



ORIGINAL ARTICLE

Crayfish hemocyanin on chitin bone substitute scaffolds promotes the proliferation and osteogenic differentiation of human mesenchymal stem cells

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Abstract

Crustacean chitin–hemocyanin–calcium mineral complexes were designed as bone biomimetics, with emphasis on their ability to bind or release calcium ions. Chitin scaffolds were prepared by dissolving chitin flakes in LiCl/dimethylacetamide, followed by gel formation and freeze-drying. Some of these scaffolds were modified by incorporation of CaCO₃. In some of the chitin–CaCO₃ scaffolds, macroporosity was introduced by HCl treatment. Hemocyanin from the crayfish *Cherax quadricarinatus* was used to further modify the chitin scaffolds by dip coating. Cytocompatibility, cellular adherence and proliferation of human mesenchymal stem cells (hMSCs) were evaluated in terms of cell number as reflected in lactate dehydrogenase activity. The chitin, chitin–CaCO₃, and porous chitin–CaCO₃ scaffolds were all found to facilitate cell attachment. Hemocyanin dip-coating of these scaffolds led to increased initial cell adhesion, enhanced proliferation, and osteogenic differentiation. Since the hemocyanin loading of the scaffolds was impaired by sterilization by gamma-irradiation (as required for biomedical applications), the hemocyanin loading was performed on previously sterilized scaffolds. All scaffolds facilitated osteogenic differentiation of osteoblasts, with the highest cell ALP-activity being found on hemocyanin-modified porous chitin–CaCO₃ scaffolds. Thus, chitin–hemocyanin scaffolds enhanced the initial stages of bone cell development and could serve as promising biomaterials for bone regeneration.

KEYWORDS

calcium carbonate, chitin, crayfish, hemocyanin, human osteoblasts

1 | INTRODUCTION

This study focuses on modified chitin as a bio-scaffold for bone tissue engineering. Chitin is the second-most abundant polysaccharide in

nature after cellulose, but as a biomaterial for medical applications, it is not as common as its deacetylated derivative, chitosan. Nonetheless, there is a continuously growing body of research on chitin-based biomaterials (Deepthi, Venkatesan, Kim, Bumgardner, & Jayakumar, 2016); for example, chitin has been processed into membranes (Nagahama et al., 2008), nanofibers (Jayakumar, Prabakaran, Nair, &

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Tamura, 2010), and hydrogels (Tamura, Nagahama, & Tokura, 2006), including injectable (Arun Kumar et al., 2016) and scaffold (Jayakumar et al., 2011) hydrogels. Despite the interest in chitin scaffolds as matrixes for producing bone graft materials (Deepthi et al., 2016; Muzzarelli, 2009; Tamura, Furuike, Nair, & Jayakumar, 2011), only a few studies have used plain nonmodified chitin in tissue engineering research, with most of the work being centered on cell cultures on chitosan-based materials (Aljawish, Muniglia, & Chevalot, 2015; Cho et al., 2004; Costa-Pinto et al., 2009; Dashtdar et al., 2015; Lai, Shalumon, & Chen, 2015; Lindborg et al., 2015; Omidvar, Ganji, & Eslaminejad, 2016; Zhao, Grayson, Ma, Bunnell, & Lu, 2006). Among these studies, two focused on culturing human mesenchymal stem cells (hMSCs) on chitosan composite scaffolds/hydrogels; two demonstrated proliferation and osteogenic differentiation of the hMSCs (Costa-Pinto et al., 2009; Zhao et al., 2006); and two others showed differentiation into chondrogenic (Cho et al., 2004) and adipogenic (Lindborg et al., 2015) phenotypes. However, in vitro studies on nonmodified chitin have been conducted only with cell lines (Austin, 1984; Chow & Khor, 2000; Rejinold et al., 2012; Terbojevich, Carraro, Cosani, & Marsano, 1988; Wan & Tai, 2013) or with mesenchymal stem cells (MSCs) derived from nonhuman sources (Sum Chow, Khor, & Chwee Aun Wan, 2001; Yan et al., 2010). It appears that a single exception is a tissue engineering study on the influence of hydroxyapatite modification of β -chitin membranes for culturing hMSCs (Madhumathi et al., 2009).

Two technological challenges have to be addressed in the development of chitin-based scaffolds for bone tissue engineering—the insolubility of chitin in common solvents and the macroporosity required to ensure an adequate blood supply and bone resorption and regeneration (Hing, 2004). The first challenge was solved in this study by the use of dimethylacetamide (DMA), supplemented with 5% LiCl (Austin, 1984; Chow & Khor, 2000; Terbojevich et al., 1988). This is one of the four solvents known to dissolve chitin (the other three are 12 N HCl (Kuo & Ku, 2008), methanol saturated with CaCl_2 (Tamura et al., 2006) and NaOH/urea (Chang, Chen, & Zhang, 2011)). The second challenge, namely, the introduction of macroporosity into scaffolds to be used for in vitro or in situ tissue engineering was solved by the addition of CaCO_3 to the chitin solution and a subsequent HCl treatment. The gaseous CO_2 produced by the reaction of HCl and CaCO_3 led to the formation of pores, as previously reported (Chow & Khor, 2000; Sum Chow et al., 2001), and further salt leaching of the remaining CaCO_3 crystals, which were only partially dissolved, later detached easily from the scaffold, also contributed to pore formation. Another advantage of using CaCO_3 lies in its ability to serve as a source of calcium ions, which are essential in tissue engineering applications. Calcium ions act both as an inorganic component of the bone composite material and as an osteogenic stimulant, where the extracellular calcium ion concentration has a significant influence on both MSCs (osteoblast progenitor cells) (González-Vázquez, Planell, & Engel, 2014) and monocytes (osteoclast progenitor cells) (Glenske et al., 2014). The extracellular calcium concentration in the vicinity of a transplanted biomaterial is therefore of crucial importance for the healing of damaged host bone tissue.

Indeed, several biomaterials have the ability—known as their bioactivity—to influence the calcium concentration in their environment and thus to induce a specific biological reaction at the interface between the biomaterial and the mineralized host tissue, that is, the formation of a calcium phosphate layer at the surface (Cao & Hench, 1996). High bioactivity facilitates an intimate connection between the biomaterial and the bone, but also leads to local calcium depletion, with negative effects on cell populations and their activity in the bone remodeling zone (Malafaya & Reis, 2009). In contrast, low bioactivity in calcium-containing biomaterials leads to increased extracellular calcium concentrations. In bone tissue engineering, the bioactivity of a biomaterial can thus be used to manipulate the calcium-dependent osteoblast/osteoclast ratio in vitro (Heinemann et al., 2013) and in vivo. With the above possibility in mind, we sought to build on the ability of naturally occurring chitin–hemocyanin–calcium mineral complexes to bind and release calcium ions with the aim to produce biomimetic composite materials for bone replacement. Crustacean hemocyanin, whose main role lies in oxygen transport in the hemolymph, also functions as a phenoloxidase (Glazer et al., 2013) and, as such, is involved in the crustacean immune response (Coates & Nairn, 2014). In addition, there is increasing evidence that hemocyanin is involved in the sclerotization of the new exoskeleton after ecdysis (Adachi, Endo, Watanabe, Nishioka, & Hirata, 2005), where the cuticular exoskeleton is composed of chitin in association with cuticular proteins. In the remodeling of the chitin network of the cuticular exoskeleton, these cuticular proteins play a variety of roles in processes such as chemical modification (Dixit et al., 2008) and cross-linking (Adachi et al., 2005; Andersen, 2010) of chitin microfibrils as well as in immunological defense (Rosa & Barracco, 2010) and pigmentation (Glazer & Sagi, 2012; Jasarapuria et al., 2010; Wade, Tollenaere, Hall, & Degnan, 2009). During each molt cycle, the formation of the new cuticular chitinous structure with its associated proteins, including hemocyanin, is a prerequisite for the deposition of CaCO_3 , either as crystalline calcite or as amorphous CaCO_3 (Lowenstam & Weiner, 1989; Shechter et al., 2008), stabilized by association with peptides or proteins (Luquet & Marin, 2004; Shechter et al., 2008) or with organic and inorganic ions and molecules (Aizenberg, Addadi, Weiner, & Lambert, 2018; Al-Sawalmih et al., 2008; Clarkson, Price, & Adams, 1992; Loste, Wilson, Seshadri, & Meldrum, 2003). In light of the above-described chitin–hemocyanin–calcium association and our previous study showing the potential utility of chitosan-based materials as scaffolds for bone substitution (Kruppke et al., 2017), we posited that a bio-scaffold based on a chitin–hemocyanin–calcium composite would be a suitable material for bone tissue engineering.

The present study thus aimed to develop a biomimetic chitin–calcium–hemocyanin composite as a scaffold material supporting the proliferation and differentiation of osteogenic cells in vitro. Our biomimetic engineering approach to pursuing the above goals comprised the following stages: preparation of chitin scaffolds, incorporation of CaCO_3 , enhancement of porosity, and dip coating with hemocyanin from the crayfish *Cherax quadricarinatus*, followed by cell-culture experiments with hMSCs.

2 | EXPERIMENTAL SECTION

2.1 | Preparation of chitin scaffolds

Four types of chitin scaffold were prepared: plain chitin, chitin modified with CaCO_3 (designated chitin- CaCO_3), porous chitin- CaCO_3 , and HCl-treated chitin (Table 1). For the preparation of the plain chitin scaffolds, a clear homogeneous viscous solution of 0.5% chitin was prepared by dissolving 0.5 g of chitin flakes with a particle size of 0.2 mm (Hepe Medical Chitosan GmbH) in 100 mL of 5% LiCl in DMA, as described previously with minor modifications (Chow & Khor, 2000; Sum Chow et al., 2001). Briefly, anhydrous LiCl was further dried at 130°C for 30 min and, after cooling to room temperature, the completely anhydrous salt was dissolved in DMA with magnetic stirring. The chitin flakes were then dissolved in the LiCl/DMA solvent system overnight with stirring at room temperature. Aliquots of 2.5 g or 5 g of the chitin solution were then dispensed into cylindrical glass containers of 2.5 cm diameter \times 4 cm height. The solvent was allowed to evaporate off under a fume hood at room temperature for 3 days. Solvent residues were removed from the resulting gels by soaking them several times in deionized water. After washing, the gels were dried to plain chitin scaffolds by lyophilization for 32 h in a freeze-dryer (Epsilon 2-4 LSC, Martin Christ Gefriertrocknungsanlagen).

To prepare chitin- CaCO_3 scaffolds, 1 g of CaCO_3 was added to a solution of 0.5 g of chitin in 100 mL of 5% LiCl/DMA. After allowing the solution to stand overnight, the resulting gels were dried to scaffolds as described above. Porous chitin- CaCO_3 scaffolds were obtained by treating the chitin- CaCO_3 scaffolds with 1 N HCl for 2 h, leading to the formation of gaseous carbon dioxide and hence to open-pore matrices (these scaffolds are designated chitin- CaCO_3 /HCl in respective figures) (Chow & Khor, 2000; Sum Chow et al., 2001). To obtain scaffolds for purposes of comparison to the HCl-treated porous chitin- CaCO_3 scaffolds, plain chitin gels

were also reacted with HCl. The resulting gels were washed several times with deionized water to remove solvent and HCl residues and lyophilized for 32 h in a freeze-dryer. Disk-shaped scaffolds with a diameter of 8–10 mm and a height of 1–3 mm were thus obtained (Figure 1). Scanning electron microscopy (SEM) was used to examine the morphology and surface structure of the scaffolds. For SEM observations, the samples were mounted on stubs and coated with carbon in an SCD 050 coater (Balzers). SEM was carried out on an ESEM XL 30 (Philips) in Hi-Vac mode with 3 kV high tension. The samples were cut manually with a scalpel and the cross-sectional areas were measured by means of energy-dispersive X-ray spectroscopy (EDS) at 10 keV. EDS was applied as a semiquantitative method for standardless analysis and two-dimensional visualization of oxygen, calcium, and carbon in the scaffold's cross-sections.

2.2 | Scaffold modification with crayfish hemocyanin

Hemolymph was obtained with a syringe from the base of the 5th walking legs of pre-molt *C. quadricarinatus* male crayfish at Ben-Gurion University of the Negev (Israel). Pooled hemolymph from a number of crayfish was mixed with 7% ethylenediaminetetraacetic acid (EDTA) in a 1:1 ratio, followed by the addition of 2 mM of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF, Sigma). The diluted hemolymph was centrifuged at 1500 relative centrifugal force (rcf) for 15 min to remove cells and debris. Thereafter, the hemolymph supernatant was mixed with a saturated solution of sodium bromide to a density of 1.22 g/mL for density gradient separation. Pure hemocyanin was obtained by first subjecting the brominated hemolymph to ultracentrifugation at 100,000 rcf for 48 h. The grey fraction was then collected and dialyzed against phosphate-buffered saline (PBS), filtered, and validated by using western blot analysis with a specific hemocyanin antibody or by MS/MS. The purified protein so obtained was lyophilized in several batches, each of 12.5 mg. Scaffold modification with hemocyanin was carried out as described below either before (designated presterilization modification) or after (designated poststerilization modification) sterilization with gamma-irradiation.

For the presterilization modification, plain chitin or chitin- CaCO_3 gels were washed several times with water and loaded with hemocyanin by dip coating, as follows. The washed disk-shaped gels were incubated in lyophilized hemocyanin dissolved in deionized water (0.4 mg/mL) for 3 days at 4°C. After dip coating, the samples were lyophilized for 32 h. Both hemocyanin-modified and unmodified scaffolds were then sterilized with gamma-irradiation of 25 kGy. Alternatively, for the poststerilization modification, scaffolds sterilized by exposure to gamma-irradiation were incubated for 3 days at 4°C in hemocyanin dissolved under sterile conditions in deionized and autoclaved water (0.4 mg/mL). After this dip coating, the modified scaffolds were dried again for 32 h in a freeze-dryer.

TABLE 1 Overview of the chitin-based scaffolds produced and the hemocyanin modifications used

Scaffold	Process	Hemocyanin
None (polystyrene)		100, 10, or 1 $\mu\text{g}/\text{mL}$ or without in culture medium
Chitin	Lyophilized	Dip-coating presterilization and after sterilization
Chitin/HCl	HCl-treated plain chitin scaffolds; lyophilized	Dip-coating after sterilization
Chitin- CaCO_3	Chitin modified with CaCO_3 ; lyophilized	Dip-coating after sterilization
Chitin- CaCO_3 /HCl (porous chitin- CaCO_3)	Chitin- CaCO_3 scaffolds HCl-treated, lyophilized	Dip-coating after sterilization

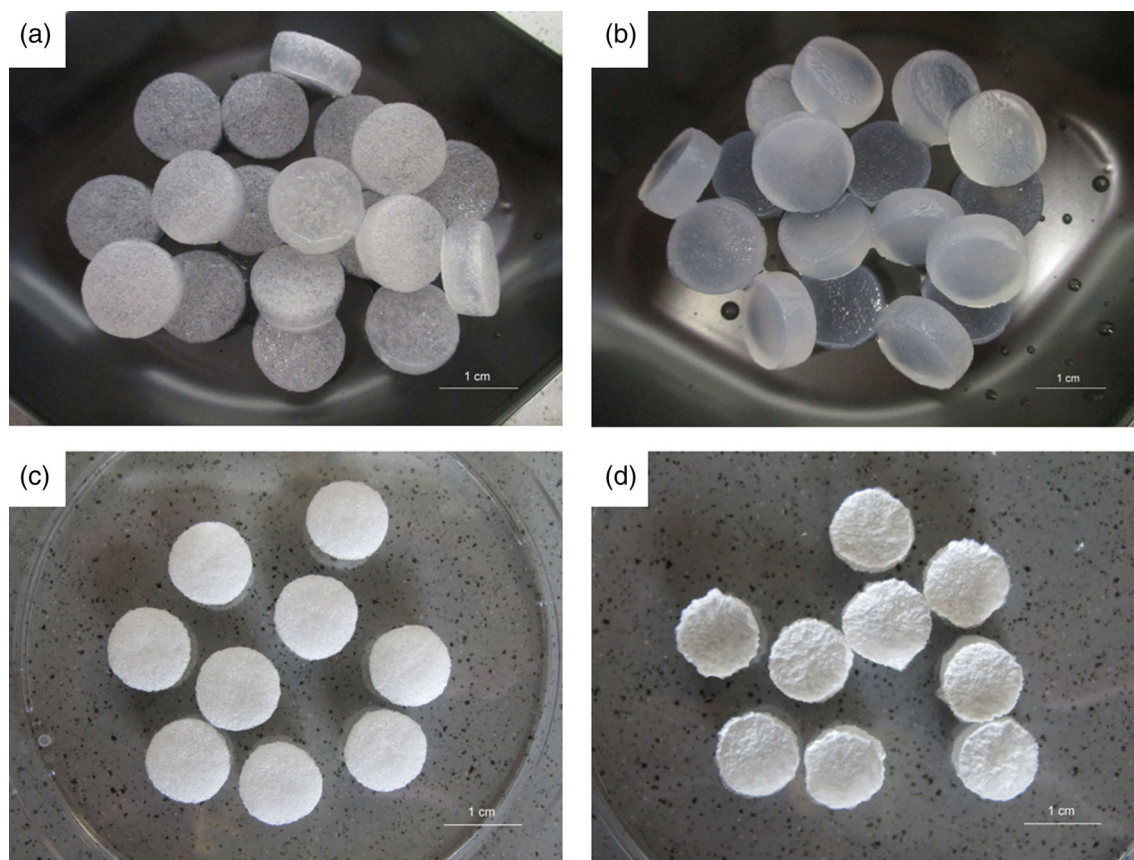


FIGURE 1 Chitin scaffolds after HCl treatment. Scaffolds with CaCO_3 (a and c) and without CaCO_3 in the chitin hydrogels (b and d). Scaffolds without lyophilization (top) and after lyophilization (bottom). Scale bar 1 cm

2.3 | Determination of immobilized hemocyanin content of the chitin scaffolds

To determine the hemocyanin content of the hemocyanin-modified chitin scaffolds, each scaffold disk was weighed, homogenized in 150 μL of TBS buffer (10 mM Tris, pH 7.6, with 150 mM NaCl) and then sonicated for 15 min. After sonication, the samples were centrifuged (12,000 rcf, 5 min), and each supernatant was transferred to new tube for separation by SDS-polyacrylamide gel electrophoresis (PAGE), as follows. The supernatant was incubated with sample buffer (Tris-HCl, pH 6.8, SDS, glycerol, β -mercaptoethanol, EDTA) for 3 min at 100°C for protein denaturation and then loaded onto a 10% SDS-PAGE gel together with several samples of hemocyanin of known concentrations. The bands on the gel were visualized by Coomassie Blue staining, and optical density was determined using Quantity One Software (BIO-RAD). From the optical density, the amount of hemocyanin in each sample was calculated according to the total homogenization volume and the actual volume loaded on the gel. It should be noted that some residual hemocyanin and other proteins might be present in the purified chitin obtained from Heppes Medical Chitosan, since its source is natural crustacean cuticle (King, Stein, Shamshina, & Rogers, 2017; Synowiecki & Al-Khateeb, 2003).

2.4 | Cell culture experiments

With the patients' consent and with the approval of the local ethics committee (10.12.2004: EK263122004), hMSCs were obtained from bone marrow aspirates at the Medical Clinic I, University Hospital Carl Gustav Carus Dresden of the Technische Universität Dresden. Cells were maintained and passaged in an expansion medium [Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (FCS), 1% penicillin-streptomycin and 2 mM L-glutamine] in a humidified atmosphere with 5% CO_2 at 37°C. For the cell-culture experiments, passage 5 was used. All the media and supplements were obtained from Biochrom Ltd.

2.4.1 | Influence of hemocyanin on hMSCs

For cytotoxicity testing, the hMSCs were seeded at a density of 50×10^3 cells in 96-well polystyrene plates (without chitin scaffolds) and incubated in a humidified atmosphere with 5% CO_2 at 37°C for 1 h. The cytotoxicity tests were started 1 h after seeding by the addition of proliferation medium [α -minimum essential medium (α -MEM) supplemented with 10% FCS, 1% penicillin-streptomycin and 2 mM L-glutamine], containing 1, 10, or 100 $\mu\text{g}/\text{mL}$ hemocyanin. To

induce differentiation of hMSCs into osteoblasts, the medium was changed 3 days after cell seeding to an osteogenic medium (α -MEM supplemented with 10% FCS, 1% penicillin–streptomycin, 2 mM L-glutamine, and the osteogenic supplements 10 nM dexamethasone, 10 mM β -glycerophosphate, and 50 μ M ascorbic acid-2-phosphate). On days 7, 14, 21, and 28, the cells were rinsed in PBS at 37°C and stored at –80°C for later biochemical analysis (described below). A control group (three replicates) was incubated in the same media but without hemocyanin under the same conditions. For all cultures, the medium used was exchanged twice a week.

2.4.2 | Adhesion, proliferation, and differentiation of hMSCs on chitin scaffolds

For the adhesion and proliferation tests, the various scaffolds were pre-incubated in cell culture medium (proliferation medium as described above) for 24 h. The hMSCs were then seeded at a density of 20×10^3 – 50×10^3 cells by drop seeding directly onto the plain chitin, HCl-treated chitin, chitin–CaCO₃, and porous chitin–CaCO₃ scaffolds either with or without hemocyanin modification. To investigate whether replacing the medium with fresh medium after the 24-h pre-incubation would have a positive impact on cell adhesion and whether it would cause partial or complete removal of hemocyanin from the scaffolds, the cells were seeded directly on one half of the pre-incubated scaffolds without changing the medium and on the other half of the scaffolds after replacing the pre-incubation medium with fresh medium. Thereafter, the scaffolds were incubated in a humidified atmosphere with 5% CO₂ at 37°C in cell-culture well plates. Four days after cell seeding, the medium was replaced with an osteogenic medium to induce osteogenic differentiation. Both types of medium, proliferative and osteogenic, were exchanged twice a week, that is, alternately after 3 or 4 days. Twenty-four hours after the initial seeding and on days 7, 14, 21, and 28 the cells were rinsed in PBS at 37°C and then stored at –80°C for later biochemical analysis.

Cell adhesion efficiency (in %) was determined as the ratio of the number of adherent cells after 24 h to the initial cell number of seeded cells \times 100. To quantify the cell adherence, the relevant scaffolds were washed in PBS to rinse off nonadherent cells. The cells on the scaffolds were fixed in 2.5% glutaraldehyde for 20 min at 4°C. The samples were stored in 0.8% glutaraldehyde at 4°C. Dehydration was performed in a graded ethanol series followed by critical-point drying using a CPD 030 apparatus (Bal-tec).

2.5 | Biochemical analysis

Cell-infiltrated chitin scaffolds (stored as described above) were thawed for 30 min on ice and subsequently incubated in 0.5 mL lysis buffer (PBS with 1% Triton X-100) for 50 min. The cellular lactate dehydrogenase (LDH) released by the lysis was determined as a quantitative marker for cell number (required for the study of

cytocompatibility and cellular adhesion and proliferation and for calculation of alkaline phosphatase levels (ALP) in vitro). For analysis of LDH, a commercial LDH-Cytotoxicity Detection Kit (Takara) was used according to the manufacturer's instructions. Absorbance of the formazan product thus created was determined with an UV/vis spectrometer (Tecan) at 492 nm. Since formazan concentrations are directly proportional to the concentration of LDH (Allen, Millett, Dawes, & Rushton, 1994), a calibration curve was constructed from 2×10^5 , 1.5×10^5 , 1×10^5 , 7.5×10^4 , 5×10^4 , 2.5×10^4 , 1×10^4 , and 0.5×10^4 cells that had been lysed as described above. Lysis buffer and scaffolds without cells were used as blanks.

ALP activity—as a marker of osteogenic differentiation—was determined spectroscopically at 405 nm using *p*-nitrophenyl phosphate (Sigma) as the substrate. After incubation of 125 μ L of 1 mg/mL substrate solution (2 mg *p*-nitrophenyl phosphate in 0.1 M diethanolamine, 0.1% Triton X-100, and 1 mM MgCl₂, pH 9.8) with 25 μ L of lysate for 30 min at 37°C, the reaction was stopped with 50 μ L 1 N NaOH. Absorption was measured with a UV/Vis spectrometer (Tecan) at 405 nm. A calibration curve was constructed from different concentrations of *p*-nitrophenol. Lysis buffer and scaffolds without cells were used as blanks. The ALP activity was normalized to the LDH activity (per cell).

2.6 | Statistics

All measurements were performed at least in triplicate and are expressed as means \pm standard deviation. One- and two-way analysis of variance (ANOVA) with Tukey post hoc test was applied for statistical analysis. *p* values <0.05 were considered significant (and are indicated by an asterisk on the figures).

3 | RESULTS

3.1 | Cytocompatibility of hemocyanin with hMSCs

To study cytocompatibility of hemocyanin per se (without the influence of chitin and/or scaffold surface morphology), hMSCs were seeded on polystyrene and incubated with three different hemocyanin concentrations, 100, 10, or 1 μ g/mL, for 28 days. A control group was cultured in medium without hemocyanin. The LDH activity was determined as a quantitative marker for cell number and for calculating relative ALP levels (Figure 2). An evaluation of the cell numbers (Figure 2a) suggested that proliferation of hMSCs did not seem to be influenced by the presence of hemocyanin, although proliferation of the cells incubated with the highest hemocyanin concentration (100 μ g/mL) was slightly slower in the first 14 days. After 21 and 28 days there were no significant differences between the cells incubated at the three different hemocyanin concentrations and the control group.

Addition of osteogenic supplements to the medium resulted in increased ALP activity, which was dependent on the hemocyanin

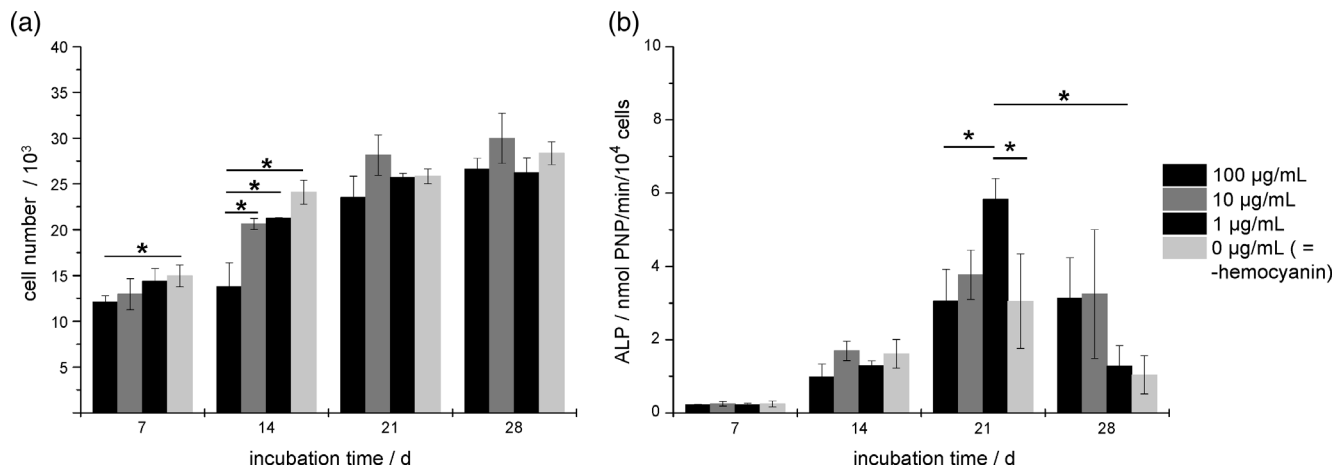


FIGURE 2 Influence of hemocyanin concentration (100, 10, or 1 µg/mL or without) in the culture medium on (a) the proliferation (LDH activity), and (b) osteogenic differentiation (ALP activity) of hMSCs seeded on polystyrene. Osteogenesis was induced 4 days after seeding. Asterisks indicate significant differences ($p < 0.05$)

concentration. Typically, maximum ALP levels—characterizing cell maturation and the beginning matrix mineralization—were obtained on day 21 for all hemocyanin concentrations. Only for a hemocyanin concentration of 1 µg/mL was there a significant increase in ALP activity on day 21 versus 10 µg/mL and versus the control (Figure 2b).

3.2 | Scaffold preparation

To obtain a processable chitin solution, we used the LiCl/DMA solvent system to produce the starting chitin scaffolds and chitin-CaCO₃ scaffolds; these scaffolds were found to be stable for more than 28 days under cell-culture conditions. SEM imaging was used to examine the structure and porosity of the chitin-CaCO₃ scaffolds. For the chitin-CaCO₃ scaffolds, Figure 3a,c suggest that the CaCO₃ had settled slightly in the solution in which the chitin and CaCO₃ were allowed to react, since the scaffolds showed a uniform, homogeneous layer of calcite crystals at the bottom of the samples, while significantly less calcite, covered by a chitin layer, was evident at the scaffold tops. For the chitin-CaCO₃ scaffolds that had been treated with HCl to obtain a porous structure, pores were detected only on the bottoms of the samples (Figure 3b,d). This was also visible in cross-sections of the scaffolds (Figure 3e,f). Those revealed that Ca was only present in chitin-CaCO₃ without HCl-treatment. Furthermore, Ca and O detection were used as an indicator for CaCO₃, which was allocated only at one half of the scaffold, the lower part during scaffold formation. The chitin-CaCO₃ scaffolds with HCl-treatment showed no Ca signal at all during EDS analysis.

3.3 | Determination of immobilized hemocyanin content on the chitin scaffolds

Table 2 presents the hemocyanin content of the modified scaffolds. Of particular importance, the Table shows that gamma irradiation had a

negative influence on the hemocyanin content of the scaffolds, since the hemocyanin densitometry values of nonmodified chitin and irradiated dip-coated chitin were very similar. It is likely that the source of the hemocyanin in the nonmodified scaffolds was the residual hemocyanin present in the commercial chitin flakes, which were obtained from a natural source (crustacean shells). In contrast, when hemocyanin modification was performed after gamma irradiation, a two- to threefold increase of scaffold hemocyanin content was achieved, which correlated with an enhanced adhesion (Figure S1). The highest content was observed for the HCl-treated porous chitin-CaCO₃ scaffolds.

3.4 | Adhesion of hMSCs on scaffolds

3.4.1 | Influence of hemocyanin on cell adhesion

The influence of the hemocyanin modification of plain chitin and porous chitin-CaCO₃ scaffolds (both HCl-treated) on the adhesion of hMSCs was evaluated by measuring LDH activity (Figure 4). Twenty-four hours after seeding 50×10^3 hMSCs on samples of both scaffold types modified with hemocyanin, 7%–30% of the cells had adhered to the scaffolds (adhesion efficiency), meaning that the remainder of the cells adhered to the plate (as shown in Figure S1) or died. The highest number of adhering cells was observed for hemocyanin-modified pure chitin scaffolds, and the lowest for porous chitin-CaCO₃ scaffolds without hemocyanin coating. In both basic scaffold types, the hemocyanin modification doubled the number of adhering cells compared to the uncoated scaffold.

3.4.2 | Combined effect of hemocyanin modification and medium change after pre-incubation

The motivation for these experiments was to investigate whether pre-incubation of the scaffolds in cell culture proliferation medium

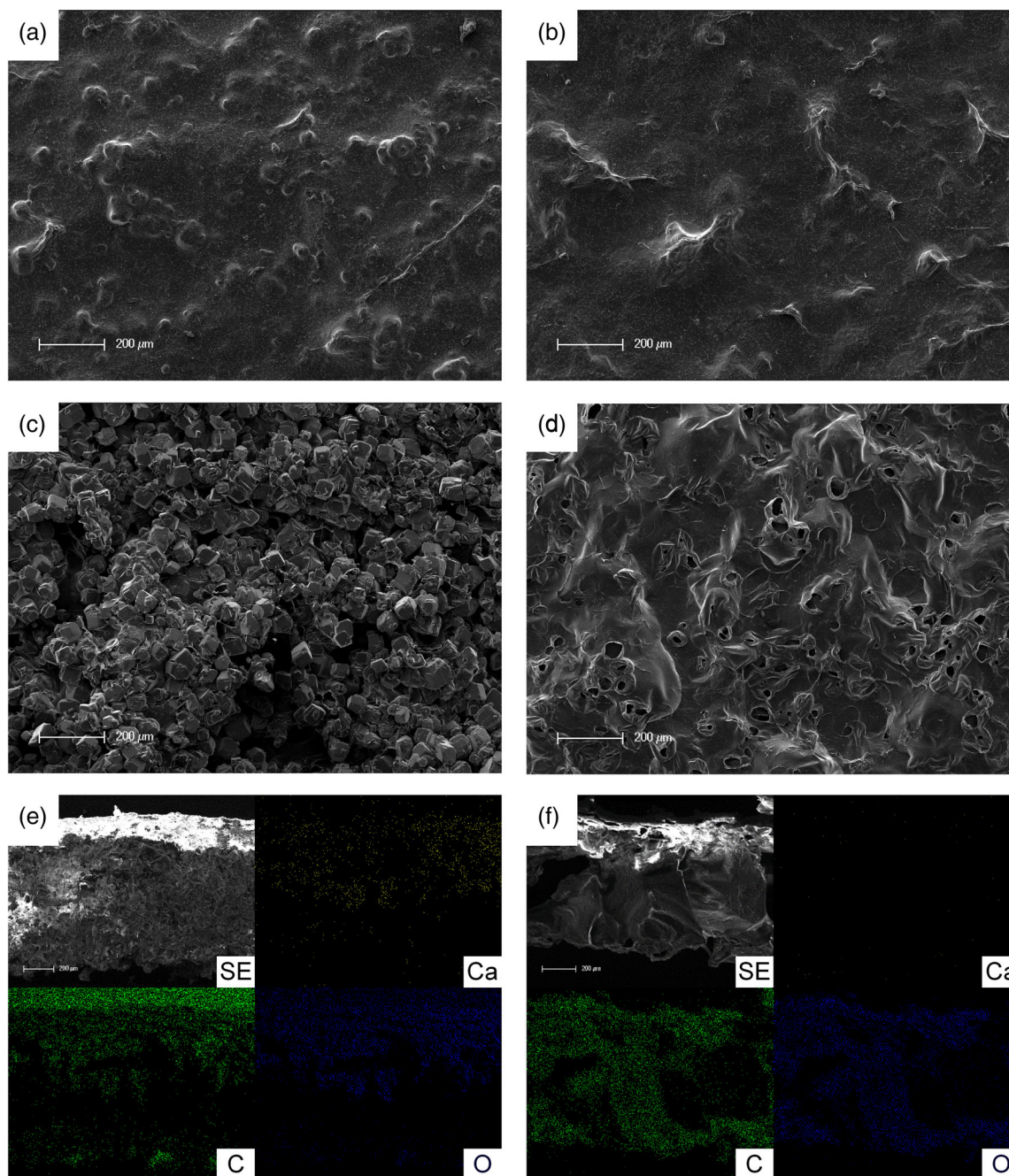


FIGURE 3 Morphology of chitin- CaCO_3 scaffolds without (a, c, e) and with (b, d, f) HCl-treatment. The surface of the scaffold samples upper sides are shown in SEM images (a) and (b), and the bottoms in (c) and (d). Cross-sections of the scaffolds are shown in (e) and (f) with two-dimensional EDS-maps of calcium (Ca), carbon (C), and oxygen (O). Scale bars 200 μm

TABLE 2 Immobilized hemocyanin content in chitin scaffolds after dip-coating

Sample	γ -Sterilization	Time of hemocyanin modification	Scaffold hemocyanin content (μg)
Chitin	+	Without hemocyanin	0.26
Chitin	-	Before sterilization	0.24
Chitin	+	After sterilization	0.4-0.64
Chitin- CaCO_3	+	After sterilization	0.64
HCl-treated porous chitin- CaCO_3	+	After sterilization	1.5

would have a positive impact on cell adhesion and whether it would cause partial or complete removal of hemocyanin from scaffolds, as described in Section 2.4.2. The influence of (a) modification of the

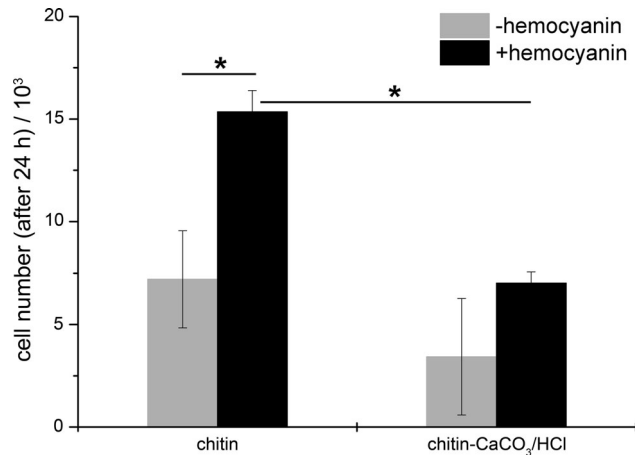


FIGURE 4 Adhesion of hMSCs (number of cells on scaffolds after 24 h) seeded on chitin and porous chitin-CaCO₃ (chitin-CaCO₃/HCl) scaffolds with and without previous hemocyanin dip-coating. Seeding cell number was 50×10^3 cells per scaffold. Asterisks indicate significant differences ($p < 0.05$)

scaffolds with hemocyanin, (b) HCl treatment during scaffold preparation (i.e., scaffold porosity), and (c) medium replacement after pre-incubation of the scaffolds on adhesion of the cells on chitin, HCl-treated chitin, chitin-CaCO₃ and porous chitin-CaCO₃ scaffolds was determined by LDH activity measurements (Figure 5). As was to be expected from the data shown in Figure 4, hemocyanin modification promoted the adhesion of hMSCs, that is, for all scaffold types, adhesion efficiency was higher on the hemocyanin-modified specimens (Figure 5), but there were slight differences in adhesion efficiency according to the different preparation methods (namely, addition of CaCO₃ or HCl treatment). For scaffolds modified with hemocyanin (black bars in Figure 5), the average adhesion efficiency was 24%, whereas without this modification it was only 14%. The highest values for cell adhesion efficiency were achieved for the chitin CaCO₃ scaffolds with a medium change after pre-incubation (Figure 5c) and for the simple chitin scaffolds without a medium change (Figure 5a), i.e., 34% and 33%, respectively. The lowest adhesion efficiency (5%) was found for HCl-treated chitin scaffolds without hemocyanin in the case where the pre-incubation medium was replaced by fresh medium before the cells were sown (Figure 5b). Remarkably, for the HCl-treated hemocyanin-modified pure chitin scaffolds, the hemocyanin remained on the scaffolds despite the replacement of the medium with fresh medium just

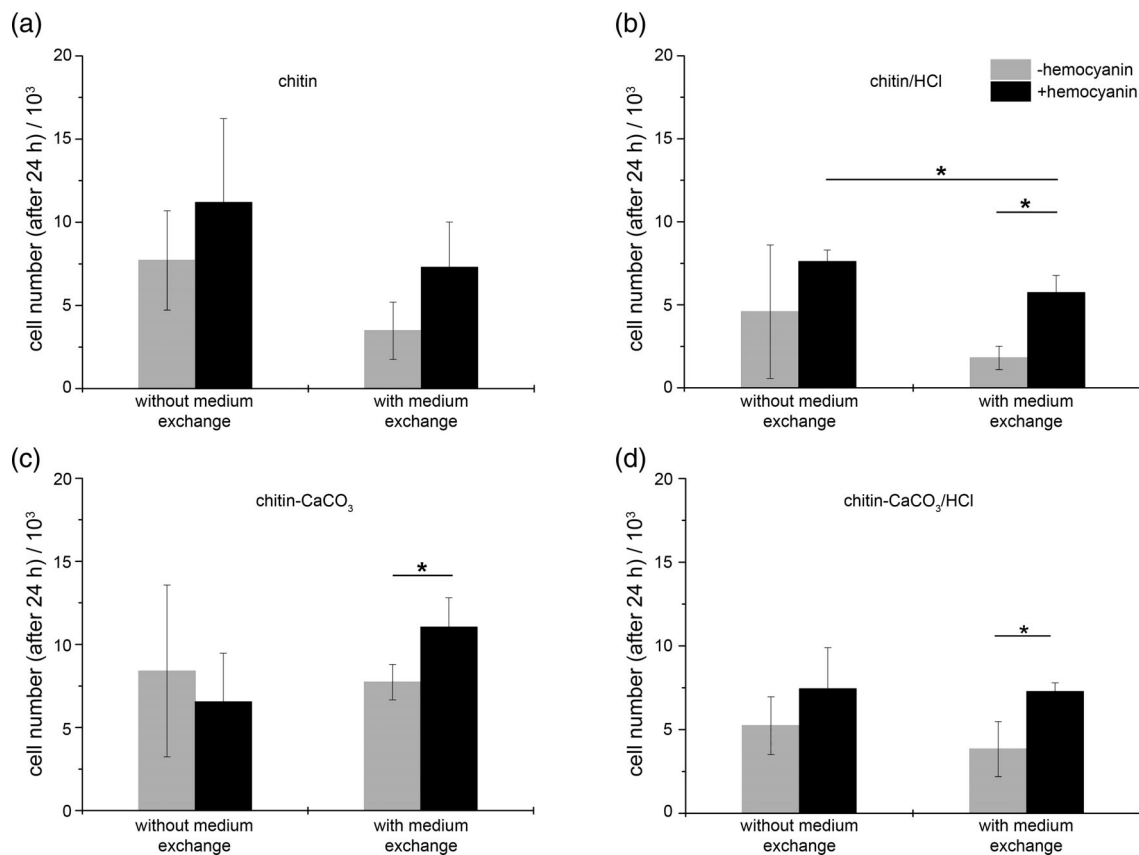


FIGURE 5 Influence of hemocyanin modification and exchange of the pre-incubation medium on adhesion of hMSCs seeded on (a) plain chitin scaffolds, (b) HCl-treated plain chitin scaffolds, (c) chitin-CaCO₃ scaffolds, and (d) porous chitin-CaCO₃ scaffolds. Seeding cell number was 35×10^3 cells per scaffold. Asterisks indicate significant differences ($p < 0.05$)

before cell seeding and thus caused a comparatively high hMSC adhesion of 17%.

As mentioned above, the adhesion efficiency with hemocyanin (dark grey bars in Figure 5) was generally higher than that without hemocyanin. This was true for the different scaffold types (chitin, chitin-CaCO₃, and porous chitin-CaCO₃), whether the medium was exchanged or not. Furthermore, for the scaffolds that had not been modified with hemocyanin, the highest adhesion efficiencies were observed for chitin-CaCO₃ scaffolds with a change of medium after pre-incubation (Figure 5c) and for chitin scaffolds without a medium change (Figure 5a). The effect of hemocyanin on adhesion efficiency was also clearly evident in the HCl-treated pure chitin, chitin-CaCO₃ and porous chitin-CaCO₃ scaffolds when the medium was exchanged after pre-incubation.

3.5 | Proliferation of hMSCs/osteoblasts on scaffolds

The influence of hemocyanin modification and of sterilization of the scaffolds with gamma-irradiation on the proliferation of hMSCs

seeded on the chitin scaffolds was evaluated (from LDH activity measurements) after culture without (Figure 6a) and with (Figure 6b) osteogenic supplements. In addition, the influence of the scaffold type—plain chitin, chitin-CaCO₃, or porous chitin-CaCO₃—on cell proliferation was investigated without (Figure 6c) and with (Figure 6d) osteogenic supplements. An increase in the number of cells of both osteogenically induced and noninduced hMSCs was observed for all scaffold types, but with different cell proliferation rates.

3.5.1 | Influence of hemocyanin modification before and after gamma irradiation on proliferation of hMSCs

The most intensive cell proliferation was observed for scaffolds subjected to gamma-irradiation after hemocyanin modification (Figure 6). On the chitin scaffolds without hemocyanin, proliferation decreased with culture time, but on the scaffolds modified with hemocyanin after sterilization the cells proliferated (threefold increase in cell number) in both proliferation and osteogenic media. Nevertheless, the addition of osteogenic supplements resulted in higher absolute cell

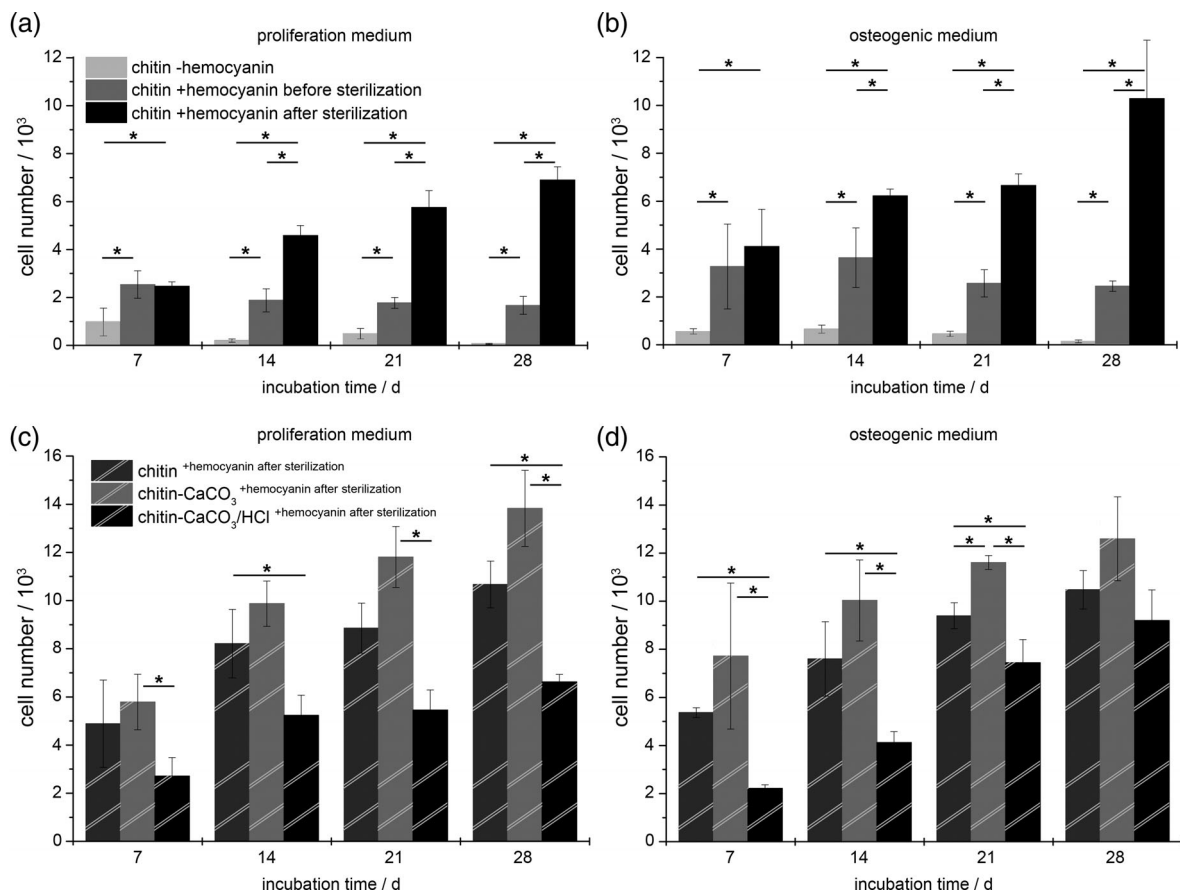


FIGURE 6 Proliferation of hMSCs seeded on chitin scaffolds. Influence of sterilization with gamma-irradiation before and after hemocyanin modification (upper row) and of scaffold type, namely, plain chitin, chitin-CaCO₃, and porous chitin-CaCO₃ scaffolds (lower row), all modified with hemocyanin and all sterilized prior to dip coating. Cells were cultured in proliferation medium (a and c) or osteogenic medium (b and d). Asterisks indicate significant differences ($p < 0.05$)

numbers after 7 days and at the end of the culture on day 28. Although samples subjected to hemocyanin modification before sterilization showed a minor decrease of cell number from day 7 to day 28, the positive effect of the hemocyanin (vs. the nonmodified chitin) could be observed.

The data in Figure 6a,b show that the only treatment giving significant proliferation of hMSCs on chitin scaffolds was hemocyanin modification after gamma irradiation. In light of these findings, all subsequent experiments were performed on scaffolds modified in this way.

3.5.2 | Influence of scaffold type (before hemocyanin modification) on proliferation of hMSCs

In the absence of osteogenic supplements, the highest increase in cell number was detected on chitin-CaCO₃ scaffolds, even though increases (doubling) in the cell number from day 7 to day 28 were also observed for chitin and porous chitin-CaCO₃ scaffolds (Figure 6). Owing to the lower adhesion efficiency on the porous chitin-CaCO₃ scaffolds, the absolute cell number after 28 days was considerably lower than that on the other scaffold types, although there were no marked differences in cell proliferation rates.

After 28 days, cells seeded on chitin-CaCO₃ scaffolds in the proliferation medium showed a higher proliferation rate than those cultured in the osteogenic medium, whereas the opposite results were obtained for porous chitin-CaCO₃ scaffolds. On the latter, there was an even higher proliferation of hMSCs after addition of the osteogenic medium. This high proliferation on the porous chitin-CaCO₃ scaffolds in the osteogenic medium led to the convergence of the cell count on day 28 on this scaffold type with the cell counts on the other two scaffold types, while the cell count on day 7 on the porous chitin-CaCO₃ scaffolds was clearly below the counts on the other two scaffold types (see Figure 5d). For chitin scaffolds, a comparison of the cell numbers in the proliferation medium with those in the osteogenic medium showed that on this scaffold type the proliferation behavior

of the hMSCs seemed not to be influenced by the presence or absence of osteogenic supplements.

3.6 | Osteogenic differentiation of hMSCs on different scaffolds

The osteogenic differentiation of hMSCs on the different scaffolds was evaluated in terms of ALP activity. The factors investigated included the influence of the timing of sterilization with gamma-irradiation, namely, before or after hemocyanin modification, on cells cultured on hemocyanin-modified plain chitin scaffolds (Figure 7a) and the influence of scaffold type on cells cultured on different types of scaffolds (Figure 7b). Similarly to cell proliferation (Figure 6b), the highest ALP values were detected for scaffolds modified with hemocyanin after gamma-irradiation (Figure 7a). For chitin scaffolds coated with hemocyanin before sterilization low ALP activity of the cultures was observed. In contrast, ALP activity of cells seeded on scaffolds modified after sterilization was significantly increased.

Addition of osteogenic supplements to the medium resulted in typically increased ALP activity for all scaffold types: plain chitin, chitin-CaCO₃, and porous chitin-CaCO₃ (Figure 7b). The typical maximum of ALP activity, indicating the differentiation around day 21, which was found for hMSC in hemocyanin solution (Figure 2b), was not achieved here. This finding could be attributed to the low adhesion efficiency of the cells on scaffolds compared to polystyrene (well plate) and thus an ongoing proliferation of the cells, which was shown in Figure 6d. The highest ALP activity of the cells was observed for the porous chitin-CaCO₃ scaffolds (for which proliferation was maximal) vs. the untreated chitin and treated chitin-CaCO₃ scaffolds.

4 | DISCUSSION

The present study demonstrates that chitin scaffolds promote adhesion, proliferation and osteogenic differentiation of hMSCs. This

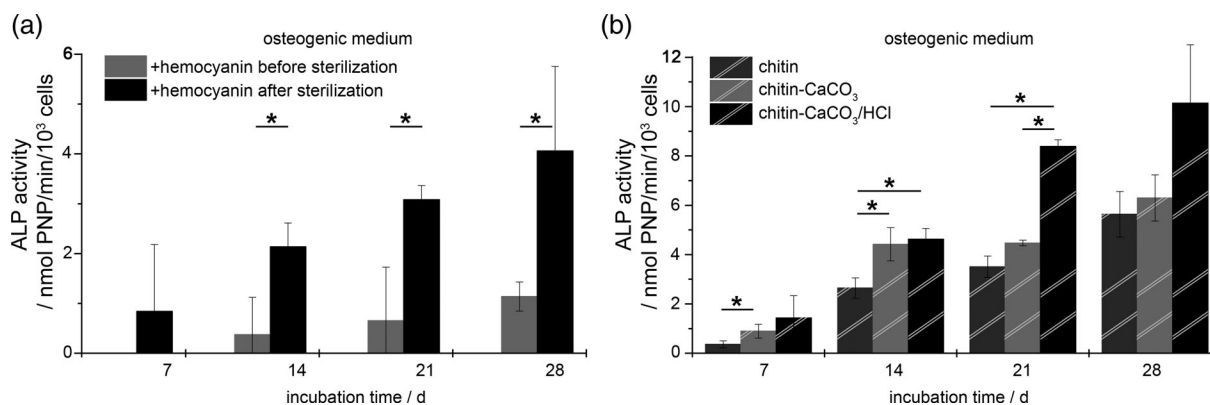


FIGURE 7 ALP activity of hMSCs seeded on chitin scaffolds. Influence of sterilization with gamma-irradiation on hemocyanin-modified plain chitin scaffolds (a) and influence of scaffold type, namely, plain chitin, chitin-CaCO₃, porous chitin-CaCO₃ (chitin-CaCO₃/HCl) modified with hemocyanin after gamma irradiation (b). Asterisks indicate significant differences ($p < 0.05$)

finding is in accordance with previous *in vitro* studies on chitin-based composites showing that chitin modification with nano-hydroxyapatite supported adhesion of a variety of cell lines (Arun Kumar et al., 2015; Kumar et al., 2011). A rare *in vivo* study demonstrated that osteoblast-loaded chitin-hydroxyapatite composites that were implanted into bone defects in a rabbit femur supported bone regeneration and facilitated the ingrowth of surrounding tissue (Ge, Baguenard, Lim, Wee, & Khor, 2004).

In vitro studies over long culture periods, such as the present study, are very rare. Nonetheless, two such studies have been reported by Arun Kumar et al. (2015) and Arun Kumar et al. (2016). In the first, they showed that rabbit adipose-derived MSCs cultured over 28 days on composite microgels comprised of nano-hydroxyapatite/chitin-poly(ϵ -caprolactone) exhibited increased ALP levels during the first 7 days of culture, indicating accelerated osteogenic differentiation and an early onset of the maturation phase compared to controls (Arun Kumar et al., 2015). In the second study, they demonstrated the same pro-osteogenic effect for the chitin hydrogels after incorporation of CaSO_4 (Arun Kumar et al., 2016). Nevertheless, it is not possible to draw parallels between their studies and ours, since neither the culture methods nor the sources of the studied cells were the same.

An important issue in any study such as ours is the cytocompatibility of the tissue engineering material with the relevant tissue. Materials such as those reported here may be regarded as "cytocompatible if both structure and functions of the tissue in direct contact with...[them] remain unchanged." (Sigot-Luizard & Warocquier-Clerout, 1993). If there is no direct contact between the materials and the tissue, then the materials exert their action mainly by binding active substances present in the extracellular space or releasing materials degradation products and immobilized substances. Therefore, there are several pathways of action on cells that have not been identified in detail in this study.

Cell adhesion was generally rather moderate on the chitin scaffolds, and large proportions of cells were found on the polystyrene (cell culture material) next to the scaffolds. Those cells were apparently not damaged by any degradation products present. Thus, it appears that both the pure chitin and the differently treated scaffolds (with CaCO_3 and/or HCl) did not exert any cytotoxic effects, as confirmed by the ability of the hMSCs to proliferate and differentiate over the entire cultivation period. Furthermore, scaffold modifications with crustacean hemocyanin and the incorporation of CaCO_3 led to increased adhesion, followed by improved proliferation and osteogenic differentiation compared to pure chitin scaffolds.

4.1 | Influence of different hemocyanin concentrations on cell culture

In the present study, the hemocyanin concentration did not seem to affect the proliferation of the hMSCs on polystyrene. ALP activity, which is an early marker of osteogenic differentiation, was significantly increased in comparison to cell culture without hemocyanin, but only at a low hemocyanin concentration (1 $\mu\text{g}/\text{mL}$). The ALP

maximum on day 21 indicated almost complete differentiation and the beginning matrix mineralization. In contrast, at higher hemocyanin concentrations (10 and 100 $\mu\text{g}/\text{mL}$), the peak in ALP activity was delayed, which indicates the influence of hemocyanin on osteogenic differentiation; these findings followed the same trend as those for cells grown on hemocyanin-modified scaffolds.

4.2 | Combined effect of hemocyanin modification and medium change after pre-incubation

Pre-incubation of scaffolds in a proliferation medium before cell seeding leads to protein adsorption and thus promotes initial cell adherence (Willumeit, Möhring, & Feyerabend, 2014). Despite previous work on the subject, it was necessary to determine for our system whether replacing the medium with fresh medium after pre-incubation of the scaffolds would be advantageous (by virtue of removing harmful ingredients, such as residues of the scaffold preparation) or detrimental (through washing out of hemocyanin released from the scaffold). We found that replacing the medium used for pre-incubation with fresh medium resulted in a decrease in the number of cells on the different scaffolds compared to cell seeding without changing the medium. Interestingly, this effect was observed irrespective of whether or not the scaffolds were modified with hemocyanin. Thus, the lower cell count obtained when the medium was exchanged cannot be attributed to washing out of the hemocyanin.

The general trend of increased adhesion due to hemocyanin modification may be caused by adsorption of hemocyanin on the chitinous material in preference to proteins from the FCS. Adsorbed hemocyanin molecules might thus serve directly as adhesion sites for hMSCs—and possibly also for FCS proteins from the medium.

4.3 | Scaffold hemocyanin content

Sterilization is a mandatory for scaffolds intended for cell culture, and it is known that the sterilization method, for example, gamma-irradiation or exposure to ethylene oxide, influences the morphology, structure, surface architecture and mechanical properties of different organic polymers (Ahmed, Punshon, Darbyshire, & Seifalian, 2013; Delgado, Pandit, & Zeugolis, 2014; Marreco, Da Luz Moreira, Genari, & Moraes, 2004). In this study, exposure of the hemocyanin-modified scaffolds to gamma irradiation adversely affected hMSC and osteoblast activities. Since this observation correlated with the results of hemocyanin content tests, it could be attributed to radiation damage to hemocyanin itself. Therefore, hemocyanin modification was carried out on process, the highest content was observed for porous chitin- CaCO_3 scaffolds. This finding could be explained by the higher specific surface area of the porous HCl-treated scaffolds. In addition, incorporation of CaCO_3 resulted in a higher amount of immobilized hemocyanin compared to plain chitin scaffolds. This is an indication of the ability of hemocyanin to bind both chitin and calcium ions.

4.4 | Influence of CaCO₃ modification of chitin scaffolds on the osteogenic differentiation of hMSCs

Chow and Khor investigated the carbonates and bicarbonates of potassium, sodium, and calcium as a source of CO₂ for introducing porosity into chitin matrixes (Chow & Khor, 2000). They reported that only CaCO₃ formed a stable suspension in the chitin solution, while the carbonates and bicarbonates of potassium and sodium underwent phase separation and concentrated in the lower part of the scaffolds. This effect was also observed to a certain degree when CaCO₃ was introduced into our chitin solutions. Therefore, both the amount of CaCO₃ in the chitin–CaCO₃ scaffolds and the number of pores in the HCl-treated chitin–CaCO₃ scaffolds were higher at the bottoms than at the tops of the scaffolds. No measures were taken to prevent this CaCO₃ precipitation during scaffold production, because a homogeneous distribution of CaCO₃ particles was nonetheless observed on the upper surfaces of the scaffolds (Figure 3a). In future studies, this effect could be prevented by increasing the viscosity of the chitin solution or by agitating the gels during solvent evaporation.

The enhanced proliferation rate of hMSCs on chitin–CaCO₃ scaffolds could be attributed to calcium ion release from the scaffolds into the culture medium. A similar effect on cell behavior was reported by Heinemann et al., who showed that the calcium concentration in the incubation medium was influenced by the bioactivity of the biomaterial (Heinemann et al., 2013). They reported that in osteoblast/osteoclast co-culture experiments, scaffolds with low bioactivity, which leave the Ca²⁺ concentration in the medium largely unaffected, stimulated cells of the osteoblast line, while those with high bioactivity, which lead to Ca²⁺ depletion in the medium, supported osteoclasts.

The HCl treatment of chitin–CaCO₃ scaffolds in the preparation process and the resulting formation of dissolved calcium led to the enhancement of hMSC proliferation and differentiation. In parallel, the dissolution of CaCO₃, as already mentioned above, led to a larger pore size in the chitin scaffolds (Figure 3d) compared to the untreated scaffolds. The immobilization of CaCO₃ in the scaffolds and HCl treatment produced a dual effect, namely, the enlargement of the macroscopic pores and later an increase of the Ca²⁺ concentration in the medium, which both promote the bone formation process. An indicator for that is the following: on the porous chitin–CaCO₃ scaffolds, the hMSCs exhibited the lowest initial seeding density (Figure 5) but the highest proliferation rate (number of adhering cells at a given time vs. the cell count after 24 h, Figure 6) compared to untreated, pore-free chitin–CaCO₃ scaffolds. The importance of adhesion efficiency on proliferation was investigated by Lode et al. in a study of the proliferation of hMSCs on three-dimensional mineralized collagen scaffolds as a function of the initial seeding density (Lode, Bernhardt, & Gelinsky, 2008). Their study revealed that the highest proliferation rate was obtained at the lowest seeding density, with the enhanced proliferation being accompanied by a stronger differentiation rate (Lode et al., 2008).

Since the ultimate aim of bone tissue engineering is the building of new bone, the most important results of this study are those that pertain to the osteogenic differentiation of hMSCs. On the porous

chitin–CaCO₃ scaffolds, the hMSCs showed enhanced proliferation in the osteogenic medium compared to the proliferation medium (Figure 6c,d). This effect may be attributed to the presence in the differentiation medium of dexamethasone and ascorbic acid, which promote proliferation of hMSCs (Choi et al., 2008; Jaiswal, Haynesworth, Caplan, & Bruder, 1997). Similarly, Heinemann et al. found higher proliferation rates of osteogenically induced hMSCs compared to non-induced cells on chitosan-based scaffolds (Heinemann et al., 2009). In the current study, ALP activity was used as a measure of early stage osteoblastic differentiation of the hMSCs cultured on chitin scaffolds. All scaffold modifications—both without hemocyanin and with postirradiation hemocyanin coating—facilitated the differentiation of hMSCs into osteoblasts. Addition of osteogenic supplements to the medium resulted in increased ALP activity without the typical maximum (Figure 7b), which could be due to low adhesion efficiency but ongoing proliferation of the cells. Cells cultured on chitin–CaCO₃ scaffolds exhibited increased ALP activity due to higher cell numbers and the pro-osteogenic effect of released calcium ions, as discussed above. The HCl treatment of the chitin–CaCO₃ scaffolds led to a further improvement of the relative ALP activity, which may be associated with the porous structure (Heinemann, Heinemann, Worch, & Hanke, 2011) of the scaffolds, but also with the higher concentration of bound hemocyanin, which was observed in the hemocyanin content test. In summary, our results suggest that the presence of CaCO₃ improves cell adhesion and proliferation and leads to increased ALP activity compared to chitin scaffolds without CaCO₃. The success of a bone substitute material depends on both its interaction with osteoblasts, which are responsible for the generation of the new bone extracellular matrix, and its influence on the differentiation and activity of osteoclasts. Therefore, future studies should focus on the influence of chitin scaffolds modified with hemocyanin on osteoclast differentiation of human monocytes as well as on the cross-talk between bone-forming osteoblasts and bone-resorbing osteoclasts. Such a co-culture model has been developed in our laboratory (Heinemann et al., 2011) and will be applied in future studies.

5 | CONCLUSIONS

Chitin scaffolds can be produced with CaCO₃ to improve bone biocompatibility in terms of hMSC proliferation and differentiation to osteoblasts. In addition, an improved function of scaffolds modified by simple dip-coating with hemocyanin promotes cell adhesion. Hemocyanin also promotes cell adhesion in FCS-containing media and even enhances the effect of the adhesion proteins contained in such media. Thus, a cooperative effect of the hemocyanin and the FCS proteins can be inferred.

Hemocyanin as additive to cell culture on polystyrene shows a general suitability for the stimulation of hMSC/osteoblasts as it

- did not affect hMSC proliferation.
- increased osteoblastic differentiation at low concentration (1 µg/mL).

The study also showed that scaffold modification with crayfish hemocyanin

- is an excellent and necessary means to achieve acceptable adhesion efficiency.
- is gamma-irradiation sensitive.
- causes increased proliferation and osteogenic differentiation of hMSCs.
- provides—with the addition of CaCO₃—the highest proliferation of hMSCs.

In conclusion, scaffolds made of chitin–hemocyanin constitute a promising biomaterial. The materials provide a starting point for detailed analysis of the basic impact of hemocyanin and other promising crustacean proteins as well as of CaCO₃ and other calcium sources with respect to the influence on bioactivity of implant materials.

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REFERENCES

- Adachi, K., Endo, H., Watanabe, T., Nishioka, T., & Hirata, T. (2005). Hemocyanin in the exoskeleton of crustaceans: Enzymatic properties and immunolocalization. *Pigment Cell Research*, *18*, 136–143. <https://doi.org/10.1111/j.1600-0749.2005.00217.x>
- Ahmed, M., Punshon, G., Darbyshire, A., & Seifalian, A. M. (2013). Effects of sterilization treatments on bulk and surface properties of nanocomposite biomaterials. *Journal of Biomedical Materials Research Part B: Applied Biomaterials*, *101*, 1182–1190. <https://doi.org/10.1002/jbm.b.32928>
- Aizenberg, J., Addadi, L., Weiner, S., & Lambert, G. (2018). Stabilization of amorphous calcium carbonate by specialized macromolecules in biological and synthetic precipitates. *Advanced Materials*, *8*, 222–226. <https://doi.org/10.1002/adma.19960080307>
- Aljawish, A., Muniglia, L., & Chevalot, I. (2015). Growth of human mesenchymal stem cells (MSCs) on films of enzymatically modified chitosan. *Biotechnology Progress*, *32*, 491–500. <https://doi.org/10.1002/btpr.2216>
- Allen, M., Millett, P., Dawes, E., & Rushton, N. (1994). Lactate dehydrogenase activity as a rapid and sensitive test for the quantification of cell numbers in vitro. *Clinical Materials*, *16*, 189–194. [https://doi.org/10.1016/0267-6605\(94\)90116-3](https://doi.org/10.1016/0267-6605(94)90116-3)
- Al-Sawalmih, A., Li, C., Siegel, S., Fabritius, H., Yi, S., Raabe, D., ... Paris, O. (2008). Microtexture and chitin/calcite orientation relationship in the mineralized exoskeleton of the American lobster. *Advanced Functional Materials*, *18*, 3307–3314. <https://doi.org/10.1002/adfm.200800520>
- Andersen, S. O. (2010). Insect cuticular sclerotization: A review. *Insect Biochemistry and Molecular Biology*, *40*, 166–178. <https://doi.org/10.1016/j.ibmb.2009.10.007>
- Arun Kumar, R., Sivashanmugam, A., Deepthi, S., Bumgardner, J. D., Nair, S. V., & Jayakumar, R. (2016). Nano-fibrin stabilized CaSO₄ crystals incorporated injectable chitin composite hydrogel for enhanced angiogenesis & osteogenesis. *Carbohydrate Polymers*, *140*, 144–153. <https://doi.org/10.1016/j.carbpol.2015.11.074>
- Arun Kumar, R., Sivashanmugam, A., Deepthi, S., Iseki, S., Chennazhi, K. P., Nair, S. V., & Jayakumar, R. (2015). Injectable chitin-poly(ϵ -caprolactone)/nanohydroxyapatite composite microgels prepared by simple regeneration technique for bone tissue engineering. *ACS Applied Materials & Interfaces*, *7*, 9399–9409. <https://doi.org/10.1021/acsami.5b02685>
- Austin, P. R. (1984). Chitin solvents and solubility parameters. In *Chitin, Chitosan, Relat. Enzym.* (pp. 227–237). Cambridge, Massachusetts, United States: Academic Press. <https://doi.org/10.1016/B978-0-12-780950-2.50020-4>
- Cao, W., & Hench, L. L. (1996). Bioactive Materials. *Ceram. Int.*, *22*, 493–507. [https://doi.org/10.1016/0272-8842\(95\)00126-3](https://doi.org/10.1016/0272-8842(95)00126-3)
- Chang, C., Chen, S., & Zhang, L. (2011). Novel hydrogels prepared via direct dissolution of chitin at low temperature: Structure and biocompatibility. *Journal of Materials Chemistry*, *21*, 3865. <https://doi.org/10.1039/c0jm03075a>
- Cho, J. H., Kim, S.-H., Park, K. D., Jung, M. C., Yang, W. I., Han, S. W., ... Lee, J. W. (2004). Chondrogenic differentiation of human mesenchymal stem cells using a thermosensitive poly(N-isopropylacrylamide) and water-soluble chitosan copolymer. *Biomaterials*, *25*, 5743–5751. <https://doi.org/10.1016/j.biomaterials.2004.01.051>
- Choi, K.-M., Seo, Y.-K., Yoon, H.-H., Song, K.-Y., Kwon, S.-Y., Lee, H.-S., & Park, J.-K. (2008). Effect of ascorbic acid on bone marrow-derived mesenchymal stem cell proliferation and differentiation. *Journal of Bioscience and Bioengineering*, *105*, 586–594. <https://doi.org/10.1263/jbb.105.586>
- Chow, K. S., & Khor, E. (2000). Novel fabrication of open-pore chitin matrixes. *Biomacromolecules*, *1*, 61–67. <https://doi.org/10.1021/bm005503b>
- Clarkson, J. R., Price, T. J., & Adams, C. J. (1992). Role of metastable phases in the spontaneous precipitation of calcium carbonate. *Journal of the Chemical Society, Faraday Transactions*, *88*, 243–249. <https://doi.org/10.1039/FT9928800243>
- Coates, C. J., & Nairn, J. (2014). Diverse immune functions of hemocyanins. *Developmental and Comparative Immunology*, *45*, 43–55. <https://doi.org/10.1016/j.dci.2014.01.021>
- Costa-Pinto, A. R., Correlo, V. M., Sol, P. C., Bhattacharya, M., Charbord, P., Delorme, B., ... Neves, N. M. (2009). Osteogenic differentiation of human bone marrow mesenchymal stem cells seeded on melt based chitosan scaffolds for bone tissue engineering applications. *Biomacromolecules*, *10*, 2067–2073.
- Dashtdar, H., Murali, M. R., Abbas, A. A., Suhaeb, A. M., Selvaratnam, L., Tay, L. X., & Kamarul, T. (2015). PVA-chitosan composite hydrogel versus alginate beads as a potential mesenchymal stem cell carrier for the treatment of focal cartilage defects. *Knee Surgery, Sports Traumatology, Arthroscopy*, *23*, 1368–1377. <https://doi.org/10.1007/s00167-013-2723-5>
- Deepthi, S., Venkatesan, J., Kim, S.-K. K., Bumgardner, J. D., & Jayakumar, R. (2016). An overview of chitin or chitosan/nano ceramic composite scaffolds for bone tissue engineering. *International Journal of Biological Macromolecules*, *93*, 1338–1353. <https://doi.org/10.1016/j.ijbiomac.2016.03.041>
- Delgado, L. M., Pandit, A., & Zeugolis, D. I. (2014). Influence of sterilisation methods on collagen-based devices stability and properties. *Expert Review of Medical Devices*, *11*, 305–314. <https://doi.org/10.1586/17434440.2014.900436>
- Dixit, R., Arakane, Y., Specht, C. A., Richard, C., Kramer, K. J., Beeman, R. W., & Muthukrishnan, S. (2008). Domain organization and phylogenetic analysis of proteins from the chitin deacetylase gene family of *Tribolium castaneum* and three other species of insects. *Insect*

- Biochemistry and Molecular Biology*, 38, 440–451. <https://doi.org/10.1016/j.ibmb.2007.12.002>
- Ge, Z., Baguenard, S., Lim, L. Y., Wee, A., & Khor, E. (2004). Hydroxyapatite–chitin materials as potential tissue engineered bone substitutes. *Biomaterials*, 25, 1049–1058. [https://doi.org/10.1016/S0142-9612\(03\)00612-4](https://doi.org/10.1016/S0142-9612(03)00612-4)
- Glazer, L., & Sagi, A. (2012). On the involvement of proteins in the assembly of the crayfish gastrolith extracellular matrix. *Invertebrate Reproduction and Development*, 56, 57–65. <https://doi.org/10.1080/07924259.2011.588010>
- Glazer, L., Tom, M., Weil, S., Roth, Z., Khalaila, I., Mittelman, B., & Sagi, A. (2013). Hemocyanin with phenoloxidase activity in the chitin matrix of the crayfish gastrolith. *The Journal of Experimental Biology*, 216, 1898–1904. <http://jeb.biologists.org/content/early/2013/02/04/jeb.080945.abstract>
- Glenske, K., Wagner, A.-S., Hanke, T., Cavalcanti-Adam, E. A., Heinemann, S., Heinemann, C., ... Wenisch, S. (2014). Bioactivity of xerogels as modulators of osteoclastogenesis mediated by connexin 43. *Biomaterials*, 35, 1487–1495. <https://doi.org/10.1016/j.biomaterials.2013.11.002>
- González-Vázquez, A., Planell, J. A., & Engel, E. (2014). Extracellular calcium and CaSR drive osteoinduction in mesenchymal stromal cells. *Acta Biomaterialia*, 10, 2824–2833. <https://doi.org/10.1016/j.actbio.2014.02.004>
- Heinemann, C., Heinemann, S., Lode, A., Bernhardt, A., Worch, H., & Hanke, T. (2009). In vitro evaluation of textile chitosan scaffolds for tissue engineering using human bone marrow stromal cells. *Biomacromolecules*, 10, 1305–1310. <https://doi.org/10.1021/bm900172m>
- Heinemann, C., Heinemann, S., Worch, H., & Hanke, T. (2011). Development of an osteoblast/osteoclast co-culture derived by human bone marrow stromal cells and human monocytes for biomaterials testing. *European Cells & Materials*, 21, 80–93 doi:vol021a07 [pii].
- Heinemann, S., Heinemann, C., Wenisch, S., Alt, V., Worch, H., & Hanke, T. (2013). Calcium phosphate phases integrated in silica/collagen nanocomposite xerogels enhance the bioactivity and ultimately manipulate the osteoblast/osteoclast ratio in a human co-culture model. *Acta Biomaterialia*, 9, 4878–4888. <https://doi.org/10.1016/j.actbio.2012.10.010>
- Hing, K. A. (2004). Bone repair in the twenty-first century: Biology, chemistry or engineering? *Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering Sciences*, 362, 2821–2850. <https://doi.org/10.1098/rsta.2004.1466>
- Jaiswal, N., Haynesworth, S. E., Caplan, A. I., & Bruder, S. P. (1997). Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. *Journal of Cellular Biochemistry*, 64, 295–312. [https://doi.org/10.1002/\(sici\)1097-4644\(199702\)64:2<295::aid-jcb12>3.0.co;2-i](https://doi.org/10.1002/(sici)1097-4644(199702)64:2<295::aid-jcb12>3.0.co;2-i)
- Jasrapuria, S., Arakane, Y., Osman, G., Kramer, K. J., Beeman, R. W., & Muthukrishnan, S. (2010). Genes encoding proteins with peritrophin A-type chitin-binding domains in *Tribolium castaneum* are grouped into three distinct families based on phylogeny, expression and function. *Insect Biochemistry and Molecular Biology*, 40, 214–227. <https://doi.org/10.1016/j.ibmb.2010.01.011>
- Jayakumar, R., Chennazhi, K. P., Srinivasan, S., Nair, S. V., Furuike, T., & Tamura, H. (2011). Chitin scaffolds in tissue engineering. *International Journal of Molecular Sciences*, 12, 1876–1887. <https://doi.org/10.3390/ijms12031876>
- Jayakumar, R., Prabakaran, M., Nair, S. V., & Tamura, H. (2010). Novel chitin and chitosan nanofibers in biomedical applications. *Biotechnology Advances*, 28, 142–150. <https://doi.org/10.1016/j.biotechadv.2009.11.001>
- King, C., Stein, R. S., Shamshina, J. L., & Rogers, R. D. (2017). Measuring the purity of chitin with a clean, quantitative solid-state NMR method. *ACS Sustainable Chemistry & Engineering*, 5(9), 8011–8016.
- Kruppke, B., Farack, J., Sommer, F., Weil, S., Aflalo, E. D., Wiesmann, H.-P., ... Hanke, T. (2017). In situ crosslinking of highly porous chitosan scaffolds for bone regeneration: Production parameters and in vitro characterization. *Macromolecular Materials and Engineering*, 302, 1700147. <https://doi.org/10.1002/mame.201700147>
- Kumar, P. T. S., Srinivasan, S., Lakshmanan, V.-K., Tamura, H., Nair, S. V., & Jayakumar, R. (2011). Synthesis, characterization and cytocompatibility studies of α -chitin hydrogel/nano hydroxyapatite composite scaffolds. *International Journal of Biological Macromolecules*, 49, 20–31. <https://doi.org/10.1016/j.ijbiomac.2011.03.006>
- Kuo, Y., & Ku, I. (2008). Cartilage regeneration by novel polyethylene oxide/chitin/chitosan scaffolds. *Biomacromolecules*, 9, 2662–2669.
- Lai, G.-J., Shalumon, K. T., & Chen, J.-P. (2015). Response of human mesenchymal stem cells to intrafibrillar nanohydroxyapatite content and extrafibrillar nanohydroxyapatite in biomimetic chitosan/silk fibroin/nanohydroxyapatite nanofibrous membrane scaffolds. *International Journal of Nanomedicine*, 10, 567–584. <https://doi.org/10.2147/IJN.S73780>
- Lindborg, B. A., Brekke, J. H., Scott, C. M., Chai, Y. W., Ulrich, C., Sandquist, L., ... O'Brien, T. D. (2015). A chitosan-hyaluronan-based hydrogel-hydrocolloid supports in vitro culture and differentiation of human mesenchymal stem/stromal cells. *Tissue Engineering. Part A*, 21, 1952–1962. <https://doi.org/10.1089/ten.tea.2014.0335>
- Lode, A., Bernhardt, A., & Gelinsky, M. (2008). Cultivation of human bone marrow stromal cells on three-dimensional scaffolds of mineralized collagen: Influence of seeding density on colonization, proliferation and osteogenic differentiation. *Journal of Tissue Engineering and Regenerative Medicine*, 2, 400–407. <https://doi.org/10.1002/term.110>
- Loste, E., Wilson, R. M., Seshadri, R., & Meldrum, F. C. (2003). The role of magnesium in stabilising amorphous calcium carbonate and controlling calcite morphologies. *J. Cryst. Growth*, 254, 206–218. [https://doi.org/10.1016/S0022-0248\(03\)01153-9](https://doi.org/10.1016/S0022-0248(03)01153-9)
- Lowenstam, H. A., & Weiner, S. (1989). *On biomineralization*. Oxford, England: Oxford University Press on Demand.
- Luquet, G., & Marin, F. (2004). Biomineralisations in crustaceans: storage strategies. *Comptes Rendus Palevol*, 3, 515–534. <https://doi.org/10.1016/j.crpv.2004.07.015>
- Madhumathi, K., Binulal, N. S., Nagahama, H., Tamura, H., Shalumon, K. T., Selvamurugan, N., ... Jayakumar, R. (2009). Preparation and characterization of novel β -chitin–hydroxyapatite composite membranes for tissue engineering applications. *International Journal of Biological Macromolecules*, 44, 1–5. <https://doi.org/10.1016/j.ijbiomac.2008.09.013>
- Malafaya, P. B., & Reis, R. L. (2009). Bilayered chitosan-based scaffolds for osteochondral tissue engineering: Influence of hydroxyapatite on in vitro cytotoxicity and dynamic bioactivity studies in a specific double-chamber bioreactor. *Acta Biomaterialia*, 5, 644–660. <https://doi.org/10.1016/j.actbio.2008.09.017>
- Marreco, P. R., Da Luz Moreira, P., Genari, S. C., & Moraes, A. M. (2004). Effects of different sterilization methods on the morphology, mechanical properties, and cytotoxicity of chitosan membranes used as wound dressings. *Journal of Biomedical Materials Research Part B: Applied Biomaterials*, 71, 268–277. <https://doi.org/10.1002/jbm.b.30081>
- Muzzarelli, R. A. A. (2009). Chitins and chitosans for the repair of wounded skin, nerve, cartilage and bone. *Carbohydrate Polymers*, 76, 167–182. <https://doi.org/10.1016/j.carbpol.2008.11.002>
- Nagahama, H., Nwe, N., Jayakumar, R., Koiwa, S., Furuike, T., & Tamura, H. (2008). Novel biodegradable chitin membranes for tissue engineering applications. *Carbohydrate Polymers*, 73, 295–302. <https://doi.org/10.1016/j.carbpol.2007.11.034>
- Omidvar, N., Ganji, F., & Eslaminejad, M. B. (2016). In vitro osteogenic induction of human marrow-derived mesenchymal stem cells by PCL fibrous scaffolds containing dexamethazone-loaded chitosan microspheres. *Journal of Biomedical Materials Research Part A*, 104, 1657–1667. <https://doi.org/10.1002/jbm.a.35695>

- Rejinold, N. S., Nair, A., Sabitha, M., Chennazhi, K. P., Tamura, H., Nair, S. V., & Jayakumar, R. (2012). Synthesis, characterization and in vitro cytocompatibility studies of chitin nanogels for biomedical applications. *Carbohydrate Polymers*, *87*, 943–949. <https://doi.org/10.1016/j.carbpol.2011.08.044>
- Rosa, R. D., & Barracco, M. A. (2010). Antimicrobial peptides in crustaceans. *Invertebrate Survival Journal*, *7*, 262–284.
- Shechter, A., Berman, A., Singer, A., Freiman, A., Grinstein, M., Erez, J., ... Sagi, A. (2008). Reciprocal changes in calcification of the gastrolith and cuticle during the molt cycle of the red claw crayfish *Cherax quadricarinatus*. *The Biological Bulletin*, *214*, 122–134. <https://doi.org/10.2307/25066669>
- Shechter, A., Glazer, L., Cheled, S., Mor, E., Weil, S., Berman, A., ... Sagi, A. (2008). A gastrolith protein serving a dual role in the formation of an amorphous mineral containing extracellular matrix. *Proceedings of the National Academy of Sciences*, *105*, 7129 LP–7134. <http://www.pnas.org/content/105/20/7129.abstract>
- Sigot-Luizard, M. F., & Warocquier-Clerout, R. (1993). In Vitro Cytocompatibility Tests. In *Test Procedures for the Blood Compatibility of Biomaterials* (pp. 569–594). Dordrecht: Springer Netherlands. https://doi.org/10.1007/978-94-011-1640-4_48
- Sum Chow, K., Khor, E., & Chwee Aun Wan, A. (2001). Porous chitin matrices for tissue engineering: Fabrication and in vitro cytotoxic assessment. *Journal of Polymer Research*, *8*, 27–35. <https://doi.org/10.1007/s10965-006-0132-x>
- Synowiecki, J., & Al-Khateeb, N. A. (2003). Production, properties, and some new applications of chitin and its derivatives. *Critical Reviews in Food Science and Nutrition*, *43*(2), 145–171.
- Tamura, H., Furuike, T., Nair, S. V., & Jayakumar, R. (2011). Biomedical applications of chitin hydrogel membranes and scaffolds. *Carbohydrate Polymers*, *84*, 820–824. <https://doi.org/10.1016/j.carbpol.2010.06.001>
- Tamura, H., Nagahama, H., & Tokura, S. (2006). Preparation of chitin hydrogel under mild conditions. *Cellulose*, *13*, 357–364. <https://doi.org/10.1007/s10570-006-9058-z>
- Terbojevich, M., Carraro, C., Cosani, A., & Marsano, E. (1988). Solution studies of the chitin-lithium chloride-N,N-di-methylacetamide system. *Carbohydrate Research*, *180*, 73–86. [https://doi.org/10.1016/0008-6215\(88\)80065-X](https://doi.org/10.1016/0008-6215(88)80065-X)
- Wade, N. M., Tollenare, A., Hall, M. R., & Degnan, B. M. (2009). Evolution of a novel carotenoid-binding protein responsible for crustacean shell color. *Molecular Biology and Evolution*, *26*, 1851–1864. <https://doi.org/10.1093/molbev/msp092>
- Wan, A. C. A., & Tai, B. C. U. (2013). CHITIN - A promising biomaterial for tissue engineering and stem cell technologies. *Biotechnology Advances*, *31*, 1776–1785. <https://doi.org/10.1016/j.biotechadv.2013.09.007>
- Willumeit, R., Möhring, A., & Feyerabend, F. (2014). Optimization of cell adhesion on Mg based implant materials by pre-incubation under cell culture conditions. *Int. J. Mol. Sci.*, *15*, 7639–7650. <https://doi.org/10.3390/ijms15057639>
- Yan, J., Yang, L., Wang, G., Xiao, Y., Zhang, B., & Qi, N. (2010). Biocompatibility evaluation of chitosan-based injectable hydrogels for the culturing mice mesenchymal stem cells in vitro. *Journal of Biomaterials Applications*, *24*, 625–637. <https://doi.org/10.1177/0885328208100536>
- Zhao, F., Grayson, W. L., Ma, T., Bunnell, B., & Lu, W. W. (2006). Effects of hydroxyapatite in 3-D chitosan-gelatin polymer network on human mesenchymal stem cell construct development. *Biomaterials*, *27*, 1859–1867. <https://doi.org/10.1016/j.biomaterials.2005.09.031>

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