Crosslinking of Apatite-Gelatin Nanocomposites as Basis for Dentine Replacement Materials

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Abstract:

A novel approach for the production of a bioinspired dentine replacement material is introduced. Apatite-gelatin nanocomposite material was crosslinked with various crosslinkers. These nanocomposites have a high resemblance to mammalian dentine regarding its composition and properties. A precipitation reaction was used to produce apatite-gelatin nanocomposites as starting materials. To produce dentine-like and thus tough and robust apatite-gelatin nanocomposites, crosslinking of the gelatin has to be performed. Therefore, the efficacy of various protein

crosslinkers was tested, and the resulting materials were characterized by Scanning Electron Microscopy; Transmission Electron Microscopy, Powder X-Ray diffraction, EXAFS as well as C,H,N,S-Analysis and tested for their mechanical performance using Vickers hardness measurements as well as for their dissolution stability in EDTA. Especially glutaraldehyde, proanthocyanidins, and transglutaminase gave promising results with hardness values of up to 63 $HV_{0.2}$. To further improve the material properties, the effective crosslinker transglutaminase was furthermore combined with casein, which led to an improved interconnection between the single nanocomposite platelets. By doing so, a crosslinked composite was obtained, which shows with 76 $HV_{0.2}$ even higher hardness values than human dentine. The combination of apatite-gelatin nanocomposites with an effective crosslinker resulted in a bioinspired material with composition and properties close to human dentine.

INTRODUCTION

Caries always was and still is, with more than 2 billion annual incidents, one of the most abundant global diseases.[1] Much effort is put into the development of caries prevention and treatment strategies. Biomimetic and bioinspired approaches represent two of the most promising ways to provide biocompatible, non-inflammatory dentine replacement materials.[2-3] By mimicking the natural composition and structure of the dental hard tissue, tooth filling materials with excellent properties as well as biocompatibility can be produced.

Dentine is a hybrid material consisting of about 70 wt% of calcium-deficient apatite-(CaOH) and–different proteins from which 90 wt% are collagens.[4] The protein matrix is of particular importance as a template for the apatite nucleation during the formation of the tooth as well as for its mechanical properties.[5] Dentine has with more than 150 MPa a remarkably high compressive strength, a high hardness, and, regardless, a high flexural strength.[6-8] To mimic the dentine

structure, a system consisting of calcium phosphates in combination with gelatin, which is denatured collagen, was used. In contrast to collagen, gelatin is well soluble in water, cheaper, and is easily industrially scalable.[9]

Collagen and gelatin, in combination with calcium phosphates both, are already used in a wide variety of applications and compositions, but due to their properties, mainly in bone defect repair.[2, 10-11] Three main routes are typically used for the production of bone scaffolds.[2] The first and most straightforward way is to blend collagen or gelatin with previously synthesized calcium phosphate particles.[10] The second method is the production of a protein network by, for example, freeze casting, which is afterwards mineralized in simulated body fluid.[10, 12-13] The third option is the direct precipitation of calcium and phosphate precursors within a protein-containing solution followed by freeze casting.[10, 13]

To synthesize the basic bioinspired materials from gelatin and calcium phosphates, a precipitation reaction was applied within this work.[14] This approach allows to synthesize nanoplatelet-like apatite-gelatin composite materials, which have a high analogy to mammal dentine. Despite its high compositional similarity, the most crucial discrepancy between the biomimetic material and its natural counterpart is that the used gelatin, in contrast to collagen, is well water-soluble.[15] Thus, the resulting composite might swell or even dissolve in contact with water. In order to reach sufficient mechanical stability for medical applications, the gelatin needs to be crosslinked. Since the prepared composite material has a high mineral content of at least 80 wt%, crosslinking is not as straightforward as for pure gelatin. Therefore, different known protein crosslinkers were tested for their performance within this apatite-gelatin nanocomposite system, and the resulting materials were characterized in detail. Using this approach, we were able

to identify the most suitable crosslinkers for possible tooth-filling materials with compositions and properties close to human dentine.

EXPERIMENTAL SECTION

Preparation of Apatite-(CaOH)-Gelatin nanocomposites: Apatite-gelatin nanocomposites were prepared in an aqueous precipitation reaction modified from Kollmann et al.[14] 3 g gelatin (bloom 200-300; pigskin; Gelitta Medella Pro <600) were dissolved in 250 ml MilliQ water followed by the addition of 24.51 g, $CaCl_2 \cdot 2H_2O$ (0,167 mol; p.a.; Carl Roth) at room temperature followed by adjustment to pH 9 using 1 M NaOH. The obtained solution was pre-structured for 30 minutes under continuous stirring at 300 rpm. During this pre-structuring period, a complexation between the gelatin and the Ca-ions occurs.[14] Simultaneously, a solution containing 13.80 g $NaH_2(PO_4) \cdot H_2O$ (0.10 mol; Reag.; Merck) in 250 ml MilliQ water was prepared. After the pre-structuring period, the $NaH_2(PO_4) \cdot H_2O$ solution was added with 3 mL/min under continuous stirring at 300 rpm while the pH was kept at 9 ± 0.1 by continuous addition of 1 M NaOH using an automated setup (Masterflex L/S 77200-80; Consort R305). After the addition was completed, the pH of the solution was kept constant for further 24 h to achieve a complete conversion. To clean the obtained milky precipitate from remaining unbound gelatin and further byproducts, washing steps by centrifugation were performed at 4000 rpm followed by addition of 200 ml of MilliQ water at 55°C and stirring for 3 minutes followed by the next centrifugal step. 4 rounds of washing and centrifugation were performed to obtain a pure product. The white viscous composite material was freeze-dried for analysis or cooled until further use.

Crosslinking of the prepared nanocomposites: In order to receive materials with composition and properties close to natural dentine, the apatite-(CaOH)-gelatin nanocomposites were

crosslinked after their synthesis. Therefore, different known protein crosslinkers were tested on their performance within the nanocomposite materials. An overview of the different crosslinkers is shown in Table 1.

The crosslinkers were analyzed at different concentrations and according to standard protocols from the literature.

Caffeic acid (Alfa Aesar Germany)was used at concentrations ranging from 0.1 % up to 1 % (w/w) due to its limited solubility at ambient temperature.[16-17] 0.01 g, as well as 0.1 g Caffeic acid was therefore diluted to 10 g with MilliQ water at 70 °C and the resulting solutions were cooled to room temperature. The pH was adjusted to pH 9 with 1 M NaOH followed by the addition of 2 g of the previously prepared composite material. The mixtures were stirred and bubbled with oxygen for 30 min. and kept on a multi-flask shaker for 24 h before centrifugation at 9000 rpm for 10 min. The obtained composites were dried at 60 °C and cut for mechanical analysis.

1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide-HCl (EDC) (Carl Roth Germany) was used at concentrations of 0.1 % and 1 % (w/w).[18] Typically, 0.01 g (0.052 mmol) or 0.1 g (0.52 mmol) EDC, respectively, were diluted to 10 g with MilliQ water. The resulting solutions were mixed with 2 g of the previously prepared composite material at pH 7. The mixtures were stirred for 1 min and kept on a multi-flask shaker for 24 h before centrifugation at 9000 rpm for 10 min. The obtained residues were dried at 60 °C and cut for mechanical analysis.

EDC/ N-Hydroxysuccinimide (NHS) (Acros Organics) coupling was carried out at a fixed threefold molar excess of EDC compared to NHS using between 0.1 % and 1 % (w/w) of EDC.[19-20] 0.01 g of EDC (0.052 mmol) was mixed with 0.002 g of NHS (0.017 mmol) and diluted to 10 g with MilliQ water. For the second sample, 0.1 g of EDC (0,52mmol) was mixed with 0.02 g of NHS (0.17 mmol) and diluted to 10 g with MilliQ water, followed by the addition of 2 g of the

previously prepared composite material. The pH was adjusted to 7 using 0.1 M HCl. The mixtures were stirred for 1 min and kept on a multi-flask shaker for 24 h followed by centrifugation at 9000 rpm for 10 min, and drying at 60 °C.

Paraformaldehyde (Sigma Aldrich) was used at a concentration of 5 % (w/w).[21] Therefore, 0.5 g of paraformaldehyde was dissolved in 10 ml of MilliQ water at 60°C and adjusted to pH 7, to produce a 1.67 M Formaldehyde solution. After cooling to room temperature, 2 g of the previously prepared composite were added, and the pH was kept at pH 7. The mixture was stirred for 1 min and kept on a multi-flask shaker for 24 h before centrifugation at 9000 rpm for 10 min, followed by drying at 60 °C.

Genipin (CBC Challenge Bioproducts) is a plant extract from the flower *Genipa Americana*, which is known as an excellent natural protein crosslinker.[22-23] Genipin has been used at concentrations from 0.1 % to 1 % (w/w) due to its lack of solubility in water. To solubilize the genipin, the required amount (0.01 g; 0.05 g; 0.1 g) was dissolved in Ethanol and afterward diluted with MilliQ water to 10 ml followed by pH adjustment to pH 7 using 0.1 M HCl. The resulting solutions were mixed with 2 g of the previously prepared composite material, and the pH was again adjusted to 7 to prevent polymerization of genipin. The resulting solution was stirred for 1 min and kept on a multi-flask shaker for 24 h before centrifugation at 9000 rpm for 10 min and drying at 60 °C.

Because of its high reactivity and easy applicability, **glutaraldehyde** is the most frequently used crosslinking agent for proteins.[24] Glutaraldehyde (Alfa Aesar Germany) has been used in concentrations between 1 % and 50 % (w/w). For crosslinking, a 50 wt. % stock solution was diluted to the required concentration by use of MilliQ water. Afterward, 10 ml of glutaraldehyde solution were mixed with 2 g of the previously prepared composite material. The mixture was

stirred for 1 min and kept on a multi-flask shaker for 24 h followed by centrifugation at 9000 rpm for 10 min and drying at 60 °C.

Grape seed extract contains a mixture of different **proanthocyanidins**, which are known to crosslink proteins, especially collagen.[25-26] Grape seed extract has been used in concentrations between 0.1 % and 6 % because of its low solubility limit in water. The needed amount of Grape seed extract was filled up with MilliQ water to 10 g followed by heating to 70°C for 3 hours under continuous stirring. The resulting solutions were cooled to room temperature and centrifuged at 9000 rpm to eliminate insoluble fractions of the extracts. The resulting supernatant was mixed with 2 g of previously prepared composite material followed by stirring for 1 minute. The mixture was kept on a multi-flask shaker for 24 h before centrifugation at 9000 rpm for 10 min, and drying of the residue at 60 °C.

Transglutaminases are enzymes, which catalyze the transamidation between glutamine and primary amines resulting in an isopeptide bond.[27-28] There are different known types of transglutaminases that can be calcium-dependent or calcium-independent. In this approach, a calcium-independent microbial transglutaminase was used. Transglutaminase (AJINOMOTO Activa WM) was first dissolved in water and ultrafiltrated by Millipore 10,000 g/mol filters five times to remove remaining byproducts and additives. The received supernatant was freeze-dried and used in concentrations between 0.1 % and 10 % (w/w) with MilliQ water. 10 g of the resulting solutions were mixed with 2 g of previously prepared composite material, stirred for 1 min, and kept on a multi flask shaker for 24 h before being centrifuged at 9000 rpm for 10 min, followed by drying of the residual at 60 °C.

Casein furthermore was selected as a bridging agent between the composite particles.[29-30] Therefore, different proportions of casein and transglutaminase were mixed to find the most

suitable composition. Casein (Sigma Aldrich Germany) has been used in concentrations between 0 % to 3 % (w/w) and transglutaminase in concentrations between 0 % and 5 % (w/w). 10 g of the prepared solutions were thereupon mixed with 2 g of previously prepared composite, stirred for 1 minute, and left on a multi flask shaker for 24 h. The resulting solutions were centrifuged at 9000 rpm for 10 minutes, and the residual was dried at 60 °C.

Analysis of the Apatite-Gelatin nanocomposites

The crystalline inorganic phase of the obtained composite material was analyzed using powder X-ray diffraction (PXRD). The samples were ground in an agate mortar prior to analysis, and the patterns were recorded using a Bruker D8-Discovery (CuK_{α}-radiation) equipped with a Vantec detector.

Furthermore, potential changes in calcium short-range order triggered by the gelatin incorporation within the composite material were investigated via EXAFS at the calcium K-edge (4.0381 keV). Measurements at the calcium K-edge were undertaken at the Elettra synchrotron Italy,[31] operating at an energy of 2 GeV and a current of 300 mA. Samples were ground in an agate mortar, diluted with graphene powder, and compacted into thin pellets. An optimal sample thickness of ~300 μm and optimal calcium concentration after dilution with graphene powder was calculated based on the calcium content of the neat samples in order to obtain an X-ray absorption at the Ca K-edge yielding a theoretical edge jump of $\Delta(\mu x) = 0.80$. Full scans of the X-ray absorption near edge structure (XANES) and extended X-ray absorption fine structure (EXAFS) were collected in steps of 5 eV in the pre-edge region (from 3738.43 eV to 4028.53 eV) and 0.2 eV in the edge region (from 4061.51 eV to 4061.71 eV), gradually increasing to 2.6 eV in the post-edge region (4061.71 eV to 4589.30 eV) up to k=13.

Three scans per sample were collected in transmission mode using a FMB-OXFORD ion chamber detector and then averaged in order to improve the signal-to-noise ratio. Samples were mounted on a copper sample holder and maintained at a temperature of 80 K using a liquid-N_2-cryostat, to minimize thermally induced structural disorder and any potential beam damage. Energy calibration was performed based on the first inflection point of a Sn L_3 edge (3929 eV) reference metal foil.

Alignment, energy calibration, and deglitching were performed on every single scan before averaging, using built-in features of the Athena software package.[32] Normalization and background removal were performed identically for all samples. The energy origin E_0 was fixed at the maximum of derivative of the edge jump, and the R_{bkg} value used for background removal of high-frequency oscillations within the software algorithm was set at 1.1.[33] The pre-edge region was fit with a linear function, and the post-edge background to a 3rd polynomial segment.

Previous solid-state NMR spectroscopy studies showed the presence of a thin boundary layer of an amorphous organic-containing phase within the apatite-gelatin nanocomposite system.[34-35] Since these results are transferable to our received apatite-gelatin composites, no further NMR experiments have been conducted within this study.

The gelatin content was determined using C,H,N,S-analysis (CHNS-analyser Vario MICRO Cube; Elementar). By CHNS-analysis, it was possible to determine the gelatin's nitrogen content to be 16.5% (w/w). Using this value, it was possible to calculate back the gelatin content of the received nanocomposites by comparing their nitrogen content to the nitrogen content of pure gelatin.

The size and morphology of the nanocomposites were determined using Transmission Electron Microscopy (TEM) using a Zeiss Libra120 electron microscope operating at 120 kV acceleration

voltage. Furthermore, electron diffraction was used to identify the phase composition of the nanocomposite material.

To further determine the structure of the composites, especially after crosslinking, and to investigate the organic components within the composite material, Scanning Electron Microscopy (SEM) and Energy-Dispersive X-ray spectroscopy (EDX) was used on a Zeiss CrossBeam 1540XB as well as on a Zeiss Gemini 500 equipped with an EDX Ultim Max 100 for EDX-Analysis. The received samples were either analyzed *per se*, or the inorganic components were dissolved using 0,2 M Ethylenediaminetetraacetic acid (EDTA) for three weeks, followed by freeze-drying for analysis of the remaining organic structure. The obtained samples were sputtered with 6 nm of platinum prior to investigation.

The mechanical properties of the composite material were investigated using Vickers hardness measurements. The obtained materials were cut in 1 mm thick platelets using a diamond saw and polished on sandpaper. Afterward, the samples were analyzed for their mechanical properties using a Leitz Miniload 2 Vickers hardness device equipped with a camera in combination with Hardsoft V1.3 Ueye software. The polished platelets were indented with a Vickers indenter using 0.2 kg of weight. All samples were measured five times, and their mean value, together with their standard deviation, were furthermore used in the analysis.

RESULTS AND DISCUSSION

Characterization of Apatite-Gelatin nanocomposites

The morphology of the synthesized nanocomposite particles is displayed in the TEM and SEM images in Fig. 1 A and B. Platelet-like particles with diameters between 30 and 100 nm and a thickness below 10 nm were produced and are consistent with the previous results of Kollmann et

al.[14]. The PXRD patterns discernible in Fig. 1 C, as well as the electron diffraction and EXAFS measurements in Fig. 1 D, reveal clear evidence that the mineral component of the nanocomposites is apatite-(CaOH).

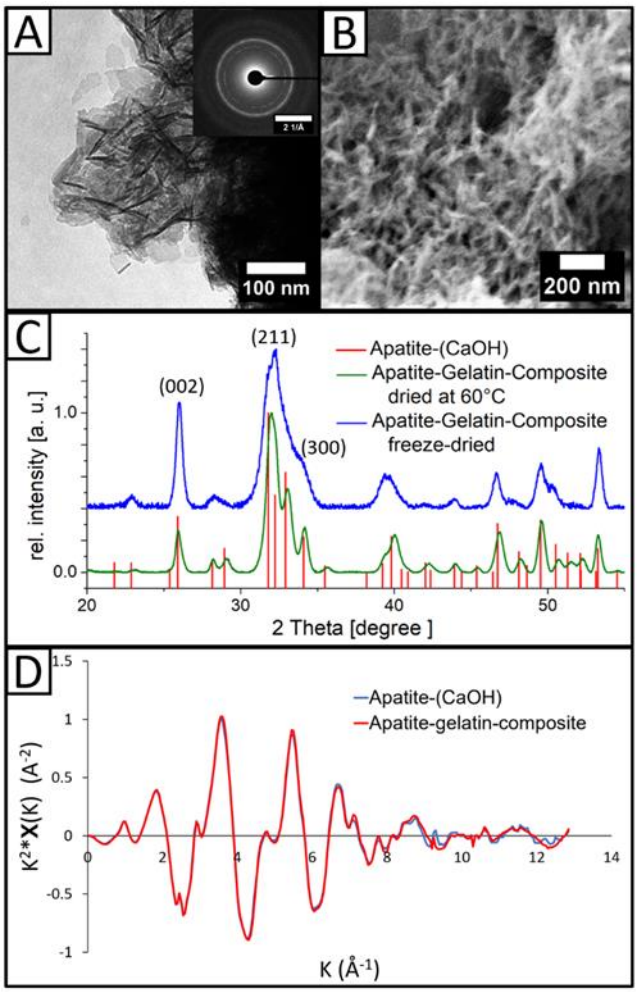

Figure 1. (A) TEM image of freeze-dried apatite-gelatin nanocomposites with corresponding electron diffraction indicating crystalline apatite. (B) SEM image of freeze-dried apatite-gelatin nanocomposites. (C) pXRD Patterns of freeze-dried and 60 °C dried apatite-gelatin nanocomposites in comparison to apatite-(CaOH) from Ref. 96-901-0052. (D) K-space EXAFS signal of apatite-(CaOH) and apatite-gelatin-nanocomposites, synthesized under the same conditions.

The EXAFS measurements show no significant difference between the average short-range order of apatite-(CaOH) and apatite-gelatin nanocomposites when synthesized under the same conditions indicating the presence of apatite primarily on the surface of the particles. The PXRD analysis reveals the difference between both investigated drying methods. Freeze-drying resulted in broad signals, which stem from the low crystallinity and the thin platelet-like structure extending only a few unit cells. In comparison, the sample dried at 60 °C shows much sharper reflexes which probably originate from the longer reaction times and the drying process leading to larger coherent scattering domains. The gelatin content of the composite material was determined to be 15 % (w/w) using CHNS-analysis, which represents a volume proportion of about 45 % (v/v) based on the lower density of gelatin. The thus obtained protein content is comparable to mammal dentine, where average protein contents of about 20 wt% are reported.[4]

Crosslinking of Apatite-Gelatin nanocomposites

Two of the most important factors for the usability of biomimetic tooth filling materials are their mechanical performance and especially their durability. Therefore, Vickers hardness testing was used to find the most suitable crosslinkers.

Table 1. Overview of the used possible gelatin crosslinkers in the apatite-gelatin nanocomposite material.

Crosslinking agent	**Crosslinking mechanism**
Caffeic acid	Crosslinking of two primary amines due to dimerization of two caffeic acids.[16-17]
EDC	Zero length crosslinker between carboxylate and amine groups.[18]
EDC/N-Hydroxysuccinimide	Zero-length crosslinker between Carboxylate and amine groups. With higher yields than pure EDC.[19-20]
Formaldehyde/ Paraformaldehyde	Methylene bridge between primary and secondary amines.[21]
Genipin	Possibly crosslinking of two primary amines. [22-23]
Glutaraldehyde	Bridging between two primary amines.[24]
Proanthocyanidins/ Grape seed extract	Possibly hydrogen bonding or covalent interactions.[25-26]
Transglutaminase	Enzymatic zero-length crosslinking between a primary amine and glutamine.[27-28]
Transglutaminase + Casein mixture	Enzymatic zero-length crosslinking between a primary amine and glutamine[27-28] combined with casein as a linker between particles.[29-30]

Furthermore, the mechanical properties of the materials can be used to get a hint into the crosslinker's performance. The hardness of the material can be set in context to the fraction of crosslinks inside the material since the correlation between hardness and crosslinking density is known for gelatin[36-37] as well as for further polymeric materials.[38-39]

Table 1 summarizes the different crosslinkers used for the crosslinking process, as well as the molecular working principle during the crosslinking. The comparison of the varying hardnesses resulting from the different crosslinkers reveals some trends. While some crosslinkers, which are known to crosslink pure gelatin, are not improving the material properties of the nanocomposites

at all, some could more than double the hardness of the plain apatite-gelatin-nanocomposites. The resulting hardnesses of the crosslinked apatite-gelatin-nanocomposites are displayed in Fig. 2

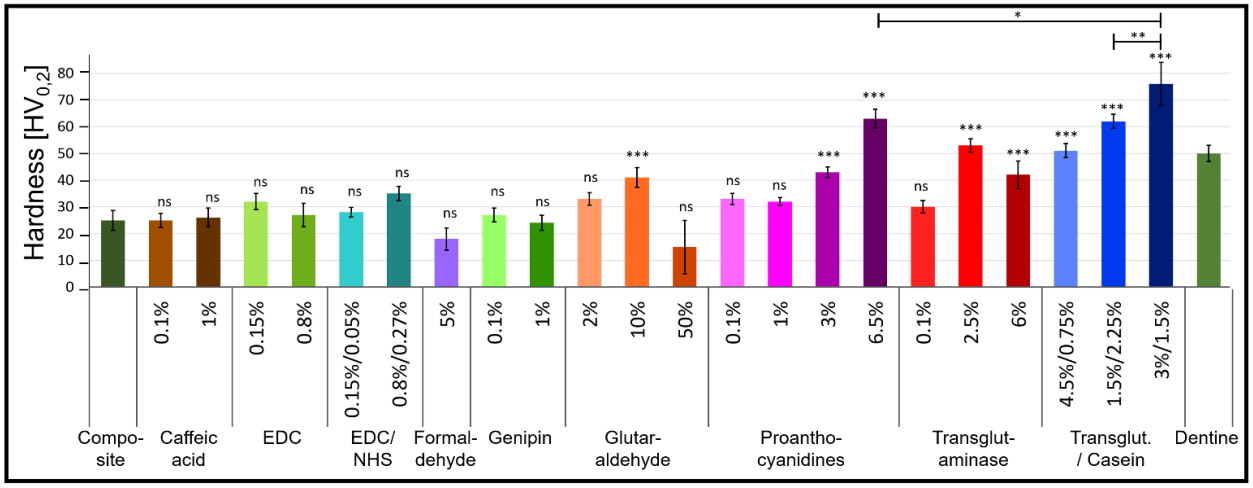

Figure 2. Overview of the different achieved Vickers hardness values using various crosslinkers in varying concentrations, compared to uncrosslinked apatite-gelatin nanocomposite and the hardness of human dentine.[8] Error bars represent the SD of five repeated measurements. Asterisks (*) indicate significant differences of the hardness of the non-crosslinked composite to the crosslinked pendants by student one-way ANOVA with ns= not statistically significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Caffeic acid, with a maximum value of 25 ± 3 HV0.2, didn't improve the hardness of the composite regardless of its concentration. Since caffeic acid is an effective protein crosslinker only in contact with oxygen,[16] the dense packing of the apatite-gelatin nanoplatelets might have circumvented the crosslinking between the different nanoplatelets blocking the oxygen diffusion. Furthermore, no oxygen bubbling could be carried out during the 24 h ripening time on the multi-flask shaker, which shortened the possible reaction times. Both effects could have led to ineffective crosslinking.

EDC could increase the hardness of the dried composite by 28 % (32 ± 3 HV0.2), but it was effective only in low concentrations of 0.15 wt.%. The impact was weakened using higher amounts of EDC and was furthermore shown to be not significant by Tuckeys post hock test. This minor increase in hardness might be caused by the multiple possible side reactions that occur during the crosslinking process using EDC in higher concentrations and especially when used at neutral pH, which was mandatory as acidic pH could lead to the dissolution of the apatite.[18]

The combination of **EDC** and **NHS** gave more promising results. At higher concentrations using 0.8 wt.% EDC (50 mM) and 0.27 wt.% NHS (17 mM) a hardness of 35 ± 3 HV0.2 could be achieved, translated into an effective hardness increase of about 40%. This shows the EDC and NHS combination‘s advantage to prevent side reactions of the crosslinking reaction, which is already known in literature.[19-20] But still, the results of the EDC/NHS crosslinked material showed no statistical significance compared to the hardness of the uncrosslinked composite, which can also be traced back to the unsuitable neutral pH during crosslinking.

In contrast, **formaldehyde** deteriorates the hardness of the composite (18 ± 4 HV0.2). No useful crosslinking could be realized within this system. It appeared that the formaldehyde even dissolved some parts of the composite material, which led to a partly porous or gum-like consistency after crosslinking. Therefore, crosslinking with formaldehyde was not further investigated.

Regardless of its high reputation, **genipin** also gave poor results in the crosslinking process, and no increase in hardness of the composite material could be recorded (27 ± 3 HV0.2). Here the alkaline environment of the composite, might have induced polymerization of genipin itself, which could have led to a deterioration of its crosslinking performance.[40]

Glutaraldehyde showed promising results under the applied conditions. Due to its good miscibility with water, it was possible to use glutaraldehyde in concentrations of up to 50 wt.%.

However, higher concentrations didn't improve the properties of the material. Amounts of 10 wt.% gave the highest hardness values. Using 10 wt.% glutaraldehyde, a hardness of 41 $HV_{0.2}$ could be realized, which corresponds to a significant improvement in hardness of 64 %. Using higher concentrations of glutaraldehyde did not further improve the hardness of the material. This observation might be explained by the fast reaction rates of glutaraldehyde, leading to an inhomogeneously crosslinked material at higher concentrations.

Even more-promising results could be achieved using **proanthocianidins** from grape seed extract. A continuous increase in the hardness of the material is visible from low concentrations to high concentrations of the crosslinker. While 1 wt.% of proanthocyanidins only leads to a statistically ambiguous increase in hardness of 28 %, the use of 3 wt.% already leads to a significant increase of 72 %.

The best results could be achieved close to the proanthocyanidins solubility limit of 6.5 wt.% with a hardness of 63 HV0.2 which corresponds to a significant increase in hardness of more than 150 %. The continuous increase in the hardness shows the good crosslinking qualities of proanthocyanidins as an apatite-gelatin-nanocomposite crosslinker. The only drawback of using proanthocyanidines as crosslinkers is their coloring effect, as they produce a brownish color within the material, which is not beneficial for tooth filling materials.

Transglutaminase also gave excellent hardness values, especially within a concentration of about 2.5 wt.%. Here the Vickers harness could be significantly increased up to 53 HV0.2 corresponding to a hardness increase of 112%. Higher concentrations, however, lowered the values. This could be due to the remaining transglutaminase residues within the apatite-gelatin-composite. These protein residues could decrease the overall hardness of the material, due to their bad integration in the protein network.

To further improve the properties of the composite material, further experiments were performed with the addition of **casein** to the **transglutaminase** crosslinking solution. This combination was intended to act as a glue between the single apatite-gelatin nanocomposite particles. Casein was chosen because of its well-known properties in reactions with transglutaminase, as this combination is frequently used as protein glue to produce meat products.[30] Additional casein is known to improve the material properties of transglutaminase crosslinked gelatin gels when blended in the mixture.[29] In this way, casein could also give benefits in the crosslinking reaction with apatite-gelatin-nanocomposites.[29] Furthermore, casein is known to have a stabilizing effect on calcium and phosphate ions and thus has an influence on the crystallization and stability of apatite.[41]

Therefore, different amounts of casein and transglutaminase were mixed in different ratios in the range of 0.3 wt.% to 3 wt.% for casein and 0.6 wt.% to 6 wt.% for transglutaminase. An exemplary overview of different combinations and the resulting hardnesses are displayed in Fig. 3. It is discernible that the combination of casein and transglutaminase enhances the hardness of the composites, especially using concentrations of casein about 1.5 wt.% in the crosslinking solution. Further results indicated that higher amounts of transglutaminase also lead to higher hardness values. Especially the combination of 3 wt.% transglutaminase with 1.5 wt.% casein gave superior results with 76 HV0.2. This result is highly encouraging for the future development of tooth filling material as it presents hardness values higher than natural human dentine.

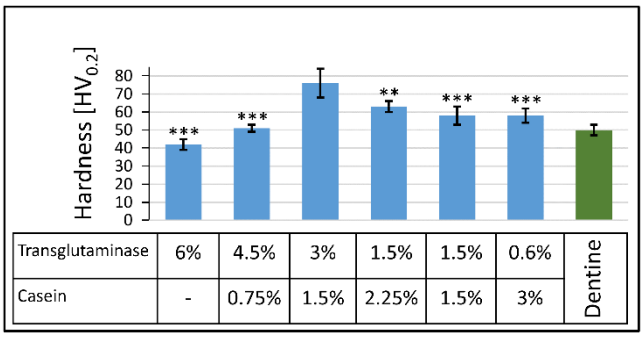

Figure 3. Comparison of the hardness of different combinations of casein and transglutaminase in comparison to natural dentine.[8] Error bars represent the SD of five repeated measurements. Asterisks (*) indicate significant differences of the hardness of the 3 % transglutaminase/ 1.5 % casein crosslinked sample to all other concentrations by student one-way ANOVA with ns= not statistically significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Analyzing the transglutaminase/ casein crosslinked composite material for its structure using SEM imaging, a outstandingly high proximity between the crosslinked composite (Fig 4 C) and the fibrous structure, which is also known for mammal intertubular dentine, is revealed.[42] This could be an explanation for the superior mechanical results received using crosslinkers within the composite since the mineral structure of dentin could be mimicked on the lower hierarchical levels. Figure 4 B shows a dried transglutaminase/ casein crosslinked sample molded in a solid structure. By precisely controlling the crosslinking and drying process it is in this way possible to synthesize samples in controlled shapes even in the centimeter range. Since the shrinking of the material complicates the precise reaction control, also blocks of the material can be synthesized and afterwards cut into the desired shapes.

In order to compare the crosslinked composites on a more quantitative base, we tried to determine the average molar mass of the gelatin chains between two crosslinks using the Flory-Rehner theory.[43-45] However, the crosslinked samples did not swell to a considerable degree

indicating a high crosslinking degree not only by chemical crosslinking by the added organic crosslinkers but also by physical crosslinking by Ca^{2+} and the apatite nanoparticles.

The most promising crosslinkers were subsequently further analyzed for their dissolution behavior. Using this experiment, we were able to identify if the composites' hardness values also correspond to their dissolution stability, as well as to have a look at the distribution of the crosslinked organics inside the composite material. Therefore, samples of Glutaraldehyde-, Transglutaminase- and Transglutaminase/Casein- crosslinked apatite-gelatin nanocomposites were investigated for their dissolution times in 0.2 M EDTA solutions. Furthermore, the dissolved samples were used for SEM measurements after freeze-drying of the remaining crosslinked organic structure to get insights into the composition and distribution of the crosslinked organic remains.

Comparing the influence of the different used crosslinkers, a similar impact on the dissolution behavior than on the hardness becomes discernible. Figure 4 A shows that the crosslinkers leading to the highest hardness values also showed with more than 30 days the most extended dissolution times in 0.2 M EDTA solutions. While the uncrosslinked composite is fully dissolved within two days, the crosslinked samples did not undergo complete dissolution, evident by a remaining transparent gelatin mold. By crosslinking, the gelatin network is stabilized against swelling and dissolution. So, dissolution in this context must be seen as a dissolution of the apatitic phase inside the composite until only the crosslinked gelatin network remains.

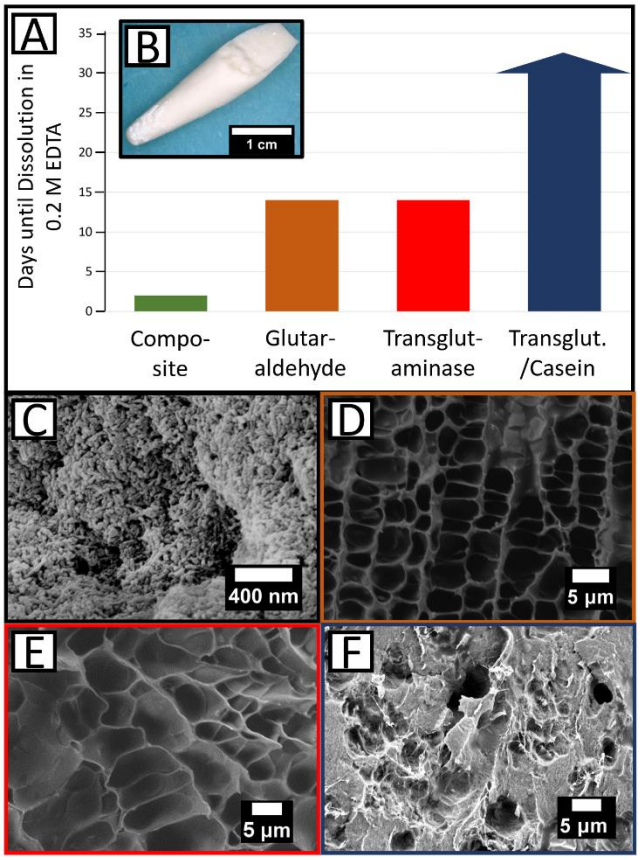

Figure 4. (A) Overview of the dissolution times of some crosslinked apatite-(CaOH)-gelatin composites in 0.2 M EDTA solutions. (B) Exemplary image of a 2.5 wt.% transglutaminase crosslinked composite after drying at 60°C. (C) SEM analysis of a 2.5 wt.% transglutaminase crosslinked composite after drying at 60°C. The image is exemplary for all transglutaminase crosslinked and dried composites. (D; E; F) SEM analysis of 3 weeks 0.2 M EDTA-treated crosslinked composites. (D) 10 wt.% Glutaraldehyde crosslinked composite. (E) 2.5 wt.% transglutaminase crosslinked composite. (F) 3 wt.% transglutaminase/ 1.5 wt.% casein crosslinked composite.

Comparing the dissolution times of the different crosslinked composites, it became apparent that crosslinking slows down the dissolution of the apatite phase due to the protection of the inorganic components by the stabilized organic network. This relation is not only observable by the dissolution within the composite disks but can also be determined by comparing the SEM images

of the differently crosslinked composites after three weeks of EDTA treatment (Fig. 4 D-F). The glutaraldehyde and the transglutaminase crosslinked composites, which both have a hardness of about 40 HV0.2, show a complete dissolution of their inorganic phase as observable in their freeze-dried structure displayed in Fig. 4 D and E. In these SEM images, the crosslinked gelatin shows pores in the size range of 5 µm, corresponding to the typical structure of pure crosslinked and freeze-dried gelatin gels.[46]

The transglutaminase/ casein crosslinked composite, in contrast, which exhibited the highest hardness values of 76 HV0.2, only shows surficial alterations (Fig 4 F). This was also observable by the solid consistency of the composite disc remaining intact for more than 30 days. Here the diffusion of the EDTA molecules inside the composite material seems to be almost suppressed by the high crosslinking density. Only small spots are observable where parts of the apatite were dissolved by EDTA before its diffusion was limited by the highly crosslinked gelatin network. Therefore, a correlation between hardness, dissolution time, and crosslinking density can be determined.

CONCLUSION

Different crosslinkers for apatite-gelatin nanocomposites have been tested for their crosslinking performance and suitability to produce a suitable dentine replacement material. Optimal crosslinkers could be identified by analyzing the mechanical properties of the crosslinked composite materials. On top of that, the dissolution stability of the resulting materials in EDTA solutions confirmed the previously achieved conclusions. Using these experiments, several possible crosslinkers for the apatite-gelatin nanocomposites could be identified from glutaraldehyde, proanthocyanidines, and transglutaminase. Although further crosslinkers like

GelMa exist,[47] the tested selection of crosslinkers showed clear differences and optimal crosslinkers could be identified. Furthermore, the mechanical analysis indicated the advantage of the combination of a fast and reliable crosslinker like transglutaminase in combination with a bridging polymeric material like casein. Here, casein could act as a glue between two single nanocomposite particles and thus improve its mechanical stability. Using this approach, Vickers hardness values higher than human dentine were attained. Values of up to 76 HV0.2 could be achieved, which corresponds to an increase of 190% compared to the uncrosslinked apatite-gelatin nanocomposite. Furthermore, it could be demonstrated that the crosslinked composite's dissolution stability depends on the crosslinkers quality in a comparable way like the hardness. It was revealed that the 3 wt.% transglutaminase/ 1.5 wt.% casein crosslinked materials did outperform every other used crosslinker over all experiments. In conclusion, a bioinspired material with properties and compositions close to human dentine could be produced. This could lead to the development of the first completely biomimetic tooth filling material.

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