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Compressive mechanical properties and cytocompatibility of bone-compliant, linoleic acid-modified bone cement in a bovine model

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Abstract

Adjacent vertebral fractures are a common complication experienced by osteoporosis patients shortly after vertebroplasty. Whether these fractures are due to the bone cement properties, the cement filling characteristics or to the natural course of the disease is still unclear. However, some data suggests that such fractures might occur because of an imbalance in the load distribution due to a mismatch between the elastic modulus (E) of the bone–cement composite, and that of the vertebral cancellous bone. In this study, the properties of bone-compliant linoleic acid-modified bone cements were assessed using a bovine vertebroplasty model. Two groups of specimens (cement-only and bone–cement composites), and four subgroups comprising bone cements with elastic moduli in the range of 870–3500 MPa were tested to failure in uniaxial compression. In addition, monomer release as well as time and concentration-dependent cytocompatibility was assessed through the cement extracts using a Saos-2 cell model. Composites augmented with bone-compliant cements exhibited a reduction in E despite of their relatively high bone volume fraction (BVF). Moreover, a significant positive correlation between the BVF and E for the composites augmented with 870 MPa modulus cements was found. This was attributed to the increased relative contribution of the bone to the mechanical properties of the composites with a decrease in E of the bone cement. The use of linoleic acid reduced monomer conversion resulting in six times more monomer released after 24 hours. However, the cytocompatibility of the bone-compliant cements was comparable to that of the unmodified cements after the extracts were diluted four times. This study represents an important step towards introducing viable bone-compliant bone cements into vertebroplasty practice.

1. Introduction

Poly(methyl methacrylate) (PMMA)–based cements have gained and kept an important place as orthopedic biomaterials ever since their first use for prosthesis fixation in 1958 and later for vertebroplasty in the 1980's^{1,2}. Vertebroplasty refers to the percutaneous injection of bone cement into a vertebral body and is typically prescribed for patients with vertebral compression fractures³. These fractures can be caused by different pathologies such as hemangioma⁴, multiple myeloma⁵, osteolytic metastases⁶ and primary or secondary osteoporosis⁷. Acrylic bone cements have succeeded as orthopedic biomaterials since vertebroplasty in patients with vertebral compression fractures has shown good results⁸⁻¹⁰. Acrylic bone cements are non-degradable and hydrophobic making it difficult for the implant to integrate to the surrounding tissue. A fully hardened PMMA–based implant can be considered as bioinert since it does not chemically interact with the host tissue¹¹ or induces a negative host response¹². Moreover, high strength and non-degradability permit the incorporation of relatively high amounts of insoluble inorganic radiopacifiers, which is required to monitor the delivery and positioning of the implant, without significantly compromising its mechanical integrity or risking a release of the radiopacifier.

Most commercial acrylic bone cements exhibit a compressive strength and elastic modulus in the range of 85-114 MPa and 1700-3700 MPa, respectively^{13,14}. The apparent compressive strength and elastic modulus of cancellous bone, on the other hand, is typically in the ranges of 0.1-15 MPa and 10-900 MPa¹⁵⁻¹⁷, respectively, encompassing osteoporotic to healthy bone. Multiple experimental studies have shown that, upon curing, cement and bone form a bone–cement composite whose apparent mechanical properties are closer to that of the cement than to that of healthy cancellous bone¹⁸⁻²⁰. This is a consequence of the much higher cement volume fraction than bone volume fraction (0-20% for vertebral cancellous bone)^{16,21}, but the lack of bonding between bone and PMMA also results in a simple rule of mixture not being applicable for predicting the mechanical response of the composite^{20,22}. The mismatch of apparent properties between healthy cancellous bone and the bone–cement composite has raised concerns, since adjacent vertebral fractures occurring shortly after vertebroplasty have been reported²³⁻²⁵. Other causes of fractures may be related to bulging of the adjacent endplates, higher volume of injected cement, and cement leakage into the disc²⁶⁻²⁹. However it is still debated, whether adjacent vertebral fractures can simply be attributed to the progression of the underlying disease or because of an altered load pattern in the spine after the augmentation³⁰.

These concerns have stimulated material scientists to develop new bone cement formulations that more closely match the mechanical properties of cancellous bone, in an attempt to reduce the occurrence of adjacent vertebral fractures. Boger et al.^{31,32} modified the macrostructure of acrylic

bone cements by inducing macroporosity through the incorporation of a hydrogel phase into the cement; however, increased levels of particle release³³ seem to have prevented this formulation from further advance. Another approach consisted of physically modifying the cements by partially substituting the monomer with a lactam structure organic plasticizer³⁴ to reduce the elastic modulus of the cements. To the author's knowledge, no further assessment of the cytocompatibility of this formulation was reported. Recently, we introduced the use of a triglyceride oil as a method of producing compliant bone cements; however, relatively high amounts of the additive were required to produce a significant reduction in the elastic modulus, and potential interference of the additive with the polymerization resulted in a negative cytocompatibility outcome³⁵. A similar outcome was reported by Lam et al.³⁶, who modified cements intended for joint fixation with strontium-substituted hydroxyapatite-nanoparticles and linoleic acid (15 vol% of the liquid phase). In a previous study (data to be published), we evaluated different fatty acids and triglyceride oils as potential mechanical property modifiers for acrylic bone cements. We found that lower amounts of linoleic acid than those used by Lam et al. (~6 vol% of the liquid phase) could help to reduce the elastic modulus of bone cement for vertebroplasty, Osteopal®V, by 75%. Because of the relatively small amounts required to reach a Young's modulus in the range of cancellous bone and its low cytotoxicity according to our preliminary tests, linoleic acid was chosen for this study.

Small concentrations of linoleic acid (≤ 50 mM), which is naturally occurring in living organisms, might lack a cytotoxic effect on human osteoblast-like cells and have been suggested to be beneficial for bone formation³⁷. The incorporation of linoleic acid to tailor the mechanical properties, however, requires an *in vitro* cytocompatibility assessment prior to any pre-clinical studies³⁸. A consensual methodology to assess the cytocompatibility of acrylic bone cements for vertebroplasty is not established in the literature. The ISO-10993-11 standard "biological evaluation of medical devices" suggests a method to evaluate the biocompatibility of potential biomaterials through their extracts, namely, an extraction media in which the material is soaked for a certain period of time³⁹. However, this standard does not establish the time that injectable cements should be cured prior to extraction. In addition, the cytocompatibility of cement extracts is highly dependent on the concentration of residual monomer and the critical monomer concentrations are not well defined. Therefore, if not neglected, different protocols are often reported in the literature^{35,40,41}. To resemble *in vivo* conditions, regular medium changes and dilutions should be implemented in the protocols, since marrow transport would attenuate potential cytotoxic effects due to unreacted remnants.

In an attempt to assess the feasibility to use the material, we defined two aims for the present work: first, to test in uniaxial compression a vertebroplasty model consisting of bovine bone cores

augmented with bone-compliant, linoleic acid-modified bone cements to verify it will lead to a significant reduction in the bone–cement composite stiffness compared to controls; second, to assess the cytotoxicity of bone-compliant, linoleic acid-modified bone cements, as well as a time-dependent release of toxic by-products, through monomer release and an indirect *in vitro* assay based on the evaluation of the cement extracts at three different concentrations.

2. Materials and methods

2.1 Preparation of the bone cores

Eight bovine tibias were obtained from a local slaughterhouse and stored in a freezer at -20°C until specimen preparation. Bone blocks (ca. 20 mm thick) were obtained from the proximal part of the frozen tibiae, perpendicular to the anatomical axis, distal to the growth plate, using a Bizerba FK 23 Bandsaw (Bizerba GmbH & Co. KG, Balingen, Germany). Cylindrical cancellous bone specimens were cored out from the blocks at a constant speed of 490 rpm, using a diamond-coated coring tool ($\varnothing_i=10.6$ mm, 20-001-10009, STRATEC Medical, Umkirch, Germany) driven by a table top Promac 212 drilling machine. The cores were visually inspected and excluded if found to have a defect such as apparent damage, severe pore heterogeneity, main trabecular orientation deviating substantially from the specimen longitudinal axis, or presence of large voids. The bone marrow was removed by placing the cores in vials with phosphate buffered saline (PBS), inside an ultrasonic water bath for 20 minutes, and subsequently washing it with a high-pressure pulse lavage OptiLavage[®] system (Biomet Cementing Technologies AB, Malmö, Sweden). This process was carried out twice for all of the specimens and three times for specimens that exhibited incomplete marrow removal. Held in a custom-made clamping device, the specimen ends were cut plane-parallel using an IsoMet[®] 5000 precision saw (Buehler GmbH Düsseldorf, Germany) prior to μ CT-scanning. Between procedures, the specimens were wrapped into PBS-soaked tissue paper, put into plastic bags and stored in a freezer at -20°C. A total of 56 bone cores were prepared using this method, and their initial height and diameter were determined using a digital caliper (Mettler Toledo, Greifensee, Switzerland).

2.2 Material preparation

Osteopal[®]V (OP, Heraeus Medical GmbH, Hanau, Germany) radiopaque bone cement for vertebroplasty was used as the base cement. The powder phase contained 54.6 wt% poly(methyl acrylate-*co*-methyl methacrylate), 45.0 wt% zirconium dioxide, 0.4 wt% benzoyl peroxide, and chlorophyll VIII. The monomer phase contained 92 wt% methyl methacrylate, 2 wt% N,N-

dimethyl-*p*-toluidine and 6 wt% other additives including chlorophyll VIII and hydroquinone. Modified cements were prepared by dissolving the corresponding amounts of 9-*cis*,12-*cis*-linoleic acid ($\geq 99\%$, Sigma-Aldrich, St. Louis, MO, USA) in the monomer phase. The modified monomer phases were homogenized using a Vortex-Genie 2 mixer (Scientific Industries, Bohemia, NY, USA) prior to cement preparation. To maintain a constant liquid-to-powder ratio, the amount of monomer phase was adjusted to the amount of LA according to equation (1), in which L is the volume of OP liquid phase (μl), m_{LA} is the amount of linoleic acid (g), P is the amount of OP powder phase (g), and 384.6 is Osteopal[®]V's recommended liquid-to-powder ratio ($\mu\text{l/g}$).

$$L = -1108.6 \cdot m_{LA} + 384.6P \quad (1)$$

Each batch of bone cement was prepared by adding the monomer phase to the powder phase inside 50 mL Falcon tubes, and then mixed for 25 seconds with a Vortex mixer, and for 5 seconds with a metal spatula, to ensure a homogeneous mixture.

2.3 Augmentation of bone cores

The bone cores for the composite group were divided into four subgroups of 14 specimens each. Before augmentation, the bone specimens were snugly fit into cylindrical Teflon molds ($\varnothing_i = 10.70 \pm 0.05$ mm) that were previously sprayed with a liquid Teflon solution to facilitate disassembly upon cement curing. In addition, a cement-only group, divided into four subgroups of 10 specimens each, was prepared. Upon mixing the cement components, the curing mixtures were scooped out of the Falcon tube into two separate polypropylene syringes, whose nozzles were cut. The cement was injected simultaneously into two molds to minimize the influence of viscosity changes on the outcome of the augmentation. The specimens were set for approximately 1 h at room temperature prior to demolding and cutting them plane with the precision saw. Following this procedure the specimens were dried until excess water had been removed. The final height, diameter, and weight were determined using a digital caliper and a precision scale (Mettler Toledo, Greifensee, Switzerland), respectively. The specimens were individually stored in PBS at 37°C for 24 h prior to the mechanical testing.

2.4 Cross-sectional analysis

The cross-sections of the composite group were examined by scanning electron microscopy (SEM) to assess the cement microstructure and the bone–cement interface. The images were acquired on a

LEO 1550 scanning electron microscope (Carl Zeiss AG, Oberkochen, Germany) operated at an acceleration voltage of 5 kV, with a secondary electron detector for topographic contrast, at a working distance of 10 mm. The specimens were sputter-coated with palladium prior to SEM imaging.

2.5 Bone morphology analysis

To assess the morphology of the bone specimens, the cores were μ CT-scanned in PBS using a μ CT 50 Scanner (Scanco Medical AG, Bassersdorf, Switzerland) at a nominal resolution of 14.8 μ m (70 kVp, 113 mA). The bone volume fraction (BVF) for the specimens was determined based on segmentation of the μ CT image data, and the nominal volume of the specimens. The cement volume fraction (CVF) was determined from the difference in estimated weight of the specimens, before and after augmentation, divided by the measured density of the cement-only specimens. The weight of the specimens prior to augmentation was based on the BVF, assuming a bone tissue density of 2.0 g/cm³⁴². The pore volume fraction (PVF) was calculated according to equation (2).

$$PVF=1-BVF-CVF \quad (2)$$

2.6 Uniaxial compression testing

The cement-only and composite groups were tested to failure in compression under displacement control at 6 mm/min in an Instron E10000 materials testing machine (Instron, Norwood, MA, USA). Five preconditioning cycles (0.1–0.5% strain)²⁰ were applied before the specimens were loaded to failure in the sixth cycle. Failure was defined as a 100 N drop in the load as recorded by the load cell. Load and displacement data were acquired at 100 Hz. Four subgroups of cements including one unmodified (OP) and three modified with 0.5 (OP-0.50), 0.75 (OP-0.75), and 1.50 (OP-1.50) wt% LA (with respect to the total weight) were tested. Mechanical testing was carried out at 21°C.

Post-processing of the data was carried out in Matlab v.2011a (Mathworks, Inc., Natick, MA, USA). The mechanical properties were calculated from the final cycle data. The displacement data was corrected for machine compliance prior to the post-processing. The stress was defined as the load applied on the specimen divided by the nominal cross-sectional area of the augmented specimens. The strain (ϵ) was defined as the displacement, measured between the platens, divided by the initial specimen length. The elastic modulus (E) was defined as the slope of the stress–strain relationship between 0.2 and 0.4% strain. The yield stress (σ_y) and the yield strain (ϵ_y) were defined by the 0.2% offset method. The ultimate stress (σ_u) was defined as the stress at maximum load, and

strain energy (U) as the area under the stress–strain curve up to the measured ultimate strain (ε_u) for each of the specimens.

2.7 Methyl methacrylate release

Extracts were prepared from two subgroups OP and OP-1.50, which corresponded to the non-modified and the most modified material (worst case scenario). Four specimens ($\varnothing=6\text{mm}$, $h=12\text{mm}$) from each subgroup were prepared. Extractions were done in 10 mL PBS for 30 min, 1, 2, 4, 6, and 24 h, since a burst release was expected during the first few hours followed by a decrease in the amount of unreacted monomer released from the specimens. After each time point, 10 mL extract was collected and replenished with fresh 10 mL PBS. The four extracts from each time point were combined to measure the average released monomer in the 40 mL solution. Afterwards, 2 mL aliquots from each solution were introduced in a headspace vial and closed hermetically. The vials were incubated at 80°C during 30 min. The analysis was performed by headspace gas chromatography-mass spectrometry (HS-GC/MS), by injecting 1 mL of the vapor phase through a special syringe kept at 85°C. A Trace GC Gas Chromatograph with Triplus injector coupled to a DSQII mass spectrometer (ThermoFisher Scientific, Waltham, MA) was used. A ZB-624 column (60 m $\times$ 0.32 mm $\times$ 1.8 m) with a helium flow of 1.8 mL/min was used for separation. The oven temperature programme consisted of 2 min hold at 60°C, followed by 8°C/min ramp to 220°C and a 5 min hold at 220°C. The temperature of the injector, interface, and ionization source was set at 220, 260, and 200°C, respectively.

2.8 Cytotoxicity assay

For the cytocompatibility tests, extracts from subgroups OP, OP-0.75, and OP-1.50 were prepared. Human osteoblast-like Saos-2 cells (HPACC) were used as cell model. The cells were maintained in cell culture flasks in an incubator with a humidified atmosphere of 5% CO₂ in air at 37 °C. DME/F medium (Thermo Scientific HyClone, ref. n. SH300023.01, Logan, UT, USA) supplemented with 1% L-glutamine/penicillin/streptomycin (PAA Laboratories, ref. n. P11-013, Pasching, Austria) and 10% foetal bovine serum (PAA Laboratories, ref. n. A15-101, Pasching, Austria) was used as culture medium. The medium was exchanged every second day. Upon confluence, cells were detached with a minimum amount of trypsin 0.25% in EDTA (Thermo Scientific HyClone, ref. n. SH30042.02, Logan, UT, USA) that was inactivated with complete DME/F medium after 10 min. 6500 Saos-2 cells were seeded in 96-well plate (cells/surface ratio of 20000 cells/cm²) and were cultured for 24 h before starting the cytotoxicity assays.

The cytotoxicity of the bone cements was evaluated by an indirect method, using cement extracts. Bone cement specimens ($\varnothing=13$ mm, $h=2$ mm) were prepared as described in the material preparation section and set in air for 30 min. The specimens were subsequently unmolded and immersed in phosphate buffered saline (PBS) for 24 h at 37°C. Cement extracts were then prepared by soaking specimens with cell medium at a surface-to-volume ratio of 3 cm²/ml, as indicated in ISO-10993-11 standard ³⁹. To investigate a time-dependent release of toxic by-products, the individual media were withdrawn after 1, 6, 12, and 24 h and replaced by fresh medium. Recent studies assessing the cytotoxicity of PMMA-based materials reported the use of 6.25-100% extracts ^{43,44}. In this study, the extracts were sterilized by filtration using a 0.2 μ m pore membrane, and were tested undiluted (100% extract), diluted 4-fold (25% extract) and diluted 10-fold (10% extract). Fresh medium was used as a control and wells without cells were used as blank. Four measurements were made per sample. The cells were incubated with extracts for 24 and 72 h. At each time point, cell viability was quantified using the AlamarBlue[®] assay. Cells were washed with PBS and then incubated in AlamarBlue[®] (Invitrogen, ref. n. DAL1100, Carlsbad, CA, USA) diluted 20-fold with MEM media (Life Technologies, Gibco, ref. n. 51200, Carlsbad, CA, USA). After 1 h of incubation in the dark, fluorescence was monitored on a microplate reader (Tecan, Männedorf, Switzerland) at 560 nm excitation and 590 nm emission. The results were converted to cell numbers using a calibration curve.

2.9 Statistical analysis

Statistical analysis was done in IBM SPSS Statistics 19 (IBM, Chicago, IL, USA) using one-way ANOVA at a significance level of $\alpha=0.05$. Tamhane's post hoc test was used as equal variances could not be confirmed for all groups. Additionally, any significant correlation between BVF and the mechanical properties was evaluated using a significance test of the Pearson correlation coefficient.

3. Results

3.1 Cross-sectional analysis

The cross-sections of the augmented bone cores were analysed by SEM (Figure 1). Only OP and OP-1.50 are shown for comparison. Despite the differences in mechanical properties, as will be shown later, the cements modified with linoleic acid exhibited a similar microstructure to that of OP. The cements appeared to be fully cured and limited bonding between the cancellous bone and the cement was observed.

Figure 1 Representative scanning electron micrographs of bone-cement composite cross-sections for A) OP and B) OP-1.50 subgroups prior to mechanical testing. Higher magnification micrographs are shown for C) OP and D) OP-1.50 with arrows indicating the lack of bonding between the bone and the cement. 1: bone cement, and 2: bony tissue.

3.2 Bone morphology analysis

The results of the morphological analysis are listed in Table 1. No statistically significant differences in BVF were found between the subgroups (OP-0.50, OP-0.75, OP-1.5) and their respective control subgroup OP. A statistically significant difference in BVF ($p=0.01$) was found only between the subgroups OP-0.75 and OP-1.50. No statistically significant differences in PVF were found between subgroups. No statistically significant differences in CVF were found between the subgroups (OP-0.50, OP-0.75, OP-1.5), and their respective control subgroup OP. A significant difference in CVF ($p=0.01$) was found only between the subgroups OP-0.75 and OP-1.50.

3.3 Uniaxial compression testing

Results corresponding to incompletely augmented specimens ($PVF \geq 10\%$) were excluded from post-processing of the data, since these most typically resulted in a mechanical response that severely deviated from that of other specimens in the same subgroup. The results from the uniaxial compression testing and the morphological analysis are summarized in Table 1. For the composite group, significant differences ($p < 0.05$) were found for all the mechanical properties (except ϵ_y)

Table 1 Summary of experimental compressive properties and morphological parameters. $\varnothing$: diameter; H: height; G: weight; BVF: bone volume fraction; PVF: pore volume fraction; CVF: cement volume fraction; E : elastic modulus; ε_y : yield strain; σ_y : yield stress; ε_u : ultimate strain; σ_u : ultimate stress; U : strain energy. Results are given as mean $\pm$ standard deviation, with the range given in the parentheses. Number of replicates (n) shown for each group. (*) : significantly different ($p < 0.05$) from OP (cement-only group). (**): significantly different ($p < 0.05$) from OP (composite group). (§): significant positive correlation ($p < 0.05$) with BVF.

	Cement-only group					Composite group				
	OP (n = 10)	OP-0.50 (n = 10)	OP-0.75 (n = 10)	OP-1.50 (n = 10)	OP (n = 11)	OP-0.50 (n = 11)	OP-0.75 (n = 10)	OP-1.50 (n = 13)		
$\varnothing$ [mm]	10.58 $\pm$ 0.09 (10.44 – 10.67)	10.52 $\pm$ 0.07 (10.44 – 10.66)	10.57 $\pm$ 0.09 (10.46 – 10.77)	10.44 $\pm$ 0.03 (10.40 – 10.50)	10.70 $\pm$ 0.03 (10.65 – 10.74)	10.68 $\pm$ 0.05 (10.58 – 10.75)	10.63 $\pm$ 0.02 (10.59 – 10.66)	10.68 $\pm$ 0.04 (10.56 – 10.72)		
H [mm]	19.68 $\pm$ 1.04 (17.44 – 20.73)	19.79 $\pm$ 1.68 (17.70 – 22.67)	19.58 $\pm$ 1.10 (18.31 – 21.37)	19.66 $\pm$ 0.48 (18.76 – 20.38)	18.67 $\pm$ 1.18 (17.02 – 20.19)	18.81 $\pm$ 0.78 (17.66 – 19.70)	18.39 $\pm$ 0.95 (17.24 – 19.59)	19.64 $\pm$ 0.69 (18.48 – 20.56)		
G [g]	2.71 $\pm$ 0.13 (2.42 – 2.85)	2.70 $\pm$ 0.21 (2.44 – 3.10)	2.67 $\pm$ 0.15 (2.53 – 2.91)	2.64 $\pm$ 0.07 (2.54 – 2.74)	2.60 $\pm$ 0.22 (2.30 – 2.90)	2.60 $\pm$ 0.14 (2.37 – 2.81)	2.57 $\pm$ 0.13 (2.35 – 2.73)	2.77 $\pm$ 0.12 (2.59 – 2.96)		
BVF [%]	-	-	-	-	24.2 $\pm$ 6.1 (13.8 – 36.2)	21.9 $\pm$ 5.5 (11.5 – 28.9)	26.3 $\pm$ 3.5 (19.5 – 31.7)	19.9 $\pm$ 4.4 (12.6 – 29.1)		
PVF [%]	-	-	-	-	6.1 $\pm$ 2.7 (2.5 – 9.8)	5.1 $\pm$ 2.1 (2.5 – 9.7)	3.5 $\pm$ 2.1 (0.9 – 7.8)	3.6 $\pm$ 0.7 (2.2 – 4.7)		
CVF [%]	-	-	-	-	69.6 $\pm$ 7.7 (58.4 – 83.7)	73.0 $\pm$ 6.7 (64.6 – 84.6)	70.2 $\pm$ 3.8 (62.5 – 75.8)	76.5 $\pm$ 4.8 (66.4 – 84.9)		
E [MPa]	3571 $\pm$ 176 (3355 – 3833)	2703 $\pm$ 230 ^(*) (2341 – 3165)	1878 $\pm$ 290 ^(*) (1232 – 2150)	872 $\pm$ 92 ^(*) (689 – 981)	3246 $\pm$ 729 (2422 – 4942)	2834 $\pm$ 518 (1799 – 3622)	2597 $\pm$ 491 ^(§) (1969 – 3346)	2274 $\pm$ 455 ^(**§) (1401 – 2869)		
ε_y [%]	2.03 $\pm$ 0.10 (1.86–2.20)	1.95 $\pm$ 0.13 (1.75 – 2.13)	1.82 $\pm$ 0.15 ^(*) (1.59–2.09)	1.73 $\pm$ 0.16 ^(*) (1.50–2.12)	1.43 $\pm$ 0.15 ^(§) (1.31 – 1.80)	1.49 $\pm$ 0.20 (1.25 – 1.85)	1.45 $\pm$ 0.22 (1.13 – 1.77)	1.34 $\pm$ 0.07 (1.23 – 1.52)		
σ_y [MPa]	65.2 $\pm$ 3.3 (58.6 – 69.2)	47.3 $\pm$ 3.4 ^(*) (41.8 – 51.8)	30.4 $\pm$ 4.9 ^(*) (21.3 – 38.7)	13.2 $\pm$ 0.8 ^(*) (11.7 – 14.6)	39.6 $\pm$ 8.6 (29.7 – 55.0)	35.8 $\pm$ 4.7 (27.2 – 41.6)	31.8 $\pm$ 3.7 (25.0 – 37.3)	25.7 $\pm$ 4.7 ^(**§) (17.7 – 32.0)		
ε_u [%]	5.40 $\pm$ 0.31 (4.88 – 5.86)	4.71 $\pm$ 0.47 ^(*) (3.99 – 5.24)	3.57 $\pm$ 0.35 ^(*) (3.23 – 4.37)	3.10 $\pm$ 0.09 ^(*) (2.93 – 3.18)	4.65 $\pm$ 0.60 (3.82 – 5.59)	4.60 $\pm$ 0.48 (3.88 – 5.25)	4.02 $\pm$ 0.73 (2.88 – 5.05)	3.24 $\pm$ 0.17 ^(**) (3.01 – 3.70)		
σ_u [MPa]	99.3 $\pm$ 6.4 (87.0 – 107.6)	70.3 $\pm$ 8.2 ^(*) (56.5 – 82.5)	40.4 $\pm$ 7.1 ^(*) (26.8 – 52.7)	17.1 $\pm$ 1.1 ^(*) (15.5 – 18.7)	63.4 $\pm$ 12.2 (46.4 – 81.4)	56.8 $\pm$ 8.8 (37.2 – 66.6)	47.5 $\pm$ 5.2 ^(**) (38.2 – 58.0)	35.1 $\pm$ 5.4 ^(**§) (25.9 – 42.8)		
$U \cdot 10^{-3}$ [J/mm ³]	4.08 $\pm$ 0.55 (3.19 – 4.96)	2.47 $\pm$ 0.57 ^(*) (1.59 – 3.20)	1.07 $\pm$ 0.27 ^(*) (0.64 – 1.60)	0.38 $\pm$ 0.03 ^(*) (0.33 – 0.41)	2.32 $\pm$ 0.53 (1.53 – 3.07)	2.04 $\pm$ 0.42 (1.10 – 2.48)	1.66 $\pm$ 0.39 ^(**) (1.10 – 2.18)	0.90 $\pm$ 0.17 ^(**§) (0.65 – 1.21)		

between the subgroup augmented with OP and the subgroup augmented with OP-1.50 (Table 1). In addition, significant differences ($p<0.05$) were found for σ_u and U between the subgroup augmented with OP and the subgroup augmented with OP-0.75 (Table 1). For the cement-only group, significant differences ($p<0.05$) were found for all the mechanical properties (except ε_y for OP-0.50) between the subgroup augmented with OP-0.50, OP-0.75, and OP-1.50 and the control subgroup augmented with OP (Table 1). A significant positive correlation ($p<0.05$) was found between BVF and the following properties: E , σ_y , σ_u , and U for the subgroup augmented with OP-1.50 (Table 1). An additional positive correlation was found between BVF and E for subgroup augmented with OP-0.75 (Table 1). Typical stress-strain curves for cement-only and composite specimens augmented with OP, OP-0.75, and OP-1.50 are shown in Figure 2.

Figure 2 Representative stress-strain curves for A) cement-only and B) composite group. The embedded pictures (not to scale) illustrate one specimen prior to testing as well as the typical failure modes for the different groups and subgroups after testing.

The E and σ_y decreased as the concentration of linoleic acid increased up to 1.50 wt%. For the cement-only group, E and σ_u decreased by 76% and 83%, respectively. For the composite group, E and σ_y decreased by 33% and 47%, respectively. The cement-only group exhibited compositional-dependent differences in the failure mode. The OP specimens plastically deformed past yield exhibiting a characteristic bulging whereas the OP-1.50 specimens did not bulge and exhibited partial elastic recovery upon removal of the load. The composite specimens, however, generally failed in a more brittle manner apart from some of the specimens with the lowest BVF, which exhibited considerable load bearing capacity beyond the ultimate load, similarly to the cement-only group. Figure 3 shows the linear regression for the most important properties, elastic modulus and ultimate compressive strength. The variation of the mechanical properties for both cement-only and composite groups was also composition-dependent and

showed a linear correlation with the concentration of linoleic acid.

Figure 3 Mechanical properties A) Elastic modulus; B) Ultimate stress and their linear correlation with the concentration of linoleic acid. The grey area represents the range of values reported in the literature for human cancellous bone.

3.4 Methyl methacrylate release

Methyl methacrylate release from OP and OP-1.50 was evaluated using HS-GC/MS. Figure 4 shows the cumulative release of MMA over a period of 24 h. The analyses revealed that OP-1.50 released 6 times more monomer than OP after 24 h. In addition, 71% and 82% of the total accumulated MMA was released over the first 6 h for OP and OP-1.50, respectively.

3.5 Cytotoxicity assay

The influence that the cement formulation and the extraction time had on cell viability for both undiluted (Figure 5A), 4-fold diluted (Figure 5B) and 10-fold diluted extracts (Figure 5C) was evaluated. Special attention was given to compare the cell number between each extract and fresh medium (control). Regarding the cells incubated in undiluted extracts for 1 day (Figure 5A), OP-1.50 significantly decreased the cell number in comparison to the OP group for each extraction time. The extraction time did not significantly affect the cell number for the OP group and only the 24 h extracts significantly reduced the cell viability for OP-0.75. For OP-1.50, the 1 h extracts reduced the cell viability significantly less than any of the other extraction times. When comparing the number of cells in fresh medium (control) to that of extracts, none of the extracts from OP significantly reduced the cell number and only the 24 h extracts from OP-0.75 caused a significant reduction; however, all extracts from OP-1.50 gave a significant decrease in cell number compared to fresh medium. Similar trends were observed after incubating the cells in undiluted extracts for 3 days. The lower the elastic modulus of the cement the lower the

number of viable cells. Moreover, the cell number was significantly reduced when incubated in the 24 h extracts from OP and OP-0.75 than the shorter extraction times; and the 1 h extracts from OP-1.50 reduced significantly less the cell number compared to the longer extraction times. Finally, when comparing the cell number in fresh medium to that of the extracts, the 24 h extracts from all the cement formulations and all the extracts from OP-1.50 reduced cell viability.

Figure 4 Cumulative concentration of methyl methacrylate over time during the initial 24 hours.

Regarding the cells incubated in 4-fold diluted extracts for 1 or 3 days (Figure 5B), the cell number was not significantly modified by using different cement types or extraction times. Interestingly, none of the extracts decreased significantly the cell number in comparison with the fresh medium. Similar results were obtained when the cells were incubated with 10-fold diluted extracts, in which cell viability was not significantly different between the fresh medium and any of the extracts (Figure 5C).

4. Discussion

In this study linoleic acid was used to produce an acrylic bone cement whose mechanical properties better matched the apparent mechanical properties of healthy cancellous bone. Linoleic acid can act as a plasticizer³⁶ or chemically modify the polymer⁴⁵. Chemical modification might occur due to methacrylation of the linoleic acid via chain transfer mechanism during polymerization⁴⁵.

Figure 5 Viability of Saos-2 cultivated in 1, 6, 12, and 24 h extracts from OP, OP-0.75, and OP-1.50 for 1 and 3 days. A) Undiluted extracts (100% extract); B) 4-fold diluted extracts (25% extract); C) 10-fold diluted extracts (10% extract). Letters indicate differences between cement formulations at each preparation extract time, numbers identify differences between preparation extract times within the same cement formulation and symbol (§) indicates differences between samples and control ($p < 0.05$).

Previous studies assessing a similar type of reaction⁴⁵ have indicated that, due to the presence of the methyl group adjacent to the vinyl bond, the methyl methacrylate (MMA) radicals are likely

to graft via abstraction of the alpha-hydrogen in the linoleic acid. Thereby, linoleic acid presumably reduces the modulus of the cement by grafting and formation of stable radicals that retard the polymerization and diminish the molecular weight ⁴⁵. In both cases, the presence of these large molecules will increase the free volume and facilitate the relative movement of the PMMA chains, thereby decreasing the glass transition temperature, and lowering the elastic modulus of the material. Moreover, the increased presence of unreacted MMA may have contributed to the lower modulus of the modified material since the monomer itself may act as a plasticizer ⁴⁶. Hence, we found that by modifying Osteopal[®]V with linoleic acid, there was a significant composition-dependent reduction of the cement elastic modulus with respect to the unmodified cement. The OP-1.50 specimens had elastic moduli (E = 689 - 981 MPa) and ultimate strengths (σ_u = 15.5 - 18.7 MPa) in the range of healthy cancellous bone (E =10-900 MPa; σ_u =0.1-15 MPa for osteoporotic to healthy bone) ¹⁵⁻¹⁷.

The first aim of the study was to test in uniaxial compression a vertebroplasty model consisting of bovine bone cores augmented with linoleic acid–modified bone cements, to verify that the modified cements would lead to a significant reduction of the composites' elastic moduli compared to the unmodified ones. By augmenting bovine bone cores with linoleic acid–modified cements, we were able to produce composite specimens with an average stiffness decrease of 30% for composites augmented with OP-1.50, compared to composites augmented with OP. The idea of producing bone–compliant cements for vertebroplasty is based on the assumption that, ideally, the elastic modulus of the resulting bone–cement composite after augmentation should match the apparent elastic modulus of healthy bone. The higher end of the elastic modulus data reported in the literature is e.g. 700-900 MPa ¹⁶. In highly osteoporotic regions, almost no cancellous bone can be found and, upon augmentation, the properties as a composite of these regions should theoretically be similar to those of the cement-only. Ideally the strength of the cement should not be inferior to that of cancellous bone (0.1-15 MPa). This condition is fulfilled even by the lowest modulus cement (OP-1.50), which had an average ultimate strength of 17.1 ± 1.1 MPa, in agreement with the higher end of values reported in the literature ¹⁵. The strain at ultimate stress for the OP-1.50 formulation was also within the range of agreement with the literature ⁴⁷. Moreover, compression testing of the OP-1.50 specimens past 5% strain, indicated that these specimens would stay intact even after extreme deformation i.e. engineering strains of up to 40-50% (data available but not shown); this is almost one order of magnitude larger than the fracture strains typically reported for cancellous bone under compression ⁴⁷. This is a unique and potentially promising characteristic. In addition, the elastic modulus is also temperature-dependent and can be overestimated by as much as ~10% ¹⁹ when tested at room temperature.

Assuming that such a temperature dependency applies for OP-1.50 would put the elastic modulus within the target range (10-900 MPa).

It is worth noting that, in the current study, relatively small amounts of linoleic acid (1.5 wt% $\approx$ 5.9 vol%) were required to lower E and σ_y by 74% and 83%, respectively. This is a lower amount compared to another study³⁶ that reported a 56% reduction in E , after modification with 15 vol% linoleic acid in combination with 20 wt% Sr-substituted hydroxyapatite particles. Additional studies, required even more drastic modifications such as up to 12 wt% castor oil to reduce E and σ_y by 70% and 83%, respectively³⁵; up to 50% monomer replacement with hyaluronic acid-based hydrogel to reduce E and σ_u by 96-97% and 95-99%, respectively^{31,32}; and up to 60% monomer replacement with N-methyl-2-pyrrolidone to reduce E and σ_y by 97% and 98%, respectively. However, it should be mentioned that it is not always feasible to directly compare the mechanical properties between different studies since these values depend, to some extent, on experimental parameters such as the properties of the base material, the curing conditions, specimen size, mechanical testing protocol, and the protocols used to calculate the properties from the raw data.

The elastic modulus of the bone–cement composite groups was found to be, on average, higher than what previously we¹⁷ and Fields et al.²¹ reported, based on apparent density, for healthy human cancellous bone from the vertebrae. This was attributed to the following: firstly, bovine cancellous bone have a higher average BVF than human vertebrae²¹, whose influence will be discussed later; secondly, the cements were injected under idealized controlled conditions, after marrow removal and discarding of high PVF specimens, which might systematically bias the data towards lower average PVF and thus higher stiffness than expected in an *in vivo* situation.

When comparing our results for the bone–cement composites with other studies^{18-20,48,49} it is worth mentioning that most studies, except that by Williams and Johnson¹⁸ who also tested bovine bone cores with a similar BVF, have not found the elastic modulus to correlate with BVF. Interestingly we found a positive correlation between BVF and the mechanical properties (except ε_y and ε_u) only for the composite subgroup augmented with OP-1.50, and between the elastic modulus and the BVF for the composite subgroup augmented with OP-0.75 (Table 1). This could be attributed to the increased relative contribution of the bone to the mechanical properties of the composites when decreasing the elastic modulus of the bone cement as is clearly seen in Figure 3.

To the authors' knowledge, only Boger et al.⁴⁸ have reported on the properties of composites consisting of bone augmented with bone-compliant bone cement. By modifying Vertecem bone cement with 35% of a hyaluronic acid-based hydrogel, they produced composites with elastic moduli down to 620 MPa, from an initial cement-only elastic modulus of 480 MPa; in the

current study, the OP-1.50 group produced composite specimens with elastic modulus down to 2274 MPa, from an initial cement-only elastic modulus of 872 MPa (Table 1). The lower elastic modulus found by Boger et al.⁴⁸ can be attributed to the lower BVF and elastic modulus of human cancellous bone, as well as the high macroporosity of their hydrogel-modified bone cements. Using the modulus-apparent density relationship (Equation 3) introduced by Morgan et al.¹⁶ and assuming a dense bone density of 2.0 g/cm³⁴², the average estimated elastic modulus of the bovine bone cores in the OP-1.50 composite group would be 1123 MPa, meaning that according to this estimate the bone contribution explains nearly 49% of the average measured stiffness.

$$E=4730 \cdot \rho_{app}^{1.56} \quad (3)$$

In addition, our data was corrected against the compliance of the materials testing machine. Furthermore, considering that the elastic modulus of acrylic bone cements is lower at body temperature than at room temperature, we believe that, in a clinical setting, the application of OP-1.50 into osteoporotic vertebra would result in a bone–cement composite with an elastic modulus more closely matching the apparent elastic modulus of healthy cancellous bone.

The second aim of this study was to assess the cytotoxicity of the acrylic cements through the amount of monomer released and an indirect *in vitro* assay based on the evaluation of the cement extracts. The material with the lowest modulus OP-1.50 released 6 times more MMA than the unmodified material OP; however, after an initial burst that occurred during the first 6 hours, the release of MMA from OP-1.50 leveled out with the release from OP. Also, clear trends were observed regarding the effect of the material as well as the extraction times when osteoblastic-like Saos-2 cells were incubated in undiluted extracts (Figure 5A). On one hand, the lower the modulus of the material the lower the number of viable cells, correlating with an increased monomer release from the OP-1.50, which gave a significant drop in the cell number compared to the OP. On the other hand, the extracts that were in contact with the cements for longer times were more cytotoxic, indicating a higher concentration of cytotoxic components. Cells were able to grow with time when incubated with all extracts from OP, and extracts from OP-0.75 (except the 24 h extracts), which is an indication of the innocuousness of these extracts. However, cells were not able to proliferate with time when cultivated in extracts from OP-1.50, which indicates a cytotoxic effect under undiluted conditions.

Even though MMA is the less cytotoxic among the most common acrylates and methacrylates⁵⁰ it has been described as genotoxic⁵¹ and is an important unreacted constituent since is present in significantly higher concentrations than any other liquid component in bone cements. In spite of

this, the permitted amount of monomer released from acrylic implants has not yet been defined. Different brands of commercial bone cements release different amounts of unreacted monomer, which may vary from 70 to 300 $\mu\text{g/g}$ during the first 24 h⁵². Therefore, the amount of monomer released from linoleic acid-modified cements will depend upon the base formulation and may be further adjusted by decreasing the liquid-to-powder ratio. However, attempting to reduce the elastic moduli of commercial bone cements using unsaturated fatty acids is likely to result in lower monomer conversion and reduced reaction rates⁴⁵. Besides, cements with a lower elastic modulus are likely to exhibit a lower glass transition temperature, which can result in earlier vitrification⁴⁶. In fact, there will always be unreacted monomer since monomer conversion is limited by vitrification⁴⁶. Therefore, low-modulus formulations produced by this method need to be optimized in order to minimize the presence of unreacted monomer. Unreacted monomer can hardly diffuse through a glass⁴⁶, and any cytotoxic effect may be assumed to be due to unreacted monomer remaining close the surface of the implant. Also, it can be hypothesized that such unreacted monomer does not accumulate around the implant but is diluted away by the bone marrow that flows through the porous structure of cancellous bone within the vertebra^{53,54}. This partly explains the success of acrylic cements despite of the intrinsic presence of unreacted monomer. Flow occurs because of pressure differences due to physiological processes⁵⁵ and depends on several factors such as the marrow rheological properties, the permeability of the bone structure, and bone strain. To account for the transport phenomena of marrow within the vertebra, the extracts were diluted 4-fold and 10-fold. Other studies report dilutions above 16-fold^{41,43}. The lower dilution (4-fold, Figure 5B) already showed to be enough to reduce the cytotoxicity of every extract to levels similar to those given by the control, regardless of the composition or the extract time. As expected, a further dilution of the extracts (10-fold, Figure 5C) gave the same results, with no statistically significant differences in the cell number between the control and the extract samples.

5. Conclusion

Modifying a commercial bone cement for vertebroplasty (Osteopal[®]V) with small amounts of linoleic acid (≤ 1.5 wt%) is an effective way to produce bone-compliant acrylic bone cements that result in elastic moduli matching those of healthy cancellous bone. Augmenting bovine bone with bone-compliant linoleic acid-modified cements, under controlled laboratory conditions, produced composites with a significantly lower elastic modulus compared to composites augmented with the base cement. A positive correlation between the bone volume fraction and the mechanical properties of the bone-cement was found, especially for composites augmented with bone-compliant cements. Finally, it was found that the bone-compliant cement containing

1.5 wt% linoleic acid released 6 times more unreacted methyl methacrylate monomer than the unmodified cement, and that 82% percent of the total monomer released during the initial 24 h burst out during the first 6 h. However, the cytocompatibility of the bone-compliant linoleic acid-modified cements was comparable to that of the base cement already for 4-fold diluted extracts. We believe that upon further adjustment of the formulation in order to minimize the presence of unreacted monomer, the present approach is a significant step towards introducing viable bone-compliant bone cements into the clinical practice.

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