRL condition.

Evaluating the ratio between major axis/minor axis (aspect ratio - AR) of the cells it is possible to better appreciate the cell morphology. AR results showed no significance difference among the studied conditions in AR index. Interestingly it is possible to appreciate a bigger cell area mean of MC3T3 seeded on treated ATZ and ZTA even if there is no significance.

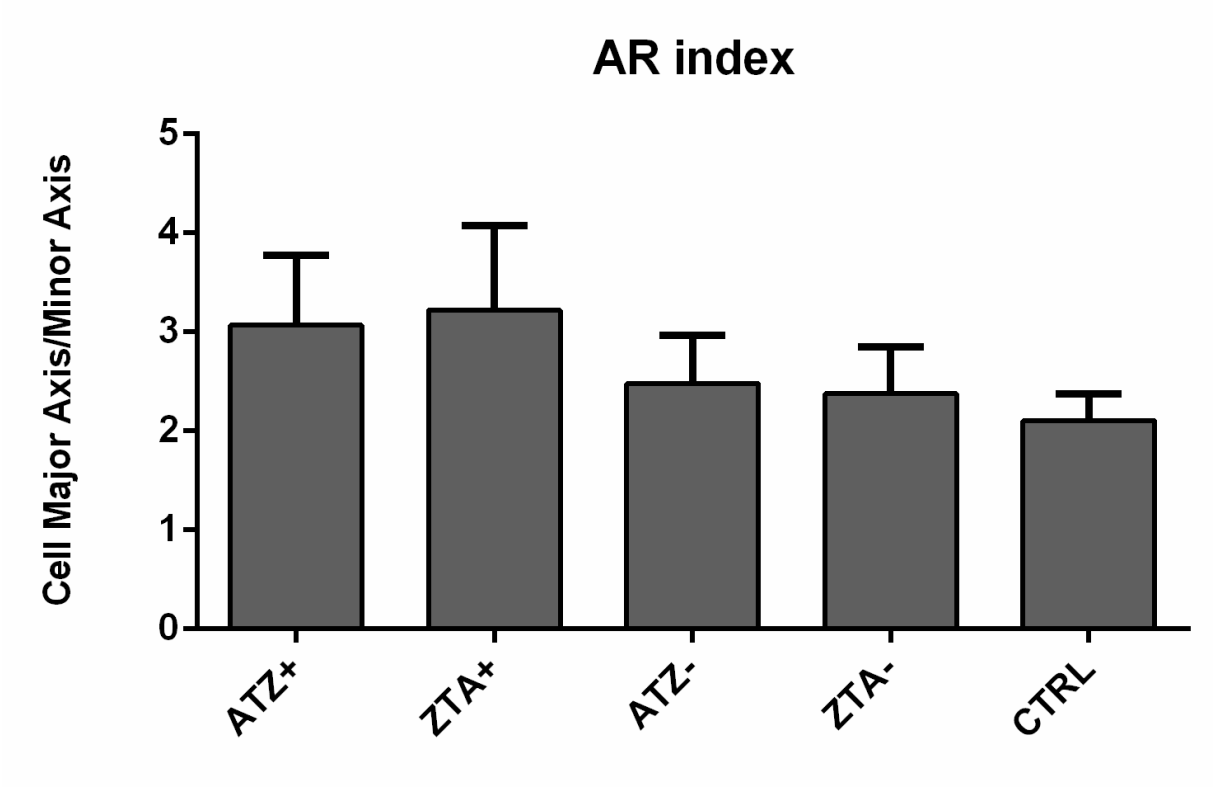


Fig. 9. AR measurements at 24h of MC3T3-E1 seeded on ATZ+, ZTA+, ATZ-, ZTA- and CTRL condition.

Cercamic surfaces enhance Focal Adhesion Density

In order to better understand the mechanisms behind cell morphology and cell adhesion, a focal adhesion analysis has been carried out at 24 h. In figure 10 are shown representative picture of MC3T3-E1 seeded on the considered surfaces.

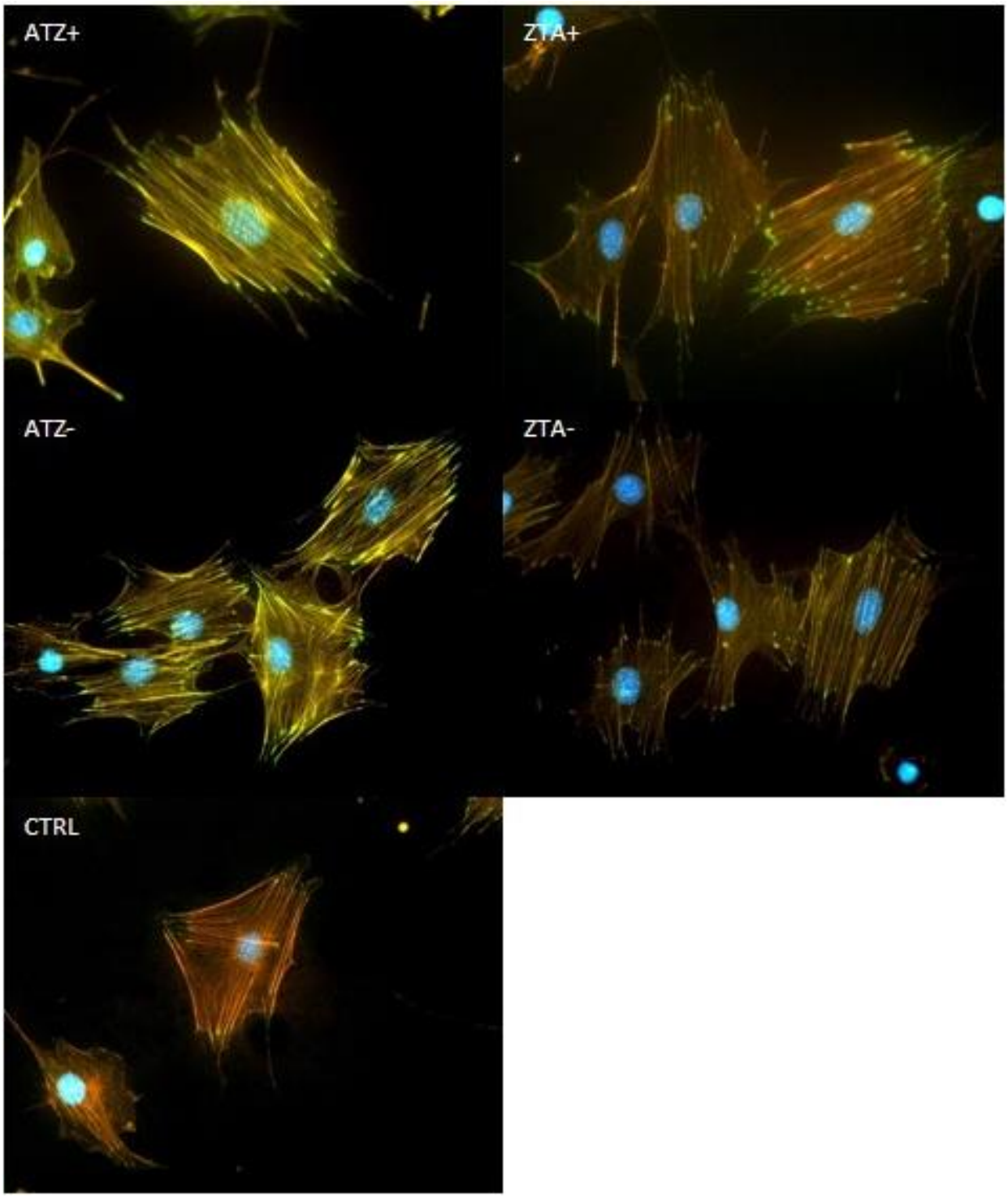


Fig. 10. Focal adhesion of MC3T3-E1 seeded on on ATZ+, ZTA+, ATZ-, ZTA- and CTRL condition. In red is show the cytoskeleton (actin), in green the focal adhesion (paxillin) and in blu the nuclei (DAPI).

Focal adhesions density was evaluated normalizing the number of detected focal adhesions to cell area. As it possible to appreciate from figure 17, all ceramics show a significantly higher density of focal adhesions. Mann-Whitney test was used as scoring method.

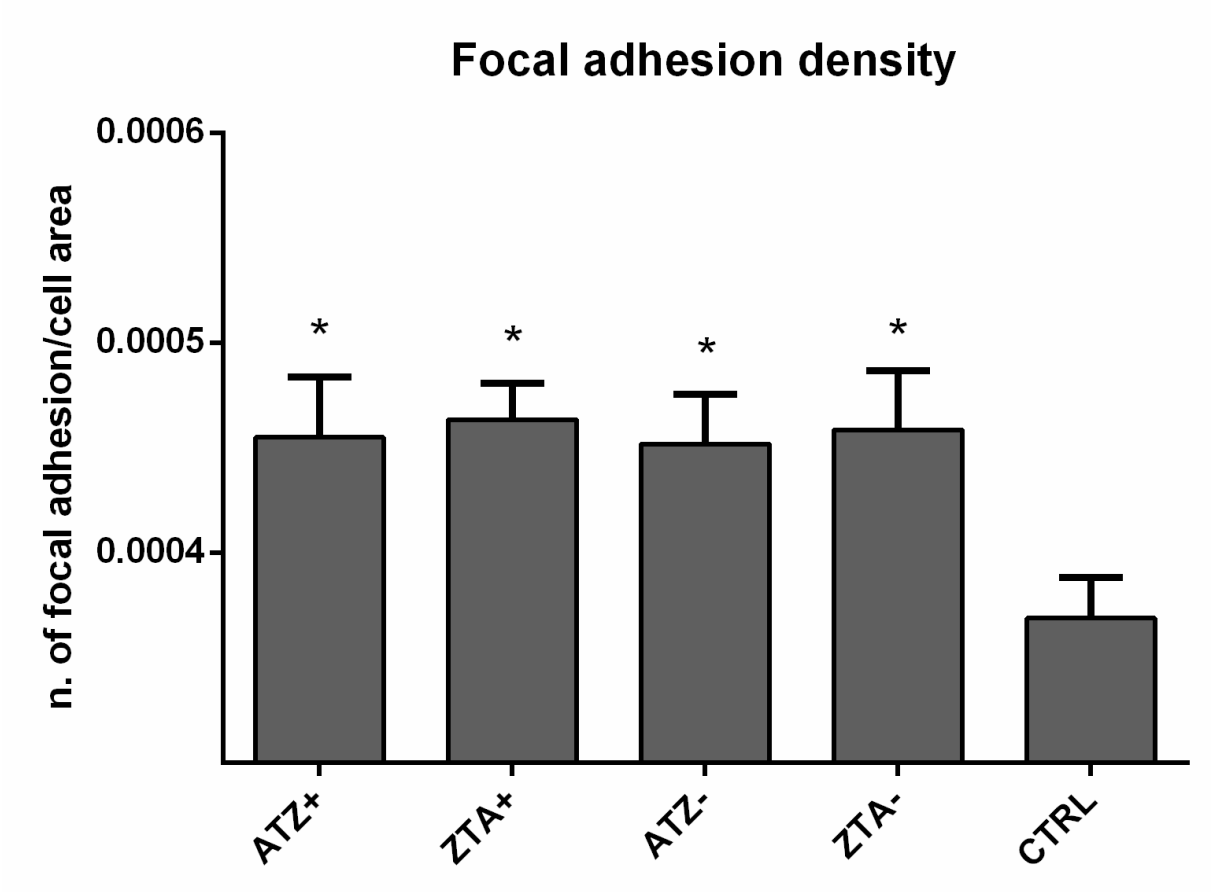


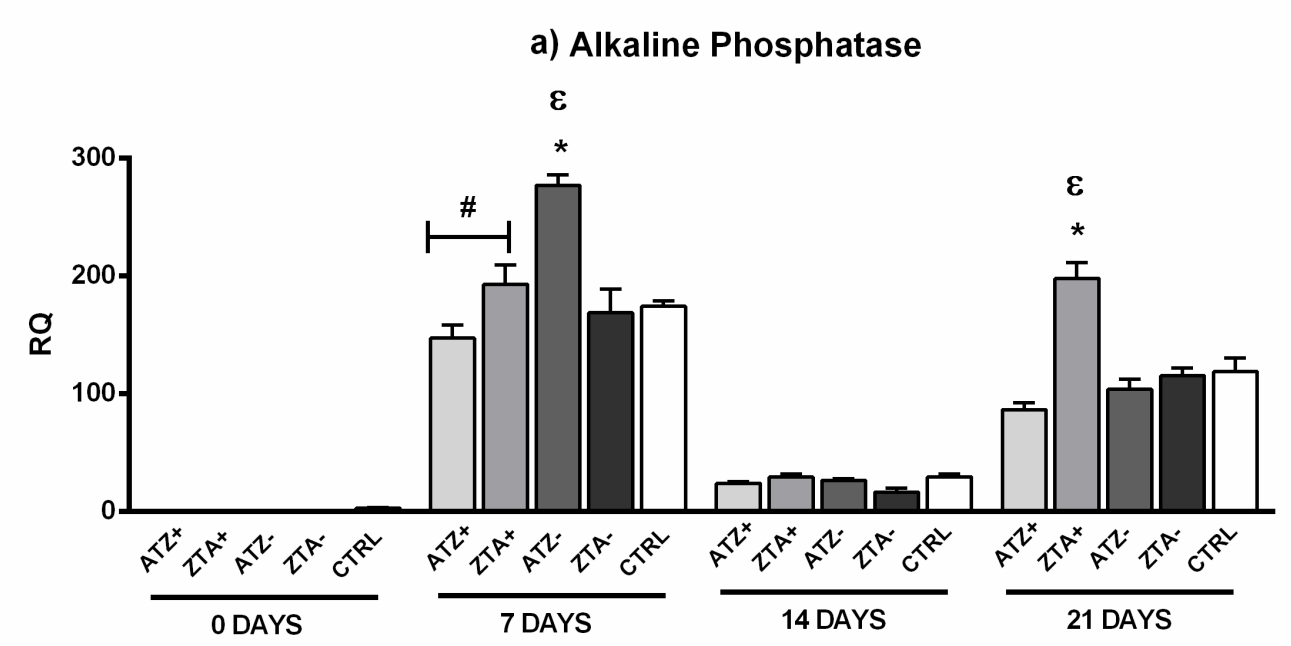
Fig. 11. *Focal adhesion density of MC3T3 normalized to cell area. The symbol * means a significant difference versus CTRL condition considering a p-value <0.05 using a Mann–Whitney test.*

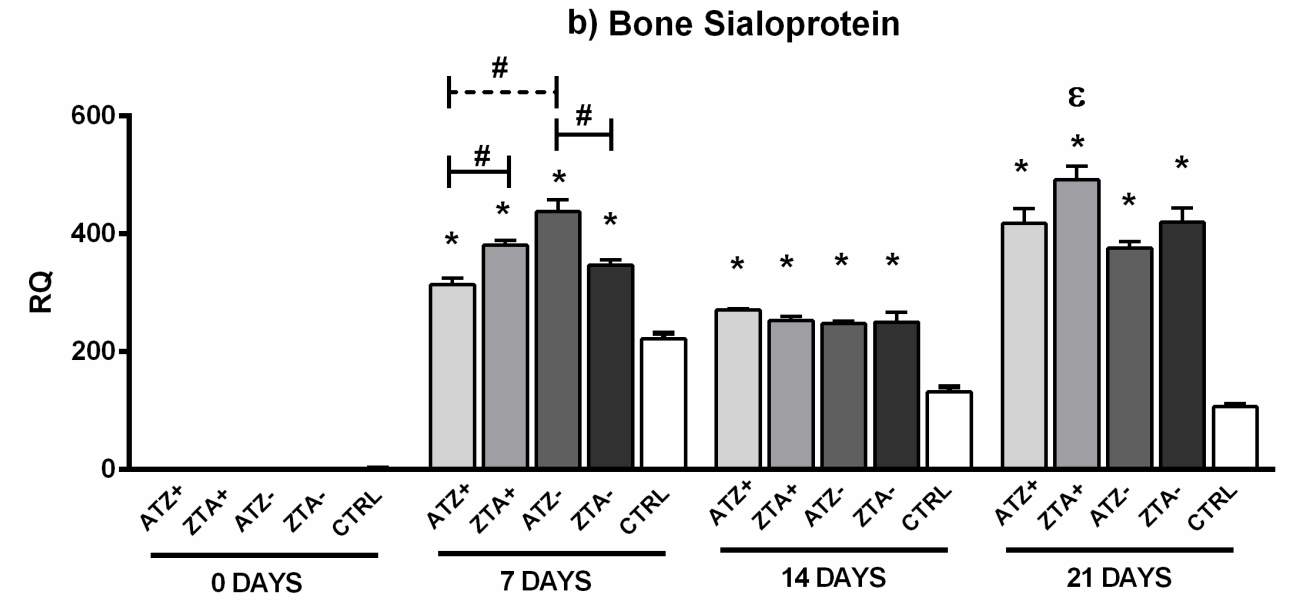
Osteoinductive proprieties of ceramic surfaces

Osteoblast differentiation markers expression

Firstly, differentiation was investigated evaluating expression of some osteoblast differentiation markers: Alkaline Phosphatase (ALP), Bone Sialoprotein (BSP), Osteocalcin (OC). Real time PCR results are showed in fig. 12 (a, b and c).

In all cases, at day 0 the gene expression is absent. ALP and BSP have a fluctuating expression as they have an higher expression after 7 and 21 days of differentiation than after 14 days of differentiation. Instead, OC has a growing expression as differentiation time passes. MC3T3 cells cultured on ZTA+ show a significant higher ALP expression than cells cultured on ATZ+ after 7 and 21 days from the differentiation induction. After 7 days of differentiation cells have a higher expression of ALP when cultured on ATZ- compared to all other surfaces. However, after 21 days from the differentiation induction, ZTA+ induces the highest ALP expression on cells. As regards Bone Sialoprotein, the trend is similar to that of Alkaline Phosphatase expression. At 7 days of differentiation, ZTA+ induces higher expression on MC3T3 cells compared to ATZ+ while cells plated on ATZ- show a higher expression of BSP than cells plated on ATZ+ and ZTA-. After 21 days of differentiation, ZTA+ has the highest BSP expression. CTRL has significant lower values of BSP expression after 7, 14 and 21 days of differentiation compared to all composite ceramics. Finally, we can see from the third graph the OC expression. It is significantly higher after 7 days of differentiation in cells cultured on CTRL than in cells cultured on ATZ+. After 14 days of differentiation, cells cultured on CTRL show the highest OC expression. Moreover, cells plated on ATZ- have a significantly higher OC expression if compared to cells cultured on all other ceramics. Cells cultured on ZTA+ has a significantly higher expression than cells cultured on ATZ+. After 21 day of differentiation, cells on CTRL show a higher OC expression compared to cell on ATZ- and ZTA-. Comparing ceramics, ZTA+ induced the highest expression of OC, followed by ATZ+, ATZ- and ZTA-, that shows the lowest OC expression. Analysis of variance (ANOVA) followed by Bonferroni test were used as scoring tests. Three replicates for each experimental point were used.


a) Alkaline Phosphatase
RQ
300
200
100
0
ε
*
#
ε
*
ATZ+
ZTA+
ATZ-
ZTA-
CTRL
0 DAYS
7 DAYS
14 DAYS
21 DAYS


b) Bone Sialoprotein
RQ
600
400
200
0
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ATZ+
ZTA+
ATZ-
ZTA-
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0 DAYS
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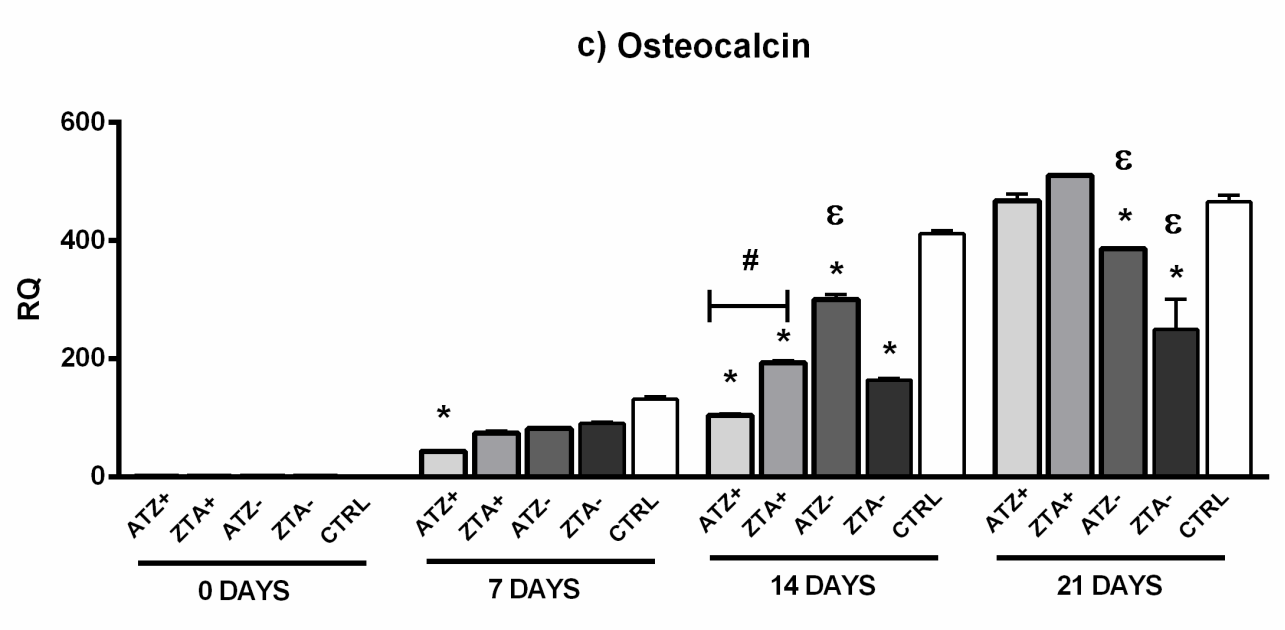

Fig. 12. *Expression of the osteogenic differentiation markers. Quantitative real-time polymerase chain reaction (RT-PCR) analysis of Alkaline Phosphatase (a), Bone Sialoprotein (b) and Osteocalcin (c) transcript level. ANOVA test followed by Bonferroni test was used as scoring test. The symbol ***** means a significant difference versus CTRL considering a p-value < 0.05; the symbol **ε**< 0.05 means a significant difference versus all other treated or untreated ceramics considering a p-value < 0.05 ; the symbol **#** means a significant difference versus due different ceramics considering a p-value < 0.05 (continuous line = consecutive ceramics; dashed line = non consecutive ceramics).*

ALP activity evaluation

To evaluate the differentiation of osteoblasts an enzymatic assay, ALP activity assay, was employed. The activity of ALP enzyme after 14 days of culture on ATZ+, ZTA+, ATZ-, ZTA- and control is shown in Fig. 13. As we can see, the quantity of ALP is significantly increased when osteoblasts are cultured on ZTA+ and ATZ+ compared with control. Instead, there isn't a significant difference between treated and not treated ceramics, even if ATZ+ and ZTA+ seem to permit an higher ALP activity in osteoblasts especially compared to ATZ-. Mann-Whitney test was employed as scoring test.

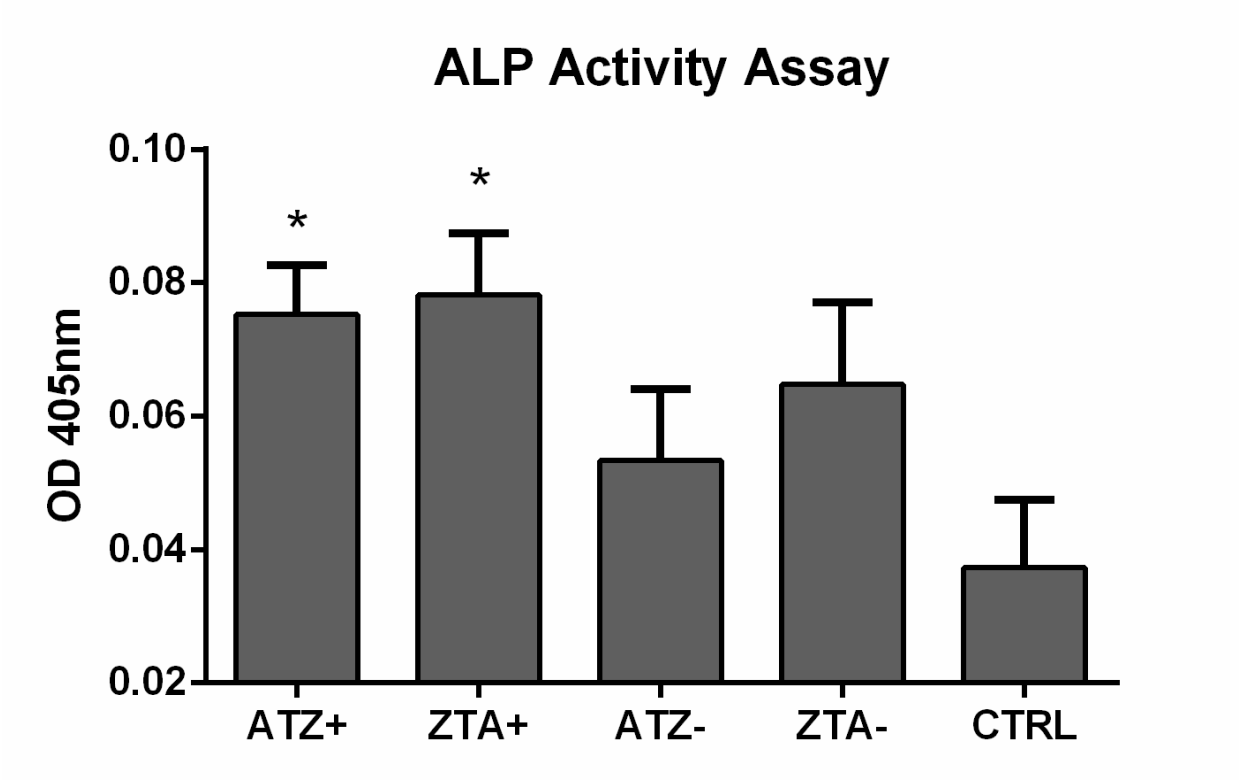

Fig.13. *The effect of ATZ+, ZTA+, ATZ-, ZTA- and control on ALP activity of MC3T3. The symbol * means a significant difference versus CTRL considering a p-value <0.05 using a Mann–Whitney test.*

Bone mineralization evaluation

Mineral deposition was observed by day 21 of differentiation as shown in figure 14. Calcium deposition was significantly affected as a function of the substrate used. All ceramics composite induced a significant enhancement in solubilized Alizarin Red, which means an increase in mineralization. However, ATZ+ induced the highest mineralization with a significant increase compared to untreated ATZ and ZTA.

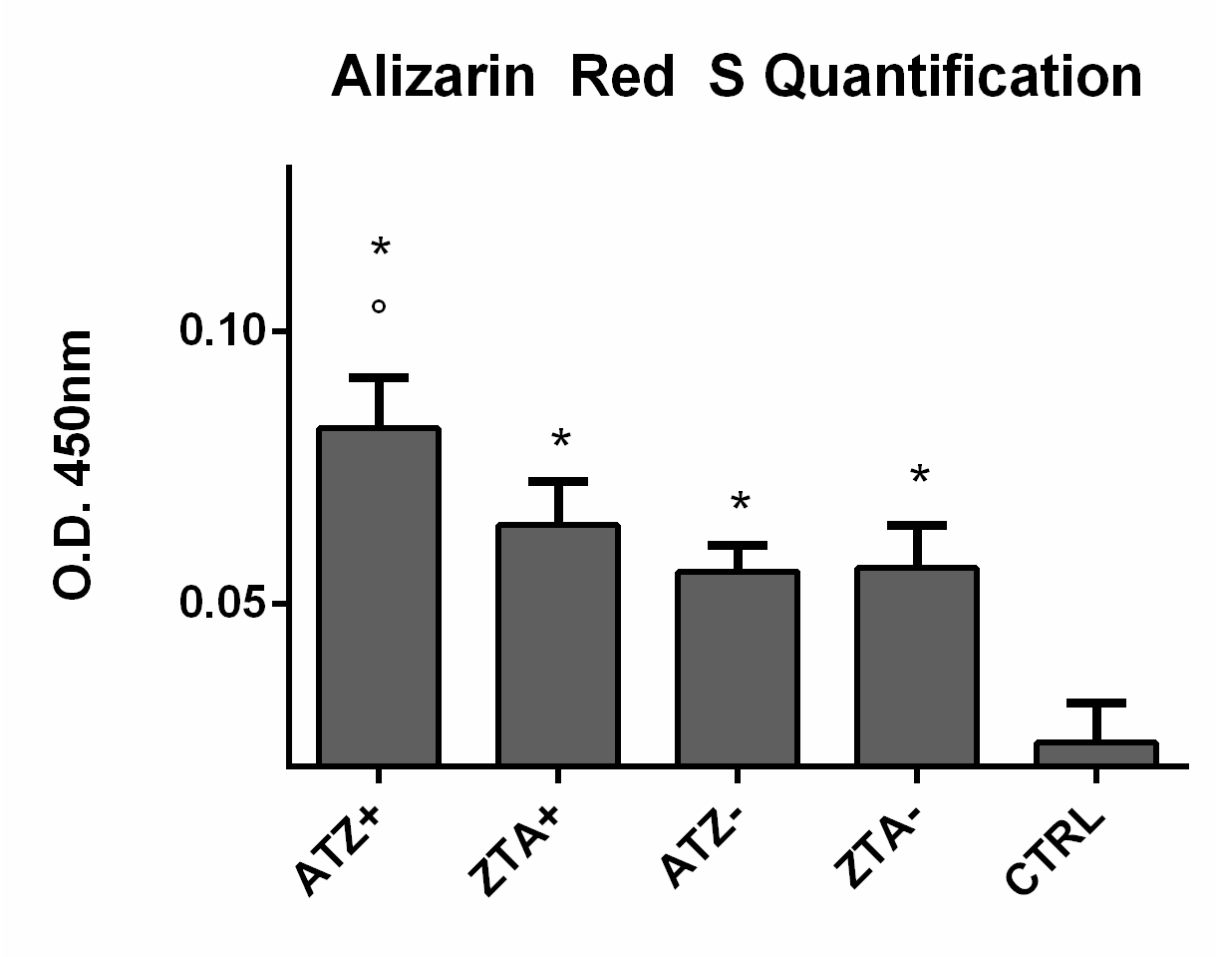

Fig. 14. *Alizarin red staining performed on day 21 post induction of differentiation, which indicates the mineralization of osteoblasts. The symbol * means a significant difference versus CTRL condition considering a p-value <0.05 using a Mann-Whitney test ; the symbol ° means a significant difference versus ATZ-, ZTA- considering a p-value <0.05 using a Mann-Whitney test.*

In order to confirm the mineralization obtained with Solubilized Alizarin Red a calcium assay content was performed as shown in figure 15. As confirmed by this assay, all ceramics composite induced a significant enhancement in mineralization compared to CTRL condition. Moreover, ATZ and ZTA treated showed a significant increase compared to untreated ceramics.

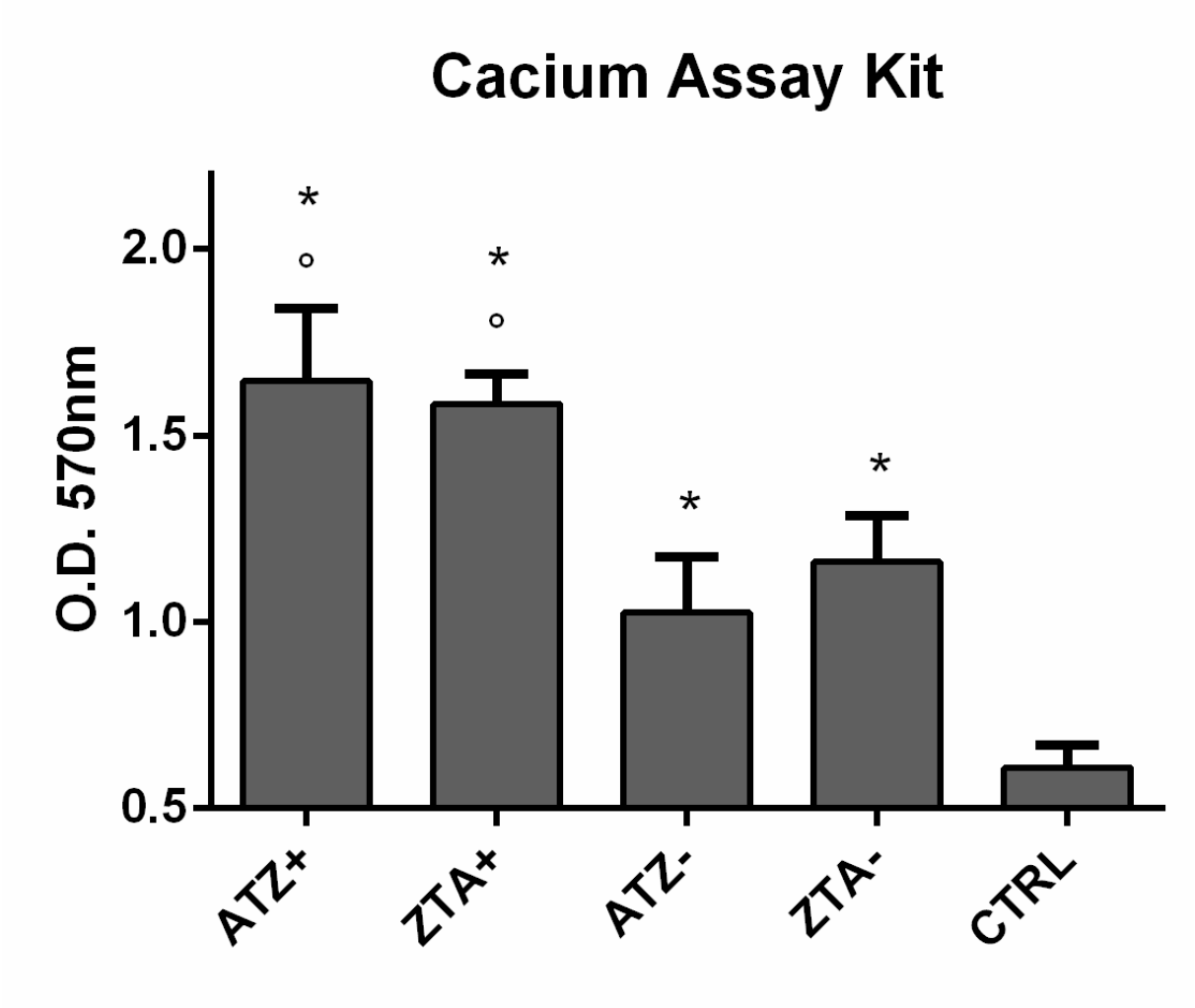

Fig. 15. *Calcium Assay Kit on day 21 post induction of differentiation, which indicates the mineralization of osteoblasts. The symbol * means a significant difference versus CTRL condition considering a p-value <0.05 using a Mann-Whitney test; the symbol ° means a significant difference versus ATZ-, ZTA- considering a p-value <0.05 using a Mann-Whitney test.*

Discussion

From the second half of the XVIII century, titanium has been employed as material for bone interface material. Its physical features and great biocompatibility are so suitable that titanium is considered the “gold standard” today in clinical applications. However, some limits concerning this biomaterial are the reason that bring scientist to study new biomaterials that could be used as bone interface materials.

Scientific evidence on alumina and zirconia composites supports their reputation of suitability for manufacturing orthopedic prosthetics, due to their satisfactory biological response (Roualdes et al. 2010) and mechanical properties superior to those of monolithic oxides (De Aza et al. 2002; De Aza et al. 2003; Affatato et al. 2006).

Among oxide ceramics materials, alumina exhibits a high wear resistance and excellent mechanical properties. However, it has high fracture rates (Piconi & Maccauro 1999) and due to its bioinert behavior it is not favorable for applications. Zirconia is more resistant than alumina to crack growth, but case studies have shown that delayed failure can occur in vivo (Masonis et al. 2004; Rinke et al. 2015). The development of mixed oxides ceramic materials may be able to combine the properties of both alumina and zirconia, emphasizing specific properties, and could be the key for good alternative bearing surfaces. The addition of a fraction of zirconia to alumina or vice versa results in a “composite” material of increased toughness. Two kinds of composites can be prepared in the alumina – zirconia system: a zirconia matrix reinforced with alumina particles (alumina-toughened zirconia, ATZ) or an alumina matrix reinforced with zirconia particles (zirconia-toughened alumina, ZTA). Also, since the visual characteristics of these ceramic materials lead to advantageous aesthetic features, which are appreciated especially in dentistry, alumina–zirconia composites may be proposed as materials for dental implant fabrication. Thus, ATZ and/or ZTA might become a feasible alternative to the monolithic zirconia devices that are already clinically used (Oliva et al. 2010), despite some concerns have recently arisen regarding their possibly low survival rate (Kohal et al. 2012; Rinke et al. 2015).

The mechanical stability is not the only requirement a good implant material should possess, since bioactivity is necessary to ensure proper osseointegration. When dealing with bone

bonding materials, bioactivity can be described as the ability to grow bonelike apatite on the material surfaces. Apatite formation in simulated body fluid (SBF) is preferentially induced whenever particular hydroxyl sites are on the surface, which can be achieved through acidic and/or alkali treatments. Consistently, Faga et al. (Faga et al. 2012) described the formation of acicular hydroxyapatite crystals onto the surface of ATZ samples treated hydrothermally. Furthermore, alumina zirconia ceramics may elicit slightly better biological responses than the commercially pure titanium usually employed for dental implants.

Moreover, in literature, there is a vast presence of researches on functionalization of biomaterials for improving its ability (Seol et al. 2006; Schliephake et al. 2005; Liu et al. 2005). A work on absorption of laminin on ATZ and ZTA has recently been published (Vallée et al. 2014) and it demonstrates that ceramics samples functionalized with laminin show a better cellular activation than untreated specimens. In future studies, it could be interesting to study the functionalization also on ATZ and ZTA treated with hydrothermal cycles.

To study the in vitro effect of this hydrothermal treatment (TO2012A000029) we firstly studied the cell viability at 24, 48 and 72 hours. MC3T3-E1 properly grew on all surfaces. Therefore, results suggest that composite ceramics were not cytotoxic and that treated or not ATZ and ZTA induced proliferation in the same amount of the control.

In order to evaluate the early cellular biological response to these surfaces we studied the protein adsorption and cell adhesion at 10 minutes. Interestingly, the patented hydrothermal treatments significantly increase the measured level on both assays. This data are in accordance since the protein adsorption strongly influence the cell adhesion.

To evaluate the effect on cellular morphology we performed a software-aided image analysis with ImageJ on nuclei, cytoskeleton and focal adhesions labeled with fluorescence. Notably no significant differences was observed among the considered conditions in terms of cell's shape and cell area. Notably, the density of focal adhesions measured by paxillin, gives some information about proper attachment of osteoblasts on surfaces. Indeed, all the treated or not ceramics showed a significantly higher focal adhesion density compared to the control. However, there wasn't a significant difference neither among the various ceramics nor among treated or not ceramics. This data suggest a stronger adhesion of MC3T3-E1 on ceramics surfaces compared to control condition.

To evaluate osteoinductive effect of ceramics composite and hydrothermal treatment, we evaluated the expression of specific key marker, the activity of ALP and the production of mineralized matrix. Real-time PCR (that allow to measure expression of osteoblast differentiation transcripts) results on ALP, BSP, OC seem difficult to unambiguously elucidate. Still, we can say that at 21 days ZTA+ seemed to globally improve the expression of differentiation markers better than other ceramics. At 7 or 14 days, ATZ- has globally higher values compared with other ceramics. However, we can take into account that these transcripts are late differentiation markers and therefore that data after 21 days of differentiation might be more reliable.

Next we performed a more functional investigations by means of measuring the alkaline phosphatase (ALP) activity and the bone production efficiency.

ALP activity was significantly increased when osteoblasts were cultured on treated ceramics compared with control. Also, ZTA+ and ATZ+ ceramics seem to enhance ALP activity compared with ATZ- and ZTA- but there was no significant difference.

Mineral deposition was evaluated using two different assay for a more reliable analysis. In particular we measured the bone matrix deposition with Alizarin Red S and with a kit for calcium quantification. Globally results showed that calcium deposition was significantly enhanced in all ceramics compared with control. Hydrothermal treatment seem to increase the bone production, indeed in Alizarin Red S assay ATZ+ also in comparison with all other ceramics, and in calcium deposition Assay, both treated ATZ and ZTA induced a significant higher mineralization compared to all the other surfaces.

Conclusion

The objective of the present study is to investigate the biological *in vitro* response to two ceramic composite, in particular, zirconia matrix reinforced with alumina particles (ATZ) or an alumina matrix reinforced with zirconia particles (ZTA), studying the possible positive role of a specific hydrothermal treatment (TO2012A000029).

Based on the present study it's possible to affirm that composite ceramics increase the *in vitro* biological response compared to control condition. Moreover the hydrothermally treated surfaces strongly ameliorate both early cellular response and ostoinductive response to ceramic composites. Indeed, both cell adhesion and MC3T3-E1 differentiation are increased, suggesting that this could be a promising treatment for ceramics as bone interface materials.

Obviously more studies are necessary to confirm the obtained data, and it would be of great interest to study the effect of this treatment on other cell models and on different patterns of surface modification in terms of roughness.

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