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Antimicrobial photodynamic active biomaterials for periodontal regeneration

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Abstract

Objectives

Biomaterials for periodontal regeneration may have insufficient mechanical and antimicrobial properties or are difficult to apply under clinical conditions. The aim of the present study was to develop a polymeric bone grafting material of suitable physical appearance and antimicrobial photodynamic activity.

Material and methods

Two light curable biomaterials based on urethane dimethacrylate (BioM1) and a tri-armed oligoester-urethane methacrylate (BioM2) that additionally contained a mixture of β -tricalcium phosphate microparticles and 20 wt% photosensitizer mTHPC (PS) were fabricated and analyzed by their compressive strength, flexural strength and modulus of elasticity. Cytotoxicity was observed by incubating eluates and in direct-contact to MC3T3-E1 cells. Antimicrobial activity was ascertained on *Porphyromonas gingivalis* and *Enterococcus faecalis* upon illumination with laser light (652 nm, 1 x 100 J/cm², 2 x 100 J/cm²).

Results

The compressive strength, flexural strength and elastic modulus were, respectively, 311.73 MPa, 22.81 MPa and 318.85 MPa for BioM1+PS and 742.37 MPa, 7.58 MPa and 406.23 MPa for BioM2+PS. Both materials did not show any cytotoxic behavior. Single laser-illumination (652 nm) caused total suppression of *P. gingivalis* (BioM2+PS), while repeated irradiation reduced *E. faecalis* by 3.7 (BioM1+PS) and 3.1 (BioM2+PS) log-counts.

Significance

Both materials show excellent mechanical and cytocompatible properties. In addition, irradiation with 652 nm induced significant bacterial suppression. The manufactured biomaterials might enable a more efficient cure of periodontal bone lesions. Due to the mechanical properties functional stability might be increased. Further, the materials are antimicrobial upon illumination with light that enables a trans-mucosal eradication of residual pathogens.

Keywords: biomaterial, laser, photodynamic therapy, periodontitis, periodontal bone regeneration, aPDT, photosensitizer, *Enterococcus faecalis*, *Porphyromonas gingivalis*

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1. Introduction

Periodontitis is a chronic and highly prevalent inflammatory disease of bacterial origin that is characterized by a progressive destruction of the alveolar bone, periodontal ligament and root cementum.

To prevent inflammation and further destruction of the periodontal hard and soft tissues, the initial treatment is mainly aimed at the destruction and removal of supra- and subgingival microbial biofilms. In general, this is accomplished by a professional dental cleaning followed by mechanical debridement and smoothing of the root surface. The procedure is often accompanied by a systemic administration of antibiotics or local application of antiinfective agents [1-3].

Especially in times of growing antibiotics resistance, the search for alternative antiinfective approaches is of major concern. In periodontitis treatment, measures such as the antimicrobial Photodynamic Therapy (aPDT) are therefore increasingly applied as an adjunct to the common scaling and root planning procedures. In case of aPDT, microbes are lethally damaged by highly reactive oxygen radicals that are formed upon illumination of a so-called photosensitizer (often a deep colored dye) with light of its absorption wavelength [4]. As already shown in numerous studies, aPDT is capable in suppressing oral bacteria and fungi sufficiently [5-12].

However, in periodontitis therapy, patients that have experienced the initial anti-inflammatory treatment often suffer from persistent alveolar bone defects and decreased tissue levels [13-15]. In order to support regeneration of the injured periodontium grafting materials might be inserted [16]. These include a whole range of materials such as barrier membranes, autografts, demineralized and freeze-dried bone allografts, bovine-derived xenografts and the combination of membranes and filler substances as well as the local application of growth factors such as Emdogain® or GEM 21S® [15, 17-19].

All of these measures are likely to support periodontal regeneration. But, if the applied materials do not meet the clinical requirements exactly, problems might arise.

In those cases, the implanted grafts might be rejected, delocalized or encapsulated by fibrous tissue causing a restriction of the materials' biological function [20].

Sometimes, surgeons are also confronted with insufficient mechanical and antibacterial properties or the application of the material is time consuming and to complicated.

However, up to now, there is no grafting material available that unites osteoinductive and -conductive characteristics with local antimicrobial activity and satisfactory physical stability. In this regard, our group already published data showing that polymers of poly(vinyl butyral-co-vinyl alcohol-co-vinyl acetate, urethane methacrylate or functionalized oligolactones are of promising characteristics [21].

Based on these results, the aim of the present study was to design an innovative synthetic grafting material that can easily be applied during periodontal surgery, shows mechanical properties similar to alveolar bone and is of sufficient antimicrobial activity. In order to turn the material antiinfective, the potent photosensitizer meta-tetra(hydroxyphenyl)chlorin (mTHPC) was incorporated that can be activated by illumination with red light of 652 nm and already demonstrated efficient antimicrobial activity [5, 7].

2. Materials and methods

2.1 Chemicals

D,L-lactide was purchased from Purac Biochem (Gorinchem, The Netherlands). Urethan dimethacrylate (UDMA), a mixture of two isomeric monomers (Fig. 1) and polyethylene glycol 400 (PEG 400) extended UDMA (P-UDMA) were received from Esstech (Essington, USA).

2-isocyanatoethyl methacrylate (IEM), stannous octoate, dibutyltin dilaurate (DBTL), camphorquinone, ethyl 4-dimethylaminobenzoate and several solvents were obtained from Sigma-Aldrich Chemie (Taufkirchen, Germany).

β -Tricalcium phosphate (β -TCP Cerasorb®) was supplied by Curasan (Frankfurt am Main, Germany).

2.2 Photosensitizer mTHPC

The photosensitizer mTHPC (also known as Temoporfin), which was kindly provided by biolitec research (Jena, Germany), is a very potent second-generation photosensitizer (for its chemical structure: see figure 3) and is the active ingredient in the drug Foscan® which is already approved for photodynamic treatment of non-melanoma skin cancer (biolitec, Jena, Germany). The photodynamic reaction of mTHPC is activated by irradiation with light of 652 nm.

In the present study, the hydrophobic photosensitizer mTHPC was mixed with β -tricalcium phosphate microparticles (β -TCP) at a concentration of 20 wt% and added to the polymeric base materials (also see below). Specimens without photosensitizer and β -TCP served as controls. The mixture of mTHPC and β -TCP is abbreviated as 'PS' throughout the entire study.

The added mTHPC is expected to generate sufficient antibacterial activity upon irradiation with red laser light at 652 nm, while β -TCP mainly functioned as porogen and was used as carrier for the photosensitizer.

2.3 Biomaterial 1 (BioM1)

Biomaterial 1 (BioM1) is based on UDMA (Fig. 1) mixed with a photoinitiator system consisting of camphorquinone and ethyl 4-dimethylaminobenzoate in a ratio of 1:1. Both components of the photoinitiator system were applied as 1 molar solution.

For sample preparation, 990 mg UDMA was mixed with 50 μ l of the photoinitiator system and 10 mg of the mTHPC / β -tricalcium phosphate mixture (PS).

Specimens without PS served as controls. For these samples, only 40 μ l of the photoinitiator system was added to the UDMA. Pilot studies have shown that 40 μ l of the photoinitiator system caused the most efficient polymerization of samples without mTHPC.

On the day of testing, the materials were freshly prepared and stored in the dark at room temperature until use.

2.4 Biomaterial 2 (BioM2)

For BioM2, a glycerol oligo-D,L-lactide based tri-armed urethane methacrylate was prepared as a trifunctional monomer in a two-step synthesis.

Briefly, as a first step, ring-opening oligomerization of D,L-lactide in the presence of glycerol was performed followed by end-capping of the terminal hydroxy groups of the tri-armed oligolactone with IEM. The chemical structure of this monomer is shown in Fig. 2. The synthesis of the glycerol-oligo-D,L-lactide was performed similarly as described previously [22]:

Briefly, a mixture of glycerol (2.5 g, 27.15 mmol), D,L-lactide (58.68 g, 407.17 mmol) and 140.6 μ l (0.43 mmol) stannous octoate, in the presence of 6 ml toluene, was stirred under nitrogen at 140°C. After 30 and 60 min, the stannous octoate solution was repeatedly added and the mixture was stirred for 4 h, cooled to room temperature and dissolved in 50 ml of dichloromethane. The solution was filtered and the crude product was precipitated twice into 700 ml of heptane. Finally, the isolated product was dried under vacuum at room temperature to constant weight.

The glycerol-oligo-D,L-lactide (8 g, 3.55 mmol) and 8.4 μ l (0.014 mmol) DBTL were dissolved in 40 ml of dichloromethane. After 10 minutes, IEM (1.51 ml, 10.64 mmol) dissolved in 2 ml dichloromethane, was slowly added and the reaction mixture was stirred for 40 h at 50 °C under nitrogen. The mixture was cooled to room temperature, extracted twice with 25 ml of 2 N NaOH solution and several times with distilled water until neutral pH. The organic layer was dried over Na₂SO₄. The solvent

was evaporated and the resulting residue was dried under vacuum, yield: 8.30 g, (86 %).

For BioM2 specimen preparation, 790 mg of the tri-armed glycerol-oligolactide-urethane methacrylate was mixed with 200 mg P-UDMA, 50 μ l of the photoinitiator system (refer to 2.3) and 10 mg of the mTHPC / β -tricalcium phosphate mixture (PS). For specimens without PS (controls) only 40 μ l of the photoinitiator system was applied as described in 2.3.

The freshly prepared BioM2 with and without PS was stored in the dark at room temperature until use.

2.5 Analytical methods

In order to obtain more detailed information about the structural character, IR spectra were recorded in the region of 4000-400 cm^{-1} using the FTIR spectrometer FTS 175 (Bio-Rad, München, Germany) in ATR (attenuated total reflection) mode. ^1H and ^{13}C NMR spectra were performed on a Bruker Avance DRX 500 NMR spectrometer in CDCl_3 at 25 °C.

The glass-transition temperature (T_g) was determined by dynamic mechanical analysis (DMA). In brief, particles were chipped off the photo-polymerized specimens and collected (50 mg). Subsequently, the particles were transferred and analyzed using the dynamic mechanical analyzer DMA7e (Perkin Elmer Corporation, Norwalk, USA) in the temperature range between -20 and 200 °C. The heating rate was set to 5 K/min. The obtained results were adjusted by applying the tangent delta ($\tan \Delta$).

2.6 Specimen preparation and mechanical behavior

Mechanical tests were performed to obtain information on the compressive strength, modulus of elasticity and flexural strength of the materials with and without PS.

For each test series, 15 specimens containing PS and 15 specimens without PS were prepared. The non-polymerized materials were transferred to silicone molds (7 x 5 mm of cylindrical shape for the compression test; 20 x 4 x 5 mm of rectangular shape for the three-point flexural test) and polymerized using a commercially available dental light curing unit (Profil Lux[®], 590 mW/cm², Voco, Cuxhaven, Germany). To ensure total polymerization of the materials each side of the specimen was irradiated 3 times for 20 s. Before testing, excess material was removed and the geometrical dimensions of the specimens were checked.

The compressive strength of the biomaterials was tested using the Instron Testing System 4469 (Instron, Darmstadt, Germany) at a temperature of 25 °C and a humidity of 50 %. The maximum force of the measuring cell was set to 50.00 kN at a loading speed of 12 mm/min. Beside the compressive strength, the elastic modulus was also determined:

$$S_{compression} = E \bullet e$$

where [S] represents the compressive stress, [E] the elastic modulus and [e] the strain. The compressive stress is defined as quotient of the test force [F] and surface [A]:

$$S_{compression} = \frac{F}{A}$$

The strain [e] is defined as ratio between change in length [Δl] and initial length of the specimen:

$$e = \frac{\Delta l}{l_0}$$

The elastic modulus was defined as:

$$E = \frac{S}{e}$$

For analyzing the flexural strength a Texture Analyser TA-XT 2i (Stable Micro Systems, Godalming, UK) equipped with a three point bend rig HDP/3PB at a support length of 20 mm was used. The test was performed at a temperature of 25 °C and at a humidity of 50 %. Results were calculated applying the following formula:

$$B = \frac{M}{W}$$

The flexural strength [B] is defined as quotient of the flexural moment [M] and the resistance moment [W]. The flexural moment is defined as:

$$M = \frac{F \cdot l}{4}$$

where [F] represents the compressive strength and [l] the support length of the HDP/3PB bend rigs. The resistance moment was obtained as follows:

$$W = \frac{a \cdot h^2}{6}$$

where [a] represents half of the side length and [h] the heights of the specimen.

2.7 Water degradation study

An accelerated degradation test, based upon weight loss of specimens in distilled water at 70 °C, was performed to obtain information about the degradation behavior of the biomaterials in vitro. Cylindrical specimens (7 x 7 mm, n = 12) were fabricated by polymerizing the respective monomers into silicon molds. Each specimen contained 0.5 g monomer that was mixed with the mTHPC / β -tricalcium phosphate proportion as described in sections 2.3 and 2.4. Specimens without PS were also formulated. All specimens were light-cured for 60 s and subsequently rinsed several times with distilled water. The weight of each specimen was recorded.

The degradation test was performed in 5 ml distilled water for 20 weeks at a constant temperature of 70 °C.

The distilled water was changed every 14 days and the weight of the specimens was recorded. At given time points the specimens were removed and dried to a constant weight at 65 °C. Weight loss of the specimens was gravimetrically determined. The experiments were run in triplicate.

2.8 Cytotoxicity – eluates

Specimens of BioM1 and BioM2 with and without PS of cylindrical shape (9 x 4mm, n = 48) were prepared as described (see 2.3 and 2.4) and sterilized by UV radiation for 2 h. Immediately afterwards, the specimens were placed in a 24-well plate and incubated with α -MEM (Biochrom, Berlin, Germany) containing 10 % FBS and 1 % PenStrep. Eluates were collected daily from day 1 to 7 and subsequently at days 14, 21 and 28. The eluates were frozen at -20 °C and stored in the freezer until use. Subsequently, each specimen was rinsed with 1 ml PBS and fresh culture medium was added to the wells.

In the second part of the experiment, MC3T3-E1 cells (DSMZ ACC210, DSM, Braunschweig, Germany) were incubated with eluates obtained from the biomaterials and checked for cytotoxicity. Therefore, MC3T3-E1 cells were seeded into 96-well microtiter plates at densities of 10.000 cells/well and cultivated for 24 h under standard atmospheric conditions. After 24 h the cells were incubated with the collected eluates (100 μ l) for 2 d at 37 °C under aerobic standard conditions. The test was repeated 8 times for each biomaterial specimen no matter if it contained PS (n = 8) or not (n = 8). Controls (n = 8) were incubated with 100 μ l cell culture medium, only. After 48 h of incubation, all cells were subjected to MTT viability assay (Roche,

Mannheim, Germany) according to the manufacturers' instructions. The absorption of purple colored formazan was recorded in PBS using an absorbance spectrometer at 570 nm and cell viability was estimated as follows:

$$\text{Viability (\%)} = 100 \times (\text{absorption}_{\text{test-group}} / \text{absorption}_{\text{control-group}})$$

2.9 Cytotoxicity – direct contact

MC3T3-E1 cells (DSMZ ACC210, DSM, Braunschweig, Germany) were cultivated in Dulbecco's modified Eagle's medium (DMEM) (Biochrom, Berlin, Germany) supplemented with 10 % FCS, containing 50 U ml⁻¹ of penicillin, 0.05 mg ml⁻¹ of streptomycin at 37 °C under 5 % CO₂ atmosphere. Cylindrical specimens (5 x 5 mm, height x diameter) of biomaterial BioM1 and BioM2 with and without PS were prepared and transferred each into a separate well of a 24 well cell culture plate. After sterilization of the specimens with UV radiation for 2 h, 25.000 cells suspended in 1 ml culture medium were seeded into each well covering the biomaterial specimens. The culture medium was renewed each second day. At day 4, the specimens were carefully transferred to microscopic slides and the cells were stained with 0.05 ml of staining solution (final concentrations: 15 µg ml⁻¹ fluorescein diacetate (FDA), 4 µg ml⁻¹ GelRed® in PBS). Green and red fluorescence of the cells, respectively, was monitored using an Axiotech microscope (Carl Zeiss, Jena, Germany) equipped with filter sets 09 and 14. Photomicrographs were recorded using a CCD fluorescence microscope imager MP 5000 (Intas, Göttingen, Germany). Imaging was supported by Image-Pro Plus software (Media Cybernetics, Silver Spring, MD, USA).

2.10 Antimicrobial photodynamic activity

The antimicrobial photodynamic activity of the biomaterials was tested on the oral pathogenic species *Porphyromonas gingivalis* (ATCC 33277) and *Enterococcus faecalis* (DMSZ 20376).

Each bacterial strain was grown in Schaedler fluide media (0.1 % Konakion (wt/vol)) (Oxoid Deutschland, Wesel, Germany) for 24 h under anaerobic standard conditions. Subsequently, the bacteria were pelleted by centrifugation and dissolved in PBS to an optical density (OD_{546 nm}) of 0.05.

Prior to testing, defined amounts (35 µl) of monomeric BioM1 and BioM2 substituted with PS were transferred to 96-well microtiter plates and light-cured for 60 s using the dental curing light Polofil Lux® (Voco, Cuxhafen, Germany). Specimens of BioM1 and BioM2 without PS were arranged as controls.

The surfaces of the polymerized specimens were rinsed with distilled water and sterilized by treatment with ultraviolet radiation for 20 min. Subsequently, 50 µl of the respective bacterial solution was added on top of the polymerized material and the cells were sedimented by centrifugation (3500 rpm, 3 min). Afterwards, all specimens were incubated for 1 h at 37 °C under anaerobic standard conditions in the dark.

Irradiation of the specimens was performed by using a GaAs diode laser (652 nm, Ceralas PDT, CeramOptec, Bonn, Germany), operating in continuous mode. The respective microtiter plate was placed on a computer-controlled laser scanning stage and irradiated from above. The light from the laser device was guided by a flexible optical fiber (Medlight FD1, Medlight, Ecublens, Switzerland) equipped with a frontal light distributor. For efficient illumination of each well, the optical fiber was firmly attached to the laser scanning stage at a distance of 10 mm to the surface of the plate. Specimens were irradiated for 33 s either once (1 x 100 J/cm²) or twice (2 x 100 J/cm²) with an interval of 3 min. After irradiation, the bacterial solution (50 µl) was collected from each well and mixed with 950 µl PBS. Subsequently, dilution series from 10⁻¹ to 10⁻⁵ were arranged and 100 µl aliquots of each dilution were plated on sterile Schaedler agar (Oxoid, Wesel, Germany). After cultivation for 2 to 5 days under anaerobic standard conditions the number of colony forming units (CFU/ml) was determined by counting. For each specimen 10 runs were performed.

2.11 Statistical analysis

The results of the mechanical characterization were analyzed by student's t-test. Significance in the antibacterial photodynamic assays was checked by applying the non-parametric Mann-Whitney-U-test. The level of significance was set to $\alpha = 0.05$. For statistical analysis the PC program SPSS version 19 (IBM, Chicago, USA) was used. In subsequent figures an iconic star is shown to indicate statistically significant differences between groups.

3. Results

In the present study, two in-situ curable biomaterials, BioM1 and BioM2 were prepared as support for photosensitizers potentially usable in photodynamic antimicrobial therapy.

3.1 Monomer characterization

The newly synthesized glycerol oligo-D,L-lactide- based tri-armed urethane methacrylate monomer (Figure 2) of BioM2 was structurally characterized by IR, ^1H NMR and ^{13}C NMR spectroscopy.

The ^1H -NMR spectrum showed typical signals at 6.06 ppm, 5.56 ppm ($\text{C}=\text{CH}_2$) and 1.91 ppm (CH_3) of the methacrylate groups as well as 5.27 ppm, 4.83 - 4.34 ppm (CH) and 1.56 - 1.52 ppm (CH_3) of lactide units. The signals of the glycerin starter were localized at 5.27 ppm (CH-O), 4.34 ppm and 4.19 ppm for the $\text{CH}_2\text{-O}$ moiety. The CH_2 groups in the neighborhood of the urethane bonds displayed proton signals at 4.26 ppm and 3.47 ppm.

Complete urethane formation was confirmed by the absence of an OH absorption in the FT-IR spectra at $3300 - 3600\text{ cm}^{-1}$. The introduction of urethane methacrylate function into the tri-armed monomer is illustrated in the FT-IR spectrum by the appearance of the N-H absorption at 3420 cm^{-1} and a C=C stretching band of the methacrylate group at 1636 cm^{-1} .

The glass transition temperature (T_g) and the tangent delta of both tested materials are listed in Table 1. BioM1 showed a higher glass transition temperature ($111\text{ }^\circ\text{C}$) compared to BioM2 ($31\text{ }^\circ\text{C}$). Additional application of PS to BioM1 and BioM2 did not result in any significant change in the T_g .

3.2 Mechanical behavior

For both biomaterials the compressive strength, flexural strength and compressive modulus of elasticity was estimated (Figures 4-6).

The results show that in specimens without PS, a mean compressive strength of 432.14 MPa was obtained for BioM1 and 517.86 MPa for BioM2. Addition of PS resulted in significant different compressive strengths. In detail, 311.73 MPa was measured for BioM1 + PS and 742.35 MPa for BioM2 + PS (Fig. 4).

For BioM1 a flexural strength of 29.45 MPa was obtained. When PS was added to BioM1 the mean flexural strength decreased to 22.81 MPa, which was statistically not significant (Fig. 5). For BioM2, a mean flexural strength of 9.96 MPa was measured. Application of PS to BioM2 caused no significant reduction in the flexural strength (7.58 MPa). By comparing both PS-containing materials, BioM1 + PS showed a significant greater flexural strength compared to BioM2 + PS.

However, the modulus of elasticity was almost identical for both materials without PS (Fig. 6). Incorporation of PS caused a significant drop in the elastic modulus from 519.88 to 318.85 MPa ($p < 0.05$) in BioM1. By contrast, application of PS to BioM2 caused an increase in the elastic modulus from 335.19 to 406.23 MPa, which was statistically not significant.

In summary, additional application of PS to the polymeric base materials did not negatively influence the mechanical properties. Only in BioM1, incorporation of PS induced a significant decrease in the modulus of elasticity.

3.3 Weight loss (degradation) in water.

The results of the degradation study in distilled water at 70 °C are presented in figure 9. During the entire examination period BioM1 without PS degraded by only 1.5 % of weight. If PS was added to BioM1 a mean degradation of 4.8 % was estimated.

In contrast, BioM2 without PS lost weight by 66.8 % within the first 28 days already, without any further degradation afterwards. If PS was added a loss in weight by 74.1 % was determined during the first 28 days of incubation without any further significant changes in weight any time after.

3.4 Cytotoxic behavior

Beside their physical properties, the cytotoxic behavior of the materials w/o PS was also studied on MC3T3-E1 cells. The cells were incubated with collected eluates or cultivated in direct contact to the biomaterials.

Over a course of 28 days, no cytotoxic effects of the collected eluates were observed (Fig. 7). During the entire examination period, viability of the cells remained at high levels.

Cultivation of the MC3T3-E1 cells in direct contact to the materials (containing PS or not) did not cause any negative effect (Fig. 8). In the applied live/dead assay the vast majority of cells appeared in green fluorescence which indicates high integrity of the cell walls. Additionally, the cells showed a uniform growth pattern throughout the entire biomaterial surfaces.

In summary, none of the tested biomaterials, whether they contained PS (test-group) or not (control-group) showed any cytotoxic reaction towards MC3T3-E1 cells.

3.5 Antimicrobial photodynamic activity

Irradiation of the specimens with red laser light at 652 nm that additionally contained mTHPC caused significant microbial suppression (Figures 10, 11). The results show that both tested bacterial strains were susceptible to the photodynamic reaction. Single irradiation (100 J/cm^2) of PS containing BioM1 caused suppression of *P. gingivalis* by 4 log-counts and induced total bacterial killing in case of BioM2 + PS (test-group). When laser light was applied twice ($2 \times 100 \text{ J/cm}^2$) *P. gingivalis* was eliminated completely within both materials (Fig. 10).

With *E. faecalis* (Figure 11), single irradiation of the materials + PS resulted in only minor bacterial suppression, which was not statistically significant. However, when the specimens were irradiated twice ($2 \times 100 \text{ J/cm}^2$) bacterial reduction by 3.7 log-counts (BioM1 + PS) and 3.1 log-counts (BioM2 + PS) was observed. By contrast, irradiation of materials without PS did not result in any bacterial reduction of either *P. gingivalis* or *E. faecalis*. Furthermore, incubation of the PS containing biomaterials in the dark also caused no significant toxic effect.

4. Discussion

The aim of the present study was to develop an innovative polymeric grafting material for periodontal regeneration. According to the clinical requirements, it was intended to design an easy to use material of sufficient mechanical stability with local antimicrobial activity that can be switched on at any time, on demand, apart from the action of antibiotics. Further, the materials should also be of high cytocompatibility and osteoinductive activity.

To the best of our knowledge, there is currently no grafting material available that meets all of these requirements. The present study focused on polymeric and hybrid materials because they have the most promising characteristics to integrate all the mentioned requirements [23]. Due to their chemical structure, polymers and polymeric composites are practicable and show biomechanical characteristics that are superior to many other grafting materials [24].

In a recent study, our group published data which showed that polymers based on poly(vinyl butyral-co-vinyl alcohol-co-vinyl acetate), functionalized oligolactone-based networks and urethane methacrylates have excellent cytocompatible properties [21]. Accordingly, the present study focused on urethane methacrylate-based starting materials; that is, urethane dimethacrylates and a tri-armed oligoester-urethane methacrylate. Normally, urethane dimethacrylate-based materials show rather low tendency to degrade. In our specific clinical applications, degradable materials are advantageous, so we incorporated oligoester units into the structure of BioM2 to modulate the rate of degradation. The polymerizable monomers were further enriched with a photoinitiator system and a mixture consisting the photosensitizer mTHPC and β -TCP (PS). Both biomaterials were then subjected to different tests in order to obtain detailed information about the mechanical behavior, degradation rate, cytotoxicity and antimicrobial photodynamic activity.

BioM1 was composed of different urethane dimethacrylates. The biomechanical properties of urethane dimethacrylates are highly influenced by the type of building-blocks and the molecular structure in general. The additional appearance of rotatable ester bonds within the molecule, however, enhances the material's elastic behavior, although a dense polymeric network is formed during light-polymerization [25, 26].

Unlike monomethacrylates, dimethacrylates are often characterized by stronger hydrophobicity which is reflected by low water sorption [27]. The hydrophobic

character of dimethacrylates was also evident in the present study. As expected, BioM1 exhibited a rather low degradation rate in water. By contrast, BioM2 incorporated a more hydrophilic PEG-extended UDMA monomer and oligoester-based urethane methacrylate with potentially water-cleavable ester bindings. Over the course of 140 days the BioM1 material lost weight by only 4.7 %. BioM2 specimens without PS degraded by only 1.5 %. Compared to BioM2 which showed a degradation of 74.1 %, BioM1 can therefore be considered a rather inert material.

In regenerative therapy and tissue engineering, various grafts composed of lactide polymers are already widely applied [28, 29]. Biomaterials manufactured from lactides are usually reabsorbed by the body to a high extent [30]. It is conceivable that inert materials like BioM1 + PS may be applied to defects where a long term spacer is needed [31, 32].

In the present study β -TCP was additionally added to create pores of different dimensions. β -TCP is also widely used in regenerative therapy and tissue engineering [33-37]. Because of their osteoinductive characteristics calcium phosphate cements are therefore often applied [38].

It is well known that the porosity of a grafted material has a great impact on the incorporation and subsequent stage of vascularization [39-41]. In detail, it was found that an additional application of β -TCP increased neo vessel formation by 1.8 fold and also showed positive effects on the mechanical properties of synthetic grafting materials [42, 43].

In order to observe the impact of the photosensitizer / β -TCP mixture (PS) on mechanical properties, the biomaterials were characterized for their compressive strength, flexural strength and modulus of elasticity. As shown by the results, additional application of PS in the polymeric material did not negatively influence the mechanical properties, except in the case of BioM1 where a significant reduction in the modulus of elasticity was apparent amongst specimens containing PS.

The mechanical tests revealed mean compressive strengths of 311.73 MPa (BioM1 + PS) and 742.37 (BioM2 + PS), probably sufficient for the materials to resist normal masticatory forces [44].

In the three-point flexural tests, different strengths were recorded for both materials. BioM1 + PS showed a significant stronger resistance towards bending (22.81 MPa) compared to BioM2 + PS (7.58 MPa). Studies, however, have shown that there should be no significant discrepancy between the mechanical properties of the

implanted material and the host tissue in order to achieve sufficient regeneration. If there are any mismatches, stress shielding and local bone resorptions are likely to occur [45].

As already mentioned, application of PS caused a significant reduction in the modulus of elasticity in BioM1. For the test-group specimens, values of 318.85 MPa (BioM1 + PS) and 406.23 MPa (BioM2 + PS) were estimated. However, the results show that both biomaterials compared to the elastic modulus of natural alveolar bone with values as high as 9.6 GPa are less likely to resist elastic deformation [46].

The glass-transition temperatures T_g of the materials were also evaluated. The glass-transition temperature marks the reversible transition from a hard and brittle state to a rubbery-like state in polymers. BioM1 + PS had a glass-transition temperature of 120 °C and BioM2 + PS of 35 °C. Addition of PS only increased the glass-transition temperature by a few degrees compared to the materials without PS (9 °C for BioM1 and 4 °C for BioM2). If grafted into the body, fully polymerized BioM2 + PS with a T_g of 35 °C is more likely to have a rubbery consistency, whereas BioM1 + PS appears to be more solid. According to these results, BioM2 + PS might be grafted into smaller defects, while BioM1 + PS might be used in larger lesions.

Besides the biomechanical properties, the cytotoxicity of the materials was also evaluated. Incubation of the collected eluates with MC3T3-E1 cells did not cause any loss in viability. During the entire examination (28 days), the viability of the MC3T3-E1 cells remained at very high levels (above 95%). Even when the cells were cultivated in direct contact to the materials, no cytotoxic reaction was observed. As shown previously, the cytotoxicity of a polymeric material is highly influenced by the polymer type [47, 48].

In the present study the photosensitizer mTHPC was incorporated into the biomaterials to achieve antimicrobial activity upon illumination with red light at 652 nm. Up to now, there are only a few reports available, where photosensitizers were used to create antimicrobial activity on the surface of solid materials [49, 50]. In photodynamic therapy, application of suitable light causes a photosensitizer to enter a triplet state which subsequently leads to the formation of highly reactive oxygen radicals that cause lethal damage to the microbes.

In the present study, the antimicrobial photodynamic action of the fabricated biomaterials was tested on both *P. gingivalis* and *E. faecalis*.

P. gingivalis is often associated with severe forms of periodontitis, causing inflammation and constant degeneration of alveolar bone [51, 52]. Bacteria of the periodontal region may also originate from infected dental pulps. Those combined lesions enable bacteria to spread across different environments. In this context, the grampositive bacterial species *E. faecalis* is of major concern. Primarily, the species is associated with refractory root canal infections, but can also affect the integrity of the apical periodontium causing osteolysis and abscesses. Because the species is able to inactivate neutrophil granulocytes and aggregates with periodontopathogenic *Fusobacterium nucleatum*, it is believed to play a distinct role in the pathogenesis of refractory and chronic forms of periodontitis [53, 54].

Our recent studies show that *E. faecalis* in planktonic solution and also in biofilm mode can be suppressed sufficiently by photodynamic treatment using mTHPC [5, 7]. In the present study, *E. faecalis* was more resistant to the photodynamic treatment compared to *P. gingivalis*. While *P. gingivalis* was killed completely after only one application of light (with BioM2), single irradiation of *E. faecalis* did not cause any significant reduction. However, if illumination was applied twice ($2 \times 100 \text{ J/cm}^2$), *E. faecalis* was reduced by $3.7 \log_{10}$ in BioM1 + PS and $3.1 \log_{10}$ in BioM2 + PS. By comparison to the control specimens, which did not contain PS, it was shown that application of laser light alone did not lead in any significant bacterial reduction. Therefore, the strong antibacterial effect can clearly be attributed to the photodynamic mechanism. A minor bacterial suppression caused by thermal heat during light application cannot be excluded, since the surface temperature during light application was not recorded. But earlier published data confirm that the rise in temperature caused by laser irradiation during aPDT doses not significantly influence bacterial life [5].

The results of the photodynamic setting can be compared to an article published by McCoy et al.. The group used different photosensitizers in combination with polymers. Irradiation of the specimens was performed using two 230 W halogen bulbs situated at a distance of 24 cm from the specimens, providing 1.34 W of light across the specimen surfaces, for two hours. The results showed that *S. aureus* was suppressed by 3.62 and *E. coli* by only $1.51 \log \text{ CFU/ml}$ [49].

In another study, indocyanine green (ICG) was embedded into polyether type polyurethane with 0.015 or 0.105 wt% of the specimens and the antibacterial photodynamic action was observed on methicillin-resistant *Staphylococcus aureus*,

Escherichia coli, *Pseudomonas aeruginosa* and *Staphylococcus epidermidis*. Irradiation with 808 nm for 15 min (at energy density of 31.83 J/cm²) caused reduction in *S. aureus* and *S. epidermidis* by 2 log₁₀ and *E. coli* and *P. aeruginosa* by 0.5 log₁₀ with the specimens of higher ICG content [50]. In the case of ICG, the antibacterial effect is mainly caused by thermal heating (photothermal therapy) rather than by a photodynamic mechanism what was observed in a recent study [6].

It can be concluded that the photodynamic antibacterial activity of the manufactured mTHPC containing biomaterials was superior to the results presented in the above mentioned studies. Only one single irradiation (irradiation time: 33 s) caused total elimination of the gram-negative *P. gingivalis* (BioM2 + PS), while a 2-fold irradiation (2 x 33s) reduced *P. gingivalis* completely within both biomaterials (BioM2 + PS and BioM1 + PS) and *E. faecalis* by 3.1 log₁₀ units (with BioM2 + PS) or 3.7 log₁₀ (with BioM1 + PS). It seems to be that there is actually no grafting material available exhibiting a more sufficient antimicrobial photodynamic activity.

The results show that our group successfully manufactured two different photodynamically active biomaterials with sufficient mechanical, cytocompatible and antimicrobial properties. The performance of the materials and their antimicrobial activity in vivo still needs to be investigated.

5. Conclusions

Both tested biomaterials show excellent mechanical and cytocompatible properties. In addition, irradiation with red light at 652 nm caused strong antimicrobial behaviour. The manufactured biomaterials might enable clinicians to regenerate periodontal bone lesions more efficiently. If applied properly, the materials might also increase the primary stability of the afflicted teeth. Furthermore, the antimicrobial activity upon irradiation with light is useful to counter residual pathogens or to prevent the implant side from further microbial infections. Nevertheless, the performance of the materials still needs to be assessed in vivo.

Acknowledgements

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Biomaterial	Tg [°C]	tan Δ
BioM1	111	0.27
BioM2 + PS	120	0.20
BioM2	31	0.48
BioM2 + PS	35	0.55

Table 1:

Glass transition temperature of the materials BioM1 and BioM2 with and without the mTHPC/ β -TCP mixture (PS)

Legends

Table 1:

Glass transition temperature of the materials BioM1 and BioM2 with and without the mTHPC/ β -TCP mixture (PS)

Figure 1:

Chemical structure of the urethane dimethacrylate isomers of UDMA (used in BioM1)

Figure 2:

Chemical structure of the glycerol oligo-D,L-lactide based tri-armed urethane methacrylate monomer (used in BioM2)

Figure 3:

Chemical structure of the photosensitizer mTHPC (Temoporfin) (adapted from www.ChemDrug.com)

Figure 4:

Compressive strength of BioM1 and BioM2 with and without PS. Each group comprised n = 15 samples.

Figure 5:

Flexural strength of BioM1 and BioM2 with and without PS. Each group comprised n = 15 samples.

Figure 6:

Modulus of elasticity of BioM1 and BioM2 with and without PS. Each group comprised n = 15 samples.

Figure 7:

Cytotoxicity of eluates collected from BioM1 and BioM2 with and without PS on MC3T3-E1 cells determined by MTT viability assay

Figure 8:

Live/dead assay using FDA (green fluorescence indicating viable cells) and GelRed® (red fluorescence indication nuclei of dead cells) in direct contact of BioM1 and BioM2 with and without PS to MC3T3-E1

Figure 9:

Weight loss of BioM1 and BioM2 with and without PS in water at 70 °C

Figure 10:

Antimicrobial activity of BioM1 and BioM2 with and without PS upon irradiation with laser light (652 nm) on *Porphyromonas gingivalis*. Arrows show total bacterial elimination. Stars indicate statistically significant differences.

Figure 11:

Antimicrobial activity of BioM1 and BioM2 with and without PS upon illumination with laser light (652 nm) on *Enterococcus faecalis*. Stars indicate statistically significant differences.

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Cytotoxicity - Eluates

Figure 7:

Cytotoxicity of eluates collected from BioM1 and BioM2 with and without PS on MC3T3-E1 cells determined by MTT viability assay

Cytotoxicity – Direct contact

Figure 8:

Live/dead assay using FDA (green fluorescence indicating viable cells) and GelRed® (red fluorescence indication nuclei of dead cells) in direct contact of BioM1 and BioM2 with and without PS to MC3T3-E1

Degradation in Water (70 °C)

Figure 9:

Weight loss of BioM1 and BioM2 with and without PS in water at 70 °C

Photodynamic activity - *Porphyromonas gingivalis*

Figure 10:

Antimicrobial activity of BioM1 and BioM2 with and without PS upon irradiation with laser light (652 nm) on *Porphyromonas gingivalis*. Arrows show total bacterial elimination. Stars indicate statistically significant differences.

Photodynamic activity - *Enterococcus faecalis*

Figure 11:

Antimicrobial activity of BioM1 and BioM2 with and without PS upon illumination with laser light (652 nm) on *Enterococcus faecalis*. Stars indicate statistically significant differences.