A new method using insert-based systems (IBS) to improve cell behavior study on flexible and rigid biomaterials

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Received: 28 October 2015 / Accepted: 7 March 2016 / Published online: 25 March 2016
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Abstract In vitro studies about biomaterials biological properties are essential screening tests. Yet cell cultures encounter difficulties related to cell retention on material surface or to the observation of both faces of permeable materials. The objective of the present study was to develop a reliable in vitro method to study cell behavior on rigid and flexible/permeable biomaterials elaborating two specific insert-based systems (IBS-R and IBS-F respectively). IBS-R was designed as a specific cylindrical polytetrafluoroethylene (PTFE) system to evaluate attachment, proliferation and morphology of human gingival fibroblasts (HGFs)

on grade V titanium and lithium disilicate glass-ceramic discs characteristics of dental prostheses. The number of cells, their covering on discs and their morphology were determined from MTS assays and microscopic fluorescent images after 24, 48 and 72 h. IBS-F was developed as a two components system to study HGFs behavior on guided bone regeneration polyester membranes. The viability and the membrane barrier effect were evaluated by metabolic MTS assays and by scanning electron microscopy. IBS-R and IBS-F were shown to promote (1) easy and rapid handling; (2) cell retention on biomaterial surface; (3) accurate evaluation of the cellular proliferation, spreading and viability; (4) use of non-toxic material. Moreover IBS-F allowed the study of the cell migration through degradable membranes, with an access to both faces of the biomaterial and to the bottom of culture wells for medium changing.

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Keywords Biomaterials · Cytocompatibility ·
Insert · Human gingival fibroblasts · Membranes ·
Dental prostheses

Introduction

In vitro studies related to biological properties of biomaterials, such as cytocompatibility, are frequent and essential tests performed before preclinical or clinical experimentations (Lauer et al. 2001; Wataha 2012). Yet cell cultures encounter some difficulties

related to cell retention on material surfaces, which can engender bias when evaluating cellular viability, proliferation or spreading. Additionally, some biomaterials are rigid while others are flexible and thinner, and thus require different procedures to be maintained in the bottom of culture wells. Moreover, there are a large variety of biomaterials, with various applications, which require specific evaluations. For example, some thin biomaterials can be intentionally more or less permeable to cell migration and thus the observation of both faces of the material is necessary, which is not possible when the material lies in the bottom of the well. It may also be interesting to isolate one surface of the material if it is different from the rest of the sample, as it is the case with coated biomaterials.

Among biomaterials, dental biomaterials represent a large diversity of materials in terms of properties and indications. Most of them are directly in contact with gingival or bone tissues, such as materials used for dental implant components and prostheses, or membranes used in bone regeneration procedures. Membranes are typically thin and flexible biomaterials, which can also be permeable, while implant and prostheses materials are thicker, rigid and non permeable. Many studies were dedicated to cytocompatibility evaluation of those different types of biomaterials, using various cytotechnology methods, which comprise some disadvantages regarding the aforementioned challenges. These disadvantages are described below, for dental implant and prostheses biomaterials on the one hand, and for guided bone regeneration membranes on the other.

A dental implant is a titanium artificial root, placed in the maxillary bone to replace a missing tooth. Prosthetic teeth are fixed on implants via transgingival components called abutments. Those abutments are classically manufactured in metal alloy, such as titanium, or in polycrystalline ceramic, such as zirconia. Recently, lithium disilicate glass-ceramic abutments have been introduced (Lin et al. 2014). The interaction of the gingival soft tissue with the implant and the abutment material influences the stability of the gingival level around the crown. Particularly, as abutments connect the intrabony implant to the oral environment, the attachment of gingival fibroblasts and keratinocytes to the material is an important requirement to create a hermetic barrier able to protect peri-implant structures from bacterial penetration. If this biological seal is compromised, it generates a

bone resorption around the implant, which might compromise its long-term stability. Thus, the biocompatibility and surface properties of transgingival components are crucial regarding attachment, proliferation and spreading of fibroblasts and keratinocytes on their surface (Abrahamsson et al. 1997, 1998; Rompen 2012). Yet the study of cell behavior on such rigid biomaterials often encounters difficulties related to retention of cells on the surface of the samples. Only a few studies describe how cells are kept on the surface of the discs. A research group used a film of sterilized wax around the specimen (Cochran et al. 1994). However, an accurate and reproducible wax deposit is complicated and time consuming. Moreover, wax nature and composition could be suspected to influence cell behavior. Another group used biomaterial samples in the form of cylinders. The cylinders were embedded in a silicone support, and cells were seeded on the sample surface, resulting in an individual well containing the cylinder cross-section as the well bottom (Nothdurft et al. 2014). This model allows cell retention on the sample surface but its disadvantage lies in the design of the samples (cylinder), which is complex and expensive to manufacture. Additionally, like wax, the silicone pad could also be suspected to influence cell behavior.

Guided tissue regeneration was first described in the 1980s (Karring et al. 1985; Nyman et al. 1982). Nowadays, it is a reliable technique in oral surgery particularly to regenerate bone tissue (Guided Bone Regeneration) in order to place dental implants (Kamoi et al. 2006). In this application, membranes are used to exclude gingival fibroblasts and epithelial cells from the bone defect site and allow bone cells to proliferate. GBR membranes need to be biocompatible and to have cell-exclusion properties (barrier effect), while currently most membranes are designed to progressively resorb after the bone healing process (Scantlebury 1993). The morphology, the attachment and the proliferation of fibroblasts on guided bone regeneration membranes has been studied for more than 25 years, but standardized protocols are needed to improve experimentation (Behring et al. 2008). Indeed, the main problem encountered when performing such kind of tests is the difficulty in fixing membranes, which are thin and flexible materials, in the bottom of a well or a plate of culture. Then seeded cells are not kept on one selected face and can migrate all around the material. Several home-made

sophisticated devices were described in the literature. Yet they do not allow the evaluation of both faces of the membrane independently, and thus the barrier effect. For example several authors use double-faced adhesive tape on the floor of the wells (Kasaj et al. 2008; Takata et al. 2001a; Wang et al. 2002). Some other authors used either minucell-sheet-rings (Minucells and Minutissue Vertriebs GmbH, Bad Abbach, Germany) used in 24-well plates (Rothamel et al. 2004), or membranes under agarose, or half-covered with paraffin (Payne et al. 1996; Takata et al. 2001b). Only one study introduced an in vitro model for the evaluation of the barrier effect (Milella et al. 2001). The experimental device consisted of a glass vial filled with culture medium. The membrane was positioned over the vial and the vial was sealed. This model allows the surface evaluation on both sides of the membrane and has the advantage to include two compartments (with and without cells) to measure the barrier effect. Yet the system does not provide an access to the bottom of the bottle for medium changing and consequently does not allow the long-term measurement of the barrier effect. Moreover, analyses of cells fell in the bottom of the glass vial are not possible.

Consequently, the objective of the present study was to develop two reliable in vitro methods to study cell behavior on rigid biomaterials on the one hand, and on flexible and permeable biomaterials on the other. Two specific insert-based systems (IBS-R and IBS-F) were, respectively, designed for biomaterials characteristics of dental prostheses and implant components, such as rigid biomaterial, and for degradable polymeric membranes for guided bone regeneration, such as flexible and permeable biomaterial. The presented models aimed to facilitate the study of cell behavior promoting (1) easy and rapid handling; (2) cell retention on biomaterial surface; (3) accurate evaluation of the cellular proliferation, spreading and viability of Human Gingival Fibroblasts (HGFs); (4) use of non-toxic material. For membranes, the method was also designed to enable an access to both faces of the biomaterial and to the bottom of culture wells.

Materials and methods

All manipulations were made under sterile conditions (L2 laminar flow hood). The cells were cultured under

the condition of 5 % CO₂ and 37 °C (Binder Incubator, Tuttlingen, Germany).

Culture of human gingival fibroblasts

Primary cultures of human gingival fibroblasts were established using the explant technique. The gingival tissue samples were obtained from healthy adult patients undergoing dental surgery at the oral and maxillofacial surgery department at the University Hospital of Liège. For tissue harvest, informed consent was obtained from the patients and the protocol was approved by the Ethics Committee of the University Hospital of Liège, Belgium (approval number: B707201317973). Explants were immediately stored at 4 °C in a transport medium composed of Dulbecco's Modified Eagle Medium (DMEM high glucose, GlutaMAXTM Supplement, pyruvate, Gibco, Invitrogen, Paisley, UK) supplemented with 10 % fetal bovine serum (FBS, Gibco), 500 µg/ml gentamicin (Gibco), 200 IU/ml penicillin-200 µg/ml streptomycin (Lonza, Verviers, Belgium) and 25 µg/ml fungizone (Gibco). Explants were minced into small fragments (1 mm³) using a blade and were distributed on a Petri dish. 5 ml of complete medium composed of DMEM (DMEM high glucose, GlutaMAXTM Supplement, pyruvate, Gibco) supplemented with 10 % FBS (Gibco), 50 µg/ml ascorbic acid (Sigma Aldrich, St Louis, USA) 100 IU/ml penicillin-100 µg/ml streptomycin (Lonza), were gently added, taking care to not detach the fragments. The cultures were kept for 2 to 4 weeks in the incubator until near confluence. Cells were washed with phosphate-buffered saline (PBS) solution without calcium and magnesium (DPBS, Lonza) and detachment was performed with 0.05 % trypsin-EDTA solution (Gibco). Cells were expanded in T75 flasks by passing a 1:3 split. Stocks from different patients were stored in liquid nitrogen (complete medium dimethylsulfoxide 10 %). All experiments with IBS systems were conducted at 3rd to 6th passage.

Models

A model using a specific insert was developed for each type of biomaterial, respectively: rigid and flexible. The models were called IBS-R (IBS, insert-based system) for rigid biomaterials and IBS-F for flexible biomaterials.

Rigid biomaterials: IBS-R

Materials Discs of commercially grade V titanium (Procera, Nobel Biocare, Göteborg, Sweden) (Ti), and lithium disilicate glass-ceramic (IPS e.max Press, Ivoclar Vivadent, Schaan, Liechtenstein) (eM), 13.5 mm in diameter, were tested. Discs were sequentially ground with 800-, 2400- and 4000-grit silicon carbide discs (Planopol 3[®], Struers, Ballerup, Denmark) to a 1 mm ± 0.02 mm thickness. They were ultrasonically cleaned (Elmasonic One Clean Box, Elma Schmidbauer GmbH, Singen, Deutschland) in a detergent (RBS T105[®] 2 %, Chemical Products, Brussels, Belgium) for 10 min, rinsed with phosphate-buffered saline solution with Ca²⁺ and Mg²⁺ (PBS, Lonza Group Ltd., Basel, Switzerland), rinsed 4 times with purified water (Milli-Q[®] system, Merck Millipore Corporation, Billerica, MA, USA) and then ultrasonically cleaned in ethanol 80 % for 10 min. Finally discs were surface sterilized by UV-C exposure in laminar flow hood for 30 min per face.

Insert Specific cylindrical inserts were made to ensure an efficient seal around discs. These inserts were made of medical-grade polytetrafluoroethylene (PTFE, Fluteck[®] P2000, Sirris, Liège, Belgium) and were machined to measure with a lathe (Hardinge Model HLV-H Precision, Hardinge Inc., Elmira, NY, USA). Inserts were 2 mm thick, 15.5 mm high and 15.5 mm in diameter. In the thickness of the cylinder, a 1 mm high and 1 mm thick bevel was milled in order to receive, with a light friction, the different discs (Fig. 1).

Culture Three incubation time points were tested (24, 48 and 72 h). IBS-R were clipped on discs. Nine systems per material were assembled: 3 systems (technical triplicates) for each incubation time. They were then distributed in 24-well plates culture. HGFs were seeded at a concentration of 5×10^3 cells in 1 ml of culture medium on discs and in polystyrene (PS) wells without disc, as a control for each incubation time. This test was triplicated with cells coming from 3 different patients (biological triplicates).

Optical microscopy After the different incubation times, the PTFE inserts were removed from the discs. To evaluate the sealing effectiveness of the system, the bottoms of culture wells were checked with an inverted phase contrast microscope (Nikon, Tokyo, Japan).

Cellular viability To assess the cellular viability, a viability test was performed on discs removed from the IBS-R to evaluate the living cell population on them. Cellular viability of 100 % was attributed to the HGFs cells grown in PS wells without disc, for each incubation time respectively. Cellular viability on discs was quantified by a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (inner salt MTS, Promega, Madison, WI, USA). MTS solutions were prepared according to the manufacturer's instructions. Discs were rinsed with 1 ml of DMEM/F-12 (Gibco) and 1 ml of new DMEM/F-12 with MTS solution (10 %) was applied on. The plates were incubated in CO₂ incubator in the absence of light for 45 min. Following this, the plates were shaken for 15 s. 200 µl

Fig. 1 Drawing (a) and picture (b) of the IBS-R for rigid biomaterials

Table 1 Membrane characteristics

	Inion	Epi-guide	Bioflex
Type	Resorbable	Resorbable	Non-resorbable
Use	Periodontology	Periodontology	Cell culture
Composition	Poly(lactic acid), poly(glycolic acid) and poly(trimethylene carbonate). Plasticizer: <i>N</i> -methyl-2-pyrrolidone solution	Poly(lactic acid)	Flexible silicone elastomer membrane: non treated
Barrier effect stated	8–12 weeks porosity unknown	20 weeks porosity unknown	Unlimited porosity of 0.4 μm

of supernatant was removed from each well and placed in 96 well microplates. The absorbance of supernatant aliquots was read at 492 nm using the Powerwave X microplate spectrophotometer (Biotek instrument Inc., Winooski, VT, USA) and the viability was calculated and normalized from the absorbance of control samples taken as 100 % (PS).

Immunostaining After the MTS assay, the discs were rinsed with PBS and the cells were fixed with 4 % paraformaldehyde (Sigma-Aldrich) at room temperature for 20 min. An immuno-staining was then performed and the number of cells, their covering on discs and their morphology were determined from microscopic fluorescent images. Cell permeabilization was performed with 0.5 % Triton X-100 (Sigma) at 4 °C for 20 min. Blocking was performed with 1 % BSA (Sigma) in PBS at 37 °C for 1 h. Actin was stained with Alexa fluor 488-labeled phalloidin (Life Technologies, Carlsbad, CA, USA). The incubation was performed in 0.05 % Tween-20 (Sigma) in PBS solution with corresponding dilution (1:40) at 37 °C for 1 h. A nuclear stain dye DAPI (4',6-diamidino-2-phenylindole dihydrochloride) (D8417-1MG, Sigma)/PBS 1:5000 was added and allowed to set in at room temperature for 10 min. The stained sample surface was observed with an IX81 optical inverted microscope equipped with an UPlanFL objective at $\times 10$ magnification and with an XCite-iris IX fluorescence unit and a C-BUN-F-XC50 charge-coupled-device camera (Olympus Optical Co., Ltd). An image analysis software “CellSens” (Olympus) was used to quantify the number of cells and the covering surface. 350 pictures ($\times 10$ magnification) per sample were

performed to allow the analysis of the entire disc surface.

Statistical analysis For the cellular viability, the number of cells and their covering on discs, a statistical analysis was performed. For every variable, the data were treated by an analysis of variance with respect to fixed effects (materials, time and interactions between them) and random effects. Materials were compared two by two by multiple comparisons of Scheffe. The level of significance was set at 5 % ($p < 0.05$). The calculations were realized by means of the software SAS version 9.4 (SAS Institute, Cary, NC, USA).

Flexible biomaterials: IBS-F

Materials The Inion membrane (Inion Ltd., Tampere, Finland) and the Epi-Guide membrane (Kensey Nash, Eschborn, Germany), which are resorbable membranes for GBR, were used. The Inion membrane was treated with *N*-methyl-2-pyrrolidone (NMP) (Inion Ltd.) to achieve a rubber like consistency and to allow easy handling (Pirhonen et al. 2006). A non-resorbable membrane used for cell culture (Bioflex, Flexcell International, Hillsborough, England) was only used as a positive control for the barrier effect. Membranes composition and characteristics are shown in Table 1.

Insert The home-made device was composed of three parts: a 10 mm diameter small tubular silanized clear glass vial (Screw Neck vials ND8, La-Pha-Pack GmbH, Langerwehe, Germany) customized by cutting its flat bottomed lower part (Fig. 2b); the 8 mm

Fig. 2 Drawing (a) and picture (b, c, d, e) of the IBS-F for flexible biomaterials: the customized vial is sealed with its cap (b, c) reversed in the insert (d, e)

diameter cap of the vial, which had a 6 mm perforation in the center (Fig. 2b); an insert designed for a 12 well plate (ThinCert™ inserts, Greiner bio-one, Vilvorde, Belgium) (Fig. 2d). The original polycarbonate filter of the insert was removed. The insert functioned as a container in which the customized vial sealed with its cap is reversed, the cap down (Fig. 2b, e). The representation of the IBS-F is illustrated in the Fig. 2. The studied membrane was positioned to cover the 6 mm diameter hole located in the cap. This membrane isolated an upper and a lower compartment (Fig. 2a).

Silanization of the vials was performed in order to avoid cellular attachment to the glass. Glass samples were first cleaned with the Piranha mixture (1/3 H_2O_2 + 2/3 H_2SO_4 , v/v; Merck, Darmstadt, Germany) for 30 min. They were subsequently rinsed 5 times in demineralized water and dried at 120 °C overnight. The samples were then immersed in a 50 mM solution of trimethylchlorosilane (Merck) in dried cyclohexane (Merck). After 5 h of reaction at room temperature, samples were rinsed in cyclohexane and finally dried under nitrogen. The vials and the caps were autoclaved.

Membrane incubation and cell culture Before incubation, Inion membranes were plasticized according to the manufacturer's instructions. All membranes were placed in the incubator for 24 h in order to reach their maximal swelling in PBS solution. Following this, the membranes were cut into 8 mm diameter punches, positioned on the bottom of the caps and screwed onto the vials. The constructs were set in the inserts and the devices were installed onto a 12 well plate intended for incubation. The lower compartment of the plate was filled with 2 ml complete DMEM medium, whereas the upper compartment of the IBS-F was filled with 200 μl of complete DMEM medium.

Regarding degradation, chosen time-points for Inion and Epi-guide membranes were, respectively, 6-8-13 and 16-20-23 weeks. The choice was made according to barrier effect times declared by the manufacturer. During these periods, the culture medium was replaced twice a week. The non-resorbable Bioflex membrane was selected to serve as a control for a barrier effect of 100 % (porosity of 0.4 μm , no permeability to cells). Forty-eight hours before the termination of the incubation, media were removed. The lower compartment of the well was filled with 2 ml fresh complete DMEM medium, whereas the upper compartment of the IBS-F was filled with 200 μl of complete DMEM medium containing 4×10^4 HGFs. PS wells without IBS were filled with 2 ml fresh medium and 4×10^4 HGFs to serve as a control for 100 % viability for each incubation time. Following the 48 h of incubation, membranes were removed from the IBS-F and placed in new wells for cell viability assay or SEM analyses on membrane alone.

Barrier effect To evaluate the barrier effect of the membranes, the bottoms of culture wells were checked with an inverted phase contrast microscope (Nikon Diaphot, Tokyo, Japan) and the presence of the cells on both membrane faces was assessed using scanning electron microscopy. Cells cultured on the membranes were fixed by classical SEM methods using glutaraldehyde (2.5 %) in sodium cacodylate buffer 0.1 M, pH 7.3, desiccated to critical point and shadowed with platinum. The SEM used was a Jeol JSM-840A microscope (Jeol, Tokyo, Japan) operated at 22 kV.

Cellular viability Viability tests were then performed on the membranes to evaluate the living cell population on them and in the bottoms of culture wells, to evaluate the living cell population of cells, which fell resulting from membrane degradation. The

Fig. 3 DAPI-staining of a titanium disc with 5×10^3 HGFs, at 72 h of cultivation (magnification $\times 2$). The 1 mm-thick cell-free circle is illustrated between both red lines. (Color figure online)

viability test (MTS) was performed as described above. Cellular viability of 100 % was attributed to the HGFs cells grown in control PS wells without membrane, for each incubation time, respectively.

Statistical analysis For each condition, three replicates were evaluated including the calculation of means and standard deviations accordingly. Statistical evaluation was performed using a one-way analysis of variance (ANOVA). The level of significance was set at 5 %.

Results

Rigid biomaterials: IBS-R

The IBS-R was able to fit accurately to the discs, resulting in individual wells where the upper face of the disc acts as the well bottom.

The optical microscope observations did not reveal the presence of cells in the bottom of culture wells after the IBS-R removal. This was confirmed by the immunofluorescence microscopy images, which showed the absence of cells below the insert level. Figure 3 shows the typical 1 mm-thick cell-free circle in the periphery of the disc after the insert removal.

Regarding cellular viability (Fig. 4a), the analysis of the variance highlighted a linear effect of time

($p < 0.0001$) and of the interaction time-material ($p = 0.018$), the increase of viability being faster for Ti than for eM. For the number and the covering of cells (Fig. 4b, c), a quadratic effect of time ($p = 0.0050$, 0.015 , respectively) and of the interaction time-material ($p = 0.023$ in both cases) were observed, the increase of the number and covering of cells being faster for Ti than for eM from 48 h.

Immunofluorescence microscopy images of cell morphology with dual staining with DAPI for nuclei (blue) and phalloidin for actin filaments (green) are shown in Fig. 5. At 24 h, cells were polygonal or round. With time cell morphology changed by development of actin fibers, HGFs were denser and very elongated with multiples extensions of filopodia. On titanium discs cells were more spread and the filopodia were more visible than on glass-ceramic discs.

Flexible biomaterials: IBS-F

The IBS-F was able to fix the membrane and to create two compartments with an easy access to both faces of the membrane, as to the under-membrane space for medium changing.

The viability tests and the optical microscope observations did not reveal the presence of cells in the bottom of the wells containing the Bioflex and the Inion membranes for all time-points (Fig. 6a). SEM

Fig. 4 HGFs on Ti and eM discs: viability (a); number of cells (b); covering of cells (c)

Fig. 5 Immunofluorescence microscopy images of HGFs morphology with dual staining with DAPI for nuclei (blue) and phalloidin for actin filaments (green) (magnification $\times 10$): HGFs on a Ti disc at 24 h (a), HGFs on a Ti disc at 48 h (b),

HGFs on a Ti disc at 72 h (c), HGFs on an eM disc at 24 h (d), HGFs on an eM disc at 48 h (e), HGFs on an eM disc at 72 h (f). (Color figure online)

analysis on the Inion membranes showed an absence of cells on the membranes lower face (Fig. 7). However, the viability tests and the optical microscope observations revealed the presence of cells in the bottom of the wells containing the Epi-guide membranes after 23 weeks (Fig. 6a).

The results of HGFs viability on the upper face of Inion and Epi-guide membranes were found to be, respectively, from 5 to 20 % and from 30 to 40 % of the cellular viability found in control PS wells. Moreover, the viability on the upper face of Epi-guide was significantly higher than on Inion ($p = 0.001$) but did not significantly vary between the three time-points for both membranes ($p > 0.2$) (Fig. 6b).

Discussion

For the rigid biomaterials, the PTFE insert was able to accurately fit to the discs, resulting in individual wells where the upper face of the disc acts as the well bottom. The absence of cells in the bottom of culture wells and the 1 mm-thick cell-free space in the periphery of the discs after the insert removal confirmed that all cells were kept on the upper surface of the sample. IBS-R avoids cell loss in the bottom of culture well, which can generate bias in the measurement of the cytotoxicity, the attachment and the proliferation of cells. On the contrary to other systems previously described for dental biomaterials (Cochran

Fig. 6 Cellular viability % in the bottom of culture wells containing the Inion and Epi-guide membranes at different time points (a); Cellular viability % at the upper face of Inion and Epi-guide membranes at different time points (b)

Fig. 7 SEM of Inion membrane portion at 13 weeks with 4×10^4 HGFs seeded on the upper face 48 h before (magnification $\times 1000$): lower face (a); upper face (b)

et al. 1994; Nothdurft et al. 2014), IBS-R allows the keeping of the cells on the surface of the samples in a rapid and reproducible way, using a simple sample design and a medical-grade PTFE insert, which cannot

be suspected of influencing cell behavior, such as wax or silicon pad previously described (Cochran et al. 1994) (Nothdurft et al. 2014). As expected in the present study the cellular viability, the number of cells and the surface occupied by cells were shown to increase with time, showing a very good cellular attachment and proliferation on titanium. The results confirmed the good biocompatibility of titanium found in numerous previous studies (Cochran et al. 1994; Eisenbarth et al. 1996; Gomez-Florit et al. 2013; Grossner-Schreiber et al. 2006; Hormia and Kononen 1994; Kononen et al. 1992; Moon et al. 2013; Nothdurft et al. 2014; Pae et al. 2009). Actually, titanium is a gold-standard material in the field of dental implant prosthodontics mainly due to its high corrosion resistance, low toxicity, very low allergenic potential and good biocompatibility properties (Jorge et al. 2013). Regarding lithium disilicate glass-ceramic, HGF behavior on this material has been poorly studied (Brackett et al. 2008; Pabst et al. 2014; Tetè et al. 2014). The present results showed a good cellular attachment on lithium disilicate glass-ceramic but a slower proliferation than on titanium. To author's knowledge there is no study comparing the attachment and proliferation of HGFs on titanium and on lithium disilicate glass-ceramic. Tetè et al. (2014) reported that lithium disilicate glass-ceramic shows a lower cytocompatibility than zirconia and other authors observed that this material shows cytotoxic effects (Brackett et al. 2008; Pabst et al. 2014).

Immunostaining of the samples after the MTS assay was shown to be a reliable method, which did not damage cell morphology. A counting after detachment of cells from the surface by trypsin could engender

cellular death and counting inaccuracies (Kokoti et al. 2001). IBS-R can be used to study cell behavior on all rigid biomaterials, such as modern ceramic and composite materials used for dental and implant prostheses. Different cell types could be analyzed with this system: for dental applications it will also be used with gingival keratinocytes.

For the flexible biomaterials, IBS-F was shown to fulfill the requirements needed to study cell behavior and barrier effect of membranes. Indeed, the present study demonstrated its ability to (1) fix membranes in wells; (2) divide wells into two compartments (with and without cells) allowing the evaluation of the barrier effect; (3) create an easy access to both faces of the membrane for evaluation; (4) allow access to the under membrane space for several medium changing and long-term evaluation, with the possibility of adding cells at chosen times. Other models previously described in the literature ensured the fixation of the membrane, but they did not allow the evaluation of both faces of the membrane independently and thus the evaluation of the barrier effect (Kasaj et al. 2008; Payne et al. 1996; Rothamel et al. 2004; Takata et al. 2001a, b; Wang et al. 2002). Only one model was proposed to measure this effect, but it does not permit medium changing and long-term evaluation (Milella et al. 2001). The absence of cells in the bottom of the wells with non-resorbable membranes confirmed the cell-occlusive property of the system for several weeks. The limitation of the model is the rigidity of the membrane. Indeed, the biomaterial needs to be sufficiently flexible to fit within the insert and to prevent cell passing.

In the present study, HGFs were chosen in order to get results closer to normal phenotype behavior in comparison with immortal cell lines (Fukuda et al. 2000; Hillmann et al. 2002; Locci et al. 1997; Payne et al. 1996; Simain-Sato et al. 1999). Optical microscopy of the bottoms of wells and SEM analysis of the Inion membranes lower face confirmed the absence of cells and a positive barrier effect until 13 weeks, while the manufacturer states an 8–12 weeks in vivo barrier effect. For Epi-guide, the results showed a positive barrier effect until 20 weeks but after 23 weeks the presence of cells in the bottom of wells indicated a loss of this barrier effect. These results confirm manufacturer data, which describe an in vivo barrier effect of 20 weeks; however, an in vivo study has shown an early stage of Epi-guide degradation after 6 weeks (Vernino et al. 1995).

The viability of HGFs seeded onto membranes was significantly lower than the cellular viability found in control PS wells, and there was no significant difference of viability between the three time-points. Several authors obtained the same results, with limited attachment and proliferation of various cellular types on different guided bone regeneration membranes (Gabriel et al. 1996; Payne et al. 1996; Rothamel et al. 2004; Takata et al. 2001a; Wang et al. 2002). Only Locci et al. 1997 reported opposite findings. A small, but not statistically significant, viability decrease was observed on Inion after 13 weeks. It could be explained by a change in the surface composition and/or texture of the membrane with time. Another hypothesis is the progressive adsorption of proteins from the culture medium (such as albumin) on the membrane, which could compete with the adsorption of specific proteins necessary for cell adhesion. The viability on the upper face of Epi-guide was significantly higher than on Inion, which can be explained by the influence of composition, microstructure and topography of the different membranes.

IBS-F was also used successfully with biomaterials for ophthalmology. Particularly it allowed the measurement of fluorescent protein adsorption and lens epithelial cell colonization on non-resorbable soft intraocular lens (Bertrand et al. 2014; Huang et al. 2014).

Conclusions

In vitro studies related to biological properties of biomaterials, such as cytocompatibility, are frequent and essential screening tests to be performed before preclinical or clinical experimentations. The present work introduces two reliable in vitro methods to study cell behavior on rigid biomaterials on the one hand, and on flexible and permeable biomaterials on the other. Two specific insert-based systems (IBS-R and IBS-F) were, respectively, designed and were shown to promote (1) easy and rapid handling; (2) cell retention on biomaterial surface; (3) accurate evaluation of the cellular proliferation, spreading and viability; (4) use of non-toxic material. Moreover, IBS-F allowed the study of the cell barrier effect of membranes, and enabled an access to both faces of the biomaterial as to the bottom of culture wells for medium changing.

Besides the presented dental applications, these systems are applicable to a wide variety of biomaterials: rigid or flexible, thick or thin, more or less permeable to cell migration, or even coated. They can be used for short or long-term (>3 months) studies about cell barrier efficacy, membrane degradation, detection of toxic products into conditioned media, protein adsorption or adequacy for desired cell adhesion and growth. These characteristics could then be related to physico-chemical and mechanical properties, as well as surface topography of samples in order to develop biomaterials in view of their specific applications.

Acknowledgments We would like to express our thanks to Inion Ltd (Tampere, Finland), Kensey Nash (Eschborn, Germany), Nobel Biocare (Göteborg, Sweden), and Ivoclar Vivadent (Schaan, Liechtenstein) for sample supply. We would also like to thank Sirris (Liège, Belgium) for their assistance in the development of PTFE inserts.

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