

## ***In Vitro* and *Ex Vivo* Analysis of Collagen Foams for Soft and Hard Tissue Regeneration**

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**Abstract.** *Background/Aim: The aim of this study was the conception, production, material analysis and cytocompatibility analysis of a new collagen foam for medical applications. Materials and Methods: After the innovative production of various collagen sponges from bovine sources, the foams were analyzed ex vivo in terms of their structure (including pore size) and in vitro in terms of cytocompatibility according to EN ISO 10993-5/-12. In vitro, the collagen foams were compared with the established soft and hard tissue materials cerabone and Jason membrane (both botiss biomaterials GmbH, Zossen, Germany). Results: Collagen foams with different compositions were successfully produced from bovine sources. Ex vivo, the foams showed a stable and long-lasting primary structure quality with a bubble area of 1,000 to 2,000  $\mu\text{m}^2$ . In vitro, all foams showed sufficient cytocompatibility. Conclusion: Collagen sponges represent a promising material for hard and soft tissue regeneration. Future studies could focus on integrating and investigating different additives in the foams.*

Collagen is the most abundant protein in the human body and constitutes around 25-30% of the total amount of protein (1, 2). Up to now, 28 different types of collagen have been

discovered (3). As an essential part of the extracellular matrix (ECM), different collagen types can be found in bones, cartilage, tendons and skin, as well as in teeth, cornea and blood vessels (4-6). Collagen is biocompatible and completely biodegradable by endogenous human proteases (7, 8). In addition, it is characterized by its ability to positively influence cell adhesion, cell proliferation, and differentiation (1, 4, 9). These qualities can be further increased by adding growth and differentiation factors to the collagen matrix (10, 11). Antibacterial properties can be developed by adding nanoparticles such as AgNP (12, 13). By additional physical as well as chemical cross-linking (6-8, 14) or the combination of different types of collagen with and without additional bioabsorbable materials, the usually short-lasting degradation time of natural collagen can be further extended, which ensures a sufficient durability (*e.g.*, in wound dressings) (15, 16).

These properties make collagen as one of the most promising biomaterials in modern medicine. Depending on the area of application, it is obtained autogenously, allogeneically or xenogeneically (17, 18). Collagen is widely used as a wound dressing in the treatment of acute or chronic wounds (19), burn wounds (20, 21) or sites of skin donation and skin grafts (22), through its ability of shielding the wound from infection and contamination, reducing scarring, absorbing wound exudate, and promoting the skin's natural regeneration ability (19, 23, 24). In addition, collagen is able to bind platelets and thus activate the coagulation cascade (25, 26), which makes it very suitable for acute use in wound care. Resorbable barrier membranes made of collagen are of great importance in guided bone regeneration (GBR) for dentistry and oral and maxillofacial surgery, in order to shield the regenerating bone from the ingrowth of the surrounding tissue (27-29). Furthermore, different compositions of collagen can be used as tissue grafts in

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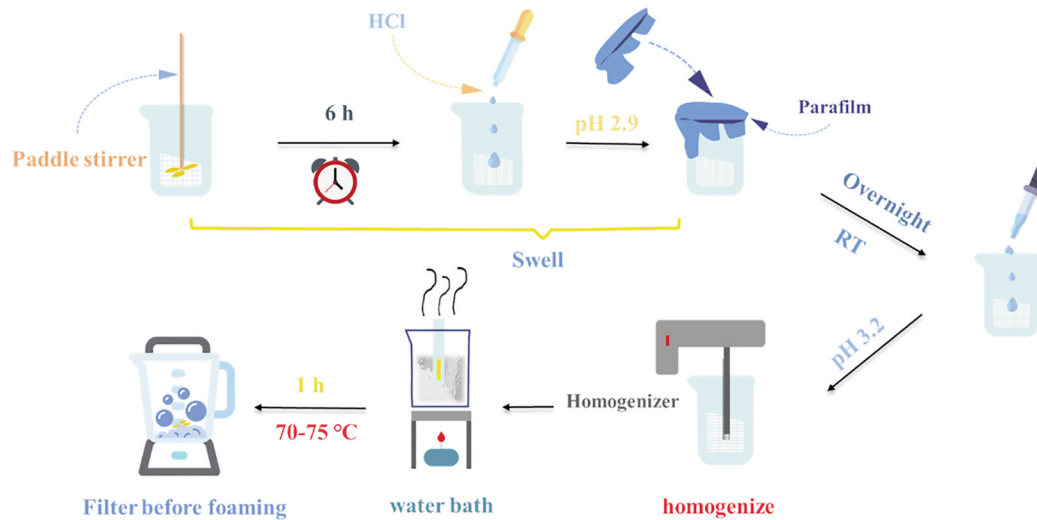


Figure 1. Processing of collagen foam.

peripheral nerve regeneration, vascular prostheses and arterial reconstruction (30-33). In addition, by promoting cell growth and adhesion, collagen is ideally suited as a substance in tissue engineering, where it can be used as part of bioinks to encapsulate cells (34, 35) or as basic substance for scaffold production (2, 22). Thereby, it is essential for the development of artificial skin implants (36, 37).

The present study aimed to develop a new collagen foam based on bovine split skin for tissue regeneration. The regeneration-promoting properties of collagen are extensively described in literature (4, 15, 38). Especially for wound regeneration, collagen foams appear to be a promising approach, since a foam can optimally adapt to a wound bed in terms of area and volume and has both a shielding and cushioning effect on the tissue (19). The approach of using biomaterials as applicable foams in wound management has already been implemented in a different context. The wound foams that have been used up to now are primarily designed for the care of moist or weeping chronic wounds, as their base materials have good exudate-absorbing characteristics (19).

With the use of collagen and the creation of a moisturizing instead of a dehumidifying environment in the wound bed, the foam presented in this study aims to positively influence the self-healing of soft tissue by using the natural regenerative properties of this biomaterial. The newly developed collagen-based foam has the potential to be a new and innovative biomaterial for use in soft tissue regeneration and could play a major role in the care of patients with acute or chronic wounds.

The focus of this preclinical study was to analyze the material characteristics and cytocompatibility of different novel collagen foams. For this purpose, pore size, density and surface structure of the foams were examined and described *ex vivo* using a dynamic foam analyzer (DFA) and

cryo-SEM. In addition, *in vitro* cytocompatibility studies were carried out in accordance with ISO 10993-5/-12, as already described in previous work (24, 39, 40).

## Materials and Methods

**Collagen foam preparation.** For fabricating the collagen foam, bovine split skin was homogenized *via* serial mechanical treatment steps, as illustrated in Figure 1. Briefly, split skin was thawed at 4°C. Then, the split skin was rinsed in double-deionized water (ddH<sub>2</sub>O) which was added at a ratio of 6.5:1 (w/w) to the split skin. The split skin was then rinsed under agitation using a paddle mixer (IKA® Digital 20, IKA® Works, Inc., Wilmington, NC, USA) at 70 rpm for 3 h and pre-homogenized at 720 rpm for another 3 h. The pH value was adjusted to 2.9 with HAc (acetic acid water solution). The suspension was stored at room temperature overnight before further processing. After adjusting the pH value to 3.2, the split skin suspension was homogenized with an IKA® Ultra-Turrax homogenizer (IKA® Works, Inc.) (about 12,000 rpm) for 1 min. The homogenization was then treated in a water bath at 75° for 1 h. The treated suspension was further diluted using 0.1% HAc at a ratio of 1:1 and filtered *via* a Buchner Funnel. The filtered suspension was then foamed using a foaming paddle to form liquid collagen foam. Foams with a final protein concentration ranging from 10 to 50 mg/ml were prepared.

**Ex vivo analyses. Pore structure and foam characterization.** For determination of the bubble size and size distribution of the collagen foams, a dynamic foam analyzer (DFA100, Krüss GmbH, Hamburg, Germany) equipped with a CCD-camera was applied. Size and amount of the bubble fractions were determined *via* the “bubbles’ different projected area” method (Figures 2 and 3).

**Cryo focus ion beam/Scanning electron microscopy (SEM).** Collagen foams with a final concentration of 20 mg/ml were analyzed by cryo-SEM. Briefly, the sample was frozen in liquid nitrogen. Without removing the sample from the liquid nitrogen, it was mounted onto a cooled sample holder. The holder was

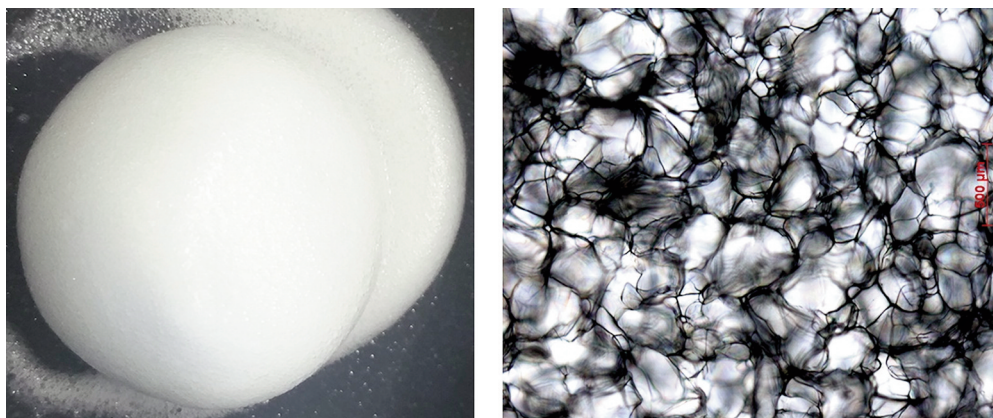


Figure 2. Macroscopic and microscopic imaging of the collagen foam. (A) Freshly prepared collagen foam. (B) Freeze dried foam for light-microscopic slides. As the foam was lyophilized, the pore size was not directly correlated to the pore size in foam.

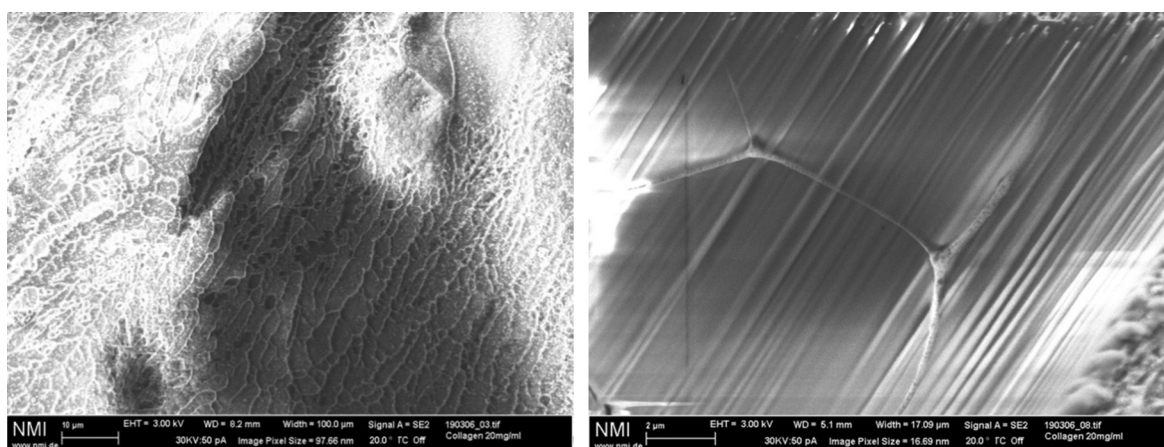


Figure 3. FIB-cryo-SEM imaging of the foam. (A) Image with a full width of 100  $\mu\text{m}$ . The structure of the collagen foam can be observed. The bubbles were organized in an oriented structure during foam formation. (B) Image with a full length of 17.09  $\mu\text{m}$ . The bubble shown in the image has a diameter of about 14  $\mu\text{m}$  and the foam lamella can be observed clearly.

transferred into a vacuum shuttle (Leica EM VCT100, Leica Microsystems GmbH, Wetzlar, Hessen, Germany). After loading, the shuttle was connected immediately to a sputter coater (Balt-Tec SCD 500, Leica Biosystems Division of Leica Microsystems Inc., Buffalo Grove, IL, USA) and evacuated so that the sample could be transferred to the cryo stage of the electron microscope (Zeiss LEO 1540XB with cryo stage, Carl Zeiss Microscopy Deutschland GmbH, Oberkochen, Baden-Württemberg, Germany). The sample was then imaged with the electron beam and the stage was heated until sublimation of the ice matrix could be observed, which occurred around  $-100^{\circ}\text{C}$ . The sample was transferred after coating with about 10 nm of platinum. Using a focused ion beam, the cross section of the samples was prepared and imaged with SEM.

*In vitro experiments.* The cytocompatibility analysis was conducted according to the DIN EN ISO 10993-5: 2009/-12: 2012 regulations as previously published (24, 27, 39). In brief, each of two collagen foam samples with final concentrations of 10 mg/ml or 30 mg/ml were used

for the extract assays. The samples were extracted for  $72 \pm 2$  h in extraction medium at  $37^{\circ}\text{C}$ , 5%  $\text{CO}_2$  and 95% humidity. The extraction medium was then transferred to L-929 mouse fibroblasts, purchased from the European Collection of Cell Cultures, ECACC (Salisbury, UK) and incubated with the cells at standard cell culture conditions;  $37^{\circ}\text{C}$ , 5%  $\text{CO}_2$  and 95% humidity for 24 h. Viability, proliferation and cytotoxicity determinations were carried out using the XTT assay (Roche Diagnostics, Mannheim, Germany), BrdU ELISA (Roche Diagnostics) and LDH assay (BioVision, Milpitas, CA, USA) in four determinations for each test sample. As comparative materials with expectable biocompatibility, Jason<sup>®</sup> membrane and Cerabone<sup>®</sup> (both from botiss biomaterials GmbH, Zossen, Germany) were used for additional extract assays and examined under the same conditions, as already described above. Blank values (only medium without cells, also in quadruple determination) were subtracted from all values. Furthermore, RM-A test samples [polyurethane film with 0.1% zinc diethyldithiocarbamate (ZDEC) (Hatano Research Institute, Food and Drug Safety Center, Hadano, Japan) were used as positive

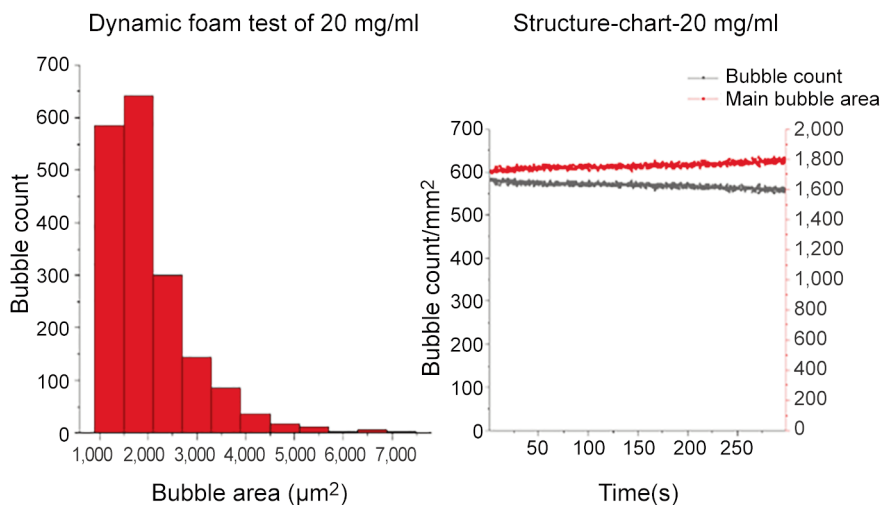


Figure 4. Bubble counts and distribution. The main fraction of the collagen foam has a bubble area of 1,000 to 2,000  $\mu\text{m}^2$ , which is equivalent to an average diameter of about 36 to 50  $\mu\text{m}$ .

control materials. As negative control, grade 4 titanium plates were incubated under the above-described extraction conditions.

**Statistics.** An analysis of variance (ANOVA), which enabled comparison of the data from the study groups *via* the GraphPad Prism 8.0 software (GraphPad Software Inc., La Jolla, CA, USA) was conducted for statistical analysis. Statistical differences were designated as significant if the  $p$ -values were less than 0.05 ( $*p \leq 0.05$ ), and highly significant if the  $p$ -values were less than 0.01 ( $**p \leq 0.01$ ) or less than 0.001 ( $***p \leq 0.001$ ). Finally, the data are shown as mean  $\pm$  standard deviation.

## Results

**Results of the *ex vivo* measurements.** DFA bubble structure and distribution depending on collagen density.

The number of bubbles and the size distribution of the collagen foam bubbles were analyzed *via* DFA and shown in Figure 4. The left panel shows the real-time images of the bubbles within the foams. The green dots show the relative uniform sizes of the produced foam. Projection areas of the most foam bubbles were measured between 1000  $\mu\text{m}^2$  and 2000  $\mu\text{m}^2$ , indicating diameters ranging from 46  $\mu\text{m}$  to 94  $\mu\text{m}$ . The largest bubble population had 28-48  $\mu\text{m}$  diameter (20 mg/ml). The black line and red line for bubble count and mean bubble area, depending on time, showed the relatively high stability and uniformity of the prepared foam.

**Results of the *in vitro* measurements.** According to ISO 10993-5:2009, non-toxic ranges are defined for values  $>70\%$  of the blank sample for BrdU and XTT assays and for values  $<130\%$  of the blank sample for LDH assays. The collagen foam showed satisfactory biocompatibility in both concentrations tested (Figure 5). Thereby, a significant

difference ( $p \leq 0.001$ ) compared to the positive control was shown in all three assays. In the BrdU and XTT assays, only a minor significant difference ( $p \leq 0.01$ ) compared to the negative control was measured at the concentration of 10 mg/ml. At the concentration of 30 mg/ml, however, there was no significant difference compared to the negative control. Similar results were obtained from the LDH assay, where no significant difference between the negative control and the collagen foam with a concentration of 30 mg/ml was detected. Instead, there was a significantly greater difference ( $p \leq 0.001$ ) between the negative control and the collagen foam with a concentration of 10 mg/ml. The results of Cerabone<sup>®</sup> and Jason<sup>®</sup> membrane should also be mentioned. While the Jason<sup>®</sup> membrane reached the areas defined as non-toxic in all three assays carried out and therefore, demonstrated convincing biocompatibility, Cerabone<sup>®</sup> reached the required limit values in BrdU and XTT assay, but showed an increase in the LDH assay, which is clearly in the cytotoxic range and even exceeds the value of the positive control, suggesting possible deficits in biocompatibility. In addition, the noticeably low value of the Jason<sup>®</sup> membrane in the LDH assay has to be considered.

## Discussion

Collagen-based biomaterials are an essential element for soft tissue management with special focus on wound care. For example, collagen materials are frequently used for acute and chronic wounds as well as burns after surgical interventions (15, 16, 19, 20). The advantage of collagen is based on its proliferation-, differentiation- and adhesion-promoting properties, which favor early vascularization and therefore rapid tissue regeneration (1, 4, 9). In addition,

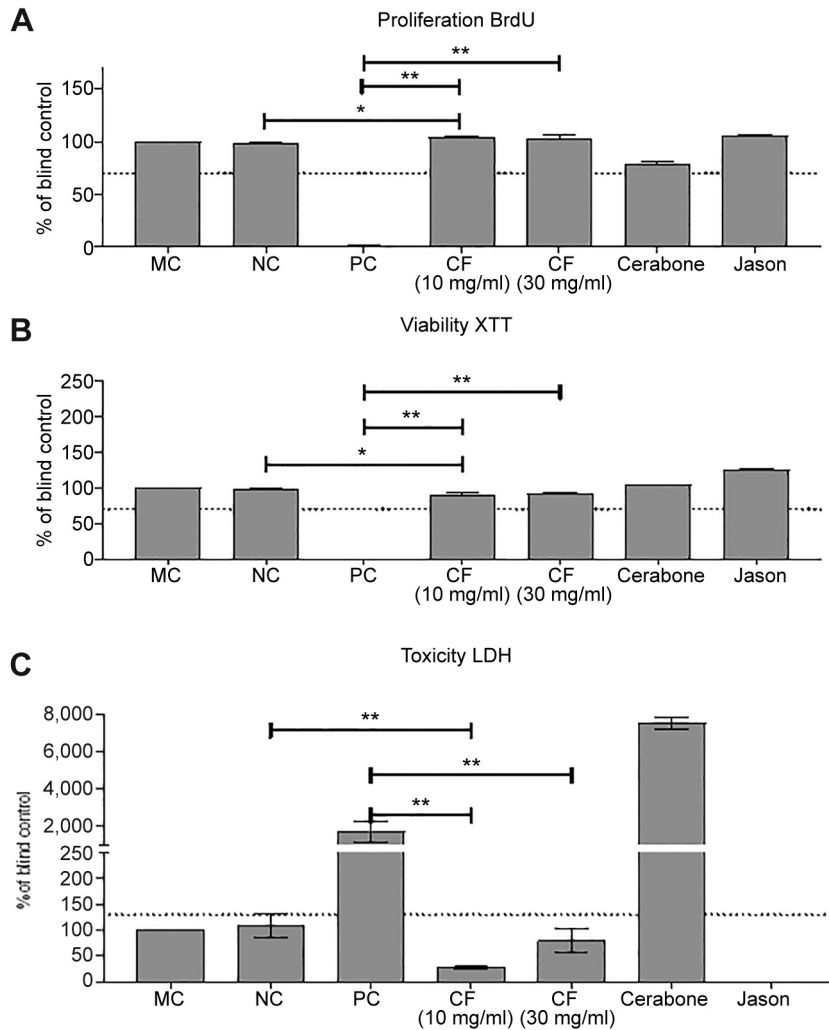


Figure 5. BCytocompatibility results using L929 cells in the different assays. (A) proliferation measured by BrdU assay; (B) viability measured by the Sodium 3,3'-[1(phenylamino)carbonyl]-3,4-tetrazolium]-3-is(4-methoxy-6-nitro) Benzene Sulfonic acid Hydrate (XTT)-assay; (C) cytotoxicity measured by the Lactate Dehydrogenase (LDH) assay. Values were normalized against blind control. Means with error bars indicate standard deviations. Dotted line indicates thresholds which should not be exceeded (LDH) or undershot (XTT, BrdU). Significant differences are declared (\* $p \leq 0.01$ , \*\* $p \leq 0.001$ ). MC: Medium control; NC: negative control (titanium grade 4); PC: positive control; CF: collagen foam.

collagen-based biomaterials are able to absorb liquids many times of their own weight and bind reactive oxygen as well as nitrogen species, which could also interfere with tissue regeneration (41, 42).

Until today, biomaterials for soft tissue regeneration like wound foams consist mainly of polyurethanes, hydro fibers or mixtures of these two materials, which absorb wound exudate in large volumes and thereby, create favorable conditions for wound healing (43, 44). Depending on the manufacturer, some foam dressings are additionally coated with silicone layers, which fixate the foam in the wound bed. Furthermore, foam dressings provide thermal isolation and protect the wound from bacteria and infections (43, 44). Another favorable advantage of current foams is their ability

to completely fill out the wound beds, which reduces the remaining dead space for bacterial colonization and possible infections (45-47).

However, current available foam dressings also feature some disadvantages. Due to their strong fluid-absorbing properties, they are suitable for use on moderately to severely exudative wounds, but are contraindicated *e.g.*, for use in very dry or necrotic wound areas (47). Most foams can be left on the wound bed for up to a week before they need to be removed (48). However, with removal there is always the risk of additional shear stress to the already agitated lesion, especially if newly formed tissue has already grown into the foam. The same applies to patients with very sensitive skin and the use of additional fixations for the foam

dressings, which have to be removed with change of the dressing and thus can damage the newly formed tissue.

By using collagen as the basic material for a new type of wound foam, it could be possible to combine the regeneration-promoting properties of collagen with the advantages of a flexible foam, being able to adapt to any size and volume of the wound bed. A major advantage would be the ability to integrate the collagen directly into the wound bed as part of the newly formed ECM, which could make subsequent removal unnecessary and constantly supports tissue regeneration. Furthermore, by adjusting the liquid content of the foam, a moistening environment can be achieved for special indications like dry wounds. Therefore, the aim of the present study was to examine the macro- and micro-structure of an innovative collagen foam *ex vivo* and to investigate its cytocompatibility *in vitro* according to DIN ISO 10993. This new foam dressing could address and improve wound management for a broad range of applications.

First of all, the structure of the newly created collagen foam with a final concentration of 20 mg/ml was examined *ex vivo* using FIB-cryo-SEM and DFA. It could be shown that the bubbles in the foam were mainly uniform in diameter (~36-50  $\mu\text{m}$ ) and area (~1,000-2,000  $\mu\text{m}^2$ ) and also distributed homogeneously within the foam (~600/mm<sup>2</sup>). Furthermore, the number and size of the bubbles remained constant over the total observation period of 5 min. These results suggest that the process used to produce the collagen foam can create a uniform microstructure within it. Collagen, as a natural component of human ECM, has binding sites for adhesion of fibroblasts, macrophages and epithelial cells, which is utilized in the creation of wound sponges or scaffolds in 3D-printing, both with defined pore sizes (49, 50). It is assumed that a constant pore size between 100-200  $\mu\text{m}$  is optimal to enable the surrounding cells to proliferate and adhere to the surrounding porous structure (49, 50). These observations suggest that collagen foams should also provide this ideal pore size and distribution, with additional beneficial effect on tissue regeneration. In reference to our own measured values for the projection area and diameter of the bubbles formed, we assume similar advantageous attributes for enabling the foam to have a positive effect on the adhesion and ingrowth of the surrounding connective tissue cells. These possibilities should be further addressed in future studies. Furthermore, bubble size seems to decrease with an increasing viscosity of the foam. Since a higher viscosity could ensure a better hold within the wound and thus make the use of additional fixation systems redundant, it is important to carry out further investigations addressing more closely with the above-mentioned hypotheses.

*In vitro*, the foam showed sufficient cytocompatibility in all colorimetric assays for both tested concentrations. It could be shown that there were no significant differences to

the negative control for the higher concentration (30 mg/ml), while mild significant differences ( $p \leq 0.05$ ) to the negative control were found for the lower concentration (20 mg/ml), which, however, appear to be negligible. The results of the reference materials for soft and hard tissue regeneration, Cerabone® and Jason® membrane, showed both good biocompatibility in the BrdU and XTT assays. However, unusual values were measured for both materials in the LDH assay. While the Jason® membrane did not seem to produce evaluable values in the assay, Cerabone® showed values that are even above the positive control and thus suggest clearly cytotoxic reactions. This might be explained by soluble non-toxic components of the materials, which interfere with the LDH assay. This can be prevented in the future by testing the materials with and without cells by subsequent subtraction of the values without cells from the values with cells as already shown by Jung *et al.* (51).

The results obtained for the collagen foam in the present study are congruent with previous observations, revealing that collagen, as natural and ubiquitous component of ECM, shows little to no damaging effects on the surrounding tissue, thus having sufficient biocompatibility (17, 52, 53). However, additional *in vitro* assays could be used in order to analyze the properties and regenerative qualities of the foam in an even more differentiated manner. For example, it would be possible to further survey the differentiation processes of stem cells or primary cell lines that are potentially induced by the foam (54, 55). In addition, quantitative and qualitative measurement of the release of pro- or anti-inflammatory cytokines by immunologically active cell lines would give additional information about inflammation processes in the presence of the collagen foam. Nevertheless, the presented *in vitro* results require future *in vivo* investigations in order to analyze tissue reactions of the foam using histological, histomorphometrical and immunohistochemical assays. In this context, additional loading of the foam, *e.g.*, with silver nanoparticles or platelet-rich plasma (PRP), could be an interesting approach. The noticeable values of the reference materials in the LDH assay could be explained by the high sensitivity of the assay, which could be also considered as possible reason for the high values shown for Cerabone®. Another reason for this measurement value can be found in the high release of calcium and phosphate ions, which might have a slight cytotoxic effect, even in case of the used static cell culture plates. This material has manifoldly been proved to be biocompatible in both preclinical and clinical studies, so that it is assumable that the ion release might not interfere with the bone healing process in *in vivo* situations due to the implant bed vascularization and the related tissue perfusion.

Altogether, the present study shows satisfactory *in vitro* compatibility of the newly developed collagen foams. The analysis of the microstructure reveals the effectiveness of the manufacturing process to create a homogeneous microstructure

of the foam with mostly consistent bubble size and diameter. Taken together, these results suggest that collagen foams show the potential to be a promising new biomaterial for tissue regeneration, especially for wounds. Future additional studies, particularly addressing tissue responses *in vivo*, are required to emphasize the present results.

### Conflicts of Interest

The Authors declare no conflicts of interest in relation to this study.

### Authors' Contributions

Conceptualization, O.J., M.B. and X.X.; methodology, O.J., M.B. and X.X.; software, X.X.; validation, O.J. and M.B.; formal analysis, O.J. and S.P.; Preparation of collagen foams and mixture with BSM, C.F.Z and F.K.; the DFA analysis and data calculation, C.F.Z and F.K.; investigation, O.J. and M.B.; resources, O.J. and M.B.; data curation, S.P.; writing—original draft preparation, O.J. and S.P.; writing—review and editing, M.B. and X.X.; visualization, S.P.; supervision, O.J. and M.B.; project administration, O.J., M.B. and X.X.; funding acquisition, O.J., M.B. and R.K.

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