*Acta Odontologica Scandinavica.* 2014; 72: 321–330

informa
healthcare

ORIGINAL ARTICLE

# Cytotoxicity of coated and uncoated fibre-reinforced composites

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**Abstract**

***Objective.*** Currently, there are many fibre-reinforced composites (FRCs) available which differ in the type and volume fraction of fibres, pre-treatment of fibres and matrix composition. The aims of this *in vitro* investigation were to determine whether there is a difference in biocompatibility of FRCs and if coating FRCs with resin composites influences their cytotoxic potential. ***Materials and methods.*** Five different FRC materials were tested which were either uncoated or coated with flowable or viscous resin composite. Artificial saliva extracts were prepared according to USP-XXIII and *ISO*-10993 to determine cytotoxicity by testing cell viability and growth of primary human gingival fibroblasts (HGF) using MTT assay, LIVE/DEAD® assay and cell proliferation assay. The influence of eluates on fibres of the cytoskeleton was investigated by vimentin, tubulin and actinin immunostainings. A two-way ANOVA followed by Scheffe's post-hoc test, which included the factors FRC material and coating procedure, was performed to assess cytotoxicity. ***Results.*** All extracts of FRC materials displayed minor cytotoxic potential on HGF cell viability, cell proliferation and integrity of the cytoskeleton. The type of FRC material significantly influenced cell viability (MTT assay) ($p < 0.0001$), whereas neither the presence of a coating nor the type of coating material resulted in altered cell viability. Distribution and organization of cytosolic fibres was not affected after HGF exposure to eluates. ***Conclusions.*** There is a lack of knowledge about the leaching behaviour of commonly available fully pre-impregnated FRCs and their interactions with coating materials. The coating of FRCs with resin composite materials did not impact biocompatibility.

**Key Words:** *Artificial saliva, cell proliferation assay, cytotoxic potential, extracts, fibre-reinforced composites, immunocytochemistry, LIVE/DEAD® assay, MTT assay*

## Introduction

Fibre-reinforced composites (FRCs) are known to have excellent mechanical properties and are, therefore, used in shipbuilding, aviation, vehicle construction, generation of wind energy and numerous fields of engineering [1]. In dentistry, FRCs are currently applied in diverse fields such as to enhance shear bond strength of brackets in orthodontics [2], to close single tooth gaps with resin-bonded fixed dental prostheses and to reinforce denture bases in prosthodontics [3–5], to strengthen core buildups in endodontics [6] and for tooth splinting in periodontal applications [7]. Additionally FRC implants and bone graft materials with osteoconductive capacity are showing good biocompatibility paired with excellent mechanical properties [8–15].

There are many FRC materials available containing different volume fraction and types of fibres, e.g. silicate glass (E-glass (electrical glass), S-glass (strength glass), R-glass (resistance glass) and polyethylene fibres in descending frequency [16]. To ensure a reliable bond to the resin, matrix fibres need to be pre-treated with a silane coating [17]. The resin matrices of different pre-impregnated FRC materials also differ in their composition. They either consist of dimethacrylate monomers (e.g. Bis-GMA, UDMA, TEGDMA) polymerizing to a cross-linked polymer (CLP matrix) or they are composed of two polymers (e.g. Bis-GMA and PMMA) polymerizing into a semi-interpenetrating polymer network (semi-IPN) (Table I) [18].

To avoid wear and degradation of the FRC in the oral cavity the manufacturers recommend covering the FRC with a layer of flowable or viscous resin

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*(Received 1 February 2013; accepted 9 July 2013)*

ISSN 0001-6357 print/ISSN 1502-3850 online © 2014 Informa Healthcare
DOI: 10.3109/00016357.2013.826381

Table I. Material data sheet information of FRCs used in this study.

| Name | everStick® PERIO | GrandTec® | Tender Fiber® | Splint-it® | Dentapreg® PFU |
|---|---|---|---|---|---|
| Code | ES | GT | TF | SI | DP |
| Manufacturer | StickTech Ltd., Turku, Finland | Voco, Cuxhaven, Germany | Micerium, Avegno, Italy | Pentron Clinical Technologies, Orange, CA | ADM, Brno, Czech Republic |
| Type of matrix | Semi-IPN | CLP | CLP | CLP | CLP |
| Resin matrix | PMMA Bis-GMA | UDMA TEGDMA | Bis-GMA + Siliciumdioxide (0.012 μm) | Bis-GMA | not specified |
| Type of Fibers | E-glass | R-glass | Glass fibres | Silicate glass fibres and polyethylene fibres | S2-glass |
| Fibre volume fraction (vol%) | 60% wt%* | 35-40% | 54.8% wt%* | 38–40% | 35–45% |
| Number of fibres | 2000 | 4000–4500 | 6400 | Not specified | 8300 |
| Direction of fibres | Unidirectional | Unidrectional | Unidirectional | Unidirectional | Unidirectional |
| Coating of fibres | Epoxy silane | Silane | Silane | Not specified | Plasma-enhanced chemical vapour deposition (PECVD) |
| LOT | 20130322EP05 | 1114411 | 2011001424 | 166340, 4144566 | PFU_09 _072011 |

E, Electric–glass; S, Strength–glass; R, Resistance–glass; Semi-IPN, semi-interpenetrating polymer network; CLP, cross-linked polymer.
*Exact fibre volume fraction was not provided by manufacturer.

composite. In the clinical setting, small vertical or horizontal interspace and/or occlusal contacts may constrain composite application. Consequently, only thin layers of composite resin or even exposed FRCs which might come into direct contact with oral mucosa and saliva are present [19].

It was shown that dependent on their matrix composition and the degree of conversion (DC %), composite resins release residual monomers and additional components (initiators, activators, inhibitors) into aqueous solutions [15,20–24]. In addition, after long-term intervals, wear and hydrolysis of the siloxane network which bonds the glass fibres to the polymer matrix lead to the release of ions and hydrolysis products [25]. There are numerous *in vitro* and *in vivo* investigations identifying cytotoxic substances from dental resin composites, including the most frequently detected methacrylates Bis-GMA, UDMA, TEGDMA and HEMA [26,27]. However, data on the biocompatibility of FRC are rare [8–15,19,28,29].

To assess the biocompatibility of materials, various test systems and methods are listed in USP XXIII and *ISO* 10993. The conformity of the FRC products with applicable regulations has been thoroughly tested by the manufacturers. Therefore, this study aimed to identify possible differences between various FRC materials (see below): Here, MTT assay [30,31], cell proliferation assay [14] and fluorescence-based cell viability assay (LIVE/DEAD assay) and immunocytochemistry were selected to examine possible cytotoxicity of the materials.

Five different FRC materials used in restorative dentistry (Table I) were chosen for testing and included either uncoated (group 1), coated with a flowable composite (group 2a, Tetric Evo Flow (TEF), Ivoclar Vivadent, Schaan, Liechtenstein) or coated with a viscous resin composite (group 2b, Tetric Evo Ceram (TEC), Ivoclar Vivadent, Schaan, Liechtenstein, Table II).

The following research hypotheses were formulated: (1) there is no difference in cytotoxicity between FRCs; (2) there is no difference in cytotoxicity between the uncoated and coated FRCs; and (3) there is no interaction between the factors material and veneering procedure.

## Materials and methods

### *Specimen preparation*

Figure 1 shows the schematic illustration of sample preparation. Five FRC materials used in restorative dentistry were selected, one semi-IPN FRC (Ever-Stick PERIO®, StickTech Turku, Finland) and four CLP-FRCs (GrandTEC®, VOCO, Cuxhaven, Germany; TenderFibre®, Micerium Avegno, Italy; Splint-It®, Pentron Clinical Technologies, Orange, CA; Dentapreg PFU®, ADM, Brno, Czech Republic; Table I). Under sterile conditions, 5 mm of each FRC material was flattened gently between two sterile microscope slides and light polymerized (SmartLite PS, DENTSPLY DeTrey GmbH, Konstanz, Germany; wavelength 450–490 nm, 890(10) $mW/cm^2$) according to the manufacturer's instructions. In group 1, specimens were left uncoated. In group 2a, specimens were covered entirely with a

Table II. Material data sheet information of dental resin composites used in this study (wt% = weight percentage). Data on sorption and solubility of TEC were taken from Sideridou et al. [40].

| Name | Tetric Evo Ceram® | Tetric Evo Flow® |
|---|---|---|
| Code | TEC | TEF |
| Manufacturer | Ivoclar Vivadent AG<br>FL-9494 Schaan,<br>Liechtenstein | Ivoclar Vivadent AG<br>FL-9494 Schaan,<br>Liechtenstein |
| Resin matrix | Bis-GMA<br>UDMA,<br>Ethoxilated Bis-EMA | Bis-GMA<br>UDMA<br>Decanoldimethacrylate |
| Resin matrix content (wt%) | 17–18% | 38% |
| Type of fillers | Barium glass<br>Ytterbium fluoride<br>Mixed oxide<br>Pre-polymer | Barium glass<br>Ytterbium trifluoride<br>Highly dispersed silicone dioxide<br>Mixed oxide and copolymer |
| Filler content (wt%) | 82 3% | 62% |
| Filler particle size<br>(mean) | 40–3000 nm<br>(500 nm) | 40–3000 nm<br>(550 nm) |
| Wave length range | 400–500 nm | 400–500 nm |
| Sorption of water<br>(30 days) | 23.0 (0.40) µg/mm$^3$ | 21.0 µg/mm$^3$ |
| Sorption of artificial saliva (30 days) | 23.3 (0.80) µg/mm$^3$ | Not specified |
| Solubility in water<br>(30 days) | 1.84 (0.31) µg/mm$^3$ | 0.1 µg/mm$^3$ (7 days) |
| Solubility in artificial saliva (30 days) | 1.98 (0.11) µg/mm$^3$ | Not specified |
| LOT | N46591 | P85060 |

0.5 mm layer of flowable resin composite (Tetric Evo Flow (TEF), Ivoclar Vivadent, Table II) and subsequently light cured according to the manufacturer's instructions. In group 2b, the coating was carried out with a 1 mm thick resin composite layer (Tetric Evo Ceram (TEC), Ivoclar Vivadent). Immediately after preparation, the specimens were incubated in 1 ml of artificial saliva (AS, 16 mM KCl, 14 mM NaCl, 1.9 mM $KH_2PO_4$, 1.35 mM $CaCl_2$, pH 6.8) for either 24 h or 7 d. Due to the different shape, size and diameter of FRCs, the exposed surface allowing for leaching could not be standardized. Therefore, the surface area (mm$^2$) of each specimen was calculated and used for further analysis.

*Cell culture*

Gingival tissue was obtained from patients who gave written informed consent. The harvest of these tissues was approved by the Ethics Committee (Medical Faculty, University of Heidelberg). Gingival fibroblasts were established from explant culture of gingival connective tissue as previously described [32]. Primary human gingival fibroblasts were routinely cultured in DMEM medium (PAA, Cölbe, Germany) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, antibiotics and antimycotics at 37°C in a humidified 5% $CO_2$ incubator.

*Cell viability*

*Part A: MTT assay.* Cell viability of primary human gingival fibroblasts (HGF) was determined using the MTT assay. Mitochondrial dehydrogenases of viable cells are able to convert the yellow MTT (3-(4, 5-dimethylthiazol-2, 5-diphenyltetrazolium bromide) into a blue formazan stored in the cytoplasm. $3 \times 10^3$ HGF cells/well were seeded in 96-well plates (Greiner Bio-One GmbH, Frickenhausen, Germany) and cultured in DMEM, 10% FCS at 37°C, 5% $CO_2$ for

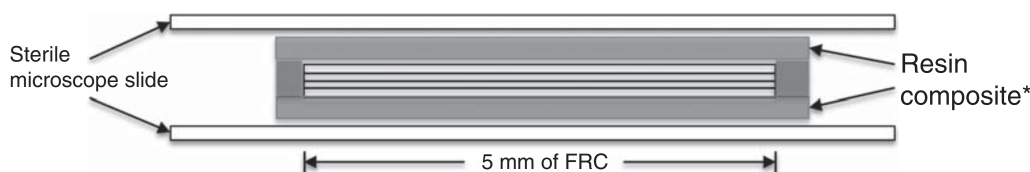



Figure 1. Schematic drawing of sample preparation. * In group 1, FRC strands were uncovered. In groups 2a and 2b, entire FRC strands were covered with 0.5 mm of flowable resin composite (TEF) or 1 mm of viscous resin composite (TEC).

24 h. Artificial saliva extracts of the five different FRCs (coated and uncoated) were mixed with equal volumes of 2 × DMEM; 20% FCS fibroblasts and HGFs were incubated in 100 μl of the mixture for 24 h or 48 h (37°C, 5% $CO_2$), respectively, to obtain different release kinetics. The mixture was removed and the cells and MTT reagent (Promega, Mannheim, Germany) were incubated for 4 h at 37°C, 5% $CO_2$. The solubilization mix was added (incubation time 1 h) and thoroughly mixed before recording the absorbance at 570 nm using a multiwell reader (GeniosPro, Tecan, Crailsheim, Germany). The relative viability of cells is presented as the ratio of the absorbance of the test specimens compared with the positive control (HGF cell culture medium) and corrected for the surface size of the test specimen adapted by surface size of the test specimens. The experiments were performed in triplicate and repeated twice.

*Part B: Fluorescence microscopy using the LIVE/DEAD® assay*. The principle of the LIVE/DEAD® Viability/Cytotoxicity Kit (Molecular Probes®, Life Technologies GmbH, Darmstadt, Germany) is the relative permeability of cell membranes when the cell is alive (green) or dead (red). The fluorescent dye, calcein-AM, passes through the cell membranes of intact cells indicating intracellular esterase activity by green fluorescence, whereas membrane-impermeable ethidium homodimer-1 labels nucleic acids only of membrane-compromised cells with red fluorescence.

We used extracts of artificial saliva after 7 days incubation with specimens and mixed these with equal volumes of 2 × DMEM supplemented with 20% FCS. HGFs were incubated in 100 μl of this mixture for 24 h at 37°C, 5% $CO_2$ in 96-well plates (μCLEAR, 96-well plate, TC, black, Greiner Bio-One GmbH, Frickenhausen, Germany). Cells were stained with 50 μl of LIVE/DEAD assay and counted under a fluorescence microscope (Zeiss Cell Observer, HXP 120, Carl Zeiss, Jena, Germany) at 10 × magnification. Five regions of interest per well were evaluated by two independent researchers. HGFs treated with 10 mM CHX 2% for 6 h served as a negative control. Mean ratios of dead cells vs live cells were determined using digital imaging software (WCIF Image J, Version 1.37c).

### Cell proliferation assay

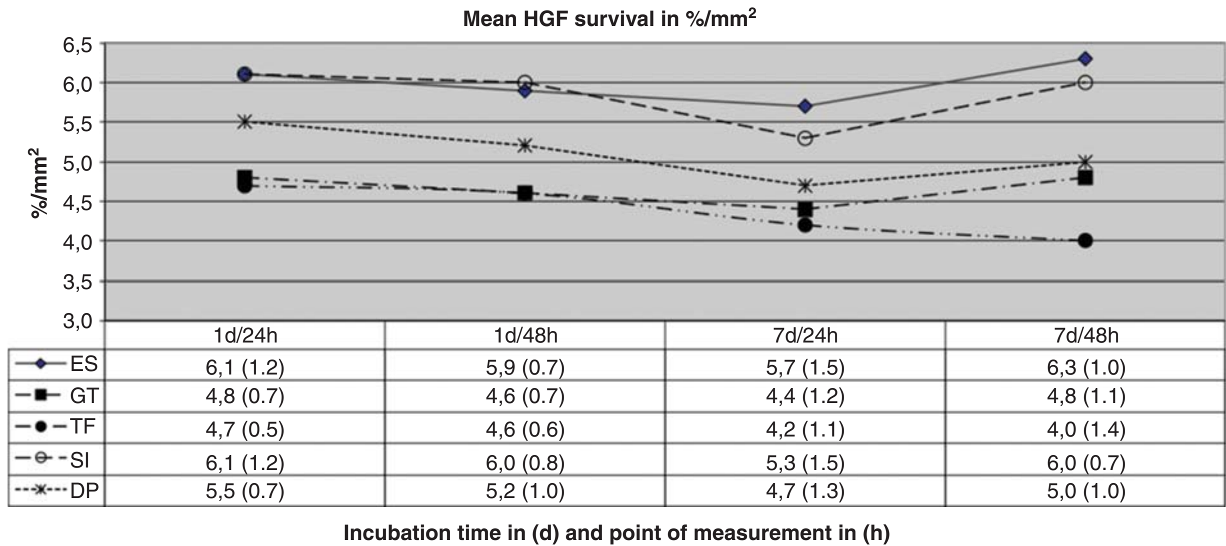
Proliferation of human gingival fibroblasts was evaluated for FRC materials shown in Table I. Culturing was done in 96-well plates (Greiner Bio-One GmbH, Frickenhausen, Germany) with 3 × $10^3$ HGF cells/well. Cells were cultured in DMEM, 10% FCS at 37°C, 5% $CO_2$. Artificial saliva extracts after 7 days incubation of the five different FRCs were mixed with equal volumes of 2 × DMEM. Cells were counted after 1, 2, 3 and 7 days using the MTT reagent (Promega, Mannheim, Germany) with the procedure described above. The relative viability of cells is presented as the cell number (ratio of the absorbance of the test specimens in relation to the positive control as a percentage) and corrected for the surface size of the test specimens. The experiments were performed in triplicate and repeated twice.

### Immunocytochemistry

The influence of eluates of FRC materials on cytosolic fibres was investigated using vimentin, tubulin and actinin immunostainings. HGFs were grown on chamberslides (Fisher Scientific, Schwerte, Germany) and exposed to FRC eluates (7 days incubation) for 48 h. Cells grown in DMEM, 10% FCS served as controls. Cells were fixed for 10 min in ice-cold acetone:methanol (1:1), blocked with blocking solution (0.1% casein, 0.1% bovine serum albumine in PBS) and incubated overnight at room temperature (RT). with the primary antibodies diluted in blocking solution (vimentin (clone: VI-RE/1, 1:200); tubulin (clone YL1/2, 1:1000) and $\alpha$-actinin (clone BM-75.2, 1:200)). Alexa Fluor 488 conjugated secondary antibodies (Jackson Immuno, Suffolk, UK) were used for detection. Cells were mounted in anti-fade reagent with DAPI (4', 6-diamidino-2-phenylindole) as a counterstain. Microphotographs were taken using a Leica DMRE microscope equipped with a digital camera (DFC300 FX, Leica, Bensheim). Image acquisition and processing was done using the Leica application suite software (Leica, Bensheim).

### Sample size and statistical analysis

Sample size was determined to be $n = 24$ for each FRC (group 1 (uncoated) $n = 8$; group 2a (coated with TEF) $n = 8$; group 2b (coated with TEC) $n = 8$). For analysis of the MTT assay and the proliferation assay the ratio of specimen absorbances vs positive control (%) was divided by the surface area (1/$mm^2$). Values are reported as arithmetic mean and standard deviation (SD). Effects of the parameters, FRC material, presence of coating and type of coating on cytotoxicity were evaluated by analysis of variance (ANOVA). For the results of MTT assay ANOVA was performed separately for incubation time (1 and 7 days) and for point of measurement of cell viability (24 and 48 h). Post-hoc multiple comparisons were performed using Scheffe's test. Two-sided $p$-values $< 0.05$ were considered statistically significant. Statistical analysis was carried out using SAS statistical software package 9.2 (SAS Institute Inc., Cary, NC).




| | 1d/24h | 1d/48h | 7d/24h | 7d/48h |
|---|---|---|---|---|
| ES | 6,1 (1.2) | 5,9 (0.7) | 5,7 (1.5) | 6,3 (1.0) |
| GT | 4,8 (0.7) | 4,6 (0.7) | 4,4 (1.2) | 4,8 (1.1) |
| TF | 4,7 (0.5) | 4,6 (0.6) | 4,2 (1.1) | 4,0 (1.4) |
| SI | 6,1 (1.2) | 6,0 (0.8) | 5,3 (1.5) | 6,0 (0.7) |
| DP | 5,5 (0.7) | 5,2 (1.0) | 4,7 (1.3) | 5,0 (1.0) |

Figure 2. Standardized absorbance in relation to the surface area (%/mm$^2$) and standard deviation (SD) of FRC materials after 1 and 7 days incubation time of specimens in artificial saliva and measurements after 24 and 48 h.

## Results

### *Part A: MTT assay*

Overall, the results of the MTT assay indicated low cytotoxicity (higher values indicated increased viability and therefore lower cytotoxicity). In general, cytotoxicity increased slightly with incubation time (Figure 2). The analysis of 1 day extracts of specimens revealed a decrease in HGF cell viability after 48 h, whereas in the 7 day extracts, HGF cell viability increased slightly after 48 h. However, values did not differ significantly.

Table III shows the results of the analysis of variance (ANOVA) for the three tested research hypotheses. The first hypothesis was rejected showing that the parameter FRC material significantly influenced ($p < 0.0001$) cell viability for each extraction time (1 and 7 days) and incubation time (24 and 48 h). Hypotheses (2) and (3) could not be rejected. Rank orders (Scheffe's post-hoc test) are given in Table IV. After 1 day of incubation, SI was least cytotoxic followed by ES and DP. After prolonged incubation (7 days) the ES material displayed lowest cytotoxicity followed by SI and DP.

### *Part B: LIVE/DEAD® assay*

The results of the fluorescence-based LIVE/DEAD assay are shown in Table V and Figure 3. Fluorescence microscopic analysis of cells after LIVE/DEAD® staining revealed low values in dead cells ranging from 1.40–2.96% in all groups. The lowest mean percentage of dead cells (< 2%) was shown for SI followed by ES. The GT, TF and DP materials yielded slightly higher values (≤ 3%).

### *Cell proliferation assay*

The results of cell proliferation assay are shown in Figure 4. Cells numbers were monitored for 7 days. In the first 24 h of the observation period there was a slight decrease in fibroblast proliferation for all materials. After 3 days the cell number of all

Table III. Results of ANOVA ($p$-values) with regard to influence of FRC material (1), coating procedure (2) and type of composite (3) on human gingiva fibroblast cell numbers in relation to surface area of the FRC materials (1/mm$^2$) for different incubation times and points of measurement.

| | 1 day/ 24 h | 1 day/ 48 h | 7 days/ 24 h | 7 days/ 48 h |
|---|---|---|---|---|
| (1) FRC material | < 0.0001 | < 0.0001 | 0.0014 | < 0.0001 |
| (2) coated vs uncoated | 0.7443 | 0.7527 | 0.9354 | 0.8616 |
| (3) Flowable vs viscous resin composite | 0.9738 | 0.9999 | 1.0000 | 0.7048 |

Table IV. Results of Scheffe's test and ranking into sub-groups ($\alpha = 0.05$). Means with the same letter are not siginficantly different.

| Sub-groups ($\alpha$ = 0.05) | 1 day/ 24 h | 1 day/ 48 h | 7 days/ 24 h | 7 days/ 48 h |
|---|---|---|---|---|
| A | SI<br>ES | SI | ES | ES<br>SI |
| A/B | DP | ES | SI<br>DP | — |
| B | GT<br>TF | DP | GT<br>TF | DP |
| B/C | — | GT | — | GT |
| C | — | TF | — | TF |

Table V. Results of fluorescence microscopy. Mean percentage (SD) of dead cells in LIVE/DEAD assay. Ratio was calculated after counting cells alive (green) and dead (red) in five regions of interest ($n$ = 5).

| | ES | GT | TF | SI | DP |
|---|---|---|---|---|---|
| Uncoated (group 1) | 1.64 (0.93) | 2.96 (0.96) | 2.78 (1.15) | 1.40 (0.80) | 2.82 (0.91) |
| Coated with TEF (group 2a) | 1.54 (0.88) | 1.86 (1.31) | 2.08 (0.31) | 1.42 (0.81) | 2.04 (0.44) |
| Coated with TEC (group 2b) | 1.70 (0.98) | 2.22 (1.57) | 2.54 (0.90) | 1.48 (0.99) | 1.72 (0.99) |

materials reached the level of the positive control. At day 7 a decrease of 7–23% in cells was detected compared to the control. However, the differences between the FRC materials and compared to the control were not statistically significant at all time points of measurements. Extracts of all FRC materials showed a minor impact on HGF proliferation.

*Immunocytochemistry*

Cytotoxic effects of resin monomers might cause disintegrations of cytosolic fibres of the cytoskeleton. To assess the potential impact of leached components of coated and uncoated FRCs, HGFs exposed to FRC eluates for 48 h were stained for vimentin, a member of the intermediate filament protein family and specific for cells of mesenchymal origin, α-actinin, an actin associated protein found along actin microfilaments, and tubulin, involved in the formation of microtubules. We could not detect indications of redistributions, rearrangements or disintegrations of cytosolic fibres after exposure to FRC eluates. Staining patterns for vimentin, tubulin and actinin were comparable to controls (Figure 5). Leached components of FRCs did not reveal an impact on the integrity of the cytoskeleton of HGFs after 48 h of exposure.

## Discussion

To date, within the field of dental fibre technology, several biocompatibility studies have been published with regard to residual monomer release [14,15,19,28,29], bone tissue response to FRC implants [8,9] and bone defect repair with bioactive FRC granule [12,13]. Yet, there is no investigation on biocompatibility of light-polymerizing FRC materials used in restorative dentistry in the literature. Since the chemical composition of light polymerizing FRCs is similar to that of light polymerizing composite resins it is assumed that their leaching behaviour might be similar.

Here, the extracts of the FRC materials were analysed by MTT assay, a fluorescence-based cell

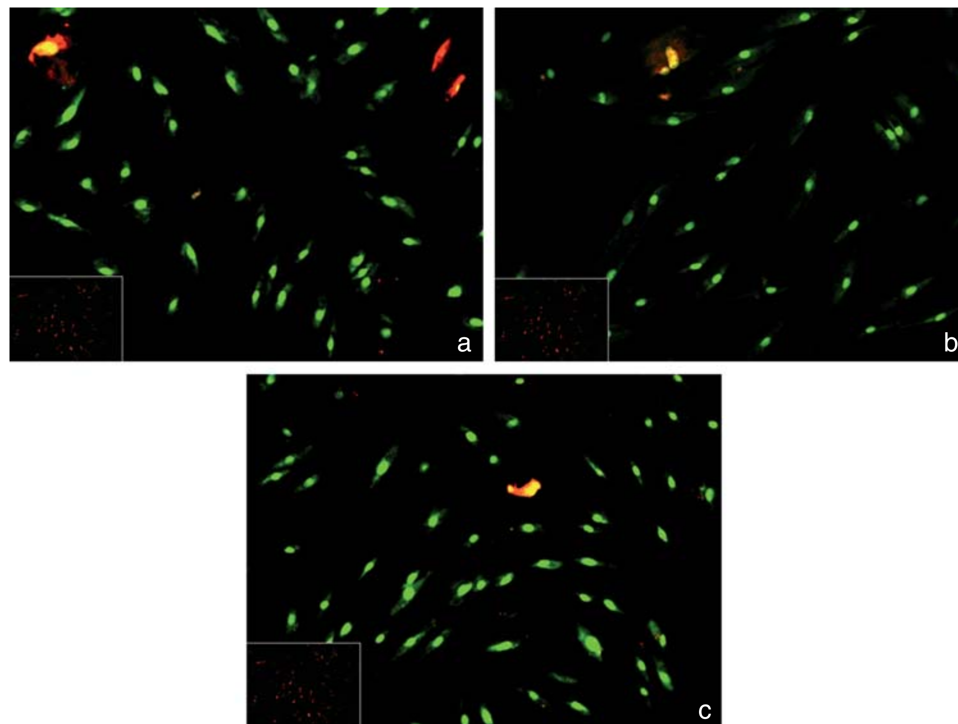



Figure 3. Examples of fluorescence microscopy using LIVE/DEAD assay for determining cell viability of artificial saliva extracts from specimens: (A) uncoated, (B) coated with TEF, (C) coated with TEC. Insert shows CHX treated (10 mM, 6 h) HGFs.

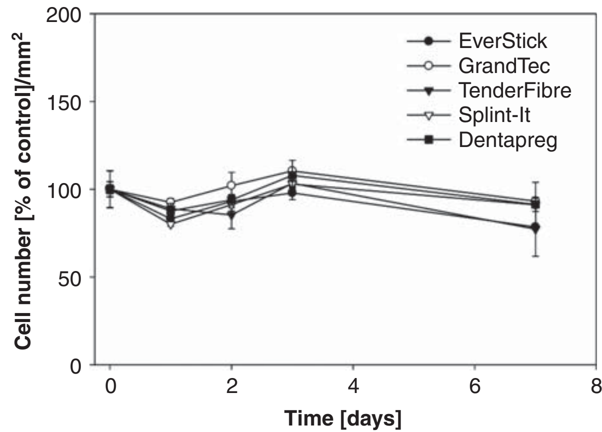



Figure 4. Results of proliferation assay. Mean percentage of cell number in relation to the positive control was calculated and values were corrected for the surface size of the test specimens.

viability assay (LIVE/DEAD assay) and a proliferation assay with the primary end-point of cell survival. Intracellular alterations of microtubules and microfilaments were visualized using immunocytochemistry technique (ICC) [33].

The results of our investigation showed minor cytotoxicity for coated and uncoated FRC materials, yet the type of FRC material significantly influenced cell viability (MTT assay) ($p < 0.0001$).

Various noxes, including resin monomers, have been shown to have an impact on the integrity of the cytoskeleton [34,35]. Periodontal cells, among them gingival fibroblasts, revealed severe changes of the cytoskeleton, for example, after the exposure to nicotine [33,36]. We have therefore investigated the possible impact of FRC eluates on the integrity of the cytoskeleton of HGFs. Eluates of FRC materials induced no morphological alteration in cytoskeletal structures. The eluates had no primary effect on intermediate filament distribution, as assessed by vimentin staining. No rearrangements of microtubules (tubulin) or depolymerizations of microfilaments ($\alpha$-actinin) were observed (Figure 5). In addition, vimentin staining proofed the mesenchymal (fibroblastoid) origin of the cells used in this study.

The test methodology in the present study focuses on testing the influence of FRC restorations on the actual intra-oral targets in close contact with these FRCs, namely human saliva and gingival fibroblasts. The extraction of leachable components in artificial saliva represents more closely the *in vivo* situation than extractions in ethanol-water mixtures and is, therefore, considered a strength of the method. With regard to different extraction media, Polydorou et al. [37] could show that, except for the elution of TEGDMA, ethanol 75% and acetone solutions are not suitable *in-vitro* substitutes to saliva. Despite hydrophilic polymeric compounds being less soluble in artificial saliva and cell culture media than in ethanol-water mixtures [38], artificial saliva is preferable because a covering of specimen surfaces with albumin and fibronectin can be avoided here [31].

For the preparation of extracts, a short-term 1 day incubation period was performed. In addition, a prolonged incubation period (7 days) was chosen to obtain a larger quantity of eluted substances. Incubation periods longer than 7 days were not considered here, as Franz et al. [39] showed that cytotoxicity and substance release of most materials decreases after long incubation periods. Therefore, the 7-day extracts

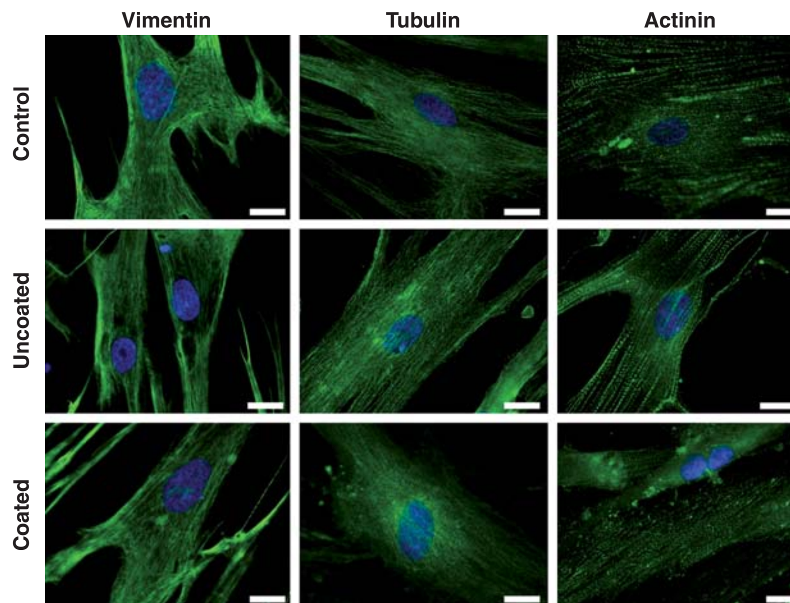



Figure 5. Immunostaining for vimentin, tubulin and actinin (green fluorescence) on HGFs. Representative microphotographs of cells treated with eluates from uncoated and coated FRCs reveal no visible effect on the integrity of the cytoskeleton compared to control cells. Counterstaing of nuclei with DAPI (blue). Bar = 20 μm.

were chosen for visualization and quantification of HGF cell viability using fluorescence-based methodology (LIVE/DEAD assay), proliferation assay and ICC technique.

As this was the first investigation on the biocompatibility of fully pre-impregnated FRC materials used in restorative dentistry, basic test methods regarding cell viability and cytokeletal alteration of HGF cells were performed. The MTT assay showed that cell viability was significantly different between the FRC materials ($p < 0.0001$, Table III), yet for the presence of a resin composite coating and the type of coating (flowable vs viscous) significance could not be shown. In the statistical analysis, the ranking of the FRC materials clearly shows that the SI and the ES materials displayed the lowest cytotoxic potential followed by the DP material (SI showed best results after 1 day incubation time, ES after 7 days, Table IV). In addition, the LIVE/DEAD assay and cell proliferation assay, performed with extracts after 7 days incubation, confirmed a generally low cytotoxicity in all groups. The ranking of the FRC materials as determined by MTT (Table IV) was confirmed by the fluorescence-based LIVE/DEAD assay (Table V).

### *Why is cytotoxicity also present when coating with resin composite is performed?*

We saw no difference in cell viability between groups 1 (no coating), 2a (coating with flowable resin composite) and 2b (coating with viscous resin composite), suggesting that both coating materials (TEC and TEF) contain unbound monomers which are released after prolonged incubation in artificial saliva [21]. In our evaluation, cytotoxicity increased slightly from 1 to 7 days incubation time for all three groups. The slight increase in cytotoxicity following 7 days of leaching is in accordance with Miettinen and Vallittu [29] and Väkiparta at al. [15]., who showed that release of residual monomers into water from glass fibre-reinforced composite materials occurred within the first days. For coated FRC materials (groups 2a and 2b) it can be assumed that no difference in substance release was found, as both materials (TEC and TEF) displayed comparable values for the sorption of water (23 and 21 $\mu g/mm^3$, respectively, Table II) [40].

### *Why do the FRC materials have different cytotoxic potential?*

From the data provided by the manufacturers, there are main differences in the matrix composition of the five test materials (Table I). Either the matrix consists of cross-linking dimethacrylate-monomers (e.g. Bis-GMA, TEGDMA or UDMA) or it consists of two polymers forming a semi-IPN matrix (PMMA and Bis-GMA). Hence, the semi-IPN network differs from a cross-linked polymer network because it consists of two separate polymer networks that are not chemically linked [18,41]. Despite all FRC materials being handled following manufacturer's instructions, there might be differences regarding their degree of conversion (DC%) and leaching behaviour. It was shown that different curing sources are influencing the DC% and the residual monomer release of resin-based dental biomaterials [15,21,23,24]. In addition the relation of base monomers and polymerization initiator/inhibitor obviously differs in FRC brands (Table I) and might cause different polymerization rates and DC%, which influence quantitites of released residuals [22].

During the dissolution process (such as in the aqueous oral environment), the solvent diffuses into the polymer network where microvoids have formed during light polymerization, which allow solvent adsorption and the release of monomers. Furthermore, the bonding between the matrix of the fully pre-impregnated FRC material and the flowable and viscous resin composites may display material-specific differences. Vallittu [41] showed that reinforcing fibres are difficult to impregnate with a resin matrix of high viscosity. Such highly viscous light-curing resins are those with particulate fillers. The TF material, which yielded highest cytotoxic potential in the MTT assay, contains, in contrast to all other FRC materials, silicium dioxide with a mean particle size of 0.012 μm as a filler. In contrast, the ES and SI materials with the lowest cytotoxic potential consist either of a cross-linked Bis-GMA matrix without filler content (SI) or of a semi-IPN matrix (ES).

### *Outlook*

Since the tests performed in this study show, for the first time, insight into the biocompatibility of uncoated and coated FRC materials, further, already ongoing, studies are needed to fully test FRC materials with regard to composition, interfacial bond strength to different veneering resin composite systems and substances released dependent on the DC% in aqueous solvents, respectively. Taken together, we see significant clinical importance in evaluating these materials to determine optimal resin matrix compositions for FRC materials and suitable flowable and viscous resin composites for clinical application in the intra-oral cavity.

## Conclusions

*In-vitro* cytotoxicity tests are designed to determine how a material affects target cells. In general, extracts of FRCs in artificial saliva displayed minor cytotoxic potential on HGF cell viability, cell proliferation and integrity of the cytoskeleton. The results of the MTT assay yielded differences between the cytotoxicity of

the five tested FRC materials after incubation in artificial saliva. Coating of the FRC material with flowable or viscous resin composite did not increase cell viability. The coating of a FRC material, however, seems to be useful for protecting the fibres from wear and degradation in the oral cavity. There is a lack of knowledge about the material composition of commonly available fully pre-impregnated FRCs and their interactions with dental resin composite systems and bonding agents. Further studies are needed to gain information on the biocompatibility of FRC materials to optimize their intrinsic properties to accommodate clinical demands.

## Acknowledgements

The authors would like to thank the Imaging and Cytometry Core Facility (light microscopy unit) of the German Cancer Research Center for providing technical equipment and expertise for fluorescence microscopy.

***Declaration of interest*:** The authors declare that they have no financial or other relationships that might lead to actual or potential conflict of interest. This study was self-funded by the authors and their institutions in its major parts. Ivoclar Vivadent, Schaan Liechtenstein provided the dental resin composites and VOCO, Cuxhaven, Germany; GC EUROPE, Leuven, Belgium and ADM, Brno, Czech Republic provided the FRC materials for this investigation.

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