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Hard Tissue Augmentation of Aged Bone by Means of a Tin-Free PLLA-PCL Co-Polymer Exhibiting in vivo Anergy and Long-Term Structural Stability

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Keywords
Regeneration · Mesenchymal stem cells · 3D culture · Hard tissue

Abstract
Background: Due to aging, tissue regeneration gradually declines. Contemporary strategies to promote tissue-specific regeneration, in particular in elderly patients, often include synthetic material apt for implantation primarily aiming at upholding body functions and regaining appropriate anatomical and functional integrity. ***Objective:*** Biomaterials suitable for complex reconstruction surgical procedures have to exert high physicochemical stability and biocompatibility. ***Method:*** A polymer made of poly-L-lactic acid and poly-ε-caprolactone was synthesized by means of a novel tin-free catalytic process. The material was tested in a bioreactor-assisted perfusion culture and implanted in a sheep model for lateral augmentation of the mandible. Histological and volumetric evaluation was performed 3 and 6 months post-implantation. ***Results:*** After synthesis the material could be further refined by cryogrinding and sintering, thus yielding differently porous scaffolds that exhibited a firm and stable appearance. In perfusion culture, no disintegration was observed for extended periods of up to 7 weeks, while mesenchymal stromal cells readily attached to the material, steadily proliferated, and deposited extracellular calcium. The material was tested in vivo together with autologous bone marrow-derived stromal cells. Up to 6 months post-implantation, the material hardly changed in shape with composition also refraining from foreign body reactions. ***Conclusion:*** Given the long-term shape stability in vivo, featuring imperceptible degradation and little scarring as well as exerting good compatibility to cells and surrounding tissues, this novel biomaterial is suitable as a space filler in large anatomical defects.

Introduction

Many tissues suffer from age-related decline in regenerative capacity [1]. Various strategies have been conceived and propagated to promote tissue-specific regeneration with much emphasis given to adult and aging patients. Synthetic material implantation is but one strategy which is implemented to uphold body functions and regain appropriate anatomical integrity also in older age. Biomaterials suitable for complex reconstruction surgical procedures need to show high physicochemical stability

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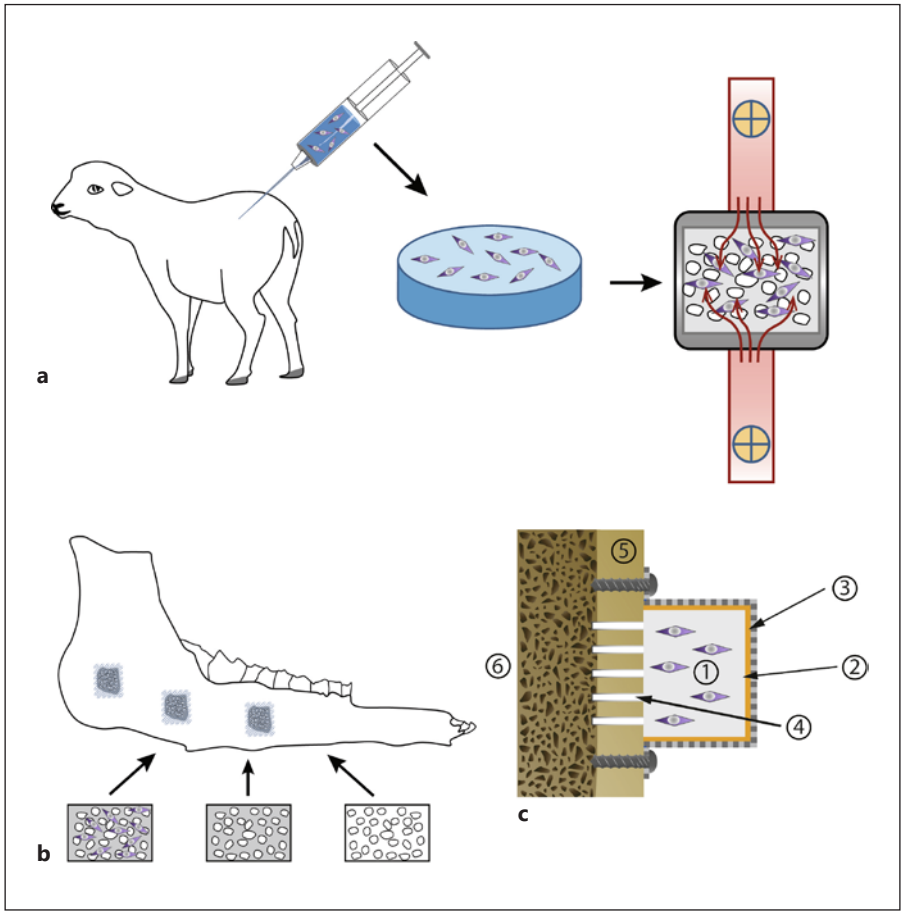

Fig. 1. Experimental Approach and Surgical Technique: PbP was tested for mandibular augmentation in sheep (n = 11) in comparison to TCP ceramic. **a** Stromal cells were harvested from bone marrow by aspiration and tested in 2D and 3D culture. **b** PbP was implanted either acellularly or seeded with cells using a 3D bioreactor method (PbP +/– cells in grey and b-TCP in white). **c** Schematic drawing of the augmentation at the outer cortex of the mandible: (1) scaffold containing cells was covered with a PTFE membrane (2) and fixed and stabilized via a titanium cage (3) on the cortical mandible (5). Drill holes (4) into the cortical bone (6) were performed to enhance nutrient supply.

and biocompatibility. Reconstruction and recontouring after complicated osseous injuries, in particular in cases of facial or cranial deformation after falls, or tumor resection are challenging endeavors in regenerative medicine. These procedures require highly evolved surgical skills, which to a great extent also involve advanced technology. Increasingly often these days is the use of biomaterials considered pivotal for satisfactory restoration. This is pertinently important, for above all humans regardless of age identify themselves with their individual facial characteristics [2]. It is also related to daily pain, physical symptoms, and self-efficacy, with activity overall associated with physical and psychological functioning [3]. Almost always, be it due to disease, trauma, or owing to unsuccessful repair attempts, clearly apparent changes induce significant psychological distress [4–6].

Reconstructive strategies not only stipulate careful selection and planning of surgical procedures and techniques, but also frequently make use of implants or include the incorporation of biomaterials, for instance in the oral cavity [7, 8]. High demands are material geometrical shape, primarily because filling the space by replacing lost tissues within the patient's body optimally is key. In this particular context, long-term in vivo stability and functional integrity are valued material characteristics and thus widely investigated [9, 10].

To date, beta-tricalcium phosphate (TCP) ceramics are routinely applied in trauma surgery. This type of material is osteoinductive, promotes bone remodeling, refrains from inducing rejection as a side reaction, and exhibits relatively fast degradation in situ [11]. However, carving TCP at the operational site in order to yield distinctly shaped scaffolds for contour reconstruction is still below state of the art [12]. Seeking better alternatives, we chose to employ poly-L-lactic acid (PLLA) and poly-ε-caprolactone (PCL), abbreviated in this contribution to PLLA-b-PCL polymer (PbP), particularly due to its known biocompatibility [13, 14]). The PbP which was used in this study (Fig. 1) was designed and manufactured by PPPolymer AB, Vällingby, Sweden (tradename PP

Sorb™), in particular exploring its potential application for cell cultivation in 3D and facial reconstruction after trauma or tumor resection.

Materials and Methods

Production of PbP

Scaffold synthesis was undertaken using the precursors PLLA and PCL, employing the ring-opening polymerization procedure to obtain homopolymers of PLLA and PCL with controlled molecular weight. Notably in this context, ring-opening polymerization was carried out by means of a tin-free catalyst, in order to provide material that is nontoxic to cells. The materials used in these studies were obtained by blending homopolymers of PLLA and PCL of well-controlled molecular sizes. The molecular weight of the homopolymers used in this study were approximately 22 kg/mol for PLLA and 34 kg/mol for PCL. The resulting polymer blend was 75 weight% PLLA and 25 weight% PCL. The PbP blend was further processed by cryogrinding and sintering, yielding compact material with differing porosity regarding pore morphology and pore connectivity (tested here: PbP 125: 41–42% porosity, PbP 126: 45–48%, PbP 140: 34%). Polymers were characterized with respect to molecular size and chemical composition. Resorbability was evaluated according to the standard ISO13781 and in vitro cytotoxicity tests according to ISO10993-5 standard. The scaffold remained stable upon gamma sterilization. For research purposes, PbP can be obtained from PPPolymer, Vällingby, Sweden (contact Swaraj Paul, pppolymer.se)

MSC Isolation and Cultivation

Human bone marrow-derived mesenchymal stem cells (MSC) were isolated, characterized, differentiated, and expanded as described previously [15]. The procedure yielded also good results when applied to isolate sheep cells. Briefly, ovine MSCs were isolated from harvested bone marrow aspirate. Individual MSC lines were established from all sheep that were included in the animal trial. The aspirate was pushed through a cell strainer and subsequently diluted 1:1–1:4 in sterile Dulbecco phosphate-buffered saline (PBS). Ficoll-Paque™ PLUS was prepared and the aspirate layered on top with a volume ratio of 1:1. All other steps were performed closely following the published standard protocol used to culture expand human MSC (Klepsch et al. [15]). Briefly, isolated cells were seeded at a density of 200,000/cm^2 in minimal essential medium containing GlutaMAX™-I (Gibco #41090) supplemented with 20% fetal calf serum (Invitrogen #10499044), 100 units mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin (Difco #5854-59) at 37 °C. While ovine cells were cultivated at 20% O_2, 5% CO_2, human cells were expanded at 3% O_2 (Thermo 3131). The medium was chanced every 2–3 days and cells were split at 70–80% confluence using 0.5% trypsin/1 mM EDTA (Gibco #25200-056). Cells were harvested not later than passage 2 and stored for subsequent use and analysis in aliquots of 0.5–1 × 10^6 cells, first cooled down using a freezing container (NALGENE #5100, –1 °C/min) at –80 °C for 24 h, and subsequently transferred into a liquid N_2 tank for long-term storage. Prior to applying the explanted cells in animal experiments the primary ovine bone marrow-derived MSC were evaluated in culture. The cells revealed a spindle-like morphology with long processes and showed indistinguishable cell proliferation and colony formation when cultivated at 3% or 20% O_2, yet exhibited slower rates when compared to human bone marrow-derived MSC [15, 16]. Colony formation was determined by seeding cells in 58 cm^2 cell culture dishes at a density of 10 cells/cm^2. For evaluation, cells were washed with PBS twice and fixed with 4% paraformaldehyde, and thereafter rinsed with PBS and stained with 2 mL 2% crystal violet in double distilled water per dish for 20 min on a shaking platform. Staining solution was recovered, and the dishes were carefully rinsed with tap water and imaged. Due to the lack of species-specific antibodies the cells could only be evaluated for a small set of surface markers. They were positive for CD44 and found negative for CD45, CD11b, CD31, CD14, and CD105, and exhibited tri-lineage differentiation potential. Assays revealing cellular differentiation were performed according to Rentsch et al. [16]. Osteogenic differentiation was induced by adding 0.5 μM L-ascorbic acid phosphate (Fluka #49752), 1 mM β-glycerol phosphate (Fluka #50020), and 100 nM dexamethasone (Sigma #D4902) to standard growth medium.

3D Bioreactor

PbP scaffolds (8 × 4 mm) were placed into a U-CUP™ bioreactor (Cellec Biotek AG, Basel, Switzerland) and incubated at 37 °C, 5% CO_2, and 3 or 20% O_2. Valve inlets were wiped with 70% ethanol, and 10 mL growth medium were injected with a syringe equipped with a soft plastic injector into the lower U-CUP™ valve to allow wetting of the scaffold material and for replacement of air. After equalizing medium levels in the front and rear tubes, 0.5 × 10^6 ovine MSC or human MSC in 2 mL growth medium were injected into the upper valve. A 5-mL syringe was attached to the front tube to de-gas the U-CUP™ via pumping and pushing until all bubbles underneath the scaffold disappeared. Then the rear tube was attached to a syringe pump (Harvard Apparatus PHD ULTRA™). Cell seeding was proceeded at 400 μL/min pushing 4 mL medium back and forth for 24 h. Thereafter, medium exchange cell cultivation was performed at 1 mL/min flow rate for up to 6 weeks. Cell differentiation was carried out with media compositions as outline above. To end cultivation the scaffolds were rinsed within the bioreactor with warm PBS for 10 min. The scaffold was quartered for further analysis. The presence of cells was validated in one quarter by first washing, fixation, and then staining with crystal violet as outlined before. The scaffold was then

Fig. 2. The tin-free co-polymer PbP. **a–d** Characterization, physical shape, and degradation of PbP was compared in reference to PLLA polymer at 70 °C for 8 days, and at 37 °C for up to 110 days. **e** The newly developed biomaterial can be further processed to form different geometries by applying sintering technology. **f** 0.1 × 10^6 ovine MSC (stained with the fluorescent membrane dye DIO) were seeded on 1-mm-high cylinders; these were made from starting polymer comprising different particle sizes yielding either low (<30%) or high porosity (40%). Cellular attachment was accounted at the indicated time points after seeding.

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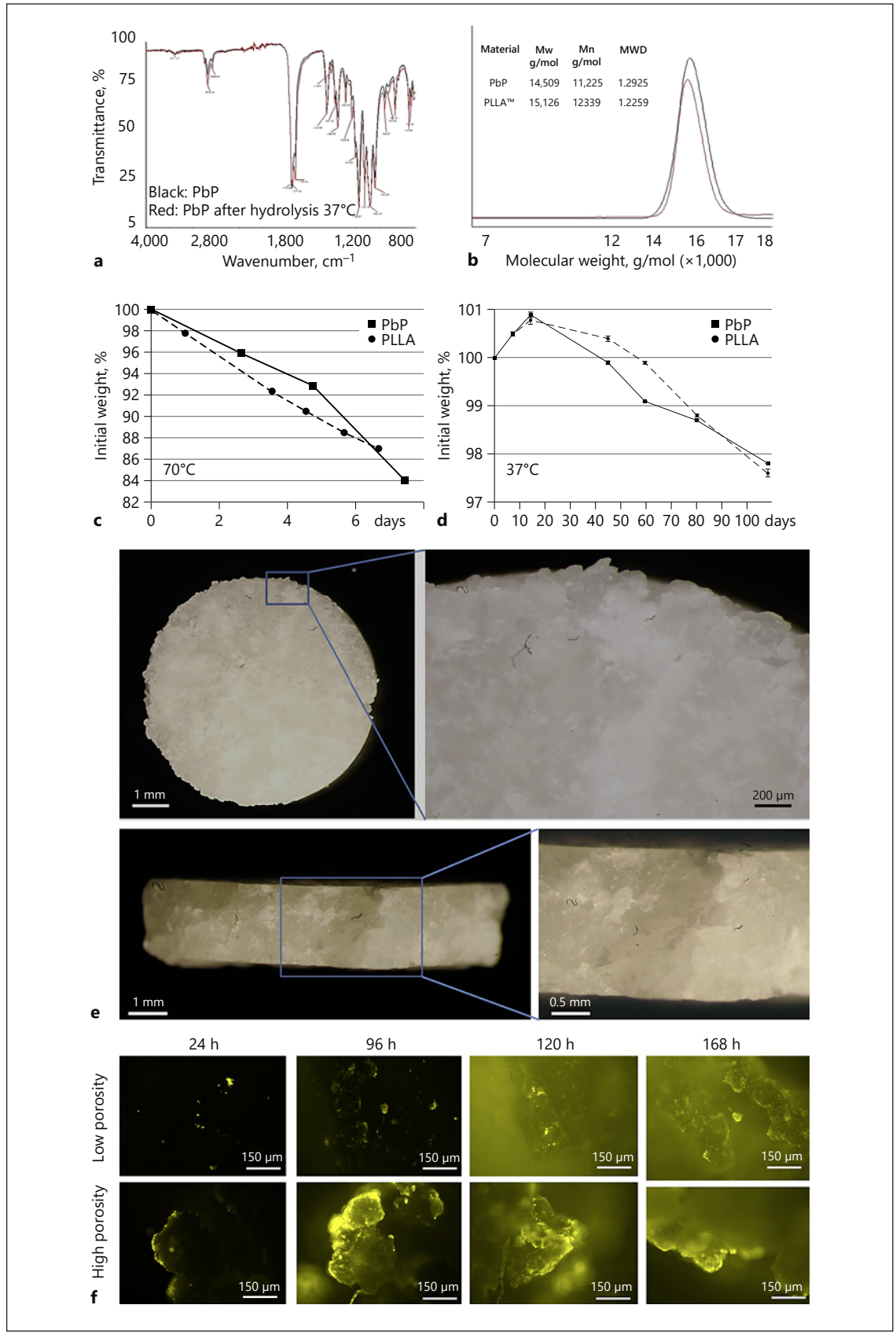

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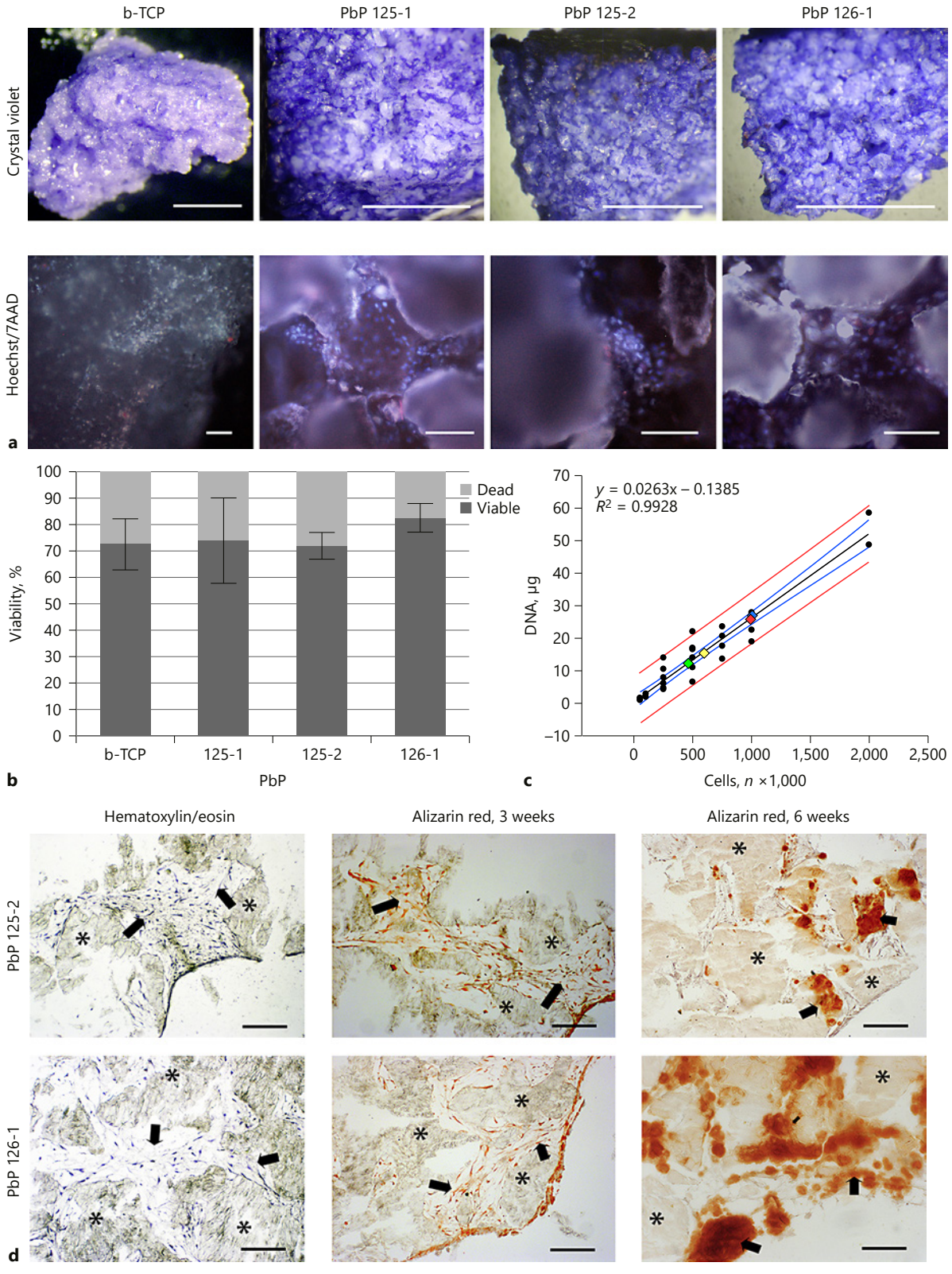

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carefully rinsed with water and photographed under a dissection microscope. Viability staining was performed by incubating the scaffold with 1 μL/mL pre-dissolved fluorescent dyes Hoechst 33342 and 7-amino actinomycin D in D-PBS or growth medium for 15 min at 37 °C, 5% CO_2, and 3 or 20% O_2. Viable and dead cells of at least 3 randomly chosen frames were counted.

Animal Experiment

The polymer was tested in 6 female merino sheep (3–6 years old, weight: 71–92 kg) applying a lateral augmentation procedure (permission was granted by the Austrian Ministry of Science and Cultural Affairs: BMWF-66.009/001-WF-V-3b). The animals were housed according to the guidelines of the local authorities in a certified surrounding. Reporting was according to the ARRIVE guidelines [17]. During experimentation, all animals showed no signs of side effects, inflammation, or pain and started chewing immediately after surgery.

Before surgery, cefazolin (2 g) was administrated preoperatively to reduce infections and wound healing disorders. The operation was initiated with thiopental (15 mg/kg) and intravenously applied fentanyl. Upon intubation, anesthesia was conducted with fentanyl 0.2 mL/kg/h, Tracrium 0.03 mL/kg/h, and 2% propofol (Diprivan) 0.5 mL/kg/h. All surgical procedures were performed by the same surgeon together with a trained team. After skin shaving and skin disinfection (Betaisodona), a submandibular approach was performed. After incision of the skin and subcutaneous tissue the masticatory muscles were dissected. The mandible was subperiosteally denuded and positions for implantation were specified. The cortical bone surface was preconditioned for the lateral augmentation with 1-mm drill holes.

PbP with and without cells were implanted in reference to TCP cylinders of the same size (Curasan, Germany). Prior to application, all scaffold materials were sterilized by γ-irradiation. Cells were seeded onto the scaffolds employing the described bioreactor technology (for seeding procedure of polymers with autologous MSCs see 3D Bioreactor). Prior to implantation empty PbP and TCP were soaked with blood. The materials were implanted at randomly selected positions separated at least 1 cm apart to reduce the risk of potential interactions or contamination (see Fig. 1b). The cylindrical implant had a diameter and a height of 1 cm. The implants were covered with a polytetrafluorethylene (PTFE) membrane (Cytoplast™ TST 200, 25 × 30 mm, Lot 06273; Curasan) in order to prevent expansion of adjacent extraosseous tissue into the scaffold and nucleation of soft tissue therein. They were stabilized and fixed to the perforated cortical bone by customized titanium cages (MatrixNEURO™ mesh 0.4 mm; Synthes Austria) with at least 4 screws (MatrixMIDFACE™; Synthes Austria). The approach was closed in layers with resorbable sutures. For postoperative analgesia and antibiotic shielding, the animals received fentanyl 3 μg/kg/h and benzyl penicillin 15 mg/kg) 3 or 6 months after the operation. Three sheep were sacrificed under general anesthesia by applying phenobarbital 300 mg/kg (WDT, Garbsen, Germany). Mandibles were dissected via a submandibular approach. After fixation in 4% neutral-buffered paraformaldehyde, specimens together with surrounding tissue were explanted and bone formation was assessed.

Cone Beam Computed Tomography

For a radiologic evaluation cone beam computed tomography (KaVo 3D eXam; KaVo Dental GmbH, Bieberbach, Germany) was used. For grey value referencing a hydroxyapatite phantom (Scanco Sc5053, Lot K35-05-83, Switzerland) was scanned in parallel. Results were watched and analyzed with the aid of a standard DICOM viewer (IcoView 4.1.1, Austria). In vivo volume stability was measured indirectly by assessing the spatial density of the remaining, nondissolved material. Area measurement was carried out using ImageJ Software (version 1.50c4; National Institutes of Health, USA).

Histology

Processing of fixed and embedded specimens was performed as described by previously [18]. In detail, the specimens were fixed in 4% neutral-buffered formalin and subsequently dehydrated using increasing alcohol concentrations and embedded in Technovit 9100NEW (Heraeus Kulzer, Hanau, Germany). The polymerization process was performed at 4 °C to avoid heat-induced damage. The samples were mounted in order to cut coronal planes. Each sample was divided into three equal parts yielding two cutting planes by sawing (Exact 3000 CP; Exact Apparatebau GmbH, Germany). The specimens were grinded to a thickness of 20 μm (Exact AW 110; Exact Apparatebau GmbH) and mounted on microscopic slides (Thermo Scientific, Menzel Gläser, Braunschweig, Germany). Technovit was dissolved in methyl glycol (Merck, Germany), and rehydrated specimens were stained with toluidine blue (Carl Roth, Karlsruhe, Germany). Photomicrographs were taken and analyzed with the aid of a Nikon Eclipse 80i microscope and NIS Elements BR 3.10 (both Nikon GmbH, Austria).

Fig. 3. In vitro cellular colonization and ossification of scaffold material. **a** 0.5×10^6 cells were applied for cellularization of scaffold material employing the U-CUP bioreactor technology. Seeding efficiency and cellular distribution throughout the material was determined by crystal violet staining. Cellular viability was assessed by distinguishing Hoechst 33342 stain in viable cells (blue) from 7AAD positivity of dead cells (red/purple) after 14 days of cultivation. PbP scaffolds differ with respect to their porosity (25% 125-1, 30% 125-2, and 40% 126-1). Scale bars, 500 μm in the upper and 100 μm in the lower photographs. **b** At least 5 images per scaffold were used to count the number of dead and viable cells showing 10% increased viability in PbP 126 compared to TCP. **c** DNA isolated from scaffolds used for 3D MSC cultivation allowed the estimation of cell numbers in the scaffolds. The amount of DNA correlated well with the number of cells used for isolation (n = 2–6). Linear regression analysis was performed yielding $R^2 = 0.99$ (black line: mean of standard curve values; blue lines: 95% confidence band; red line: 95% prediction band; green diamond: PLLA; yellow diamond: PbP 126; orange diamond: PbP 125-1; blue diamond: PbP 125-2). **d** Evaluation 0.5×10^6 cells in PbP 125 and 126 after being cultivated for 1 week in growth medium and subsequent induction in osteogenic medium for 3 or 6 weeks. Histological sections were stained with hematoxylin and eosin in order to visualize cells, or with alizarin red to detect extracellular calcium deposition. Asterisks indicate scaffold material and arrows point at cells residing within the scaffold's cavities. Scale bars, 100 μm.

Statistical Analysis

Statistical analysis was carried out using SPSS (version 24; IBM, Chicago, IL, USA).

Results

Chemical Specification of PbP

The biomaterial tested here is a newly developed polymer blend scaffold made from PLLA and PCL and abbreviated in this contribution to PbP. It is described here for the first time. It exhibited good mechanical and chemical stability in the course of all tests performed during this study. The material was used to prepare scaffolds of specific dimensions with controlled pore morphology. Cylindrically shaped polymer scaffolds (1 × 8 or 4 × 8 mm) were investigated for their potential use in facial contour reconstruction. A facile and rapid single measurement carried out at 70 °C indicated that PbP degrades comparably to PLLA, losing 16% of its initial weight within 8 days. However, it remained highly stable at 37 °C, only exhibiting 1.5% degradation within 110 days (Fig. 2a–d). The molecular weight of PbP was 14,509 g/mol, thus comparable to PLLA (15,126 g/mol). It has the same whitish color as PLLA, yet the processed material forms a scaffold with a firm and stable appearance (Fig. 2e). Cryogrinding prior to sintering allowed manufacturing of PbP with different pore size and connectivity.

Cell Attachment and Proliferation on PbP

Attachment of ovine bone marrow-derived MSC and proliferation on the material surface was largely dependent on pore size. Material with 20–30% porosity resulted in a distinctly weaker cellular response compared to PbP exhibiting 40% porosity (Fig. 2f). Cells on PbP commenced growth in the form of colonies shortly after seeding, while on the commercially available TCP ceramics Cerasorb® M (Curasan) [19–21], cells proliferated in a dispersed manner. The results of these initial tests prompted us to primarily test scaffold material with high porosity, which in due course of the experimental evaluation was also selected for in vivo experimentation.

In order to gain cell attachment also within the scaffold core, both PbP and TCP were mounted in a bioreactor (U-CUP; Cellec Biotek AG) (Fig. 3a–c). When seeding 0.5×10^6 MSC, cells were consistently found throughout the scaffold after 7 days, also displaying at a good rate of cellular viability. Within TCP, 71% of the cells were found alive. One week after cell seeding, osteogenic differentiation was started, and 3–6 weeks thereafter calcified nodules could be revealed. Due to their inherent fragility and brittleness, the TCP scaffolds were actually unsuitable for histological sectioning. The degree of osteogenesis and cellular colonization was thus determined in PbP only employing 8-µm histological sectioning and staining of calcium deposits with alizarin red (Fig. 3d). A parallel 7-week cultivation without osteogenic induction showed extended cellular proliferation and general occupation of the porous scaffold core in all PbP scaffolds used in this study. Cultivated with osteogenic medium, the onset of osteogenesis could already be revealed after 3 weeks, yet 6 weeks of osteogenic induction showed prominent calcium deposition within a great majority of scaffold pores.

Bone Augmentation by Implantation of PbP in a Sheep Mandible

PbP was implanted in subperiosteally denuded mandibular bone of female merino sheep. In order to enhance lateral augmentation, the site was further processed by drilling 1-mm perforations to ease the transfer of vessels, cells, and extracellular material from the bone marrow cavity to the augmentation site. Polymer was cellularized with autologous stromal cells prior to implantation and was, as such, implanted alongside with empty PbP and TCP (Curasan). Biomaterials were covered with a PTFE membrane. The composite was stabilized by titanium cages, which were screwed to the perforated cortical bone. Apparent manifestations of sustaining inflammation or foreign body reactions could not be observed over the entire observational period. Screws underwent stable osseointegration. No screw fracture or titanium cage deformity due to masticatory forces was detected. The cages holding PbP remained

Fig. 4. Mandibular augmentation in sheep. **a** Radiologic examination of mandibles with mounted implants showing PbP, PbP with ovine MSC (oMSC), or TCP scaffold after 3 and 6 months. **b** Toluidine blue staining of histological sections of PbP with and without MSC and TCP scaffolds 3 and 6 months post-implantation (bone is stained in brown, soft tissue in blue, PTFE membrane in orange partly aligned with titanium mesh residues in black, and scaffold material is brownish-black. The white stars indicate bone that enclosed the drilled osseous defects. **c** Histological sections were stained with an antibody raised against TNF-α. No signs of inflammation or rejection were detectable either at 3 or 6 months post-implantation.

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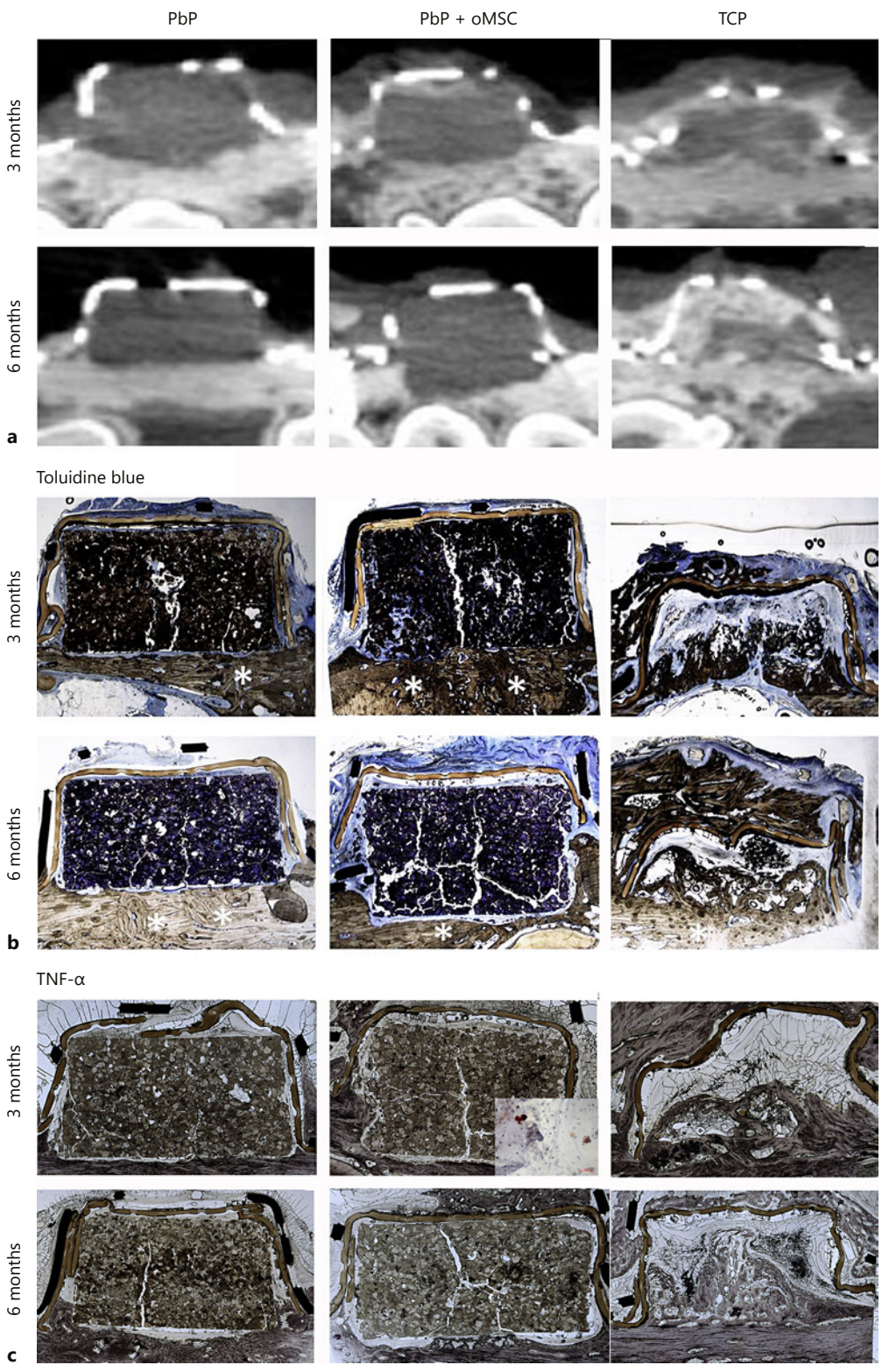

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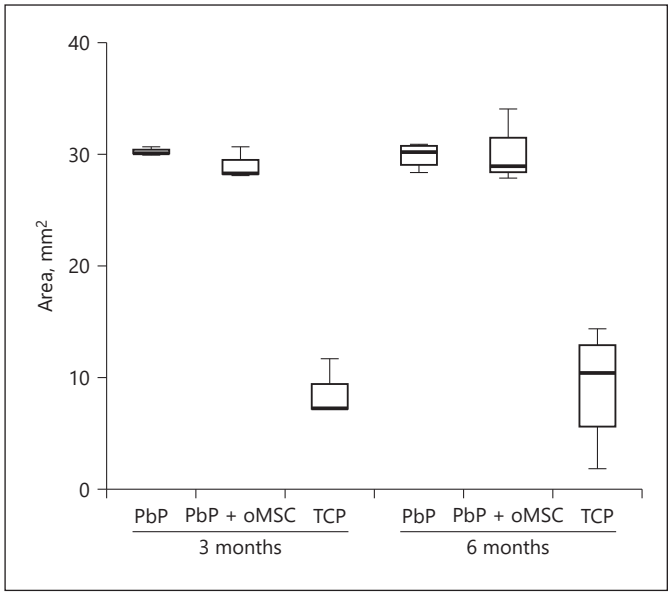

Fig. 5. Cross-sectional area of implanted biomaterial: resilient content of implanted PbP co-polymer, PbP containing autologous ovine mesenchymal stroma cells (oMSC), and beta-tricalcium phosphate (TCP), which were implanted for 3 or 6 months in sheep mandible.

radiolucent and structurally stable over the entire period of 6 months' inspection time and showed no obvious morphological changes. As anticipated, TCP commenced with degradation already during the first 3 months post-implantation.

Implantation material was taken into histological analysis after 3 and 6 months in order to characterize composition, integrity, cellular content, or osseous ingrowth (Fig. 4). PbP hardly changed in shape and composition during 3 and 6 months of implantation. The polymeric molds exhibited a consistent rectangular shape and the surrounding PTFE membrane remained immaculate, while the TCP scaffold rapidly degraded. Together with the TCP content the membranes collapsed within the cages, being successively replaced by solid bone. These changes were quantified after 3 and 6 months post-implantation (Fig. 5), demonstrating that PbP filled 30.1 mm^2 (SD 0.36), cellularized PbP filled 28.3 mm^2 (SD 1.42), while TCP shrank to 7.2 mm^2 (SD 2.56) during 3 months. Later time points corroborated the preliminary observations as the volume of PbP remained at 30.2 mm^2 (SD 1.14), cellularized PbP at 28.9 mm^2 (SD 3.3), yet TCP was almost completely degraded with the emerging space being replaced by osseous material at 10.7 mm^2 (SD 5.36). Besides spatial stability, immunological characteristics of PbP were investigated. Immune histological assessment of TNF-α expression within the specimen suggested absence of a foreign body reaction, thus indicating good anergic properties of the structurally stable biomaterial.

Discussion

Materials that are designed and made for implantation need to meet the highest standards (e.g., as laid down in the recent amendments of the European regulations 2017/745 on medical devices, regarding Directive 2001/83/EC and Regulation (EC) No. 178/2002 and 1223/2009). Chemical and functional stability is pivotal, yet seemingly important considerations are given to in vivo degradation of implant materials, in particular involving kinetics and formation of degradation end products that may provoke adverse reactions [22, 23]. A dichotomy resulting from these two demands is that wanted scaffold material should exhibit stability for a desired time period followed by degradation kinetics that greatly obey the physiological needs of a changing, in most cases healing, or restructuring environment in vivo [24]. Ideally, material applicable for implantation should fulfil several requirements: uncomplicated handling during surgical procedures, individualized and easy shaping at the site of operation to exactly suit the patient's case, supporting cellular migration, proliferation, and differentiation, and last but not least, a basic stability in combination with distinct degradation kinetics without causing allergic or toxic reactions. Research on materials meeting these, or at least a broad combination of these, demands occupies many academic centers and industrial cooperation [25, 26]. Of important note in this particular context is the role of tin complexes. These are often employed to start the polymerization of polyesters. Although known to exert very low toxicity in food, tin is considered a potential hazard when present in biodegradable implantable polymers. However, ring opening to start polymerization can also be accomplished by tin-free measures, which allowed us to produce PbP with good yields and without the need of extensive post-production purification to reliably reduce tin content below the currently allowed 20-ppm limit.

Material properties are highly selective upon development of biological interfaces [27–31]. In lieu of powerful predictive modeling, emergence of discriminative effects can yet not be predicted, thus the field greatly relies on empirical studies. The prime intention of this refined

synthesis of a PLLA/PCL blend was designed to provide a novel type of biomaterial that is suitable for in vitro cell testing, tissue engineering applications, and implantation. The material property testing here was by and large in reference to TCP, a material which is routinely used in various clinical reparative medicinal approaches as well as in tissue engineering approaches [20, 32]. The novel PbP blend is a cream-white, geometrically stable, sturdy and brittle material, and can be sintered to take specific shape thereby taking differently porous structures. Following tissue engineering concepts, for which organ-specific cells are brought together with scaffold materials in conjunction with bioactive factors under physiologic conditions [33], we tested whether stromal cells readily attach to this type of surface without the need of prior coating or cell-special treatments. We could show that the surface provided a favorable substrate for growth and differentiation of mesenchymal cells. In addition, after cellularization PbP was found to be perfectly suitable for paraffin-based histology, which, in stark contrast to ponderous and laborious characterization of scaffolds such as pristine PLLA or ceramics, eases experimental analysis, in particular when intending to apply immune histochemistry. PbP serving as a novel substrate for adherent cells in conjunction with quick histological analysis thus provides further grounds for resolving basic cell biological questions. It is generally believed that cells behave very differently when grown in 3D, as cell-cell contacts or production and deposition of matrix will greatly differ compared to 2D cell culture in plastic dishes [34]. Straightforward unsophisticated biomaterial-assisted growth could be the first step towards experimental analysis of organotypic cell biology [35, 36]. Noteworthy in this context is the fact that treatment with serum-containing culture media caused no apparent reduction of long-term stability. This indicates that PbP can be further useful for studying biological restrictions of long-term biomaterial stability (e.g., interactions with osteoclasts) [37]. PbP may be further expedient in establishing conditions to study activated stroma in combination with cancerous cells or when modeling a proinflammatory condition that would actually propel fibrosis [38].

We experienced that cell seeding could be consistently accomplished within the highly porous material. Seeding was aided by employing an inexpensive, simple, and reliably working bioreactor technology. Cellularization provides good grounds for two important branches of biomedical applications: implantation surgery supporting tissue repair and ex vivo tissue engineering [39, 40]. Due to its permissive activity on osteogenic differentiation and calcification, we assumed that PbP cellularized with multipotent mesenchymal progenitor cells may potentiate ossification after implantation into the vicinity of bone. Within the designated period of 3–6 months, which is considered sufficient for bone augmentation in nonsevere cases, TCP quickly disintegrated while implanted PbP remained intact. Furthermore, the implant also restrained from uncontrolled cellular responses such as excessive superficial cellular growth or fibrosis, the latter being particularly important in order to reduce the risk of implant failure due to fibrotic capsule formation. This specifically argues for application in plastic and recontouring surgery, in particular when displacement of tissue with anergic material is necessary. Albeit biocompatible at large, the highly porous PbP scaffolds will not provide enough mechanical strength for load-bearing applications such as mandibular reconstructions. Likewise, very delicate constructs will easily disintegrate due to mechanical strains and deformations, thus clearly limiting the usage of the biomaterial to specific therapeutic interventions.

Conclusion and Outlook

PbP is a promising material for a variety of applications. New materials are pivotal as few to none of the currently available biomaterials comparable to PbP are meeting all the requirements for potential application in facial reconstruction. Of prime importance is long-term toxicity, which compared to other materials could be greatly reduced due to the successful production of PbP material through introduction of a novel tin-free polymerization method. The so-produced scaffolds were chemically stable and refrained from disintegration during cell culture tests and harsh treatments in organic solvents during histological evaluation.

For in vivo use with mesenchymal stroma cells, the scaffolds provided a highly compatible substrate with respect to proliferation and osteogenic differentiation also during extended periods, suggesting that the material is not only applicable for long-term 3D cell cultivation but also for complex approaches in the biotechnological processes and fermentation. Further along these lines, 3D testing of human cell types under conditions closely resembling in vivo conditions may help to reduce animal experiments currently performed to test and confirm functionality and compatibility of medical drugs.

Results gained during and after material implantation demonstrated that PbP holds great potential to be used as a space filler in large anatomical defects, which are often experienced in adult patients after severe trauma. A specific further clinical need is supporting optimal restoration and regeneration. This can be enhanced by spatial modeling on the basis of volumetric data derived from the surgical site and 3D anatomical measures of the surrounding soft and hard tissue. According to such data, the material could be precisely shaped. For instance, in the case of reconstructing lost hard tissue, such as the nose tip in diseased or traumatized patients [41] which is also often needed after tumor resection [42], PbP, which is actually suitable for laser-guided sintering [12], could be specifically molded according to given facial contours prior to implantation. It is well conceivable that in cases other than those exemplified here the material will offer means for supporting regeneration by providing long-term shape stability, with little degradation and scarring, while exerting good compatibility to cells and surrounding tissues.

Acknowledgments

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Statement of Ethics

Permission for the animal experiment was granted by the Austrian Ministry of Science and Cultural Affairs (BMWF-66.009/001-WF-V-3b).

Disclosure Statement

All authors declare no conflict of interest regarding the content disclosed in this work.

Author Contributions

The paper was written through contributions from all authors. All authors gave approval to the final version of the paper.

References

1 Yun MH. Changes in Regenerative Capacity through Lifespan. Int J Mol Sci. 2015 Oct; 16(10):25392–432.
2 Nielsen BR, Linneberg A, Christensen K, Forman JL, Schwarz P. The Influence of Clinical Experience and Photographic Presentation on Age Assessment of Women. Gerontology. 2016;62(2):191–9.
3 Curtis RG, Windsor TD, Mogle JA, Bielak AA. There's More than Meets the Eye: Complex Associations of Daily Pain, Physical Symptoms, and Self-Efficacy with Activity in Middle and Older Adulthood. Gerontology. 2017;63(2):157–68.
4 De Sousa A. Psychological issues in acquired facial trauma. Indian J Plast Surg. 2010 Jul; 43(2):200–5.
5 van den Elzen ME, Versnel SL, Hovius SE, Passchier J, Duivenvoorden HJ, Mathijssen IM. Adults with congenital or acquired facial disfigurement: impact of appearance on social functioning. J Craniomaxillofac Surg. 2012 Dec;40(8):777–82.
6 Knowles LM, Skeath P, Jia M, Najafi B, Thayer J, Sternberg EM. New and Future Directions in Integrative Medicine Research Methods with a Focus on Aging Populations: A Review. Gerontology. 2016;62(4):467–76.
7 Balic A. Biology Explaining Tooth Repair and Regeneration: A Mini-Review. Gerontology. 2018;64(4):382–8.
8 Yen AH, Yelick PC. Dental tissue regeneration - a mini-review. Gerontology. 2011; 57(1):85–94.
9 Gotz W, Papageorgiou SN. Molecular, Cellular and Pharmaceutical Aspects of Synthetic Hydroxyapatite Bone Substitutes for Oral and Maxillofacial Grafting. Curr Pharm Biotechnol. 2017;18(1):95–106.
10 Wang W, Yeung KW. Bone grafts and biomaterials substitutes for bone defect repair: A review. Bioact Mater. 2017 Jun;2(4):224–47.
11 Guillaume B. Filling bone defects with β-TCP in maxillofacial surgery: A review. Morphologie. 2017 Sep;101(334):113–9.
12 Chia HN, Wu BM. Recent advances in 3D printing of biomaterials. J Biol Eng. 2015 Mar; 9(1):4.
13 Ajami-Henriquez D, Rodríguez M, Sabino M, Castillo RV, Müller AJ, Boschetti-de-Fierro A, et al. Evaluation of cell affinity on poly(L-lactide) and poly(epsilon-caprolactone) blends and on PLLA-b-PCL diblock copolymer surfaces. J Biomed Mater Res A. 2008 Nov;87(2):405–17.
14 Saini P, Arora M, Kumar MN. Poly(lactic acid) blends in biomedical applications. Adv Drug Deliv Rev. 2016 Dec;107:47–59.
15 Klepsch S, Jamnig A, Trimmel D, Schimke M, Kapferer W, Brunauer R, et al. Isolation of mesenchymal stem cells from human bone and long-term cultivation under physiologic oxygen conditions. Methods Mol Biol. 2013; 976:99–109.
16 Rentsch C, Hess R, Rentsch B, Hofmann A, Manthey S, Scharnweber D, et al. Ovine bone marrow mesenchymal stem cells: isolation and characterization of the cells and their osteogenic differentiation potential on embroidered and surface-modified polycaprolactone-co-lactide scaffolds. In Vitro Cell Dev Biol Anim. 2010 Jul;46(7):624–34.
17 Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. J Pharmacol Pharmacother. 2010 Jul;1(2):94–9.
18 Donath K, Breuner G. A method for the study of undecalcified bones and teeth with attached soft tissues. The Säge-Schliff (sawing and grinding) technique. J Oral Pathol. 1982 Aug;11(4):318–26.
19 Yang DJ, Jeon JH, Lee SY, An HW, Park KO, Park KB, et al. Effects of Collagen Grafting on Cell Behaviors in BCP Scaffold with Interconnected Pore Structure. Biomater Res. 2016 Jan;20(1):3.

20 Tanaka T, Komaki H, Chazono M, Kitasato S, Kakuta A, Akiyama S, et al. Basic research and clinical application of beta-tricalcium phosphate (β-TCP). Morphologie. 2017 Sep; 101(334):164–72.
21 Guyton GP, Miller SD. Stem cells in bone grafting: trinity allograft with stem cells and collagen/beta-tricalcium phosphate with concentrated bone marrow aspirate. Foot Ankle Clin. 2010 Dec;15(4):611–9.
22 Anderson JM, Rodriguez A, Chang DT. Foreign body reaction to biomaterials. Semin Immunol. 2008 Apr;20(2):86–100.
23 Sheikh Z, Najeeb S, Khurshid Z, Verma V, Rashid H, Glogauer M. Biodegradable Materials for Bone Repair and Tissue Engineering Applications. Materials (Basel). 2015 Aug; 8(9):5744–94.
24 R. P-L. Shirzaei Sani E, N. A: Biomimetic Orthopedic Materials. In: Webster T, editor. Orthopedic Biomaterials. Cham: Springer; 2017.
25 Narayan RJ. The next generation of biomaterial development. Philos Trans A Math Phys Eng Sci. 2010 Apr;368(1917):1831–7.
26 Vallet-Regí M, Izquierdo-Barba I, Colilla M. Structure and functionalization of mesoporous bioceramics for bone tissue regeneration and local drug delivery. Philos Trans A Math Phys Eng Sci. 2012 Mar;370(1963):1400–21.
27 Leferink AM, van Blitterswijk CA, Moroni L. Methods of Monitoring Cell Fate and Tissue Growth in Three-Dimensional Scaffold-Based Strategies for In Vitro Tissue Engineering. Tissue Eng Part B Rev. 2016 Aug;22(4): 265–83.
28 Akazawa K, Iwasaki K, Nagata M, Yokoyama N, Ayame H, Yamaki K, et al. Cell transfer technology for tissue engineering. Inflamm Regen. 2017 Oct;37(1):21.
29 Cecoltan S, Stancu IC, Drăguşin DM, Serafim A, Lungu A, Ţucureanu C, et al. Nanocomposite particles with improved microstructure for 3D culture systems and bone regeneration. J Mater Sci Mater Med. 2017 Aug; 28(10):153.
30 Jiang J, Papoutsakis ET. Stem-cell niche based comparative analysis of chemical and nanomechanical material properties impacting ex vivo expansion and differentiation of hematopoietic and mesenchymal stem cells. Adv Healthc Mater. 2013 Jan;2(1):25–42.
31 Li J, Wang Q, Gu Y, Zhu Y, Chen L, Chen Y. Production of Composite Scaffold Containing Silk Fibroin, Chitosan, and Gelatin for 3D Cell Culture and Bone Tissue Regeneration. Med Sci Monit. 2017 Nov;23:5311–20.
32 Lin S, Cui L, Chen G, Huang J, Yang Y, Zou K, et al. PLGA/β-TCP composite scaffold incorporating salvianolic acid B promotes bone fusion by angiogenesis and osteogenesis in a rat spinal fusion model. Biomaterials. 2018 Apr;S0142-9612(18)30243-6.
33 Bunpetch V, Zhang ZY, Zhang X, Han S, Zongyou P, Wu H, et al. Strategies for MSC expansion and MSC-based microtissue for bone regeneration. Biomaterials. 2017 Nov; S0142-9612(17)30755-X.
34 Duval K, Grover H, Han LH, Mou Y, Pegoraro AF, Fredberg J, et al. Modeling Physiological Events in 2D vs. 3D Cell Culture. Physiology (Bethesda). 2017 Jul;32(4):266–77.
35 Reitinger S, Schimke M, Klepsch S, de Sneeuw S, Yani SL, Gaßner R, et al. Systemic impact molds mesenchymal stromal/stem cell aging. Transfus Apheresis Sci. 2015 Jun;52(3):285–9.
36 Davies JA. Synthetic Biology: Rational Pathway Design for Regenerative Medicine. Gerontology. 2016;62(5):564–70.
37 Pietschmann P, Mechtcheriakova D, Meshcheryakova A, Föger-Samwald U, Ellinger I. Immunology of Osteoporosis: A Mini-Review. Gerontology. 2016;62(2):128–37.
38 Adjei IM, Blanka S. Modulation of the tumor microenvironment for cancer treatment: a biomaterials approach. J Funct Biomater. 2015 Feb;6(1):81–103.
39 Naranjo JD, Scarritt ME, Huleihel L, Ravindra A, Torres CM, Badylak SF. Regenerative Medicine: lessons from Mother Nature. Regen Med. 2016 Dec;11(8):767–75.
40 Scarritt ME, Pashos NC, Bunnell BA. A review of cellularization strategies for tissue engineering of whole organs. Front Bioeng Biotechnol. 2015 Mar;3:43.
41 Park SS. Nasal reconstruction in the 21st century—a contemporary review. Clin Exp Otorhinolaryngol. 2008 Mar;1(1):1–9.
42 Parthasarathy J. 3D modeling, custom implants and its future perspectives in craniofacial surgery. Ann Maxillofac Surg. 2014 Jan; 4(1):9–18.