1. Introduction

In modern bone surgery cements are commonly used as bone substitutes and as implant materials to fixate prostheses. Since the development of the first bone cement based on polymethylmethacrylate (PMMA) by Charnley and Smith in the early 1960s, various bone cements and fillers have been developed, such as PMMA based cements for orthopedic applications, bioactive glasses for dental surgery and repair of bone defects, and calcium phosphate cements (CPC) for bone tissue replacement. All these biomaterials have specific properties and have been approved for different application areas. Nevertheless, none of them is without any shortcomings. In case of PMMA cement, radiolucent fibrous tissue is observed at the bone/cement interface, primarily consisting of fibroblasts, macrophages and foreign body giant cells. This fibrous interface is a result of high polymerization temperatures combined with release of toxic methylmethacrylate (MMA) monomers, both damaging the surrounding tissue. Failure of the bone/cement interface in cemented joint prostheses is the main contributor to implant loosening. Furthermore, PMMA cements have a dense structure, which does not allow bone cells to grow into the cement, and they show inadequate elasticity in relation to natural bone, especially to spongiosa. Calcium phosphate cements, however, show good biocompatibility and osteoconductivity, but on the other hand they have inappropriate mechanical properties, low biodegradation in vivo, high brittleness, relatively long final setting times and lower flexibility than natural bone. During the last decade, many research groups tried to improve commercially available biomaterials, but no breakthrough has been achieved so far.

The aim of the present studies was to investigate mechanical properties and biocompatibility of a newly developed biomaterial, a platform technology named COOL. COOL is a variable composition of commonly accepted bioceramics and chemically modified PMMA. This technology is reported to combine the advantages as release of inorganic components and high mechanical strength by avoiding the disadvantages such as high temperature, monomer release and/or low bending strength. Furthermore, COOL is said to fill the gap between CPCs and PMMA based cements.

2. Materials and Methods

2.1. Composition of materials

COOL Bone Cement (COOL) is a two component system of powder and liquid. The powder component contains several bioceramics: the bioresorbable glass ceramic GB14, tetra calcium phosphate (TETRA), a mixture of fluorapatite (FA) and calcium zirconium phosphate (CZP), and zinc oxide (ZnO). All bioceramics have defined particle sizes in the range of 2 to 200 μm. COOL contains 50 to 70 wt. % of inorganic components. The liquid contains acid-modified polymethylmethacrylate (PMMA) dissolved in a mixture of ethylacetocetate and ethanol (1:1). The powder component was sterilized by heat sterilization for 2 hours at 200 °C, whereas no sterilisation of the liquid component is required because of the solvent’s composition. Tested formulations of COOL are listed in Table 1.

Refobacin® Bone Cement R (Biomet, Berlin, Germany) was used as a reference because of its broad application and acceptance as gold standard. This bone cement is a mixture of a powder containing PMMA, zirconium dioxide, benzoyl peroxide, gentamycin sulphate...
and a solution consisting of methylmethacrylate and N,N-dimethyl-p-toluidine.

2.2. Mechanical strength

Determination of four point bending strength was carried out according to DIN standard 54343 utilizing a Dynstat device. Specimens with a dimension of 15 x 10 x 2 mm were manufactured using Teflon® moulds. Series of ≥10 specimens per formulation were investigated after 24 hours of storage in water at 37 °C followed by 24 hours of drying at room temperature. The comparability of four point bending test according to DIN 54343 and three point bending test after ISO 5833 was demonstrated by Kuehn1.

Compressive strength was measured according to ISO standard 5833. Specimens with 6 mm in diameter and 12 mm in height were manufactured using Teflon moulds. After storage in water at 37 °C for 24 hours and subsequent drying for 24 hours, ≥5 specimens were measured for each formulation using the universal test device ZWICK 010 (Ulm, Germany) with a traverse speed of 21 mm/min.

Differing from the usual setting procedures described above test specimens of the standard formulation were also stored in air at 37 °C for up to 10 days and in water at 37 °C with 24 hours drying period for up to 7 days.

2.3. Setting behavior

The initial time of setting was determined using a Gillmore needle according to ASTM standard C 266–04. Cement was poured into moulds with 11 mm in diameter and 14 mm in height and surface was flattened. Moulds were stored in water at 37 °C. Time measurement started when PMMA solution contacted the ceramic powder. For every collection of data 3 specimens with maximum 6 indentations were used.

2.4. Porosity

For determination of pore proportion, density of selected examples was measured using the helium pycnometer AccuPyc 1330 (Micromeritics, Münchenladbach, Germany). After determining volume (V) and weight of the specimens, they were grinded with a ball mill and 2 to 3 g of the powder were used for investigation. Powder volume was calculated from the determined density and the weight of the powders.

Porosity was calculated using the formula:

$$\text{Porosity} = \frac{V_{\text{specimen}} - V_{\text{powder}}}{V_{\text{specimen}}} \times 100\% \quad (1)$$

2.5. Scanning electron microscopy (SEM)

SEM micrographs (Maxim, Cambridge, UK) were performed under low vacuum conditions (10 mbar) with 20 kV acceleration energy of primary electrons using back scattered imaging mode. Braking edges of COOL specimens, which were used for determining bending strength, were photographed for analyzing pore structure.

2.6. X-ray opacity

Using the X-ray device TransX (Schütz-Dental, Rosbach, Germany), COOL formulation A was investigated for radiopacity in relation to 2 different reference materials: Refobacin® R bone cement containing zirconium dioxide (ZrO₂) and a titan implant sample with 8 mm in diameter and 2 mm in thickness, as representative for metallic implant materials.

2.7. In Vitro cell culture and experimental procedure

In Vitro biocompatibility testing of COOL formulation A was examined according to ISO and DIN 10993-5 in a culture model of primary human osteoblasts (pHOB). pHOB cells, collected from bone samples of a 66 year old female (donor 1) and a 72 year old male (donor 2), both undergoing total hip surgery, were purchased from Provitro (Berlin, Germany). Osteoblasts from both donors were characterized for osteocalcin expression. pHOB cells from each donor were cultured separately on 12-well plates at a concentration of 5 x 10⁴ per well in Osteomed Growth Medium supplemented with 4 mM L-glutamine, 50 µg.mL⁻¹ gentamycin, 50 ng.mL⁻¹ amphotericin B, 20 mM HEPES and 10% heat-inactivated fetal calf serum (purchased as complete medium from Provitro, Berlin, Germany). Cultures were incubated in a humidified atmosphere containing 5% CO₂ at 37 °C with replacement of medium every 2 to 3 days. After allowing the cells to attach to the surface of the wells for 24 hours, bone cement specimens with a surface area of 0.38 cm² were prepared and added to the cell monolayer either directly after preparation or after 5 and 24 hours of setting in culture medium at 37 °C. At day 7 of culture the medium was supplemented with 10 nM dexamethasone, 5 x 10⁻⁸ M 1,25(OH)₂D₃, 50 µg.mL⁻¹ L-ascorbic acid, 10 mM β-glycerolphosphate and 1.5 mM CaCl₂ in order to induce osteogenic differentiation. All osteogenic supplements were purchased from Sigma-Aldrich (Deisenhofen, Germany).

2.8. Cell morphology and viability

During the entire culturing period, morphology and density of pHOB cells were observed by light microscopy and photographed.
For determination of cell viability/proliferation Alamarblue™ assay (Biozol, Eching, Germany) was performed every 2 to 3 days according to the manufacturer’s instructions. Briefly, 1 mL of culture medium together with 100 µL of Alamarblue™ reagent were added to each well and the cells were incubated in this medium/Alamarblue™ mix under humidified 5% CO₂ conditions at 37 °C. After 3 hours of incubation, absorbance was analyzed spectrophotometrically at 570 nm with 595 nm as a reference.

2.9. Alkaline phosphatase (ALP) activity

ALP activity was assessed in cell lysates by determining the release of p-nitrophenol (PNP) from p-nitrophenyl phosphate (PNPP) using the Phosphate Substrate Kit from Perbio Science (Bonn, Germany). The substrate solution contained 1 mg.mL⁻¹ of PNPP dissolved in 1 M diethylamine substrate buffer (pH 9.8). After an incubation period of at least 20 minutes (which was in the linear range) at 37 °C, the reaction (volume 110 µL) was stopped with 50 µL 2N NaOH. Results of ALP activity were extrapolated from a standard curve of PNP (Sigma-Aldrich, Deisenhofen, Germany) and related to cell viability.

2.10. Statistics

All the data are expressed as means ± SD. Statistical differences were analyzed using one-way analysis of variance (ANOVA). P values < 0.05 were considered statistically significant.

3. Results

3.1. Mechanical strength

Depending on the formulation of the cement bending strengths from 9.9 ± 2.4 to 26.4 ± 3.0 MPa and compressive strengths from 3.6 ± 0.8 to 26.2 ± 1.8 MPa were achieved 48 hours after mixing. Bending strength (Figure 1) and compressive strength (Figure 2) increased significantly if ZnO (up to 7%) was added to the different formulations. COOL formulation B contained CZP instead of CZP/
FA mix. In formulations C to E 40 wt. (%) of resorbable bioceramic GB14 were substituted by another ceramic component as described in Table 1. Not any of the substitutions led to substantial differences of bending or compressive strength.

Compressive and bending strength was higher if tested after 7 and 10 days, respectively. Compressive strength increased during the first 3 days of storage in water as well as in air. Values for water stored specimens stayed constant within the following 7 days (33.2 ± 1.6 MPa), whereas those for air stored specimens were still increasing (62.8 ± 1.3 MPa) after 10 days of storage. Bending strength increased to 26.4 ± 1.3 MPa if stored in air for 7 day, but decreased slightly (18.5 ± 3.0 MPa) if stored in water.

Reference material showed bending and compressive strength after 48 hours of 57.8 ± 2.8 MPa and 74.1 ± 2.5 MPa, respectively.

Figure 4. X-ray opacity of COOL formulation A 1) in comparison to Refobacin®; 2) and titanium specimen; and 3) (exposure time 0.5 seconds).

Figure 5. Light microscopic analysis of pHOB cell morphology (donor 1) during incubation with specimens of COOL formulation A (d-f) in comparison to cells of the control (a-c) and those with reference material (g-i) at day 2, 5 and 16 of culture. Bone cement specimens were added to the cell cultures immediately after preparation (magnification 100x).
3.2. Setting behavior of COOL

By mixing the viscous dissolution of modified PMMA with the mixture of bioceramics, a creamy adhesive compound is formed. Setting takes place through cementation at body temperature. A significant decrease in initial setting time was observed if ZnO was added to the composite. An initial setting time of 26 minutes was measured for COOL standard formulation A. In contrast, COOL formulation A/2 lacking ZnO showed initial setting after 107 minutes. Reference material had an initial setting time of less than 7 minutes.

3.3. Porosity

Dependent on the formulation of COOL, high variation in pore sizes was observed by using SEM. All fracture surfaces showed a large amount of pores, which differed in shape and size. As an example, Figure 3 shows differences in the appearance between the breaking edges of COOL formulations containing ZnO (A) and those that lack ZnO (A/2). Compared to formulation A, formulation A/2 clearly showed larger amounts of pores. Furthermore, pore diameters were higher in formulation A/2. In general, all formulations lacking ZnO revealed more and bigger pores compared to similar formulations containing ZnO. Majority of the pores of all formulations were > 50 µm.

Except for formulation A/2, which had a porosity of 43%, all other formulations (A – E) showed porosity between 31 and 37%.

3.4. X-ray opacity

The specimens could be displayed with exposure times up to 0.5 sec. Radiopacity of COOL formulation A and titan specimen were comparatively high, whereas radiopacity of reference material was rather low (Figure 4).

3.5. Biocompatibility of COOL

In contrast to Refobacin® R, COOL revealed high biocompatibility In Vitro independent of setting time. Figure 5 shows morphology of pHOB cell cultures from donor 1 after 2, 5 and 16 days of incubation with specimens of COOL formulation A (Figure 5d-f) or Refobacin® R (Figure 5g-i) added to the cell monolayer directly after preparation. At day 2, control cells (Figure 5a) and cells of the COOL group (Figure 5d) showed typical long-stretched fibroblast-like appearance during early proliferation. Cells incubated with reference material, however, showed no fibroblast-like appearance at all (Figure 5g) and a large part of them was lost from the surface of the culture discs. Cell density intensively increased between day 2 and day 16 of culture and the pHOB cells became a polygonal shape (Figure 5a-f). At day 16, a comparatively high cell density could be observed in cultures incubated with COOL (Figure 5f) and those of the control (Figure 5c). In cultures incubated with reference material, however, all cells died after 5 days in culture (Figure 5h-i). Determination of cell viability revealed similar results. Differences in cell proliferation rates were measured between the control and cultures incubated with COOL and Refobacin® R. Cell viability slightly decreased in all these groups until day 2 (Figure 6a). Between day 5 and day 7, cell proliferation rates rapidly increased, except for cells incubated with Refobacin® R. In this group, proliferation rapidly decreased over time, resulting in total lost of cells after 5 days in culture. In case of adding bone cement specimens after 5 hours (Figure 6b) and 24 hours (data not shown) of setting no significant differences in proliferation behavior could be determined anymore between the three different groups.

Comparable results in cell density, morphology and viability could be observed with pHOB cells derived from donor 2 (results not shown).

Figure 6. Proliferation of pHOB cells derived from donor 1 incubated with COOL formulation A or reference material in comparison to the control during the entire cultivation period of 16 days. a) Cement specimens were added to the cell monolayers directly after preparation and b) 24 hours after mixing. Results are the mean ± SD, n = 3. Significant differences between COOL and reference are marked with * (P < 0.05).

Figure 7. ALP activity in pHOB cell cultures derived from donor 1 incubated with specimens of COOL formulation A or reference material at day 16, calculated as percentage of the control. Cement specimens were added to the cell cultures directly after preparation (0 hour) and after setting for 5 or 24 hours. Results are the mean ± SD (n = 3). Significant differences between the groups are marked with * (P < 0.05) or ** (P < 0.01).
3.6. Osteoinductivity of COOL

Figure 7 shows the results of ALP activity measurements at day 16 in pHOB cell cultures of donor 1 incubated with COOL formulation A or Refobacin® R in relation to the control. Independent of setting time, ALP activity was considerably higher in cultures incubated with COOL compared to those incubated with reference material. If added to the cultures directly after preparation, pHOB cells incubated with reference material died after 5 days in culture, whereas COOL cultures survived, thereby showing a 50% higher ALP activity as cultures of the control. Compared to COOL, ALP activity was reduced by 40% in cultures incubated with Refobacin® R if added to the cell monolayers after 5 hours and by nearly 60% if added after 24 hours of setting. After 5 and 24 hours of setting, however, no significant differences in ALP activity could be determined anymore between COOL and control cultures.

ALP activity measurements with pHOB cells derived from donor 2 revealed similar results. (data not shown).

4. Discussion

This study describes material properties and In Vitro biocompatibility of the novel platform technology COOL, which is a composite of biostable as well as biodegradable ceramics and dissolved, chemically modified PMMA.

Setting of COOL takes place via a cementation reaction at room or body temperature. This reduces stress to the surrounding tissue compared to PMMA based cements where high temperature of up to 110 °C and corresponding heat necrosis were reported. Humidity or direct contact to water or other fluids do not influence the cementation reaction of COOL. Metallic ions are integrated into the modified PMMA structure due to their high complex formation capability by interactions with free electron pairs from the carboxyl group. Bending and compressive strength of COOL can be varied in a relatively broad range by adding ZnO which is interesting to achieve properties needed for different indications.

COOL did not achieve bending and compressive strength as seen for classical PMMA based cements, but it should be considered that no mechanical mixing system was applied. It should also be considered that if standard PMMA based cements were in contact with blood during polymerization, bending strength would be reduced to less than 25 MPa[31]. This raises the question if current test methods (ISO 5833) are suitable to predict mechanical strength in vivo and whether 25 instead of 50 MPa bending strength would also be sufficient for fixation of hip joint prostheses.

Compared to PMMA based cements mechanical strength of CPCs is weaker. Depending on the composition final compressive strength can vary at a large scale between 4 and 80 MPa. Moreover, CPCs possess high brittleness and low bending strength[32] which was not observed for COOL with a bending strength of up to 26.4 MPa.

Dependent on the field of application resorption of cements within different time frames and to a different extent is desirable. COOL is partially resorbable. Extent and timeframe are dependent on the ceramic components. Since the mechanical properties of different formulations are comparable, resorption time can be varied by changing proportions of fast resorbable (GB14), slow resorbable (TETRA) and biostable (CZP/FA) bioceramics[33]. In order to achieve good resorption and ingrowth of cells into bone cements, porosity and a minimum pore size are required. The porosity of COOL is between 31 and 37% which is less than the range described for CPCs[34] to 60%. Healing of large bone defects requires macro pores (> 50 µm) to enable initial ingrowth of bone cells into the artificial material followed by its resorption and substitution by newly generated bone in a moderate time interval. Classical CPCs like Norian SRS have pores with a diameter of 30 nm. COOL shows a mixture of micro and macro pores with a diameter of up to 350 µm which render the possibility of ingrowths of osteoblasts. Effects of these large pores and combination of the different bioceramics on resorption behavior of COOL have to be investigated more extensively In Vitro and in vivo.

In orthopedic bone cement application usually proceeds within a time span of up to 10 minutes after mixing. Large defects and revision of position need more time. Therefore longer setting times might be mandatory as well. In vertebroplasty, longer setting times are preferential in order to ensure optimal filling of the vertebral body. Setting times between 1 (4) and > 20 (17) minutes are reported for standard CPCs (PMMA)[35]. COOL possesses a setting time of 20 minutes which can be prolonged by changes in the composition. The latter is favorable for e.g. vertebroplasty in agreement with Chavali et al.[36] who used ice cooling to prolong the application time and to delay the polymerization reaction. In other cases, faster setting can be beneficial requiring further investigations on the composition of COOL.

Obviously during application, the cement has not yet completely hardened, which may provoke adverse biological effects associated with the reaction mechanism as found for PMMA cements[37,38]. So, for testing biocompatibility of new biomaterials to bone cells In Vitro, it is important to use a method that matches the in vivo situation as closely as possible. For that reason, in this study In Vitro biocompatibility of COOL was determined in pHOB cells at different time points after mixing. Except for some small donor-dependent variations, no cytotoxic effects on pHOB cell density, morphology and viability could be observed during incubation with COOL formulation A independent on the setting time. In accordance to Siggelkow et al.[39], pHOB cells from both donors showed a typical long-stretched fibroblast-like appearance during early proliferation, which switched to polygonal during continuing proliferation. In contrast, pHOB cells incubated with Refobacin® R added to the cultures directly after preparation showed extremely high death rates, probably due to chemical necroses caused by the release of free, not polymerized, toxic MMA monomers.

However, differences in cell density, morphology and viability between cultures incubated with COOL or Refobacin® R disappeared completely when the specimens were added 5 or 24 h after mixing, respectively. These results confirm the findings of another group, which analyzed cell proliferation of various acrylic bone cements added at different time intervals after polymerization[40,41]. They found that cytotoxic effects of these cements are inversely proportional to their polymerization time, which means that toxicity of PMMA-based cements decreases with proceeding polymerization time. In the future, adaptation of the general test system should be considered in order to resemble the in vivo situation more closely.

COOL revealed significant higher ALP activity even when viability was comparable indicating that COOL has osteoinductive properties compared to Refobacin® R.

GB14, one of the bioceramics used, conveys cells to adhere to the biomaterial based on the mechanism described by Knabe and Berger[42]. So, GB14 strongly contributes to the osteoconductive properties of COOL.

Another component of the ceramic mixture is CZP, which is biostable[43,44] and increases the radiopacity of COOL. It accounts for more than 10 wt. (%) and is well integrated into the PMMA matrix. In all In Vitro studies independent of application time, COOL reached at least cell viability and ALP activity comparable to control. CZP is generally described as biocompatible proven in In Vitro as well as in vivo studies[45]. Only recently, CZP coated implants were found to reduce bone mineralization in vivo[46]. Since a special thermal treatment (flame spray) was applied during the coating process, these findings can neither be generalized nor assigned to COOL.
ZnO is an important component of COOL, since it increases the mechanical properties. Zinc is an essential element and plays an important role in bone metabolism\(^a\). Zinc deficiency can lead to reduced levels of alkaline phosphatase (ALP) activity in bone\(^b\),\(^c\) which results in reduced bone formation. Another positive feature of zinc is that it works antiestrogenically\(^d\),\(^e\). The effects are a question of the concentration used since at higher concentrations of Zinc powder it was found to be cytotoxic somehow\(^f\),\(^g\). The amounts of ZnO used in the COOL formulations did not have any negative effect on biocompatibility as shown. Taken together with the rationale that Zn ions are integrated into the PMMA matrix no negative effects are expected for COOL, but further studies sought to be carried out.

Although COOL did not reach the bending and compressive strength for endoprosthetic applications according to ISO 5833, stability and toughness of healthy spongy and trabecular bone were achieved. Lower bending and compressive strength might even be a possibility to reduce stress between implant and bone. In case the powder-liquid ratio of PMMA based cements were changed, a reduction of strength up to 24\% would be caused without any remarkable influence on the patient outcome\(^h\). High radiopacity, good biocompatibility, compressive and bending strength of \(>20\text{ MPa}\) are warranted for vertebroplasty\(^i\),\(^j\) and can be provided by COOL.

5. Conclusion

In conclusion, COOL demonstrates good mechanical properties and high biocompatibility In Vitro. COOL presents a good balance between porosity and pore size on the one hand and mechanical properties on the other hand and reaches bending and compressive strength comparable to trabecular bone. In contrast to traditional PMMA cements, COOL exhibits less mechanical strength, but cures at body temperature. It releases components of bioceramics, exhibits macropores for ingrowth of osteoblasts and has high radiopacity. In contrast to CPCs, COOL possesses higher bending strength which improves the applicability, is pH-neutral and water stable. Overall COOL should be further investigated in different in vivo models for different applications areas such as endoprosthetics and vertebroplasty.

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