

Aus der Klinik und Poliklinik für Dermatologie und Venerologie

Universitätsmedizin Rostock

Direktor: Prof. Dr. med. Steffen Emmert

„Cytocompatibility and biocompatibility approaches with analysis of the *in vivo*
immune responses of collagen-based and synthetic materials for bone regeneration“

Kumulative Inauguraldissertation
zur
Erlangung des akademischen Grades
Doktor der Medizinwissenschaften
der Universitätsmedizin Rostock

vorgelegt von

Yanru Ren, geb. am 17.01.1996 in China

aus Berlin

Rostock, 13.03.2025

Gutachter:

Univ.-Prof. Dr. med. Dr. biol. hom. Volker Alt, Universitätsklinikum Regensburg, Klinik und Poliklinik für Unfallchirurgie

PD Dr. rer. nat. habil. Mike Barbeck, Universitätsmedizin Rostock, Klinik und Poliklinik für Dermatologie und Venerologie

PD Dr. Dr. Michael Dau, Universitätsmedizin Rostock, Klinik und Poliklinik für Mund-, Kiefer- und Gesichtschirurgie

Jahr der Einreichung: 2026

Jahr der Verteidigung: 2026

DEDICATION

This doctoral thesis is dedicated to my parents, my sister Yanli Ren, and my partner Fengliang Tian, whose unwavering support has been a constant source of strength throughout my studies and professional journey.

Based on the following publications

Ren, Y.; Liu, L.; Xiong, X.; Krastev, R.; Smeets, R.; Rimashevskiy, D.; Schnettler, R.; Alkildani, S.; Emmert, S.; Jung, O.; et al. (2023) Suitability of *R. pulmo* Jellyfish-Collagen-Coated Well Plates for Cytocompatibility Analyses of Biomaterials. *Int. J. Mol. Sci.* 24, 3007.

Barbeck, M., Alkildani, S., Mandlule, A., Radenković, M., Najman, S., Stojanović, S., **Ren, Y.**, et al. (2022). *In Vivo* Analysis of the Immune Response to Strontium- and Copper-doped Bioglass. *In Vivo.* 36, 2149–2165.

Ren, Y., Jung, O., Batinic, M., Burckhardt, K., Görke, O., Alkildani, S., et al. (2024). Biphasic bone substitutes coated with PLGA incorporating therapeutic ions Sr^{2+} and Mg^{2+} : cytotoxicity cascade and *in vivo* response of immune and bone regeneration. *Front. Bioeng. Biotechnol.* 12, 1408702.

Ren Y., Alkildani S., Burckhardt K., et al. (2024). The influence of different crosslinking agents onto the physical properties, integration behavior and immune response of collagen-based barrier membranes. *Front. Bioeng. Biotechnol.* 12:1506433

Alkildani, S., **Ren, Y.**, Liu, L., Rimashevskiy, D., Schnettler, R., Radenković, M., et al. (2023). Analyses of the Cellular Interactions between the Ossification of Collagen-Based Barrier Membranes and the Underlying Bone Defects. *Int. J. Mol. Sci.* 24, 6833.

Publications Authored or Co-Authored During the Doctoral Research

Ren, Y., Fan, L., Alkildani, S. et al. Barrier Membranes for Guided Bone Regeneration (GBR): A Focus on Recent Advances in Collagen Membranes. *International Journal of Molecular Sciences.* 2022, 23, 14987.

Fan, L., **Ren, Y.**, Burkhardt, C., et al. Evaluation of Injectable Composite Material Comprising Biphasic Bone Substitutes and Crosslinked Collagen. *Advanced Engineering Materials.* 2023. 25: 2300508.

Fan, L., **Ren, Y.**, Emmert, S. et al. The Use of Collagen-Based Materials in Bone Tissue Engineering. *International Journal of Molecular Sciences.* 2023, 24, 3744.

Bielenstein, J., Radenković, M., Najman, S., Liu, L., **Ren, Y.** et al. In Vivo Analysis of the Regeneration Capacity and Immune Response to Xenogeneic and Synthetic Bone Substitute Materials. *International Journal of Molecular Sciences.* 2022, 23, 10636.

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Abbreviations

GBR	Guided bone regeneration
BSMs	Bone substitute materials
FBR	Foreign body response
FBGCs	Foreign body giant cells
PTFE	Polytetrafluoroethylene
BCP	Biphasic calcium phosphate
HA	hydroxyapatite
β-TCP	β-tricalcium phosphate
VEGF	Vascular endothelial growth factor
MNGCs	Multinucleated giant cells
ECM	Extracellular matrix
EC/NHS	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/N-hydroxysuccinimide
PC	Proanthocyanidins
HMDI	Hexamethylene diisocyanate
GA	Glutaraldehyde

1. Introduction

1.1 Medical device in Guided bone regeneration

Guided Bone Regeneration (GBR) is a well-established technique that facilitates new bone formation by preventing the invasion of non-osteogenic tissues into the defect site, creating an environment conducive to the natural healing process ¹. The success of GBR depends on the combined use of both bone substitute materials (BSMs), which serve as scaffolds for new bone growth, and barrier membranes, which segregate the bone defect from surrounding tissues. This combination has become a key approach in various clinical applications, including oral and maxillofacial surgery, periodontal regeneration, and orthopedic procedures ².

BSMs are designed to replace missing bone tissue and provide a structural framework that promotes osteoregeneration, supporting the integrity and function of the original bone. Autologous bone grafts are considered the "gold standard" due to their superior osteoinductive, osteoconductive, and osteogenic properties ³. However, their clinical application is often restricted by limited donor tissue availability, donor site morbidity, and potential complications. These limitations have driven the growing interest in alternative materials, particularly synthetic BSMs mainly based on natural or synthetic calcium phosphates and similar compounds, which offer advantages such as greater availability, reduced risk of disease transmission, and the ability to tailor material properties to meet specific clinical needs ⁴.

However, despite their promising potential, current synthetic BSMs often fall short of matching the regenerative capacity of autologous bone grafts, particularly in the areas of osteogenesis, osteoinduction, and the promotion of vascularization—critical factors for long-term success in bone regeneration. One promising strategy to improve the performance of synthetic BSMs is the incorporation of bioactive ions. Cations such as magnesium (Mg^{2+}), zinc (Zn^{2+}), copper (Cu^{2+}), and strontium (Sr^{2+}) have demonstrated potential in stimulating osteogenic differentiation, improving bone mineralization, and promoting angiogenesis ^{5,6}. Magnesium, for example, plays a crucial role in bone metabolism and has been shown to enhance osteoblast activity ⁵. Copper and strontium are known to promote both osteogenesis and angiogenesis, which are essential for the formation of new bone and the integration of graft materials into host tissue ^{7,8}.

In conjunction with BSMs, barrier membranes are integral to the GBR process. These membranes act as a physical barrier to prevent the infiltration of soft tissue into the bone defect, allowing for undisturbed bone regeneration ⁹. Resorbable membranes have gained increasing prominence over non-resorbable alternatives, such as polytetrafluoroethylene (PTFE) membranes, primarily due to their ability to degrade over time, thereby eliminating the need for secondary surgical removal ¹⁰. Among bioresorbable membranes, collagen membranes have emerged as a leading choice due to their biocompatibility, biodegradability, and ability to support cellular functions critical for bone regeneration, such as cell adhesion, migration, and differentiation ¹¹. Interestingly, recent studies have highlighted that the molecular microenvironment

induced by barrier membranes may also influence molecular events in the adjacent bone regeneration process^{12,13}. Consequently, collagen membranes are now expected not only to provide a physical barrier but also to "actively" support bone healing.

However, one of the primary limitations of natural collagen membranes is their relatively short degradation time, which may be insufficient for long-term tissue regeneration. For instance, in periodontal tissue regeneration, the barrier function needs to be maintained for 4-6 weeks, while in bone augmentation procedures, it may need to persist for 16-24 weeks⁹. To address this challenge, several chemical cross-linking strategies have been developed to enhance the stability and prolong the functional lifespan of collagen membranes.

1.2 Preclinical testing of medical devices

Despite their critical role in clinical treatments, the application of medical devices is not possible without different challenges and associated risks. Patients may experience a range of reactions, from mild tissue irritation to more severe complications, such as infections or implant failure. The primary factor influencing the long-term success and safety of these implants is their biocompatibility, which is defined as "the ability of the implant material to integrate with surrounding biological tissues without eliciting adverse responses"¹⁴. Ensuring biocompatibility is vital not only for the functionality of the device but also for the understanding and prevention of material-related inflammatory reactions, tissue necrosis, and immune responses that can compromise the implant's success.

Given the importance of biocompatibility, regulatory bodies worldwide impose stringent requirements. The International Organization for Standardization (ISO) has established the ISO-10993 series, a globally recognized framework that outlines the methods and processes for testing the biocompatibility of medical devices to guide standardized preclinical testing¹⁵. These standards include a range of tests, such as cytotoxicity, sensitization, irritation, and long-term implantation studies, aimed at thoroughly assessing the biological safety of the materials used. The specific testing requirements for biomaterials depend on their composition and intended clinical application. *In vitro* cytotoxicity testing is typically the foundation of preclinical evaluation, followed by subcutaneous implantation studies in animals to assess tissue responses under physiological conditions. However, achieving biocompatibility is not the endpoint in material development. Biomaterials must also demonstrate functionality in relevant models, such as those evaluating bone regeneration in standardized bone defect models. Building on this, the 3R principle—focused on the reduction, refinement, and replacement of animal testing—also should be emphasized in preclinical research¹⁶. Optimizing *in vitro* testing systems and models to more accurately simulate *in vivo* outcomes is a significant step forward.

1.3 Foreign Body Response of implants

A so-called "Foreign Body Response (FBR)" is the term refers to the series of cellular events triggered by the introduction of medical implants or biomaterials into the body¹⁷. This response involves a cascade of cellular events, predominantly orchestrated by

immune cells such as macrophages, monocytes, and neutrophils, which interact with proteins and release cytokines to guide the immune response. Central to this process are macrophages, which play a pivotal role in regulating the cellular dynamics of the response. Based on their molecular expression profiles, macrophages are generally classified into two phenotypes: the pro-inflammatory M1 phenotype and the anti-inflammatory M2 phenotype¹⁸. The M1 phenotype dominates the early stages of tissue healing, mediating the degradation of implanted materials *in vivo*. As the acute inflammatory phase subsides, macrophages polarize toward the M2 phenotype, which expresses factors that promote tissue repair¹⁹. A balanced transition toward an M2-dominant response is essential for favorable tissue regeneration; however, chronic pro-inflammation can result in fibrotic encapsulation, leading to implant failure. Another hallmark of the FBR is the formation of foreign body giant cells (FBGCs), which arise from the fusion of activated macrophages in response to persistent stimulation from the implant surface²⁰. These multinucleated giant cells are often found at the interface between the implant and surrounding tissue, and their presence is a key indicator of chronic inflammation. FBGCs attempt to degrade the implant through phagocytic activity and the release of degradative enzymes, but their prolonged activity can also lead to tissue damage and fibrosis, compromising the long-term success of the implant.

In addition to immune cell recruitment, vascularization is a critical factor in determining the outcome of the FBR. Adequate blood vessel formation is essential for delivering oxygen and nutrients to the site of implantation, supporting tissue regeneration, and maintaining the health of surrounding tissues²¹. However, the chronic inflammatory environment associated with FBR can hinder proper vascularization, leading to hypoxia and impaired healing. Macrophages and FBGCs have been shown to influence angiogenesis by releasing factors such as vascular endothelial growth factor (VEGF), which promotes blood vessel formation^{13,22}. Nevertheless, the balance between inflammatory and pro-regenerative signals remains delicate, and dysregulation can result in poor integration of the implant. Consequently, understanding the interaction between biomaterial properties and the underlying mechanisms of bone regeneration is a critical focus in preclinical research.

2. Aim of the study series

This study series adheres to the standards outlined in DIN EN ISO 10993-5/-12 and ISO 10993-6 to evaluate the cellular and biocompatibility properties of synthetic bone substitute materials incorporated with metal ions, as well as collagen-based barrier membranes. These evaluations were conducted using both subcutaneous or calvaria implantation models, with particular emphasis the materials-related macrophage-dominated inflammatory patterns and tissue regeneration processes. The objective is to further elucidate the immunomodulatory properties of these biomaterials and their relationship with tissue regeneration. Additionally, in accordance with the 3R principles, the study explores the potential of jellyfish collagen in optimizing *in vitro* cell culture systems, aiming for improving the accurate predictions of cellular compatibility.

Theses:

- (1) Collagen type 0 derived from jellyfishes is a suitable substrate for *in vitro* cytocompatibility analyses of biomaterials due to its nativity.
- (2) The *in vitro* cytocompatibility results using mammalian collagen or jellyfish collagen are comparable.
- (3) Metal ions as an additive of bone substitute materials hold promise for influencing bone regeneration processes by modulating immune responses and vascularization.
- (4) Strontium/magnesium-doping of biphasic calcium phosphate-based bone substitutes have influence on macrophage polarization and bone tissue regeneration.
- (5) Copper- and strontium-doped bioactive glasses have influence on macrophage polarization and bone tissue regeneration.
- (6) Chemical cross-linkers enhance collagen membrane stability by improving mechanical strength and enzymatic resistance through intermolecular and intramolecular bonds.
- (7) The cross-linking of collagen membranes induces an increase of the material-related foreign body response (FBR) to biomaterials including a higher occurrence of multinucleated giant cells and pro-inflammatory macrophages
- (8) The cross-linking of collagen membranes can induce a fibrotic tissue response.
- (9) Collagen barrier membranes play an active biological role in bone healing by modulating the molecular micromilieu.
- (10) Membrane-induced pro-inflammatory macrophages can influence the vascularization process within adjacent bone defect regions.

3. Publications

3.1 Suitability of *R. pulmo* Jellyfish-Collagen-Coated Well Plates for Cytocompatibility Analyses of Biomaterials

3.1.1 Introduction

Collagen has long been regarded as an ideal substrate for cell culture. Its interaction with various cell surface receptors—including integrins, discoidin domain receptors (DDR), glycoprotein VI, osteoclast-associated receptor (OSCAR), leukocyte-associated immunoglobulin-like receptor 1 (LAIR-1), and uPARAP/Endo180—plays a crucial role in regulating cell adhesion, proliferation, and differentiation²³. These properties make collagen a vital substrate for *in vitro* cell cultures, especially as most cells from normal tissues exhibit anchorage dependence, requiring attachment to a surface for survival and growth.

For decades, bovine and porcine sources have served as the primary origins of xenogeneic collagen in biomedical applications²⁴. However, the use of these animal-

derived collagens faces significant limitations, including concerns over the transmission of infectious diseases and religious concerns. Jellyfish-derived collagen has emerged as a promising alternative, offering a unique solution to these limitations. Studies suggest that jellyfish collagen can elicit similar cellular responses to mammalian collagen, including cell adhesion, proliferation, and migration²⁵. Notably, jellyfish collagen has been shown to induce a long-term anti-inflammatory macrophage response, a critical factor for minimizing adverse immune reactions and supporting tissue regeneration²⁶. Furthermore, *in vivo* studies have demonstrated that jellyfish collagen promotes favorable vascularization, which is essential for effective bone tissue regeneration²⁷.

In this study, *in vitro* cytotoxicity tests were conducted on L-929 fibroblasts and MC3T3 pre-osteoblasts using well plates coated with jellyfish collagen, bovine collagen, and porcine collagen. The aim of this study is to evaluate the suitability of Jellyfish collagen as a culture substrate for *in vitro* biocompatibility testing by comparing its performance to that of bovine and porcine collagens.

3.1.2 Materials and Methods

Jellagen® (a jellyfish collagen derived from *Rhopilema pulmo*) was utilized to prepare collagen-coated well plates. A combination of cell viability (XTT assay), cytotoxicity (LDH assay), and cell proliferation (BrdU assay) analyses were conducted in accordance with the protocols outlined in DIN EN ISO 10993-5: 2009/-12: 2012 to assess cellular compatibility. Additionally, LIVE/DEAD staining was conducted to observe the growth status of L-929 fibroblasts on well plates coated with different types of collagen. For more specific details on the preparation and evaluation of the materials, refer to the work published by Ren et al.²⁸.

3.1.3 Results

The cell viability of L929 fibroblasts and MC3T3 pre-osteoblasts was assessed using the XTT assay (Figure 1A and D). Results indicated that viability was significantly higher for both L929 and MC3T3 cells cultured on Jellagen® collagen-coated plates compared to those on porcine collagen-coated plates (##### $p < 0.0001$) (Figure 1A). The viability values for the other two groups were close to the 70% threshold defined by the DIN ISO standard. Additionally, the MC3T3 cell viability on the Jellagen® collagen coating was notably higher than that on the porcine collagen coating. BrdU assay results showed significantly higher L929 cell proliferation on the Jellagen® collagen coating compared to the porcine collagen coating group (## $p < 0.01$, Figure 1B and E). However, MC3T3 cell proliferation on the Jellagen® coating was significantly lower than on both the porcine and bovine collagen coatings. Furthermore, cytotoxicity analysis via LDH assay revealed that L929 cells on the Jellagen® collagen coating exhibited significantly lower cytotoxicity values compared to those on the porcine and bovine collagen coatings (## $p < 0.01$ and # $p < 0.05$, respectively). In contrast, MC3T3 cells displayed significantly higher cytotoxicity on the Jellagen® collagen coating (Figure 1C and F).

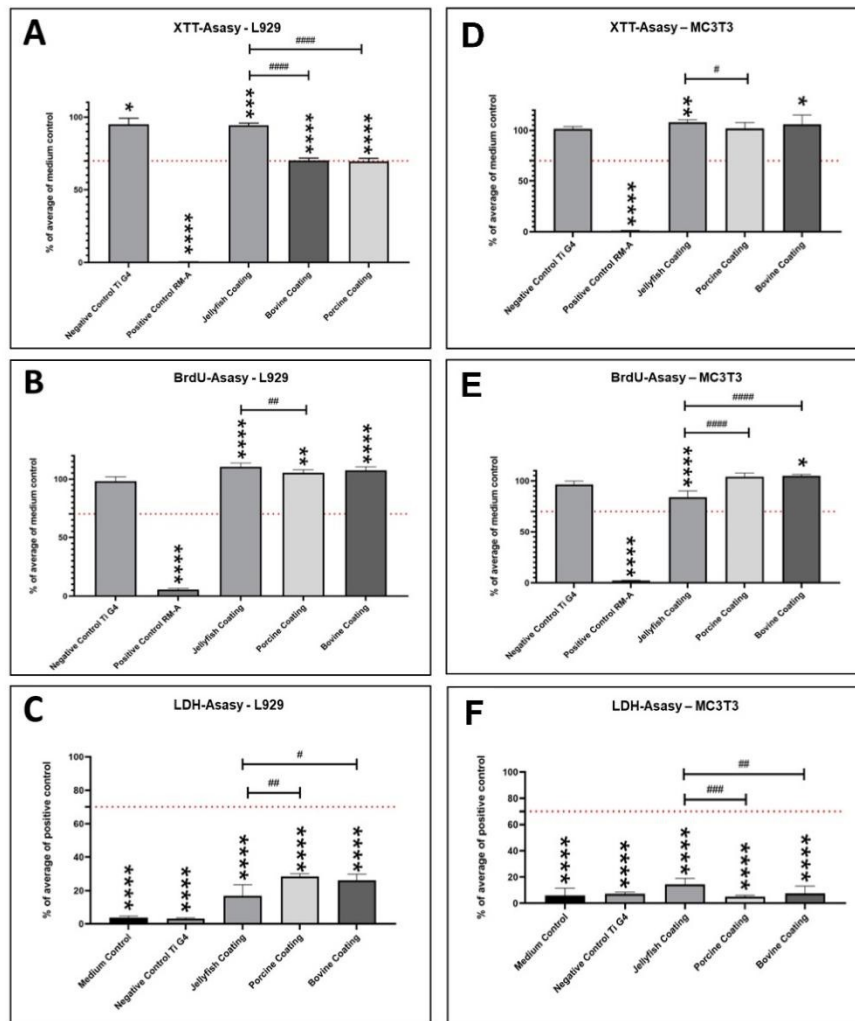


Figure 1. Cytocompatibility analysis of various coatings on L929 fibroblasts and MC3T3 pre-osteoblasts. Dotted lines represent the respective thresholds that should not be exceeded (LDH) or fall below (XTT, BrdU). Significant differences are indicated as follows: #/ $*$ $p < 0.05$, ##/ $**$ $p < 0.01$, ###/ $***$ $p < 0.001$, ####/ $****$ $p < 0.0001$.

3.1.4 Discussion

In this study, the co-culture of L929 or MC3T3 cells with jellyfish, porcine, and bovine collagen-coated well plates provided important insights into the effects of different collagen sources on cell biocompatibility. XTT assay results showed that the viability of L929 cells decreased in all collagen groups compared to the medium control. Although the value of cell cytotoxicity was significantly higher in all collagen groups than that in the medium control, Jellagen® collagen coatings exhibited lower cytotoxicity relative to porcine and bovine collagen coatings. This difference may be attributed to the variation in fibrillar precipitation types resulting from distinct collagen sources and their processing methods. Moreover, LIVE/DEAD staining revealed that L929 cells adhered in smaller numbers to the porcine collagen-coated surface compared to other collagen groups, suggesting differential adhesion kinetics based on collagen type. Taking together, these findings indicate that Jellagen® collagen coatings offer more favorable conditions for L929 cell adhesion, growth, and proliferation compared to the other collagen sources tested.

The MC3T3 cell line, widely regarded as a robust model for evaluating cytocompatibility and *in vitro* osteogenesis, also exhibited differential responses to the collagen coatings. While all collagen groups supported adequate MC3T3 cell viability, proliferation, and cytotoxicity, the Jellagen® group demonstrated significantly lower proliferation and higher cytotoxicity compared to the porcine and bovine collagen groups. However, it is essential to interpret these findings with caution, as previous studies have shown that Jellagen® collagen 3D scaffolds yield minimal cytotoxicity for MC3T3 cells, even outperforming medium and negative controls ²⁹. This discrepancy may be related to differences in experimental setups, such as 2D versus 3D environments, which can greatly influence cell behavior and responses.

In conclusion, this study demonstrates that Jellagen® collagen (derived from *R. pulmo*, a species of jellyfish) exhibits excellent cytocompatibility with L929 fibroblasts and MC3T3 pre-osteoblast cells, comparable to that of porcine and bovine collagen. These findings suggest that Jellagen® collagen can serve as a viable alternative for *in vitro* cytocompatibility assessment of biomaterials or medical devices.

3.2 *In Vivo* Analysis of the Immune Response to Strontium- and Copper-doped Bioglasses

3.2.1 Introduction

Bioactive glass has garnered significant attention as bone substitute materials (BSMs) for its unique properties, offering a combination of bioactivity, biocompatibility, and mechanical adaptability. Bioactive glass is characterized by its ability to bond directly with bone tissue, a feature that distinguishes it from many other synthetic biomaterials. This bonding occurs due to the formation of a hydroxycarbonate apatite (HCA) layer on the glass surface when it comes into contact with body fluids, mimicking the mineral phase of natural bone ³⁰. One of the most valuable characteristics of bioactive glass is its capacity to serve as a carrier for a wide range of bioactive molecules and ions ³¹.

45S5 bioactive glass (commonly known as Bioglass®) is one of the most widely studied and utilized in clinical applications. It exhibits a high degree of bioactivity, with the ability to rapidly form an HCA layer upon exposure to physiological environments ³². Despite its many advantages, 45S5 bioactive glass presents some challenges related to its processing window and tendency to crystallize during thermal treatments ³³. The narrow processing window means that precise control over temperature is required during the fabrication process to prevent crystallization, which can significantly reduce the material's bioactivity and mechanical properties. In response to these limitations, Elgayar and colleagues developed ICIE16 bioactive glass ³⁴. ICIE16 contains a higher percentage of silica (SiO₂), leading to the formation of a more stable silicate network that reduces the risk of crystallization during thermal processing ³⁵. The increased SiO₂ content also imparts greater network flexibility, allowing for easier incorporation of various additives.

To explore the further potential of ICIE16 bioactive glass for BTE, this study builds on its composition by introducing 5 wt% copper (CuO) or strontium (SrO), producing two new bioactive glasses: ICIE16-Cu and ICIE16-Sr. For comparison, pure ICIE16 and

45S5 bioactive glass were used as control materials in the study. The primary aim of this investigation is to assess the effect of strontium and copper doping on the induction of M1 and M2 macrophages, as well as vascularization.

3.2.2 Materials and Methods

Four different bioactive glass systems were synthesized using the melt-quenching method, including 45S5 (composition by weight: 45% SiO₂, 24.5% CaO, 24.5% Na₂O, and 6.0% P₂O₅), ICIE16 (composition by weight: 48.0% SiO₂, 6.6% Na₂O, 32.9% CaO, 2.5% P₂O₅, 10.0% K₂O), and ICIE16 doped with 5 wt% copper (ICIE16-Cu) or 5 wt% strontium (ICIE16-Sr). 45S5 and ICIE16 were used as control materials. The synthesized bioactive glasses were characterized for their surface morphology using scanning electron microscopy (SEM), and the chemical composition was analyzed using energy-dispersive X-ray spectroscopy (EDX). Inductively coupled plasma optical emission spectrometry (ICP-OES) was employed to measure the release of metal ions in simulated body fluid, with measurements taken on days 3, 7, and 14 for each sample.

The four prepared bioactive glass materials were then implanted into a standardized rat subcutaneous model to evaluate their tissue response. A total of 32 female rats (3 to 4 months old, weighing 255±15 grams) were selected for the subcutaneous implantation study and randomly divided into groups based on bioactive glass type and time points (n=4 per group at each time point). Histopathological and histomorphometric evaluations were performed on the materials, following the method described by Lindner et al., with a particular focus on the number of macrophages and patterns of vascularization. Detailed protocols for material preparation and characterization can be found in the published work by Barbeck et al.³⁶

3.2.3 Results

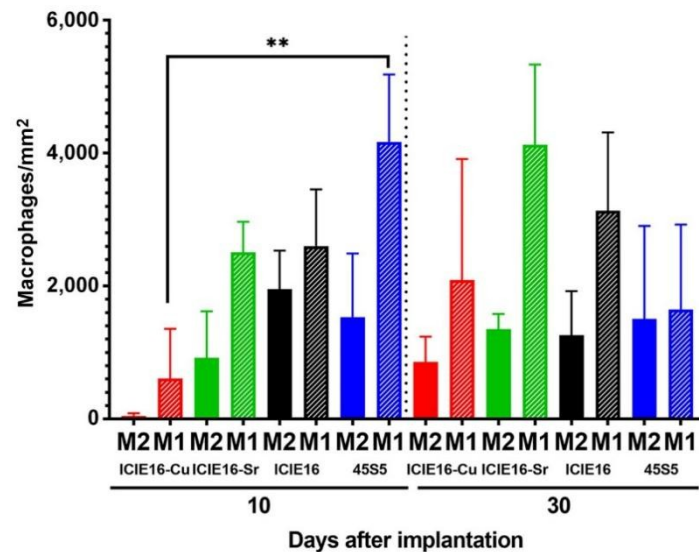


Figure 2. Histomorphometric analysis of pro-inflammatory and anti-inflammatory (M1 and M2) macrophage subtypes (cells/mm², n=4, significance ***p* < 0.01).

Figure 2 shows the histomorphometric results for macrophages. On day 10 post-implantation, ICIE16-Cu group exhibited fewer macrophages in the implant bed compared to the other groups, particularly CD163-positive M2 macrophages. Additionally, the number of M1 macrophages in this group was significantly lower than that in the 45S5 group (** $p < 0.01$). No significant differences in macrophage subtype occurrence were observed between the other study groups. By day 30 post-implantation, the number of M1 macrophages increased in the ICIE16-Sr and pure ICIE16 groups and was higher than the number of CD163-positive M2 macrophages. In contrast, both macrophage subtypes were present in comparable numbers in the ICIE16-Cu and 45S5 bioglass groups.

Figure 3 shows the vascularization results induced by the implanted bioglass in the implantation bed. At both 10- and 30-days post-implantation, although no statistically significant differences were observed, the ICIE16-Cu group exhibited the lowest number of blood vessels. The other three groups showed higher levels of vascularization, with no significant differences between them. Additionally, the comparable vascularization of all groups was observed at both time points.

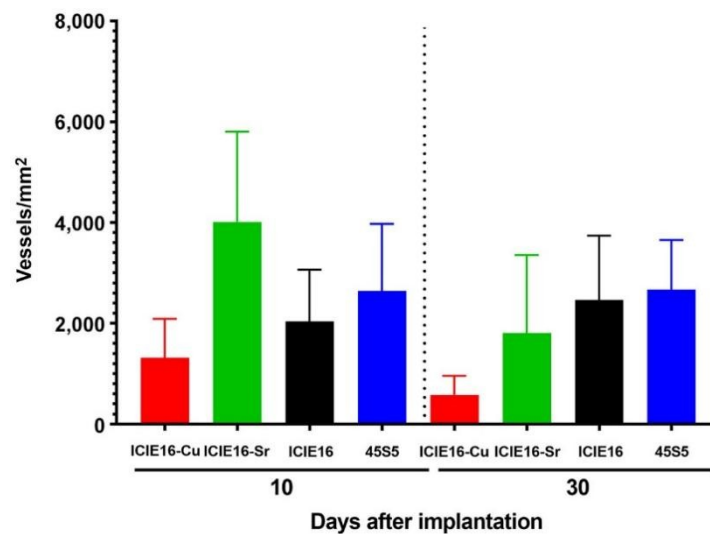


Figure 3. Histomorphometric analysis of vessel density (vessels/mm², n=4).

3.2.4 Discussion

In this publication, a rat subcutaneous implantation model was utilized to assess the effects of strontium and copper-doped bioactive glasses on macrophage activation and vascularization. Findings revealed that all ICIE16 groups exhibited a decrease in granulation tissue intensity and phagocytic cell numbers, along with mild fibrosis. Conversely, the 45S5® bioactive glass group demonstrated the highest levels of M1 macrophages, suggesting that 45S5® more effectively elicited an immune response linked to biodegradation. These results were further supported by histomorphometrical analysis, particularly on day 10 post-implantation, when the accumulation of M1

macrophages was significantly higher in the 45S5 group compared to the ICIE16-Cu group. This could potentially be attributed to a temporary rise in pH levels during the early stages of 45S5 implantation induced by its higher sodium content. Another possible explanation for the observed tissue response pattern could be the broader sintering window of ICIE16 bioactive glass, which may result in a lower crystallization tendency, potentially influencing tissue responses. In this context, the initial binding of proteins to the surface of biomaterials is crucial for initiating the foreign body reaction and coordinating subsequent tissue responses ³⁷. The surface characteristics of biomaterials determine the types of proteins that bind and their conformation, which exposes specific binding sites for different cell receptors and cell types, thus driving the foreign body reaction cascade ¹⁷.

Although copper ions have been reported to have a positive effect on angiogenesis, no significant differences in vascularization were observed at the implant site in this study. Neither the ICIE16-Cu nor the ICIE16-Sr groups enhanced the vascularization of the implants, which contradicts predictions based on the angiogenic potential of copper observed *in vitro*. This result is surprising, as both ions have been described as influencing molecular cascades related to immune responses and angiogenesis, but it aligns with another *in vivo* study, which also analyzed copper-doped silicate-based materials and found no impact ³⁸.

Overall, the bioactive glass materials investigated demonstrated biocompatibility with a foreign body response typical of synthetic bone substitute materials (BSM). However, given the prolonged process of bone regeneration, additional studies are required within the bone microenvironment to fully assess the osteogenic potential of these ion-doped bioactive glass materials.

3.3 Biphasic Bone Substitutes Coated with PLGA incorporating therapeutic ions Sr^{2+} and Mg^{2+} : Cytotoxicity Cascade and *In Vivo* Response of Immune and Bone Regeneration

3.3.1 Introduction

Biphasic calcium phosphate (BCP), a composite material consisting of hydroxyapatite (HA) and β -tricalcium phosphate (β -TCP), has gained widespread use as a bone substitute material due to its exceptional biocompatibility and its structural resemblance to natural bone ³⁹. Hydroxyapatite (HA) is the primary inorganic component of human teeth and bones, well-known for its impressive mechanical strength and stability within biological environments. However, one of the limitations of HA is its slow degradation rate. When implanted, HA can persist in the body for several years, potentially impeding complete bone regeneration and delaying the replacement of the biomaterial by native bone ⁴⁰. In contrast, β -TCP is a highly bioresorbable and biocompatible material that typically degrades completely within 13 to 20 weeks post-implantation ⁴¹. Given these opposing characteristics, researchers have combined HA and β -TCP to create biphasic bone substitutes (BBS). The combination of HA and β -

TCP in BBS enables a synergistic effect, where the slower degradation of HA provides long-term structural support, while the faster resorption of β -TCP facilitates early-stage bone healing ⁴².

One promising avenue for improving BSMs is the incorporation of bioactive ions such as magnesium (Mg^{2+}), zinc (Zn^{2+}), copper (Cu^{2+}), and strontium (Sr^{2+}). In addition to ion incorporation, the surface modification of BSMs with polymer coatings represents another promising strategy for enhancing the biological performance of bone substitutes ⁴³. Among the various polymers, poly(lactic-co-glycolic acid) (PLGA) stands out as an FDA-approved biodegradable material with excellent biocompatibility and mechanical properties ⁴⁴.

In this study, the biocompatible biphasic calcium phosphate material Maxresorb® was used as the base to create several variants incorporating combinations of Sr^{2+} and Mg^{2+} ions, along with PLGA coatings. Pure Maxresorb® served as the control group. The primary aim of this investigation is to explore the effects of therapeutic ion doping on bone regeneration patterns and inflammatory responses.

3.3.2 Materials and Methods

In this study, the biphasic bone substitute (BBS) material Maxresorb® (Botiss biomaterials GmbH, Berlin, Germany), consisting of 60% hydroxyapatite (HA) and 40% β -tricalcium phosphate (β -TCP), was used as the base inorganic material. The BBS was further functionalized with bioactive cations (Sr^{2+}/Mg^{2+}) and PLGA coatings through a dip-coating process. Cytotoxicity of the eluates from 18 BBS variants was assessed using XTT assays with L929 and NHOst cells, following the DIN EN ISO 10993-5 guidelines. Two variants, BBS_PLGA_ Sr^{2+}/Mg^{2+} and BBS_ Sr^{2+} , demonstrated satisfactory cytocompatibility and were selected for further material characterization and *in vivo* studies. Surface morphology, chemical composition, and release kinetics of the selected variants were characterized using scanning electron microscopy with energy-dispersive X-ray spectroscopy (SEM-EDX), Fourier transform infrared spectroscopy (FTIR-ATR), X-ray diffraction (XRD), and inductively coupled plasma optical emission spectroscopy (ICP-OES).

A total of 42 male Wistar rats, aged 10 to 12 weeks, were randomly assigned to two study groups: BBS_ Sr^{2+} and BBS_PLGA_ Sr^{2+}/Mg^{2+} . At three designated time points (10, 30, and 90 days), Wistar rats ($n = 7$ per group) were sacrificed for analysis. Histopathological and histomorphometric assessments were then conducted to examine the implanted materials, following the methodologies described by Lindner et al. and Ghanaati et al.⁴⁵. The analyses primarily focused on bone regeneration, the presence of macrophages, multinucleated giant cells, and vascularization patterns within the implant site. Additionally, correlation coefficients were calculated to explore the relationship between BBS and bone regeneration. For more specific details on the preparation and characterization of the materials, refer to the work published by Ren et al.⁴⁶

3.3.3 Results

As shown in Figure 4A, significant increases in bone formation were observed over time within the groups. All groups demonstrated significantly enhanced bone regeneration by day 90 compared to day 10, (## $p < 0.01$, ##### $p < 0.0001$, Figure 4A). By day 30 post-implantation, a significant increase in bone formation was detected exclusively in the BBS_Sr²⁺ group when compared to day 10 (# $p < 0.05$, Figure 9A). At day 90 post-implantation, both the control BBS group and the BBS_PLGA_Sr²⁺/Mg²⁺ group exhibited a notable rise in bone regeneration relative to day 30 (# $p < 0.05$ and ## $p < 0.01$, Figure 4A). Interestingly, bone regeneration was comparable across all experimental groups at each respective time point. Additionally, osteoconductivity analysis showed no significant differences between groups at any time point, but a significant increase was observed in the BBS and BBS_PLGA_Sr²⁺/Mg²⁺ groups at day 90 compared to day 10.

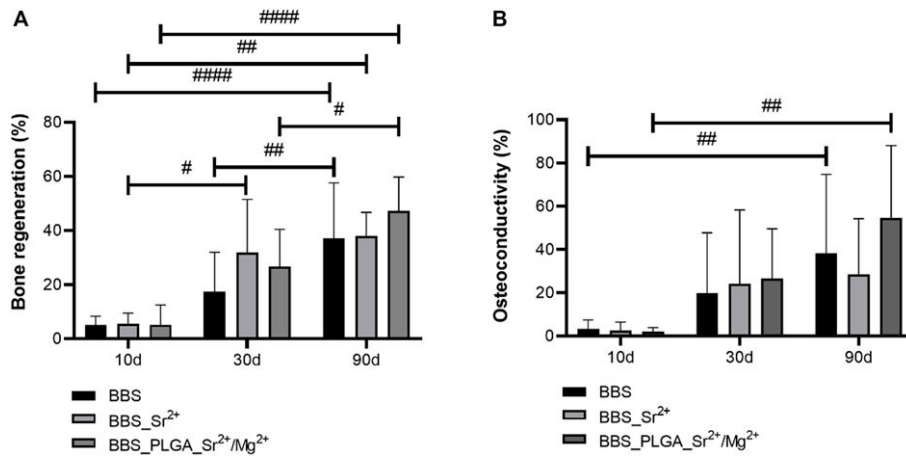


Figure 4. Histomorphometric evaluation of (A) bone regeneration and (B) osteoconductivity (intraindividual significance levels: # $p < 0.05$, ## $p < 0.01$, ##### $p < 0.0001$).

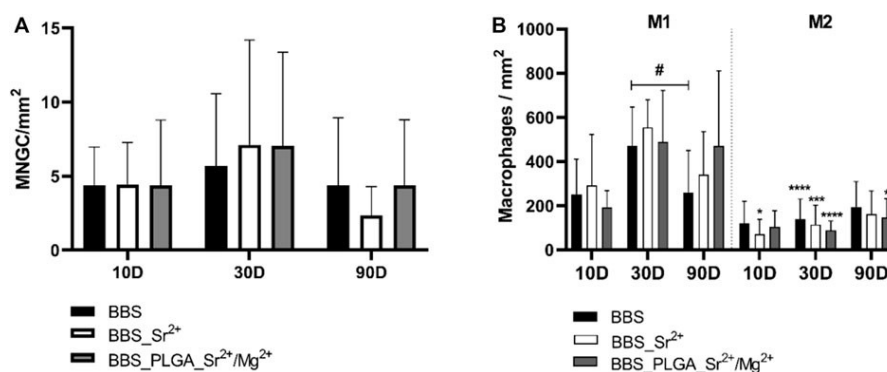


Figure 5. Histomorphometric evaluation of (A) CD11c-positive multinucleated giant cells and (B) pro-inflammatory (M1) and anti-inflammatory (M2) macrophage subtypes (intraindividual comparisons: #/* $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$, with *denoting significant differences between M1 and M2).

Figure 5A exhibited the histomorphometric evaluation of MNGC. By day 30 post-implantation, all study groups exhibited an upward trend in the presence of CD11c-positive multinucleated giant cells when compared to day 10. This trend was followed by a decline at day 90, with the most pronounced reduction observed in the BBS_Sr²⁺ group. Analysis of macrophage subtypes revealed that a predominantly pro-inflammatory immune response was induced in all groups at each time point (Figure 5B). In particular, at day 30 post-implantation, the number of pro-inflammatory macrophages was significantly greater than the anti-inflammatory subtype in all groups (***p < 0.001, ****p < 0.0001, Figure 5B). At day 10 post-implantation, the BBS_Sr²⁺ group exhibited a significantly higher count of pro-inflammatory macrophages compared to anti-inflammatory ones, while at day 90, this pattern was observed in the BBS_PLGA_Sr²⁺/Mg²⁺ group (*p < 0.05). Notably, only in the BBS group was there a significant reduction in pro-inflammatory macrophages at day 90 relative to day 30 (##p < 0.01, Figure 5B).

3.3.4 Discussion

In this publication, the biocompatibility and bone regeneration potential of biphasic calcium phosphate loaded with Sr²⁺/Mg²⁺ ions and coated with PLGA were investigated in a rat calvarial model. Among the 18 initially prepared BBS variants, two groups, BBS_PLGA_Sr²⁺/Mg²⁺ and BBS_Sr²⁺, exhibited satisfied NH₄Ost cell viability in XTT assay and were selected for further physicochemical experiments and *in vivo* studies. Pure BBS group was served as control group. SEM and EDX analyses confirmed the successful incorporation of magnesium and strontium ions into the BBS. The *in vivo* histopathological analysis revealed a consistent moderate foreign body reaction across all groups of implanted bone substitute particles. Similar to previous studies on bioglass, this response was predominantly driven by M1 macrophages. Inflammatory cells, including macrophages, express molecules like vascular endothelial growth factor (VEGF), which alongside vascularization at the implant site, supports the foundation for tissue regeneration^{47,48}. Based on these observations, the occurrence of multinucleated giant cells (MNGCs) was also examined. Histomorphometric analysis showed a trend towards increased MNGC numbers across all groups by day 30 post-implantation, comparable to the M1 macrophage response, although no significant differences were found within or between individuals. Correlation analysis further indicated a negative relationship between MNGCs macrophages and osteoconductivity on day 30, whereas M2 were found to be positively correlated with osteoconductivity at the same time point. These findings suggest that pro-inflammatory cells, such as macrophages and MNGCs, are mainly involved in the degradation of materials, while anti-inflammatory macrophage phenotypes likely play a role in regulating molecular cascades that promote tissue regeneration.

Bone regeneration analysis demonstrated effective and comparable bone regeneration in all groups. As expected, gradual bone formation was observed in all test groups up to 90 days post-implantation, with significant increases compared to day 10. Interestingly, the BBS_Sr²⁺ group showed earlier bone regeneration, reaching a plateau by day 30, while the BBS and BBS_PLGA_Sr²⁺/Mg²⁺ groups exhibited more

pronounced regeneration in the later stage. However, since all groups displayed similar macrophage responses, these results may be incidental, particularly given the potential involvement of anti-inflammatory macrophages in tissue repair. Neither the BBS_Sr²⁺ group nor the BBS_PLGA_Sr²⁺/Mg²⁺ group showed a significant effect on final bone regeneration or macrophage response when compared to the BBS group. This observation contradicts the initial hypothesis but aligns with previous research involving bioglasses loaded with copper and strontium ions. Metal ion concentration may be an explanation of this phenomenon. Studies by Shen et al. demonstrated that higher strontium doping levels promote M2 macrophage polarization and rapid bone formation, whereas lower concentrations, such as 0.1 mmol/L of strontium, showed no significant effect on hMSC proliferation or osteogenic differentiation^{49,50}. This suggests that the metal ion concentrations used in the present study may not have been optimal for modulating bone remodeling. Future studies should investigate the impact of different metal ion concentrations on immune responses and tissue regeneration.

3.4 Analyses of the Cellular Interactions between the Ossification of Collagen-Based Barrier Membranes and the Underlying Bone Defects

3.4.1 Introduction

In recent years, several studies have suggested an ideal membrane should not only act as a barrier but also actively contribute to the regeneration process by creating a favorable environment for bone healing⁵¹. In this context, research has emphasized that the molecular microenvironment induced by the membrane can influence the regenerative processes of the surrounding bone tissue^{12,13}. This effect is particularly relevant when considering the inflammatory response following implantation, which is a key factor in determining the success of bone regeneration. For instance, natural collagen membranes, derived from various animal and tissue sources, have been shown to elicit a stronger anti-inflammatory response¹⁸. This response is often characterized by an increase in M2 macrophages, a cell type associated with tissue repair and regeneration.

Among the various sources of collagen, membranes derived from porcine pericardium have demonstrated particularly promising results. These membranes have been shown to induce a mild and predominantly anti-inflammatory tissue reaction in the implantation bed⁵². This reaction not only minimizes the risk of chronic inflammation but also supports key processes such as cell migration, which are essential for tissue regeneration. Furthermore, porcine pericardium-based membranes have demonstrated superior durability, maintaining their structural integrity for several months post-implantation without evidence of degradation or fragmentation⁵³.

Building on these findings, this study aims to further explore the biological properties of collagen membranes. A standardized rat calvarial defect model was employed to investigate the cellular interactions between the ossification of collagen-based barrier membranes derived from pericardium and the underlying bone defects.

3.4.2 Materials and Methods

In this study, a pericardium-based collagen membrane (Jason® membrane, Botiss Biomaterials GmbH, Zossen, Germany) was combined with the bone substitute material cerabone® plus (Botiss Biomaterials GmbH, Zossen, Germany) to cover and fill cranial bone defects. A total of 21 male Wistar rats (N = 7 per group), aged 10–12 weeks and weighing 230 ± 20 grams, were selected for standardized cranial implantation surgery in accordance with the DIN ISO 10993-6 guidelines. The animals were randomly assigned to three experimental groups, with assessments conducted at 2-, 8-, and 16-weeks post-implantation.

Histopathological and histomorphometric analyses were performed to evaluate the implanted materials, following the protocol outlined by Lindner et al.⁴⁵ These evaluations focused primarily on bone regeneration, macrophages and vascularization patterns within the implantation bed. For further details regarding the preparation and characterization of the materials used, refer to the published work by Alkildani et al.⁵⁴.

3.4.3 Results

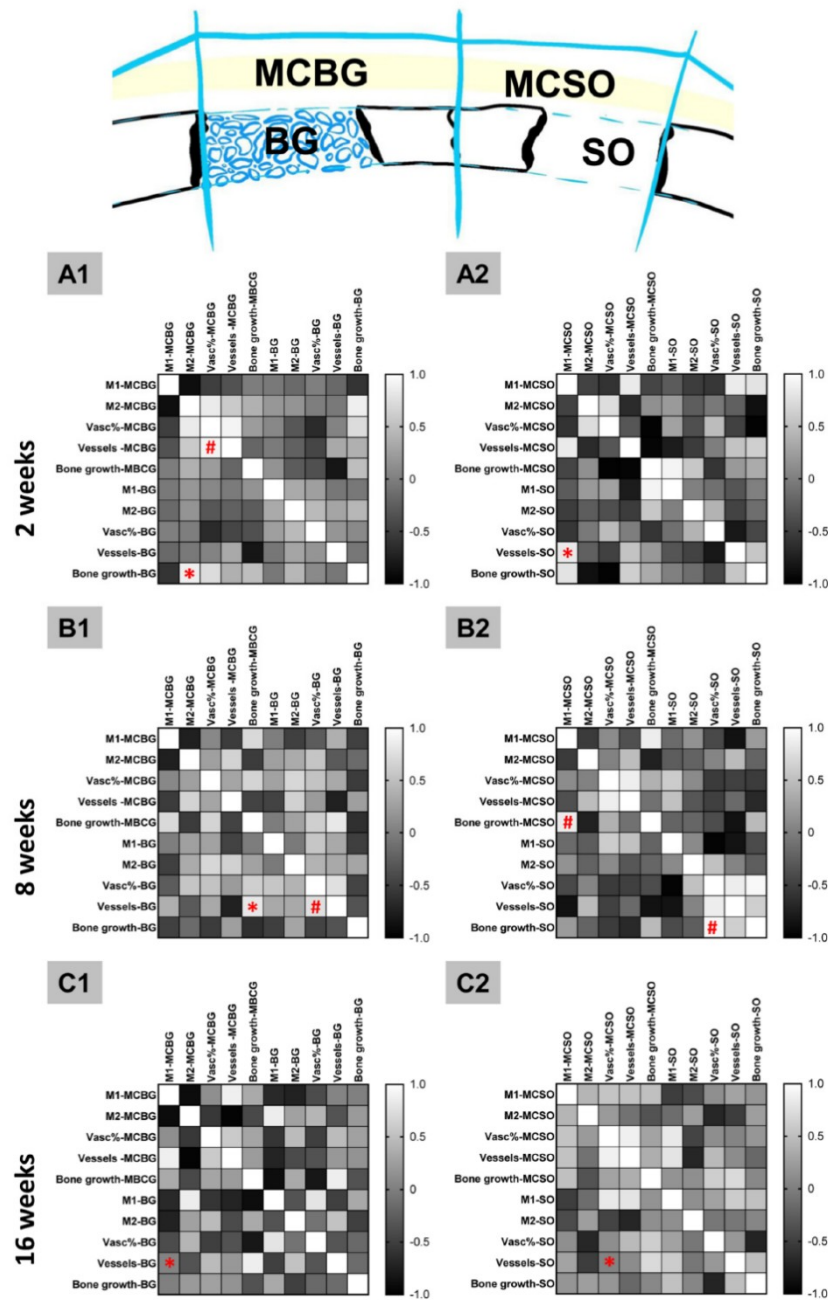


Figure 6. Correlation matrices of all measured variables within and between the ipsilateral compartments at 2-, 8-, and 16-weeks post-implantation. Left columns (A1 – C1): correlation matrices within and between the MCBG and BG compartments. Right columns (A2 – C2): correlation matrices within and between the MCSO and SO compartments. Asterisks indicate significant differences between compartments, while pound signs indicate significance within compartments. MCBG: membrane-covered compartment over the bone graft-filled defect; MCSO: membrane-covered compartment over the sham-operated defect; BG: bone graft-filled defect; SO: sham-operated bone defect.

Pearson correlation analysis was conducted to investigate positive correlations between two measured parameters within and between ipsilateral compartments (i.e.,

MCBG and BG compartments or MCSO and SO compartments). At 2 weeks post-implantation, a positive correlation was observed between bone growth in the BG compartment and the presence of M2 macrophages in the MCBG compartment ($p = 0.023$) (Figure 6). Additionally, vascular density in the SO compartment was found to be positively correlated with the presence of M1 macrophages in the MCSO compartment ($p = 0.041$) (Figure 6-A2). By 8 weeks post-implantation, a significant positive correlation was detected between vascular density in the BG compartment and bone growth in the MCBG compartment ($p = 0.009$) (Figure 6-B1). Moreover, bone growth in the MCSO compartment was positively correlated with the presence of M1 macrophages in the same area ($p = 0.017$) (Figure 6-B2). Additionally, a positive correlation between bone growth and the percentage of vascularization in the SO compartment was observed ($p = 0.018$) (Figure 6-B2). At 16 weeks post-implantation, vascular density in the BG compartment was positively correlated with the presence of M1 macrophages in the MCBG compartment ($p = 0.021$) (Figure 6-C1).

3.4.4 Discussion

In this published study, the bioactivity of collagen barrier membranes was examined using a rat calvaria defect model, focusing on their role in promoting bone regeneration in the underlying defects. Both histological and histomorphometric analyses showed comparable bone formation in the membrane compartments at later stages of implantation. During the implantation period, the collagen membrane was progressively resorbed and replaced with bone tissue or matrix. Remarkably, a distinct bone layer, referred to as an "ossification membrane," was observed to form over the defect. This ossification of the collagen membrane was an unexpected outcome, with only a limited number of studies reporting similar findings. A plausible explanation is that the collagen matrix promotes bone growth, acting both as an osteoconductive scaffold and as a supportive framework for osteogenesis. The pericardium collagen membrane analyzed in this study showed the formation of "ossification nuclei" accompanied by active osteoblasts and matrix deposition, primarily occurring along the collagen fiber bundles, similar to physiological endochondral ossification. In this context, it is conceivable that the natural pericardium collagen membrane tested in this study demonstrated a combination of osteoconductive and osteogenic properties, potentially accelerating bone regeneration within the defect area. However, in studies like Zubery *et al.*, natural collagen membranes used as controls did not induce similar ossification processes^{55,56}. Additionally, the ossification of ribose-crosslinked collagen membranes tested in that study occurred only in areas directly in contact with residual bone. Thus, further studies are needed to validate the mechanisms underlying collagen membrane ossification and to assess its effects on bone regeneration in the defect area beneath the membrane.

The correlation analysis provided further insight into how the molecular microenvironment created by the barrier membrane influences adjacent bone regeneration. At 8 weeks post-implantation, a relationship was identified between bone growth and the percentage of vascularization within the SO compartment. This finding aligns with the well-established role of vascularization as a fundamental process in

bone regeneration, ensuring the delivery of essential nutrients, growth factors, minerals, oxygen, and cells needed for tissue repair ²¹. Interestingly, at this same time point, bone growth also correlated with the presence of M1 macrophages in the MCSO compartment, which was unexpected. Typically, biomaterials that elicit an anti-inflammatory response are considered more conducive to (bone) tissue regeneration ¹⁹. Nevertheless, this correlation may be explained by the vascularization pattern, where M1 macrophage-driven biodegradation linked to the membrane also associates with the formation of new vasculature, potentially supporting bone growth ⁵⁷. Similar correlations were observed during weeks 2 and 16, where vascular density in the SO compartment corresponded with the presence of M1 macrophages in the MCSO compartment.

Additionally, at 8 weeks, a notable correlation was found between vascular density in the BG compartment and bone formation in the MCBG compartment. This is particularly interesting given that nutrient transport is constrained by limited diffusion distances, suggesting that direct effects between adjacent compartments are not easily explained. However, it is conceivable that an indirect relationship between vascularization and bone formation exists between these compartments. Ample vascularization could enable cell migration between compartments. Furthermore, at 16 weeks, a correlation was observed between the vascular density in the SO compartment and the percentage of vascularization in the MCSO compartment, suggesting that despite the physical separation imposed by the membrane, these vertically adjacent compartments may share a complex, interconnected vascular network. In conclusion, this study provides detailed evidence supporting the interaction between the barrier membrane and underlying bone regeneration. The molecular environment within the membrane compartments likely plays a potential role in promoting osteoconductive bone growth in neighboring implantation regions.

3.5 The influence of different crosslinking agents onto the physical properties, integration behavior and immune response of collagen-based barrier membranes

3.5.1 Introduction

One of the main limitations of collagen membranes is their relatively rapid degradation rate, which often occurs within a few days to several weeks post-implantation ⁵⁸. This rapid breakdown can compromise the barrier function of the membrane, potentially affecting the regenerative outcome. To overcome these limitations, researchers have focused on improving the physicochemical and biological properties of collagen membranes through cross-linking techniques. Cross-linking agents, including glutaraldehyde (GA), proanthocyanidins (PC), hexamethylene diisocyanate (HMDI), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/N-hydroxysuccinimide (EC/NHS), have been widely explored for their ability to enhance membrane stability ^{59–62}. These agents work by introducing covalent and non-covalent bonds between collagen molecules, thereby increasing resistance to enzymatic degradation and mechanical stress.

Despite the promising advancements in cross-linking techniques, the challenge remains to balance the improved stability of collagen membranes with their biocompatibility and biological function⁶³. Excessive cross-linking may alter the natural properties of collagen, leading to reduced cell adhesion or potential inflammatory responses. This study aims to explore the impact of various cross-linking methods on the structural and biological properties of collagen membranes, particularly focusing on tissue integration, angiogenesis and immune pattern. By comparing different cross-linking strategies, this research seeks to provide insights into developing next-generation barrier membranes with enhanced clinical potential for GBR and GTR applications.

3.5.2 Materials and Methods

Porcine-derived bilayer collagen barrier membranes were produced through a lyophilization technique and subsequently cross-linked using agents such as glutaraldehyde (GA), proanthocyanidins (PC), hexamethylene diisocyanate (HMDI), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/N-hydroxysuccinimide (EC/NHS). A non-crosslinked membrane was employed as a reference control (RF). The mechanical characteristics of these cross-linked membranes, including tensile strength, denaturation temperature, swelling behavior, and collagenase resistance, were evaluated.

An *in vitro* cytotoxicity test was then conducted, followed by (immune-) histochemical and histomorphometric analyses to examine the cellular inflammatory responses, tissue integration, and vascularization patterns at 10-, 30-, and 90-days post-implantation in a subcutaneous rat model. For more specific details on the preparation and characterization of the materials, refer to the work published by Ren *et al.*

3.5.3 Results

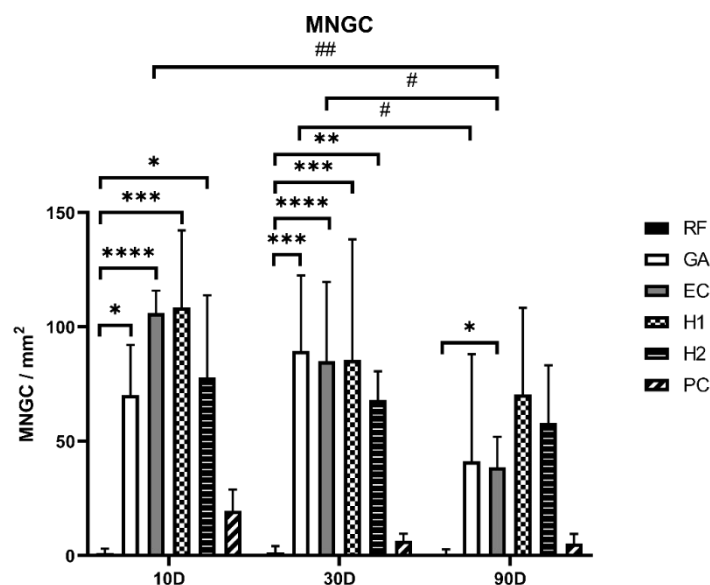


Figure 7. Histomorphometrical results of MNGCs within the implanted bed of the tested collagen membranes. Statistical significance: */# $p < 0.05$, **/## $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 7 showed the histomorphometrical results of MNGCs within the implanted bed. The PC membrane resulted in a slightly higher count of multinucleated giant cells (MNGCs) compared to the RF membrane group, though the difference was not statistically significant (Figure 7). Conversely, at both day 10 and day 30 post-implantation, all other cross-linked membranes elicited a significantly greater number of MNGCs relative to the RF group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). By day 90, only the EC and RF membrane groups showed a statistically significant difference (* $p < 0.05$).

Intraindividual analysis further revealed significant changes in MNGC counts within the GA and EC groups over time. In the EC group, the MNGC count showed a continuous decline throughout the study, with a marked reduction at day 90 compared to both day 10 and day 30 (# $p < 0.05$, ## $p < 0.01$). Similarly, in the GA group, MNGC levels observed at day 90 were significantly lower than those recorded at day 30 (# $p < 0.05$).

The interindividual comparison of histomorphometric analysis for macrophage subtype responses was presented in Figure 8. At 10 days post-implantation, the GA membrane, used as a positive control, triggered significantly higher levels of M1 macrophages compared to the RF membrane (**** $p < 0.0001$, Figure 8A). Similarly, the H1 and EC membranes also showed significantly elevated M1 macrophage levels compared to RF (*** $p < 0.001$, * $p < 0.05$). Additionally, M1 levels for the PC and H2 membranes were significantly lower than those observed in the GA group (•• $p < 0.001$, • $p < 0.01$). Notably, all cross-linked membranes, except RF, predominantly induced an M1 macrophage response (# $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, #### $p < 0.0001$), while the RF group displayed comparable levels of M1 and M2 macrophages.

At 30- and 90-days post-implantation, the M1 macrophage levels in the PC group were similar to those in the RF group, while all other cross-linked membranes exhibited significantly higher M1 levels compared to RF (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, Figure 8B). Furthermore, the M2 macrophage levels induced by the GA and H1 membranes were significantly lower than their corresponding M1 levels at 30 days. (# $p < 0.05$, ##### $p < 0.0001$). At 90 days post-implantation, the RF membrane group showed significantly higher M2 macrophage levels compared to M1 (## $p < 0.01$). In the PC group, both M1 and M2 macrophage levels were comparable at this time point, while in the other cross-linked membrane groups, M2 levels remained significantly lower than the corresponding M1 levels (# $p < 0.05$, ### $p < 0.001$, #### $p < 0.0001$).

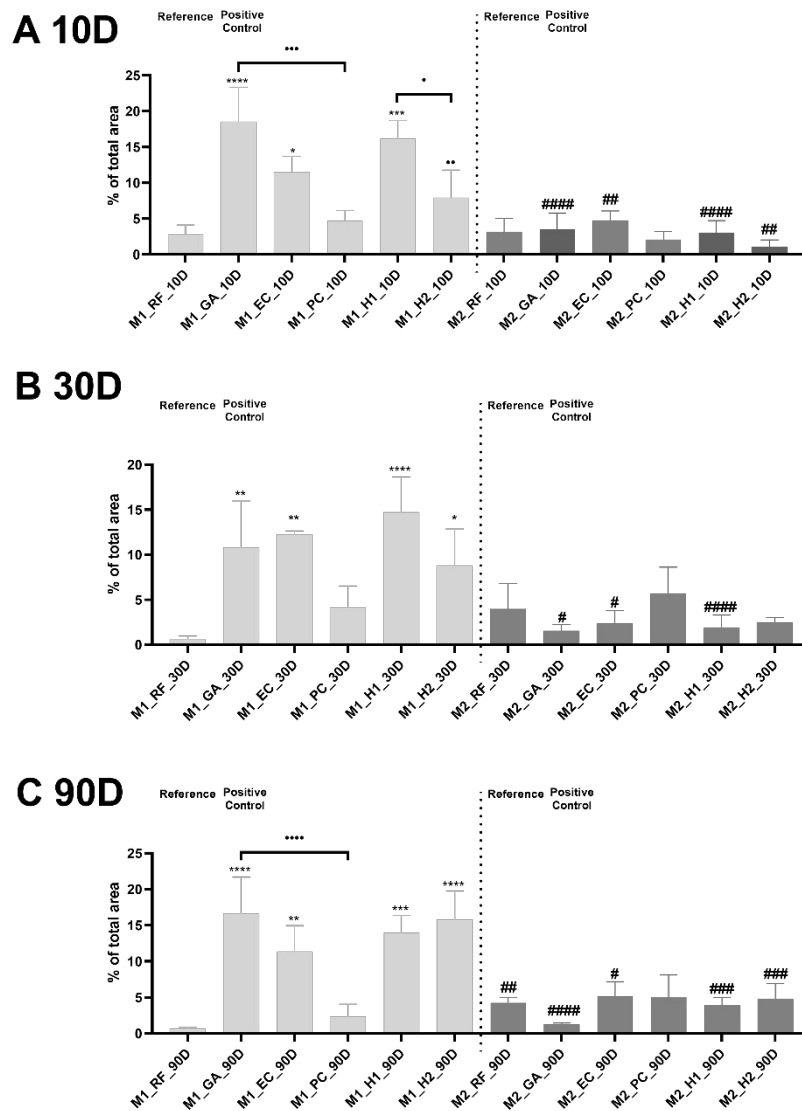


Figure 8. Interindividual comparison of histomorphometric analysis for macrophage subtype responses to the evaluated collagen membranes. Statistical significance across groups: */# $p < 0.05$, **/## $p < 0.01$, ***/### $p < 0.001$, ****/•••/##### $p < 0.0001$. * denotes comparison with the reference group (RF), while # indicates intraindividual significance between M1 and M2 macrophages.

3.5.4 Discussion

In this study, the biological and material properties of four collagen membranes, modified using different cross-linking agents, were evaluated through *in vitro*, *ex vivo*, and *in vivo* experiments using a rat subcutaneous implantation model. Compared to the RF group, all cross-linked membranes demonstrated enhanced enzymatic resistance and prolonged *in vivo* retention. Histopathological analysis indicated that the RF group primarily triggered a mononuclear cell response, suggesting that the native collagen structure in this group was more susceptible to degradation by mononuclear cells. In contrast, the cross-linked membranes, with the exception of the PC group, exhibited a robust tissue response characterized by a significant infiltration of multinucleated giant cells (MNGCs). Consistent with previous studies, the presence

of MNGCs and M1 macrophages correlated with the degradation of the membranes over time, resulting in fragmentation and structural collapse^{13,51,58}. This supports the established role of MNGCs and pro-inflammatory M1 macrophages in the degradation of implanted materials.

Among the crosslinked membranes, the EC group maintained structural integrity for up to 30 days, comparable to the GA group (negative control), while the other groups exhibited earlier breakdown behaviors. Notably, the collagen fibers in the EC group were surrounded by fibrous connective tissue, which limited the number of surrounding cells and enhanced vascularization. This characteristic might have contributed to the prevention of epithelial cell ingrowth, supporting the membrane's barrier function. However, fibrosis represents the final stage of the foreign body response, where surrounding tissues fully encapsulate the biomaterial, hindering further interaction. This suggests that membranes inducing such responses may be less suitable for guided bone regeneration (GBR) applications. Membranes cross-linked with low and high HDMI concentrations (H1 and H2, respectively) exhibited lower resistance to enzymatic degradation, as demonstrated in collagenase tests. The *in vivo* results confirmed these findings, membrane degradation in these two groups was observed as early as day 30 post-implantation. By day 90, only scarred remnants of the membranes were detected, surrounded by granulation or connective tissue, indicating the loss of barrier functionality in these groups. Compared to other cross-linked groups, the PC group demonstrated significantly fewer MNGCs and M1 macrophages, approaching levels observed in the RF group, due to their anti-inflammatory properties, effectively reduced the foreign body response *in vivo*. However, its *in vivo* durability was shorter than that of the EC and GA groups. This can be explained by its physical properties, as the PC membrane exhibited a higher elongation at break during tensile testing, indicating a higher susceptibility to mechanical deformation.

This study also revealed the membrane-induced vessel pattern in the implantation bed. The GA, EC, and H1 membranes exhibited significantly lower vascular density and area fractions compared to the RF group at 10 days post-implantation. This observation may be attributed to the elevated levels of M1 macrophages induced by these membranes. Previous research has highlighted that increased M2 macrophage activity or a more pronounced transition from M1 to M2 macrophages is typically associated with enhanced vascularization⁴⁷. However, this correlation is not universally applicable, as both M1 and M2 macrophages play essential regulatory roles in the angiogenic process⁵⁷. Therefore, the M2/M1 ratio may be a more reliable indicator than the absolute quantities of each macrophage subtype. The results from the PC group further support this notion, as despite similar M1 and M2 levels to those found in the RF group, the PC group exhibited a lower M2/M1 ratio and correspondingly reduced vascularization. In summary, the EC membrane demonstrated the highest potential for long-term barrier functionality as a potential GBR membrane. However, given the unique "fibrotic" tissue response observed, further investigation using bone implantation models is required to confirm its ability to support effective (bone) tissue regeneration.

4. Summary Discussion

Biocompatibility remains a primary focus in preclinical studies as the biological response of host tissues to an implanted material directly influences the overall success of GBR procedures¹⁴. A positive host response fosters proper tissue integration and enhances bone tissue regeneration, whereas negative outcomes—such as inflammation, fibrotic encapsulation, or implant failure—can hinder or entirely prevent the therapeutic effectiveness of the device¹⁹. Consequently, a comprehensive understanding of the biological interactions between the implant material and the surrounding tissue environment is essential for advancing GBR technologies. By investigating these interactions, researchers can more accurately predict and improve the biological performance of GBR devices, ultimately leading to more effective clinical outcomes in bone regeneration therapies.

In vitro testing is a foundational step in preclinical biocompatibility studies and provides an essential reference for subsequent *in vivo* trials. Given the ethical emphasis on the 3R principles for animal testing, *in vitro* methods for biomaterial evaluation have become increasingly important¹⁶. This shift has led to a growing focus on improving the predictive accuracy and quantitative capabilities of cell culture systems. As a key component of the extracellular matrix (ECM), collagen plays a critical role in creating a microenvironment that closely mimics *in vivo* conditions, promoting more accurate cell growth studies²⁴. The first study demonstrated that jellyfish-derived collagen offers distinct advantages in the culture of L929 fibroblasts and MC3T3 osteoblasts. This finding suggests that jellyfish collagen could serve as a viable alternative in *in vitro* biocompatibility testing, offering the potential to enhance the reliability of cell compatibility assessments.

While *in vitro* models are invaluable for early-stage screening and provide insights into cellular behavior in response to biomaterials, they fall short of capturing the full range of biological processes that ultimately determine the success or failure of an implant. Many studies have revealed significant discrepancies between *in vitro* and *in vivo* outcomes, highlighting the limitations of *in vitro* systems in replicating the complexity of biological environments⁶⁴. For instance, in the final study, glutaraldehyde (GA)-treated collagen membrane, which served as the positive control, exhibited strong cytotoxicity in *in vitro* tests. However, when implanted subcutaneously in a rat model, the same material induced only a moderate inflammatory response, with no adverse effects such as fibrotic encapsulation. This finding exemplifies the unpredictable nature of translating *in vitro* cytotoxicity results to *in vivo* contexts, particularly when evaluating tissue integration and long-term outcomes. As a result, *in vivo* experiments remain indispensable for evaluating critical outcomes such as tissue regeneration, integration, and the long-term biocompatibility of biomaterials.

Macrophages have been identified as key players in the foreign body response (FBR) triggered by implanted materials¹⁷. They exhibit remarkable plasticity, allowing them to adopt different functional phenotypes depending on the local microenvironment and specific signals they encounter¹⁹. This plasticity is most notably characterized by their ability to polarize into two primary phenotypes: the classically activated, pro-

inflammatory M1 macrophage and the alternatively activated, anti-inflammatory M2 macrophage. M1 macrophages are typically associated with the initiation of inflammation, promoting the release of pro-inflammatory cytokines and reactive oxygen species that can drive tissue damage and hinder implant integration. On the other hand, M2 macrophages play a more reparative role, facilitating tissue remodeling, promoting wound healing, and resolving inflammation¹⁸. The balance between these two macrophage phenotypes is therefore critical for determining the outcome of the FBR. As observed in the fourth and fifth studies, both the natural porcine pericardium and porcine skin collagen membranes induced an immune response predominantly characterized by M2 macrophages, which represents a typical immune pattern where a mild M1 macrophage reaction is initially triggered to participate in material degradation, followed by a transition to an M2 phenotype that facilitates tissue regeneration. This shift from M1 to M2 is crucial for creating a favorable environment for tissue repair and can be considered a paradigm of a beneficial immune response induced by implants.

In contrast, for the majority of non-natural implants, a pro-inflammatory tissue response is more commonly observed. In the implantation sites of metal ion-loaded bioactive glass and biphasic calcium phosphate, a significantly higher presence of M1 macrophages was detected. A similar immune profile was also noted in the study involving the cross-linked collagen membrane. This is typically regarded as an unfavorable or excessive foreign body reaction, indicating a more aggressive inflammatory response that could impede tissue regeneration⁶⁵. However, recent studies have shown that this pro-inflammatory M1 macrophage response may not be entirely detrimental. Instead, it has been increasingly associated with material degradation, particularly in the case of synthetic and bioactive implants^{46,48}. On the one hand, M1 macrophages exert oxidative stress and secrete lytic enzymes, such as nitric oxide and reactive oxygen species, which actively contribute to the breakdown of implanted materials⁶⁶. On the other hand, the localization of these macrophages on the surface of the implant suggests that they play a key role in the phagocytosis of material debris¹⁹.

Multinucleated giant cells (MNGCs), another marker of a strong foreign body response, are often found alongside high levels of M1 macrophages during the degradation process. The co-localization of MNGCs with the degrading materials, and their exclusive expression of pro-inflammatory cytokines, further supports the view that a "pro-inflammatory phenotype" is instrumental in material degradation. This relationship was particularly evident in the third study, which demonstrated a positive correlation between M1 macrophages and the degradation of metal ion-loaded BPC bone substitutes. These findings support a hypothesis that M1 macrophages may serve as precursors to MNGCs, which then fuse to further facilitate material breakdown. In this context, the early inflammatory phase could serve as a critical step in the overall remodeling process, ensuring that the implant is properly degraded and replaced by native tissue.

Interestingly, although many studies have reported the beneficial effects of specific metal ions in modulating immune responses and enhancing vascularization at the implantation site, the second study found that therapeutic ions such as copper (Cu) and strontium (Sr) did not produce any significant biological effects on immune response or vascularization. Similarly, in the cranial model used in the third study, neither magnesium (Mg) nor Sr influenced bone regeneration outcomes. These findings are surprising, as they contradict a substantial body of *in vitro* research that demonstrates the positive effects of these ions on tissue regeneration. This discrepancy may be due to the dose-dependent nature of metal ions, which exert different effects at varying concentrations. As a result, determining the optimal concentrations of metal ions for tissue regeneration remains an area for further investigation.

Collagen barrier membranes, commonly used with bone substitutes, face the challenge of potential rupture due to tissue infiltration and vascularization, which can compromise their barrier function and overall effectiveness⁵¹. Jason and BioGide membranes as the most clinically used membranes have demonstrated controlled tissue infiltration involving only mononuclear macrophages, which represents a beneficial tissue integration pattern for guided bone regeneration (GBR) membranes. However, in the final study, porcine collagen (PC) membranes induced a similar inflammatory response but exhibited rapid degradation, likely due to their higher extensibility. This highlights the importance of the membrane's physical properties in influencing its degradation *in vivo*.

Conversely, membranes treated with hexamethylene diisocyanate (HMDI), ethyl carbodiimide (EC), and the GA positive control group showed extensive infiltration by M1 macrophages and MNGCs, leading to severe membrane fragmentation within 30 days and loss of barrier function. This pattern of tissue integration is generally considered unfavorable. An intriguing observation from the EC-treated membranes was that their collagen fibers were surrounded by fibrous connective tissue, with minimal cellular infiltration but increased vascularization. This suggests that fibrotic encapsulation may delay tissue invasion and extend the membrane's barrier function. However, fibrosis is typically regarded as the endpoint of an adverse foreign body reaction, indicating poor tissue integration¹⁷. As such, the development of GBR membranes based on this response pattern remains questionable.

The study of tissue responses induced by collagen membranes offers important insights not only into the ideal patterns for tissue integration but also into how these membranes can influence the molecular microenvironment, potentially affecting adjacent bone regeneration. In the fourth study, a correlation between the vascularization of different compartments was identified, confirming molecular crosstalk between the compartments. These findings suggest that bone regeneration should be understood as a complex process regulated by both the bone substitute and the barrier membrane, with their interactions playing a crucial role in determining clinical outcomes.

5. Conclusion

This study series, grounded in established preclinical cyto- and biocompatibility testing methods, reveals the complex relationship between immune responses and tissue regeneration, offering valuable insights into the clinical application of GBR devices. Jellyfish-derived collagen shows promise in enhancing the reliability of early-stage cytocompatibility testing and advancing *in vitro* cell culture systems, though *in vivo* studies remain essential for evaluating long-term outcomes. A balanced immune response, particularly the transition from pro-inflammatory M1 macrophages to anti-inflammatory M2 macrophages, is crucial for promoting tissue regeneration. While non-natural implants tend to elicit stronger M1-driven inflammation, this response is increasingly recognized as vital for initiating material breakdown and tissue remodeling. Additionally, the molecular microenvironment induced by collagen membranes and bone substitute materials plays a complex and critical role in bone regeneration at defect sites. Future advancements in GBR technologies will depend on optimizing the balance between immune modulation, material degradation, and tissue integration to improve clinical outcomes. Moreover, in line with the 3R principles, the development of advanced *in vitro* testing systems remains an important focus for preclinical evaluation.

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List of Scientific Publications

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Appendix

a Affidavit

I hereby declare that the present dissertation was written by me independently and that no other aids than those indicated have been used. The dissertation has not yet been submitted to any other faculty or scientific institution.

I also declare that I have not yet completed any doctoral procedure without success.

Rostock, 13.03.2025

Yanru Ren

Hiermit erkläre ich, dass die vorliegende Dissertation von mir selbstständig verfasst wurde und keine anderen als die angegebenen Hilfsmittel benutzt worden sind.

Die Dissertation ist bisher keiner anderen Fakultät, keiner anderen wissenschaftlichen Einrichtung vorgelegt worden.

Ich erkläre ferner, dass ich bisher kein Promotionsverfahren erfolglos beendet habe.

Rostock, 13.03.2025

Yanru Ren

b Declaration of own contribution to the dissertation

The work was carried out at the Clinic and Polyclinic for Dermatology and Venereology at the University Medical Center Rostock under the supervision of Priv.-Doz. Dr. rer. nat. habil. Mike Barbeck. The study was designed in collaboration with Priv.-Doz. Dr. rer. nat. habil. Mike Barbeck, and with support of Dr. rer. nat. Jens-Wolfgang Pissarek, Managing Director and CTO Collagen Technologies at Biotrics Bioimplants AG.

The *in vitro* experiments were carried out under the supervision of Aenne Foth. The *in vivo* experiments were carried out after training by the laboratory members Sanja Stojanovic, Department of Biology and Human Genetics, Faculty of Medicine, University of Niš and Priv.-Doz. Dr. rer. nat. habil. Mike Barbeck, and I carried out this study independently with the support of Prof. Stevo Najman, Department of Biology and Human Genetics, Faculty of Medicine, University of Niš.

The histological and histomorphometric analyses as well as the statistical analyses were carried out by me under the instructions of Priv.-Doz. Dr. rer. nat. habil. Mike Barbeck. I confirm that I have written the manuscript independently and have not used any sources other than those specified by me.

c Curriculum vitae**Yanru Ren**Personal data

Name	Yanru Ren
Date of birth	17.01.1996 in China
Address	Elisenstraße 6, 12169, Berlin
Telephone	015228187238
Email	yanruren_sky@163.com

Education

2011-2014	Northern Heavy Industries No. 3 Middle School, Nei Mongol, China Degree: General higher education entrance qualification
2014-2018	Nanjing University of Posts and Telecommunications, Nanjing, China Degree: Bachelor of Science in Material physics
2018-2021	Beijing University of Chemical Technology, Beijing, China Degree: Master of Engineering in Bioengineering
2021- 2026	Universitätsmedizin Rostock, Rostock, Germany Degree: Dr. rer. hum. in medical biotechnology

d Acknowledgements

I would like to begin by expressing my heartfelt gratitude to Prof. Dr. Steffen Emmert for his invaluable scientific support and guidance throughout the entire course of this project.

My sincere thanks also go to Priv.-Doz. Dr. Mike Barbeck, for his unwavering advice and support, both scientifically and personally. Their significant contributions were instrumental in the realization of this work, from its initial conception to its successful completion.

I am also deeply grateful to the biotrics team and the BerlinAnalytix team, with special appreciation to Said Alkildani for his exceptional assistance in the design and evaluation of multiple aspects of this work.

I would also like to express my gratitude and deepest appreciation to the members of the DFG Research Unit “FOR 5250”. Their constant commitment, both through their scientific know-how and by providing the necessary resources for research and publication, was indispensable for the success of my work.

Finally, I would like to extend my deepest gratitude to my beloved family, whose constant support, patience, and understanding have been a cornerstone of my academic journey.

e original research articles

Article

Suitability of *R. pulmo* Jellyfish-Collagen-Coated Well Plates for Cytocompatibility Analyses of Biomaterials

Yanru Ren ¹ , Luo Liu ² , Xin Xiong ³ , Rumen Krastev ⁴ , Ralf Smeets ^{5,6} , Denis Rimashevskiy ⁷, Reinhard Schnettler ⁸, Said Alkildani ^{1,9} , Steffen Emmert ¹ , Ole Jung ^{1,†} and Mike Barbeck ^{1,9,*} 

- ¹ Clinic and Policlinic for Dermatology and Venereology, University Medical Center Rostock, 18057 Rostock, Germany
- ² Beijing Advanced Innovation Center for Soft Matter Science and Engineering, College of Life Science and Technology, Beijing University of Chemical Technology, Beijing 100013, China
- ³ NMI Natural and Medical Sciences Institute, University of Tübingen, 72770 Reutlingen, Germany
- ⁴ Faculty for Applied Chemistry, Reutlingen University, Alteburgstraße 150, 72762 Reutlingen, Germany
- ⁵ Department of Oral and Maxillofacial Surgery, University Medical Center Hamburg-Eppendorf, 20246 Hamburg, Germany
- ⁶ Department of Oral and Maxillofacial Surgery, Division of Regenerative Orofacial Medicine, University Medical Center Hamburg-Eppendorf, 20246 Hamburg, Germany
- ⁷ Department of Traumatology and Orthopedics, Peoples' Friendship University of Russia, 117198 Moscow, Russia
- ⁸ University Medical Centre, Justus Liebig University of Giessen, 35390 Giessen, Germany
- ⁹ BerlinAnalytix GmbH, 12109 Berlin, Germany
- * Correspondence: mike.barbeck@med.uni-rostock.de; Tel.: +49-(0)-176-81022467
- † These authors contributed equally to this work.



Citation: Ren, Y.; Liu, L.; Xiong, X.; Krastev, R.; Smeets, R.; Rimashevskiy, D.; Schnettler, R.; Alkildani, S.; Emmert, S.; Jung, O.; et al. Suitability of *R. pulmo* Jellyfish-Collagen-Coated Well Plates for Cytocompatibility Analyses of Biomaterials. *Int. J. Mol. Sci.* **2023**, *24*, 3007. <https://doi.org/10.3390/ijms24033007>

Academic Editor: Dario Puppi

Received: 21 December 2022

Revised: 27 January 2023

Accepted: 1 February 2023

Published: 3 February 2023



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Abstract: Cytocompatibility analyses of new implant materials or biomaterials are not only prescribed by the Medical Device Regulation (MDR), as defined in the DIN ISO Norm 10993-5 and -12, but are also increasingly replacing animal testing. In this context, jellyfish collagen has already been established as an alternative to mammalian collagen in different cell culture conditions, but a lack of knowledge exists about its applicability for cytocompatibility analyses of biomaterials. Thus, the present study was conducted to compare well plates coated with collagen type 0 derived from *Rhizostoma pulmo* with plates coated with bovine and porcine collagen. The coated well plates were analysed in vitro for their cytocompatibility, according to EN ISO 10993-5/-12, using both L929 fibroblasts and MC3T3 pre-osteoblasts. Thereby, the coated well plates were compared, using established materials as positive controls and a cytotoxic material, RM-A, as a negative control. L929 cells exhibited a significantly higher viability (#### $p < 0.0001$), proliferation (## $p < 0.01$), and a lower cytotoxicity (## $p < 0.01$ and # $p < 0.05$) in the Jellagen® group compared to the bovine and porcine collagen groups. MC3T3 cells showed similar viability and acceptable proliferation and cytotoxicity in all collagen groups. The results of the present study revealed that the coating of well plates with collagen Type 0 derived from *R. pulmo* leads to comparable results to the case of well plates coated with mammalian collagens. Therefore, it is fully suitable for the in vitro analyses of the cytocompatibility of biomaterials or medical devices.

Keywords: jellyfish collagen; cytocompatibility; collagen coating; well plate; in vitro; medical devices; biomaterials

1. Introduction

Cell culture systems are of value in testing the biocompatibility of medical devices and biomaterials before they are introduced into further preclinical in vivo trials and clinical studies. In recent years, in vitro methods for assaying biomaterials have gained importance owing to the growing concern over the use of animals for testing biomaterials. Significant effort is therefore being focused toward developing predictive and quantitative—but also

simple and reliable—methods of testing using cultured cells. At present, a number of methods for measuring both the cytotoxicity and the specific cytocompatibility of different materials are available [1–3]. The usefulness of these systems is no longer confined to screening new materials as they can be used to study the mechanisms of action of cell or tissue/material interactions of various biomaterials.

Collagen has long been considered an ideal substrate for culturing cells [4–6]. It accounts for more than 25% of total mammalian protein and provides structural and strength support for the body as a major component of the extracellular matrix [7,8]. Moreover, collagen is naturally biologically active as an endogenous component of the body to optimally mimic the in vivo environment [9]. Collagen has been shown to be involved in the regulation of cell adhesion, proliferation, and differentiation by binding to at least five different receptors (integrins, DDR, Glycoprotein VI, Osteoclast-associated receptor (OSCAR), LAIR-1, and uPARAP/Endo180) [9,10]. This is crucial for in vitro cell culture because all cells from normal tissues are anchorage-dependent, except for cells from the hematopoietic system and tumor cells [11]. In other words, cells must be anchored to an extracellular-matrix-like substrate and interact with their surroundings to grow and proliferate normally, otherwise they will experience growth arrest and anoikis [12]. The natural pores of collagen also support the attachment and migration of cells on its surface, which is essential for the normal physiological activities of cells [13].

For decades, cows and pigs have been the main sources of heterologous bovine and porcine collagen. This is largely influenced by human meat consumption habits [4,14]. However, both sources of collagen face limitations due to infectious diseases (e.g., bovine spongiform encephalopathy, foot-and-mouth disease, and swine flu) and religious factors [4]. In this case, additional evaluation and purification procedures are necessary due to safety concerns, which lead to higher costs and face the challenge of preserving the “natural” collagen structure during the complex purification process [15]. In addition, as complex organisms, differences in the origin, age, sex, and size of mammals may cause inevitable inconsistencies between different batches of collagen, which can sometimes be detrimental to the research [4,16,17]. Therefore, a more reliable, alternative source of collagen is urgently sought.

Jellyfish-derived collagen has gradually entered the limelight in recent years [8,18]. As ancient invertebrates, jellyfish have a simple physiological structure consisting mainly of water and a collagen-rich mesoglea [19]. Jellyfish-derived collagen is homogeneous with mammalian types I, II, III, V, and IX, and is therefore also known as “type 0 collagen” [20]. The desirability of jellyfish-derived collagen as an emerging alternative source is based on (1) the avoidance of complex mammalian tissues, (2) its low cost and improved carbon footprint, (3) fewer infectious disease and religious concerns, (4) batch-to-batch consistency, and (5) benefits for marine waste utilization and ecological management [5,21,22]. However, it should be noted that jellyfish collagen is less cross-linked than mammalian collagen due to its lower proline and hydroxyproline content, which makes jellyfish collagen products face stability concerns [19].

The jellyfish species currently available for collagen extraction are *Somolophus meleagris* jellyfish [22,23], *Rhizostomous* jellyfish [24], and *Chrysaora sp.* jellyfish [16]. Among these species, collagen from *Rhizostoma pulmo* has been shown to have a sequence homology with the vertebrate collagen type I [19]. The biocompatibility of jellyfish collagen has been confirmed by many studies, including cytotoxicity, adhesion tests, and the secretion of pro-inflammatory factors [19,22,24,25]. Jellyfish collagen is thought to elicit cellular responses similar to mammalian collagen involving adhesion, proliferation, and migration [19]. In addition, jellyfish collagen has been shown to induce a long-term, anti-inflammatory macrophage response as well as excellent vascularization in vivo to support bone tissue regeneration [26,27].

The Jellagen[®] collagen from *R. pulmo* (JC) involved in this study has been reported to have potential in culturing human iPSC-derived Microglia (iMGL) and ovarian cancer (OvCa) cell lines [24,28]. In this context, Jellagen[®] collagen is of interest as a culture medium

for a cytocompatibility analysis, which is an extensively used tool in the evaluation of biomaterials. Our previous studies have demonstrated that fibroblasts and osteoblasts exhibit good proliferation behaviour on Jellagen®-collagen-coated multi-well culture plates and jellyfish 3D scaffolds [25]. However, it remains unclear whether Jellagen® collagen is comparable to porcine- and bovine-derived collagen as a cell culture substrate for in vitro biocompatibility analyses.

In the present study, we report a comparison of well plate coatings based on Jellagen® collagen and bovine and porcine collagen to assess their effects on culturing L-929 fibroblasts and MC3T3 pre-osteoblasts. Cell viability (XTT assay), cytotoxicity (LDH assay), and cell proliferation (BrdU assay) analyses were combinatorially conducted to evaluate the usability of the collagen coating for cytocompatibility analyses. Supplemental LIVE/DEAD staining was used to visualize the growth status of L-929 on well plates with different collagen coatings. The aim of this study was to assess the suitability of Jellagen® collagen as a culture matrix for in vitro biocompatibility analyses in comparison with porcine- and bovine-derived collagen.

2. Results

2.1. Cytocompatibility Results—L929-Fibroblasts

The analysis of the cell viability of the L929 fibroblasts via XTT assay revealed that the coating using the Jellagen® collagen induced a significantly lower ($*** p < 0.001$) fibroblast viability compared to the medium control (Figure 1A). Moreover, both the porcine and the bovine coatings showed even significantly lower values ($**** p < 0.0001$) (Figure 1A). Thus, the viability values based on the Jellagen® collagen coating were significantly higher ($#### p < 0.0001$) when compared to the porcine and the bovine coatings (Figure 1A). Altogether, the viability values in the group of the Jellagen®collagen coating were well above the 70% limit defined by the DIN ISO standard, while the values in the other two groups were close to this limit. The XTT analysis also showed that the viability values in the group of the negative control (titanium, grade 4) were significantly lower ($* p < 0.05$) when compared to the medium control. Additionally, the positive control material, RM-A, induced a significantly lower ($**** p < 0.0001$) cell viability when compared to the medium control (Figure 1A).

The proliferation analysis of the L929 fibroblasts via BrDU assay showed that all collagen coatings induced significantly higher values ($** p < 0.01$ and $**** p < 0.0001$) compared to the medium control (Figure 1B). Additionally, the proliferation values in the group of well plates coated with Jellagen® collagen were significantly higher ($## p < 0.01$) when compared to the values in the group with the porcine collagen coating, while no significant difference was found when compared to the values in the group with the bovine collagen coating (Figure 1B). Additionally, the BrDU analysis confirmed that the negative control (titanium, grade 4) showed no significant difference compared to the medium control, while the positive control material, RM-A, induced a significantly lower ($**** p < 0.0001$) cell proliferation value when compared to the medium control (Figure 1B).

Finally, the cytotoxicity analysis via LDH assay revealed that the values in all coating groups and in both control groups, i.e., the medium control group and the group with the grade 4 titanium material, were significantly lower ($**** p < 0.0001$) when compared to the values of the positive control group (RM-A) (Figure 1C). Furthermore, the values in the group with the Jellagen®collagen coating were significantly lower ($## p < 0.01$ and $# p < 0.05$) when compared to the values in the groups with the porcine and bovine collagen coatings (Figure 1C).

The LIVE/DEAD staining results additionally confirmed the data via the afore mentioned assays (Figure 2). The cells in the medium control were, for the very most part, viable, and formed a dense layer with a partial, spindle-shaped form, with only a few dead cells detectable (Figure 2). In the case of the cytotoxic control (RM-A), only a few cells were detectable, of which a few cells were viable, and the majority showed signs of acute cell death (Figure 2). In case of the cytocompatible control material (titanium), a denser

layer formation of the L929 fibroblasts was observed, and more cells showed a spindle-like form (Figure 2). In the groups with the Jellagen®collagen coating and the bovine collagen coating, an even higher cell density was detected, and most of the L929 fibroblasts demonstrated a spindle-like form. In the group with the porcine collagen coating, a slightly lower cell density was found, while most of the fibroblasts also showed a spindle-like shape (Figure 2).

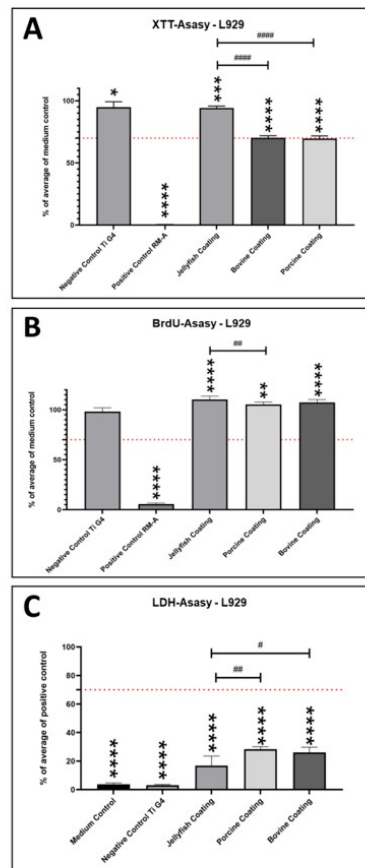


Figure 1. Cytocompatibility results of the different coatings using L929 fibroblasts. (A) Viability measured by the XTT assay, (B) proliferation measured by the BrdU assay, and (C) cytotoxicity measured by the LDH assay. Dotted lines show the respective thresholds that should not be exceeded (LDH) or undershot (XTT, BrdU). Significant differences: #/ $p < 0.05$, ##/ $p < 0.01$, *** $p < 0.001$, ####/ $p < 0.0001$.

2.2. Cytocompatibility Results—MC3T3-Pre-osteoblasts

The analysis of the cell viability of the MC3T3 pre-osteoblasts via XTT assay showed that both the bovine and Jellagen® collagen coatings induced a significantly higher ($p < 0.05$ and $p < 0.01$) viability compared to the medium control, while no differences were found between the values in the group with the porcine coating and the medium control (Figure 3A). Moreover, the viability values in the group of the Jellagen® collagen coating were significantly higher ($p < 0.05$) when compared to the porcine coating but

not to the values in the group with the bovine coating (Figure 3A). The XTT analysis confirmed that the viability values in the group of the negative control (titanium, grade 4) were comparable to the medium control. Additionally, the positive control material, RM-A, induced a significantly lower ($**** p < 0.0001$) cell viability when compared to the medium control (Figure 3A).

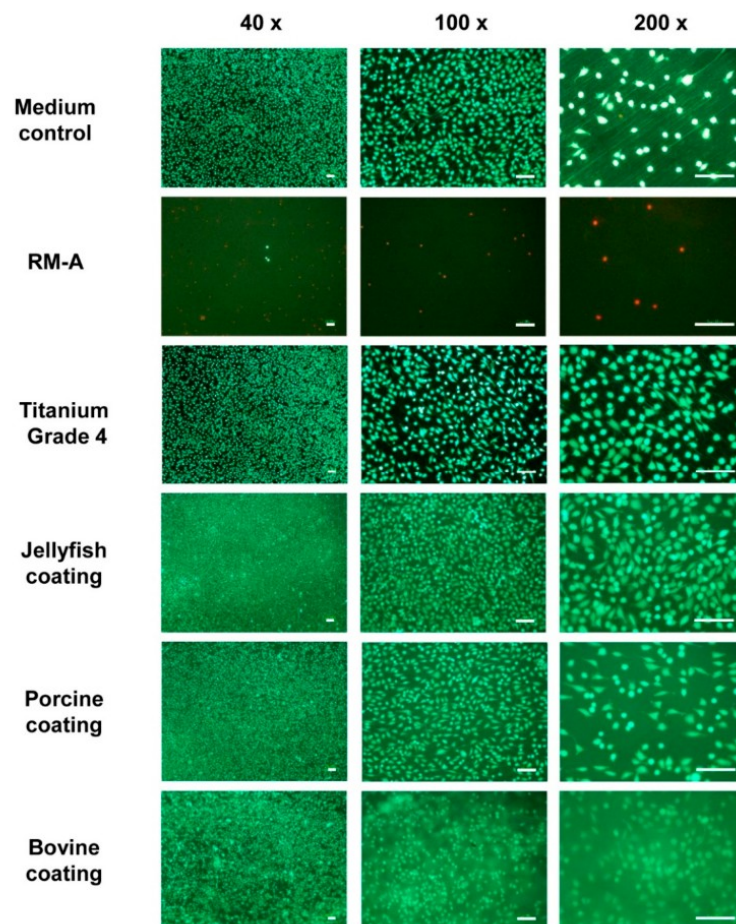


Figure 2. LIVE/DEAD-staining of the controls (rows 1–3) and test samples (rows 4–6). Green: viable cells; red: dead cells (**left column**: $4\times$ magnifications; **middle column**: $100\times$ magnifications, **right column**: $200\times$ magnifications; scale bars = $100\ \mu\text{m}$).

The proliferation analysis of the MC3T3 pre-osteoblasts via BrDU assay showed that only the bovine collagen coating induced significantly higher values ($*p < 0.05$) compared to the medium control, while no differences were found between the values in the group of the porcine coating and the medium control (Figure 3B). Additionally, the proliferation values in the group of well plates coated with Jellagen[®] collagen were significantly lower ($**** p < 0.0001$ and $#### p < 0.0001$) when compared to the values in the group of the medium control and the coatings with porcine and bovine collagen (Figure 3B). The BrDU analysis also confirmed that the negative control (titanium, grade 4) and the porcine coating

were comparable to the medium control, while the positive control material, RM-A, induced a significantly lower ($**** p < 0.0001$) cell proliferation compared to the medium control (Figure 3B).

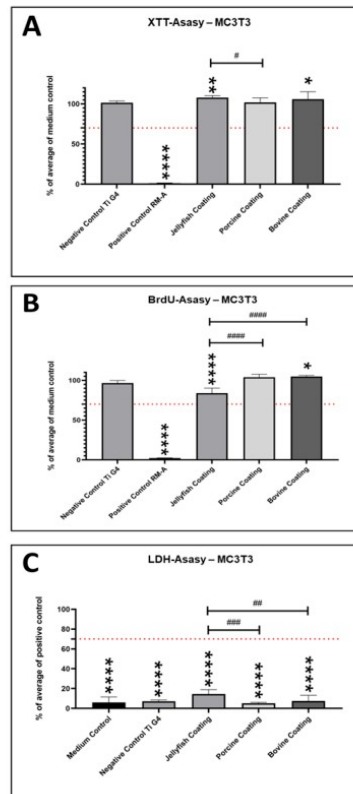


Figure 3. Cytocompatibility results of the different coatings using MC3T3 pre-osteoblasts. (A) Viability measured by the XTT assay, (B) proliferation measured by the BrdU assay, and (C) cytotoxicity measured by the LDH assay. Dotted lines show the respective thresholds that should not be exceeded (LDH) or undershot (XTT, BrdU). Significant differences: #/* $p < 0.05$, ##/** $p < 0.01$, ### $p < 0.001$, ####/**** $p < 0.0001$.

Finally, the cytotoxicity analysis via LDH assay revealed that the values in all coating groups but also in the both control groups, i.e., the medium control group and the group with the grade 4 titanium material, were significantly lower ($**** p < 0.0001$) when compared to the values in the group of the positive control (RM-A) (Figure 3C). Furthermore, the values in the group with the Jellyfish[®] collagen coating were significantly higher ($## p < 0.01$ and $### p < 0.001$) compared to the values in the group of the porcine and bovine collagen coatings (Figure 3C).

3. Discussion

In vitro cell culturing is not an easy task. The normal physiological activities of most human cells depend on the three-dimensional network of the extracellular matrix, which provides structural and physiological support for the cells [29]. Therefore, simulating a similar physiological environment in vitro is crucial for a successful cell culture. Well

plates are the most widely used tools for in vitro cell experiments, especially in the fields of biomaterials and tissue engineering. Proper surface treatment for well plates is necessary prior to cell inoculation due to their hydrophobic polystyrene surface [30]. As the dominant structural component of the extracellular matrix, collagen provides natural bioactivity to well plates [31]. Collagen surfaces naturally possess ligands that bind to a variety of receptors to regulate cell adhesion, proliferation, and migration [10,13,19]. In addition, collagen substrates support cells in producing their own extracellular matrix during growth [13,32]. Not surprisingly, collagen matrices have become the matrix of choice for in vitro cell cultures.

The collagen currently utilized for cell cultures is mainly derived from mammalian sources such as bovine, porcine, and murine [6,11]. As the most abundant protein, collagen is widely represented throughout the tissues in the body, such as the skin, pericardium, cartilage, and tendons, which can be easily obtained from slaughterhouse by-products [4,14,33,34]. The manufacturing process to obtain collagen is quite complex due to the structural and compositional complexity of mammalian tissues [17]. However, even if every step of the process is strictly regulated, batch-to-batch inconsistencies can occur due to individual mammalian variability [35]. In addition, current purification methods are also considered insufficient to eliminate the risk of disease infection, such as bovine spongiform encephalopathy (BSE) [36]. However, researchers desire a consistent, disease-free substrate that provides an optimal protocol for in vitro cell culturing.

Jellyfish collagen, derived from marine animals with a higher economic efficiency, is expected to provide an optimized approach to this gap. Unlike mammalian collagen, Jellagen[®] collagen is free of residual contaminants such as prions, proteins, and polysaccharides. It also brings reduced off-target effects due to its inert, non-specific miRNA. In addition, centuries of conserved evolutionary structure and simple jellyfish physiology ensure that Jellagen[®] collagen exhibits excellent batch-to-batch consistency [24]. Another remarkable aspect of jellyfish collagen is its homogeneity with mammalian types I, II, III, V, and IX due to the ancient chemical lineage [19,37]. As a so-called “type 0 collagen”, collagen derived from *Rhizostoma pulmo* in particular is expected to culture every cell type [24,25,28].

In this context, jellyfish collagen is of great interest as a cell culture matrix for in vitro cytocompatibility experiments in the field of biomaterials and tissue engineering. It is also intriguing whether the cytocompatibility of Jellagen[®] collagen can be compared to that of porcine and bovine collagen when co-incubated with fibroblasts and osteoblasts. Therefore, in this study, L929 and MC3T3 cells were co-incubated with the extracts of Jellagen[®], porcine, and bovine collagen-coated well plates. XTT, BrdU, and LHD assays, which are also described in the DIN ISO norms 1099-5/-12 for cytocompatibility analyses of medical devices, were used to determine cell viability, multiplication, and cytotoxicity, respectively. As the most used fibroblast for in vitro cytocompatibility, the L929 cells used in this study showed a decreased cell viability in all collagen and control groups when compared to the medium control. It is worth noting that the viability of L929 cells in Jellagen[®] collagen group was significantly higher in comparison to the bovine and porcine collagen groups. In contrast, both the proliferation and cytotoxicity of all collagen groups were significantly higher than the values in the group of the medium control. However, the Jellagen[®] collagen coating showed a significantly lower cytotoxicity compared to the porcine and bovine collagen coatings. The above results thus reveal that the Jellagen[®] collagen coating greatly supports the growth and proliferation of L929 cells as a culture substrate.

In this study, the lower cell viability of L929 cells was conspicuous after co-incubation with extracts of the porcine and bovine collagen coatings for 24h when compared to the other groups. In this context, what triggered these lower values can only be conjectured, as little is known about the exact origin and especially the processing of the collagen. For example, the user does not know which collagen type and which chemicals, or which collagen fibril type, was used for the production of the respective coatings. It is precisely in this area that there is still a need for clarification, since it is known that the different fibrillar precipitation types also cause different cellular responses [17,38]. However, the

research community and the manufacturers have only recognized these issues in the last few years, which is why there is little knowledge in this area and why significant research is still needed regarding these issues. In contrast, the porcine and bovine collagen coatings did not affect the metabolic activity of the L929 fibroblasts. The LIVE/DEAD staining data showed that, in contrast to the acute death of most cells in the positive control group (RM-A), the L929 fibroblasts had a spindle-like shape and formed a dense cell layer in all collagen groups. It is worth noting that slightly fewer cells adhered to the porcine collagen coating than to the other collagen coatings. These results appear to be consistent with the study of Böhm et al., which showed that fibroblasts display sparse initial adhesion on both porcine and equine collagen surfaces compared to bovine collagen [39]. This is attributed to the fact that cells adhere to different surfaces at different initial rates. Consideration should also be given to the incomplete adhesion of the cells due to the poor wettability of the coating (cells were thus washed off during the staining process). Altogether, the above results conclude that the Jellagen® collagen from *R. pulmo* is fully biocompatible and is even more recommended than collagen from porcine and bovine sources to be used as a device for the in vitro testing of the cytocompatibility of biomaterials.

The MC3T3 cell line is well-known as a model for studying the cytocompatibility of bone substitute materials as well as the process of osteogenesis in vitro [32,40,41]. The results show that all collagen groups induced a satisfactory cell viability, proliferation, and cytotoxicity, suggesting that all collagen coatings allowed for the excellent growth and proliferation of MC3T3 cells and can be used as an alternative device for in vitro analysis models. Interestingly, the Jellagen®-collagen-coated group exhibited a significantly lower proliferation of MC3T3 cells when compared to the porcine- and bovine-collagen-coated groups. Similar results were also reported in a previous in vitro study on Jellagen® coating and jellyfish 3D scaffolds [25]. It is necessary to note that only the 24h post-culture results of the MC3T3 cells with Jellagen®-collagen-coated extracts were reported in the present study. However, MC3T3 cells typically exhibit a higher rate of DNA synthesis and a gradual increase in cell numbers during the initial phase of in vitro culture up to 9 days [32]. At this stage, MC3T3 does not express alkaline phosphatase and there is no accumulation of the extracellular collagen matrix [42]. At around day 9, cell proliferation reaches a plateau and growth arrests [32]. Therefore, the cell proliferation data should be considered to be a result of experimental limitations rather than a hasty conclusion that the Jellagen® collagen inhibited MC3T3 proliferation at the initial stage when compared to porcine and bovine collagen.

Moreover, the MC3T3 in the Jellagen®-collagen-coated group exhibited a slightly higher cytotoxicity compared to all other groups in the LDH assay. However, these data should not be overestimated as, in the previous study, MC3T3 demonstrated the lowest cytotoxicity on Jellagen® collagen 3D scaffolds, even when compared to the medium control and negative control [25]. This low cytotoxicity can be attributed to the 3D jellyfish scaffold structure, which provides additional orientation for cell growth. Once cells grow into and adhere to the internal pores, the 3D scaffold provides an optimal simulation of the in vivo extracellular matrix (ECM) environment, regulating more complex biological functions, including transcriptional regulation, cell migration, proliferation, and differentiation, by recreating cell–matrix interactions via complicated cellular signals [9,24]. It is also worth mentioning that a combination of three different assays was used to assess the cytocompatibility of collagen coatings at different levels. The results of all tests must therefore be considered together in order to draw reliable conclusions. In this context, Jellagen® collagen is therefore recommended as an alternative culture substrate for MC3T3 cells.

Taken together, the reported results indicate that Jellagen® collagen is expected to be a mainstream source of collagen products in the future. The most notable advantage of jellyfish collagen is its high homology to mammalian collagen, particularly type I, which is the basis for its consideration as an alternative to mammalian collagen [5,21]. Mammalian collagen has long faced difficulties with batch-to-batch inconsistency in laboratory studies, which is critical for reproducibility of experiments. The high batch-to-batch consistency

of jellyfish collagen could ameliorate this dilemma and provide more reliable data for studies. In addition, jellyfish collagen provides an alternative source for countries and regions where religious restrictions prevent the use of biological products from porcine or bovine sources. Taken together, this study shows that Jellagen[®], a jellyfish collagen from *R. pulmo*, has excellent cytocompatibility comparable to porcine or bovine collagen as a culture medium for L929 fibroblasts and MC3T3 pre-osteoblasts. It is recommended as an alternative device for the in vitro cytocompatibility evaluation of biomaterials or medical devices.

4. Materials and Methods

4.1. Preparation of the Well Plates

The preparation of the well plates was conducted following a specialized protocol [43]. All collagen solutions and coating procedures were comparatively conducted for compatibility of the coatings. Jellagen[®] (JCP96W) was purchased from Jellagen[®] Limited, Cardiff, U.K. In brief, the respective collagen was initially combined with acetic acid to obtain a 0.1% (*w/v*) collagen solution and then diluted 10-fold to achieve a concentration of 0.01%. For the coating procedure, a concentration of 6–10 µg/cm² was chosen and the well plates were dried at room temperature for several hours to allow the collagen to bind to the surfaces. After an UV-mediated sterilization, the coated well plates were rinsed with sterile, tissue-culture grade water before introducing the cells and media.

4.2. Cell Seeding

All coated well plates and controls were incubated with media at 37 °C and 5% CO₂ for 72 hours. Blank controls comprised the corresponding media and were subtracted from the results. The extraction of all collagen-coated well plates was carried out according to EN ISO 10993-12:2012. Afterwards, fibroblasts and pre-osteoblasts were seeded into the extracts separately and incubated for 24 h at 37 °C and 5% CO₂. The extracts were seeded with 1×10^4 cells/100 µL for the in vitro assays, and 2.4×10^5 cells/1 mL for the LIVE/DEAD staining.

4.3. Cytocompatibility Analyses

The cytocompatibility analyses were applied in accordance to protocols of the DIN EN ISO 10993-5: 2009/-12: 2012, as previously published [25,44]. In brief, L-929 mouse fibroblasts and MC3T3 pre-osteoblasts, purchased from the European Collection of Cell Cultures, ECACC (Salisbury, U.K.) were used under standard cell culture conditions, i.e., 37 °C, 5% CO₂, and 95% humidity for 24 h. Blank values that included determination only with a medium but without cells were used as so-called medium controls and were also measured in triplicate determination. These values were additionally subtracted from all other values. RM-A materials, which included polyurethane film with 0.1% zinc diethyldithiocarbamate (ZDEC) (Hatano Research Institute, Food and Drug Safety Center, Hadano, Japan), were used as positive control. Finally, grade 4 titanium plates were applied as a negative control.

Test kits for the analyses of the viability, proliferation, and cytotoxicity were conducted in triplicates, i.e., the Sodium 3,3'-[1(phenylamino)carbonyl]-3,4-tetrazolium]-3-is(4-methoxy-6-nitro) Benzene Sulfonic acid Hydrate (XTT)-assay (Roche Diagnostics, Mannheim, Germany), Bromdesoxyuridin (BrdU) ELISA (Roche Diagnostics, Mannheim, Germany), and Lactate Dehydrogenase (LDH) assay (BioVision, Milpitas, CA, USA). All assays were carried out according to the manufacturer's instructions. The protocols are briefly described as follows.

XTT assay. Initially, the electron coupling reagent was mixed with the XTT labelling reagent in a ratio of 1:50. A total of 50 µL of this mixture was then added to the cells and incubated for 4 hours under standard cell culture conditions. Afterwards, the absorbance of 100 µL aliquots in a new 96-well plate was assessed by a scanning multi-well spectrophotometer (ELISA reader) with filters for 450 and 650 nm.

BrdU assay. Briefly, the cells were co-incubated with BrdU for 2 hours under cell culture conditions. Following this, FixDenat reagent was used to fix the cells at room temperature. The cells were then treated with anti-BrdU peroxidase (POD) antibody for 1 hour, followed by washing (three times for 5 min). After adding tetramethylbenzidine (TMB) to react with the substrate for 20 min at room temperature, 25 μ L 1M H₂SO₄ was added to stop the reaction. Finally, absorbance was measured at 450 and 690 nm using a scanning multiwell spectrophotometer (ELISA reader) with a filter.

LDH assay. Briefly, 10 μ L of cell supernatant containing LDH was incubated with 100 μ L of LDH reactive solution for 30 minutes at room temperature. Afterwards, the absorbance was analysed at 450 and 650 nm by scanning multiwell spectrophotometer (ELISA reader).

4.4. LIVE/DEAD-Staining

The LIVE/DEAD-staining procedure was conducted following the corresponding DIN EN ISO protocol, as 2.4×10^5 cells in 1 ml of cell medium were added into each well of a 12-well-plate so that the surface area/medium ratio was 5.65 cm²/mL. The staining was applied after 24 h of incubation under standard cell culture conditions, i.e., 37 °C, 5% CO₂, and 95% humidity, via a standardized kit (Thermo Fisher, Waltham, MA, USA). Directly after the application of the staining, the cells were analysed using an inverted fluorescence microscope (Nikon ECLIPSE Ti-S/L100, Nikon GmbH, Düsseldorf, Germany). Thereby, the cell culture microscope was combined with a filter for the simultaneous detection of red and green fluorescence. The images were taken via a connected digital camera (Axiocam 208 color, Carl Zeiss AG, Oberkochen, Germany).

4.5. Statistical Analysis

The statistical analysis was conducted using ANOVA tests combined with Tukey's multiple comparisons test by means of the GraphPad Prism 9.4 software (GraphPad Software Inc., La Jolla, CA, USA). The resulting differences were stated as significant if the *p*-values were less than 0.05 (* *p* ≤ 0.05) or as highly significant for *p*-values less than 0.01 (** *p* ≤ 0.01) or less than 0.001 (***) *p* ≤ 0.001). The data were finally graphed as mean values ± standard deviations.

5. Conclusions

Stable and reliable 2D and 3D in vitro cell culture models are important preliminary assessment tools for the cytocompatibility of biomaterials. Collagen, a key regulator of cell growth and proliferation, has proven to be an ideal substrate for cell culture. Marine organisms represent a very attractive source of collagen. Particularly, collagen products derived from *R. pulmo* have been shown to support cell growth, adhesion, and interaction. Its potential as a culture substrate for human iPSC-derived microglia and ovarian cancer cells has been demonstrated.

This study provides preliminary results comparing well plates coated with Jellagen[®] collagen and mammalian collagen (porcine and bovine) as in vitro cell culture devices for the analysis of the cytocompatibility of medical devices with fibroblasts and osteoblasts. Both L929 fibroblasts and MC3T3 pre-osteoblasts showed significantly higher cell viability after co-incubation with extracts of Jellagen[®] collagen coating when compared to that of porcine and bovine collagen coatings. Both cells also showed satisfactory proliferation and cytotoxicity in the group with Jellagen[®] collagen coating. L929 cells also formed the densest cell layer on the Jellagen[®] collagen coating and demonstrated healthy behaviour. Overall, Jellagen[®] collagen is a fully biocompatible cell culture substrate suitable for in vitro cytocompatibility analyses in the field of biomaterials and tissue engineering.

Author Contributions: Conceptualization, R.S. (Ralf Smeets), O.J. and M.B.; methodology, R.S. (Ralf Smeets), O.J. and M.B.; software, S.A., O.J. and M.B.; validation, Y.R., S.A., O.J. and M.B.; formal analysis, O.J. and M.B.; investigation, Y.R., S.A., O.J. and M.B.; resources, L.L., X.X., R.K., R.S. (Reinhard Schnettler), D.R., R.S. (Ralf Smeets), S.E., O.J. and M.B.; data curation, Y.R., S.A., O.J. and M.B.; writing—original draft preparation, Y.R., S.A., O.J. and M.B.; writing—review and editing, Y.R., S.A., O.J. and M.B.; visualization, Y.R., S.A., O.J. and M.B.; supervision, L.L., X.X., R.K., R.S. (Reinhard Schnettler), D.R., R.S. (Ralf Smeets), S.E., O.J. and M.B.; project administration, O.J. and M.B.; funding acquisition, O.J. and M.B. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Federal Ministry of Economics and Climate Protection of the Federal Republic of Germany (BMWK, FKZ: ZF4289207AJ8), the Federal Ministry of Education and Research (BMBF, Germany, FKZ: 13GW0400A and C) and the State Ministry of Baden-Württemberg for Economic Affairs, Labour, and Tourism. Additionally, the authors gratefully acknowledge the funding by the German Research Foundation (Deutsche Forschungsgemeinschaft, DFG) for the subprojects 2, 5 and 6 within the Research Unit 5250 “Permanent and bioresorbable implants with tailored functionality” (No. 449916462).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All relevant data are included in the manuscript.

Acknowledgments: The authors want to thank Judith Schreiber for her excellent technical support.

Conflicts of Interest: The authors declare no conflict of interest.

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In Vivo Analysis of the Immune Response to Strontium- and Copper-doped Bioglass

MIKE BARBECK^{1,2}, SAID ALKILDANI^{1,2}, ARMANDO MANDLULE³, MILENA RADENKOVIĆ⁴,
STEVO NAJMAN^{4,5}, SANJA STOJANOVIĆ^{4,5}, OLE JUNG¹, YANRU REN^{1,6}, BAOYI CAI^{1,6},
OLIVER GÖRKE³, DENIS RIMASHEVSKIY^{6*} and FRANZISKA SCHMIDT^{7,8*}

¹Clinic and Policlinic for Dermatology and Venereology, University Medical Center Rostock, Rostock, Germany;

²BerlinAnalytix GmbH, Berlin, Germany;

³Department of Ceramic Materials, Institute for Materials Science and Technologies, Technical University of Berlin, Berlin, Germany;

⁴Department for Cell and Tissue Engineering, Faculty of Medicine, University of Niš, Niš, Serbia;

⁵Department of Biology and Human Genetics, Faculty of Medicine, University of Niš, Niš, Serbia;

⁶Department of Traumatology and Orthopedics, Peoples' Friendship University of Russia, Moscow, Russia;

⁷Department of Prosthodontics, Geriatric Dentistry and Craniomandibular Disorders, Charité-Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin,

Humboldt-Universität zu Berlin, Berlin, Germany

⁸Berlin Institute of Health, Berlin, Germany

Abstract. Background: Bioglass is a highly adoptable bone substitute material which can be combined with so-called therapeutic ions. However, knowledge is poor regarding the influence of therapeutic ions on immune reactions and associated bone healing. Thus, the aim of this work was to investigate the influence of strontium- and copper-doped bioglass on the induction of M1 and M2 macrophages, as well as vascularization. Materials and Methods: Two types of alkali glass were produced based on ICIE16 bioglass via the melt-quench method with the addition of 5 wt% copper or strontium (ICIE16-Cu and ICIE16-Sr). Pure ICIE16 and 45S5 bioglass were used as control materials. The ion release and chemical composition of the bioglass were investigated, and an in vivo experiment was subcutaneously performed on

Sprague–Dawley rats. Results: Scanning electron microscopy revealed significant differences in the surface morphology of the bioglass materials. Energy dispersive X-ray spectroscopy confirmed the efficiency of the doping process by showing the ion-release kinetics. ICIE16-Cu exhibited a higher ion release than ICIE16-Sr. ICIE16-Cu induced low immune cell migration and triggered not only a low number of M1 and M2 macrophages but also of blood vessels. ICIE16-Sr induced higher numbers of M1 macrophages after 30 days. Both bioglass types induced numbers of M2 macrophages comparable with those found in the control groups. Conclusion: Bioglass doping with copper and strontium did not significantly influence the foreign body response nor vascularization of the implantation bed in vivo. However, all the studied bioglass materials seemed to be biocompatible.

*These Authors contributed equally to this study.

Correspondence to: Mike Barbeck, Clinic and Policlinic for Dermatology and Venereology, University Medical Center Rostock, 18057 Rostock, Germany. Tel: +49 17681022467, e-mail: mike.barbeck@med.uni-rostock.de

Key Words: Bioglass, ion release, hydroxyapatite deposition, bone tissue regeneration, macrophages, vascularization, copper doping, strontium doping, 45S5, ICIE16, DIN EN ISO 10993-6.



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Bone substitute materials (BSMs) are a small group including biomaterials mainly based on natural or synthetic calcium phosphates and similar compounds (1). Bioactive glass is a specific subgroup which has been shown to be a good BSM due to its optimal bonding to newly formed bone tissue and due to the formation of a hydroxycarbonate apatite layer which supports bone regeneration (2). Since the invention of bioactive glass materials in the 1970s by Professor Larry L. Hench, with 45S5[®] bioglass being one of the first developed materials, much research has been conducted on bioactive glass (3). Silicate-based bioactive glass and glass ceramics, such as Perioglas[®] (4) and Ceravital[®] (5, 6), have been clinically used as BSMs for several decades. Nevertheless,

45S5® has some disadvantages, such as the small processing window, which can lead to crystallization during the production process and thus to a reduction of bioactivity due to the presence of crystalline and amorphous phases (7, 8). Furthermore, the original composition includes a large amount of sodium to reduce the melting temperature (9), which leads to cell death under *in vitro* conditions due to a burst release and subsequent fast increase in pH (10). Because of its high crystallization tendency, 45S5 is difficult to shape it into glass fibers or amorphous scaffolds without changing its initial properties and thus its ability to bond to bone. In contrast, ICIE16 (by weight 48.0% SiO₂, 6.6% Na₂O₃, 2.9% CaO, 2.5% P₂O₅, 10.0% K₂O) was developed by Elgayar and co-workers (11). It has a lower tendency to crystallize and a greater sintering window (12), which makes it a promising agent for producing various shapes, including amorphous scaffolds (13).

One of the most worthwhile features of bioactive glass is the ability to incorporate vast quantities of additives into them, such as therapeutic ions (*e.g.*, strontium, copper, zinc, or fluorine) or drugs (14). In this context, ICIE16 is an ideal candidate for incorporation of therapeutically active ions into scaffolds, which can be used for controlled degradation (13, 15). Therapeutic ions can be added in the form of metallic ions and are also naturally present in the body. Although they occur in the body at lower concentrations in the form of trace elements (16), higher quantities released from bioactive glass have stimulatory effects on certain cells, without being toxic (16-18). Thus, therapeutic ions are able to enhance the biological impact of bioactive glass and, therefore, strengthen bone formation due to their stimulating effects (19). Like any other biomaterial, bioactive glass induces an inflammatory reaction within their implant bed (16, 20). This reaction cascade, also known as the 'foreign body reaction to biomaterials' includes macrophages as central control elements, which significantly control material-specific inflammation through the expression of a variety of signaling molecules (21). The consensus is that a biomaterial should induce an anti-inflammatory tissue response, and thereby also a predominance of M2 macrophages, in order to support tissue regeneration optimally (22). The addition of strontium leads to stimulation of osteoblasts and reduction of osteoclast activity (19, 23). Its inclusion induces new bone formation, and it is therefore widely used as a therapeutic ion for osteoporosis (24). Furthermore, copper is known for its promotion of angiogenesis and (related) stimulation of bone regeneration (25, 26). However, it is completely unknown what influence these so-called therapeutic ions have on inflammatory tissue reactions that are strongly connected with tissue regeneration (16, 19). Thus, it was supposed that their inclusion might be used to control the material-triggered healing process by regulating the induction of M2 macrophages. In this context, it was shown that copper, zinc,

and silver have anti-inflammatory and antimicrobial properties (27). The use of therapeutic ions in dental applications may therefore provide advantages compared to both purely inorganic calcium phosphate-based bone substitute materials and to organic biomolecules (*e.g.*, growth factors). Altogether, the addition of therapeutic ions to bioactive glass might be expected to lead to a better bone-healing process by adaption of the associated and underlying inflammatory reactions.

Thus, the aim of this work was to investigate the influence of the release of strontium and copper ions from bioactive glass-based BSM on the induction of M1 and M2 macrophage subtypes.

The objectives of the present study were: (a) The synthesis of bioactive glass 45S5 (by weight: 45% SiO₂, 24.5% CaO, 24.5% Na₂O, and 6.0% P₂O₅) and ICIE16 (by weight: 48.0% SiO₂, 6.6% Na₂O, 32.9% CaO, 2.5% P₂O₅, 10.0% K₂O); (b) incorporation of 5 mol% strontium or copper ions into ICIE16; (c) examination of the influence of strontium and copper on the resulting structure and material properties; (d) evaluation of the influence of strontium and copper on the bioactivity of the synthesized glass and the ability to form a hydroxycarbonate apatite layer; (e) assessment of inflammatory tissue reaction induced by ICIE16 and the strontium- and copper-doped glass after subcutaneous implantation for 10 and 30 days using previously established and published methodologies (14, 28, 29). Thereby, both pure ICIE16 and 45S5 bioglass implants served as control materials. 45S5 Bioglass was used as a control due to its proven biocompatibility in literature (14).

Materials and Methods

Materials. Four different bioglass alkali systems were synthesized by melt quenching, namely 45S5 (by weight: 45% SiO₂, 24.5% CaO, 24.5% Na₂O, and 6.0% P₂O₅), ICIE16 (by weight: 48.0% SiO₂, 6.6% Na₂O, 32.9% CaO, 2.5% P₂O₅, 10.0% K₂O), 5 mol% Cu-doped (ICIE16-Cu) and 5 mol% Sr-doped (ICIE16-Sr) ICIE16. 45S5 and ICIE16 were prepared as control materials. Compared to 45S5, ICIE16 has a lower crystallization tendency during sintering and remains amorphous up to 700°C (30). Table 1 provides the molecular composition of each bioglass.

Per batch, 100 g of bioglass were prepared *via* a melt-quench process. High-purity powders [SiO₂, Na₂CO₃, Ca₃(OH)₁₃P₃, Ca(OH)₂, K₂CO₃, CuCO₃, and SrCO₃] were weighed out according to the desired relative ratios, dried at 100°C for 24 h, melted at 1,400°C for 2 h, quenched at room temperature after 2 h, dried at 100°C overnight, milled to 100-315 µm particle size for 3 min *via* dry ball-milling, and finally pressed into pellets (diameter: 1 mm, height: 0.5 mm) using pressure of 15 bars for 3 min. Finally, the materials underwent gamma-sterilization.

Bioglass characterization. In vitro: The surface morphology of the prepared bioglass materials was characterized *via* scanning electron microscopy (SEM) with a LEO Gemini 1530 SEM (Carl Zeiss SMT GmbH, Oberkochen, Germany) after preparation by means of a

Table 1. Nominal composition of the investigated bioglass types (mol%).

Material		SiO ₂	P ₂ O ₅	Na ₂ O	CaO	K ₂ O	CuO	SrO
Control	45S5	46.1	2.6	24.4	26.9	0	0	0
	ICIE16	49.46	1.07	6.6	36.27	6.6	0	0
Test	ICIE16-Cu	46.99	1.02	6.27	34.46	6.27	5	0
	ICIE16-Sr	46.99	1.02	6.27	34.46	6.27	0	5

washing step with ethanol and drying at 50°C. The samples were then carbon- and gold-sputtered and examined at accelerating voltages of 10–20 kV. Furthermore, energy-dispersive X-ray (EDX) spectra (K α line) of disc surface areas of 1×1 μm^2 were recorded at 10 and 20 kV in field emission gun SEM to analyze the chemical composition and level of contamination qualitatively.

Inductively coupled plasma optical emission spectrometry (ICP-OES): The simulated body fluid (SBF) was prepared according to Kokubo *et al.* (31). The bioglass samples were immersed in the SBF and incubated at 37°C for 3, 7, and 14 days, under static conditions. After incubation, the samples were removed, and ICP-OES measurements were performed using Ultima-2 (Horiba, Kyoto, Japan) at 252.851 nm to determine the amounts of ions which were dissolved from the bioglass samples into the SBF. For each sample, three measurements were conducted at days 3, 7, and 14.

In vivo study. Experimental design. Subcutaneous implantation was performed on 32 female Sprague–Dawley rats, 3 to 4 months old, weighing 255±15 g, (Military Medical Academy, Belgrade, Serbia) that were randomly assigned to groups according to bioglass type and time point (n=4 per each group and time point). The experimental animals were anesthetized *via* an intraperitoneal injection of ketamine (100 mg/kg of body weight) and xylazine (5 mg/kg of body weight). A midline incision was made in the skin of the scapular region, followed by a blunt incision to create a subcutaneous pocket. Then 100 mg of bioglass was implanted in the subcutaneous pocket under sterile conditions and the wound was stitched with nonabsorbable suture materials. At 10 or 30 days post implantation, animals were sacrificed using an overdose of the anesthesia to analyze both the early and late tissue responses to the biomaterials in accordance with previous publications (28, 32). The biomaterials were extracted with their surrounding tissue and placed in formaldehyde solution for preservation for 24 h. Experiments were authorized by the Local Ethical Committee of the Faculty of Medicine (University of Niš, Serbia) based on the approval of the Veterinary Directorate of the Ministry of Agriculture, Forestry and Water Management of the Republic of Serbia (approval number 323-07-01762/2019-05/9; date of approval: 01 March 2019).

Histological workup. The explants were processed with an initial dehydration through increasing ethanol concentrations and cleared in xylene. Afterwards, the explants were treated with 10% Tris-buffered ethylenediaminetetraacetic acid for 2 weeks for decalcification to allow subsequent sectioning. The explants were then embedded in paraffin. The paraffin blocks were sectioned at 3–5 μm thickness. Serial sections were used for histochemical staining (*i.e.*, hematoxylin and eosin) and immunohistochemical staining, *i.e.*, against CD11c for detection of M1 macrophages, CD163 for detection of M2 macrophages, and CD31 for detection of

endothelial cells). All antibodies were obtained from Abcam (Cambridge, UK). Immunohistochemical staining has been thoroughly described by Lindner *et al.* (28). In brief, the histological sections were treated with citrate buffer and proteinase K at pH 8 for 20 min in a water bath at 96°C, followed by equilibration using TBS-T buffer. Subsequently, the slides were prepared by H₂O₂ and avidin and biotin blocking solutions (Avidin/Biotin Blocking Kit; Vector Laboratories, Burlingame, CA, USA). Incubation with the respective primary antibody for 30 min was conducted, followed by incubation with the secondary antibody (goat anti-rabbit IgG-B, sc-2040, 1:200; Santa Cruz Biotechnology, Shandon, CA, USA). Afterward, the avidin–biotin–peroxidase complex (ThermoFisher Scientific, Dreieich, Germany) was applied for 30 min, and counterstaining by bluing was conducted.

Histological and histomorphometrical analysis. Histopathological evaluation was conducted using a conventional diagnostic microscope (Axio Imager M2; Zeiss, Oberkochen, Germany). The evaluation included determining the state of the implantation bed and biomaterial, by assessment of granulation tissue, types of immune cells [granulocytes, lymphocytes, macrophages, and biomaterial-associated multinucleated giant cells (BMGCs)], vascularization, fibrosis, hemorrhage, and necrosis. Photographs were taken using an Axiocam 506 color connected to its software ZEN Core (Zeiss).

To conduct the quantitative histomorphometrical analysis, the immunohistochemically stained slides were initially digitized using a scanning microscope (M8; PreciPoint GmbH, Munich, Germany). The frequency of M1 and M2 macrophages was analyzed using a plugin in ImageJ (National Institutes of Health, Bethesda, MA, USA) that was developed by Lindner and colleagues (28). This plugin allows measurement of the number of positively stained cells per mm^2 and the vascularization pattern (vessels/ mm^2 and percentage vascularization).

Evaluation of the local biological effects of bioglass implantation based on semiquantitative histopathological scoring. Histopathological scoring of the local effects of bioglass implantation was conducted based on ISO 10993:2016, Part 6, Annex E (33). All microscopic analysis was performed by a single experienced pathologist who was blinded through coded slides. A semiquantitative histological analysis was performed on each slide from which six high-power fields (×400) were scanned according to the area of interest, without any overlap, establishing a count of the surveyed cells and resulting in a score value indicating predominance among them. The biological response parameters at the bioglass interface were evaluated and irritancy/reactivity was scored as shown in Table II. The mean and the standard deviation of the scoring results of the six visual fields were calculated for each test material

Table II. Histological evaluation system for irritancy/reactivity—cell type/response. The overall irritancy score was calculated using the individual component scores: (polymorphonuclear cells+lymphocytes+plasma cells+macrophages+giant cells+necrosis)×2+(neovascularization+fibrosis+fatty infiltrate).

Response	Component score				
	0	1	2	3	4
Polymorphonuclear cells	0	Rare, 1-5/hpf	6-10/hpf	Heavy infiltrate	Packed
Lymphocytes	0	Rare, 1-5/hpf	6-10/hpf	Heavy infiltrate	Packed
Plasma cells	0	Rare, 1-5/hpf	6-10/hpf	Heavy infiltrate	Packed
Macrophages	0	Rare, 1-5/hpf	6-10/hpf	Heavy infiltrate	Packed
Giant cells	0	Rare, 1-2/hpf	3-5/hpf	Heavy infiltrate	Packed
Necrosis/osteolysis	0	Minimal	Mild	Moderate	Marked
Neovascularization	0	Minimal capillary proliferation focal, 1-3 buds	Groups of 4-7 capillaries with supporting fibroblastic structures	Broad band of capillaries with supporting structures	Extensive band of capillaries with supporting fibroblastic structures
Fibrocytes/fibroconnective tissue, fibrosis	0	Narrow band	Moderately thick band	Thick band	Extensive band
Fatty infiltrate	0	Minimal amount of fat associated with fibrosis	Several layers of fat and fibrosis	Elongated and broad accumulation of fat cells about the implant site	Extensive fat completely surrounding the implant

Hpf: High-powered field (×400).

and the control material (45S5 bioglass). Finally, according to Annex E, Table E of DIN EN ISO 10993-6:2016 (33), for each test material the relative irritancy was calculated as: Irritancy score of the test material – irritancy score of the control material, while a negative difference was assigned a score of 0. Irritancy status was assigned according to the values listed in Table III.

Statistical analysis. Quantitative data from the histomorphometrical analysis are presented as means with standard deviation. The data were analyzed using analysis of variance tool via GraphPad Prism 9.3.1 software (GraphPad Inc., La Jolla, CA, USA). Statistical differences were designated as significant when *p*-values were less than 0.05. Both inter and intra-individual significances were calculated.

Results

Bioglass characterization. SEM analysis. The analysis via SEM revealed significant differences in the surface morphology of the (Figure 1). The surface morphology ranged from an almost smooth surface of the 45S5 bioglass to predominantly crystalline surface structures of ICIE16 and ICIE16-Sr, to a vesicular surface pattern in the case of ICIE16-Cu (Figure 1).

EDX spectroscopy. EDX spectroscopy was used to determine the qualitative chemical composition of the samples. EDX analyses revealed the presence of Si, Na, Ca, P, and O in the 45S5 samples, as well as of additional K in ICIE16 samples (Figure 2). Moreover, it confirmed the presence of Cu and Sr in the ion-doped ICIE16 bioglass materials (Figure 2).

Table III. Irritancy/reactivity grade [adapted from DIN EN ISO 10993-6 (33)].

Overall irritancy score	Irritancy/reactivity status
0.0 to 2.9	Minimal or no reaction (non-irritant)
3.0 to 8.9	Slight reaction (slight irritant)
9.0 to 15.0	Moderate reaction (moderate irritant)
>15.1	Severe reaction (severe irritant)

ICP-OES. The release of ions from the ICIE16-Cu and ICIE16-Sr materials was measured before and after immersion in SBF for 3, 7, and 14 days (Figure 3). The release of both ions consistently significantly increased with time (*p*<0.001) (Figure 3). Moreover, a significantly higher copper release was found at every time point compared to strontium (Figure 3).

In vivo histopathological analysis. Histological analysis showed that the different types of bioglass were detectable within the subcutaneous connective tissue at day 10 and 30 post implantation. Moreover, no tissue ingrowth into the bioglass pellets was detected. All bioglass materials were surrounded by inflammatory cell types, *i.e.*, mainly macrophages, as well as lower numbers of granulocytes and fibroblasts at 10 days post implantation (Figure 4). Furthermore, single multinucleated giant cells (BMGCs) were detectable at the material surfaces in all study groups. The histopathological analysis revealed comparable extents

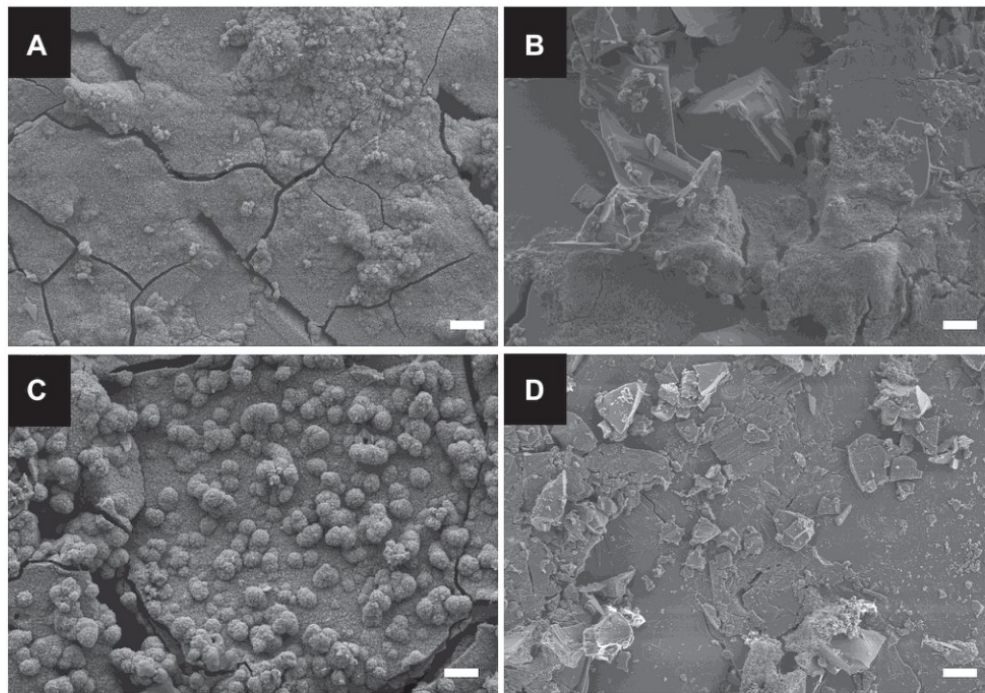


Figure 1. Exemplary scanning electron microscopy images of the surface morphology in 45S5 (A), ICIE16 (B), ICIE16-Sr (C) and ICIE16-Cu (D) bioglass (200× magnification; scale bars=10 μ m).

of inflammatory tissue reactions in all four study groups. However, immunohistochemical detection revealed fewer macrophages within the implantation beds of animals receiving ICIE16-Cu bioglass and especially lower numbers of CD163-positive macrophages in this group compared to the other groups (Figure 5). Between the three other study groups, no visible differences in the occurrence of CD163-positive cells were observed. Moreover, the analysis showed that minimally lower numbers of CD11c-positive macrophages were observed in the ICIE16-Cu bioglass group compared to the other study groups. No differences in the occurrence of this macrophage subtype were observed in the other study groups (Figure 6). Additionally, visibly lower numbers of pro-inflammatory macrophages were observable in all study groups. Furthermore, comparable numbers of small vessels were found within the reactive connective tissue neighboring each implant (Figure 7).

At day 30 post implantation, still comparable tissue reaction patterns were detected in all groups. Histopathological analyses showed that the same types of cells, *i.e.*, mainly

macrophages, as well as single granulocytes and fibroblasts were involved (Figure 8). However, the number of BMGCs at the material surface was slightly increased in all groups. Additionally, the histopathological analyses revealed that the numbers of M1 macrophages had visibly increased in the groups with ICIE16-Sr and the pure ICIE16 bioglass and their numbers were higher compared to the number of CD163-positive M2 macrophages. In contrast, in the ICIE16-Cu and 45S5 bioglass groups, comparative numbers of both macrophage subtypes were observed (Figure 5 and Figure 6). The observation of the implantation bed vascularization showed that comparable numbers of vessels were still apparent within the granulation and connective tissues surrounding all bioglass materials (Figure 7).

Results of the histopathological scoring. Histopathological scoring resulted in similar inflammation in all groups at day 10 post implantation (Table IV). The inflammatory tissue response to the bioglass included cells of the immune system, *i.e.*, mainly polymorphonuclear cells/granulocytes (moderate presence),

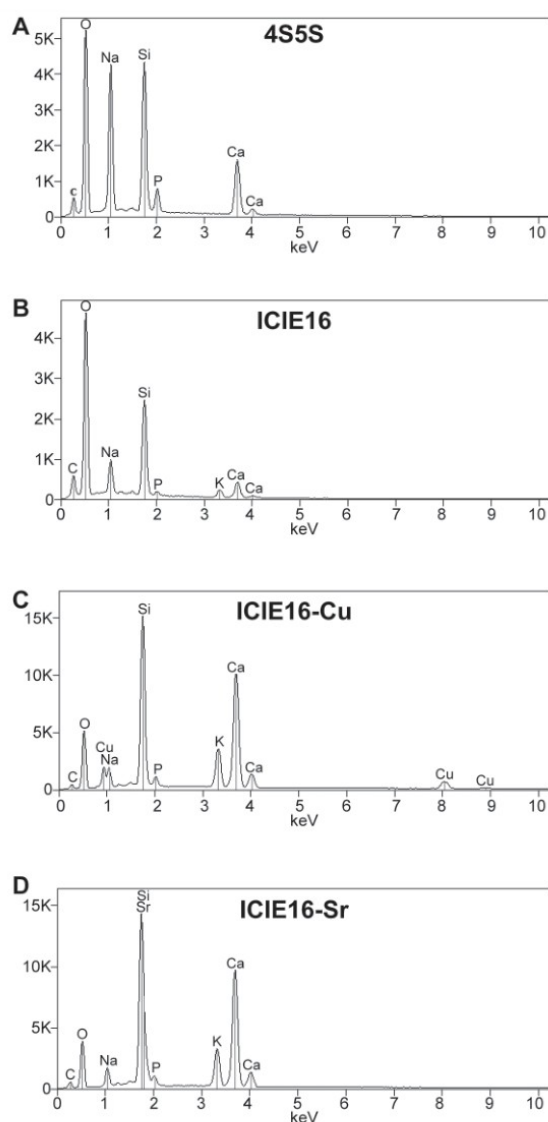


Figure 2. Energy-dispersive X-ray spectroscopy spectra of the 4S5S (A), ICIE16 (B), ICIE16-Cu (C) and ICIE16-Sr (D) bioglass materials.

lymphocytes (moderate presence), plasma cells (rare), macrophages (moderate presence) and giant cells (moderate presence), which were comparable in all groups, as was neovascularization (moderate); whereas fibrosis was moderate in all ICIE16 groups and, rare in the 4S5S group). Fatty infiltrate was not detectable in any group, nor was necrosis.

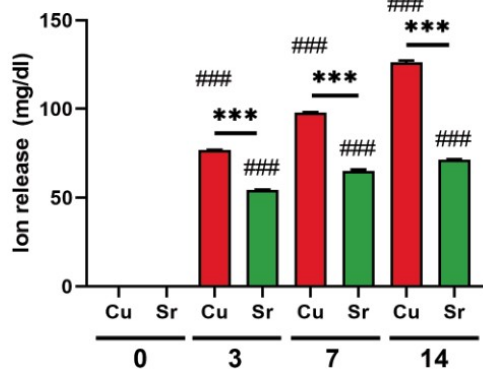


Figure 3. Release of Cu ions from ion-doped ICIE16-Cu and Sr ions from ICIE16-Sr bioglass materials after immersion in simulated body fluid (SBF) for 3, 7, and 14 days. Significantly different at $p < 0.001$; ***between metals; ###between bioglass samples of the same type.

Histopathological scoring resulted in comparable degrees of inflammation at day 30 post implantation (Table IV). The inflammatory tissue response to the bioglass included cells of the immune system, *i.e.* mainly polymorphonuclear cells/granulocytes (moderate presence, comparable in all groups), lymphocytes (moderate presence, comparable in all groups), plasma cells (rare presence, comparable in all groups), macrophages (moderate presence, comparable in all groups), giant cells (moderate presence, comparable in all groups), neovascularization (moderate, comparable in all groups), fibrosis (moderate in the groups of the ICIE16 bioglass, rare in the group of the 45S5 bioglass), fatty infiltrate (not detectable in all groups), and necrosis (not detectable in all groups).

The irritancy score was calculated based on the scoring results. The calculation showed that the ICIE16 bioglass had an average treatment irritancy of 18.5 at day 10 post implantation, and an overall irritancy score of 1.3; hence, the biomaterial was considered non-irritant at this time point (Table V). The ICIE16-Cu bioglass had an average treatment irritancy of 12.5 at day 10 post implantation, and an overall irritancy score of 0.0; hence, the biomaterial was considered non-irritant at this time point. The ICIE16-Sr bioglass had an average treatment irritancy of 13.7 at day 10 post implantation, and an overall irritancy score of 0.0; hence, the biomaterial was considered non-irritant at this time point.

At day 30 post implantation, the treatment irritancy score of the ICIE16 bioglass had a total of 18.6, and an overall irritancy score of 2.8; hence, the biomaterial was considered

non-irritant at this time point (Table V). The ICIE16-Cu bioglass had an average treatment irritancy of 17.3 at day 30 post implantation, and an overall irritancy score of 1.4; hence, the biomaterial was considered non-irritant at this time point. The ICIE16-Sr bioglass had an average treatment irritancy of 14.7 at day 30 post implantation, and an overall irritancy score of 0.0; hence, the biomaterial was considered non-irritant at this time point.

Histomorphometrical analysis of the immune response. At 10 days post implantation, ICIE16-Cu induced lower numbers of pro-inflammatory M1-macrophages (612.7 ± 429.5 cells/mm²) than detected in the 45S5 group, which showed the highest values for pro-inflammatory macrophages ($4,167 \pm 1,015$ cells/mm²). The evaluation furthermore revealed that in the ICIE16-Sr ($2,508 \pm 460.3$ cells/mm²) and ICIE16 ($2,603 \pm 851.6$ cells/mm²) groups, comparable numbers of M1 macrophages were found (Figure 8). Furthermore, the number of M1 macrophages was higher in the ICIE16-Sr group compared to the ICIE16-Cu group, but only the number of M1 macrophages in the 45S5 group was significantly higher than in the ICIE16-Cu group ($p < 0.01$). In the ICIE16 group ($1,953 \pm 581.1$ cells/mm²) and the 45S5 group ($1,529 \pm 961.6$ cells/mm²) the number of M2 macrophages was highest followed by the ICIE16-Sr group (919.1 ± 701.9 cells/mm²) and the ICIE16-Cu group (47.73 ± 39.12 cells/mm²), without any significant differences.

At 30 days post implantation, increasing numbers of M1 macrophages were detected in the ICIE16-Cu ICIE16-Sr and ICIE16-groups ($2,091 \pm 1,820$, $4,126 \pm 1,207$ and $3,133 \pm 1,177$ cells/mm², respectively). Furthermore, the number of M1 macrophages in the 45S5 group decreased from 10 to 30 days ($1,648 \pm 1,278$ cells/mm²).

The number of M2 macrophages remained at a relatively low level in the of the ICIE16-Cu group (859.2 ± 380.8 cells/mm²). In the ICIE16-Sr ($1,350 \pm 230.4$ cells/mm²), the ICIE16 ($1,260 \pm 662.2$ cells/mm²) and the 45S5 ($1,504 \pm 1,401$ cells/mm²) groups, comparable higher numbers of M2 macrophages were found. Thereby, only in the latter group were similar numbers of M1 and M2 macrophages detected.

Histomorphometrical analysis of the implantation bed vascularization. At 10 days post implantation, the lowest numbers of vessels were found in the ICIE16-Cu group ($1,316 \pm 774$ vessels/mm²), while higher numbers were determined in the ICIE16 ($2,040 \pm 1,025$ vessels/mm²), 45S5 ($2,643 \pm 1,331$ vessels/mm²) and ICIE16-Sr ($4,012 \pm 1,791$ vessels/mm²) groups (Figure 9).

At day 30 post implantation, the lowest vessel numbers were still found in the ICIE16-Cu group (582.5 ± 372.9 vessels/mm²) followed by higher numbers in the ICIE16-Sr ($1,806 \pm 1,548$ vessels/mm²), ICIE16 ($2,461 \pm 1,280$ vessels/mm²) and 45S5 ($2,669 \pm 985.5$ vessels/mm²) groups (Figure 9). Thus, comparable

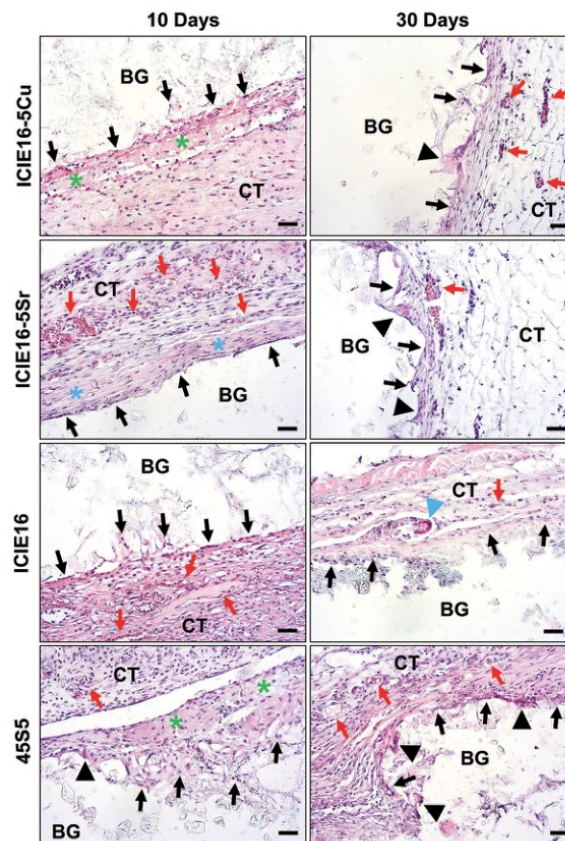


Figure 4. Exemplary histological images showing the tissue reactions to four bioglass (BG) types within subcutaneous connective tissue (CT) at 10 days (left) and 30 days (right) after implantation. Black arrows: Mononuclear cells at the material–tissue interface; black arrowheads: multinucleated cells at the material surface; red arrows: blood vessels; green stars: fibrin network; blue arrowhead: multinucleated giant cell associated with a fragment of ICIE16 bioglass (hematoxylin and eosin staining, 20× magnification; scale bars=20 μ m).

implantation bed vascularization was found at both study time points in all groups (Figure 9).

Discussion

It is still unknown what influence so-called therapeutic ions have on inflammatory tissue reactions that are strongly associated with tissue regeneration (34). Thus, it was supposed that their inclusion can be used to control material-triggered healing process by regulating the induction of M2 macrophages (35, 36). Interestingly, the research in this field

revealed immunomodulatory characteristics of different therapeutic ions indicating they lead to the reduction of the migration of macrophages, the formation of biomaterial-associated multinucleated giant cells (MNGCs) and fibrous capsules, as well as the production of inflammatory cytokines around the implanted materials (36). It was concluded that ion addition might allow regulation of immune responses by altering the ionic microenvironment (37). The use of therapeutic ions in dental applications therefore provides many advantages compared to both purely inorganic bone substitute materials and also to organic

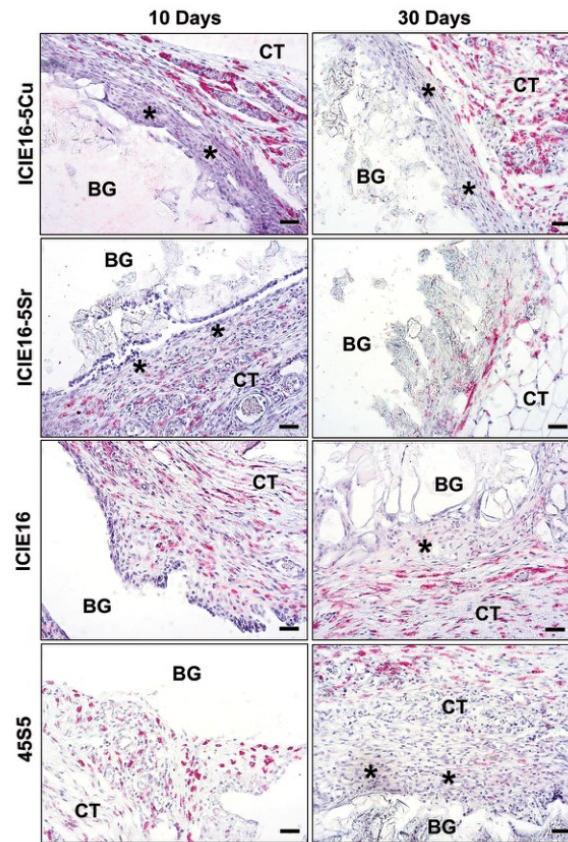


Figure 5. Exemplary histological images showing CD163-positive M2 macrophages (red staining) within the subcutaneous connective tissue (CT) at 10 days (left) and 30 days (right) after implantation of the four bioglass (BG) types. Stars: Cell walls at the material surface that were free of M2 macrophages (CD163 immunostaining, 20× magnification; scale bars=20 μm).

biomolecules (*e.g.*, growth factors). Altogether, the addition of therapeutic ions to BSMs, such as bioglass, might lead to a better bone-healing process by adaption of the underlying inflammatory reactions. Thus, the present study was conducted to investigate the chemical and *in vivo* influence of doping ICIE16 bioglass with 5 wt% copper, and with 5 wt% strontium ions.

Initially, the examination of the chemical composition of the bioglass *via* EDX analysis revealed that the ion-doped bioglass exhibited peaks reflecting the normal elemental distribution of the pure bioglass material and confirmed the presence of the added ions within the respective bioglass.

To determine the release of both therapeutic ions, ICP-OES analysis was conducted. Copper and strontium ions were found to be released from ICIE16 in an increasing manner over time. Additionally, it was observed that the release of both ions consistently significantly increased with time and at every time point, a significantly higher copper release was found compared to strontium. In this context, it should be mentioned that both ions are considered microelements on the basis of their relative abundance in biological cells/tissues and physiological fluids, *i.e.*, concentration >100 mg/dl and <100 mg/dl, respectively (38, 39). High concentrations of these metals can be toxic (36, 40). In the case of the bioglass

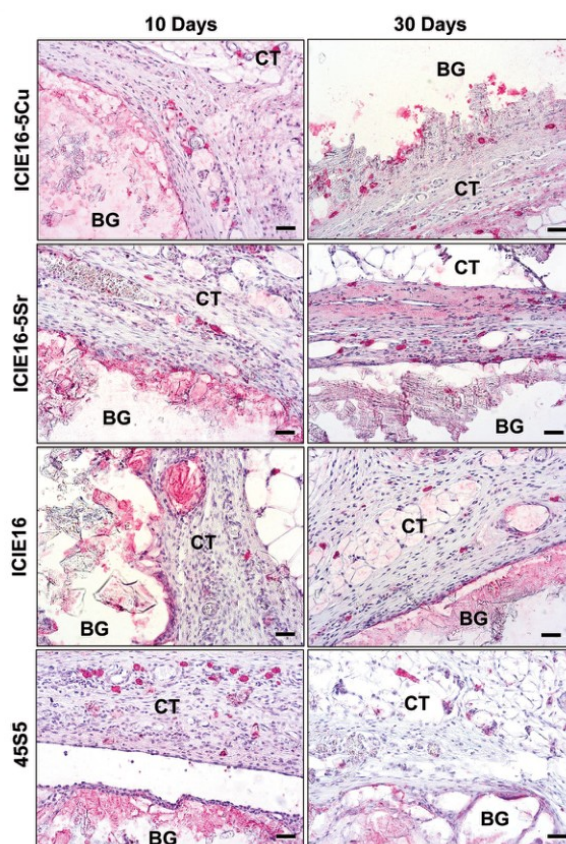


Figure 6. Exemplary histological images showing CD11c-positive M1 macrophages (red staining) within the subcutaneous connective tissue (CT) at 10 days (left) and 30 days (right) after implantation of the four bioglass (BG) types (CD11c immunostaining, 20× magnification; scale bars=20 µm).

materials studied here, the ion release was found to be below the above thresholds with only one exception, ICIE16-Cu at 14 days. Based on these values, an increased inflammatory reaction might be triggered by ICIE16-Cu.

The histopathological analysis showed that all bioglass types induced moderate foreign body reactions whose manifestation is usually seen in case of a broad variety of BSMs (29, 41). This reaction pattern includes formation of a granulation tissue involving mainly macrophages, biomaterial-associated MNGCs and lymphocytes, as well as blood vessels, normally starting at day 10 post implantation. Signs of (cellular) degradation were also observed beginning on day 10 post implantation, including mononuclear phagocytes within the body of the implant and the presence of biomaterial-associated MNGCs at the material

surfaces. Interestingly, the intensity or cell number of the granulation tissue and thus of phagocytes decreased in all ICIE16 groups almost to slight fibrosis but not in case of the 45S5[®] bioglass, which suggests that the 45S5[®] bioglass continuously triggers a biodegrading immune reaction. This leads to the conclusion that the ICIE16 bioglass triggered the observed tissue reaction and in general underwent a lower degree of cellular biodegradation compared to that with 45S5[®] bioglass. Moreover, these results also showed that the addition of the therapeutic ions did not seem to influence the inflammatory tissue reaction. An explanation for the latter observation may be found in the material characteristics of both bioglass types. Thus, the larger sintering window of ICIE16 bioglass due to its composition may result in a lower tendency

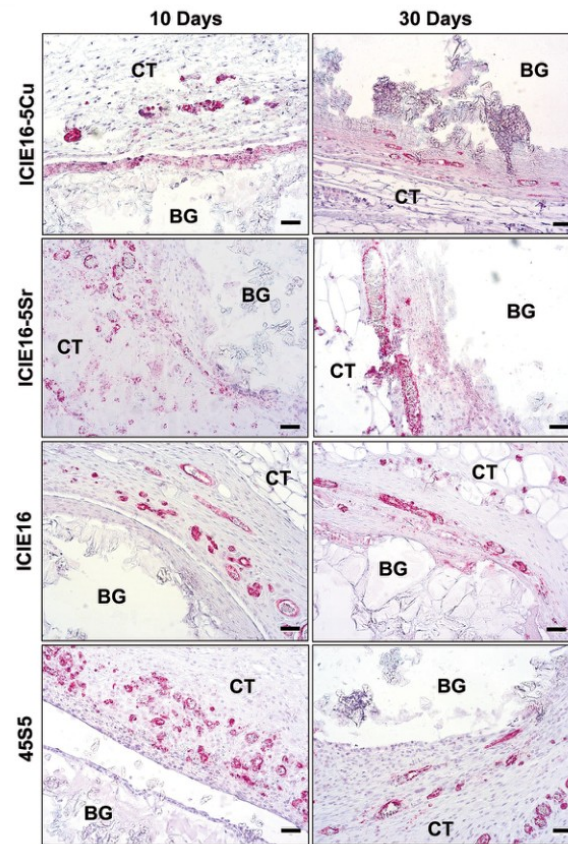


Figure 7. Exemplary histological images showing blood vessels (red staining) within the subcutaneous connective tissue (CT) at 10 days (left) and 30 days (right) after implantation of the four bioglass (BG) types. (CD31 immunostaining; 200 $\times$, scale bars=20 μ m).

to crystallize, which may have influenced the tissue reaction at the molecular level (42, 43). In this context, it has been described that the initial binding of proteins onto biomaterial surfaces is of high importance for the overall alignment of the following tissue reaction (44). Thereby, the surface pattern of the biomaterial dictates both (a) the type of proteins bound to the surface and (b) its conformation, which will expose specific binding sites for different cell receptors and cell types and thus also dictate the foreign body cascade (45). In this context, it is conceivable that the ICIE16 bioglass induced higher expression of transforming growth factor β (TGF β), especially isoform 1, mainly by macrophages and biomaterial-associated MNGCs (46). This finding was already shown by Hernandez-Pando *et al.*, who observed that injected nitrocellulose particles initially

caused biomaterial-associated MNGCs to express interleukin-1 α , tumor necrosis factor α , and TGF β (47); interestingly, TGF β was the most persistently expressed cytokine over time and its expression was associated with extensive chronic fibrosis. An additional explanation for the observed pattern of tissue reaction might be found in the fact that the ICIE16 bioglass contains a considerably lower amount of sodium oxide; the higher sodium content of the 45S5 bioglass has been shown to induce a strong increase in local pH, which, at least in part, accounts for its dose-dependent cytotoxic effects (48). Thus, it is conceivable that the higher pH within the implantation bed of the 45S5 bioglass did not lead to fibrosis but was a trigger for faster cellular biodegradation and served as an inducer of phagocyte accumulation.

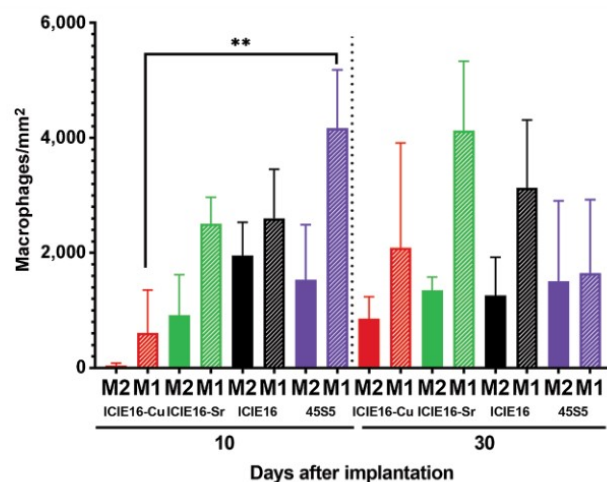


Figure 8. Results of the histomorphometrical analysis of CD11c-positive M1 and CD163-positive M2 macrophages (cells/mm²). ***Significantly different at $p < 0.001$ ($n=4$).

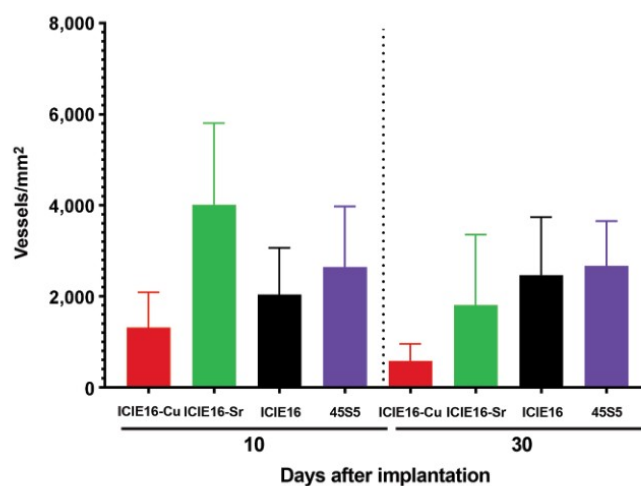


Figure 9. Results of the histomorphometrical analysis of vessel density (vessels/mm²) at 10 and 30 days after implantation of four bioglass types ($n=4$).

However, the histopathological scoring showed that the ICIE16-based bioglass can be considered as non-irritant as it induced a slightly increased inflammatory tissue reaction involving a granulation tissue, although its appearance was

similar to that observed in the control group of the 45S5 bioglass at day 10 and day 30 post implantation. Moreover, the histomorphometrical analysis revealed no significant differences between the numbers of M1 and M2 macrophages

Table IV. Results of the evaluation of irritancy scoring of the four bioglass types. Scoring is detailed in Table II. Data are the mean scores $\pm$ standard deviation.

Parameter	ICIE16		ICIE16-Cu		ICIE16-Sr		4S5S (Control)	
	10 Days	30 Days	10 Days	30 Days	10 Days	30 Days	10 Days	30 Days
Polymorphonuclear cells	2.0 $\pm$ 0.2	2.4 $\pm$ 0.4	2.7 $\pm$ 0.2	2.8 $\pm$ 0.3	2.8 $\pm$ 0.2	1.8 $\pm$ 0.1	2.3 $\pm$ 0.2	1.3 $\pm$ 0.2
Lymphocytes	1.7 $\pm$ 0.5	2.0 $\pm$ 0.2	1.3 $\pm$ 0.1	1.0 $\pm$ 0.2	1.2 $\pm$ 0.3	0.9 $\pm$ 0.2	1.5 $\pm$ 0.2	1.6 $\pm$ 0.3
Plasma cells	0.3 $\pm$ 0.1	0.3 $\pm$ 0.2	0.1 $\pm$ 0.1	0.1 $\pm$ 0.1	0.3 $\pm$ 0.1	0.1 $\pm$ 0.1	0.3 $\pm$ 0.2	0.3 $\pm$ 0.2
Macrophages	3.0 $\pm$ 0.0	3.0 $\pm$ 0.0	3.0 $\pm$ 0.0	3.0 $\pm$ 0.0	3.0 $\pm$ 0.0	3.0 $\pm$ 0.0	3.0 $\pm$ 0.0	3.0 $\pm$ 0.0
Giant cells	1.2 $\pm$ 0.3	0.7 $\pm$ 0.2	0.3 $\pm$ 0.1	0.6 $\pm$ 0.3	0.8 $\pm$ 0.4	0.5 $\pm$ 0.3	1.0 $\pm$ 0.2	1.1 $\pm$ 0.7
Neovascularization	1.4 $\pm$ 0.1	1.1 $\pm$ 0.1	1.2 $\pm$ 0.2	1.3 $\pm$ 0.2	1.2 $\pm$ 0.2	1.1 $\pm$ 0.3	1.2 $\pm$ 0.2	1.1 $\pm$ 0.1
Fibrosis	0.8 $\pm$ 0.2	0.8 $\pm$ 0.3	0.8 $\pm$ 0.2	0.8 $\pm$ 0.2	0.9 $\pm$ 0.3	1.0 $\pm$ 0.0	0.1 $\pm$ 0.1	0.2 $\pm$ 0.1
Fatty infiltrate	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
Necrosis	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0

at the different study timepoints – with only one exception for M1 macrophages for the 4S5S bioglass group compared to the ICIE16-Cu group at day 10 post implantation. This finding is also in line with the aforementioned higher phagocyte accumulation in the case of 4S5S bioglass and additionally shows its higher inflammatory potential. Interestingly, higher M1 macrophage accumulation was only detected at day 10 post implantation; this might have been the result of an increase in pH with 4S5S bioglass being restricted to the early phase after implantation. This assumption is based on the fact that is well known that an apatite layer forms at the surface of bioglass that can prevent pH shifts (14). This observation might thus be comparable with findings made after implantation of magnesium-based biomaterials (49). Recent advances in the understanding of corrosion mechanisms have shown that corrosion of magnesium continues despite the presence of an oxide layer. This layer has been shown to prevent the corrosion process marginally, but may fail to adequately protect magnesium-based implants in acidic and neutral environments, where it can corrode rapidly exposing the metal underneath to further attack. However, in our case, no influence of the addition of copper in ICIE16 was found, as the ratio of M1 and M2 macrophage numbers in the ICIE16-Cu-treated group did not significantly differ compared to the other groups.

Interestingly, the numbers of pro-inflammatory macrophages in bioglass-treated animals tended to be higher. This phenomenon is very interesting even in view of the biocompatibility of the analyzed bioglass materials. Even in the case of other BSMs, higher M1 macrophage numbers were found within their implantation beds, which is believed to be an unnatural immune response to such materials, even natural materials, such as collagen membranes, amongst others (50, 51). It was also proposed that this pro-inflammatory cell type is associated with the inflammation-based biodegradation pattern (52). In this context, various studies have revealed a dependency of the biodegradation capacity of macrophages,

Table V. Irritancy scores and irritancy status. For each test material, the relative irritancy was calculated as: Irritancy score test material – irritancy score control material; a negative difference was assigned a score of 0. Irritancy status was assigned according to Table III.

Time point	Study group	Overall irritancy score	Relative irritancy score	Irritant status
10 Days	ICIE16	18.5	1.3	Non-irritant
	ICIE16-Cu	12.5	0 (–4.7)	Non-irritant
	ICIE16-Sr	13.7	0 (–3.5)	Non-irritant
	4S5S (Control)	17.2	-	-
30 Days	ICIE16	18.6	2.8	Non-irritant
	ICIE16-Cu	17.3	1.4	Non-irritant
	ICIE16-Sr	14.7	0 (–1.1)	Non-irritant
	4S5S (Control)	15.8	-	-

and oxidative degradation (53). For example, it was shown that pro-inflammatory macrophages are capable of exerting oxidative stress and release lytic enzymes, such as nitric oxide and reactive oxygen species (54, 55). Moreover, high levels of reactive oxygen species were associated with high expression of pro-inflammatory cytokines in pro-inflammatory macrophages (56). Furthermore, a study by Wissing *et al.* demonstrated that lipopolysaccharide/interferon- γ treatment of macrophages led to a pro-inflammatory gene-expression profile and induced significant up-regulation of lipid peroxidation, overexpression of the oxidative genes NADPH oxidase 2 (NOX2) and nuclear factor kappa-light-chain-enhance of activated B-cells (NFkB) and increased enzymatic activity (57). Thus, the comparable numbers of M1 macrophages with the use of all bioglass types studied here may also hint at their comparable biodegradation pattern. Another interesting finding is the pattern of localization of the M1 and M2 macrophages found in all study groups. The immunohistochemical study

showed M2 macrophages remained within the surrounding granulation and connective tissues but not on the surface nor within the implant body. In contrast, pro-inflammatory M1 macrophages appeared on the surface of all implants (and also within them) starting from day 10 post implantation. These results suggest that both macrophage subtypes have different functionalities within the foreign body cascade, as already suggested (58, 59). Proinflammatory cells seem to engage in cell-mediated resorption, while anti-inflammatory cells seem to be more involved in the regeneration of the surrounding tissue – even in the time frame of the present study. However, no significant differences between the M1 and M2 macrophage subtypes were found, leading to the conclusion that all the bioglass types analyzed are biocompatible as overall, they induced balanced numbers of M1 and M2 macrophages.

Even though copper has been reported to have angiogenic potential (60-62), no significant differences were observed in the vascularization of the implantation bed. Contradicting the *in vitro*-based predications of the angiogenic potential of copper (26, 63), neither ICIE16-Cu nor ICIE16-Sr enhanced implant vascularization in this study.

Altogether, neither of these therapeutic ions had any biological influence on the immune response or the vascularization pattern. This result is surprising as both ions have been described to influence both molecular cascades but are in line with another *in vivo* study including an analysis of silicate-based copper-doping that also showed no influence and, more importantly, the materials in fact hindered the regeneration process (62, 64).

One limitation of this study was the short-term *in vivo* experiment. As bone regeneration occurs over a longer time period, further studies within the bony microenvironment have to be conducted in order to verify the osteogenic potential of these ion-doped bioactive glass materials. A further *in vivo* trial using bone implantation models may show another tissue reactivity of the analyzed materials. Another point to consider is that the *in vitro* investigation of ion-release in SBF can only be seen as a limited result compared to the ion-release *in vivo*. That is because SBF differs from body fluids in the following aspects: i) body fluid is a dynamic system while the ion-release measurement was conducted in a static environment; ii) body fluids are 'open' systems, which means that their components are constantly replaced/renewed whereas SBF was not changed during the analysis; and iii) SBF does not include all the components of body fluids (*e.g.*, proteins, enzymes, and ions). Thus, an *in vivo* analysis of ion release can provide more insights into the concentrations of the ions within the surrounding tissue and the overall dissolution behavior.

Another analysis method that could provide further understanding is the quantitative measurement of the nanoscale build-up of apatite and whether a correlation exists with histomorphometrical results.

Overall, the studied bioactive glass materials seemed to be biocompatible, with an acceptable foreign body reaction that is typical of synthetic BSMs. Finally, and in contradiction to published *in vitro* evaluations, copper- and strontium-doping did not significantly influence macrophage polarization, nor vascularization.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

Conceptualization: FS and MB; methodology: SN, SS, OG, DR, FS and MB; software: OG, FS and MB; validation: SA, FS and MB; formal analysis: AM, MR, SN, SS, FS and MB; investigation: SA, AM, YR, BC, OG, FS and MB; resources: SN, SS, OG, FS and MB; data curation: SN, SS, MR, OG, FS and MB; writing—original draft preparation: SA, SN, SS, OG, DR, FS and MB; writing—review and editing: SA, SN, FS and MB; visualization: SA, FS and MB; supervision: SN, FS and MB; project administration: SN, FS and MB; funding acquisition: SN and MB. All Authors read and agreed to the published version of the article.

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Received June 28, 2022

Revised July 28, 2022

Accepted August 9, 2022



OPEN ACCESS

EDITED BY
Shen Liu,
Shanghai Jiao Tong University, China

REVIEWED BY
Ahmed El-Fiqi,
National Research Centre, Egypt
Nenad L. Ignjatovic,
Institute of Technical Sciences (SASA), Serbia

*CORRESPONDENCE
Mike Barbeck,
✉ mike.barbeck@med.uni-rostock.de

[†]These authors have contributed equally to this work

RECEIVED 28 March 2024
ACCEPTED 27 May 2024
PUBLISHED 24 June 2024

CITATION
Ren Y, Jung O, Batinic M, Burckhardt K, Gölke O, Alkildani S, Köwitsch A, Najman S, Stojanovic S, Liu L, Prade I and Barbeck M (2024), Biphasic bone substitutes coated with PLGA incorporating therapeutic ions Sr²⁺ and Mg²⁺: cytotoxicity cascade and *in vivo* response of immune and bone regeneration. *Front. Bioeng. Biotechnol.* 12:1408702. doi: 10.3389/fbioe.2024.1408702

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Biphasic bone substitutes coated with PLGA incorporating therapeutic ions Sr²⁺ and Mg²⁺: cytotoxicity cascade and *in vivo* response of immune and bone regeneration

Yanru Ren¹, Ole Jung¹, Milijana Batinic², Kim Burckhardt¹, Oliver Gölke², Said Alkildani³, Alexander Köwitsch⁴, Stevo Najman^{5,6}, Sanja Stojanovic^{5,6}, Luo Liu⁷, Ina Prade^{8†} and Mike Barbeck^{1,3*†}

¹Clinic and Policlinic for Dermatology and Venereology, University Medical Center Rostock, Rostock, Germany, ²Institute of Materials Science and Technology, Chair of Advanced Ceramic Materials, Technical University Berlin, Berlin, Germany, ³BerlinAnalytix GmbH, Berlin, Germany, ⁴Biotrics Bioimplants AG, Berlin, Germany, ⁵Department of Biology and Human Genetics, Faculty of Medicine, University of Niš, Niš, Serbia, ⁶Scientific Research Center for Biomedicine, Department for Cell and Tissue Engineering, Faculty of Medicine, University of Niš, Niš, Serbia, ⁷College of Life Science and Technology, Beijing University of Chemical Technology, Beijing, China, ⁸FILK Freiberg Institute, Freiberg, Germany

The incorporation of bioactive ions into biomaterials has gained significant attention as a strategy to enhance bone tissue regeneration on the molecular level. However, little knowledge exists about the effects of the addition of these ions on the immune response and especially on the most important cellular regulators, the macrophages. Thus, this study aimed to investigate the *in vitro* cytocompatibility and *in vivo* regulation of bone remodeling and material-related immune responses of a biphasic bone substitute (BBS) coated with metal ions (Sr²⁺/Mg²⁺) and PLGA, using the pure BBS as control group. Initially, two cytocompatible modified material variants were identified according to the *in vitro* results obtained following the DIN EN ISO 10993-5 protocol. The surface structure and ion release of both materials were characterized using SEM-EDX and ICP-OES. The materials were then implanted into Wistar rats for 10, 30, and 90 days using a cranial defect model. Histopathological and histomorphometrical analyses were applied to evaluate material degradation, bone regeneration, osteoconductivity, and immune response. The findings revealed that in all study groups comparable new bone formation were found. However, during the early implantation period, the BBS-Sr²⁺ group exhibited significantly faster regeneration compared to the other two groups. Additionally, all materials induced comparable tissue and immune responses involving high numbers of both pro-inflammatory macrophages and multinucleated giant cells (MNGCs). In conclusion, this study delved into the repercussions of therapeutic

ion doping on bone regeneration patterns and inflammatory responses, offering insights for the advancement of a new generation of biphasic calcium phosphate materials with potential clinical applicability.

KEYWORDS

bioactive ions, magnesium, strontium, biphasic bone substitute, biocompatibility, bone remodeling, immune response, *in vivo*

1 Introduction

Although bone tissue possesses the capacity to repair and regenerate after injury or surgical treatment, the natural healing can be a slow process that often fails to restore the original structure and functionality of bone (Giannoudis et al., 2005; Ansari, 2019). Large maxillofacial bone defects, resulting from trauma injuries, cancer treatments, or tooth loss, frequently necessitate the application of bone grafting procedures. (Weiss et al., 1999; Karhuketo et al., 2022). Autologous bone transplants continue to be considered as the most effective substitute of choice for bone regeneration (Albrektsson and Johansson, 2001; Karhuketo et al., 2022). However, a limited amount of autologous bone and the donor site morbidity are factors that limit its application, which has been promoting the development of bone substitute materials (BSMs) within the last decades (Henkel et al., 2013). Synthetic BSMs, particularly calcium phosphates like hydroxyapatite (HA) and beta tri-calcium phosphate (β -TCP) ceramics, are widely used due to their biocompatibility and resemblance to natural bone (LeGeros, 2008; Bellucci et al., 2016). Hydroxyapatite (HA), a key component of teeth and bone, boasts impressive mechanical strength but degrades at a slower rate, which is usually maintained at least several years after implantation, partially hindering bone regeneration up to a complete *restitutio ad integrum* (Bellucci et al., 2016; Fernandez de Grado et al., 2018). β -TCP is also a biocompatible and bioresorbable material, which can be completely degraded in approximately 13–20 weeks after implantation and is then often replaced by remodeled new bone but its fast degradation profile can lead to decreased osteoconductive properties (Chazono et al., 2004). Based on their relatively opposite resorption behaviors, HA is often combined with β -TCP in form of biphasic bone substitutes (BBS). The mixture of HA and β -TCP combines the advantages even in view of the cellular degradability and solubility and avoids the disadvantages of these two components (Ghanaati et al., 2012). Although, BBS present biocompatibility with improved osteoconductivity and bone regeneration, there still remains significant room for improvement of synthetic BSMs to reach regenerative capacities of the “golden standard” autologous bone transplants, especially in view of osteogenesis, osteoinduction, and vascularization (Fernandez-Bances et al., 2013; Jeong et al., 2019).

In response, increasing attention has been shifted to BSM with bioactive cations, such as magnesium (Mg^{2+}), zinc (Zn^{2+}), copper (Cu^{2+}), strontium (Sr^{2+}), and others. In particular, Mg^{2+} and Sr^{2+} in wide concentration ranges do not generate distinct cytotoxicity (Bracci et al., 2009). Sr^{2+} is beneficial in improving osteoblast proliferation, and hence promoting bone formation by increased matrix synthesis through the calcium-sensing receptor and the Extracellular Signal-Regulated Kinase (ERK) signaling pathway

(Li et al., 2020; Chen et al., 2022). In addition, it has been confirmed, that Mg^{2+} , known as the fourth most abundant element in the human body, has shown to provide great advantages as a bioactive substance in biomaterials for repairing bone defects (Hung et al., 2019; Ballouze et al., 2021). The released Mg^{2+} has proved to promote new bone formation and to increase the expression of osteogenic markers, and thus enhance osteogenesis and osseointegration (Ballouze et al., 2021; Fan et al., 2022). Furthermore, adaptations of BSMs even in view of the material-induced immune response, especially on pro- and anti-inflammatory cells such as macrophages but also multinucleated giant cells (MNGCs), and the combined immunomodulatory properties have been in the focus of biomaterials research over the last decade (Zheng et al., 2018; Yang et al., 2022; Zhao et al., 2022). In this context, bioactive cations have shown to be capable of regulating the material-associated inflammatory process, which is another important regenerative factor for bone healing (Rolvien et al., 2018; Abels et al., 2021; Barbeck et al., 2021; Barbeck et al., 2022; Alkildani et al., 2023). Hence, exploring the inflammatory response provoked by bone substitute materials containing metal ions, and their impact on modulating bone regeneration *in vivo*, is a matter of significant interest.

Moreover, polymer coatings also have been cited as an effective technique for modifying the bio-functionality of tissue engineering materials (Maadani et al., 2023). Poly-lactic-co-glycolic acid (PLGA), a group of FDA-approved biodegradable polymers, holds great promise for drug encapsulation, biomaterial degradation modulation and mechanical properties modulation owing to its exceptional physical strength and high biocompatibility (Klose et al., 2008). In the present study, 18 types of surface modifications with metal ions (Sr^{2+} and/or Mg^{2+}) and PLGA on basis of an already approved and biocompatible BBS (maxresorb, botiss biomaterials GmbH, Zossen, Germany) were prepared. The pure BBS was applied as a control group in this study (Koneremann et al., 2014; Trajkovski et al., 2018; botiss biomaterials GmbH, 2024). It was hypothesized that the addition of the bioactive divalent cations Sr^{2+} and/or Mg^{2+} will affect the inflammatory tissue reaction to the biomaterials as well as improve bone tissue regeneration, leading to more satisfactory bone repair outcomes. To evaluate these hypotheses, an *in vitro* cytotoxicity study with the above mentioned 30 modified BBS materials was initially conducted according to the DIN ISO 10993-5 protocol. Based on the *in vitro* results the most cytocompatible materials were chosen for further characterizations using Scanning electron microscopy with energy dispersive X-ray spectroscopy (SEM-EDX) and inductively coupled plasma optical emission spectroscopy (ICP-OES). Thereafter, *in vivo* investigations were carried out with the focus on material degradation, bone formation, osteointegration and the material-

associated immune response during the bone healing process using established and standardized methodologies (Sieger et al., 2019; LINDNER et al., 2020a; Oberdiek et al., 2021; Pröhl et al., 2021). Finally, the correlation coefficient between the above parameters was calculated to uncover the relationship between the BBS and bone regeneration.

2 Materials and methods

2.1 Material preparation

The biphasic bone substitute (BBS) material *maxresorb*[®] (Botiss biomaterials GmbH, Berlin, Germany) composed of 60% hydroxyapatite (HA) and 40% β -tricalcium phosphate (β -TCP) is applied as the basis inorganic material in this study (Weiss et al., 1999). The manufacturing process of *maxresorb*[®] included a controlled precipitation process of aqueous solutions of calcium and phosphate as well as subsequent cold isostatic pressing into mechanically stable objects. This process results in an interconnecting porous system with defined pore diameters ranging from 200 to 800 μ m, as well as micropores of 1–10 μ m (Weiss et al., 1999; Barbeck et al., 2020). The further functionalization of BBS with bioactive cations and PLGA was achieved by dip-coating.

Briefly, SrCl₂ solutions and MgCl₂ solutions in different concentrations (0.01 M and 0.1 M) were prepared with ddH₂O. BBS materials were incubated with each salt solution (0.2 g/mL) for 16 h at room temperature with gentle shaking, followed by drying in the drying oven at 120°C until the solution evaporated.

Sample containing PLGA coating were prepared using the following procedure: 22.5 mL of PLGA solution in acetone (2 mg/mL) were added with 1.25 mL of SrCl₂ solutions and MgCl₂ solutions (0.1 M), then homogenized by vortex mixing. The final concentration of Mg²⁺/Sr²⁺ in PLGA solution was 0.005 M 0.4 g BBS materials were infiltrated once/twice-/three times with 2 mL PLGA-Sr²⁺, PLGA-Mg²⁺, PLGA-Sr²⁺/Mg²⁺ solutions. Afterwards, materials were dried in the oven at 80°C until the solution evaporated.

2.2 Cell cultivation

Primary normal human osteoblasts (NHOst; Lonza) were cultured in OGM (OBM supplemented with OGM Single Quots; Lonza) and grown as monolayer cultures in T75 flasks (Greiner Bio-One). Trypsin/EDTA (Lonza) and TNS solution (Lonza) was used to subculture the cells twice a week. L929 fibroblasts (CLS Cell Lines Service GmbH) were cultivated in monolayer culture with DMEM media containing 10% FBS and supplemented with 1% glutamine (all: Sigma Aldrich). When reaching 80%–90% confluence, cells were trypsinized and subcultured. Both cell types were incubated at 37°C in an atmosphere containing 5% of CO₂ and with a relative humidity of 95%. The cell number was determined with a Neubauer counting chamber (Paul Marienfeld).

2.2.1 In vitro cytotoxicity test

In vitro, the cytotoxicity of sample eluates was investigated according to DIN EN ISO 10993-5. L929 and NHOst cells were used. The extracts of all samples were prepared before XTT test.

Briefly, 100 mg of each sample was transferred to a reaction tube and then incubated in 1,000 μ L culture medium with serum for 24 h at 37°C. Afterwards, the extracts were obtained from supernatant after vortexing and centrifuging at 2000 rpm for 3 min. Undiluted and diluted extracts in a ratio of 1:1 with fresh culture medium were added to a seeded cell lawn for sodium 3,3'-[1(phenylamino) carbonyl]-3,4-tetrazolium]-3-is (4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) assay. The fresh culture medium and culture medium with 1% Triton X100 were used as negative control and positive control, respectively. For XTT-testing the PromoKine Colorimetric Cell Viability Kit III was used. Cells were seeded at a concentration of 1×10^4 cells/well in 96-well cell culture plates and then incubated overnight at 37°C and 5% CO₂. After exchanging the medium with the extracts or control-media, cells were incubated for 24 h. Afterwards, XTT labelling reagent mixed with the electron coupling reagent was added to cells and incubated for 2 h until the orange dye became visible. Finally, the absorbance was measured photometrically at a wavelength of 450 nm. Each sample was measured in triplicate determinations.

2.3 Material characteristics

2.3.1 SEM-EDX

The surface morphological characterization as well as the determination of element composition were performed with SEM-EDX by using a LEO Gemini 1,530 with a field-emission gun (Carl Zeiss AG, Jena, Germany) at the Helmholtz Zentrum Berlin (HZB). Samples were precoated with carbon before the morphological observation and elemental mapping. The applied voltage for imaging was set up to 5 kV. The Point-and-Shoot images were captured with a Thermo Noran X-ray detector and analyzed with Noran System Six (Version 4.1).

2.3.2 Release kinetics

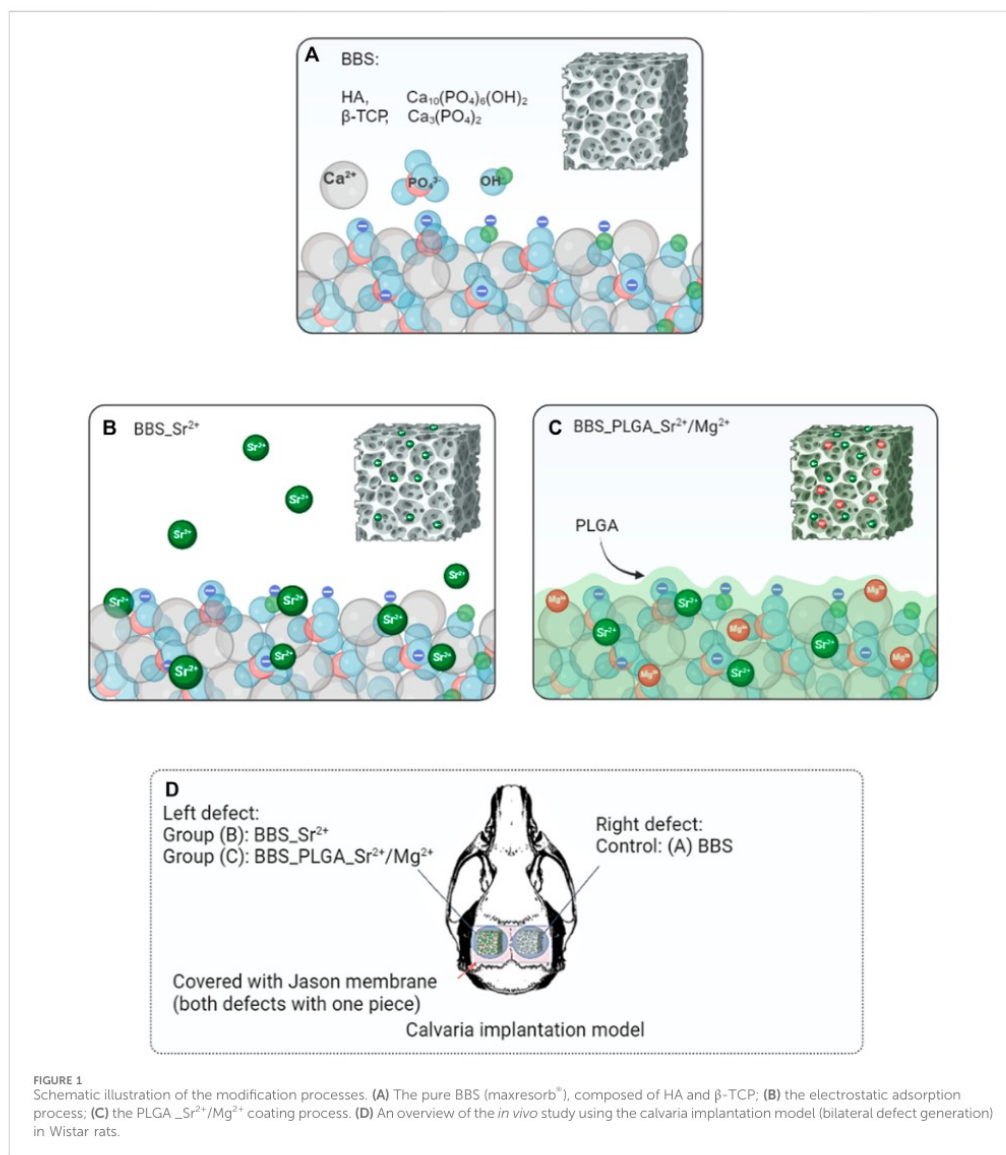
Release kinetics assessment was performed via Inductively Coupled Plasma—Optical Emission Spectrometry analyzer (Ultima 2, Horiba JY, France). Each sample was placed in a falcon ($n = 4$) and filled with 10 mL of distilled water, then kept in a drying oven at 37°C for 10 min to simulate body temperature. At the end of the time, sample were removed from the drying oven and the solution was analyzed using ICP-OES after filtering. The samples were reused for the next two upcoming time points, 24 and 168 h, under same conditions.

2.3.3 FTIR-ATR

The infrared spectroscopic measurements were made with the Bruker Vertex 70 IR spectrometer with the Golden Gate Spectra reflection unit (wavenumber 400–40; resolution of 1 cm⁻¹). The spectra were analyzed with the Bruker software OPUS Version 7.8.

2.3.4 XRD

The phase composition and crystallinity of all samples were characterized by XRD using the Bruker D8 Advance. The measurement technique is based on a copper tube with SOL-X semiconductor detector. Bragg-Brentano geometry was used. All samples were measured with Cu-K α -radiation at a wavelength of $\lambda = 1.54178$ Å (measurement parameters: stop 0,02°; 0,5 s). The diffractograms were matched with a database.



2.4 In vivo study

2.4.1 Pre- and post-implantation procedure, surgical procedure

The *in vivo* study, which accorded to calvaria implantation model, was authorized by the Local Ethical Committee of the Faculty of Medicine, University of Niš, Niš, Serbia, and was based on the approval number 323-07-00073/2017-05/7 of the Veterinary Directorate of the Ministry of Agriculture, Forestry and Water

Management of the Republic of Serbia (date of approval: 22 February 2017). A total of 42 male Wistar rats aged ten to 12 weeks were randomly assigned to two study groups (BBS_ Sr^{2+} and BBS_PLGA_ $\text{Sr}^{2+}/\text{Mg}^{2+}$, Table 1). The sample size was calculated via a power analysis with an additional drop-out rate of 5% (effect size 1.3, G*Power) (Faul et al., 2007). Each of the two groups contained 21 Wistar rats that were assigned to the groups by simple randomization method by the *in vivo* study director, as previously described (Al-Maawi et al., 2021). Wistar rats were sacrificed for each

group at 3 time point ($n = 7$; 10, 30, and 90 days). Animals were housed in standard conditions, provided with regular mouse pellets, unrestricted access to water, and subjected to a 12-h artificial light-dark cycle. Animals were obtained from and kept in the Vivarium of the Faculty of Medicine, University of Niš, Serbia.

The implantation was performed following the protocol described by Pröhl *et al.* (Figure 1) without control of confounders (Pröhl *et al.*, 2021). In brief, the surgical area was shaved and disinfected after anesthesia. The incision was then made in the sagittal plane below the midline of the skull. Subsequently, two holes were drilled in each skull without damaging the dura mater. The coated bone substitutes were implanted in the left defect, while the commercial bone substitute Maxresorb® (botiss biomaterials GmbH, Germany) was implanted in the right defect. Maxresorb® was used as a control group for this study. Afterwards, both defect holes were covered by Jason membrane (botiss biomaterials GmbH, Germany). Finally, the soft tissue was sutured via suture material (Prolene 6.0, Ethicon, Germany).

Postoperative wound openings or infections or other (inflammatory) abnormalities of the wound areas or in the behavior of the test animals, which would have been registered during the daily control of the test animals, were defined as exclusion criteria.

The explantation started after healing periods of 10, 30, and 90 days. Therefore, after the euthanasia the implanted bone substitutes were cut out with the peri-implant tissue. Each explant was finally fixed in 4% neutral-buffered formalin for 48 h.

2.5 Histological workup

Histological workup was performed by following the Technovit 9,100 protocol. Briefly, all explants were embedded in PMMA and sanded with sandpaper (80 grit) into a diamond shape. Tissue blocks were then cut with a thickness of 5–7 μm using a microtome (MicroTec Laborgeräte GmbH; Type: CUT4060E; S-Nr.: 601370). Movat Pentachrome-stainings, CD163- and CD11c-staining were performed following the intern protocols. Movat Pentachrome-stainings was used to quantitatively assess osteogenesis, osteoconduction and residual BBS. CD11c served as a marker for the anti-inflammatory M1 phenotype, while CD163 was utilized to identify the pro-inflammatory phenotype.

2.6 Histopathological analysis

The qualitative histopathological analysis was conducted by following a previously published protocol (Ghanaati *et al.*, 2010a; Barbeck *et al.*, 2015c; Barbeck *et al.*, 2015d; Barbeck *et al.*, 2016b). Parameters like cells participating in the integration and degradation process of the bone substitutes or inflammatory tissue reactions were analyzed using a light microscope (Axio Imager A2, Carl Zeiss Microscopy GmbH, Germany). Histological images were taken by using AxioCam 506 color of Carl Zeiss Microscopy GmbH, Germany.

2.7 Histomorphometrical analysis

Using ImageJ (Version 1.52t), all stained slides digitized with PreciPoint M8 microscope were analyzed to obtain results on bone growth, osteoconductivity and macrophage subtypes.

To evaluate bone growth and residual BBS, the ROI (Region of Interest) for total area of each defect area [$\text{in } \mu\text{m}^2$], newly formed bone area [$\text{in } \mu\text{m}^2$] and the bone substitute material area [$\text{in } \mu\text{m}^2$] was manually marked in ImageJ and then measured automatically. The percentage of newly formed bone and bone substitute to the total area were evaluated for each biomaterial, defect and timepoint.

To assess osteoconductivity, the total distance of new bone - bone substitute surface—interaction [$\text{in } \mu\text{m}$] was measured for each biomaterial, timepoint and defect area. The calculated values were related to the total circumference of all bone substitute granules [$\text{in } \mu\text{m}$] located in the delimited defect area.

To determine the number of M1 and M2 macrophages, the soft tissue in the defect area was delimited manually. Then, a specialized plugin was used to count the number of stained cells within delimited total area automatically, which was described by Lindner *et al.* (Lindner *et al.*, 2020b). Finally, the number of cells per mm^2 was obtained. In addition, CD11c-positive MNGCs were counted manually.

2.8 Statistical analysis

The histomorphometrical data underwent normality testing using the Shapiro-Wilk test and variance homogeneity testing using the Brown-Forsythe test.

Subsequently, the data underwent analysis of variance (ANOVA), an extension of the T-test, for statistical analysis. A Tukey post-hoc assessment followed to compare groups by using GraphPad Prism software (Version 9.0.0, GraphPad Software Inc., La Jolla, United States). The statistical differences were defined by three significance levels. If p -values were less than 0.05 ($^*p < 0.05$), the difference were considered as significant. High significant were considered, if the p -values were less than 0.01 ($^{**}p < 0.01$) or less than 0.001 ($^{***}p < 0.001$). By using GraphPad Prism software, the data were represented as average values and standard deviation.

Furthermore, multiple variable correlation analyses were conducted based on the measurements within the BBS group in the present study. Correlation matrix analyses using a two-tailed Pearson's correlation were conducted to identify any possible relationships between the two parameters within the BBS group. Pearson's correlation coefficient (r) was used to determine the strength and direction of the correlation, whether it was positive, negative, or weak. The confidence interval was set at 95%.

3 Results

3.1 *In vitro* cytocompatibility test

To evaluate the cytocompatibility of the BBS combined with 18 coatings based on the bioactive cations strontium (Sr^{2+}) and magnesium (Mg^{2+}) as well as PLGA coating, XTT cell viability assays were conducted using L929-fibroblasts and Normal Human Osteoblasts (NHOst) following the DIN ISO 10993-5 protocol as previously described (Gogele *et al.*, 2020; Schroepfer *et al.*, 2020;

TABLE 1 Overview of the study groups, presenting the number of experimental animals per group at each timepoint.

	BBS_Sr ²⁺	BBS_PLGA_Sr ²⁺ /Mg ²⁺	Control group
10 days	7	7	14
30 days	7	7	14
90 days	7	7	14
Number per study group	21	21	--
42 experimental animals in total			

TABLE 2 An overview of the results of the cytotoxicity testing using L929 fibroblasts and NHOst.

BBS with 30 coatings	Cytotoxicity (compared with negative control in %)			
	L929 fibroblasts		NHOst	
	100% of extracts	50% of extracts	100% of extracts	50% of extracts
1. BBS_MgCl ₂	55.7	76.8	30.7	52.2
2. BBS_SrCl ₂	60.8	75.8	95.7	109.0
3. BBS_MgCl ₂ -SrCl ₂	56.5	72.0	-6.4	95.7
4. BBS_MgCl ₂ (0.1x)	67.5	91.0	63.1	82.4
5. BBS_SrCl ₂ (0.1x)	80.8	96.0	61.0	78.7
6. BBS_MgCl ₂ -SrCl ₂ (0.1x)	75.6	73.5	51.7	63.7
7. BBS_MgHPO ₄	72.3	67.9	31.7	39.0
8. BBS_SrHPO ₄	71.7	66.4	53.3	66.7
9. BBS_MgHPO ₄ -SrHPO ₄	61.9	62.0	36.4	41.7
10. BBS_PLGA_MgCl ₂ _1	73.2	65.5	39.5	53.5
11. BBS_PLGA_MgCl ₂ _2	31.2	74.6	26.4	57.3
12. BBS_PLGA_MgCl ₂ _3	23.9	73.0	19.2	40.0
13. BBS_PLGA_SrCl ₂ _1	111.7	104.6	52.3	72.7
14. BBS_PLGA_SrCl ₂ _2	51.0	58.3	102.9	106.7
15. BBS_PLGA_SrCl ₂ _3	5.7	79.2	4.9	56.9
16. BBS_PLGA_MgCl ₂ -SrCl ₂ _1	77.1	89.5	72.2	88.8
17. BBS_PLGA_MgCl ₂ -SrCl ₂ _2	39.2	62.5	29.6	54.7
18. BBS_PLGA_MgCl ₂ -SrCl ₂ _3	-0.8	73.0	2.2	43.9

PLGA, poly (lactic-co-glycolic acid); 1/2/3 = Single/dual/triple coated; 0.1x = Tenfold dilution of 0.1M salt solution; Green coloring = cell viability above 70%. Green groups = selected group.

Pantermehl et al., 2022; Ren et al., 2023). Based on the cytocompatibility analyses, the material types with a viability above 70% compared to the negative control group as defined within the DIN ISO norm were considered cytocompatible and used for further material characterizations and *in vivo* studies.

The results of this initial analysis step revealed that only the material combination that combined the BBS with the single PLGA coating including MgCl₂ and SrCl₂ showed completely adequate cytocompatibility values in case of both cell lines above 70% and in both extract conditions (Table 2). Additionally, the material combination of the BBS coated with a tenfold dilution of SrCl₂

could be considered cytocompatible as it induced only low cytotoxicity for NHOst in contact with the 100% extracts, while the other values were in the non-cytotoxic range (Table 2). All other material combinations showed more or higher deviations from the non-cytotoxicity ranges for both cell types or the different extract concentrations and were therefore classified as cytotoxic (Table 2). Based on these results both material combinations, i.e., the BBS coated with a tenfold dilution of SrCl₂ (BBS_Sr²⁺) and the BBS with the single PLGA coating including MgCl₂ and SrCl₂ (BBS_PLGA_Sr²⁺/Mg²⁺), were chosen for the next study step.

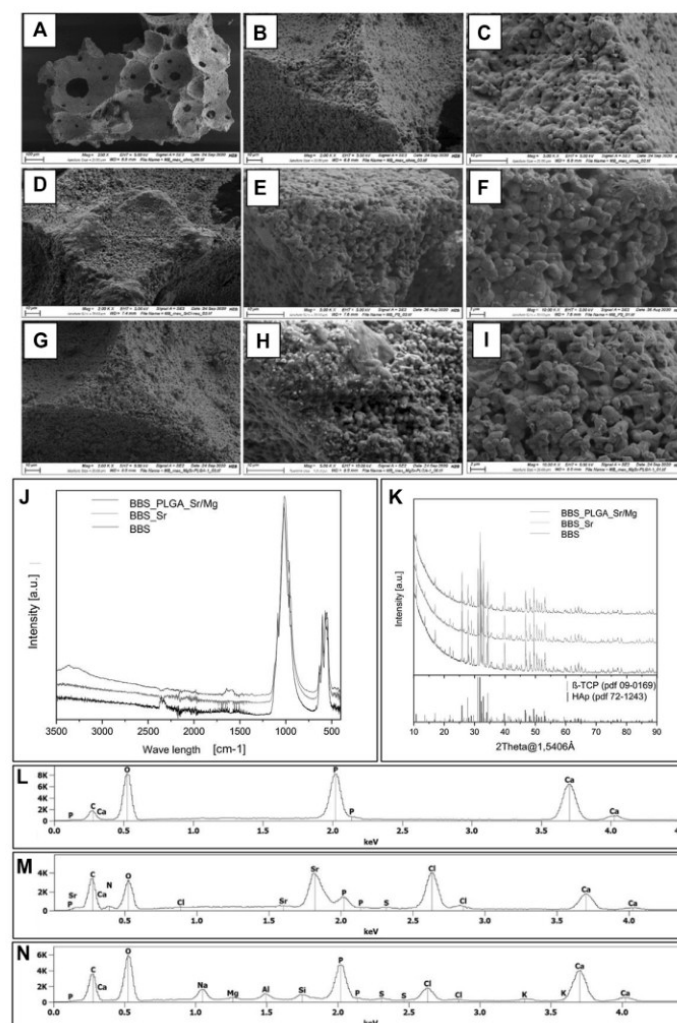


FIGURE 2 (A–I) SEM images of bare BBS (A,B,C), BBS coated with the Sr^{2+} solution (D,E,F), and the BBS combined with the coating based on PLGA, MgCl_2 and SrCl_2 (G,H,I). (J) FTIR spectra of all bone substitutes, (K) XRD diffractograms of all bone substitutes, (L) EDX spectrum of the pure BBS, (M) EDX spectrum of the BBS_ Sr^{2+} group and (N) EDX spectrum of the BBS_PLGA_ Sr^{2+} /Mg $^{2+}$ group.

3.2 Material characteristics

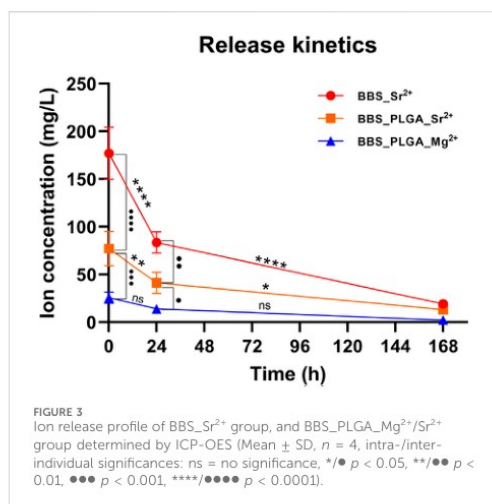
3.2.1 Scanning electron microscopy with energy dispersive X-ray spectroscopy (SEM-EDX)

SEM images of pure BBS and both coated BBS compositions, i.e., the BBS coated with a tenfold dilution of SrCl_2 and the BBS with the single PLGA coating including MgCl_2 and SrCl_2 , were depicted in Figure 2. The BBS has an open pore structure (Figures 2A–C) (Konermann et al., 2014). After the coating with the Sr^{2+} ion

solution, the material showed a more even “fused” surface structure compared to the bare BBS, which indicates an effective coating adhesion (Figures 2D–F). In addition, highly grown and superimposed elongated crystallites were observed at the material surfaces. The SEM analysis of BBS with the single PLGA coating including MgCl_2 and SrCl_2 revealed that this material composition had a smooth and continuous surface, which could be associated with the PLGA polymer layer (Figures 2G–I). Thereby, the micro-structure of the BBS granules was no longer present in form of

TABLE 3 EDX analysis of bare BBS, BBS_Sr²⁺ and BBS_PLGA_Sr²⁺/Mg²⁺.

	O-K	P-K	Ca-K	Sr-L	Mg-K	Cl-K
BBS	39.1 ± 0.24	18.2 ± 0.14	42.8 ± 0.26	—	—	—
BBS_Sr ²⁺	23.2 ± 0.23	4.8 ± 0.14	20.1 ± 0.16	27.7 ± 0.21	—	24.1 ± 0.20
BBS_PLGA_Sr ²⁺ /Mg ²⁺	40.0 ± 0.33	13.6 ± 0.12	29.2 ± 0.29	—	0.5 ± 0.04	10.8 ± 0.14



regular spherical particles as in case of the pure BBS but showed a cubic or polyhedral structure (Figure 2I). Overall, both coating techniques were effectively processed and could be verified via SEM on the surface of the BBS granules. Moreover, none of the coatings did change the porosity of the BBS.

The results of SEM-EDX elemental analysis are shown in Figures 2L–N and Table 3. Calcium (Ca), phosphate (P) and oxygen (O) were observed in all analyzed biomaterials indicating the existence of the calcium phosphate basis of the BBS. The weight percentage of Ca, P and O in the group of the bare BBS was higher than in case of the materials coated with both different methodologies. Furthermore, the BBS coated with the Sr²⁺ ion solution exhibited a high weight percentage of strontium (Sr, 27.73 ± 0.21 wt%) and chloride (Cl, 24.06 ± 0.20 wt%), suggesting the successful addition of Sr²⁺ on the surfaces of the BBS. The BBS combined with the single PLGA coating including MgCl₂ and SrCl₂ presented a higher composition of Ca, P, O than the BBS coated with the Sr²⁺ solution. Moreover, the weight percentage of Sr²⁺ present in the PLGA coated BBS was comparable lower.

3.2.2 Release kinetics

Both coated BBS materials displayed similar trends in the release of Sr²⁺ ions (Figure 3). Thus, the initial release of Sr²⁺ ions was significantly high within the first 24 h (**p < 0.01, ****p < 0.0001) in both study groups, followed by a gradual significant decrease in both study groups until 168 h (*p < 0.05, ****p < 0.0001) (Figure 3). In the group of the BBS with the PLGA coating including MgCl₂ and SrCl₂,

the concentrations of both Mg²⁺ and Sr²⁺ ions in preparation solution were 0.005 M, while the Sr²⁺ concentration in the BBS_Sr²⁺ group was 0.01 M. As a result, the group with BBS coated with the Sr²⁺ solution exhibited the greatest Sr²⁺ ion release at all three time points, significantly surpassing the levels observed in the BBS_PLGA_Sr²⁺/Mg²⁺ group (**p < 0.01, ****p < 0.0001).

Although the release of Mg²⁺ ions also showed a decreasing trend over time, no significant differences were observed at the three time points. Moreover, the release of Sr²⁺ ions in the BBS_PLGA_Sr²⁺/Mg²⁺ group was about 3 times significantly higher than that of Mg²⁺ ions (**p < 0.01, ***p < 0.001). At 168 h, the metal ions in both groups approached complete release, with no observed intergroup significances.

3.3 In vivo studies

For the *in vivo* study, the coated bone substitutes were implanted into the left defect in Wistar rats, while the commercial bone substitute Maxresorb® (botiss biomaterials GmbH, Germany) was implanted into the right defect.

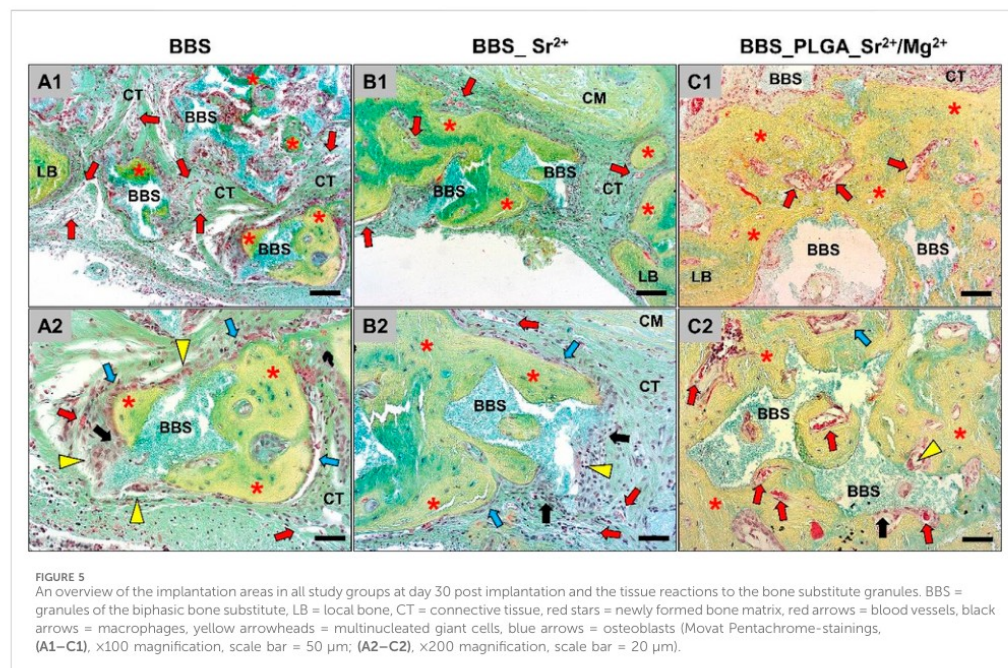
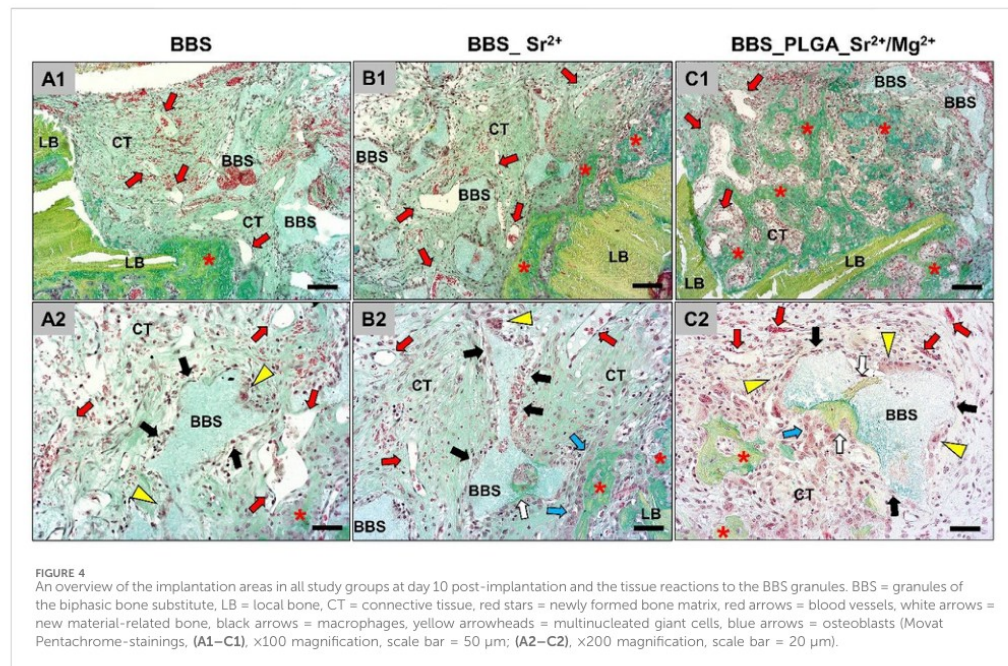
3.3.1 Histopathological analysis

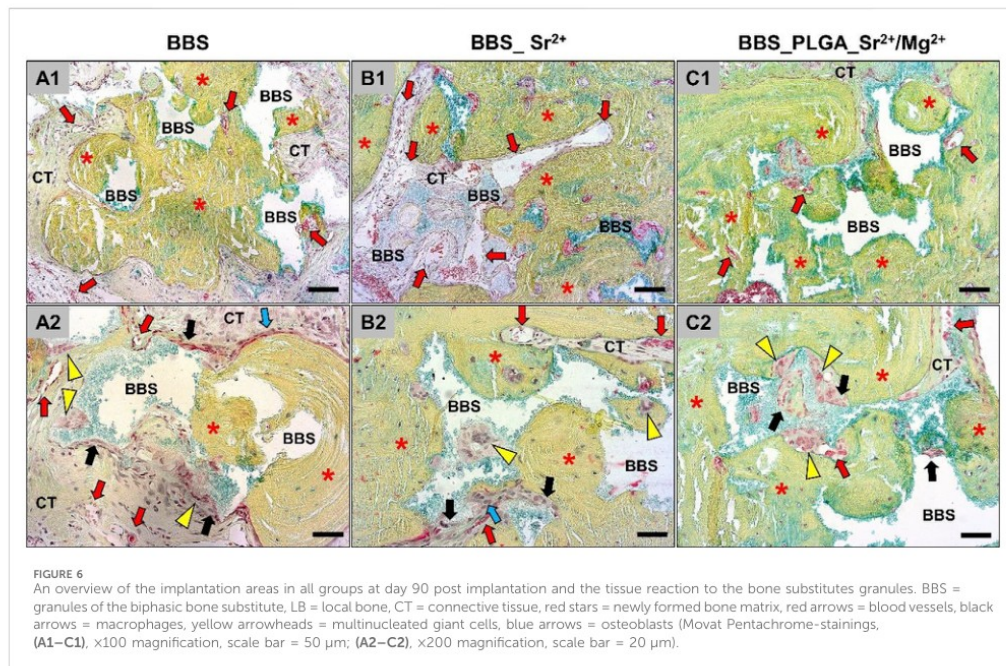
At day 10 post-implantation (Figure 4), the implanted bone substitute granules in all study groups could be localized within the defect areas and were mostly embedded within connective tissue.

Inflammatory cells, i.e., mainly macrophages as well as low numbers of multinucleated giant cells, could be identified at the surfaces of the BBS granules without visible differences between the study groups. Moderate numbers of lymphocytes and granulocytes were additionally found within the intergranular connective tissue that showed a moderate vascularization in comparable extents in all study groups. In addition, slight bone growth was visible at the defect margins in form of uncalcified bone matrix, which was associated with osteoblastic hems. Thereby, a material-dependent bone growth was less detectable than a material-independent matrix growth outgoing from the local residual bone at this early study time point. In summary, no differences in the extents of the material-related tissue reactions could be registered.

At day 30 post-implantation, the histological evaluation showed that more newly formed bone tissue could be detected particularly within the peripheral defect areas close to the defect borders (Figure 5).

In all three study groups, the material particles (BBS) were embedded in newly formed bone matrix within the defect areas showing a material-dependent bone growth. Still, less bone growth within the central defect area in all study groups was detectable. At this post-implantation time point parts of the newly formed bone matrix were not fully calcified and remained associated with many





osteoblastic hems indicating further bone tissue development. Also, a cell- and vessel-rich connective tissue was detected within the granular interspaces, which was mainly composed of macrophages between lower numbers of lymphocytes, and fibroblasts. At this time point, mainly macrophages as well as lower numbers of multinucleated giant cells could be detected at the material surfaces in all study groups in comparable extents. In summary, no differences in the extents of the material-related tissue reactions between the study groups could be observed.

At day 90 post-implantation, a higher extent of newly formed bone matrix was detectable in all three study groups up to the central defect areas (Figure 6).

In most of the defect areas a complete bony regeneration was detectable, and all granules of the biphasic bone substitutes were embedded within newly bone matrix at this late study time point. Furthermore, the histological analysis indicated that even at this late study time point, osteoblastic hems were detectable, which indicated a continued bone formation. Also, a cell- and vessel-rich connective tissue was detected, which was still mainly composed of macrophages and fibroblasts. High numbers of macrophages between lower numbers of multinucleated giant cells were still detected on the surfaces of the material granules that were neighbored to connective tissue (Figure 6). In summary, no differences in the extents of the material-related tissue reactions between the three study groups can be registered.

The histopathological results of the macrophage subpopulations revealed that considerable numbers of pro-inflammatory M1- and anti-inflammatory M2-macrophages were detectable at all implantation sites (Figure 7).

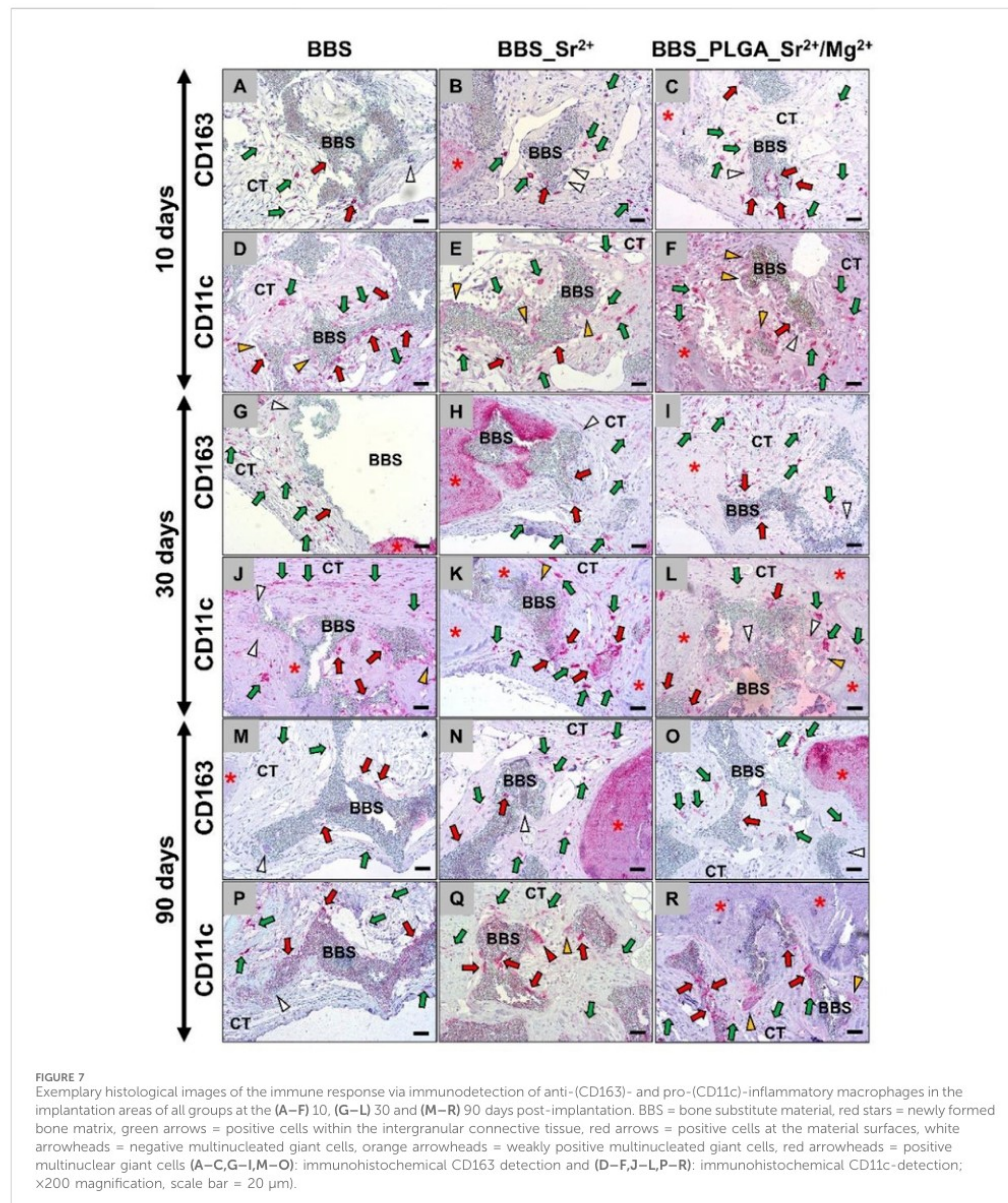
Thereby, the numbers of pro-inflammatory macrophages were tending to be higher than the numbers of the anti-inflammatory subtype at all study time points and in all groups. Only at day 30 post-implantation the occurrence of M1-macrophage was visibly higher in all study groups compared to the other study time points. Moreover, no visible differences in the induction of both macrophage subtypes in the three different study groups were seen at any observation time point. Additionally, the observation showed that none of the material-associated multinucleated giant cells showed signs of a CD163-expression in any of the study groups, while they showed low-grade signs of an CD11c-expression. However, most of these cells were not expressing CD163 nor CD11c.

3.3.2 Histomorphometrical analysis

3.3.2.1 Material degradation

The histomorphological analysis of material degradation assessed the granule area as a percentage of the total implanted region. The findings indicated a reduction of the granule sizes in both the BBS and BBS_Sr²⁺ group over the study period, whereas the granule size in the BBS_PLGA_Sr²⁺/Mg²⁺ group remained consistent throughout the implantation period (Figure 8).

In particular, the granule size in the BBS group was significantly decreasing between day 10 and 30 days ($p < 0.05$, Figure 8), while only a trend towards a material degradation within this time frame was found in the BBS_Sr²⁺ group. Between day 30 and 90 post-implantation no further material degradation was measured. Moreover, no significant or tending differences in the granule sizes were found in the BBS_PLGA_Sr²⁺/Mg²⁺ group at all three study time points.

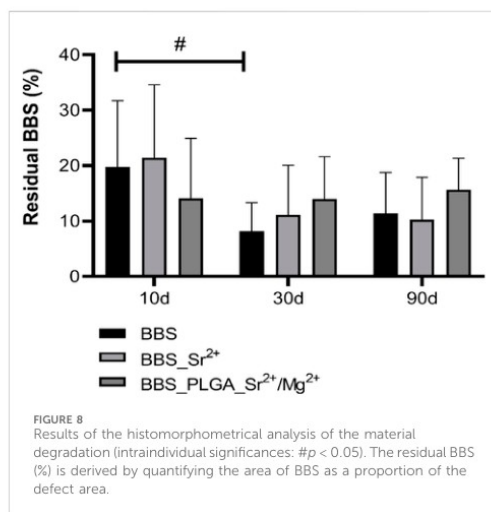


3.3.2.2 Bone regeneration and osteoconductivity

The histomorphometrical analysis of the bone regeneration, measuring the new bone area as a percentage of the total implanted region, indicated that no interindividual differences between the amounts of newly formed bone between the three

study groups were found at any of the observation time points (Figure 9A).

Comparable regeneration patterns were observed in all groups starting with small amounts of newly formed bone of approximately 5% in all groups at day 10 post-implantation. At day 30 post-



implantation also comparable bone regeneration pattern were found in all groups (Figure 9A). The highest amount of bone formation was found in the BBS_Sr²⁺ group ($31.78\% \pm 17.11\%$), while lower bone formation amount was measured in the BBS_PLGA_Sr²⁺/Mg²⁺ group ($26.76\% \pm 12.25\%$) and the lowest amount was detected in the control BBS group ($17.46\% \pm 13.08\%$) but without significant differences (Figure 9A). At day 90 post-implantation still comparable bone regeneration pattern were found in all groups with the highest bone formation in the BBS_PLGA_Sr²⁺/Mg²⁺ group ($47.36\% \pm 11.36\%$) and approximately equal lower values in the BBS_Sr²⁺ group ($38.04\% \pm 7.54\%$) and the control BBS group

($37.05\% \pm 19.52\%$) but still without significant differences (Figure 9A).

At day 30 post-implantation only in the BBS_Sr²⁺ group a significant increase of bone formation was found compared to day 10 ($#p < 0.05$, Figure 9A). At day 90 post-implantation significant increases of bone regeneration compared to day 30 were detected in the control BBS group and the BBS_PLGA_Sr²⁺/Mg²⁺ group ($#p < 0.05$ and $##p < 0.01$, Figure 9A). Moreover, all experimental groups exhibited significantly higher bone regeneration at day 90 post-implantation in comparison to the values at day 10 ($##p < 0.01$ and $####p < 0.0001$, Figure 9A).

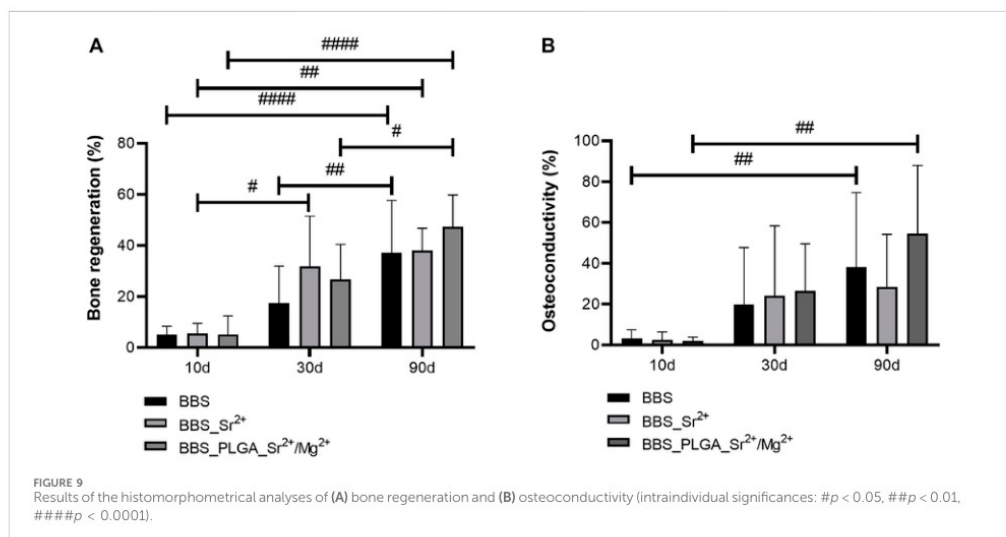
The histomorphometrical analysis of the osteoconductivity revealed that no significant differences among the three groups at any of the study time points (Figure 9B). At day 90 post-implantation in the BBS group and the BBS_PLGA_Sr²⁺/Mg²⁺ group significantly higher osteoconductivity in comparison to the values at day 10 were measured ($##p < 0.01$) (Figure 9B).

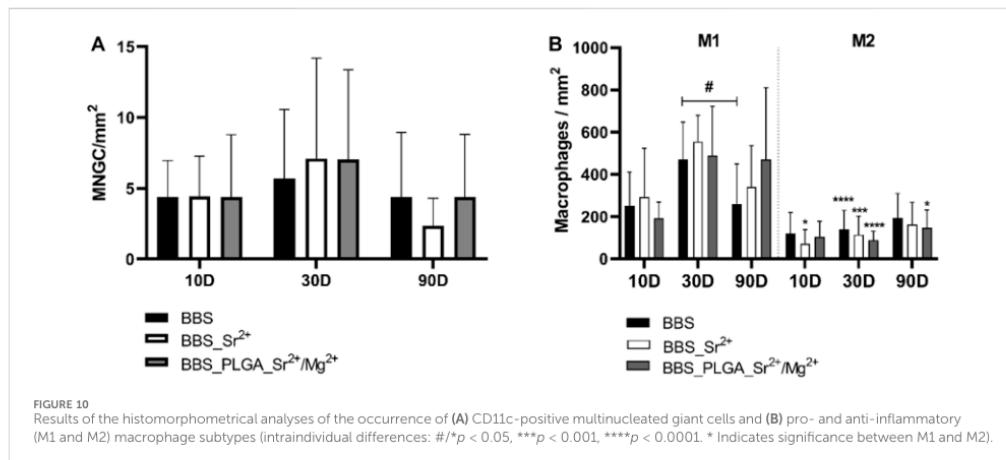
3.3.2.3 Immune response

The histomorphometrical evaluation of the occurrence of CD11c-positive multinucleated giant cells showed the absence of significant differences between the three study groups at any of the observation time points (Figure 10A).

A tendency towards an increase of the occurrence of CD11c-positive multinucleated giant cells was observed in all study groups at day 30 post-implantation, while a further trend towards a decrease up to day 90 post-implantation was also detectable—especially in the BBS_Sr²⁺ group (Figure 10A).

The histomorphometrical assessment of the macrophage subtypes revealed similar macrophage responses in all study groups and at all time points characterized by a prevalence of pro-inflammatory macrophages over the anti-inflammatory counterpart (Figure 10B). At day 10 post-implantation only in a significantly higher number of pro-inflammatory macrophages compared to the anti-inflammatory





subtype in the BBS_Sr²⁺ group ($*p < 0.05$) was measured. At day 30 post-implantation significantly higher numbers of pro-inflammatory macrophages compared to the anti-inflammatory subtypes were found in all study groups ($***p < 0.001$, $****p < 0.0001$) (Figure 10B). Finally, significantly higher values of pro-inflammatory macrophages ($*p < 0.05$) were also found in the BBS_PLGA_Sr²⁺/Mg²⁺ group at 90 days post-implantation. Only between day 30 and 90 post-implantation a significant decrease of the number of pro-inflammatory macrophages in the BBS group was found ($\#p < 0.01$) (Figure 10B).

3.3.2.4 Correlation analysis

Pearson's correlation analysis was utilized to explore the relationships between the five measured parameters. Only in the BBS groups significant correlations were found (Figure 11).

At all timepoints, a significant positive correlation ($*p < 0.05$, $**p < 0.01$) was observed between osteoconductivity (OC) and bone regeneration (BR) (Figure 11). Although not statistically significant, there was tendentially a moderate positive correlation between M1 and BR at day 10 and 30 post-implantation, but a negative correlation at 90 days.

Additionally, at day 10 post-implantation, a positive correlation between the occurrences of MNGCs and M1-macrophages was observed ($*p < 0.05$). At day 30 post-implantation, a positive correlation ($**p < 0.01$) between the occurrence of M2-macrophages and osseointegration was calculated. Additionally, different correlations between the occurrence of MNGCs and different other parameters were found (Figure 11). A positive significant correlation of the MNGC occurrence and the amount of the remaining BBS ($*p < 0.05$) was measured. Moreover, negative correlations between the MNGC occurrence and the occurrence of M2-macrophages ($**p < 0.01$) but also the osseointegration ($*p < 0.05$) were measured.

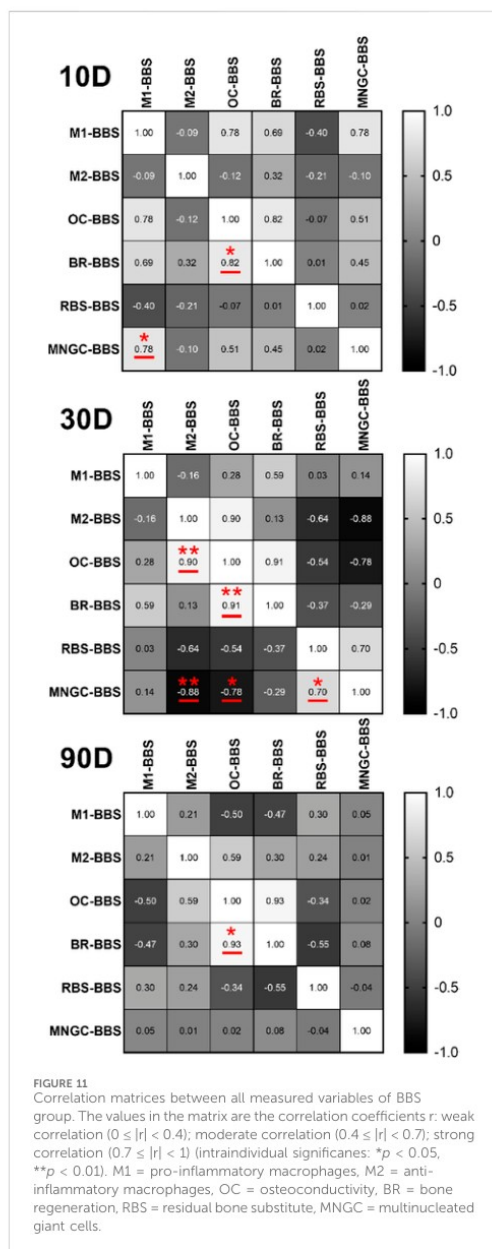
4 Discussion

Even after several decades of research in the field of bone substitutes and a broad variety of material developments and

biological studies focusing on new synthetic materials as an alternative to autologous bone transplants, this topic is still current. It is important to find material factors that combine both the osseoregenerative properties that optimally support tissue regeneration and, on the other hand, the material-induced immune responses that optimally support healing cascades but also lead to a material durability in the sense of *restitution ad integrum*.

The cytocompatibility of newly developed biomaterials is an important step to consider the further development and analysis of biomaterials for biomedical applications. The results of the XTT cell viability assay indicated that most of the coatings including the bioactive cations on BBS exhibited cytotoxicity, while only a few of the material prototypes were cytocompatible. Among the four materials selected based on suitable cytocompatibility values using L929 cells, only one material, BBS_PLGA_MgCl₂-SrCl₂, showed an adequate cell viability of NHOst cells above 70%. This result suggests that this material prototype may have the potential to promote cell growth and proliferation, making it a promising candidate for use in tissue engineering applications. Additionally, the material prototype BBS_SrCl₂ (10x) showed only slight cytotoxicity on NHOst cells, thus it was also selected for the further physicochemical experiments and the *in vivo* study part. Overall, these results highlight the importance of a safe and effecting loading strategy.

The results of the SEM analysis and the EDX elemental analysis indicate that the coating techniques were effectively processed as the coatings were detectable at the surface of the BBS but did not change the material structure. The higher Ca, P and O compositions in the BBS_PLGA_Sr²⁺/Mg²⁺ group should be regarded as a result of the PLGA coating. Furthermore, the exceptionally low strontium content in this group may be a consequence of either non-uniform distribution or concealment by the PLGA coating. The PLGA coating was originally introduced to improve the bio-performance and long-term ion release kinetic of BBS. Nonetheless, the present findings indicate that the Sr²⁺ ion release patterns in case of the BBS_PLGA_Sr²⁺/Mg²⁺ and the BBS_Sr²⁺ prepared are comparable. The BBS_PLGA_Sr²⁺/Mg²⁺



group exhibited a lower release of strontium ions at all timepoints compared to that in the BBS_Sr²⁺ group, which can primarily be attributed to the distinct initial loadings. Notably, magnesium ions are released more evenly in the BBS_PLGA_Sr²⁺/Mg²⁺ group, which was significantly lower than the release of strontium ions at all time

points. This observation may be explained on the one hand by the smaller ionic radius and higher charge density of magnesium ions, which might facilitate their enhanced adsorption onto the BBS surface and stronger binding to the carboxyl group of PLGA. On the other hand, it is also plausible that the initial loading of magnesium ions onto the BBS is lower, consequently leading to reduced release.

The histopathological findings from the *in vivo* study part revealed a comparable moderate foreign body reaction in response to implantation of all bone substitute granules. The tissue response was mainly characterized by the presence of macrophages and lower numbers of granulocytes, material-associated multinucleated giant cells (MNGCs), and lymphocytes, which is a common response to materials of this class but also to different other biomaterials mostly being a correlate of the cellular biodegradation (Ghanaati et al., 2010b; Barbeck et al., 2015a; Barbeck et al., 2015b; Barbeck et al., 2016a; Abels et al., 2021). Moreover, the presence of both macrophages and MNGCs can contribute to tissue healing processes due to their pro- and anti-inflammatory nature (Miron and Bosshardt, 2018). It is worth to mention that the formation of a well vascularized granulation tissue starting with day 10 up to day 90 post-implantation is not only a histopathological sign of the ongoing material degradation but also an optimal basis for tissue regeneration. This is attributed to molecules such as the expression of the vascular endothelial growth factor (VEGF) by the associated phagocytes and the related implantation bed vascularization (Ghanaati et al., 2010a; Barbeck et al., 2021). In this context it has been described that the β -TCP fraction of the BBS induces a rapid vascularization of the implantation bed based on a fast cellular degradation beside its high solubility (Ghanaati et al., 2012). At day 10 and 90 post-implantation mostly tendencies of M1 macrophages higher occurrence with few exceptions were found, while at day 30 post-implantation significantly higher numbers of M1-macrophages were observed. These might reflect the observations of the afore-mentioned publication by Ghanaati et al. that described the “separated” degradation pattern of both calcium phosphate compounds, i.e., the faster biodegradation pattern of the β -TCP component and the delayed reactivity of HA (Ghanaati et al., 2010a; Ghanaati et al., 2012). The histomorphometrical analysis of the residual BBS revealed that this degradation pattern was also detectable in case of the pure BBS showing a significant decrease between day 10 and 30 post-implantation, while its degradation reached a steady state up to day 90 post-implantation. Interestingly, only a tendency towards a comparable degradation pattern was found in both groups of the coated materials, but without significances. This result could be seen as an indication of an influence of the coating on the degradation behavior of the materials - initially independent of the degradation pathway, i.e., the solubility behavior and the cellular-based degradation via phagocytosis.

Based on this initial observation an analysis of and the occurrence of MNGCs was also conducted. The histomorphometrical analysis of the MNGC occurrences revealed no intra- or interindividual significances but showed a tendency towards higher numbers at day 30 post-implantation, which is comparable to the results found in case of the M1-macrophages and might underline the afore-mentioned theory of the

predominant cellular biodegradation pattern of the BBS. Moreover, the correlation analysis revealed another interesting result as the occurrence of M1-macrophages and that of (pro-inflammatory) MNGCs were positively associated. This observation in conjunction with the fact that the material associated MNGCs expressed only the pro-inflammatory factor, confirms the earlier suggestion that the “pro-inflammatory limb” of the tissue response is responsible for material degradation (Abels et al., 2021; Barbeck et al., 2022). In addition, the correlation of these two phagocyte subtypes shows that the formation of MNGCs only occurs as part of the pro-inflammatory tissue response and can therefore also be assigned to the “foreign body giant cell type” based on these observations as described before by Barbeck et al. (Barbeck et al., 2017). On the other hand, this result can of course be attributed to the fact that the macrophages are the mononuclear precursor cells of the MNGCs, which then fuse in the further tissue reaction (Miron and Bosshardt, 2018). Additionally, a positive correlation between the remaining BBS and the occurrence of MNGCs was detected, which underlines the assumption that material phagocytosis is executed by pro-inflammatory MNGCs that seem to have a higher phagocytosis capacity compared to macrophages. In summary, our study reaffirms that primarily proinflammatory phagocytes, i.e., macrophages and MNGCs, are involved in material degradation, while the anti-inflammatory macrophage subtype seem to be involved in tissue regeneration and regulating cascades being located within the surrounding tissue (Abels et al., 2021; Barbeck et al., 2022; Unger et al., 2022).

The analysis of the material-mediated bone tissue healing showed that sufficient and comparable bone regeneration was achieved in all test groups. As expected, progressive bone formation was observed in all study groups up to day 90 post-implantation that was at this late time point significantly higher compared to day 10 post-implantation. It is worth to note that osseoregeneration in the BBS_Sr²⁺ group primarily occurred during the early implantation period reaching a “steady-state” at 30 days post-implantation, whereas the BBS group and BBS_PLGA_Sr²⁺/Mg²⁺ group experienced a more pronounced regeneration in the late study period. Although in the latter group a significantly higher M1-macrophage presence was detected, no correlation was found between these both parameters, so that this result can only be interpreted as a chance finding—especially in view of the fact that the anti-inflammatory macrophage phenotype might be associated with the process of (bone) tissue healing (Pajarinen et al., 2019). This assumption is also supported by the fact that similar bone tissue regeneration patterns were determined based on the measurement of osseointegration, but no significant differences were calculated. Moreover, the results of the correlation analysis substantiated this assumption in two different points: Initially, a positive correlation between the occurrence of M2-macrophages and osseointegration was found at day 30 post-implantation, which showed the linkage between the anti-inflammatory limb of the tissue reaction and (bone) tissue repair. Secondly, the analysis revealed at the same time point a significantly negative correlation between osseointegration and the occurrence of MNGCs. Moreover, the correlation analysis uncovered further positive correlation between the processes of new tissue formation and the osseointegration of the BBS. This is not surprising, as both processes should generally correlate in the case of critical-size defects

and an osseointegrative bone substitute. These results merely confirm the material-based bone regeneration of the control group and thus the effectiveness of the BBS, which has already been tested many times both preclinically and clinically (Ghanaati et al., 2012; Bielenstein et al., 2022).

Notably, neither the BBS_Sr²⁺ group nor the BBS_PLGA_Sr²⁺/Mg²⁺ group affect the final bone regeneration and macrophages response compared to the BBS group. This result is inconsistent with the initial hypothesis but is in line with some previous *in vivo* experiments with metal ion doping. Barbeck et al. doped strontium and copper ions in bioactive glass, which also showed no effect on immune response or vascular integration patterns (Barbeck et al., 2022). It might be due to the fact that pro-inflammatory MNGCs were closely linked to material degradation, but these foreign body giant cells were not subject to regulation by Sr²⁺/Mg²⁺ ions in a manner similar to osteoclasts. Additionally, acidic degradation products of PLGA coatings have been reported to impact the deposition of calcium phosphate on the material surface (Maadani et al., 2023). Moreover, prior investigations have shown a strongly dose-dependent influence of both metal ions on bone regeneration. For example, a study conducted by Shen and others has demonstrated that high proportion of Sr doping promote M2-macrophage polarization and fast bone regeneration (Shen et al., 2022). Conversely, a concentration of 0.1 mmol/L of strontium displayed a mild effect on proliferation and osteogenic differentiation of hMSC (Schumacher et al., 2013). Moreover, it has been described that a Mg²⁺ concentration of 100 mg/L and above can promote osteogenic differentiation of MSC but also promotes the secretion of bone morphogenetic protein 2 (BMP2) by macrophages, and should induce phenotypic switching into M2-macrophages (Nie et al., 2022). Nevertheless, the most suitable ion concentrations highlighted across diverse studies exhibited considerable variation due to distinct preparation protocols, sample attributes, and experimental frameworks, thus impeding direct comparisons. While the ions loaded in this study didn't yield a substantial enhancement in biological effects on immune response or bone regeneration, it was noted that all bone materials induced balanced immune response and exhibited excellent tissue integration without any signs of fibrous encapsulation or other unfavorable tissue responses.

5 Conclusion

In conclusion, this study investigated the effects of loading of Sr²⁺ and/or Mg²⁺ ions onto BBS materials on cyto- and biocompatibility including the immune response and osteoregeneration. The results showed that two cytocompatible ion-modified material variants were identified and further tested by the *in vitro* cytocompatibility analysis.

All examined material groups provided a good *in vivo* biocompatibility with a moderate and balanced inflammatory response and significant new bone regeneration during the healing period. Notably, the BBS_Sr²⁺ group exhibited a distinct faster regeneration compared to the other two groups. The formation of new bone tissue predominantly took place during the early implantation period for this group, whereas in the other two groups, it mainly occurred during the later implantation period.

These findings align with observed macrophage patterns and osteoconductivity. However, further investigation is needed to determine the correlation between a pro-inflammatory environment and material-associated bone regeneration. Moreover, pro-inflammatory macrophages and multinucleated giant cells demonstrate important role in material degradation. However, the specific advantage of Sr^{2+} and Mg^{2+} on overall osteoregeneration could not be determined in this study. This suggests that the concentration of metal ions utilized in this study may not be optimal for regulating bone remodeling pattern. Consequently, there is an imperative to delve deeper into the immune responses associated with material and tissue regeneration induced by varying metal ion concentrations in the future.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal studies were approved by the Local Ethical Committee of the Faculty of Medicine, University of Niš, Niš, Serbia. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

YR: Data curation, Formal Analysis, Investigation, Writing—original draft, Writing—review and editing. OJ: Funding acquisition, Writing—review and editing. MB: Data curation, Formal Analysis, Investigation, Writing—original draft. KB: Data curation, Formal Analysis, Investigation, Project administration, Visualization, Writing—original draft. OG: Funding acquisition, Investigation, Methodology, Resources, Software, Supervision, Writing—original draft. SA: Data curation, Formal Analysis, Writing—original draft. AK: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing—original draft, Writing—review and editing. SN:

Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing—review and editing. SS: Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Writing—review and editing. LL: Funding acquisition, Methodology, Resources, Supervision, Writing—review and editing. IP: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing—original draft, Writing—review and editing. MB: Writing—original draft, Writing—review and editing. Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. The authors gratefully acknowledge the funding by the German Research Foundation (Deutsche Forschungsgemeinschaft, DFG) for the subproject 6 within the Research Unit 5250 “Permanent and bioresorbable implants with tailored functionality” (no. 449916462).

Acknowledgments

Figure 1 was created with BioRender.com.

Conflict of interest

Author SA was employed by company BerlinAnalytix GmbH.

Author AK was employed by Biotrics Bioimplants AG.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Article

Analyses of the Cellular Interactions between the Ossification of Collagen-Based Barrier Membranes and the Underlying Bone Defects

Said Alkildani ¹ , Yanru Ren ^{1,2}, Luo Liu ³ , Denis Rimashvskiy ⁴ , Reinhard Schnettler ⁵, Milena Radenković ⁶ , Stevo Najman ^{6,7} , Sanja Stojanović ^{6,7}, Ole Jung ^{2,†} and Mike Barbeck ^{1,2,*,†} 

¹ BerlinAnalytix GmbH, 12109 Berlin, Germany

² Clinic and Policlinic for Dermatology and Venereology, University Medical Center Rostock, 18057 Rostock, Germany

³ Beijing Advanced Innovation Center for Soft Matter Science and Engineering, College of Life Science and Technology, Beijing University of Chemical Technology, Beijing 100013, China

⁴ Department of Traumatology and Orthopedics, Peoples' Friendship University of Russia, 117198 Moscow, Russia

⁵ University Medical Centre, Justus Liebig University of Giessen, 35390 Giessen, Germany

⁶ Department for Cell and Tissue Engineering, Scientific Research Center for Biomedicine, Faculty of Medicine, University of Niš, 18000 Niš, Serbia

⁷ Department of Biology and Human Genetics, Faculty of Medicine, University of Niš, 18000 Niš, Serbia

* Correspondence: mike.barbeck@med.uni-rostock.de; Tel.: +49-176-81022467

† These authors contributed equally to this work.



Citation: Alkildani, S.; Ren, Y.; Liu, L.; Rimashvskiy, D.; Schnettler, R.; Radenković, M.; Najman, S.; Stojanović, S.; Jung, O.; Barbeck, M. Analyses of the Cellular Interactions between the Ossification of Collagen-Based Barrier Membranes and the Underlying Bone Defects. *Int. J. Mol. Sci.* **2023**, *24*, 6833. <https://doi.org/10.3390/ijms24076833>

Academic Editor: David Magne

Received: 4 March 2023

Revised: 27 March 2023

Accepted: 3 April 2023

Published: 6 April 2023



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Abstract: Barrier membranes are an essential tool in guided bone Regeneration (GBR), which have been widely presumed to have a bioactive effect that is beyond their occluding and space maintenance functionalities. A standardized calvaria implantation model was applied for 2, 8, and 16 weeks on Wistar rats to test the interactions between the barrier membrane and the underlying bone defects which were filled with bovine bone substitute materials (BSM). In an effort to understand the barrier membrane's bioactivity, deeper histochemical analyses, as well as the immunohistochemical detection of macrophage subtypes (M1/M2) and vascular endothelial cells, were conducted and combined with histomorphometric and statistical approaches. The native collagen-based membrane was found to have ossified due to its potentially osteoconductive and osteogenic properties, forming a “bony shield” overlying the bone defects. Histomorphometrical evaluation revealed the resorption of the membranes and their substitution with bone matrix. The numbers of both M1- and M2-macrophages were significantly higher within the membrane compartments compared to the underlying bone defects. Thereby, M2-macrophages significantly dominated the tissue reaction within the membrane compartments. Statistically, a correlation between M2-macrophages and bone regeneration was only found at 2 weeks post implantation, while the pro-inflammatory limb of the immune response correlated with the two processes at 8 weeks. Altogether, this study elaborates on the increasingly described correlations between barrier membranes and the underlying bone regeneration, which sheds a light on the understanding of the immunomodulatory features of biomaterials.

Keywords: collagen membrane; barrier membrane; ossification; histomorphometry; correlation matrix analysis; M1-macrophages; M2-macrophages; bone tissue regeneration; in vivo; immunohistochemical staining

1. Introduction

Today, collagen membranes are integral components of the treatment concept of guided bone regeneration (GBR) for the augmentation of the jawbone or the healing of jaw ridge defects in combination with oral implant treatment in dentistry [1,2]. In recent

decades, this material class has replaced non-degradable materials, such as polytetrafluoroethylene (PTFE)-based membranes, for most indications. This is mainly based on the fact that bioresorbable membranes prevent secondary surgery for removal. In this context, barrier functionality is still the main purpose of these medical devices in order to enable undisturbed bone-healing without potentially hindering effects outgoing from the soft tissue [3]. Thus, the application of an ideal barrier membrane must allow for the desired clinical regenerative outcome, which is bone regeneration for a following implant insertion. Interestingly, other (desired) requirements have been postulated in recent years, especially features of resorbable collagen-based barrier membranes alongside barrier functionality [3]. Among other things, a barrier membrane should “actively” support the process of bone-healing. In this context, a so-called “selective permeability” of the membrane must prevent the ingrowth of epithelial cells while promoting the proliferation of osteogenic cells. Furthermore, the optimal barrier membrane should allow the establishment of an adequate angiogenesis and vascularization for bone tissue regeneration in form of a so-called “transmembraneous vascularization” [4].

Additionally, different authors stated that the molecular micromilieu induced by a barrier membrane might also influence the molecular events of the neighboring bone tissue regeneration process [4–7]. Altogether, it has been assumed that tissue reactions to the membrane might have an active (molecular) biological role in the bone-healing process in addition to the bone substitute material (BSM) used. For example, the application of an ideal membrane could promote the recruitment and differentiation of different cell types that are involved in the bone-healing process, such as osteoblasts and precursor cells. Moreover, it has been assumed that the inflammatory tissue responses to a membrane could have a substantial influence on the underlying bone-healing process [8,9]. In this context, it has been suspected that biomaterials inducing an overall anti-inflammatory tissue reaction, including the respective macrophage subtype, i.e., anti-inflammatory M2-macrophages, may optimally contribute to the material-related tissue regeneration progress [10,11].

It has already been shown that biomaterials, including barrier membranes based on collagen from different animal and tissue sources, induce a higher anti-inflammatory cell responses, as demonstrated by the significantly higher numbers of M2-macrophages [6,7]. Specifically, a barrier membrane based on the porcine pericardium has been shown to fulfill the above-mentioned requirements as the device showed a mild and predominantly anti-inflammatory tissue reaction within the surrounding tissue and a required cell migration in combination with optimal barrier functionality without any signs of fragmentation for several months [12–14]. Additionally, it has been shown that this membrane allows for sufficient transmembraneous vascularization [4,5,14,15].

To test the hypothesis of the interaction between the bioactivity of barrier membranes and the underlying bone defects, the present study was conducted. The membrane was used in combination with a newly developed BSM (combining xenogeneic granules with hyaluronic acid) and implanted in Wistar rats at early (2 weeks), intermediate (8 weeks), and late (16 weeks) time points in a standardized calvaria implantation model [16]. As a control, a sham operation without BSM was performed at all time points. Thereby this implantation model was used to simulate the intraoral clinical indications that require GBR procedures, as the anatomical conditions (i.e., bone defects that are covered with soft tissue and epithelium) are comparable. Established histological evaluation methods in combination with histomorphometrical analyses were conducted to quantitatively examine the membrane degradation and new bone growth, as well as the immune response and vascularization [16,17]. The data were then employed in Pearson’s correlation analyses to further investigate the potential interactions between the membrane and the defect areas.

2. Results

2.1. Histological Results

At 2 weeks post implantationem, the histological analysis showed that the membrane was detectable and intact in both membrane compartments (Figure 1A). Moderate cellular

infiltration into the membrane was observed (Figure 2(A1,A3)). Furthermore, a slight ingrowth of new bone matrix outgoing from the bony defect borders was detectable in all compartments (Figures 1A and 2(A1–A4)). An osteoconductive bone growth process was found in the group of the bone graft-filled compartments, while a mixture of osteoconductive and osteogenic bone growth was visible in both membrane compartments (Figures 1A and 2(A1–A4)). Thus, an osteoconductive bone ingrowth extruding from the surrounding underlying bone tissue was visible, leading to the ossification of the membrane fibers, while independent ossification nuclei within the membrane fibers were also observable (Figure 2(A1,A3)). Thereby, a moderate inflammatory tissue response, mainly involving macrophages, alongside the lower numbers of granulocytes and lymphocytes in combination with a moderate implantation bed vascularization, was observed in all compartments. Furthermore, the low numbers of multinucleated giant cells (MNGCs) were detectable on the surfaces of the BSM granules.

The detection of M2-macrophages showed their occurrence within the membrane and within the intergranular connective tissue of the BSM granules and the surrounding tissue of both implant types (Figure S2). M2-macrophages were not seen directly on the surface of the collagen membrane or the BSM granules. The detection of M1-macrophages showed that in all compartments, this subtype was detectable at the surface of both biomaterials and within the membrane (Figure S1). Mono- and multinuclear cells expressing the CD11c molecule were also detected on the surfaces of the BSM.

Higher numbers of M1- and M2-macrophages were found in both membrane compartments than their vertically adjacent compartments. Moreover, comparable numbers of M1- and M2-macrophages were found in both membrane and bone defect compartments (Figures S1 and S2).

At 8 weeks post implantation, a significant increase in newly formed bone matrix was seen in all compartments (Figures 1B and 2(B1–B4)). In the implantation beds of the BSM and in the sham operation group, the central defect areas still contained connective tissue and thus showed no complete bone-healing, indicating a further osteoconductive bone growth process, extending from the defect borders towards the defect center (Figures 1B and 2(B2,B4)). In both membrane compartments, a further ossification of the collagen membrane took place up to a nearly complete calcification in some animals, building a “bony shield” above the bone defect areas, independent of the bone growth within the underlying compartments (Figures 1B and 2(B1,B3)). Thereby, most of the collagen fibers of the membranes were calcified, while a few ossification nuclei within the membrane fibers were still observable, hinting at ongoing osteogenic bone growth (Figure 2(B1,B3)).

A slight inflammatory tissue response, mainly involving macrophages and low numbers of granulocytes, lymphocytes, and multinucleated giant cells (MNGCs) at the surfaces of the BSM granules in combination with moderate implantation bed vascularization, was observed in all compartments. At this study time point higher numbers of both macrophage subtypes were still found in both membrane compartments, compared to their vertically adjacent compartments. Comparable numbers of the M1- and M2-macrophages were found in both membrane compartments, as well as both bone defect compartments (Figures S1 and S2).

At 16 weeks post implantation, a further increase in bone regeneration was seen in all compartments (Figures 1C and 2(C1–C4)). In the membrane compartments, only slight membrane remnants were detectable, while nearly the whole membranes were replaced by mature bone matrix, building a “bony shield” above the bone defect areas as mentioned above (Figures 1C and 2(C1,C3)). In the membrane compartment above the sham operation, minor areas of the membrane were still filled by connective tissue that was condensed, hinting at a precursor matrix for the growth of bone matrix (Figure 2(C3)). At this study time point, a slight inflammatory tissue response was still present, mainly composed of macrophages in combination with low numbers of granulocytes, lymphocytes, and multinucleated giant cells (MNGCs) at the surfaces of the BSM granules, as well as a moderate implantation bed vascularization, which were detectable in the remaining areas

of connective tissue in all compartments. At this late time point of study, higher numbers of M2-macrophages were found in both membrane compartments compared to the occurrence of M1-macrophages, while comparable numbers of M1- and M2-macrophages were found in defect compartments (Figures S1 and S2).

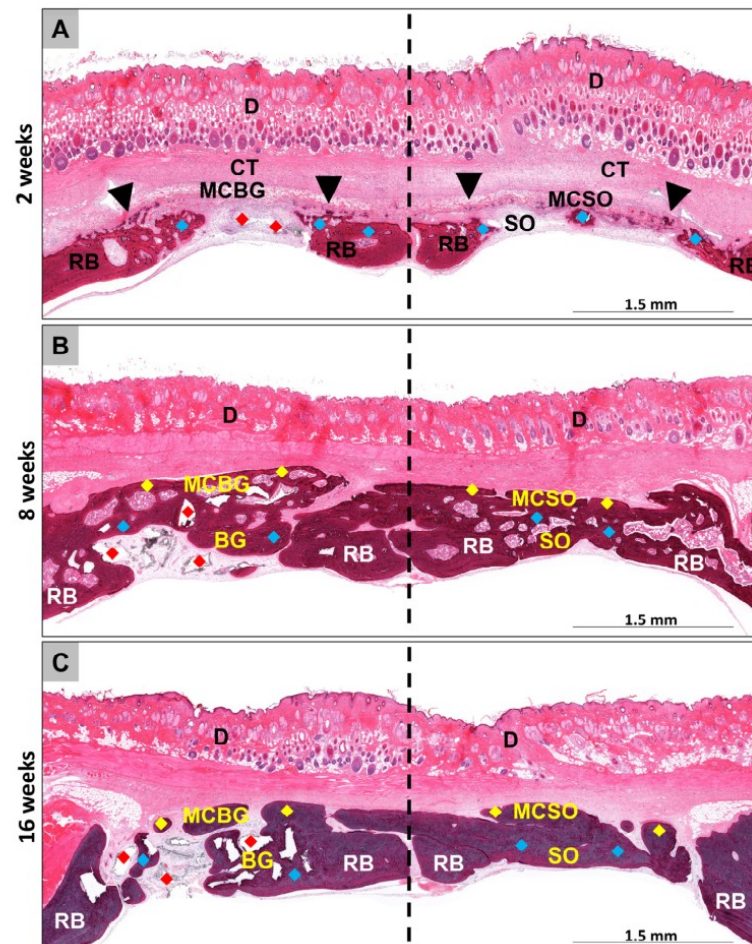


Figure 1. Overviews of the implantation beds at the three time points at (A) 2, (B) 8, and (C) 16 weeks post implantation and the progression of the bone regeneration within the four compartments, i.e., the membrane compartment above bone graft-filled defects (MCBG), the bone graft-filled defects (BG), the membrane compartment above the sham operation defects (MCSO), and the sham bone defects (SO). blue rhombus = newly formed bone tissue; red rhombus = bone substitute material; black arrowheads = bone ingrowth into the membrane; yellow rhombus = ossified collagen membrane; RB = residual bone; CT = connective tissue; D = dermis (HE staining, 100× magnifications scale bars = 1.5 mm).

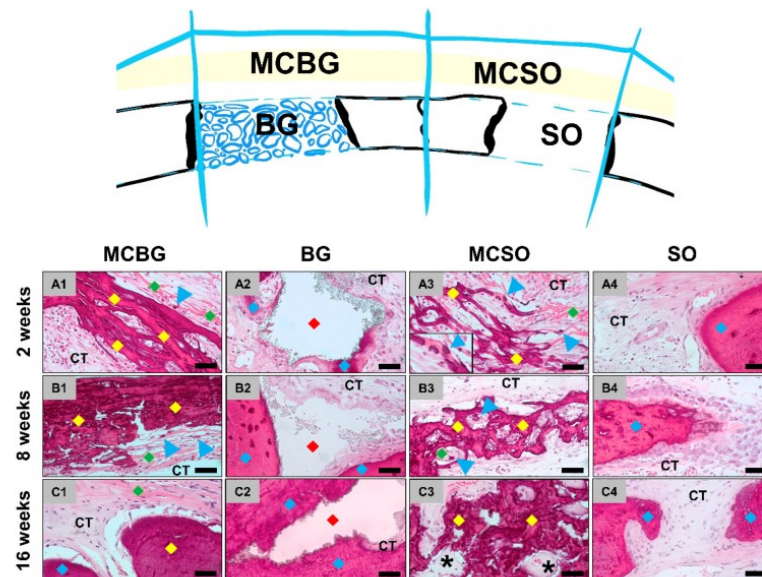


Figure 2. Histological images of the tissue responses in the four compartments at (A1–A4) 2, (B1–B4) 8, and (C1–C4) 16 weeks post implantationem. Left row: tissue responses within the membrane compartment above the bone graft-filled defect (MCBG). Left center row: tissue responses within the bone graft-filled defects (BG). Right center row: tissue responses within the membrane compartment above the sham operation defects (MCSO). Right row: tissue responses within the sham bone defects (SO). Green rhombus = collagen fibers of the membrane; blue arrowheads = ossification nuclei within the membrane fibers; yellow rhombus = ossified membrane (fibers); red rhombus = BSM granules; blue rhombus = newly formed bone with the bone graft-filled and sham operation defects; stars = areas of condensed connective tissue; CT = connective tissue (HE staining, 400× magnifications, scale bars = 20 μm).

2.2. Histomorphometrical Results

2.2.1. Bone Regeneration

At 2 weeks post implantationem, the measurements showed that in the groups of the bone graft-filled compartment and the sham operation, a comparable bone regeneration was found (Table 1 and Figure 3A). Additionally, in both membrane compartments, a slightly comparable bone ingrowth was detectable (Table 1 and Figure 3A). The analysis furthermore showed that only a significant difference ($* p < 0.05$) was found between the bone regeneration values in the bone graft-filled compartment and in both membrane compartments (Table 1 and Figure 3A). At 8 and 16 weeks post implantationem, the analysis revealed that comparable bone regeneration values in the different compartments were detectable with the exception of a significant difference ($** p < 0.01$ and $* p < 0.05$) between the amount of newly formed bone matrix in the groups of the sham operation and its related membrane compartment (Table 1 and Figure 3A).

Additionally, the measurements of the new bone within the membrane compartments revealed that a significantly higher comparable percentage ($*** p < 0.001$) of the membranes in both compartments was identifiable as only a slight comparable ossification fraction was observed at 2 weeks post implantationem (Table 1 and Figure 3B).

Table 1. Histomorphometrical measurement results of the tissue distribution. Values are the percentage of the TIA. MCBG: membrane compartment above bone graft-filled bone defects; MCSO: membrane compartment above sham operation; BG: bone graft-filled bone defect; and SO: sham operation bone defects.

	Tissue Fraction	MCBG	MCSO	BG	SO
2 weeks	Bone tissue (%)	1.6 ± 3.7	1.6 ± 2.1	22.8 ± 12.5	15.0 ± 15.3
	Biomaterial (%)	24.7 ± 12.7	28.6 ± 8.2	10.3 ± 7.5	-
8 weeks	Bone tissue (%)	35.5 ± 10.9	26.6 ± 9.4	33.4 ± 16.1	51.6 ± 25.7
	Biomaterial (%)	14.5 ± 13.1	9.9 ± 13.9	19.3 ± 9.2	-
16 weeks	Bone tissue (%)	33.4 ± 12.7	27.9 ± 17.3	34.0 ± 23.4	48.8 ± 28.6
	Biomaterial (%)	4.2 ± 2.1	9.1 ± 11.6	15.2 ± 10.4	-

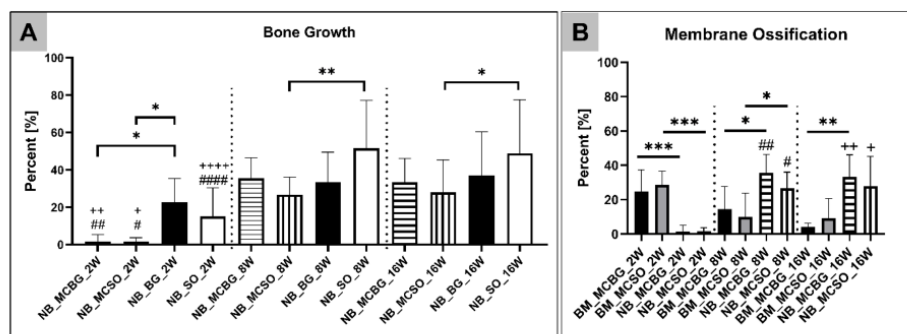


Figure 3. (A) Results of bone regeneration at 2, 8, and 16 weeks post implantationem. (B) Results of the membrane ossification measurements in comparison to membrane resorption within both membrane compartments. Intraindividual significances: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; interindividual significances: #/ $p < 0.05$, ##/ $p < 0.01$, and ####/ $p < 0.0001$. For (A,B), # indicates significant differences between the 2- and 8-week datasets and + indicates significant differences between the 2- to 16-week datasets. NB = newly formed bone; BM = biomaterial; MCBG: membrane compartment above bone graft-filled defects; MCSO: membrane compartment above sham operation; BG: bone graft-filled defect; and SO: sham operation bone defect.

At 8 weeks post implantationem, the significant percentages (* $p < 0.05$) of the membranes in both compartments were ossified (Table 1 and Figure 3B). The percentages of membrane ossification were also significantly higher (# $p < 0.05$ and ## $p < 0.01$) compared to the respective values at 2 weeks post implantationem (Table 1 and Figure 3B).

At 16 weeks post implantationem only in the group of the membrane compartment above the bone graft-filled bone defects a significantly higher membrane ossification (** $p < 0.01$) was detected (Table 1 and Figure 3B). Moreover, the ossification values at this time point were significantly higher (+ $p < 0.05$ and ++ $p < 0.01$) compared to the values at 2 weeks post implantationem (Figure 3B).

An exemplary histological image, showing the transition of collagen to bony tissue is found in the Supplementary Materials (Figure S4).

2.2.2. Immune Response

The histomorphometrical analysis of the occurrence of pro- and anti-inflammatory macrophage subtypes within the four compartments initially revealed that significantly higher numbers of M2-macrophages (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$) were detectable in all compartments and at all three time points (Table 2 and Figure 4). Furthermore, the analysis showed that the numbers of pro- and anti-inflammatory macrophages

were significantly higher ($p < 0.05$, $### p < 0.001$, and $#### p < 0.0001$) within both membrane compartments, i.e., the MCBG and the MCSO compartments, compared to the respective numbers within the bone defect compartments, i.e., the BG and the SO compartments at 2 and 8 weeks post implantation (Table 2 and Figure 4). At 16 weeks post implantation no significant differences between the numbers of M1-macrophages were detectable in all four compartments, while significantly higher numbers of M2-macrophages ($### p < 0.001$ and $#### p < 0.0001$) were found within both membrane compartments compared to both bone defect compartments (Table 2 and Figure 4).

Table 2. Macrophage subtype numbers at 2, 8, and 16 weeks post implantation. MCBG: membrane compartment above bone graft-filled defects; MCSO: membrane compartment above sham operation; BG: bone graft-filled defects; and SO: sham operation bone defect.

	MCBG	MCSO	BG	SO
CD163 (cells/mm ²)				
2 weeks	1201 ± 571.9	1006 ± 562.6	308.7 ± 146.9	371.8 ± 244.3
8 weeks	1100 ± 474.9	823.5 ± 413.8	365.3 ± 173.8	346.5 ± 189.8
16 weeks	691.5 ± 320.8	987.8 ± 558.2	241.1 ± 122.0	297.8 ± 110.1
CD11c (cells/mm ²)				
2 weeks	503.7 ± 126.8	422.6 ± 170.4	8.8 ± 10.0	7.6 ± 4.6
8 weeks	290.5 ± 190.6	212.4 ± 115.9	16.6 ± 11.8	20.0 ± 11.2
16 weeks	104.2 ± 47.0	129.5 ± 39.4	14.6 ± 11.5	11.51 ± 5.7

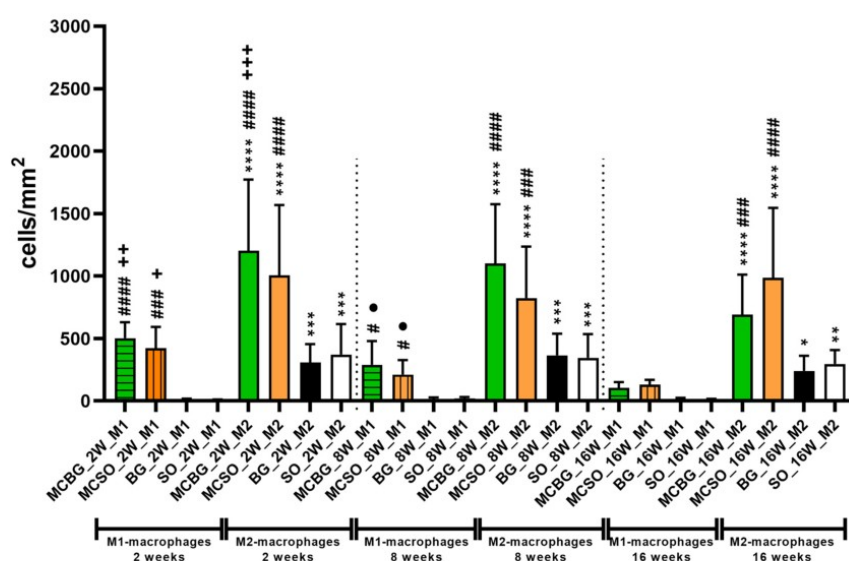


Figure 4. Results of the macrophage subtype measurements at 2, 8, and 16 weeks post implantation. Intraindividual significant differences: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. Interindividual significant differences: # $p < 0.05$, ### $p < 0.001$, and #### $p < 0.0001$, + $p < 0.05$, ++ $p < 0.01$, and +++ $p < 0.001$. + = significant differences between 2- and 16-week datasets; • = significant differences between 8- and 16-week datasets. MCBG: membrane compartment above bone graft-filled defects; MCSO: membrane compartment above sham operation; BG: bone graft-filled defect; and SO: sham operation bone defect.

Additionally, the analysis revealed that a significant decrease in the M1-macrophage numbers in the MCBG and the MCSO compartments and of the M2-macrophages within the MCBG compartment was detectable between 2 and 16 weeks post implantationem (+ $p < 0.05$, ++ $p < 0.01$, and +++ $p < 0.001$) (Figure 4). Additionally, a significant decrease in the M1-macrophage numbers was observed in the MCBG and the MCSO compartments between 8 and 16 weeks post implantationem ($p < 0.05$) (Figure 4).

2.2.3. Vascularization

The histomorphometrical analysis of the implantation bed vascularization showed that comparable vessel density values were detected in all compartments at 2 weeks post implantationem (Table 3 and Figure 5A). At 8 and 16 weeks post implantationem, only a significant difference (* $p < 0.05$) between the vessel densities within the MCSO compartment and the BG compartment was found (Figure 5A). Furthermore, the statistical analysis revealed that significant decreases in the vessel densities (# $p < 0.05$) in all compartments, with the exception of the SO compartment between 2 and 8 weeks post implantationem, were measured (Figure 5A). Additionally, significant decreases in the vessel densities (+ $p < 0.05$ and ++++ $p < 0.0001$) in the MCBG compartment and the BG compartment between 2 and 16 weeks post implantationem were found (Figure 5A). Finally, a significant decrease in the vessel density ($p < 0.01$) in the MCSO compartment between 2 and 16 weeks post implantationem was found (Figure 5A).

The analysis of the vascularization percentage, relevant to the TIA, showed that the highest values were found in the BG compartment at 2 and 8 weeks post implantationem and that were significantly higher (* $p < 0.05$ and ** $p < 0.01$) than the values in the MCBG and the MCSO compartments, while no difference was found compared to the SO compartment (Table 3 and Figure 5B). Furthermore, the statistical analysis revealed that significant decreases in the vascularization percentage (# $p < 0.05$ and ## $p < 0.01$) in all compartments between 2 and 8 weeks post implantationem were measured (Figure 5B). Additionally, significant decreases in the vascularization percentages at 2 weeks (+ $p < 0.05$, ++ $p < 0.01$, and ++++ $p < 0.0001$) in the MCBG compartment, the BG compartment, and the SO compartment were detected, compared to 16 weeks (Figure 5B). Finally, a significant decrease in the vascularization percentage ($p < 0.05$) in the BG compartment between 8 and 16 weeks post implantationem was measured (Figure 5B). The histological figures of the immunohistochemical detection of vascularization are provided in the Supplementary Materials (Figure S3).

Table 3. Vascularization values (vessel density and vascularization percentage) at 2, 8, and 16 weeks post implantationem. MCBG: membrane compartment above bone graft-filled defects; MCSO: membrane compartment above sham operation; BG: bone graft-filled defect; and SO: sham operation bone defect.

	MCBG	MCSO	BG	SO
Vessel Density (vessels/mm ²)				
2 weeks	291.2 ± 49.4	253.9 ± 42.9	323.8 ± 73.5	315.0 ± 48.8
8 weeks	184.8 ± 105.7	161.2 ± 80.7	249.7 ± 96.5	246.1 ± 55.8
16 weeks	202.8 ± 82.2	281.8 ± 83.4	183.1 ± 40.8	259.5 ± 70.7
Vascularization Percentage (%)				
2 weeks	5.2 ± 1.9	4.1 ± 1.0	7.2 ± 2.5	5.6 ± 1.6
8 weeks	2.2 ± 1.7	1.4 ± 0.8	4.6 ± 2.4	2.7 ± 1.1
16 weeks	1.9 ± 0.9	2.9 ± 1.4	2.6 ± 1.4	3.7 ± 1.5

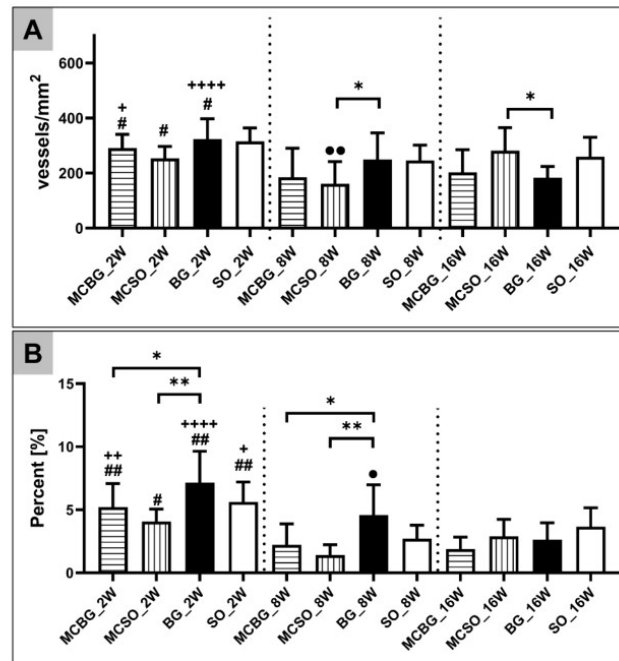


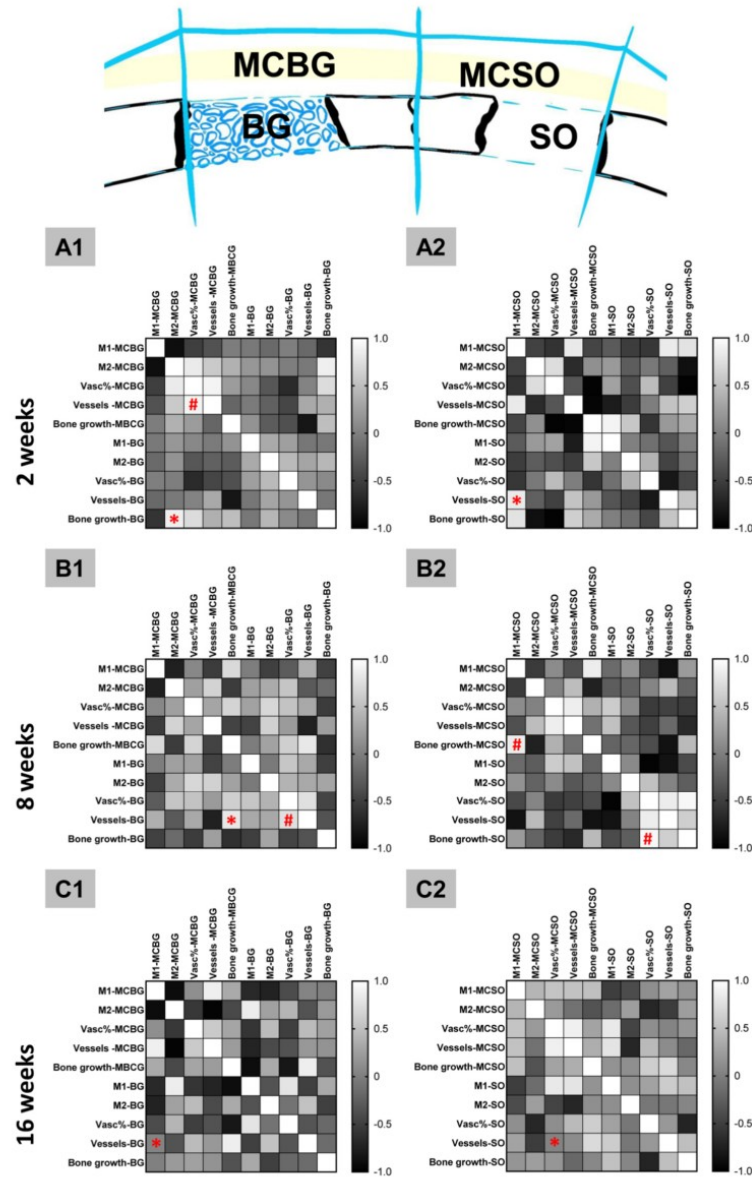
Figure 5. Results of the measurements of (A) the vessel density and (B) the vascularization percentage at 2, 8, and 16 weeks post implantation. Intraindividual significant differences are indicated as * $p < 0.05$ and ** $p < 0.01$. Interindividual significant differences are indicated as #/+/ $\bullet$ $p < 0.05$, ##/++/ $\bullet\bullet$ $p < 0.01$, and ++++ $p < 0.0001$. # = significant differences between 2- and 8-week datasets; + = significant differences between 2- and 16-week datasets; $\bullet$ = significant differences between 8- and 16-week datasets.

2.3. Correlation Analyses

Pearson's correlation analysis was conducted to explore positive correlations between two measured parameters within the same compartment or between overlying coherent compartments, i.e., the MCBG and the BG compartments or the MCSO and the SO compartments (Figure 6).

At 2 weeks post implantation, a positive correlation ($p = 0.023$) between the bone growth in the BG compartment and the occurrence of M2-macrophages in the MCBG compartment was found (Figure 6). Additionally, a positive significant correlation ($p = 0.033$) between the vessel density and the vascularization percent within the MCBG compartment was calculated (Figure 6(A1)). Moreover, a positive correlation ($p = 0.041$) between the vessel density in the SO compartment and the occurrence of M1-macrophages within the MCSO compartment was measured (Figure 6(A2)).

At 8 weeks post implantation, a positive correlation ($p = 0.009$) between the vessel density in the BG compartment and the bone growth within the MCBG compartment was measured (Figure 6(B1)). Additionally, a positive correlation ($p = 0.013$) between the vessel density and the vascularization percentage in the BG compartment was found (Figure 6(B1)). Furthermore, a positive correlation ($p = 0.017$) between the bone growth within the MCSO compartment and the occurrence of M1-macrophages within the same compartment was found (Figure 6(B2)). Finally, a positive correlation ($p = 0.018$) between the bone growth and the vascularization percent within the SO compartment was revealed (Figure 6(B2)).



At 16 weeks post implantation, a positive correlation ($p = 0.021$) between the vessel density in the BG compartment and the occurrence of M1-macrophages in the MCBG compartment was calculated (Figure 6(C1)). Finally, a positive correlation ($p = 0.022$) between the vessel density in the SO compartment and the vascularization percent in the MCSO compartment was revealed at this time point (Figure 6(C2)).

3. Discussion

The aim of the present study was to analyze the molecular interactions between the bioactivity of collagen-based barrier membranes and the underlying bone defects. To achieve this, a pericardium-based collagen membrane was used in combination with a newly developed BSM combining xenogeneic granules with hyaluronic acid in an established calvaria implantation model in Wistar rats for 2, 8, and 16 weeks post implantation [16]. This BSM, mixed with hyaluronic acid as a water-binding molecule, was investigated in a previous study by our group [16]. The addition of hyaluronic acid did not yield in any differences regarding immune response, vascularization, and bone growth but improved clinical handling; therefore, only BSM mixed with hyaluronic acid was used in this study. This implantation model was conducted to simulate the intraoral clinical indications that require GBR procedures as the anatomical conditions (i.e., bone defects that were covered with soft tissue and epithelium) are comparable.

Initially, both the histological and the histomorphometrical analyses revealed that a gradual ossification within both the two bone defect compartments (filled with the bone substitute material as well as in the control compartment) and within the two membrane compartments was detectable. Only at 2 weeks post implantation, a higher osteoconductive bone growth was found, in the bone defects compartment filled with the BSM especially, while only a very slight bone regeneration took place in both membrane compartments. However, starting from 8 weeks and up to 16 weeks post implantation, comparable bone regeneration values were detected in all four compartments. Thereby, no signs of resorption of the xenogeneic bone substitute granules were observable, as had also been previously described in the case of this material class [18–20]; however, the collagen membrane was gradually resorbed while being completely transformed into bone tissue or bone matrix based on its fiber structure. In this context, the ossified membrane formed an independent bony layer above the actual bone defect areas. The gradual bone growth around the BSM is expected as such materials have been reported to enhance bone growth in such a fashion [18,21,22]. However, the bone transversion of the “native” collagen membrane is a surprising result as this phenomenon has only been described in a very small number of studies [23–25]. For example, in a preclinical study, Taguchi et al. described the ossification of a native bi-layered collagen membrane [25]. The authors concluded that the collagen matrix allows for bone growth, serving as both an osteoconductive and an osteogenic scaffold. Comparable to the results of the present study, it was observed that new bone formation within the membrane was independent of regeneration from the bone defect cavity. Moreover, it was described that the collagen matrix induced osteoblastic differentiation as also described in the case of the pericardium-based collagen membrane analyzed in the present study showing “ossification nuclei” with associated active osteoblasts and matrix deposition along the collagen fiber bundles, which is similar in definition to the physiological endochondral bone growth. Additionally, in both studies, it was not possible to distinguish the new membrane-derived bone matrix from that which was outgoing (via osteoconduction) from the residual bone tissue. In a previous study, it was concluded that the osteogenic bone growth was driven by mediating factors, such as bone morphogenetic proteins, transforming growth factors, insulin-like growth factors, and fibroblast growth factors, that might have (indirectly) bound to the collagen fibers [25]. In another study conducted by Zubery et al., it was demonstrated that a cross-linked porcine collagen barrier membrane also ossified with time and augmented the original alveolar ridge in both preclinical and clinical studies [23,24]. In the case of this membrane, which is cross-linked via ribose, only osteoconductive bone growth was observed in areas of direct contact with

residual bone indicating that this membrane did not induce both regeneration pathways as described above. Interestingly, the above-mentioned non-cross-linked bi-layered type I and III porcine collagen membrane, which was used as control material in both studies, showed very few signs of membrane ossification. The results of the present study revealed that the analyzed pericardium-based membrane did allow for a combination of osteoconductive and osteogenic bone regeneration and did contribute to a faster and complete bony substitution within both membrane compartments compared to the previously described studies. It can also be concluded that the ossification and the associated generation of the described “bony shield” might help to regenerate the underlying bone defect by means of additional bone ingrowth and due to the protection of the underlying defect area. However, further preclinical trials, including large animal models and appropriate clinical trial settings, need to be conducted to verify these observations.

In addition to their osteoconductive properties, it has been proposed by different authors that the molecular microenvironment induced by biomaterials in general and thus by a barrier membrane might also influence the molecular events of the neighboring bone tissue regeneration process [26–29]. In this context, it has widely been assumed that the inflammatory tissue responses to a membrane and especially an overall anti-inflammatory tissue reaction, involving mainly M2-macrophages, might have a substantial influence on the underlying bone-healing process [30,31]. Moreover, it has been stated by different authors that the optimal barrier membrane allows for sufficient transmembraneous vascularization to contribute to bone regeneration [4,5,15]. However, there have been no studies to date that examine these molecular relationships. Based on study protocols that have already been published in various preliminary studies, the present study was aimed directly at this analysis [7,16,32]. In this context, the histomorphometrical analyses of the vascularization pattern and the occurrence of pro- and anti-inflammatory macrophage subtypes were conducted to correlate both parameters with the bone regeneration process. The analysis of the vascularization within the four different compartments revealed that comparable vessel densities and vascularization percentages were found within all compartments over the complete study period except for the significantly higher values of both parameters in the bone graft-filled bone defect compartment. This latter result can be explained by the higher number of multinucleated giant cells that are known to be potent sources of proangiogenic factors, such as the vascular endothelial growth factor, (VEGF) and are particularly induced in the case of bone substitute materials, such as the xenogeneic material used in the present study [33].

Furthermore, the analysis of the macrophage subtypes showed that within the four compartments significantly higher numbers of M2-macrophages were initially detectable in all compartments and at all three time points. These results show that the used biomaterials, i.e., the bone substitute material as well as the collagen membrane, can be classified as biocompatible, as they did not induce exaggerated pro-inflammatory tissue reactions. Furthermore, this result is in line with the assumption of the immunocompatibility of biomaterials, as it was stated that an optimal material should induce an overall anti-inflammatory tissue reaction and macrophage response to support (bone) tissue regeneration [34]. Additionally, the comparable values in the material compartments to the macrophage numbers within the SO compartment are noticeable and this result additionally reveals that both biomaterials, i.e., the BSM and the collagen membrane, do not induce adverse cell reaction patterns or pro-inflammatory tissue responses beyond the “basal” level, i.e., a reaction that does not exceed that usually involved in the wound-healing process.

Additionally, the numbers of pro- and anti-inflammatory macrophages were significantly higher within both membrane compartments compared to the respective numbers within the bone defect compartments. Only at 16 weeks post implantationem were no significant differences between the numbers of M1-macrophages detectable in all four compartments, while significantly higher numbers of M2-macrophages were found within both membrane compartments compared to both bone defect compartments. This measurement result can be explainable on the basis of the biodegradation patterns of both

biomaterials used in the present study. Thus, the membrane has been shown to degrade faster even in comparison to the xenogeneic BSM granules. In this context, it has already been shown that the integration and the associated biodegradation of collagen membranes are processed mainly by macrophages, which was confirmed by this study result once again [4,7]. Therefore, faster biodegradation is also accompanied by the migration of a higher number of phagocytes, such as the measured macrophages. Since the membrane was almost completely ossified after 16 weeks and also integrated or degraded, it can be assumed that the further migration of phagocytosing macrophages was no longer “required” to be induced.

Finally, the analyses of positive correlations revealed two different “dependency categories”, i.e., (a) correlations between tissue parameters within the same compartment and (b) between correlating parameters within vertically adjacent compartments (bone defect compartment and neighbored membrane compartment).

At 2 and 8 weeks post implantation, correlations between the vessel density and the vascularization percent within the MCBG compartment and the BG compartment were found, respectively. This result is not surprising because it has already been proven that the cell types involved in the (inflammatory) tissue responses to biomaterials, such as the used bone substitute material and the pericardium-based barrier membrane (mainly macrophages and MNGCs), express different mediating factors, such as different members of the VEGF family [5,33,35,36]. The different VEGF isoforms have been shown to induce various signaling pathways, also including angiogenesis and vessel maturation, so that a connection to this signaling family is conceivable [37,38]. Additionally, the combinatory effects of two or more different mediators, such as the fibroblast growth factor 2 (FGF2) and VEGF, have been shown to increase both angiogenesis and blood vessel maturation when combining both mediators with collagen–heparin scaffolds [38]. Additionally, vascular pericytes and mural cells support cells that range in phenotype from pericytes to vascular, and smooth muscle cells have been shown to be involved in the process of vessel maturation [39].

Bone growth and the vascularization percentage within the SO compartment were also found to correlate at 8 weeks post implantation. This result was also predictable as both direct and indirect interactions between vascularization pathways and the molecular processes of bone regeneration have been identified [40,41]. The achievement of bone tissue regeneration is focused on the primary role of vascularization occurrence in order to deliver enough nutrients, growth factors, minerals, oxygen, and relevant cells for tissue restoration [42]. Additionally, in this context, VEGF has been shown to indirectly stimulate osteogenesis [40].

Moreover, a correlation between the bone growth process and the occurrence of M1-macrophages within the MCSO compartment was detectable at 8 weeks post implantation. This result appears initially surprising, as it has widely been stated that biomaterials that induce a predominantly anti-inflammatory tissue response are also likely to improve (bone) tissue regeneration [8,43]. However, this correlation might also be explainable on the basis of vascularization patterns. In this context, it is well known that every biomaterial induces a material-specific inflammatory tissue response [44]. Even bioresorbable materials, as used in the present study, provoke granulation tissue, including phagocytes containing macrophages and partially containing multinucleated giant cells (MNGCs) that are involved in biodegradation [7,16]. A vascularization pattern dependent on the rate of biodegradation and associated inflammation is also an essential component of this granulation tissue—if only to guarantee the movement and clearance of phagocytes and their progenitor cells [45]. Additionally, it has been revealed by Abels et al. that the key role of the proinflammatory M1-macrophages is the phagocytic activity towards a hydroxyapatite-based BSM, together with other studies that showed the same phenomenon in the case of collagen-based biomaterials [46]. The histomorphometrical data of the aforementioned study about BSM indicate that the small BSM granules underwent a higher biodegradation—although no differences in the overall M1- or M2-macrophage numbers

compared to the other groups were measured. Transferred to the results of the present study, which showed a correlation between the occurrence of M1-macrophages and the bone growth process within the membrane compartment of the sham operation group, this result might indicate that the biodegradation via membrane-induced M1-macrophages is linked with an associated vascularization pattern, which might have supported the bone regeneration process. The question of why this phenomenon was not detectable within the membrane compartment neighbored to the bone graft-filled bone defects arises and an answer to this may be found in the interactions between the membrane and bone defect areas, which are discussed below.

Initially, this analysis part showed that a correlation between the bone growth in the BG compartment and the numbers of M2-macrophages in the related MCBG compartment were detectable at 2 weeks post implantation. This result is in line with the results of other studies that describe the regenerative role of this subtype in (bone) tissue regeneration [30,31]. In the context of bone regeneration, different pathways, e.g., the regulation of mesenchymal stem cell and osteoblast function, based on factors such as macrophage-derived microRNAs (miRNAs), have been described as contributing to positive or negative regulation [47]. However, it is surprising that this observation was made only at this early time point, since it is assumed that a switch from M1-macrophages to M2-macrophages also occurs only later. However, this can be explained by the general decrease in macrophage numbers measured in the present study.

Furthermore, the vessel density in the SO compartment and the occurrence of M1-macrophages within the associated MCSO membrane compartment correlated at this early time point. Interestingly, the same correlation was found at 16 weeks post implantation, as the vessel density within the BG compartment and the occurrence of M1-macrophages within the MCBG compartment were shown to be significantly linked. As mentioned above, it might be that the membrane-induced M1-macrophages also influenced the angiogenesis process in the associated bone defect area.

Additionally, the vessel density within the BG compartment and the bone growth within the MCBG compartment correlated at 8 weeks post implantation. On the one hand, this result is interesting, since the diffusion distance for nutrient transport (oxygen: ~50 µm, nutrients: ~200 µm) is restricted [48,49]. This means that cells that are further away from an implantation bed of a membrane or the BSM undergoes nutrient limitations, which should lead to non-optimal conditions for new bone matrix production on the basis of interactions between both compartments. Thus, a direct influence seems to be only conditionally presumable, while it is conceivable that even indirect pathways between vascularization and bone growth in both compartments might be linked. For example, it has been reported that sufficient vascularization also leads to the improved transport of progenitor cells such as pre-osteoblasts [41]. Additionally, the relevant cells are capable of local migration or “crawling” via chemotaxis [50]. In this way, a correlation between the two parameters in the two compartments can probably also be explained as an underlying factor of osteoconductive bone growth from one compartment to the neighboring implantation area.

Finally, a correlation between the vessel density within the SO compartment and vascularization percent within the MCSO compartment was measured at 16 weeks post implantation. On the one hand, it is conceivable that direct interactions between the implantation sites of both compartments also took place. However, it is also more likely in this case that indirect factors contributed to this correlation due to the distances between the two compartments. In this context, it has been described that vessel maturation, which means a higher vascularization percentage in contrast to a higher vessel density formed by vasculogenesis, requires not only the recruitment of mural cells but also support from a sufficient extracellular matrix [51]. Thus, the ongoing bone growth and bone-healing process in both compartments might be an explanation for this correlation. In this context, the blood vessel maturation might be controlled by osteoblasts (and other related cell types) based on a negative feedback loop mediated by the VEGF-induced secretion of the notch-1

receptor ligand, delta-like 4 (DLL4) [52]. An enhanced DLL4 expression downregulates the expression of VEGFR2 and thus EC proliferation. Additionally, the different VEGF isoforms secreted by osteoblasts amongst other cell types, such as VEGF121 and VEGF165, might have contributed to these measurement results [40]. In addition, the described transmembraneous vascularization of the pericardium-based barrier membrane might be an underlying factor for vessel maturation as endothelial cells have shown to trigger vessel maturation via the recruitment of pericytes to the newly synthesized branches via platelet-derived growth factor-BB (PDGF-BB) expression [53]. This is described by strictly imagining that the overlying compartments are physically separated, but in reality, these vertically adjacent compartments share an intertwined and complex vascular network.

Altogether, the present study substantiates in more detail the already widely suspected correlations between the two classes of materials [3,27,54]. Thereby, the correlations also show clear interactions between the pro- and anti-inflammatory immune responses to the biomaterials as well as the processes of implant bed vascularization and material-supported bone growth. Interestingly, the influence of M2-macrophages was only found at 2 weeks post implantation, while the pro-inflammatory limb of the immune response correlated with the two processes at 8 weeks. These results are shedding new light on the widely described development of immunomodulatory materials. This suggests that while (bone) tissue regeneration requires typical regenerative features (i.e., biocompatibility, vascularization, etc.), it should be seen as a complex process that can be modulated (and even enhanced) by the optimal balance of the immune response. Furthermore, the result substantiates the direct correlation of the vascularization process with bone tissue regeneration—also in the context of the biomaterial application. The ossification of the collagen membrane represents another crucial observation. On the one hand, this demonstrates the bone-healing-promoting effect of long-term stable collagen materials, such as the pericardium-based membrane studied here. Furthermore, it can be assumed that the ossification of the membrane also leads to significantly better bone-healing and bone defect-shielding and stability after its clinical application.

4. Materials and Methods

4.1. Biomaterials

4.1.1. Collagen-Based Barrier Membrane

The pericardium-based collagen membrane (Jason® membrane, Botiss Biomaterials GmbH, Zossen, Germany) was used in the present study to cover the bone defects. This membrane was obtained from porcine pericardium. The medical device was prepared by means of a standardized manufacturing process that begins with an initial selection process of the donor animals and was followed by a purification process, including a wet-chemical treatment and lyophilization finalized by ethylene oxide gas sterilization. The collagen membrane is composed of a natural, multilayered structure with grid-like collagen fibers. Microscopically, the membrane has a honeycomb-like porosity. The biocompatibility and clinical usability of the membrane have been tested in different studies [7,15,55,56]. For the purpose of this study, the membrane (20 × 30 mm²) was cut into four equal rectangular parts. The membrane has been reported to have thickness range of 0.05–0.35 mm [57].

4.1.2. Xenogeneic Bone Substitute Material

The bone substitute material (BSM) cerabone® plus (Botiss Biomaterials GmbH, Zossen, Germany) combines the established bovine bone graft substitute cerabone® with hyaluronic acid (high molecular weight), resulting in “sticky bone” BSM after hydrogenation [16]. The xenogeneic BSM cerabone® was harvested from the femoral heads of cattle from registered slaughterhouses in New Zealand and Germany. The potentially immunogenic components were removed in a multi-step process to ensure their safe application [58]. In this process, the bovine bone raw material undergoes an ensured three-step heating, which is free of chemical additives and includes high-temperature treatment at over 1200 °C. cerabone® plus must be hydrated before application (~0.5 mL saline solution per 1 mL

cerabone® plus) in order to result in a malleable bone grafting material, which facilitates the application and reduces particle distribution in the augmentation area. For the present study, cerabone® plus with a granule size of 0.5–1 mm was used. Further chemical and structural analyses have been previously reported [59].

4.2. In Vivo Study

4.2.1. Experimental Design

The calvaria implantation model was used to simulate the anatomical conditions of GBR procedures, i.e., bone defects with adjacent soft tissue and epithelium. Thereby, the implantations including the creation of defects within the calvaria were performed as previously described [16]. The study included 21 ($N = 7$) male 10–12-week-old male Wistar rats with a mean weight of 230 ± 20 g. Prior to the implantation procedure, the experimental animals were randomly divided into three different study groups for the respective time points, i.e., 2, 8, and 16 weeks post implantation.

Prior to the study, the in vivo experiments were authorized by the local Ethical Committee of the Faculty of Medicine (University of Niš, Serbia) based on the approval number 323-07-00073/2017-05/7 of the Veterinary Directorate of the Ministry of Agriculture, Forestry and Water Management of the Republic of Serbia (date of approval: 22 February 2017).

The calvaria implantation procedure was performed in a standardized manner in accordance with the DIN ISO norm 10993-6 [60]. After initial anesthesia by means of an intraperitoneal injection with ketamine (100 mg/kg of body weight) and xylazine (5 mg/kg of body weight), the process was as follows: the shaving and disinfection of the skull region, the sagittal incision into the skin, the stripping of the periosteum, and the induction of bilateral cranial defects with a respective diameter of 4 mm with a trephine bur (GC, Tokyo, Japan). All left-sided defects were treated with equal amounts of the BSM, while the right-sided defects were treated as controls without material implantation. Prior to suturing, the defects were covered with the pericardium-based collagen membrane. The animals were routinely checked on and their health was documented. Water and food were available ad libitum until the designated time point. After the respective healing periods, the animals were euthanized using euthasol (400 mg/mL), the defect sites were removed and transferred to 4% formalin solution. Afterwards, routine histological evaluation was performed.

4.2.2. Histological Evaluation

The explants were embedded in the polymer embedding system Technovit 9100 (Technovit 9100, Kulzer GmbH, Hanau, Germany). After initial dehydration, stepwise immersion at 4 °C with Technovit 9100 medium using specific infiltration solutions (following the manufacturer's instructions) was conducted. Afterward, the tissue blocks were trimmed into a diamond shape using a grinding machine (EcoMet 30, Bühler, Esslingen, Germany) and sectioned at 7 µm using a specialized rotation microtome for hard tissue samples (CUT4060E, microTec GmbH, Walldorf, Germany). Finally, the slides were histochemically stained with hematoxylin and eosin (H&E) and Movat Pentachrome staining. The immunohistochemical staining of the transmembrane expression of CD11c (abx231412, Abcam Ltd., Milton, UK) and CD163 (ab182422, Abcam, Cambridge, UK) was used for the detection of M1- and M2-like macrophages, respectively. CD31 antibody (ab182981 Abcam, Cambridge, UK) was used for the detection of vascular endothelial cells. The process of immunohistochemical staining has been thoroughly described by Lindner et al. [32]. The antigen retrieval sections were (pre-)treated with TRIS-EDTA pH 9 for 20 min at 96 °C to recover antigen reactivity. To prevent non-specific binding, the sections were incubated with a blocking solution for 10 min. Primary antibodies (CD11c, CD163, and CD31) were incubated at room temperature for 60 min, 40 min, and overnight, respectively. Afterward, the biotinylated secondary antibody was applied to bind several molecules of the permanent streptavidin-alkaline phosphatase (AP)-red conjugate. Upon the addition of the chromogenic solution, the target

antigen was detected via a red-pink reaction product. Unless otherwise stated, all solutions and reagents for immunohistochemical analysis were purchased from Zytomed Systems (Berlin, Germany).

4.2.3. Histological Analyses

Histological analysis was performed using an Axioscope 5 microscope (Zeiss, Oberkochen, Germany). The analyses included the observations of the tissue-biomaterial interactions, including the cell reactions as well as tissue responses involving (bone) tissue integration and vascularization. Microphotographs were taken using a scanning microscope (M8, PreciPoint, Munich, Germany).

4.2.4. Histomorphometry

To enable histomorphometry, total scans were generated using a scanning microscope (M8, PreciPoint, Munich, Germany). The histomorphometry was achieved by means of image analyses via the software ImageJ v 1.53 (National Institutes of Health, Bethesda, MD, USA) [32]. To determine the different parameters within the specific areas of the implantation beds, the margins were manually integrated to separate the different compartments after determining the total implantation area (TIA) (Figure 7A,B):

- i Membrane compartment above bone graft-filled bone defects (MCBG);
- ii Membrane compartment above sham defects (MCSO);
- iii Bone graft-filled bone defects (BG);
- iv Sham bone defects (SO).

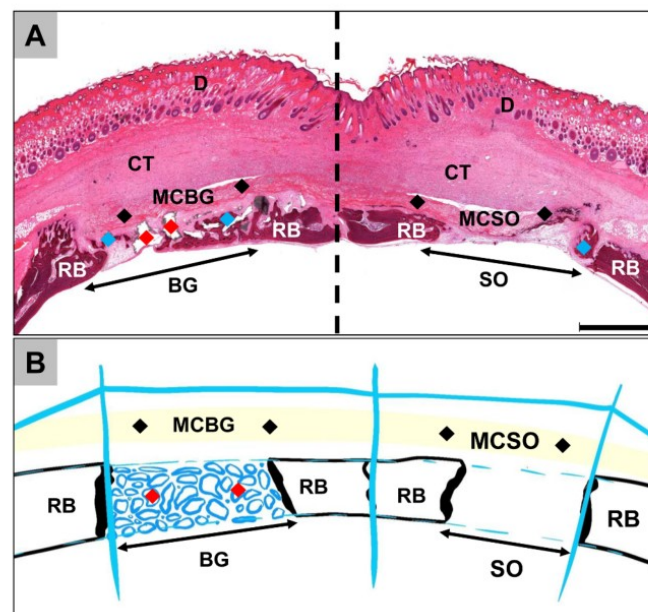


Figure 7. (A,B) Schematic overview of a calvaria, involving the four different compartments used for histomorphometrical measurement. BG and double-arrow = bone graft-filled bone defect; SO and double-arrow = sham-operated bone defect; MCBG = membrane compartment above bone graft-filled bone defect; MCSO = membrane compartment above sham defects; blue rhombus = newly formed bone tissue; red rhombus = bone substitute material; black rhombus = collagen membrane; RB = residual bone; CT = connective tissue; D = dermis. (A) HE staining, “total scan”, 100× magnification, scale bar = 5 mm.

The four compartments were then utilized to measure the respective areas of newly formed bone, remaining membrane, remaining BSM, and connective tissue. Percentages of those areas were calculated with respect to the areas of the four compartments. Moreover, the occurrence of the M1- and M2-macrophage subtypes, as well as the vascularization pattern, were analyzed using a specialized plugin on ImageJ that was developed by Lindner et al. [32,61]. This plugin allows us to measure the number of stained cells per mm² and the vascularization pattern, i.e., the vessel density (vessels/mm²) and vascularization percent (percentage of vessel areas related to the TIA). Briefly, the detection of stained areas was carried out via ImageJ, whereas vessel density was the measurement of the number of detected vessels over the assigned area. Furthermore, the percentage of vascularization was measured by measuring the area of detected vessel and dividing it over the assigned area.

4.3. Statistical Analyses

The quantitative data were initially confirmed and approved for normality via the D'Agostino–Pearson and Shapiro–Wilk normality tests by means of the GraphPad Prism 9.3.1 software (GraphPad Inc., La Jolla, CA, USA). Afterward, an ordinary one-way ANOVA test of data with equal variances and Brown–Forsythe combined with Welch's ANOVA test of data with unequal variances were conducted to compare the values and to determine statistical differences between the study groups or the different time points. All multiple comparison tests were combined with false discovery rate (FDR) correction. Statistical differences were stated as significant if *p*-values were less than 0.05 and highly significant if *p*-values were less than 0.01, less than 0.001, or less than 0.0001. Finally, the data were graphed as means and standard deviations. Intraindividual differences were symbolized with asterisks (*) (from one to four asterisks according to the *p*-value). Similarly, interindividual differences were indicated with either hashtags (#) for differences between 2- and 16-week datasets, pluses (+) for differences between 2- and 8-week datasets, or with dots (•) for differences between 8- and 16-week datasets.

Additionally, correlation analyses were performed based on the multiple variables measured in the present study. Correlation matrix analyses (two-tailed Pearson's correlation) were conducted to uncover whether a relationship existed between two parameters within the same compartment or between two compartments that lay above each other. Pearson's correlation coefficient (*r*) indicated whether the parameters were either positively, negatively, or weakly correlated. In this paper, only positive correlations were considered. In this case, the *p*-value was also taken under consideration as a measurement of how significant the correlation was, which is indicated in the heatmaps. The confidence interval for these analyses was 95%.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms24076833/s1>.

Author Contributions: Conceptualization, S.A. and M.B.; methodology, S.A. and M.B.; software, M.B.; validation, S.A. and M.B.; formal analysis, S.A., Y.R. and M.B.; investigation, S.A., Y.R., L.L., D.R., R.S., M.R., S.N., S.S., O.J. and M.B.; resources, L.L., D.R., R.S., S.N., S.S., O.J. and M.B.; data curation, S.A., Y.R. and M.B.; writing—original draft preparation, S.A., Y.R. and M.B.; writing—review and editing, S.A., Y.R., L.L., D.R., R.S., M.R., S.N., S.S., O.J. and M.B.; visualization, S.A., Y.R. and M.B.; supervision, L.L., D.R., R.S., S.N., S.S., O.J. and M.B.; project administration, L.L., D.R., R.S., S.N., S.S., O.J. and M.B.; funding acquisition, L.L., D.R., R.S., S.N., S.S., O.J. and M.B. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Federal Ministry of Education and Research (BMBF, Germany, FKZ: 13GW0400A). Additionally, the authors gratefully acknowledge the funding by the German Research Foundation (Deutsche Forschungsgemeinschaft, DFG) for the subproject 6 within the Research Unit 5250 “Permanent and bioresorbable implants with tailored functionality” (No. 449916462).

Institutional Review Board Statement: Experiments on animals were authorized by the Local Ethical Committee of the Faculty of Medicine, University of Niš, Niš, Serbia, and was based on the approval number 323-07-00073/2017-05/7 of the Veterinary Directorate of the Ministry of Agriculture, Forestry and Water Management of the Republic of Serbia (date of approval: 22 February 2017).

Informed Consent Statement: Not applicable.

Data Availability Statement: All data are included in the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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EDITED BY
Sangram Keshari Samal,
Regional Medical Research Center (ICMR), India

REVIEWED BY
Ahmed El-Fiqi,
National Research Centre, Egypt
Chaobo Huang,
Nanjing Forestry University, China

*CORRESPONDENCE
Mike Barbeck,
✉ mike.barbeck@med.uni-rostock.de

RECEIVED 05 October 2024
ACCEPTED 29 November 2024
PUBLISHED 06 January 2025

CITATION
Ren Y, Alkildani S, Burckhardt K, Köwitsch A,
Radenkovic M, Stojanovic S, Najman S, Jung O,
Liu L and Barbeck M (2025) The influence of
different crosslinking agents onto the physical
properties, integration behavior and immune
response of collagen-based
barrier membranes.
Front. Bioeng. Biotechnol. 12:1506433.
doi: 10.3389/fbioe.2024.1506433

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The influence of different crosslinking agents onto the physical properties, integration behavior and immune response of collagen-based barrier membranes

Yanru Ren¹, Said Alkildani², Kim Burckhardt²,
Alexander Köwitsch³, Milena Radenkovic⁴, Sanja Stojanovic^{5,6},
Stevo Najman^{5,6}, Ole Jung¹, Luo Liu⁷ and Mike Barbeck^{1,2*}

¹Clinic and Policlinic for Dermatology and Venereology, University Medical Center Rostock, Rostock, Germany, ²BerlinAnalytix GmbH, Berlin, Germany, ³Biotrics bioimplants AG, Berlin, Germany, ⁴Department for Cell and Tissue Engineering, Faculty of Medicine, University of Niš, Niš, Serbia, ⁵Department for Cell and Tissue Engineering, Scientific Research Center for Biomedicine, Faculty of Medicine, University of Niš, Niš, Serbia, ⁶Department of Biology and Human Genetics, Faculty of Medicine, University of Niš, Niš, Serbia, ⁷Beijing Advanced Innovation Center for Soft Matter Science and Engineering, College of Life Science and Technology, Beijing University of Chemical Technology, Beijing, China

This study investigates the mechanical properties as well as *in vitro* and *in vivo* cyto- and biocompatibility of collagen membranes cross-linked with glutaraldehyde (GA), proanthocyanidins (PC), hexamethylenediisocyanate (HMDI) and 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide/N-hydroxysuccinimide (EC/NHS). A non-crosslinked membrane was used as reference control (RF). The initial *in vitro* cytotoxic analyses revealed that the PC, EC, and HMDI crosslinked membranes were cytocompatible, while the GA crosslinked membrane was cytotoxic and thus selected as positive control in the further *in vivo* study. Cross-linking enhances the tensile strength and collagenase resistance, effectively prolonging the membrane's standing time *in vivo*. Using (immune-) histochemistry and histomorphometrical analyses, the cellular inflammatory responses, tissue integration and vascularization patterns at 10-, 30-, and 90-day post-implantation in a subcutaneous implantation model in rats were analyzed. The PC membrane elicited the mildest inflammatory cell levels, akin to the RF membrane, while other groups induced an M1-dominated macrophage response and numerous multinucleated giant cells throughout the study period. EC membranes maintained structural stability up to 30 days post-implantation, similar to the GA group, whereas others collapsed prematurely. Concurrent with membrane collapse, transmembrane vascularization occurred across all groups. Histopathological and histomorphometry results reveal the intricate interplay of inflammatory cell populations in vascularization. These findings offer valuable insights into the pivotal role of cross-linkers in modulating mechanical properties and tissue responses of collagen membranes.

KEYWORDS

guided bone regeneration (GBR), collagen membrane, barrier membrane, transmembraneous vascularization, macrophages, degradation, integration

1 Introduction

Over the past few years, barrier membranes have gained increasing prominence in the field of Guided Tissue Regeneration (GTR) and Guided Bone Regeneration (GBR) for the treatment of various defects such as horizontal and augmentation, ridge preservation and intraosseous defects. The first task for the application of a barrier membrane is the separation of the soft tissue and the bone defect area to prevent the migration of connective tissue into the intended regeneration site. Nowadays, more specific requirements for the "ideal barrier membrane" have been proposed to trigger clinical effectiveness including space maintenance, cellular occlusion, easy handling, and especially bioactive properties such as the transmembraneous vascularization or the induction of an (inflammatory) micromilieu that optimally supports bone tissue regeneration (IA et al., 2018; Omar et al., 2019; Sasaki et al., 2021).

As the most prominent structural protein of the extracellular matrix, collagen has earned its status as a very appropriate candidate for medical biomaterials for a broad variety of indications due to its exceptional biocompatibility, biodegradability, low immunogenicity, and cellular affinity (Chattopadhyay and Raines, 2014; Silvipriya et al., 2015; Pawelec et al., 2016). Collagen membranes have found widespread clinical use, expediting early wound stabilization and defect closure (Bunyaratavej and Wang, 2001; Allan et al., 2021). Additionally, their suitability for clinical procedures for GBR/GTR applications is underscored by the advantages of single-step application and low exposure rates (Gueldepfennig et al., 2020; Kumari et al., 2021).

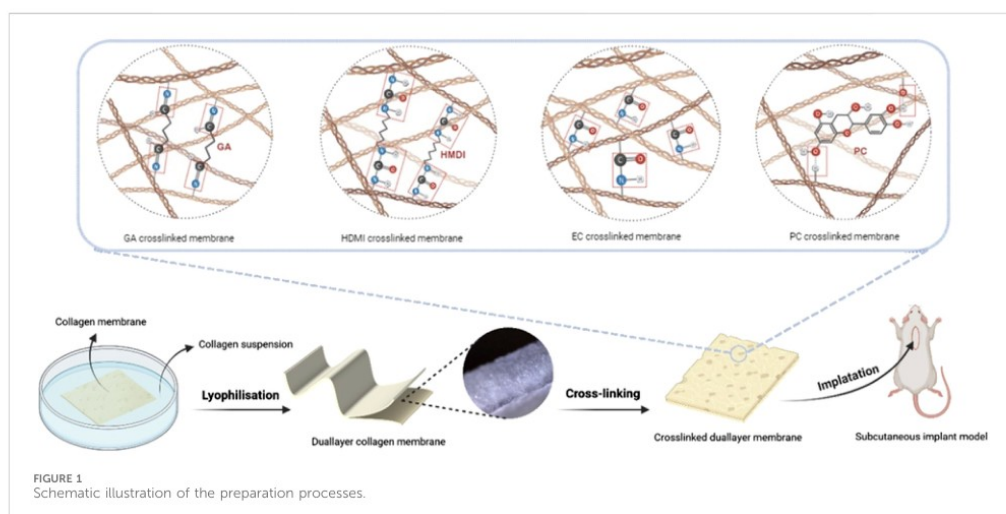
It is generally accepted that the barrier functionality should endure for 4–6 weeks in periodontal tissue regeneration and 16–24 weeks in case of bone augmentation procedures (Caballé-Serrano et al., 2019; Aprile et al., 2020; Sasaki et al., 2021). However, collagen-based materials especially derived from mammalian skin, which are most frequently used for production of barrier membranes, experience rapid degradation within a few days up to some weeks post-implantation (Sheikh et al., 2017). Multiple strategies have been introduced to increase the durability and mechanical strength of collagen membranes within the tissue, including alterations in membrane structure and collagen source (Schlegel et al., 1997; Wang et al., 2016; Noble et al., 2022). One of the most renowned commercial collagen membranes, Bio-Gide®, features a bilayer structure, with the dense layer remaining intact for up to 2 months *in vivo* (Schlegel et al., 1997; Radenković et al., 2021). Another clinically utilized membrane, Jason®, derived from porcine pericardium, boasts remarkable multidirectional tear resistance and sustained barrier function lasting 8–12 weeks (Fujioka-Kobayashi et al., 2017; Ratiu et al., 2019).

Among these strategies, cross-linking has gained significant attention for its effectiveness in enhancing the physicochemical and biological properties of collagen membranes by introducing inter- and intramolecular covalent or non-covalent bonds (Jiménez García et al., 2017; Adamiak and Sionkowska, 2020). While glutaraldehyde (GA) is a highly efficient traditional chemical crosslinker for collagen-based materials, concerns have been raised regarding its propensity to induce localized cytotoxicity and significant inflammation (Ruijgrok et al., 1994; Nashchekina et al., 2020; Shi et al., 2020). Hexamethyldiisocyanate (HMDI)

initially appeared as a promising substitute to GA, which also forms stable urea groups with the primary amine groups on collagen without toxic by-products (Olde Damink et al., 1995; Jarman-Smith et al., 2004; Sarrianiadis et al., 2021). Notably, the commercially available collagen repair patch from Zimmer employs HMDI crosslinking, demonstrating a prolonged degradation period without triggering an increase in fibrinogen levels as an indicative of inflammation (Nicholson et al., 2007). In pursuit of safer alternatives, 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide/N-hydroxysuccinimide (EC-NHS) gained prominence as a zero-length cross-linking agent (Yang, 2012; Zhang et al., 2022). Applications of EC-NHS crosslinked collagen are diverse, and it has been proven that EC-NHS crosslinked collagen membrane induced successful bone regeneration in the Beagle mandible model and the rabbit calvaria defect model (Ahn et al., 2020). Moreover, in a study by Yang et al., it was demonstrated that an increase of the EC concentration leads to a reduced swelling ratio and an enhancement in resistance to enzymatic degradation within collagen hydrogels (Yang, 2012). In addition to these advancements, proanthocyanidins (PC), a natural polyphenolic crosslinking agent, is generally considered to provide distinct advantages in terms of biocompatibility when compared to conventional chemical crosslinkers (Green et al., 2010; He et al., 2011). Moreover, PC exhibits a wide array of beneficial biological activities in the context of tissue regeneration including antibacterial, antioxidant, anti-inflammatory, and anti-tumor characteristics (Green et al., 2010; He et al., 2011; Rauf et al., 2019).

Although various cross-linking technologies for collagen implants and barrier membranes have been investigated, concerns have arisen due to crosslinker-induced foreign body reactions (Oryan et al., 2018; Adamiak and Sionkowska, 2020). The primary emphasis is consistently on enhancing the degradation pattern of a collagen membrane while minimizing foreign body reactions induced by crosslinkers. It is worth noting that the type and concentration of the crosslinker can trigger distinct immune responses and degradation patterns (Oryan et al., 2018; Sarrianiadis et al., 2021). In this context, the comparison of the characteristic and composition of the foreign body reactions induced by different cross-linking agents is a pivotal endeavor in the evaluation of suitable cross-linking technologies.

Thus, novel bilayer collagen membranes manufactured through lyophilization and crosslinking with the four different cross-linking agents including GA, EC/NHS, PC, and HMDI were analyzed in the present study. Initially, the mechanical properties of the differently crosslinked membranes were compared by testing tensile strength, denaturation temperature, swelling rate, and collagenase resistance. Furthermore, an *in vitro* cytotoxicity part followed by an *in vivo* study part including analyses of the tissue integration, angiogenesis and immune pattern using the by subcutaneous implantation model in rats up to 90 days were explored. Established and previously published methodologies especially focused on the histological and histomorphometrical analyses were used (Kapogianni et al., 2021; Radenković et al., 2021; Alkildani et al., 2023). By comparing *ex vivo*, *in vitro* and *in vivo* characteristics exhibited by the differently crosslinked collagen membranes, this study aimed to provide valuable insights for the further development of clinically optimal collagen membranes.



2 Methods and materials

2.1 Preparation of dual-layer collagen membranes

A native collagen membrane sourced from porcine dermis (Collprotect, botiss biomaterials GmbH, Zossen, Germany) served as the base layer (BL) and was initially cut into 5×5 cm dimensions for the subsequent steps. Additionally, porcine skin was crushed and subsequently homogenized in trisodium phosphate buffer utilizing an IKA Ultra-Turrax T-25 Digital Homogenizer. The collagen extracted from this process was then re-diluted with water to create a 0.75% collagen suspension at a pH of 7. The collagen suspension was poured into a mold, and the base layer was placed on top of the suspension. The final dual-layer membrane, consisting of a fleece layer made from the collagen suspension and a base layer, was then formed through freeze-drying (Figure 1).

2.2 Crosslinking processes

For the present study, four different cross-linking agents, i.e., proanthocyanidin (PC), glutaraldehyde (GA), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EC) and hexamethylene diisocyanate (HMDI) in two different concentrations (H1 and H2) were used for the preparation of new collagen membrane prototypes (Table 1). A dual-layer membrane without further cross-linking was used as reference membrane (RF) or negative control group (Table 1).

2.2.1 EC/NHS crosslinking

To produce the EC/NHS crosslinked membrane, the fleece layer of the bilayer membrane obtained in the previous step was initially soaked in 95% isopropanol (Geyer Chemsolute, Renningen, Germany) for 10 min. EC (Carl Roth, Karlsruhe, Germany) and

NHS (Carl Roth, Karlsruhe, Germany) were separately weighed in a molar ratio of 2:1. Each compound was dissolved in 40 mL of 95% isopropanol and then introduced into the vessel containing the bilayer membranes. The reaction proceeded for 24 h. Following this, the EC crosslinked membrane underwent two washing steps with 95% isopropanol for 2 min each using an ultrasonic bath. It was subsequently washed once with 95% isopropanol for 10 min and once with 100% isopropanol for 5 min under stirring. The final membrane was obtained after air drying with lint-free cloths (KIMTECH SCIENCE* Precision Wipes, Kimberly-Clark, Roswell, GA, United States) in a fume hood.

2.2.2 PC crosslinking

To create the PC crosslinked membrane, PC (Grape seed extract pure, PureBulk, Inc., Roseburg, U.S.) was accurately weighed and introduced into 300 mL of 90% isopropanol. Subsequently, the fleece layer of the membrane was immersed in the PC solution for a duration of 24 h. Following this soaking period, the membrane underwent two successive washes with 90% isopropanol for 10 min each, followed by a final wash with 100% isopropanol under continuous stirring. The ultimate membrane product was obtained after air drying using lint-free cloths (KIMTECH SCIENCE* Precision Wipes, Kimberly-Clark, Roswell, GA, United States) within a fume hood.

2.2.3 HMDI crosslinking

To prepare the HMDI crosslinked membrane layer, a 40 mg/mL HMDI (Sigma-Aldrich, St. Louis, MO, U.S.) solution in 95% isopropanol was initially prepared. Two HMDI crosslinked membranes, designated as H1 (50 wt%) and H2 (100 wt%), were then subjected to crosslinking in HMDI solutions with varying concentrations. To prevent the precipitating urea compound that inhibit the crosslinking reaction from coating on the collagen, the collagen membranes were immersed separately in 20.2 mL (for H1 membrane) and 36.1 mL (for H2 membrane) of 40 mg/mL

TABLE 1 Cross-linking details of the collagen membranes.

Group	Cross-link agent	Amount of collagen	Amount of crosslinker	Concentration
RF	-	-	-	-
PC	Proanthocyanidin	1,496 mg	1,496 mg	100 wt%/g collagen
EC	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide	1,633 mg	EC 1878.28 g NHS 451 g	6 mmol/g collagen
H1	Hexamethylene diisocyanat	1,616 mg	808 mg	50 wt%/g collagen
H2	Hexamthylendiisocyanat	1,443 mg	1,443 mg	100 wt%/g collagen
GA	Glutaraldehyde	1,617 mg	2021 mg	125 wt%/g collagen

HMDI solution for 5 min. Following this, the reaction solution was diluted to a total volume of 150 mL, and the reaction was initiated by adding NaOH solution (with a molar ratio of NaOH to HMDI as 1: 25) to the fleece layer. The reaction was allowed to proceed for 24 h. Afterward, the HMDI crosslinked membranes underwent two washes with 100% isopropanol for 2 min each using an ultrasonic bath. They were then washed once with 100% isopropanol for 20 min under continuous stirring. The final membrane product was obtained after air drying using lint-free cloths (KIMTECH SCIENCE* Precision Wipes, Kimberly-Clark, Roswell, GA, United States) within a fume hood.

2.2.4 GA crosslinking

To create the GA crosslinked membrane, GA (Thermo Fisher Scientific Inc., Waltham, Massachusetts, U.S.) was accurately weighed and dissolved in 140 mL of 95% isopropanol. This solution was then added to the fleece layer of the membrane for a 1-hour crosslinking process. Subsequently, the membrane was washed twice with 95% isopropanol for 2 min each, using an ultrasonic bath, and once with 100% isopropanol for 10 min under stirring. The final membrane was obtained after air drying using lint-free cloths (KIMTECH SCIENCE* Precision Wipes, Kimberly-Clark, Roswell, GA, United States) within a fume hood.

2.3 Ex vivo tests

2.3.1 Collagenase assay

The stock solution of collagenase (Sigma Aldrich C9891) was diluted in TESCA buffer to achieve a concentration of 1 mg/mL (PH 7.4). The needed volume per sample tube is calculated under consideration of the enzyme activity given as collagen digestion unit (CDU). The collagen samples, with and without collagenase, were accurately weighed using a Kern ABT 120-5DNM balance and placed into individual Eppendorf tubes. These samples were then hydrated in TESCA buffer (excluding collagenase) for a duration of 30 min. The calculated volume of the collagenase solution was thoroughly mixed into each sample tube (1 CDU/mg collagen), and subsequently, the tubes were placed in a preheated incubator (Thermocycler, Thermomixer C, Eppendorf) at 37°C. At different timepoints, the digestion reaction was halted by centrifuging the sample tubes for 10 min at 4°C, utilizing an Eppendorf 5424 R centrifuge. Following centrifugation, the supernatant was meticulously decanted, and the samples were washed with

deionized water, with each cycle involving centrifugation and removal of the supernatant. This washing process was repeated twice. Subsequently, the samples were left to air dry within a desiccator for 48 h before their residual collagen mass was weighed.

2.3.2 Differential scanning calorimetry (DSC)

DSC measurements were conducted using a DSC 214 Polyma instrument (NETZSCH-Gerätebau GmbH, Germany). Initially, the samples were hydrated in deionized water and then transferred into Concavus Pans (Netzsch, NGB817525). Subsequently, they were weighed using an analytical balance (ABT 120-5DNM, Kern and Sohn GmbH) and sealed with Concavus Lids (Netzsch, NGB817526). The heating rate applied during the measurements was set at 10 K/min. Before performing the sample measurements, a correction and reference measurement were carried out. The obtained results were evaluated using the *Proteus* software (Netzsch).

2.3.3 Tensile test

The tensile strength of the specimens was assessed using a tensile testing machine (RetrolineZ2.5, ZwickRoell GmbH and Co. KG). To prepare the samples, they were first cut to the required dimensions and then hydrated in deionized water for a duration of 2 min. Subsequently, the thickness of each sample was measured at three distinct points in the central region using a thickness measuring device (C110T, Kroeplin GmbH). The minimum recorded thickness value among the three measurements was utilized for calculating the tensile strength. The specimens were securely clamped, and the measurements were carried out with a 50 N load cell. The evaluation of the measurements was performed using the testXpert® II software (ZwickRoell).

2.3.4 Swelling ratio

The swelling ratio of a collagen membrane can be calculated by measuring the weight of the membrane before and after it absorbs liquid. After soaking for different times, the wet weight of membrane was recorded and then the swelling ratio was calculated according to the following equation:

$$\text{Swelling ratio} = \frac{W_{\text{wet}} - W_{\text{dry}}}{W_{\text{dry}}} \times 100\%$$

where:

W_{wet} is the weight of the membrane after swelling (wet weight),

W_{dry} is the weight of the membrane in its dry state.

2.4 In vitro experiments

2.4.1 Cell culture and extraction

Cytocompatibility assessments were carried out in accordance with ISO 10993-5/12. Briefly, L-929 mouse fibroblast cells, sourced from the European Collection of Cell Culture (ECACC) in Salisbury, UK, were cultured in standard cell culture conditions with appropriate cell culture medium. When the cell culture reached approximately 80% confluency, cells were passaged.

All test samples were extracted after a 72-hour cultural period under standard cell culture conditions, maintaining a surface-to-volume ratio of 3 cm²/mL in cell culture medium. As a control, an extraction was performed using cell culture medium alone. Afterwards, the extract medium underwent centrifugation at 14,000 rpm for 10 min. The supernatants were used for further L929 cells culture that described below.

Subsequently, 96-well plates were seeded with 1×10^4 cells per well in 100 μ L of cell culture medium and cultured for 24 h under standard cell culture conditions. Following this incubation period, the cell culture medium was aspirated, and 100 μ L of the extract solutions were added to each well. After an additional 24-hour incubation, the cells were subjected to analysis using BrdU- and XTT-assays. Simultaneously, the supernatants underwent LDH assay. Besides, parallel assays were performed for all extracts, excluding cells, to serve as a control for potential assay interference. RM-A, a polyurethane film containing 0.1% zinc diethyldithiocarbamate (ZDEC), obtained from the Hatano Research Institute, Food and Drug Safety Center in Japan, was utilized as the positive control. Absorbance values obtained from blank controls (comprising medium without cells) were subtracted from the results of all assays.

2.4.2 Sodium 3,3'-(1(phenylamino)carbonyl)-3,4-tetrazolium]-3-is (4-methoxy-6-nitro) Benzene Sulfonic acid Hydrate (XTT) assay

The Cell Proliferation Kit II from Roche Diagnostics in Mannheim, Germany, was employed following the manufacturer's guidelines. In brief, the electron-coupling reagent was mixed with the XTT labeling reagent at a 1:50 dilution, and 50 μ L of this mixture was added to the cells. Following a 4-hour incubation period under cell culture conditions, the conversion of the substrate was assessed by measuring the absorbance of 100 μ L aliquots in a new 96-well plate using a scanning multi-well spectrophotometer (ELISA reader) equipped with filters for 450 nm and 650 nm (reference wavelength).

2.4.3 Bromodeoxyuridine (BrdU) assay

The BrdU (colorimetric) test kit from Roche Diagnostics in Mannheim, Germany, was employed following the manufacturer's guidelines. Briefly, cells were incubated with BrdU for 2 h under cell culture conditions, followed by fixation with FixDenat reagent for 30 min at room temperature. Subsequently, cells were incubated with an anti-BrdU peroxidase (POD) antibody for 1 h and then subjected to three 5-minute rinses with washing buffer. The addition of Tetramethyl-benzidine (TMB) to the substrate initiated a

reaction, which was halted after 20 min at room temperature by adding 25 μ L of 1 M H₂SO₄. Finally, the resulting immune complexes were quantified using a scanning multi-well spectrophotometer (ELISA reader) at wavelengths of 450 nm and 690 nm (reference wavelength).

2.4.4 Lactate dehydrogenase (LDH) assay

The LDH Cytotoxicity Assay Kit II from BioVision in Milpitas, CA, United States, was employed following the manufacturer's instructions. Specifically, 10 μ L of cell supernatants were mixed with 100 μ L of LDH reaction mix and incubated for 30 min at room temperature. Subsequently, stop solution was added, and the absorbance was measured using a scanning multi-well spectrophotometer (ELISA reader) at wavelengths of 450 nm and 650 nm (reference wavelength).

2.5 In vivo experiments

2.5.1 Study design

The *in vivo* experiments were conducted in collaboration with the Faculty of Medicine at the University of Niš, Serbia. The animal study was approved by the Local Ethical Committee of the Faculty of Medicine, University of Niš and by the Veterinary Directorate of the Ministry of Agriculture, Forestry and Water Management of the Republic of Serbia (decision number 323-07-09101/2020-05/5; date of approval: 26 August 2020). A total of 90 male Wistar rats, aged 3–4 months, from the Vivarium of the Faculty of Medicine (University of Niš, Serbia) were divided into six study groups. Each group comprised 15 experimental animals, with five animals designated for each of the three time points ($n = 5$) at 10, 30, and 90 days in each group. These experimental animals were accommodated under standard conditions, which included access to water *ad libitum*, exposure to artificial lighting, and provision of regular rat pellets. Additionally, standard pre- and postoperative care procedures were diligently administered.

2.5.2 Subcutaneous implantation and explantation procedure

The implantation procedure closely adhered to the protocol outlined by Barbeck and colleagues (Barbeck et al., 2014; Barbeck et al., 2016; Barbeck et al., 2020; Barbeck et al., 2022; Flaig et al., 2020). Briefly, the animals underwent anesthesia through intraperitoneal injection, which consisted of ketamine [100 mg/kg of body weight] and xylazine [5 mg/kg of body weight]. Following the administration of anesthesia and subsequent preparation, including shaving and disinfection, an incision was made extending down to the subcutaneous tissue within the rostral subscapular region. Subsequently, a subcutaneous pocket was gently created using scissors, and the biomaterials were placed within this pocket. Following the implantation, the incisions were sutured.

Afterwards, following the euthanasia of the animals using an overdose of a ketamine and xylazine mixture, the implanted membranes were removed at the different timepoints, directly fixed in 4% formalin for a duration of 24 h, and then sectioned into three equal segments. For tissue processing a series of dehydration steps using increasingly concentrated alcohol

solutions and xylol were applied. Then, the samples were embedded in paraffin, and histological sections with a thickness ranging from 3 to 5 μm were prepared using a rotary microtome (Leica, Wetzlar, Germany).

2.5.3 (Immuno-) histochemical staining

From each tissue block, four sections were sliced and subsequently utilized for (immuno-) histochemical staining procedures, which included hematoxylin and eosin (H&E) staining, CD163 and CD11c staining, as well as CD31 staining. The CD163 marker is specific to the anti-inflammatory M2 phenotype of macrophages, while the CD11c marker is specific to the pro-inflammatory M1 phenotype of macrophages. Moreover, CD31 serves as a common marker for identifying blood vessels, specifically marking endothelial cells. The execution of all staining protocols strictly adhered to previously published procedures (Flaig et al., 2020; Barbeck et al., 2022).

2.5.4 Histo (patho)logical analyses

In this study, all histopathological and histomorphometrical analyses described in the following sections were conducted on the fleece layer of the dual-layer collagen membranes.

Initially, specimens of all membranes were histologically analyzed *ex vivo* to obtain more information about the material structure and further relations to the *in vivo* structure based by the cross-linking technologies as previously described (Barbeck et al., 2015; Kapogianni et al., 2021). To assess *in vivo* parameters such as cellular involvement in the integration and degradation processes of the tested membranes, as well as inflammatory tissue reactions, histopathological analyses were conducted using an Axio. Scope. A1 light microscope (Zeiss, Oberkochen, Germany). Histological images were captured with a connected AxioCam 305 color camera and processed using the ZEN Core software (Zeiss, Oberkochen, Germany).

2.5.5 Histomorphometrical analysis

Utilizing ImageJ (Version 1.52t, U. S. National Institutes of Health, Bethesda, MD, United States of America) a histomorphometrical analysis was conducted on the immunohistochemically stained slides, which had initially been digitized using the PreciPoint M8 microscope (Precipoint GmbH, Munich, Germany). The objective was to derive data pertaining to macrophage subtypes and vascularization.

To quantify the presence of M1 and M2 macrophages, the adjacent soft tissue within the defect area was manually delineated. Subsequently, a specialized plugin, as described by Lindner et al., was employed to automatically calculate the area occupied by stained cells within the delineated total area (Lindner et al., 2020). This procedure yielded the area of positive cells per square millimeter (in %). In addition, multinucleated giant cells (MNGCs) were counted manually.

To assess vascularization, a dedicated plugin also developed by Lindner et al. was utilized to independently quantify the number and area of blood vessels within both the delineated membrane area and the peri-membrane area as previously described (Barbeck et al., 2015; Lindner et al., 2020). Subsequently, calculations were performed to obtain the vessel density (number of vessels per mm^2) and vessel area fraction (%).

2.6 Statistical analyses

The data were presented as mean values along with standard deviations, utilizing GraphPad Prism software (Version 9.0.0, GraphPad Software Inc., La Jolla, United States of America). Afterwards, the statistical analysis of the data was conducted using two-way analysis of variance (ANOVA). Subsequently, a Tukey *post hoc* assessment was performed for group comparisons with the assistance of the GraphPad Prism software. Inter- and intraindividual significances were acknowledged when p -values were less than 0.05 ($*/p < 0.05$), denoting a significant difference. High significance was attributed to cases where the p -values were less than 0.01 ($*/**/p < 0.01$), 0.001 ($*/***/p < 0.001$) or less than 0.0001 ($*/***/****/p < 0.0001$).

3 Results

3.1 Morphology of the prepared bilayer collagen membranes

The native collagen membrane with natural pores served as base layer (BL) during preparation of the bilayer collagen membrane. The base layer exhibits a dense and uniform structure with a thickness of approximately 0.4 mm (Figure 2A). Once a fleece layer was produced on top of the base layer, the thickness of the bilayer membrane increased significantly (Figure 2B). No visible pores were observed on the surface of the fleece layer, while its overall structure appears much fluffier compared to the base layer (Figures 2C, D). Thereby, no gap between the two layers was observable, indicating the formation of an irreversible interlayer connection (Figures 2C, D). In summary, a homogeneous and tightly connected bilayer collagen membrane was successfully created through the lyophilization process.

The histological examination of the membranes showed that all membranes exhibited a dual-layer structure, comprising the base layer (BL), characterized by thick collagen fibers, and the upper fleece layer (RF, GA, EC, PC, H1, and H2), prepared from a collagen suspension, featuring thinner collagen fibers (Figure 3). In case of all materials, both layers displayed a honeycomb-like pore structure (Figure 3). Interestingly, the microstructure of the cross-linked membranes (Figures 3B–F) appeared to undergo minimal alteration compared to reference membrane (Figure 3A). Neither tissue nor cells were observed within these membranes.

3.2 Cytocompatibility analyses

Three different *in vitro* assays were combined used to assess the cytotoxicity of the non-crosslinked reference and crosslinked membranes. RM-A film was used as positive control *in vitro* analysis. According to the ISO 10993–5 protocol, the sample with a L929 cell viability/proliferation above 70% of the medium control in XTT/BrdU assays, and a cytotoxicity below 30% of the medium control in LDH assays, were considered cytocompatible (Jung et al., 2019). Except for the GA group, the values in all membrane groups demonstrated good cytocompatibility in all three assays (Figure 4). Thus, all membrane types allowed for viability and proliferation

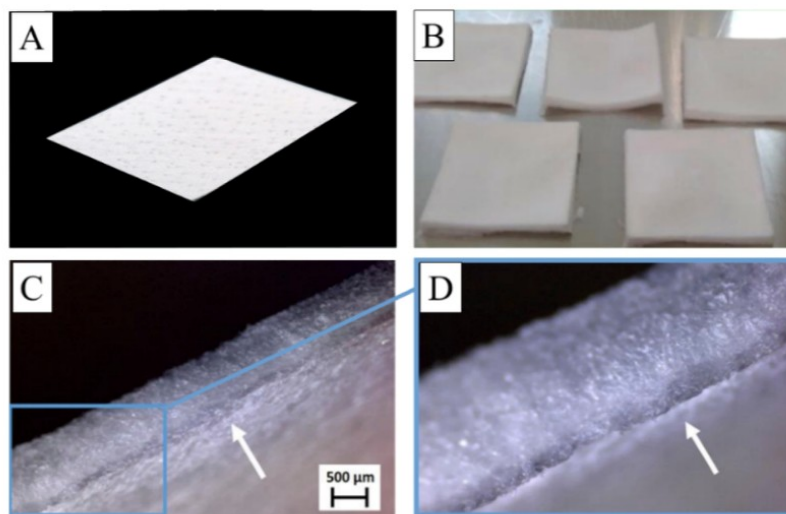


FIGURE 2
Macroscopic and microscopic morphology of the collagen membranes. (A) Native collagen membrane derived from porcine skin used as base layer; (B) prepared bilayer collagen membranes; (C, D) microscopic morphology of a reference membrane showing the non-cross-linked fleece layer on top of the native collagen membrane (white arrows).

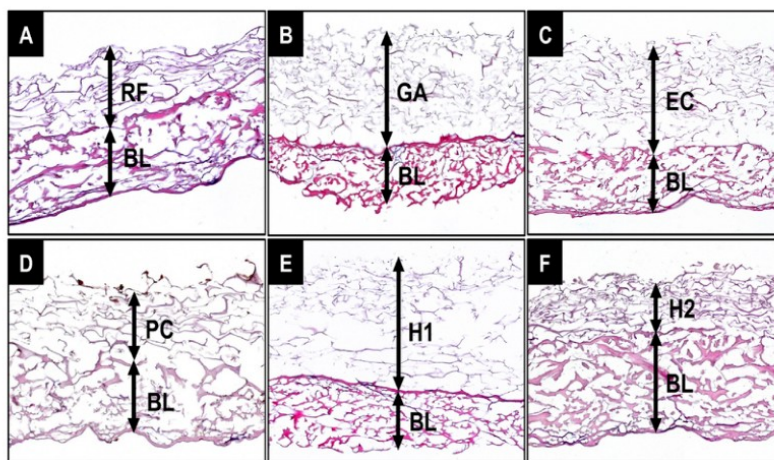
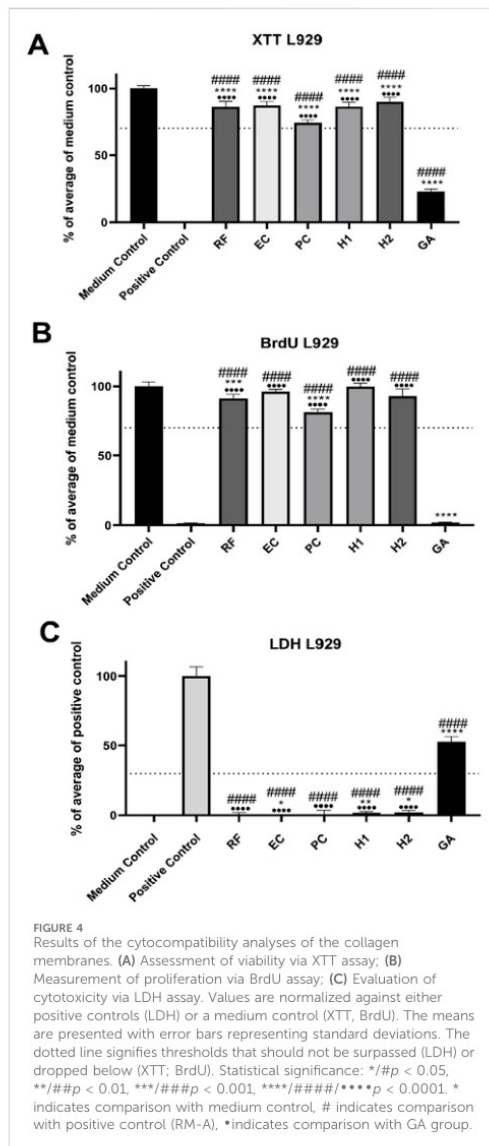


FIGURE 3
Histological visualization of the bi-layered barrier membranes consisting of the base layer (BL) and the fleece layers with and without cross-linking. (A) Reference membrane with the native non-crosslinked collagen fleece layer (RF); (B) Glutaraldehyde cross-linked fleece layer (GA); (C) EC/NHS cross-linked fleece layer (EC); (D) Proanthocyanidin cross-linked fleece layer (PC); (E, F) Hexamthylendiisocyanat cross-linked fleece layers (H1 and H2) (HE-stainings, excerpts of total scans, 100x magnifications).



above 70% (Figures 4A, B) combined with a cytotoxicity below 30% (Figure 4C).

However, the viability values in all membrane groups were significantly lower compared to the medium control (**** $p < 0.0001$) but significantly higher compared to the values in the positive control group (#### $p < 0.0001$). Finally, the values in all membrane groups were significantly higher compared to the values in the group of the GA membrane (*** $p < 0.0001$) (Figure 4A).

The proliferation measurements showed that only the values in the groups of the reference membrane and the PC membrane differed significantly from that in the medium control group (*** $p < 0.001$ and **** $p < 0.0001$), while the values in the groups of the EC membrane and both HMDI membranes were comparable (Figure 4B). Furthermore, the values in the GA group were significantly lower compared to that in the medium control group (**** $p < 0.0001$). Moreover, the values in the afore mentioned membrane groups were significantly higher compared to the values in the positive control group (#### $p < 0.0001$) and the group of the GA membrane (*** $p < 0.0001$) (Figure 4B). No significances between the values in the positive control group and the GA group were measured.

The cytotoxicity measurement indicated that all membrane types induced significantly lower values compared to the values in the positive control group (#### $p < 0.0001$) and the group of the GA membrane (*** $p < 0.0001$) (Figure 4C). Thereby, the values in the RF and PC membrane groups were comparable to that in the medium control group, while the values in the groups of the EC membrane (* $p < 0.05$) and both HMDI membranes were significantly higher (* $p < 0.05$ and ** $p < 0.01$) (Figure 4C).

3.3 Performance tests

To evaluate the mechanical properties of the crosslinked membranes, a series of tests including tensile testing, elongation at break, Differential Scanning Calorimetry (DSC), and swelling ratio were conducted.

The tensile strength tests showed that all crosslinked membranes exhibited increased mean tensile strength, but only PC membrane displayed a significant difference compared to the RF group (* $p < 0.05$) (Figure 5A). Additionally, the PC membrane also demonstrated higher elongation at break values compared to the EC membrane group (* $p < 0.05$) (Figure 5B).

The DSC analysis measuring the denaturation temperature of the tested membranes showed that all membranes exhibited an increase in denaturation temperature following crosslinking (Figure 5C). Remarkably, the denaturation temperature of the GA membrane showed the most significant improvement with a denaturation temperature significantly higher than that of all the other membranes (*** $p < 0.001$, **** $p < 0.0001$). Furthermore, the EC membrane also displayed a significantly enhanced denaturation temperature compared to the RF membrane (**** $p < 0.0001$).

The results of the swelling ratio test in Simulated Body Fluid (SBF) buffer showed that all membranes initially underwent rapid swelling within the first 5 min (Figure 5D). Afterwards, the swelling rate slowed down in all groups reaching a steady state at 20 min. In comparison to the RF membrane, the EC and GA membranes exhibited a faster swelling rate and higher swelling ratio (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). The PC membrane displayed the lowest swelling rate in the initial stage (* $p < 0.05$), but it ultimately exhibited a higher swelling ratio than the RF membrane in the later stage (** $p < 0.01$). The H1 membrane exhibited a swelling pattern most similar to the RF membrane, whereas the H2 membrane, featuring a higher crosslinker concentration, displayed the lowest swelling rate and swelling ratio (* $p < 0.05$).

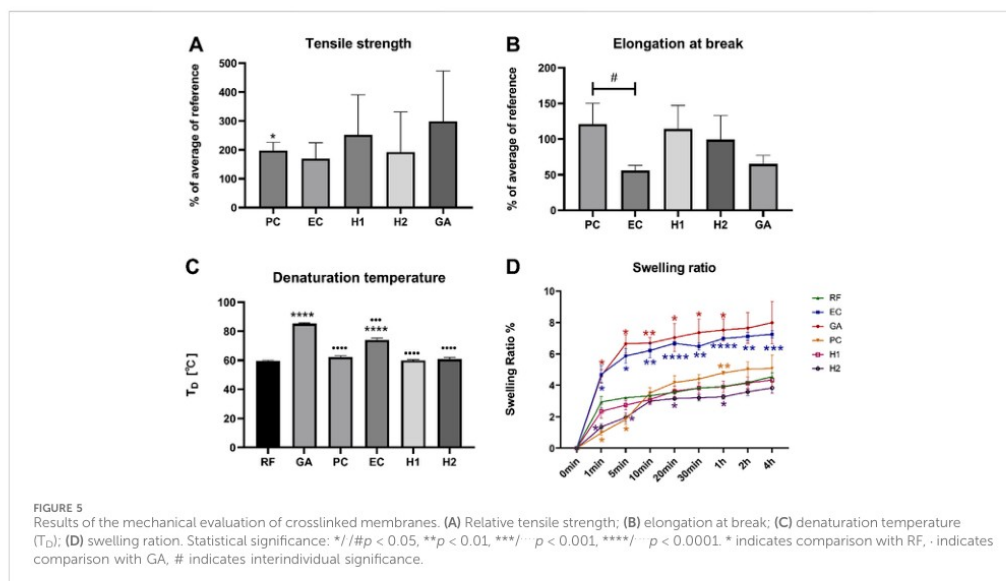


FIGURE 5 Results of the mechanical evaluation of crosslinked membranes. (A) Relative tensile strength; (B) elongation at break; (C) denaturation temperature (T_D); (D) swelling ratio. Statistical significance: */# $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. * indicates comparison with RF, # indicates comparison with GA, # indicates interindividual significance.

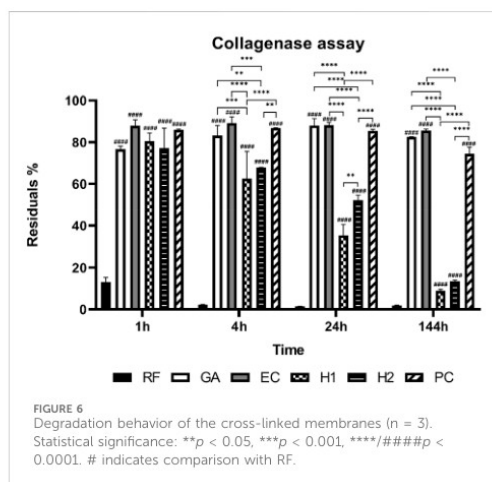


FIGURE 6 Degradation behavior of the cross-linked membranes (n = 3). Statistical significance: ** $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$. # indicates comparison with RF.

3.4 Collagenase assay

In the collagenase assay, all membranes underwent digestion in the presence of 1 CDU/mg (Collagen Digesting Units per milligram) collagenase at 37°C within a duration of 6 days (Figure 6). Evidently, the collagenase resistance of all crosslinked membranes exhibited a significant improvement.

The RF membrane experienced a mass loss exceeding 80% within the first hour of digestion, nearly undergoing complete degradation after 4 h. Notably, the HMDI crosslinking, while

enhancing the collagenase resistance of the membrane, yielded the least improvement among the crosslinked membranes. Both the H1 and H2 membranes demonstrated notably higher mass losses than the other crosslinked membranes starting from the fourth hour of digestion (** $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$). After 6 days of collagenase digestion, these two groups retained less than 20% of their initial mass. It is worth mentioning that collagenase stability exhibited a slight increase with the elevation of crosslinker concentration from H1 (50 wt% HMDI) to H2 (100 wt% HMDI) (** $p < 0.05$, after 24 h of digestion).

Conversely, the remaining three crosslinked membrane types, i.e., the GA, EC, and PC membranes, showcased higher collagenase stability throughout the study, retaining over 80% of their initial mass up to 6 days of digestion, with no significant differences observed among them.

3.5 Histopathological results

3.5.1 Comprehensive histological outcomes

The histopathological evaluation revealed that all cross-linked membranes remained detectable within the implant bed at 10 days post-implantation, eliciting an inflammatory tissue reaction (Figure 7). At this early study time point the RF membrane was already nearly completely degraded and thus observable in form of a thin layer on top of the base layer (Figure 7A). As expected, the RF membrane elicited a mild inflammatory tissue response involving mainly macrophages beside lower numbers of eosinophils and lymphocytes accompanied by a marginal neovascularization (Figure 7A).

In contrast, all of the crosslinked membranes induced a more pronounced inflammatory tissue response including moderate

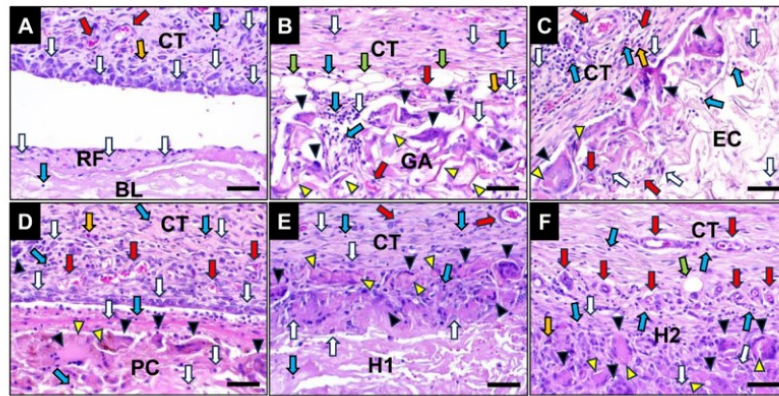


FIGURE 7
Exemplary histological images of the collagen membranes at 10 days post-implantation within the subcutaneous connective tissue (CT). (A) RF group, (B) GA group, (C) EC group, (D) PC group, (E) H1 group and (F) H2 group. White arrows: macrophages, blue arrows: lymphocytes, green arrows: fat tissue, yellow arrow: granulocytes, red arrows: vessels, yellow arrowheads: residual materials, black arrowheads: MNGCs, BL: base layer. (HE-stainings, 400x magnifications, scale bars: 20 μ m).

numbers of multinucleated giant cells (MNGCs) beside the aforementioned mononuclear cell types (Figure 7).

At 30 days post-implantation, the fleece layer of RF group was fully degraded and was thus unobservable (Figure 7A). In comparison to previous study time point, a very mild inflammation with a reduced number of inflammatory cells surrounding the base layer was detected in the RF group (Figure 7A).

Furthermore, a comparable inflammatory tissue response mainly involving MNGCs, macrophages and lymphocytes to that observed at day 10 post-implantation was detected in all cross-linked groups at day 30 post-implantation, with increased infiltration of reactive tissue into the membrane area (Figure 8). The presence of numerous membrane fragments surrounded by MNGCs and macrophages at the material-tissue interfaces especially in the GA, EC and H2 groups indicated that a fast phagocyte-driven material degradation was ongoing at this time point.

At 90 days post-implantation, no histological signs of material-induced inflammatory tissue response in the RF group were detected as no signs of this layer were detectable (Figure 9A). However, material-induced inflammatory responses including mainly MNGCs, macrophages and lymphocytes were still clearly observed in all crosslinked groups and these membranes were nearly completely fragmented (Figures 9B–F). The histopathological evaluation showed that especially in the groups of H1 and H2 membranes, the membrane region was completely penetrated by MNGCs (Figures 9E, F). The connective tissue that was observable within the interspaces of the material fragments contained high numbers of small and medium-sized vessels. Furthermore, a slight fibrosis was observed surrounding the implanted materials in the PC, H1 and H2 membrane groups, while a higher extent of fibrosis surrounding all material fibers was found in the EC membrane group.

3.5.2 Immune response

The histological analysis of the occurrence of macrophages subtypes at 10 days post-implantation revealed that especially the membranes in the GA group, the EC group and the H1 group induced a more pronounced pro-inflammatory response (Figures 10C, E, I). In contrast a balanced ratio between M1 and M2 macrophages was already detected in the RF group, the PC group and the H2 group at this early stage (Figure 10). Furthermore, comparable low numbers of M2-macrophages were found in all study groups at this study time point. In all groups the MNGCs only showed signs of a CD11c-expression (Figure 10). Additionally, a spatial distribution pattern was observable in all study groups at this early study point as the pro-inflammatory phagocytes (M1-macrophages and MNGCs) were located in direct vicinity of the membranes, i.e., at the material-tissue surfaces or within the superficial membrane areas, while M2-macrophages were predominantly located in the periphery of the implantation beds of the membranes (Figure 10).

At 30 days post-implantation, the highest numbers of M1-macrophages were detectable at the material-tissue-interfaces in the groups of the GA, EC, H1 and H2 membranes, which very visibly higher compared to the respective M2-macrophage numbers (Figure 11). In the groups of the RF and PC membranes lower M1-macrophage numbers were found, which were comparable to the respective anti-inflammatory subtype numbers. Only in the group of the RF membranes the numbers of anti-inflammatory macrophages seemed to be higher compared to the occurrence of M1-macrophages. However, comparable numbers of M2-macrophage were noticeable in all study groups at this intermediate study time point. In all groups of the crosslinked membranes CD11c-positive MNGCs were found within the implantation beds, while no MNGCs were detected in the RF group at this time point (Figure 11). Also, the afore-mentioned spatial distribution pattern

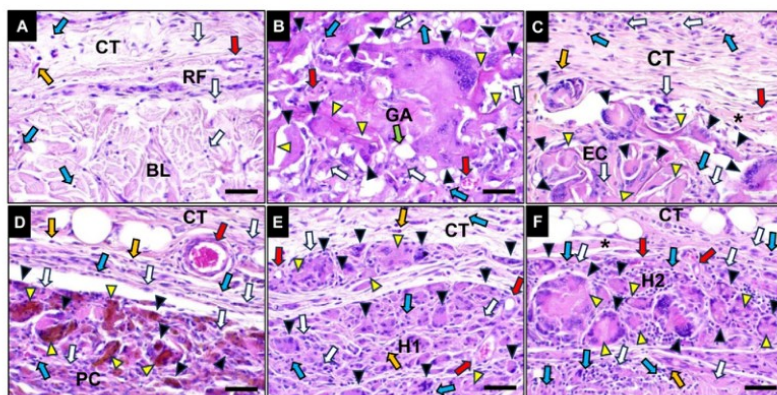


FIGURE 8
Exemplary histological images of the crosslinked collagen membranes at 30 days post-implantation within the subcutaneous connective tissue (CT). (A) RF group, (B) GA group (C) EC group, (D) PC group, (E) H1 group and (F) H2 group. White arrows: macrophages, blue arrows: lymphocytes, green arrows: fat tissue, yellow arrow: granulocytes, red arrows: vessels, yellow arrowheads: residual materials, black arrowheads: MNGCs, BL: base layer. (HE-stainings, 400x magnifications, scale bars: 20 μ m).

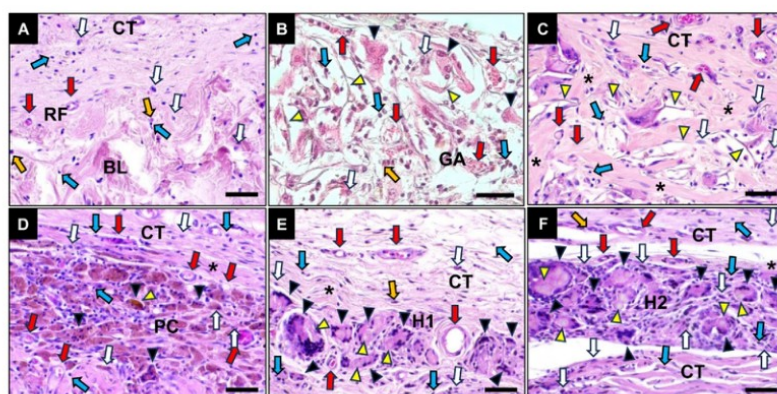
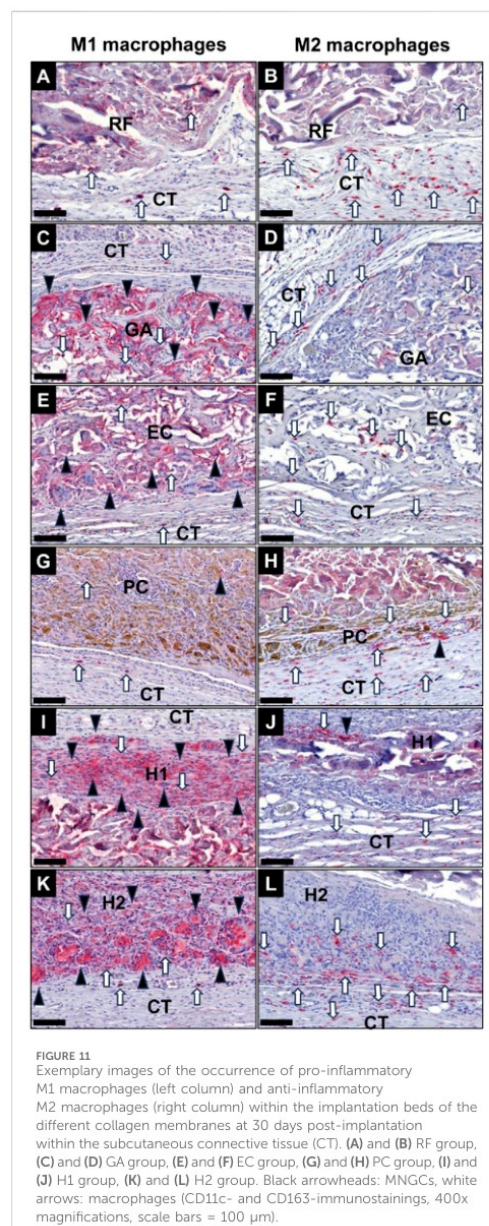
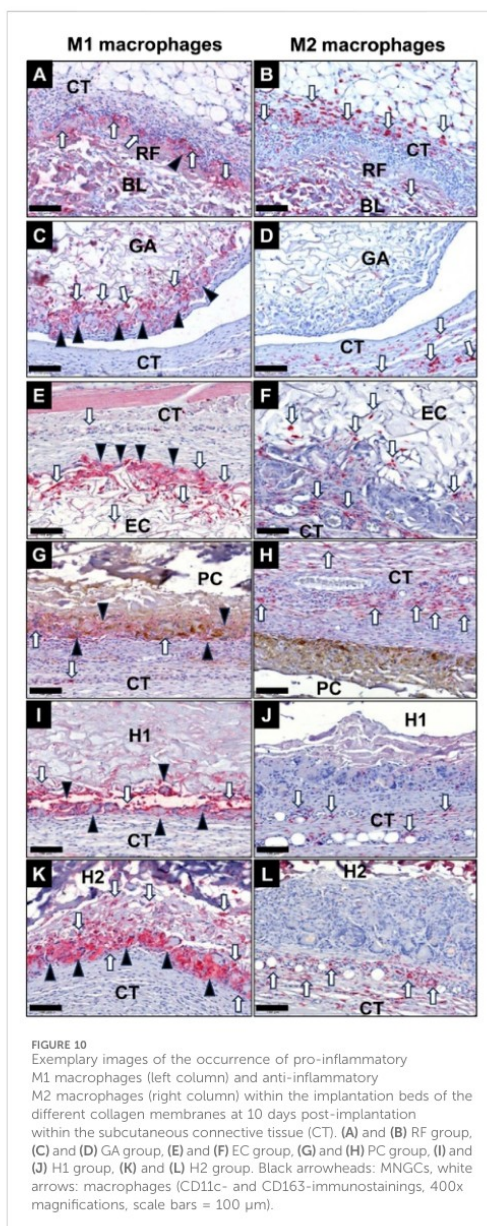


FIGURE 9
Exemplary histological images of the crosslinked collagen membranes at 90 days post-implantation within the subcutaneous connective tissue (CT). (A) RF group, (B) GA group (C) EC group, (D) PC group, (E) H1 group and (F) H2 group. White arrows: macrophages, blue arrows: lymphocytes, green arrows: fat tissue, yellow arrow: granulocytes, red arrows: vessels, yellow arrowheads: residual materials, black arrowheads: MNGCs, BL: base layer. (HE-stainings, 400x magnifications, scale bars: 20 μ m).

of pro- and anti-inflammatory immune cells was observable in all study groups at this study point.

At 90 days post-implantation, both the RF and PC membrane groups exhibited considerably lower levels of M1-macrophages and MNGCs compared to other experimental groups (Figure 12). The number of M2-macrophages in the RF and PC membrane groups was higher compared to the M1-macrophage numbers and predominantly located within the reactive inflammatory tissue surrounding the membranes. However, comparable M2-macrophage numbers were

found in all study groups. In contrast, visibly higher presence of pro-inflammatory macrophages and MNGCs was detected in the groups of the GA, EC, H1, and H2 membranes, which were also higher compared to 30 days post-implantation. Furthermore, an increased number of M2-macrophages was detected within the invading connective tissue in all groups of the crosslinked membranes, while the afore-mentioned spatial distribution pattern of pro- and anti-inflammatory immune cells was in general still observable in all study groups.

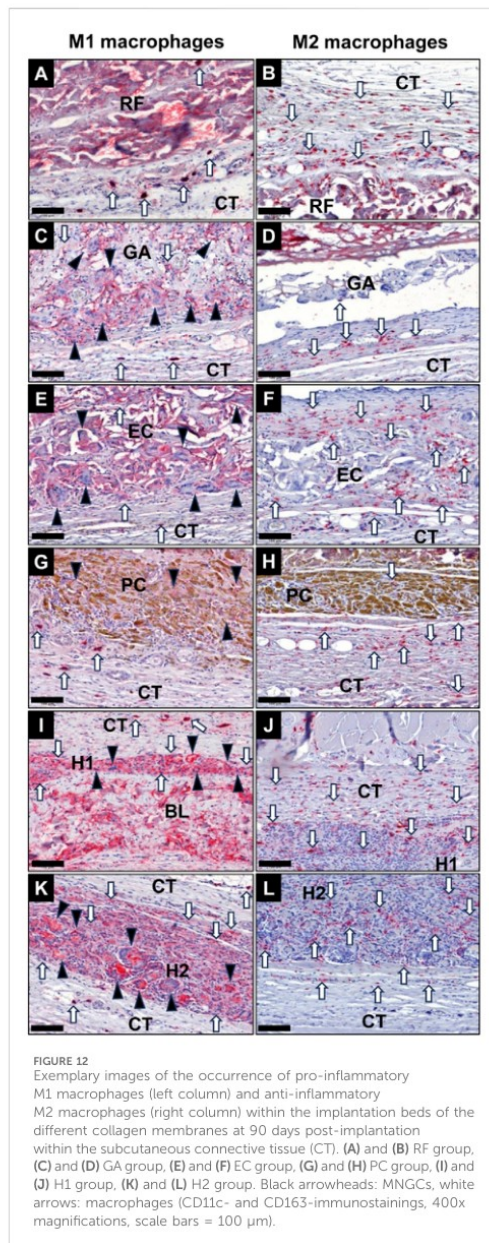


3.5.3 Vascularization pattern

The histological analysis of the implantation bed vascularization revealed that the RF membrane area was nearly completely replaced by connective tissue (Figures 13A–C). Thereby, the membrane area as well the surrounding connective tissue showed a higher vascularization pattern, i.e., more small blood vessels with higher

lumina, at 10 days post-implantation compared to the other study groups.

Stable porous membrane structures were observed in the groups of the GA, EC, and PC membranes at this early time point, accompanied by slight reactive tissue ingrowth. Due to limited tissue infiltration,



blood vessels in these three groups were mainly observed in the surrounding connective tissue of the membrane. Although the H1 membrane was still detectable at this time point, the edge region of the membrane was completely degraded due to ingrowth of reactive tissue, with only a small amount of membrane fragments observable

(Figure 13P). In the H2 group, inflammatory tissue penetration into the membrane was the most severe among all crosslinked membranes. The membrane area of H2 group was nearly completely infiltrated by macrophages and MNGCs, with abundant neovascularization observed in the infiltrated tissue (Figure 13P).

At the 30 days post-implantation, the membrane in the GA and EC groups exhibited extensive fragmentation, with connective tissue penetrating nearly completely through the membrane area (Figures 13E, H). Abundant undegraded collagen fibers and newly formed blood vessels were observable within the invading tissue. Conversely, in the PC, H1, and H2 groups, the membranes were completely fragmented at this time point, leaving behind only remnants of collagen fragments. The membrane area was entirely replaced by reactive tissue containing numerous newly formed blood vessels (Figures 13K, N, Q).

At 90 days following implantation, the membranes in the GA and EC groups exhibited complete fragmentation, while still retaining a notable presence of collagen fragments and fibers (Figures 13F, I). The inflammatory regions of PC, H1 and H2 groups had diminished compared to the 30-day post-implantation period, leaving behind minimal remnants of collagen fragments (Figures 13L, O, R). Consistent with former study time points, a substantial vascular network was evident within the invading connective tissue associated with the material in all research groups.

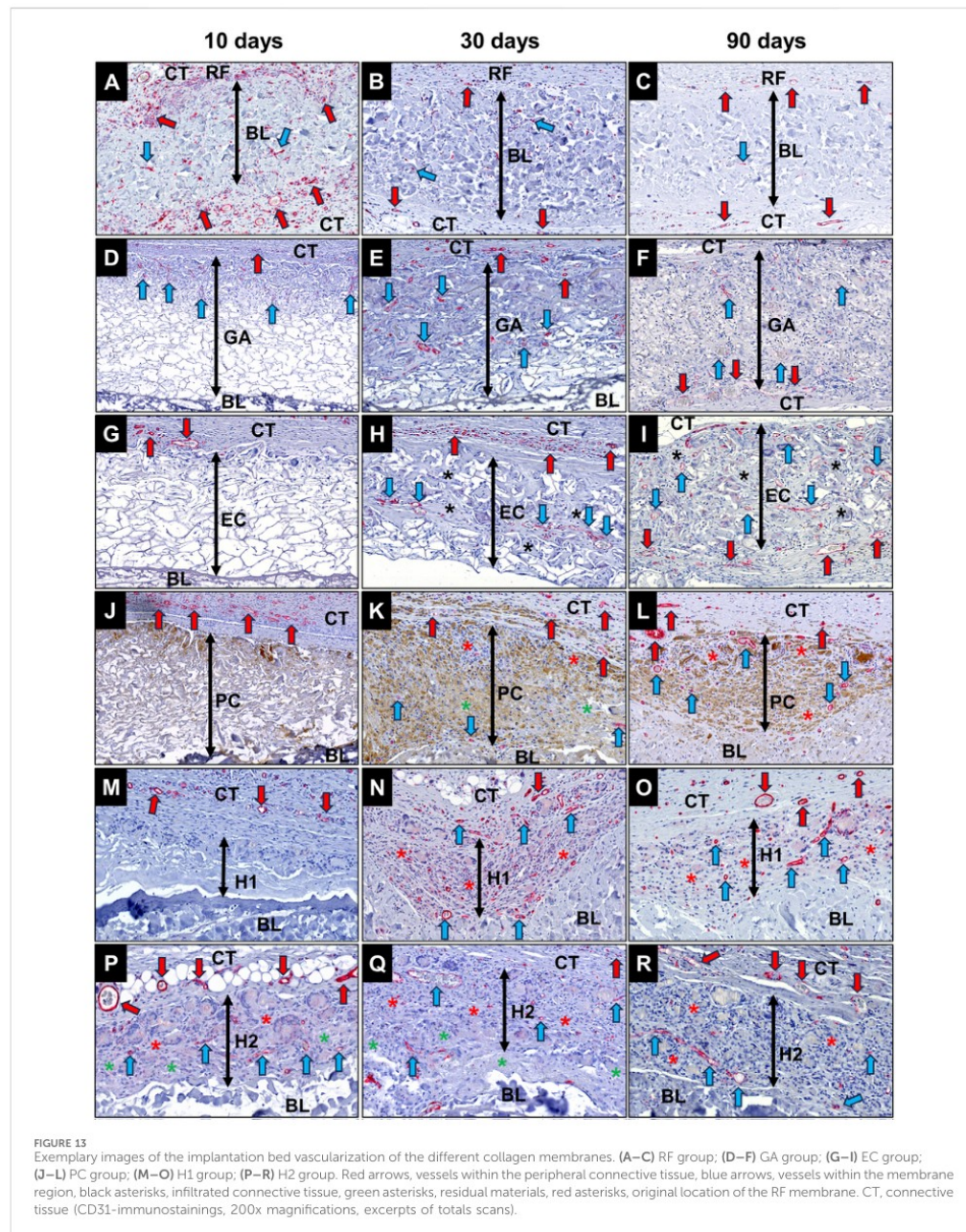
3.6 Histomorphometrical results

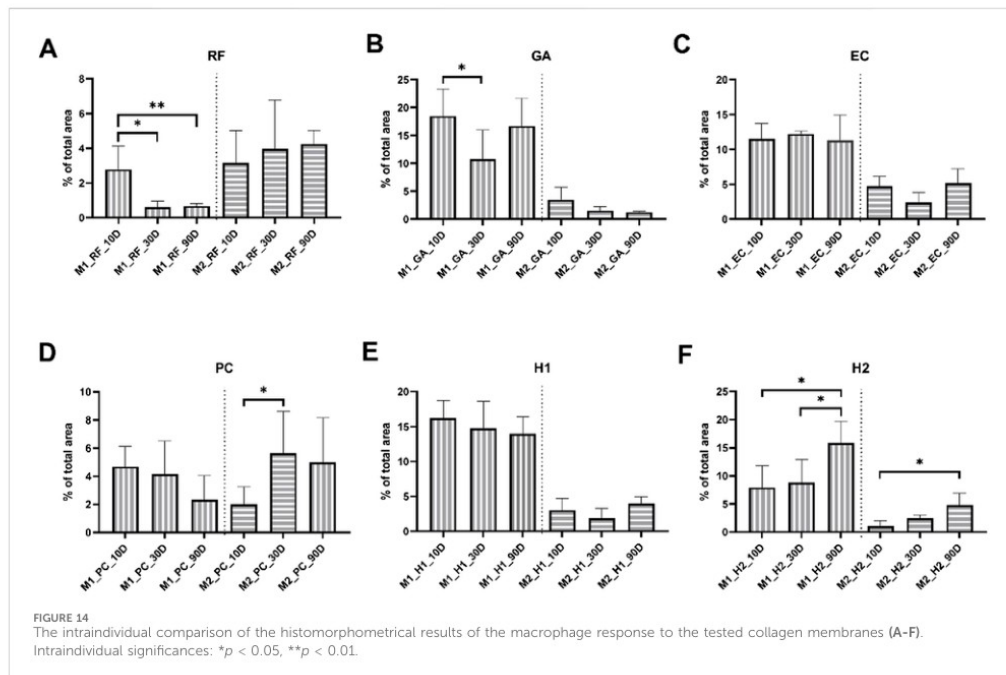
3.6.1 Occurrence of macrophage subtypes

The histomorphometrical analysis of the macrophage subtype occurrence revealed that the RF membrane induced an immune response mainly dominated by M2-macrophages (Figure 14A). Within this group, the numbers of M1-macrophages reached a peak at 10 days post-implantation followed by a significant decrease at day 30 and day 90 post-implantation ($p < 0.05$). In contrast, the numbers of the M2-macrophages remained on a constant niveau throughout the implantation period showing a minimal trend towards an increase during the time course of the study.

Throughout the entirety of the implantation period, the levels of M1 macrophages induced by the PC group were very similar compared to the values in the RF membrane group (Figure 14D). Thus, a decreasing tendency in the numbers of M1-macrophages was found starting with day 10 post-implantation up to the end of the observation period at day 90 post-implantation. In contrast, the numbers of M2-macrophages increased during this time frame with a significant increase between day 10 and 30 post-implantation ($p < 0.05$).

However, with exception of the PC membrane, all crosslinked membranes induced an immune response strongly dominated by M1-macrophages. The M1-macrophage response in the GA group also exhibited notable changes, with cell numbers significantly lower at 30 days post-implantation compared to 10 days ($p < 0.05$), followed by a subsequent increase at 90 days post-implantation (Figure 14B). In the groups of the EC and the H1 membranes no changes of the M1- or M2-macrophage numbers were found during the time course of the study (Figures 14C, E).





In contrast, the H2 membrane induced significantly lower M1 macrophage levels in the early post-implantation phase compared to the later phase (* $p < 0.05$) (Figure 14F), while also an increase of M2 macrophages over the study period with a significant difference between day 10 and 90 post-implantation (* $p < 0.05$) was found.

At 10 days post-implantation, the GA membrane as positive control triggered a significantly higher M1-macrophage level compared with the RF membrane (**** $p < 0.0001$) (Figure 15A). Apart from the values in the GA membrane group, the values of levels of M1 macrophages in the H1 and EC membrane groups were also significantly higher than in the RF group (*** $p < 0.001$, * $p < 0.05$). In contrast, the PC and H2 membranes exhibited significantly lower M1 levels than the GA membrane (*** $p < 0.001$, * $p < 0.01$). Notably, at this time point, all crosslinked membrane induced M2 macrophages levels significantly lower than M1 levels (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$) with exception of the values in the RF group that were comparable.

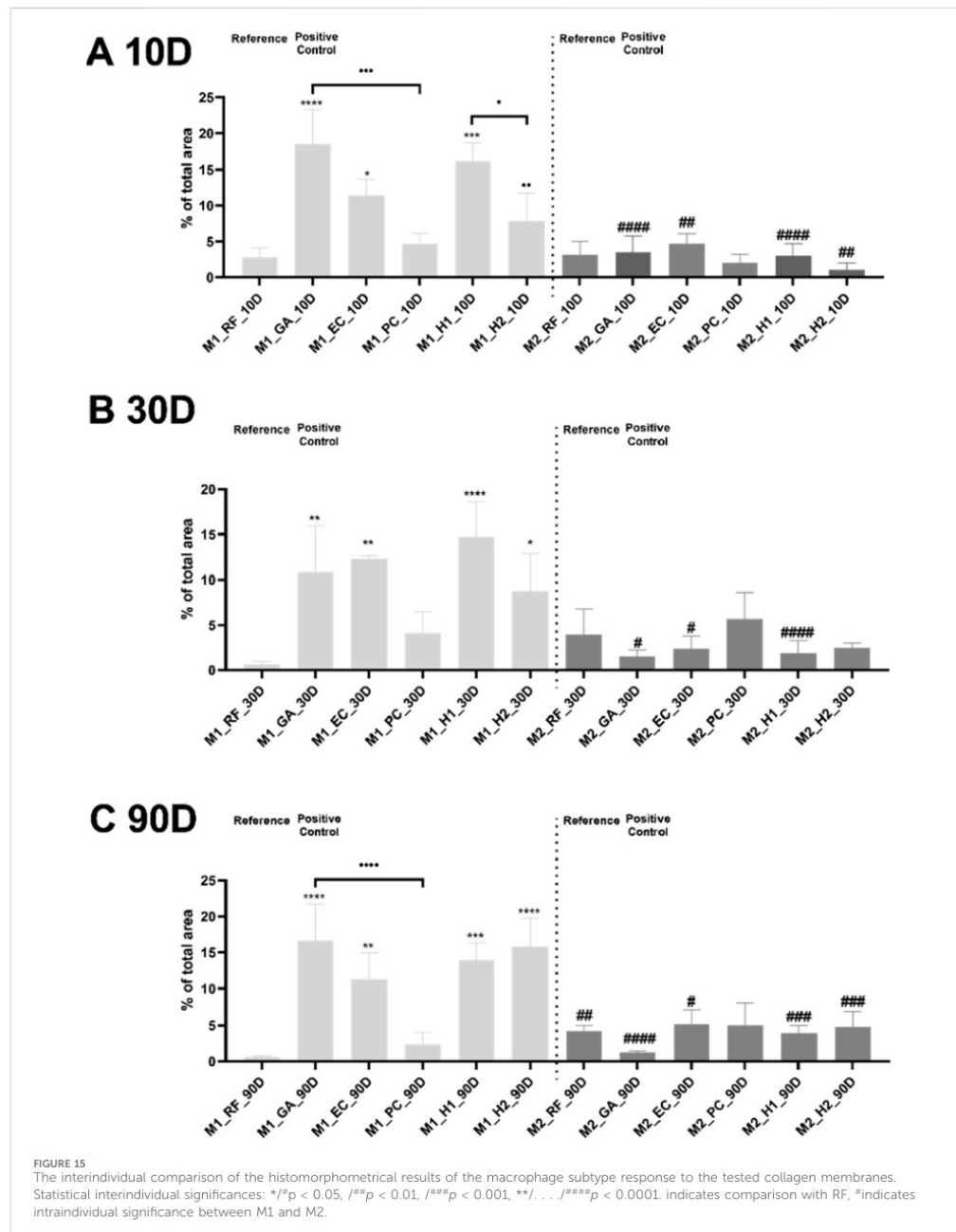
At 30 days post-implantation, except for the PC membrane, all crosslinked membrane exhibited significantly higher M1 levels than in the RF membrane group (* $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$) (Figure 15B). Notably, the H1 membrane induced the highest M1-macrophage response, even slightly surpassing the values in the GA membrane group. Although without significance, the PC membrane induced the highest M2-macrophage response out of all crosslinked membranes at this timepoint. Furthermore, the GA and H1 membranes induced M2-macrophage values that were significantly lower than the M1-macrophage levels (* $p < 0.05$, **** $p < 0.0001$).

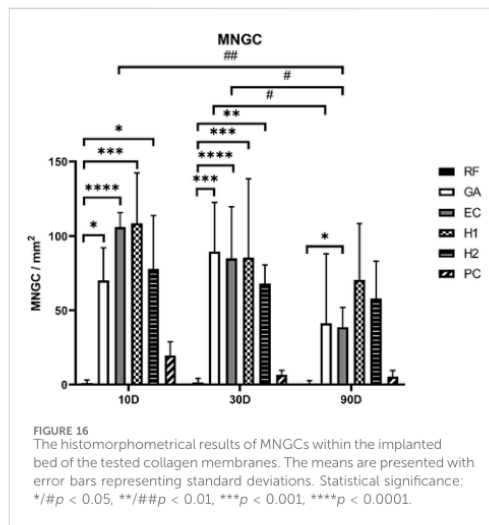
At 90 days post-implantation, the PC membrane continued to exhibit the lowest M1 levels among all membranes (Figure 15C). All crosslinked membranes induced significantly higher M1-macrophage responses than the RF membrane (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). At this timepoint, the M2-macrophage level in the RF membrane group was significantly higher than the respective M1-macrophage level (** $p < 0.01$). In the PC group, the low M1- and M2-macrophage levels did not show statistical significance. However, in the other crosslinked membrane groups, the M2-macrophage values remained significantly lower than the respective M1-macrophage levels (* $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$).

3.6.2 Occurrence of multinucleated giant cells (MNGCs)

Multinucleated giant cells (MNGCs), considered a pivotal immunological and biodegradation indicator, were also analyzed in this study. As shown in Figure 16, MNGCs triggered by the RF (reference) membrane consistently maintained very low levels throughout whole study period.

The PC membranes induced slightly more MNGCs compared to the numbers in the RF membrane group without significant differences. In contrast, all the other crosslinked membrane induced significant higher MNGC numbers compared to that found in the group of the RF membranes at day 10 and 30 post-implantation (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). At 90 days post-implantation, significant differences were only observed between the values in the EC membrane group and the RF membrane group (* $p < 0.05$).





Furthermore, intra-individual significant differences in the MNGC numbers in the GA and EC groups were also observed at different intervals. The quantity of MNGCs in the EC group exhibited a declining trend throughout the implantation period, with MNGC numbers that significantly decreased between 10- and 90-day post-implantation ($\#p < 0.01$) but also between 30- and 90-day post-implantation ($\#p < 0.05$). Similarly, in the GA group, the level of MNGCs at 90 days post-implantation was also significantly lower than at 30 days post-implantation ($\#p < 0.05$).

3.6.3 Vascularization

The vascularization within implantation area including the intra-membrane (IMR) and peri-membrane (PMR) regions, was examined focusing on two key parameters: vessel number/mm² (vessel density) and vessel area in % (vessel area fraction).

At 10 days post-implantation, the RF group exhibited the highest overall vessel density and vessel area fraction, as the values in this group were significantly higher than in the EC, GA, PC, and H1 groups ($*p < 0.05$, $**p < 0.01$) (Figures 17A, D). Thereby, only significant differences were found within the peri-membrane regions, while no significances were detectable within the intra-membrane areas. Thus, both the vessel density and vessel area fraction in the PMR area of GA group were significantly lower than that of the RF group ($*p < 0.05$, $**p < 0.01$) (Figures 17B, E). Also, the vessel area fraction in PMR area of the PC membrane group was significantly lower than that in the RF group ($*p < 0.05$) (Figure 17E). Furthermore, no significances between all groups were found within the intra-membrane region at this early study time point (Figures 17A–F).

At 30 days post-implantation, only the overall vessel density in the H₂ membrane group was significantly lower than that observed in the RF group ($**p < 0.01$) (Figure 17A). Apart from the H₂ group, no intergroup significances were observed at 30 days post-implantation. Also, no differences in the overall vessel area fraction were observed (Figure 17B). Additionally, the analysis

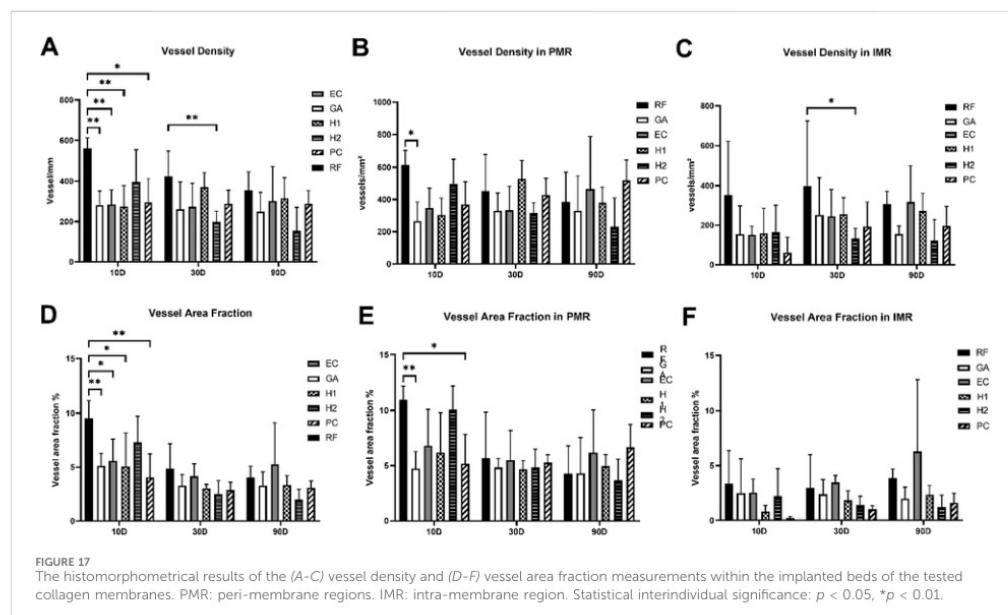
showed that no differences nor in the vessel density or the vessel area fraction between all study groups within the peri-membrane area were found at this study point (Figures 17A–F). Only, significantly lower vessel density values were found in the H₂ membrane group compared to the values in the RF membrane group within the intra-membrane region were found ($*p < 0.05$) (Figure 17C), while no other differences were calculated.

At 90 days post-implantation, no significant differences in the overall vascularization parameters nor in the different regions of the implantation beds were found between all study groups (Figures 17A–F).

4 Discussion

Barrier membranes were initially designed to form a physical barrier between soft or gingival tissue and the jaw bone defect to prevent the migration of cell types (such as epithelial cells) that might negatively interfere with the bony healing process during Guided Bone Regeneration (GBR) procedures (Yang et al., 2022). By creating a secluded space, the optimal membrane should allow specific cells such as osteoblasts and precursor cells to populate the defect site and promote the regeneration of the bone tissue (Allan et al., 2021; Ren et al., 2022). During excessive development of resorbable barrier membranes in the last decades this material class is nowadays mainly composed of xenogeneic collagen of different animal sources and tissue origins, while the optimum standing time has been determined: Resorbable barrier membranes require integrity for 4–6 weeks for periodontal tissue regeneration and 16–24 weeks for bone tissue regeneration (Caballé-Serrano et al., 2019). However, native collagen derived from porcine skin that is mainly used as tissue source falls short of meeting the requirement. In this context, chemical crosslinking of native collagen is a well-established approach to improve the mechanical properties as well as the standing time of such materials and has a long history with respect to medical applications (Oryan et al., 2018; Adamiak and Sionkowska, 2020).

Interestingly, the requirements for this type of biomaterial have also changed considerably in recent years. Thus, the requirement for a purely physical barrier functionality has been expanded to include various biological functionalities. This topic was discussed even in the last years and especially “bioactive properties” have been discussed in this context (Omar et al., 2019; Ren et al., 2022; Yang et al., 2022). Altogether, this term summarizes that the optimal barrier membrane should “actively” support the process of bone-healing, which includes also the establishment of an adequate angiogenesis and vascularization for bone tissue regeneration in form of a so-called “transmembraneous vascularization” but furthermore an optimal inflammatory tissue response to support the underlying bone healing process. In this context, Alkildani and colleagues showed in an *in vivo* study that a native collagen-based barrier membrane was found to get ossified, while inducing a high occurrence of both M1- and M2-macrophages within its implantation bed significantly dominated by the anti-inflammatory subtype. More interesting, a correlation between M2-macrophages induced by the membrane and bone regeneration in the underlying bone defects was found that hints on the interaction of the immune responses induced by the collagen membrane and the



bone healing process (Alkildani et al., 2023). These results and different other study results led to the realization that it is of great importance to understand the immunomodulatory features of collagen-based barrier membranes for their future development.

In the present study, the material and biological properties of four collagen membranes modified by different crosslinkers, i.e., GA, EC/NHS, PC, and HMDI, were analyzed via established *ex vivo*, *in vitro* and *in vivo* methodologies (Kapogianni et al., 2021; Radenković et al., 2021; Alkildani et al., 2023). These chemical crosslinking were attained via different interhelical linkages such as imine/amine bonds, amide bonds and hydrogen bonds (Tanzer, 1973; Adamiak and Sionkowska, 2020). In this context, it has already been described that GA is the most representative aldehyde crosslinker which reacts with lysine or hydroxylysine residues forming an intermediate Schiff base and its further reaction pattern leads to the formation of more stable covalent imine bonds (Ruijgrok et al., 1994; Zhang et al., 2022). However, the application of GA has limited in commercial bio-products due to the evidence that unreacted GA residues and by-products such as Schiff bases can induce cytotoxicity, inflammation, and calcification (Zhang et al., 2022). The *in vitro* study also supports above conclusion that the GA membrane exhibited high deviations from the non-cytotoxicity ranges and can thus be classified as cytotoxic. Particularly in the BrdU assay, the values of the GA membrane were extremely low and not significantly different from the positive control (RM-A). Given this concern, EC/NHS gain a growing interest as the agent itself is not incorporated into the network after crosslinking (Yang, 2012). EC activates carboxylic acid groups on collagen to form an O-acylisourea intermediate, which then react with amino groups to create covalent amide bonds (Gao et al., 2022). After reaction, EC

is transformed into water soluble urea derivatives which is easy to be removed. Another agent of interest is HMDI, which reacts with amino groups ($-NH_2$) on their lysine residues of collagen through isocyanate groups to form stable urea linkages which is highly resistant to hydrolysis (Olde Damink et al., 1995; Sarrigiannidis et al., 2021). Unreacted HMDI and its byproducts, such as urethanes, are easier to remove and exhibit lower cytotoxicity compared to that of GA. Proanthocyanidins (PC), characterized by their anti-inflammatory properties and abundance of hydroxyl groups, serve as another beneficial cross-linking agent proficient in establishing hydrogen bonds with collagen molecules (Green et al., 2010; He et al., 2011; Vidal et al., 2016). In this study, collagen membranes prepared with the above three crosslinkers demonstrated satisfactory cytocompatibility and then following the 3R-cascade, implanted into subcutaneous connective tissue to determine the integration behavior, the standing time and the related immune responses as previously described (Ghanaati et al., 2010; Lindner et al., 2020; Naenni et al., 2020). GA membranes and RF membranes were employed as the positive control and negative control, respectively, in the further *in vivo* study.

Initially, the histological and the material analyses of the pure collagen membranes showed that all membranes displayed a well-defined layer characterized by a honeycomb-like pore structure. While only slight structural alterations occur following crosslinking, the introduction of all crosslinkers enhanced the tensile strength of collagen membranes by creating additional chemical bonds between collagen molecules. Notably, proanthocyanidins and hexamethylene diisocyanate (HMDI) crosslinked membranes exhibited similar ductility and denaturation temperature with RF membrane. However, both GA and EC membranes displayed

decreased ductility, particularly the elongation at break of the EC membrane was notably lower than that of the PC membrane, which means that they may experience a propensity for lesser plastic deformation compared to the other membranes. Histological images showed consistent results that GA and EC membranes retained a bilayer structure with well-defined and interconnected pores and void spaces within the collagen matrix even after 30 days post-implantation. This stiff structure provides ample strength endure the compression and tension exerted by surrounding soft tissues and influencing the ability of membranes to absorb and retain water. This inference is further corroborated by the significantly increased swelling ratio observed in both EC and GA membranes compared to the rest groups. These two groups also displayed a stronger resistance to temperature which may indicate the forming of more robust molecular bridges between collagen fibers. These bridges contribute to a more interconnected and reinforced structure, making it harder for the collagen fibers to unravel during denaturation at high temperatures.

Moreover, the formation of molecular bridges serves to conceal collagen's cleavage sites, thereby augmenting collagen's resistance to enzymatic degradation (Oryan et al., 2018; Sarrigiannidis et al., 2021). The findings indicate that all crosslinked collagen membranes display significantly improved collagenase resistance, especially GA, EC and PC groups, which retained membrane integrity after 7 days of collagenase digestion *in vitro*. However, both H1 and H2 membranes crosslinked with Hexamethylene diisocyanate (HMDI) exhibited a significant mass loss after 4 h of collagenase digestion, persisting throughout the entire assay. Besides, the collagenase resistance of collagen materials is strongly influenced by the crosslinking protocol, such as concentration, crosslinking time and temperature (Adamiak and Sionkowska, 2020). Therefore, it is anticipated that optimizing the crosslinking scheme for HMDI-crosslinked membranes will enhance their enzyme resistance, which is supported by the fact that H2 crosslinked membrane, treated at a higher HMDI concentration, exhibited significantly higher mass residues compared to H1 crosslinked membrane after 24 h of collagenase digestion. Even digestion results of the GA membranes confirm the good suitability of this crosslinker - even apart from the problem of biocompatibility issues that can occur.

The results of the swelling ratio test showed that the EC, PC and GA membranes exhibited a significantly faster swelling rate and/or higher swelling ratio in comparison to the RF membrane. The H1 membrane exhibited a swelling pattern most similar to the RF membrane, whereas the H2 membrane, crosslinked by a higher HMDI concentration, displayed the lowest swelling rate and swelling ratio. This result is very important as it has been reported that crosslinked collagen membranes have prolonged degradation times, but their application is associated with significantly higher membrane exposure rates of up to 70.5% (Friedmann et al., 2011; Bouguezzi et al., 2020). Thereby, the premature exposure of membranes is often associated with bacterial invasion, as demonstrated by Becker et al. (2009). Even in view of the results won via the collagenase assay it can be concluded that the GA, EC and PC membranes might not be suitable for GBR procedures in patients with a thin gingiva biotype (Sarma et al., 2021), while the HMDI-crosslinked membranes might be more suitable even in this point.

The analyses revealed that the non-crosslinked reference membrane was degraded very fast until day 30 post implantation

inducing a very low immune response mainly based on macrophages and without occurrence of multinucleated giant cells (MNGCs). Initially, M1-like macrophages were activated and displayed a pro-inflammatory phenotype during early implantation stages—mainly induced by the surgery and the related tissue defect. However, by day 30 post-implantation, the population of M1 macrophages notably diminished, giving way to a predominance of anti-inflammatory M2 macrophages. This result was to be expected and also corresponds to the results of various other studies (Ghanaati, 2012; Al-Maawi et al., 2018). This expected degradation rate, and the integration behavior were therefore also the reason for selecting this membrane as a negative control and show especially the fast biodegradation, which is obviously too fast for many GBR procedures. However, this results even in view of the immunological tissue response is very interesting as it has manifoldly discussed and shown that synthetic but also different other biomaterials such as bone substitutes induce a special spatiotemporal macrophage response (Abels et al., 2021; Barbeck et al., 2022; Barbeck et al., 2023; Ren et al., 2024). In this context it has been described that M1 macrophages even in case of “unnatural” materials are located at the material surfaces in combination with MNGCs as correlate of a foreign body response that is involved in material degradation via phagocytosis (Barbeck et al., 2023). In this context, the observed tissue response of the RF membrane is in complete contrast with this pro-inflammatory tissue response and might show that this implant material or the collagen fibers has a natural constitution and is more digested by mononuclear cells of the collagen turnover as described in case of the BioGide membrane (Ghanaati, 2012; Radenković et al., 2021). As the most widely used membrane in clinical practice, BioGide has been shown to involve only controlled tissue infiltration by mononuclear macrophages, which represents a beneficial tissue integration pattern for guided bone regeneration (GBR) membranes. This means that RF membrane rightly serves or can serve as a reference material for collagen-induced cellular transition in this study.

In contrast, the GA-crosslinked membrane, which was included into the study as a negative control group, induced a pronounced inflammatory tissue response including high numbers of MNGCs but was in total present until day 90 post-implantation. Thereby, mostly single fiber bundles surrounded by mononuclear and multinucleated phagocytes, which had spread over the entire implantation area, were found starting with day 30 post-implantation. This immune response is definitely more pro-inflammatory in nature by inclusion of higher numbers M1-macrophages and M1-MNGCs reflecting the “unnatural physicochemical material properties”, revealing that this membrane served also as a “negative reference material”. Thus, this integration pattern is not in line with a real barrier functionality without cell infiltration or with minor cell infiltration as in the case of PTFE-based materials or other collagen-based membranes (Korzinskas et al., 2018; Ottenbacher et al., 2021; Radenković et al., 2021). In this context, it was shown that also a cell infiltration into the membrane structures of the currently most clinically used barrier membranes, i.e., Jason membrane and BioGide, was observable (Ottenbacher et al., 2021). However, the cell invasion in these latter cases only included macrophages and did thus not evoke such a high degradation pattern combined with a material fragmentation.

Moreover, a recently published study by Alkildani *et al.* showed that the pericardium-based Jason membrane has a long-standing time and was completely remodeled into bone tissue within several weeks after implantation by serving as an osteoconductive scaffold (Alkildani *et al.*, 2023). This leads to the conclusion that in the case of collagen-based membranes, cellular infiltration does not necessarily have to be harmful in terms of GBR functionality. As in the case of any (biodegradable) biomaterial, the material-specific tissue response, i.e., the cellular elements induced, in combination with the material properties, determines the degradation mechanisms and duration of biodegradation. The degradation behavior of the GA membrane seems thus represent not a suitable integration pattern.

In view of the integration and degradation pattern of the other crosslinked collagen membranes it was shown that the EC membrane also showed an invasion of M1-macrophages and high M1-MNGC numbers combined with a degradation pattern comparable to the GA membrane group. Histopathological analysis demonstrated that at 30 days post-implantation, the porous structure of EC membranes was still clearly visible. Subsequently, the material underwent collapse until the conclusion of the study. Interestingly, the collagen fibers were surrounded by a fiber-rich connective tissue with lower cell numbers and high vascularization, which might be a factor that can prevent the ingrowth of other tissues such as epithelial tissue and might thus be an interesting material candidate in the GBR procedure with a completely different approach. However, fibrosis is the end stage of the foreign body response to a biomaterial (Anderson *et al.*, 2008) – meaning that the recipient tissue and the immune system of the recipient organism see no other option than to completely encapsulate the biomaterial and no longer allow any further interactions. It can therefore also be assumed in the case of this material candidate that it will not be possible to successfully develop a GBR membrane on this basis.

In the case of the membranes cross-linked with both the lower and higher HDMI concentration, this disintegration and degradation pattern was already observed on day 30 post-implantation – combined with high M1-macrophage and M1-MNGC numbers. On day 90, only cell-rich connective or granulation tissue was left and only very scarred membrane fragments, so that no barrier functionality was present in these two groups either. This outcome can be attributed to the lower resistance of H1 and H2 membranes to cell-secreted degrading enzymes, consistent with the findings from the previously described *in vitro* collagenase experiments. It is noteworthy that although the collagenase resistance of H2 membranes appeared to be superior to that of H1 membranes, the histopathological analysis showed that the former exhibited most severe degradation was accompanied by a higher number of MNGCs at 10 days post-implantation. This suggests that the higher concentration of HMDI, while enhancing the collagenase resistance of the collagen membranes, induced higher quantity of multinucleated phagocytes, consequently resulting in faster cellular degradation. These results underscore the significant role of phagocytes, particularly macrophages and MNGCs of the pro-inflammatory phenotype, in material degradation. This degradation occurs through phagocytosis and the secretion of degrading mediators such as reactive oxygen species (ROS) and matrix metalloproteinases (MMPs), among others, to break down

foreign objects (Sheikh *et al.*, 2015). Consequently, mitigating inflammation post-implantation could theoretically facilitate optimal healing and extend the durability of collagen materials within the body.

However, complicating this model is the fact that PC membranes seem to successfully induce a mild inflammatory response with low MNGC numbers similar to that of RF membranes due to the incorporation of anti-inflammatory proanthocyanidins, do not demonstrate a longer standing time. This cellular response was combined with a complete fragmentation and a disintegration of the membrane into very small subunits, so that membrane functionality can no longer be assumed by day 30 post-implantation at the latest. Combined with the aforementioned mechanical property findings, this phenomenon may be attributed to the higher ductility of PC membranes. This characteristic renders PC membranes more prone to plastic deformation *in vivo* due to pressure, tensile, and shear forces exerted by the surrounding microenvironment. As a result, the increased surface area might make the membrane more vulnerable to fragmentation by cellular and enzymatic degradation. Altogether, only the EC-crosslinked membranes might be most suitable to offer barrier functionality up to day 90 post-implantation due to their special “fibrous” tissue integration pattern, but this is also very doubtful.

Macrophages also have been recognized as key regulators of vascularization, with distinct roles attributed to M1 and M2 phenotypes (Wang *et al.*, 2021). It is widely accepted that M1 macrophages kickstart vascular regeneration by releasing vascular sprouting-associated factors, such as VEGF (Sunderkötter *et al.*, 1991; Wang *et al.*, 2021). Conversely, M2 macrophages play a crucial role in vascular maturation by recruiting pericytes and mesenchymal stem cells (MCS) through PDGF-BB secretion, both of which are crucial for vascular stabilization (Sunderkötter *et al.*, 1991; Spiller *et al.*, 2014). Thus, the precise timing and sequential activation of M1 and M2 macrophages are imperative for optimal vascularization outcomes. In this study, the GA, EC, and H1 membranes exhibited significantly lower levels of both vessel density and area fraction than the RF group at 10 days post-implantation, possibly due to their higher induction of M1 macrophages. Several studies have shown that increased population of M2 macrophages or enhanced M1 to M2 transition correlate with improved vascularization (Moore *et al.*, 2018; Yin *et al.*, 2020; Yang *et al.*, 2023). Moreover, given the intricate interactions between M1 and M2 macrophages in vascularization, an increasing number of studies have shifted their focus to the M2/M1 ratio rather than the absolute population of macrophage subtypes. This notion is supported here by the findings in the PC group, where despite exhibiting M1 and M2 levels similar to those of RF, the lower M2/M1 ratio and significantly less vascularization compared to the RF group were observed.

Interestingly, the H2 group also induced a predominantly M1 macrophage response at this time point, yet it demonstrated vessel density and area fraction closest to RF. This could be attributed to their lower M1 levels and premature membrane collapse similar to that observed in RF, promoting premature growth of vascular-rich connective tissue into the membrane. Additionally, induced MNGCs also have the potential to secrete

vascular endothelial growth factor (VEGF), thus contributing to the vascularization process (Barbeck et al., 2015; Al-Maawi et al., 2018). However, this vascularization advantage was not sustained. At 30 days post-implantation, the H2 group exhibited significantly lower vessel density than RF, possibly due to ongoing molecular crosstalk between multinucleated giant cells and macrophages. Numerous studies have demonstrated that vascularization is a complex process regulated by various inflammatory cells. For instance, Spiller et al. discovered that transient presence of M1 macrophages on the first day promoted blood vessel formation, while prolonged exposure on the third day led to vessel regression (Graney et al., 2020). The above findings underscore the intricate interactions among inflammatory cells, particularly macrophage subtypes, during vascularization.

When transferring the above results to clinical scenarios, it's crucial to take the structural integrity of the membrane into account. On one hand, the fragmentation of the barrier membrane directly results in the loss of its barrier function, leading to the failure of Guided Bone Regeneration (GBR). On the other hand, premature fragmentation of the barrier membrane also expedites its degradation *in vivo*. Of the cross-linked membranes tested in this study, the EC membranes were stable materials within 30 days of implantation. Conversely, the other three membranes fragmented earlier, rendering them less suitable for application as Guided Bone Regeneration (GBR) barrier membranes. Furthermore, this study highlighted disparities between *in vitro* and *in vivo* investigations. Despite being chosen as a positive control for *in vivo* experiments due to cellular incompatibility, the GA group did not provoke adverse inflammatory reactions or fibrous capsule formation in the subcutaneous implant model. The restriction of the study lies in the subcutaneous model, which only reveals the biocompatibility and tissue response of the collagen membranes. A further study should be conducted using a bony implantation model which provides more relevant data in the context of bone tissue regeneration. However, this approach is complicated and often requires large animal testing.

5 Conclusion

The present study elucidates the material characteristics and distinct cell and tissue reactions elicited by collagen membranes cross-linked with various agents. Compared to the RF group, all cross-linking agents improved the enzyme resistance and standing time of the collagen membrane *in vivo*. In a subcutaneous implantation model, mononuclear cell responses were solely triggered in the RF group, whereas numerous multinucleated giant cells (MNGC) were observed in all cross-linked groups except for the PC group. With the emergence of abundant MNGC and M1 macrophages, membrane integrity deteriorated over the course of the experiment, leading to fragmentation and collapse. Among these, EC membranes remained structurally stable for up to 30 days, similar to GA group (negative control), whereas the other groups experienced structural breakdown earlier. Proanthocyanidins demonstrated anti-inflammatory properties, effectively mitigating foreign body reactions post-implantation. The PC group exhibited significantly reduced MNGC and M1 macrophages compared to other cross-linked groups,

approaching levels seen in the RF group. However, it still faced premature membrane collapse *in vivo*, probably due to susceptibility to plastic deformation. Moreover, as the collagen membrane fragmented, transmembrane vascularization was observed across all tested groups, with histomorphometric findings highlighting intricate interactions of inflammatory cells during vascularization. Altogether, the EC membranes were stable materials within 30 days of implantation making them the most preferable material candidate out of the cross-linked membranes tested in this study. Conversely, the other three membranes fragmented earlier, rendering them less suitable for application as Guided Bone Regeneration (GBR) barrier membranes.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was approved by the Local Ethical Committee of the Faculty of Medicine, University of Niš and by the Veterinary Directorate of the Ministry of Agriculture, Forestry and Water Management of the Republic of Serbia (decision number 323-07-09101/2020-05/5; date of approval: 26 August 2020). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

YR: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Software, Validation, Visualization, Writing—original draft, Writing—review and editing. SA: Conceptualization, Formal Analysis, Investigation, Writing—original draft. KB: Data curation, Formal Analysis, Investigation, Software, Writing—original draft. MR: Data curation, Formal analysis, Investigation, Writing—review and editing. AK: Conceptualization, Methodology, Project administration, Resources, Writing—original draft. SS: Funding acquisition, Methodology, Project administration, Resources, Writing—review and editing. SN: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing—review and editing. OJ: Methodology, Supervision, Conceptualization, Funding acquisition, Resources, Writing—review and editing. LL: Project administration, Resources, Supervision, Writing—review and editing. MB: Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Resources, Software, Supervision, Validation, Writing—original draft, Writing—review and editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. The author(s)

declare that financial support was received for the research, authorship, and/or publication of this article. The authors gratefully acknowledge the funding by the German Research Foundation (Deutsche Forschungsgemeinschaft, DFG) for the subproject 6 within the Research Unit 5250 "Permanent and bioresorbable implants with tailored functionality" (No. 449916462).

Acknowledgments

YR acknowledges the support of the China Scholarship Council (CSC, No. 202106880014). The statistical analysis of data was performed with GraphPad 9. The figure 1 was created with BioRender.

Conflict of interest

Authors SA, KB, and MB were employed by BerlinAnalytix GmbH. Author AK was employed by Biotrics bioimplants AG.

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