ORIGINAL RESEARCH ARTICLES

FUNCTIONALIZATION OF TITANIUM SURFACE WITH CHITOSAN VIA SILANATION: 3D CLSM IMAGING OF CELL BIOCOMPATIBILITY BEHAVIOUR

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Abstract

Introduction

Biocompatibility ranks as one of the most important properties of dental materials. One of the criteria for biocompatibility is the absence of material toxicity to cells, according to the ISO 7405 and 10993 recommendations. Among numerous available methods for toxicity assessment; 3-dimensional Confocal Laser Scanning Microscopy (3D CLSM) imaging was chosen because it provides an accurate and sensitive index of living cell behavior in contact with chitosan coated tested implants.
Objectives: The purpose of this study was to investigate the in vitro biocompatibility of functionalized titanium with chitosan via a silanation using sensitive and innovative 3D CLSM imaging as an investigation method for cytotoxicity assessment.

Methods

The biocompatibility of four samples (controls cells, TA6V, TA6V-TESBA and TA6V-TESBA-Chitosan) was compared in vitro after 24h of exposure. Confocal imaging was performed on cultured human gingival fibroblast (HGF1) like cells using Live/Dead® staining. Image series were obtained with a FV10i confocal biological inverted system and analyzed with FV10-ASW 3.1 Software (Olympus France).

Results

Image analysis showed no cytotoxicity in the presence of the three tested substrates after 24 h of contact. A slight decrease of cell viability was found in contact with TA6V-TESBA with and without chitosan compared to negative control cells.

Conclusion

Our findings highlighted the use of 3D CLSM confocal imaging as a sensitive method to evaluate qualitatively and quantitatively the biocompatibility behavior of functionalized titanium with chitosan via a silanation. The biocompatibility of the new functionalized coating to HGF1 cells is as good as the reference in biomedical device implantation TA6V.

Key words

Chitosan coating, Biocompatibility, Live/Dead® staining, 3D Scanning Confocal Microscopy, Titanium implants.

Résumé

Introduction

Un des critères de biocompatibilité est l'absence de toxicité des matériaux pour les cellules, selon les recommandations de la norme ISO 7405 et10993. Parmi les nombreuses méthodes disponibles pour l'évaluation de la toxicité, l'imagerie tridimensionnelle à l'aide du microscope confocal à balayage laser (3D CLSM) a été choisie en raison de sa précision et de sa sensibilité dans l'étude de la viabilité cellulaire en présence d'implants revêtus de chitosane.

Objectifs

Le but de cette étude a été d'analyser la biocompatibilité in vitro d'une surface de titane fonctionnalisée par du chitosane via une silanation. Celle-ci a été effectuée en utilisant une nouvelle méthode sensible et innovante

d'investigation de détection de la cytotoxicité: 3D CLSM.

Méthodes
La biocompatibilité de quatre échantillons (cellules de contrôle, TA6V, TA6V-TESBA and TA6V-TESBA-Chitosane) a été comparé in vitro après 24h d'incubation. L'imagerie confocale a visualisé une culture cellulaire de fibroblastes gingivaux humains marqués par la coloration Live/dead®. Les clichés ont été obtenus avec un confocal FV10i système biologique inversé et analysé avec le logiciel FV10-ASW 3.1 (Olympus France).

Résultats
L'analyse des images a démontré l'absence de cytotoxicité des trois substrats testés après 24 h. Une légère diminution de la viabilité cellulaire a été constatée dans le cas de TA6V-TESBA-CS en comparaison avec les cellules contrôles.

Conclusion
Nos travaux démontrent l'utilisation de l'imagerie confocale CLSM 3-D time-lapse comme une méthode sensible pour évaluer qualitativement et quantitativement le caractère biocompatible d'un substrat de titane fonctionnalisé par du chitosane via une silanation. La biocompatibilité de ce nouveau greffage avec les cellules HGF1 est équivalente à celle du dispositif de référence (TA6V) utilisé dans l'implantologie médicale.

Mot clés
Revêtement à base de chitosane, Biocompatibilité, Marquage Live/Dead®, Microscopie confocale à balayage, Implants en titane.

Introduction
Success rates of dental implants are relatively high[1,2]. Failures can occur principally due to peri-implant disease, which is the general term used to describe host tissue inflammatory reactions, caused by bacterial colonization of dental implants affecting the soft and the hard surrounding tissues[3,4]. Titanium and its alloys are typically used for implants because of their superior biocompatibility, first-rate corrosion resistance and high mechanical properties[5,6]. Nowadays, titanium implants do not prevent peri-implant infections[7]. Therefore, an alternative is needed using titanium implants with a bioactive coating that would have antiseptic properties. Implants should also allow for strong adhesion of peri-implant soft tissues which helps to prevent bacterial colonization and subsequent chronic inflammation[8]. That is why the implant should also allow for improved gingival cell adhesion on its surface. The use of natural polymers seems to be an attractive option because of their good biocompatibility. Chitosan is a collective name for a group of partially and fully deacetylated chitin compounds[9]. It represents is a unique biopolymer that exhibits outstanding properties, beside biocompatibility and biodegradability[10], many applications have been found either alone or blended with other natural polymers, in the food, pharmaceutical, textile, agriculture, water treatment and cosmetic industries[11,12,13]. It has been shown that chitosan can increase the growth and attachment of gingival cells[14,15]. In addition, the positive charge of the amino groups along the biopolymer chain is reported to confer unique antibacterial properties[16]. Consequently, Chitosan represents an attractive candidate for coated titanum implants. The most challenging part of this type of coating is the attachment of chitosan to a metal substrate. Studies have been conducted on chitosan simply deposited on the metal surface with a weak bond (0.5 MPa)[17]. Chitosan films can also be formed by coating the substrate with a silane molecule. A successful simplified method to coat the implant material using a silane has recently been used by our group[18]. This method involves the grafting of triethoxysilyl butyraldehyde (TESBA) which is directly linked with chitosan[19]. The biological properties of the coated-chitosan material have to be demonstrated. There is no evidence that the coating process did not affect the chitosan properties[20]. Among newly developed methods for testing dental materials biocompatibility behavior, techniques based on fluorescing agents as a powerful investigative tool have been used in different fields of dental research. These tracers with high specificity are excellent for identifying the path or the current location of a compound because most of them are stable in an aqueous environment, are easily detectable even in at low concentrations, are inexpensive and are non-toxic, enabling their use in clinical as well as laboratory investigations[21]. As an improvement over electronic microscopy techniques, scanning confocal microscopy offers the ability to visualize distinct components of cells by incorporation of fluorescent markers. This provides greater resolution than conventional imaging, yielding greatly enhanced images of biological structures[22]. The intricate and often

complicated methodology of specimen drying required for conventional electronic microscopy analysis is not necessary for confocal imaging. An additional feature of the confocal principle is that it permits visualization not only of a specimen surface, but also its subsurface thus achieving a three-dimensional image, revealing more accurate and informative structural correlations when compared to two-dimensional analyses[23,24].

The aim of the present study was to assess the in vitro biocompatibility of coated implant with TESBA as silane, using 3D CLSM imaging as a relevant and sensitive cytotoxicity assay. The CLSM method provides advantages in testing procedures over conventional methods in terms of sensitivity, accuracy and image resolution. The method presented in this paper allowed live observation and imaging of cells without changing the cell mass structure. We used The Live/Dead® cell cytotoxicity kit staining for dental implant cytocompatibility assessment. The method employed has recently been published by our group to differentiate the biocompatibility behavior of two commercially dental composites based on similar methacrylate monomers[25].

Materials and Methods

Materials

Titanium samples were supplied by Global D (France). Toluene, ethanol, low molecular weight chitosan, DMSO, HDMS, Glutaraldehyde, Sodium Cacodylate, resazurin and propidium iodide were obtained from Sigma-Aldrich (France) while TESBA were purchased from ABCR (Germany). Media, buffer solutions, Trypsine/EDTA, Triton X100 and antibiotics were purchased from PAA Company (Austria). Staining solutions were obtained from In vitrogen (USA). Fibroblasts cells were purchased from American Type Culture Collection (ATCC, USA).

Surface functionalization of titanium substrates

Titanium samples were sonicated for 30 min in a solution (acetone/ethanol, v/v, 50/50). They were left for 15 min in a fresh piranha solution (sulfuric acid/hydrogen peroxide, v/v, 70/30) at room temperature. Then metal coupons were strongly rinsed in ultra-pure water. To attach silane on surfaces, dried titanium piranha treated samples were submerged in a solution of triethoxysylilbutyraldehyde (TESBA) in extra-dry toluene (v/v, 2/98) and allowed to react for 24 h. Following the 24 h reaction time, the coupons were placed in pure anhydrous toluene and sonicated for 30 min. The procedure of sonication was repeated twice more using fresh anhydrous toluene, for a total sonication time of 90 min. To remove any residual toluene, the metal coupons were rinsed with ethanol and then dried 10 minutes at room temperature. Then, the substrates were dipped one time in a solution of 4 wt.% chitosan, 2 wt.% acetic acid, and 94 wt.% deionized water through a dip-coater (v = 3mm/s). The chitosan-coated samples were then allowed to dry at 80°C for 4 hours. The structure formulas of the coupling agent and surface modification steps are illustrated (Fig.1).

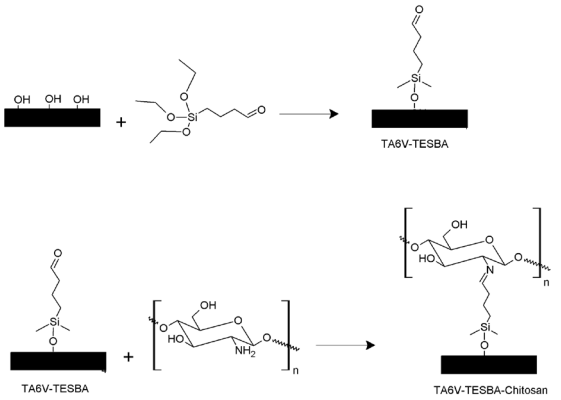

Fig.1: Reaction scheme allowing the covalent bonding of chitosan to titanium surface.

Cell culture

Human gingival fibroblast cell line (HGF1) was used in this study. This cell line has been frequently used for dental material biocompatibility evaluation. Cells were cultured in Dulbecco's modified Eagle medium (DMEM) in the presence of 10% fetal bovine serum (FBS), 2% Penicillin/Streptomycin and 1% Amphotericin B. Cultures were maintained at 37°C under a humidified atmosphere of 5% CO2 in air. The medium was changed every 3 days, and the cells were passaged every 5 days. After reaching confluence, the cells were trypsinized and resuspended in the culture medium. The cells were centrifuged at 1200 rpm/min for 5 min and counted with a Scepter handheld automated cell counter (Millipore, USA). After the removal of trypsin, the remaining cell pellets were resuspended in a fresh medium for subsequent experiments. One milliliter of cell suspension at a cell density of $2.5x10^4$cells/ml was seeded in 12-well

microplates in the presence or absence of tested substrates.

Cytocompatibility assessment via Live/Dead®

Fluorescent labelling of cells

The Live/Dead^{V} cell cytotoxicity for mammalian stain was used according to the manufacturer's instructions (Invitrogen European Headquarters, UK). The kit provides a two-color fluorescence assay of cell viability relying on membrane integrity: viable cells are stained by Calcein and fluoresce green, while damaged cells are stained by Ethidium homodimer-1 (EthD-1) and fluoresce red. The determination of cell viability depends on these physical and biochemical properties of cells. A working solution of a final concentration containing 2 µM of Calcein AM and 4 µM EthD-1(prepared by combined of the two stain reagents), this concentration was found to be suitable for fibroblasts. The stain was then added to adherent cells on the selected substrate surfaces. This short staining protocol allowed direct observation of the original cell structure and the time-lapse microscopy. No centrifugation or fixation steps were needed. Microscopic observations started 15 min after staining. Excitation/emission maxima for Calcein and EthD-1 are 495/515 and 525/635 nm, respectively. The polyanionic dye Calcein is well retained within live cells, producing an intense uniform green fluorescence in live cells. EthD-1 enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence in dead cells (Neri et al., 2001). If there are no nucleic acids in the bulk media, the background of the images remains poorly fluorescent.

Confocal Laser Scanning Microscopy (CLSM)

Image series were obtained with a FV10i confocal laser scanning biological inverted microscope (Olympus, France). Tow laser sources 473 nm (15mW) and 559 nm (18mW), were used to excite Calcein and EthD-1. The bandwidths of the detected fluorescence have been optimized for each channel to the maximum. All regular acquisitions were collected sequentially (473nm/559nm) to avoid potential cross-talking (Fig. 2). 1024×1024 pixels regular images were obtained with a 60× objective, with a 0.231μm×0.231μm pixel size.

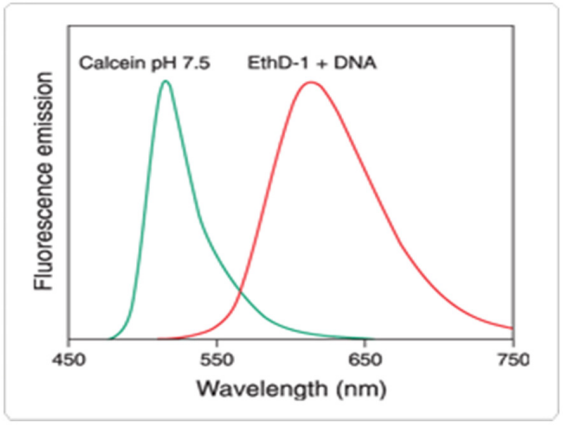

Fig.2: Fluorescence excitation and emission spectra of the two dyes used in the Live/Dead® staining.

Images were stored as12bits/pixel TIFF files and analyzed with FV10-ASW 3.1 Software (Olympus, France). Images analysis and viability rate calculation was performed on nine image stacks from the 39 captured map images (acquired with a10x objective). CLSM observations were performed with cells in the presence or absence of substrates. The cytotoxicity experiments were done with four different reactor chambers: the first one containing stain cells without substrate, the second in the presence of cells growth on TA6V substrate, the third in the presence of cells growth on TA6V TESBA substrate and the fourth one in the presence of cells growth on TA6V TESBA substrate. A chamber slide system (14220040X, PAA. France) was used. These non-fluorescent microscope slides contain 4 chambers (square wells, working volume: 0.2–0.5 mL). The chamber was placed in an incubator (inside the confocal instrument) with a 37ºC temperature, a humidity level higher than 90% and 5% of CO_2.Water was automatically supplied to the water-immersion inverted objective. During the experiments the scan was continuous running for the necessary acquisition period.

Statistical analysis

The statistical analysis of the current data was done by the application of one-way analysis of variance (ANOVA), comparing the results of exposed cells to tested substrates to control cells. The results are reported as mean ± SD. Statistical significance was accepted at $P < 0.05$.

Results

Live/Dead® staining
CLSM analysis and cell viability
The optical zoom of the confocal instrument used allowed for a fast overview of the slides before zooming in on selected cell populations with optimal magnification. The three-dimensional structure of cells was able to be visualized. Visualization of cells collected in the control chamber and of cells collected in the chamber after 24 hours of contact with the indicated substrates is presented in the Figure 3; this showed the fate of selected mass cells. Nine stacks of images (xyzt mod scan, 29 images per stacks, voxel-depth 0.99 µm) were obtained. No variation was observed for uncoated TA6V substrates compared to negative control cells. A slight decrease in green fluorescence and an increase in red fluorescence were obtained in cells in the presence of TA6V-TESBA without chitosan (Fig.3.III) and with chitosan (Fig.3.IV) respectively. Images analysis was performed on 9 different image stacks selected in different cell populations from the 19 mapping images for each tested sample. Calcein and EthD-1 fluorescence emission intensity were measured on control cells, TA6V, TA6V-TESBA and TA6V-TESBA-Chitosan substrates after 24 h of cell contact. The green/red fluorescence ratio based on integrated intensities of the green (495-515 nm) and red (620-650 nm) was calculated. The viability ratios as a function of substrate type are presented in the table1. The percentage of live cells was found to decrease by 7.1% in the presence of TA6V substrates, by 7.5 % in the presence of TA6V-TESBA with chitosan, and by 7.9 % TA6V-TESBA without chitosan coating. No significant different was observed

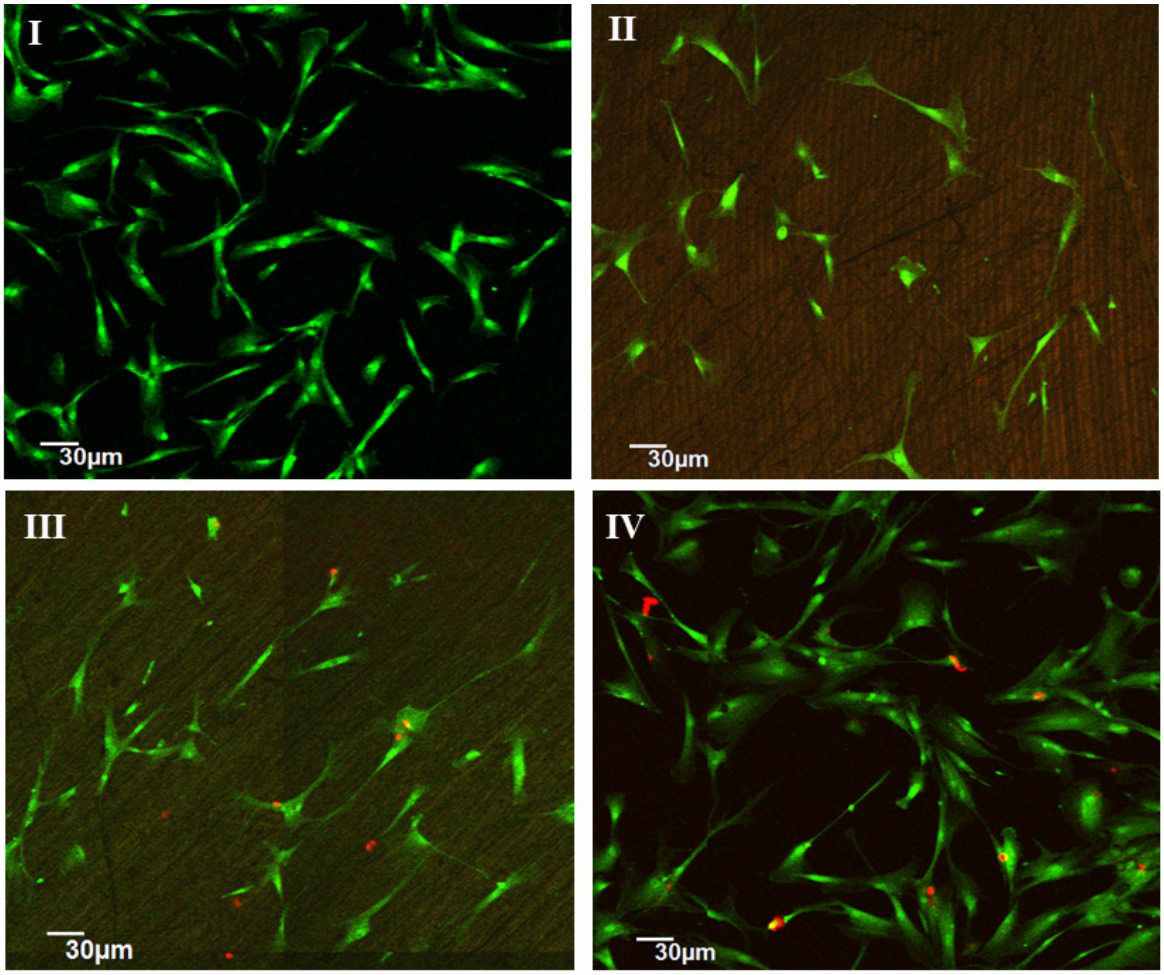

Fig. 3: Confocal laser scanning microscopy images of a population cells coming from a control chamber (I), from TA6V chamber (II), from TA6V-TESBA chamber (III) and from TA6V-TESBA-Chitosan (IV) after 24h hours of contact. Green areas are live cells and red areas are damaged cells. Live/Dead® staining.

Substrates	Cell Viability (%)
Control cells	100
TA6V	92.9 ±7
TA6V-TESBA-Chitosan	92.5 ± 5
TA6V-TESBA	92.1 ± 9

Table 1. Rate of live HGF-1 cells evolution after 24h of contact with substrates. Cell viability was assayed by staining using the Live/Dead® cytotoxicity kit. Data show mean values ± SD of nine image stacks analysis. Values are not significantly different from control cells.

between the tested substrates and the negative controls (cells without any substrates) after 24 hours of contact. The line profile of stained cells is shown in the figure 4; the green and the red signal evolution of the cells in contact with tested substrates was comparable to those of control cells. Morphology modifications

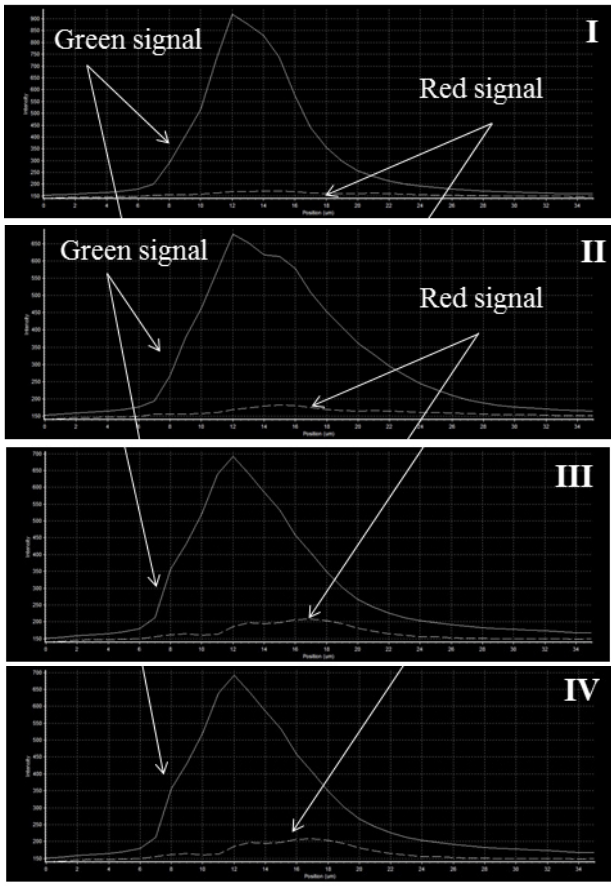

Fig.4: Line profile of cells observed in CLSM microscopy. Control cells (I), Cells in contact with TA6V (II), Cells in contact with TA6V-TESBA (III) and Cells in contact with TA6V-TESBA-Chitosan (IV) after 24h hours of contact.

are slightly more visible in the presence of chitosan coating than in the presence of uncoated substrates (Fig.5).

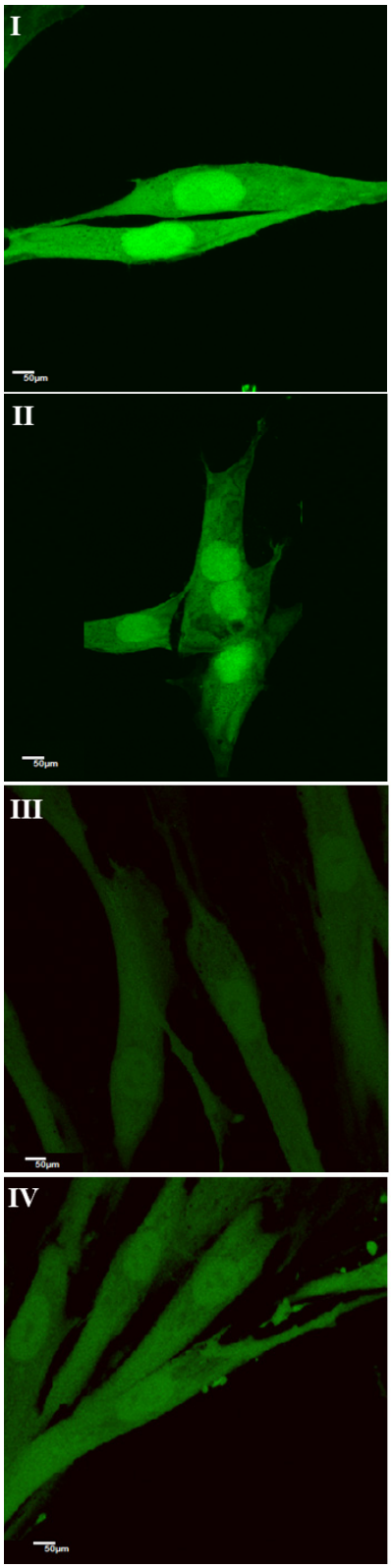

Fig. 5: Panel of CLSM images showing slight modifications cell morphology. Control cells (I), Cells in contact with TA6V (II), Cells in contact with TA6V-TESBA (III) and Cells in contact with TA6V-TESBA-Chitosan (IV) after 24h hours of contact. Live/Dead® staining.

Discussion

All biomaterials used in dentistry must be evaluated for biocompatibility using screening assays for patient health and safety[26]. The traditional concept of biocompatibility is regarded as a lack of significant adverse reaction between the oral tissues[27]. Nowadays, it is recognized that there are few materials, if any, which do not create a significant interaction with the host tissues[28,29]. Such reactions may aid the oral healing response following restorative treatment. An updated definition of biocompatibility might be the ability of a restorative material to induce an appropriate and advantageous host response during its intended clinical usage. The need of biocompatible materials for use in dental implants has generated a requirement for cytotoxicity assays to screen compounds and characterize the potentially harmful effects of a biomaterial to oral tissues prior to clinical use. Cytotoxicity screening assays provide a measure of cell death caused by materials or their extracts. Different types of screening assays are available, and it is important to understand the advantages and limitations of each assay, so that they can be selected for appropriateness and interpreted accurately. The evaluation of in vitro cytotoxicity of a biomaterial is the initial step on a biocompatibility investigation.

International standards cover specifically dental materials (ISO 7405)[30] and medical devices (ISO 10993)[31], which also include dental materials. The recommended testing methods (ISO 10993; ISO 7405) use cell counting, dye-binding, metabolic impairment or membrane integrity as endpoints of the cytotoxicity assay. The direct use of cell and colony counting as an assay endpoint is probably the least reliable method. Ideally, endpoints should conform to strict criteria for classifying results, so that the subjective element when determining a measure of cytotoxicity for a test material is minimized.

The aim of this study was the use of innovative CLSM imaging to assess and screen the cytotoxicity of a chitosan implant coating via a silanation technique. The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide MTT method using metabolic activity endpoint was previously used to evaluate the biocompatibility of the same assayed implant coating[18]. The MTT test is based on the ability of viable cells to produce formazan from the cleavage of the tetrazolium salt by functional mitochondria[32]. However, the MTT test requires killing the cells, making it impossible to follow-up cell cultures. In order to monitor more closely the behavior of the stained cells in the current study, direct observations of live cells combined with automated image analysis quantification were performed. The method involved direct observation of the original cell structure, without the need for centrifugation or fixation steps, yielding accurate measurements of changes in the fluorescence of the probes. The comparison of the various techniques used for biocompatibility investigation has demonstrated that great care needs to be taken when transferring assays normally performed for specific applications and samples. We adapted a confocal procedure recently used to assess time-dependent cytotoxicity of two dental composites[25]. Complications were faced initially when the experiment with the tested implant substrates was performed. Specifically, some difficulties were encountered relating to the development of substrate handling in the u confocal system. The tested substrates did not have the same inclination across each of their surfaces. Indeed, not all cell layers could be visualized on the test implant surfaces within the adapted confocal chamber. Thanks to our knowledge in CLSM imaging, the handling of substrates was validated in the used confocal system.

After that, cell observation and image acquisition became possible on the tested implant surfaces. The investigated CLSM method is based on membrane integrity measurement. The cytotoxicity evaluation via this endpoint seems to be a valuable marker of cell biocompatibility with tested materials and it is also approved by the ISO recommendations. This assessment method was chosen because it provides an accurate index of cell viability and informative structural correlations when compared to the two-dimensional analyses as already mentioned[33,34].

The present study assesses the biocompatibility of the chitosan coating titanium implants regarding human gingival fibroblast cell lines. Chitosan coating has been also evaluated by others regarding osteoblast precursor cell line and human embryonic palatal mesenchyme cells[35] and regarding the UMR 106 osteoblast cells[36,37]. Commonly, human gingival fibroblast cells have been used for cytotoxicity testing because they are sensitive cells, and they are in close contact with dental implants in the oral cavity. A recent study in our lab has demonstrated the good biocompatibility

behavior of the same tested substrate regarding the NIH3T3 murine fibroblast cell line 18. Some studies have assessed the effects of material placed in direct contact with cells. Others have assessed the effects through material extracts collected from the biological medium (indirect contact)[38]. In a test based on direct contact the material sample is in physical contact with the cells, this system showed more clinical relevance[39,40], especially in the case of titanium substrates testing. In different clinical situations, titanium implants could be placed on direct contact with gingival cells. For these reasons, our experimental protocol was focused on the direct contact system with test human gingival fibroblast cells.

Despite the difficulties encountered the adaptation of the recently used CLSM method for the titanium coating investigation showed sensitive measurement, compared to the traditional MTT assay. The coated chitosan substrate seemed to maintain its biocompatible properties by inducing 92% of cell viability, after 24 hours of contact. As previously reported by Dahl and collaborators, cytotoxicity responses were ranked as severe (<30%), moderate (30–60%), slight (60–90%) or non-cytotoxic (>90%) based on the activity relative to values obtained for the controls41. Therefore according to this ranking, the coated material could be ranked as non-cytotoxic and preserved the same behavior as uncoated titanium known as biocompatible.

In conclusion, when deciding which cytotoxicity assay to adopt for a particular study it is important to understand the benefits of each assay. Results highlight that CLSM imaging could be used for titanium implants biocompatibility evaluation; which can sensitively give the initial rate of living cells. Adapted confocal chamber could be developed to avoid the encountered complications. The chitosan method via silanation could be also improved with optimized coating method in order to overcome the substrates inclinations and their drawbacks. Our findings underline the use of 3D CLSM confocal imaging, as a sensitive method to assess biocompatibility behavior of a chitosan coating. Furthermore, the described method of covalent chitosan coating provides a biocompatible material with improved bioactive properties. Chitosan-coated implant material therefore has a significant potential in dental implantology and more generally in biomedical implantation devices.

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