Marius Behnecke*, Lukas Bräutigam, Alina Heß and Svea Petersen

Analytical approaches for evaluating chlorhexidine and hydroxyapatite content in 3D-printed resins for biomedical applications

https://doi.org/10.1515/cdbme-2025-0108

Abstract: The intersection of 3D printing technology and materials science has catalysed the development of bioactive materials with tailored properties, crucial for expanding applications from temporary to permanent components in fields such as dentistry and tissue engineering. This study explores the formulation and characterization of a 3D-printable resin comprised of methacrylate-based compounds filled with hydroxyapatite (HAp) and chlorhexidine (CHX). By integrating HAp and CHX, the resin aims to synergize antimicrobial activity with osteoconductivity, potentially enhancing the material's utility in biomedical applications by promoting bone cell attraction and minimizing inflammation during healing. Dimensional accuracy tests were conducted on rectangular and circular structures, revealing acceptable accuracy. HAp content was effectively quantified using a complexometric titration post-incineration. CHX extraction, despite attempts with methanol and acetic acid in both ultrasonic and Soxhlet methods, recovery rates did not exceed 55.3%, indicating significant retention within the resin possibly due to intermolecular interactions with the macromolecules. This research establishes a robust methodology for the incorporation of bioactive agents in 3D-printed materials, offering insights into optimizing resin formulations. By addressing these challenges, the study contributes to advancing 3D-printed resin technologies with potential long-term applications in the biomedical sector.

Keywords: 3D printing, modification, hydroxyapatite, chlorhexidine, drug delivery systems

***Corresponding author: Marius Behnecke:** University of Applied Science Osnabrück, Albrechtstraße 30, Osnabrück, Germany, e-mail: m.behnecke@hs-osnabrueck.de
Lukas Bräutigam, Alina Heß: University of Applied Science Osnabrück, Osnabrück, Germany
Svea Petersen: Institution University of Applied Science Osnabrück, Osnabrück, Germany

1 Introduction

Advancements in 3D printing technology have significantly impacted the field of materials science, particularly in developing bioactive materials with tailored properties. Due to the desired expansion from temporary to permanent applications, there is a growing demand for functional materials that not only succeed mechanical performance but also incorporate bioactive functions. This study investigates the formulation and characterization of a 3D printable resin that combines the properties and easy processing of methacrylate-based 3D-printing resins with bioactive functions of hydroxyapatite (HAp) and chlorhexidine (CHX). Utilisation of hydroxyapatite as a filler is reported to have an attracting effect on osteoblasts and thus an influence on the long-term anchoring of the component in the bone. Additivation with chlorhexidine, on the other hand, is intended to reduce the risk of inflammation during healing and secondary caries via a short and long-term drug release.[1] A critical challenge is ensuring the precise quantification of embedded bioactive compounds, such as CHX and HAp, in highly crosslinked acrylate matrices used in 3D printing. This study addresses the question of which extraction methods provide the most reliable results for determining bioactive components in the printed and therefore highly crosslinked material. By investigating various extraction approaches, we aim to identify the most effective method for quantifying the CHX content to understand and control its release dynamics. First step to this is the determination of intact and mobile CHX in the printed parts, as well as the HAp content. Therefore, a method for the quantification of the actual filler content in printed parts was established. By doing so, this research seeks to contribute to the optimization of 3D printed resin formulations for biomedical applications that combine structural functionality with enhanced bioactivity.

2 Experimentals

2.1 Materials

The resin formulation used in this study consisted of propane-2,2-diylbis[4,1-phenyleneoxy(2-hydroxypropane-3,1-diyl)]bis(2-methylprop-2-enoate) (Bis-GMA), 2,2-bis(4-(2-methacryloxyethoxy)phenylpropane (Bis-EMA), and triethylene glycol dimethacrylate (TEG-DMA) in a weight ratio of 25:60:15, respectively. Diphenyl(2,4,6-trimethylbenzoyl)phosphine oxide (TPO) served as the photoinitiator. The components were weighed into a beaker and immersed in a preheated water bath at 80 °C and stirred at 500 rpm for 20 minutes. 2 wt% TPO was then incorporated under continues stirring at 1000 rpm for additional 10 minutes, just like 10 wt% HAp. Separately, 2 wt% chlorhexidine acetate was dissolved in methanol (15 mL) and added to the mixture. HAp ($Ca_5(OH)(PO_4)_3$) was synthesized using the precipitation method of Tlili et al., a precipitation reaction between calcium nitrate tetrahydrate ($Ca(NO_3)_2 \cdot 4\ H_2O$; 0.5 M, Merck KGaA, Darmstadt, Germany) and diammonium hydrogen phosphate ($(NH_4)_2HPO_4$, 0.3 M, Merck KGaA, Darmstadt, Germany) at 80 °C and pH 10 [2]. After crystallization for 24 hours, the precipitate was filtered, washed, dried at 100 °C for 8 hour and calcined at 850 °C for 2 hours. Finally, the calcined material was grounded using a mortar. The successful synthesis of hydroxyapatite was verified by X-ray diffraction.

2.2 Methods

The 3D printing process was carried out using a “Photon S” LCD-3D-printer (Shenzhen Anycubic Technology Co., Ltd., Shenzen, China). The layer thickness is 50 µm, layer exposure time 8 seconds. Post-printing, the parts were cleaned using isopropyl alcohol for 2 min followed by drying and 3 min post-curing (Anycubic Wash&Cure 3.0). Samples and the filler distribution was analysed by means of light microscopy (Keyence Corporation, Osaka, Japan). Test samples were analysed using a digital light microscope of the type ‘VHX-500’. The surfaces of the samples were polished with 1 µm diamond abrasive for the best possible evaluation. Due to the lack of contrast on the surface of the unfilled samples, all images were taken using edge lighting. To determine the HAp content in 3D-printed samples, complexometric titration was employed. 1 g of material was coarsely crushed, weighed, and incinerated at 850 °C to remove organic materials and isolate mineral residues. After cooling, the residual was treated with 10 ml of 1 M nitric acid. The mixture was homogenized in an ultrasonic bath and transferred to a 50 ml volumetric flask and filled up with water. A 0.1 ml aliquot of this solution was further diluted with water, indicator and 1 ml of 25% ammonia were added, and the sample was titrated with 0.0002 mol/L EDTA. Calcium content was calculated and HAp content was calculated according to stoichiometry. To extract CHX from 3D-printed samples extraction with methanol was employed. Samples were immersed in 15 ml methanol and agitated for 45 min. The solution was then treated for 15 min in an ultrasonic bath. Ten millilitres of the medium then was replaced. This cycle was repeated for a total of 6 hours. For Soxhlet extractions, the material was grounded and 1 g was weighed into extraction thimbles. Methanol respectively 1% acetic acid in methanol was used for 2 - 12 hours extraction. Quantification of chlorhexidine was performed with an "Altus A-10" HPLC system (PerkinElmer Inc., Shelton, Connecticut, USA), equipped with a “Sonoma C18 150x4.6 mm 5 µm" column while detecting at 260 nm. An isocratic eluent mixture of 35% acetonitrile and 65% aqueous sodium acetate buffer was used. (0.03 M, pH 3.3 with glacial acetic acid + 0.2% triethylamine). The flow rate was 1 ml/min, injection volume 40 µL and column was heated to 30 °C. For CHX quantification, a dilution series was prepared, and the concentration range from 10 to 0.5 mg/L was calibrated.

3 RESULTS

Printing accuracy of the resin as percentage deviation from the nominal dimension of test structures was determined (Figure 1).

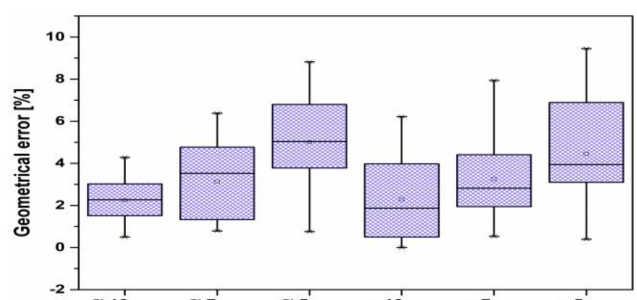

Figure 1: Percentage geometrical errors of 3D printed samples. Deviations are shown for various geometries and sizes: diameters (⌀) and rectangles (□).

The geometrical error in diameter of 10 mm structures (Figure 1, ⌀ 10mm) ranges from 0.5% to 4.3%, with the interquartile range (IQR) spanning from 1.5% to 2.8% and a median deviation of 2.3%. In contrast, ⌀ 7 mm (Figure 1) showed a broader distribution of geometrical errors, ranging from 0.8% to 6.4%, with a median of 3.5% and IQR of 1.5% to 4.6%. Data for the round 5 mm structure (Figure 1, ⌀ 5mm) exhibited deviations from 0.8% to 8.8%, with a median of 5.8% and IQR between 3.8% and 7.8%. Rectangular 10 mm structure (Figure 1, □ 10mm) deviations varied between 0%

and 6.2%, with the bulk of the values between 0.5% and 3.7% and a median of 1.2%. Rectangular 7 mm (Figure 1, □ 7mm) structures group spanned from 0.5% to 7.9%, with a median at 3.4% and interquartile limits from 1.9% to 4.8%. Rectangular 5mm structure (Figure 1, □ 5mm) revealed deviations ranging from 0.4% to a maximum of 9.5%, with a central tendency marked by a median of 3.5% and IQR from 2.9% to 5.0%. Neither HAp nor CHX showed a significant influence on geometrical errors presented here (data not shown). Microstructure of printed resin was documented by microscopy (Figure 2). The unmodified resin (Figure 2A) shows a smooth surface without features, only scratches remaining from the polishing process. Samples with CHX (Figure 2B) show a comparable surface, despite the fact that more scratches are visible. Samples modified with HAp (Figure 1C) or with HAp and CHX (Figure 1D) show irregularly shaped particles with a large size distribution. These particles are homogeneously distributed in the material, no substantial agglomerations could be identified.

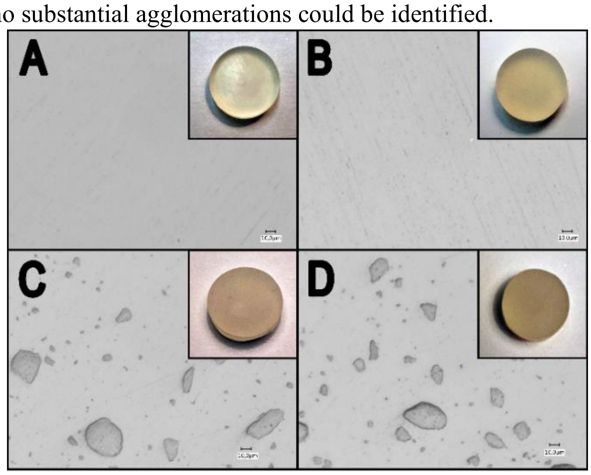

Figure 2: Microscopic images (1,000x) and photographs of the unfilled (A), CHX- additivated (B), HAp-filled (C) as well as CHX and HAp-filled printed composites (D). Scale bar (10 µm), valid for the microscopic images.

The HAp content in the printed component was determined by means of complexometric titration. The results of the quantification of the material additivated with HAp and the material additivated with HAp and CHX are shown in Figure 3. For the only HAp samples, the recovery ranges from approximately 65.0% to 82.4% compared to the theoretically added Hap content of 10 wt%. The interquartile range is bounded by approximately 70.4% and 75.5%, with a median value at around 74.1%. This reflects the fact that most samples tend to cluster around higher mass recoveries within the dataset. In comparison, the HAp+CHX samples show a recovery range from about 62.9% to 77.1%. The interquartile range is observed between approximately 68.8% and 74.8%, and the median is close to 70.3%. This dataset demonstrates a relatively similar distribution to the HAp-only samples, though with slightly lower maximum and median values, indicating a potential effect of CHX on the recovery of HAp. However, an Anova analysis (p=0.05) did not reveal any significant difference between the two sample groups.

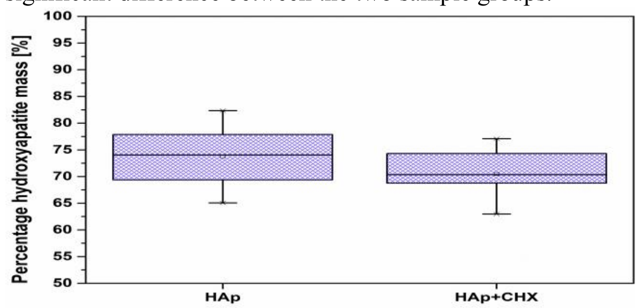

Figure 3: Illustration of the percentage mass of recovered hydroxyapatite (HAp) for samples with and without additional chlorhexidine (CHX), determined by ashing with subsequent complexometric titration. (n=5)

For CHX content determination, the percentage extraction efficiencies of three extraction methods after 6h compared to the theoretically CHX content of 2 wt% was evaluated, in solid and ground state (Figure 4).

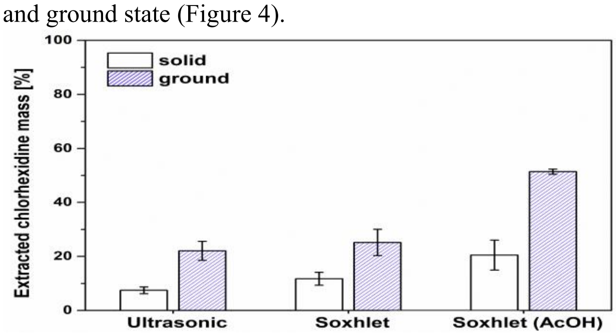

Figure 4: Percentage of CHX mass extracted, quantified by LC of extraction methods for solid and ground samples. Shown are mean values, indicators show the standard deviation. (n=3)

For ultrasonic extraction, solid samples show a mean extracted mass of 7.5%, with a standard deviation of 1.3%. The ground samples for this method exhibit a mean extracted mass of 22.1% and a standard deviation of 3.4%. In the Soxhlet extraction method, solid samples have a mean of 11.7% in extracted mass, with a standard deviation of 2.4%. Ground samples show a mean efficiency of 25.1%, with a standard deviation of 4.9%. For Soxhlet extraction with acetic acid (AcOH), solid samples show a mean extraction efficiency of 20.5%, with a standard deviation of 5.6%. Ground samples have the highest mean efficiency, based on the percentage of mass extracted, at 51.4%, with a standard deviation of 0.9%.
We furthermore investigated the time effect on extraction efficiency. During the initial 2 hours, the mean total extracted mass is 39.5%, with a standard deviation of 1.8%. From 2 to 4 hours, the extracted mass increases by 8.3 percentage points,

which results in an extraction rate of approximately 4.2% per hour. Between 4 and 6 hours, this rate decreases to an increase of 3.6 percentage points. After the 6-hour mark, the gain in extracted mass per hour diminishes further. The increase from 6 to 8 hours is 2.4 percentage points. Between 8 and 10 hours, the increase is even less with 1.0 percentage points. By 12 hours, the increase from the previous interval is only 0.5 percentage points, dropping the rate to a mere 0.25% per hour.

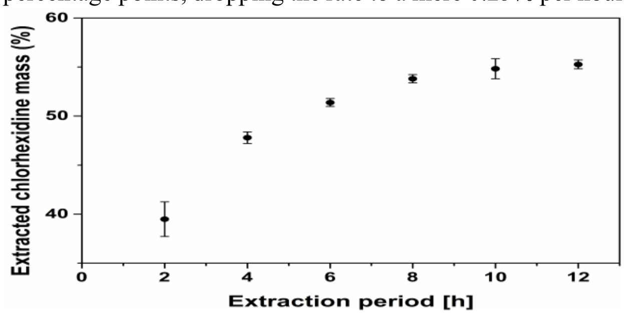

Figure 5: Percentage of CHX extracted mass of ground samples extracted with MeOH + 1% AcOH, quantified by LC over 2 to 12 hours. Shown are linearly connected mean values, indicators show the standard deviation of the respective measuring point.

4 DISCUSSION

Investigations showed that a 3D-printable resin from Bis-EMA, Bis-GMA, and TEGMA with TPO as initiator can be prepared using simple mixing methods. Bubble-free components produced from this resin exhibit acceptable dimensional accuracy, but structures smaller than 5 micrometres can deviate by up to 10%. The low TEGMA content results in higher viscosity (1000 mPa*s) compared to commercial resins, negatively affecting dimensional accuracy due to reduced flowability [3]. Adding 10% HAp or 2% CHX doesn't affect print quality due to already high viscosity. Light microscopy confirmed that filled systems (HAp and HAp+CHX) contain homogeneously distributed hydroxyapatite particles, successfully reduced to 50 micrometres by sieving. Simple dispersion suffices as no severe agglomerations were found. Complexometric titration of incinerated samples provided a method to quantify HAp content; a recovery rate of 70-75% evidenced sedimentation during printing. CHX extraction is challenging with straightforward methods achieving ≤25% recovery after 6 hours. Prior grinding of samples is essential. Using methanol with 1% acetic acid increases extraction performance to 51.4% in 6 hours; total extraction of 55.3% is reached after 12 hours. Further extraction is inefficient. Possible reasons include CHX degradation due to UV radiation and the cationic nature of dissociated CHX interacting with acrylate polymer groups, hinders extraction.[4] Further investigation is needed on CHX degradation products and methanol as a dispersing agent. Understanding these mechanisms is vital for controlling CHX release as a bioactive implant material.

5 CONCLUSIONS

The study successfully demonstrates the feasibility of preparing a 3D-printable resin from Bis-EMA, Bis-GMA, and TEGMA with TPO as an initiator using straightforward mixing methods, achieving satisfactory dimensional accuracy. Although the increased viscosity from lower TEGMA content and filler additions impaired flowability, the resin maintains print quality without significant bubble inclusion, and incorporated hydroxyapatite (HAp) and chlorhexidine (CHX) particles were well-distributed. Complexometric titration confirmed a realistic HAp recovery rate of 70-75%, acknowledging sedimentation during printing. In contrast, CHX extraction remains challenging, with maximum recovery limited to 55.3% despite extended and enhanced methods, potentially due to intermolecular binding within the resin matrix. In addition to in-vitro release studies of the differently modified systems, the investigation of the possible degradation of chlorhexidine in the printing process and the influence of intermolecular interactions of the (dissociated) active substance for further control of the release kinetics will be the subject of further investigations.

Author Statement

The authors would like to thank the European Regional Development Fund (87013560) for funding. Other conflicts of interest are not to declare.

References

[1] A. A. Campbell *u. a.*, „Development, characterization, and anti-microbial efficacy of hydroxyapatite-chlorhexidine coatings produced by surface-induced mineralization“, *J. Biomed. Mater. Res.*, Bd. 53, Nr. 4, S. 400–407, 2000, doi: 10.1002/1097-4636(2000)53:4<400::AID-JBM14>3.0.CO;2-Z.

[2] S. Tlili, B. Saida, L. Kotbia, N. Traiaia, und M. Hassani, „Synthesis and characterization of hydroxyapatite powder derived of eggshell by precipitation method“, Aug. 2023.

[3] C. Hinczewski, S. Corbel, und T. Chartier, „Stereolithography for the fabrication of ceramic three- dimensional parts“, *Rapid Prototyp. J.*, Bd. 4, Nr. 3, S. 104–111, Jan. 1998, doi: 10.1108/13552549810222867.

[4] M. Behnecke und S. Petersen, „Establishment of PEEK-Associated Drug Delivery Systems—Limits and Perspectives“, *J. Mater. Sci. Chem. Eng.*, Bd. 08, S. 32–41, Jan. 2020, doi: 10.4236/msce.2020.810004.