Received: 9 October 2023 | Revised: 6 December 2023 | Accepted: 13 December 2023

DOI: 10.1002/jbm.a.37661

RESEARCH ARTICLE

Transglutaminase enables highly hydrolytically and proteolytically stable crosslinking of collagen on titanium surfaces and promotes osteogenic differentiation of human mesenchymal stem cells

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Funding information
Deutsche Forschungsgemeinschaft, Grant/Award Number: 495328185

Abstract

Collagen with its bioactive ligand motives would be predestined as coating on bone implant surfaces like titanium hip stems to facilitate receptor-mediated cell adhesion and thereby improve early osseointegration. Unfortunately, collagen as coating exhibits very low proteolytic resistance in vivo. To overcome this limitation, different crosslinking methods of collagen (transglutaminase, GTA, EDC/NHS, riboflavin, and lysyl oxidase) with silanized titanium alloy (Ti6Al4V) were investigated in terms of degradation resistance, hydrolysis stability, tensile strength, and metabolic cell activity. The in vitro osteogenic differentiation ability of human mesenchymal stem cells (hMSCs) induced by the surface modification was evaluated by immunofluorescence of early osteogenic markers, Alizarin red staining, and energy dispersive X-ray spectroscopy. The expression of the adhesion-related protein vinculin was analyzed on the different functionalized surfaces. The results revealed that the enzymatic crosslinker transglutaminase offered high degradation resistance, tensile strength, and hydrolysis stability compared to the other crosslinking reagents tested. Remarkably, the adhesion sequences within the collagen were accessible to the hMSCs despite the transglutaminase crosslinking procedure. In conclusion, the organochemical functionalization of Ti6Al4V surfaces with collagen using transglutaminase holds great potential to facilitate an enhanced interaction with attached bone cells and thereby could potentially improve and accelerate osseointegration of a titanium-based bone implant in vivo.

KEYWORDS
collagen, crosslinker, osteogenic differentiation, proteolytic and hydrolysis stability, titanium

1 | INTRODUCTION

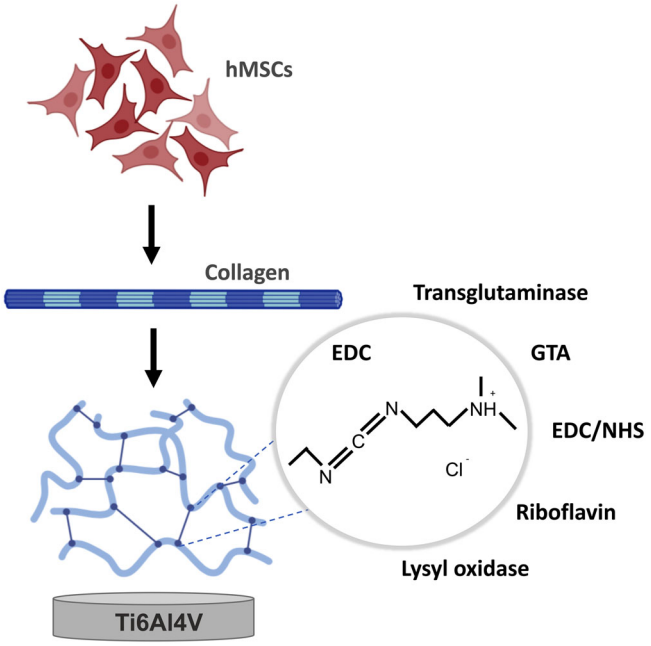
Titanium and its alloys are widely used as implant material in contact with bone due to their excellent cytocompatibility, mechanical properties, and corrosion resistance.[1–3] The corrosion resistance of these metals derives from the formation of a thin and strongly adherent oxide layer that passivates the material in vivo.[4] For this reason, titanium-based implants exhibit good osseointegration and are

frequently used in dental or orthopedic applications. Nevertheless, cell growth and differentiation capacity on the surface are limited.[5] Various functionalization strategies for titanium surfaces with bioinspired coatings have been employed in recent years to address these limitations.[6] Bioactive proteins, nucleotides, as well as peptides and antimicrobial agents have been immobilized to generate functional properties on the titanium surface.[7–10] Here, coatings that can affect cell recruitment, cell differentiation, mineral formation, antimicrobial activity, and induction of osseointegration are of great interest.[11–13] One of the most commonly used peptides to functionalize biomaterials are cell adhesion peptides containing the arginine-glycine-aspartic acid (RGD) sequence, which is present in several proteins, in particular in collagen, fibronectin, bone sialoprotein, and osteopontin.[11,14] RGD peptide-enriched materials have been shown to interact with integrin cell surface receptors and can enhance bone marrow-derived stem cell adhesion.[15,16]

Collagen is the main component of the extracellular matrix (ECM) and accounts for one third of the total body protein content in mammals.[17] To date, 28 types of collagen have been identified and characterized, revealing a similar structure.[17] Because of its excellent properties of biocompatibility, adhesion, and osteogenic induction, as well as low immunogenicity, collagen is frequently used as biomaterial.[18,19] However, biomedical applications are strongly limited due to collagen's lack of mechanical strength in aqueous media and its susceptibility to enzymatic degradation in vivo.[18]

To overcome these limitations, different cross-linking strategies have been developed to improve the properties of collagen.[18] Various strategies can be used for the binding of biomolecules to surfaces, including physical adsorption, electrostatic attraction, and polymer modification.[5,6,20] However, most of these strategies show insufficient coupling of the protein or require cytotoxic reagents for the immobilization process. A strong covalent bond between the substrate and biomolecules should provide a stable coating capable of withstanding the forces acting on the surface during surgical implantation, the aqueous environment, and enzymatic degradation in vivo.[5] Enzymatic degradation occurs due to a defense reaction against implanted foreign materials. Mainly collagens, polysaccharides (hyaluronic acids), some polyesters (e.g., polyhydroxyalkanoate), synthetic polycarbonates and proteins are degraded by this type of reaction.[21] Biochemical immobilization methods, including coupling with silanes and thiols, have been investigated to achieve a high degree of mechanical and biochemical stability of coatings made of proteins or other biomolecules.[5,22,23] Silane chemistry has already been used to crosslink biomolecules with various inorganic substrates to provide them with bioactive motifs. The addition of additional coupling agents (crosslinkers) such as EDC/NHS (1-ethyl-3-(-3-dimethylaminopropyl) carbodiimide hydrochloride/N′,N′-dicyclohexylcarbodiimide) can enable the nucleophilic reaction with the amines present on the organosilane molecule, thus ensuring stable immobilization of the biomolecules.[24]

The aim of the present study was to systematically investigate the effect of various crosslinking strategies on the adhesion and osteogenic differentiation behavior of mesenchymal stem cells on titanium-based substrates (Scheme 1). The alternative crosslinking agents used in this study included chemical (EDC/NHS, GTA), enzymatic (transglutaminase, lysyl oxidase), and photochemical (riboflavin) reagents. Due to the predominantly aqueous and enzyme-rich environment in vivo, the different crosslinker strategies were compared in terms of degradation resistance and hydrolysis stability. Furthermore, calcium production ability in hMSCs after 21 days was assessed by Alizarin red staining and energy dispersive X-ray spectroscopy (EDX). Because of the well-known effect that integrin binding receptors can be blocked through the crosslinking procedure, the expression of the focal adhesion marker vinculin was also tested.



SCHEME 1 Functionalization of Ti6Al4V with collagen using different crosslinkers. Crosslinking efficiency was analyzed in terms of enzymatic degradation, metabolic activity, and osteogenic differentiation behavior of hMSCs. The results revealed that the enzymatic agent transglutaminase triggers enhanced activity of the above mentioned parameters compared to the other established crosslinkers.

2 | MATERIALS AND METHODS

2.1 | Biofunctionalization

2.1.1 | Preparation of titanium discs

Cylindrical Ti6Al4V bars with a diameter of 9 mm was obtained from EWG E. Wagener (Heimsheim, Germany). Ti6Al4V discs (thickness 1.5 mm) were prepared by turning and subjected to a grinding process using silicon carbide grinding paper No. 80, 800, 1200, and 4000 (ATM, Mammelzen, Germany), sequentially. A roughness of <0.2 µm was adjusted on these specimens. After the grinding procedure,

the discs were cleaned by sonication in 70% ethanol (Schmittmann, Düsseldorf, Germany) and Milli-Q water (Sartorius, Göttigen, Germany).

2.1.2 | Pretreatment and silanization of titanium specimens

For the silanization procedure, the specimens were cleaned and hydroxylated by immersion in piranha solution (3:1 ratio of concentrated sulfuric acid [Sigma Aldrich, St. Louis, USA] and 30% hydrogen peroxide [Merck, Darmstadt, Germany]) for 1 min and rinsed thoroughly in Milli-Q water. After drying at room temperature, the Ti6Al4V discs were placed in a round bottom flask equipped with a magnetic stirrer and soaked in a 5 vol % 3-aminopropyldiisopropylethoxysilane (APDS, Gelest, Morrisville, USA) solution mixed with anhydrous toluene (Sigma Aldrich, St. Louis, USA). The reaction was performed under reflux conditions at 120°C for 3 h in an oil bath. Immediately afterwards, samples were thoroughly cleaned by 3-min wash steps with toluene (VWR, Radnor, USA) and Milli-Q water. The specimens were dried overnight at room temperature.

2.1.3 | Immobilization of collagen using different crosslinker

The chemical, enzymatic, and photochemical crosslinkers used within this study were contrasted to each other with respect to stable collagen attachment on titanium surfaces. In order to define a suitable degree of coupling efficiency, different concentrations and exposure times of crosslinkers were compared.

EDC, EDC/NHS

The crosslinking procedure was performed according to the protocol described in Müller et al.[4] Briefly, silanized titanium surfaces were placed in a 48 well plate and covered with 150 µL 0.4% collagen G solution (PAN-Biotech, Aidenbach, Germany) for 2 h at room temperature. After these incubation steps, the supernatant was removed from the samples and they were dried at room temperature. To accomplish the crosslinking procedure, the samples were then soaked in 500 µL PBS (Gibco, Waltham, USA) for 30 min to allow swelling of the collagen. After removal of the buffer, the samples were treated with EDC (1-ethyl-3-[3-dimethylami-nopropyl]-carbodiimide hydrochloride, Thermo Fisher Scientific, Waltham, USA) and EDC/NHS (1-ethyl-3-[3-dimethylami-nopropyl]-carbodiimide hydrochloride/N-hydroxy succinimide, Thermo Fisher Scientific, Waltham, USA) in a 4:1 ratio. For this purpose, crosslinkers were dissolved in previously prepared 2-(N-morpholino)ethanesulfonic acid (MES, Carl Roth, Karlsruhe, Germany) buffer at pH 5.5 and were incubated for 4 h at room temperature.

Glutaraldehyde

As already described above, the silanized titanium samples were placed in a 48 well plate and covered with 150 µL of 0.4% collagen G solution (PAN-Biotech, Aidenbach, Germany). After 2 h of incubation at room temperature, the remaining solution was removed from the surface and samples were dried under a fume hood. To allow the collagen to swell, the samples were incubated with 500 µL PBS. After 30 min, the buffer was removed and the glutaraldehyde (GTA, Sigma Aldrich, St. Louis, USA) was applied to the titanium samples. To accomplish that, five different concentrations (0.1%, 0.2%, 1%, 5%, and 10%) of the GTA were chosen. Finally, the crosslinking procedure took 2 h at room temperature.

Riboflavin

Riboflavin (Sigma-Aldrich, St. Louis, USA) was dissolved in sterile PBS at concentrations of 0.1%, 0.25%, and 0.5% and added to the collagen coated silanized titanium as described above. Afterwards, the 48 well plates holding the samples were placed under a UV light source (VOCO individo Light Box, Altenberge, Germany) for 5, 15, or 30 min to initialize the crosslinking process.

Transglutaminase

A total of 0.4 g of transglutaminase (Meat Glue, Chesterfield, UK) was dissolved in 100 mL PBS at 37°C under continuous stirring. For the crosslinking procedure, different concentrations (1:50, 1:500, 1:5000, and 1:50 000) of transglutaminase solution were prepared. Next, a collagen-containing buffer solution modified according to Orban et al was prepared.[25] For this purpose, 50 mM Tris–HCl (Tris-(hydroxymethyl)-aminomethane-hydrochloride, Sigma-Aldrich, St. Louis, USA), 2.5 mM calcium chloride ($CaCl_2$, Sigma-Aldrich, St. Louis, USA), and 1 mM Dithiothreitol (DTT, Fermentas, Waltham, USA) were dissolved in 15 mL collagen solution (Collagen G, Sigma-Aldrich, St. Louis, USA). Afterwards, 4 µL of each transglutaminase solution was added to 196 µL of the collagen buffer solution. In order to ensure crosslinking of collagen to the sample surfaces, the titanium samples were incubated at 37°C for 3 h. Finally, the titanium samples were dried at room temperature.

Lysyl oxidase

Lysyl oxidase (Sigma-Aldrich, St. Louis, USA) was dissolved in phosphate buffer solution (0.3 M) prepared as follows: 7.6 g of potassium dihydrogen phosphate (Sigma-Aldrich, St. Louis, USA), 3.5 g of disodium hydrogen phosphate (Sigma-Aldrich, St. Louis, USA), 15.7 g of sodium chloride (VWR Chemicals, Radnor, USA), and 50 mL of 5 mM potassium phosphate solution (Sigma-Aldrich, St. Louis, USA) were mixed in 1 L of distilled water.[26,27] Next, 0.002 g of lysyl oxidase and 16 ppm of copper(II) sulfate ($CuSO_4$, Sigma-Aldrich, St. Louis, USA) were dissolved in 5 mL of the phosphate buffer solution. The prepared lysyl oxidase stock solution was then used to prepare 0.016%, 0.053%, and 0.004% solutions by further dilution with the buffer solution. A total of 100 µL of each dilution was then mixed with 100 µL of each 0.4% collagen solution (Collagen G, Sigma-Aldrich, St. Louis, USA) for crosslinking, resulting in enzyme–substrate ratios of 1:10, 1:25, 1:75, and 1:100. Samples were incubated at 37°C for 3 h and then allowed to dry overnight at room temperature in a fume hood.

2.2 | Isolation of human mesenchymal stem cells

Bone marrow-derived MSCs were obtained with written informed consent (EK 300/13) as required by the ethics committee of the Faculty of Medicine of RWTH Aachen University from donors undergoing total hip joint endoprosthesis interventions. For the collection of hMSCs, bone marrow was flushed several times with MSC growth medium (Mesenpan, PAN Biotech, Aidenbach, Germany) containing 2% FCS (PAN Biotech, Aidenbach, Germany) and 1% penicillin/streptomycin (Gibco, Waltham, USA) using a 22 g-needle and plating the cells after centrifugation in a T75 cm^2 culture flask (VWR Chemicals, Radnor, USA). After 24 h, nonadherent cells were removed by medium change. Adherent spindle-shaped cells were recovered from the primary culture after 4–7 days. All cells were cultured at 37°C in 5% CO_2 and 90% humidity. The medium was changed twice per week and the cells were not used when older than passage three.

TABLE 1 Overview of the primary and secondary antibodies used.

Primary antibody	Dilution	Species	Company/Catalog number
Anti-Vinculin	1:5000	Mouse	Merck Millipore #MAB 3574
Anti-RUNX2	1:200	Mouse	Santa Cruz Biotechnology #sc-390,351
Anti-ALP	1:200	Mouse	Santa Cruz Biotechnology #sc-365,765
Secondary antibody	**Dilution**	**Species**	**Company**
Alexa Fluor 488	1:1000	Mouse	Thermo Fisher Scientific #A21121
Alexa Fluor 488-Phalloidin	1:250	-	Thermo Fisher Scientific #A12379
Alexa Fluor 555	1:1000	Mouse	Thermo Fisher Scientific #A31570

2.3 | MTT assay

The cell viability of hMSCs cultivated on differently modified specimens was detected by MTT colorimetric assay. Briefly, 50,000 cells/cm^2 were seeded on functionalized titanium samples in a 48 well plate for 24 h at 37°C and 5% CO_2. By the end of the culture, the cells were washed with PBS and immediately afterwards 250 µL of growth medium supplemented with 25 µL MTT reagent (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (Boster Biological Technology, #AR1156, Pleasanton, USA) was added for 4 h at 37°C. After these incubation periods, 250 µL of MTT formazan salt was pipetted into the samples, followed by another 12 h of incubation. Finally, the absorbance was measured at 570 nm using a well plate reader (SpectraMax M2/M2e, Molecular Devices, San Jose, USA).

2.4 | Immunostaining

In order to visualize the adhesion-related marker vinculin and early osteogenic markers such as RUNX2 and alkaline phosphatase (ALP) via antibody staining, hMSCs were cultured on various modified titanium surfaces. The cells were seeded onto the discs at a density of 1×10^5 cm^{-2} in serum-free medium. After 24 h/14 days incubation at 37°C and 5% CO_2, cells were fixed with 4% (wt/vol) PFA in PBS

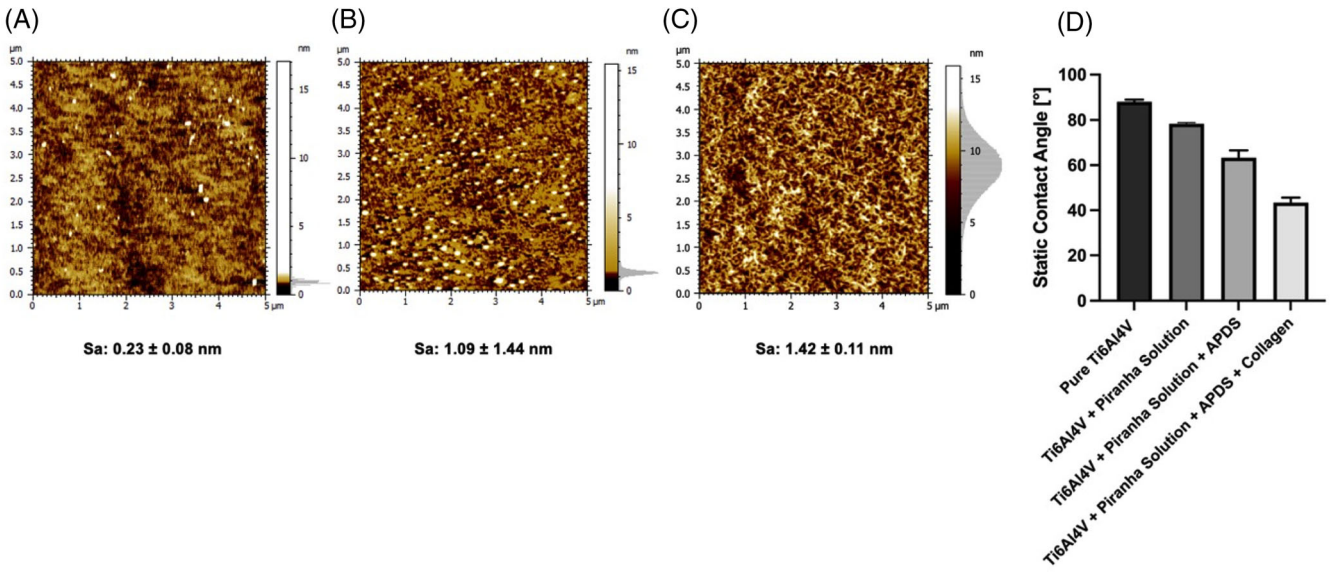


FIGURE 1 Atomic force microscopy (AFM) images of differently treated TiO_2 sapphires. AFM topography measurements of (A) untreated, (B) 3-aminopropyldiisopropylethoxysilane treated, and (C) collagen modified sapphires with a resolution of 5 µm × 5 µm. (D) The surface wettability of the individual functionalized Ti6Al4V specimens was analyzed ($n = 3$).

and permeabilized with buffered Triton X-100 solution for 5 min at room temperature. To prevent non-specific antibody binding, samples were blocked with 1% (wt/vol) BSA containing 0.3 vol % Triton X-100 for 1 h. After washing with 1% (wt/vol) BSA, specimens were incubated overnight with primary antibody (Table 1). The samples were washed as described above and incubated with the secondary antibody (Table 1) for 1 h at room temperature. After this incubation step, actin cytoskeleton was stained with Alexa Fluor 488-Phalloidin (Table 1) for 60 min under constant agitation. The cell nucleus was stained for 5 min with DAPI (4′,6-diamidine-2-phenylindole solution, dilution 1:10 000, Invitrogen, USA). After washing the specimens, imaging was conducted using a fluorescence microscope (Axio Imager.M2m, Zeiss, Oberkochen, Germany).

2.5 | Alizarin red staining

The osteogenic differentiation behavior of hMSCs was assessed for calcium deposition by Alizarin red staining. To that end, cells were seeded on Ti6Al4V samples in a density of 31,000 cells/cm^2 in a 24 well plate and covered with growth medium. After 24 h, the medium was changed to an osteogenic induction medium (OIM, DMEM [#D6046, Sigma-Aldrich, St. Louis, USA]), including 10% FCS (PAN Biotech, Aidenbach, Germany), 10 mM sodium β-glycerophosphate, 100 nM dexamethasone, and 0.05 mM l-ascorbic acid 2-phosphate (Sigma-Aldrich, St. Louis, USA). Alizarin red staining was performed after 21 days. To this end, cells were fixed with pre-cooled 100% ethanol (AppliChem, Darmstadt, Germany) for 1 h. Afterwards, samples were rinsed with distilled water and stained with 400 µL Alizarin red solution (Sigma-Aldrich, St. Louis, USA). The staining solution was filtered in advance and adjusted to a pH of 4.1 using 1 M sodium hydroxide (NaOH, Sigma Aldrich, St. Louis, USA). The specimens were incubated for 20 min at room temperature with the staining solution. To remove unbound dye, the samples were washed twice with PBS. Then, 400 µL of 10 vol % acetic acid (Sigma-Aldrich, St. Louis, USA) was transferred into each well and incubated for 30 min at room temperature. After this incubation period, the

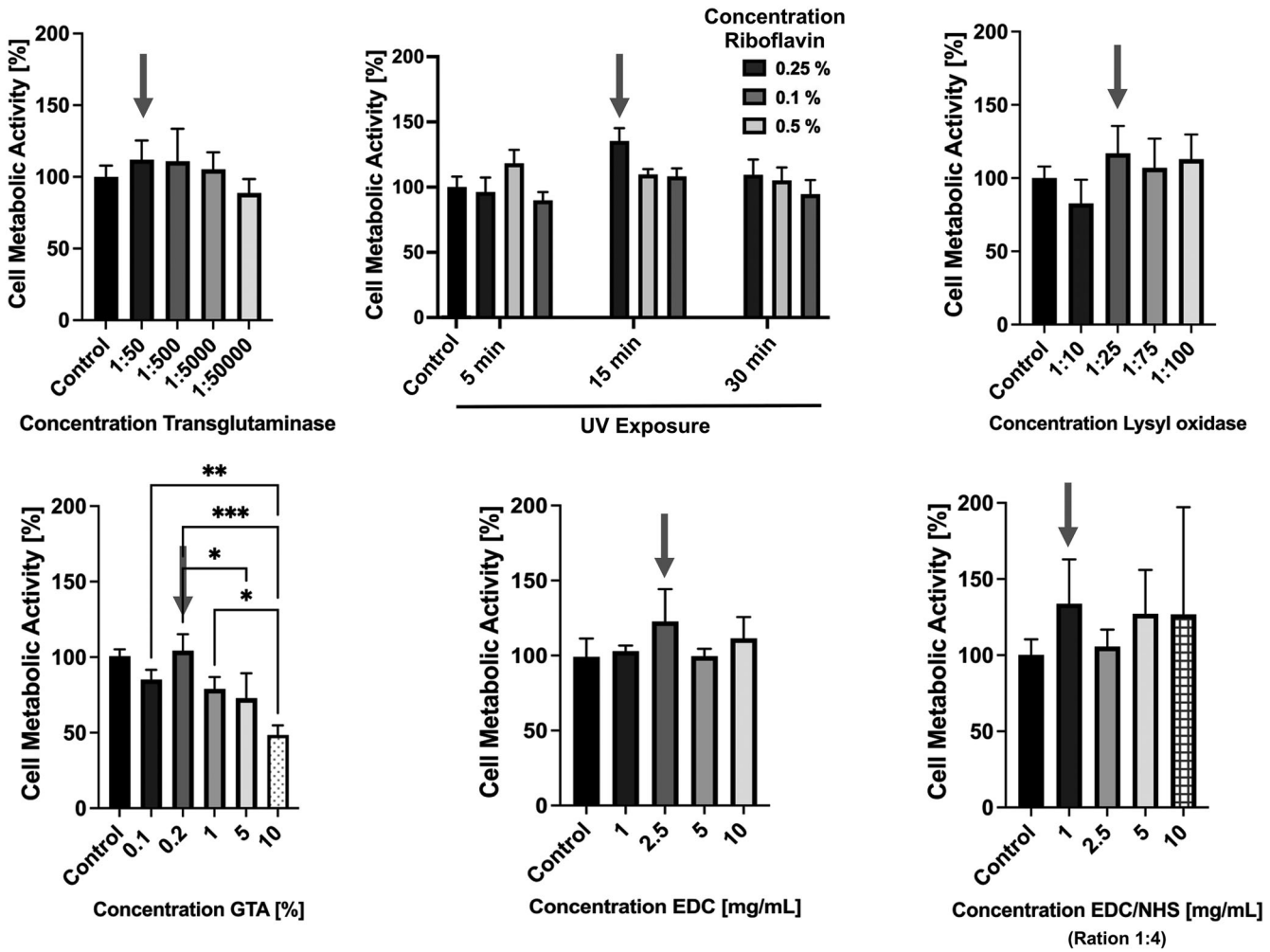


FIGURE 2 Measurement of metabolic activity of human mesenchymal stem cells (hMSCs) via MTT assay. For crosslinking of collagen on Ti6Al4V specimens, various reagents with different concentrations were tested. Afterwards, metabolic activity of hMSCs was evaluated on differently modified samples using MTT assay. Gray arrows indicate which conditions were selected for further experiments. Statistical significance values are indicated as $p < .05^*$, $p < .01^{**}$, and $p < .001^{***}$, error bars show ± standard error of the mean ($n = 3$).

supernatant was removed and transferred to 1.5 mL tubes. The tubes were heated to 85°C for 10 min in a heating block (Eppendorf, Hamburg, Germany). Immediately afterwards, the tubes were cooled on ice for 5 min and centrifuged at 200*g* for 15 min. Into each reaction tube, 100 µL of 10 vol % ammonium hydroxide was added. In order to assess the absorbance, 150 µL of the solution was transferred to a 96 well plate and measured at 405 nm in a plate reader (SpectraMax M2/M2e, Molecular Devices, San Jose, USA).

2.6 | Proteolytic stability (collagenase)

To investigate the stability of collagen, the differently crosslinked specimens were incubated in collagenase solution. Bacterial collagenase solution from *Clostridium histolyticum* (Worthington Biochemical Corporation, Lakewood, USA) was prepared by dissolving collagenase powder 1 mg/mL in PBS. The various crosslinked Ti6Al4V samples were placed in a 48 well plate, covered with 200 µL growth medium supplemented with collagenase solution, and incubated at 37°C. After 1.5, 3, 6, 24 h, and 6 days, the samples were washed with PBS for 30 min under constant agitation. Then, the remaining collagen on the samples was quantified using sirius red staining. For this purpose, each sample was covered with 200 µL of the sirius red dye (Morphisto, Offenbach am Main, Germany) for 1 h. In order to remove unbound dye, samples were rinsed five times with 200 µL of 1 mM hydrochloric acid (HCl, Sigma-Aldrich, St. Louis, USA) until no residue of dye was visible in the HCl solution. The samples were dried overnight and analyzed under a light microscope (Axio Imager M2m, Zeiss, Oberkochen, Germany). In addition, 200 µL of 0.1 M NaOH (Sigma-Aldrich, St. Louis, USA) solution was added to the samples under constant agitation for 5 min to dissolve the bound dye. The supernatant was

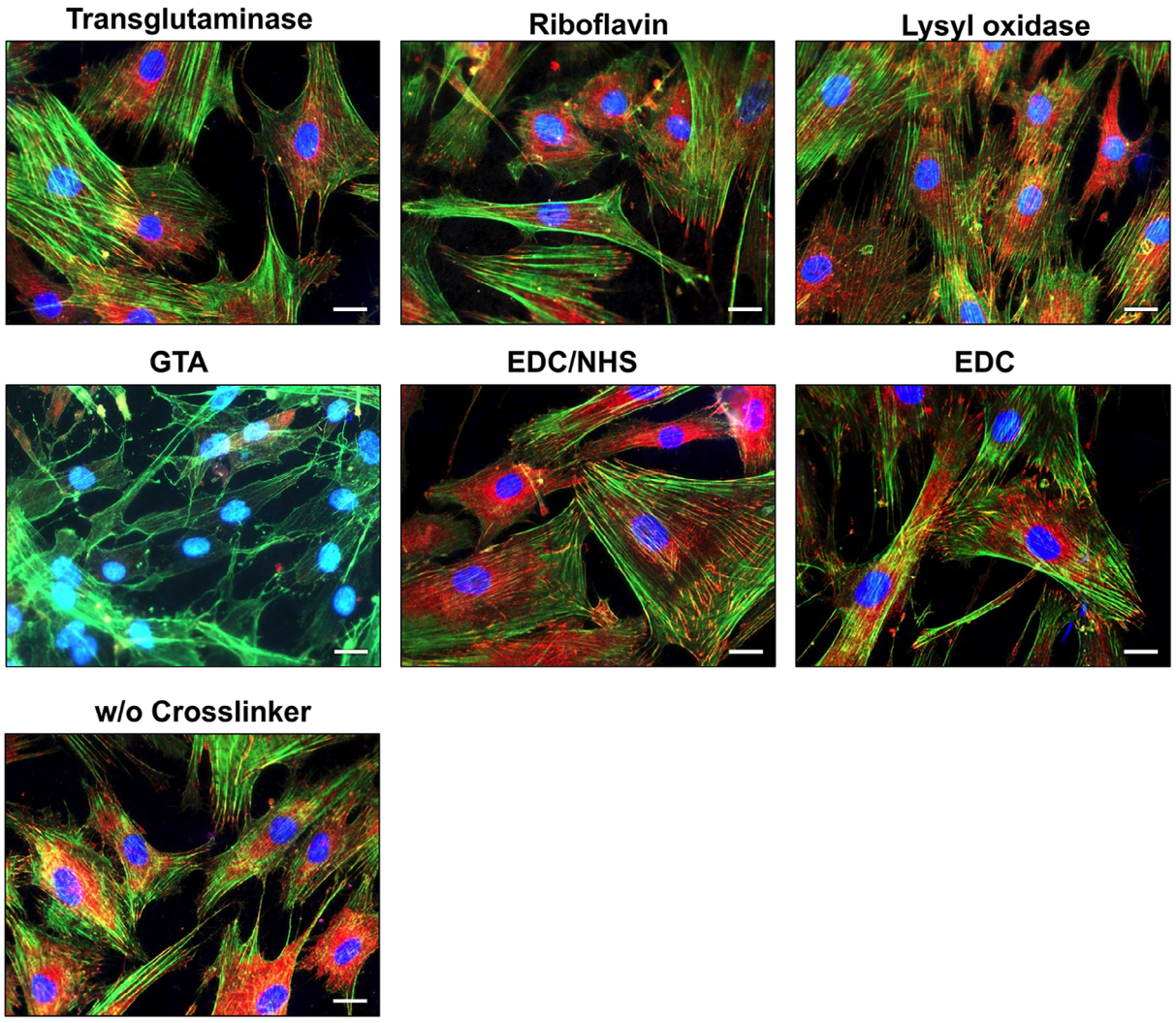


FIGURE 3 Immunostaining of human mesenchymal stem cells (hMSCs) on collagen-modified Ti6Al4V specimens using different crosslinkers. hMSCs were cultivated for 24 h on the individual samples. The actin cytoskeleton was stained with Alexa Fluor 488 Phalloidin (green), whereas the adhesion-related protein vinculin was visualized with Alexa Fluor 555 (red). Cell nucleus was stained with DAPI (blue) ($n = 3$). Scale bars: 20 µm.

transferred to a new 48 well plate and absorbance was measured at 540 nm using a plate reader (SpectraMax M2/M2e, Molecular Devices, San Jose, USA).

2.7 | Atomic force microscopy

The NanoStation II (SiS, Herzogenrath, Germany) was used in the tapping mode to assess the silane adsorption profile on titanium oxide sapphire (TiO_2,CrysTec, Berlin, Germany). The PPP-NCLR cantilever (NANOSENSORS, Neuchatel, Switzerland) was placed randomly on the individual samples and three images with a resolution of 5 µm × 5 µm were recorded. All atomic force microscopy (AFM) images were analyzed using NanoScope 9.1 software (Bruker, Billerica, USA) in order to observe the respective surface topography.

2.8 | Static contact angle

The surface wettability of native and modified Ti6Al4V specimens was analyzed by the sessile drop method. To accomplish that, 10 µL of Milli-Q water (Sartorius, Germany) was placed randomly onto the respective surfaces. Contact angles were measured directly after drop deposition. Images of each spample were taken via digital camera (Canon EOS 7D, Japan) and analyzed in Fiji with the DropSnake plugin.[28]

2.9 | Tensile strength testing

The tensile strength between the differently crosslinked collagen and underlying titanium was determined by application of poly(methyl methacryl) [PMMA] coupling agent (ESPE-Sil, 3M ESPE, Germany) to the treated surfaces. To test the hydrolysis stability of the individual samples, some of the specimens were incubated in a water bath with 100°C for 11 h. As a control, physically adsorbed collagen was used. In order to fix the PMMA-coated titanium screw to the samples, bone cement (Refobacin Bone Cement R, Biomet, Indiana, USA) was used. After 24 h, the tensile strength test was performed (Z030, Zwick, Ulm, Germany).

2.10 | Energy dispersive X-ray spectroscopy

The calcium deposition of hMSCs after 21 days was detected by an EDX (Falcon Genesis EDAX, USA). For this purpose, hMSCs were fixed after 21 days with 3% vol/vol glutaraldehyde (Agar Scientific, Germany) for 24 h at room temperature. For EDX analyses, the samples were dehydrated with ascending ethanol series (30%, 50%, 70%, 90%, and 100% vol/vol, 10 min for each step). Subsequently, the specimens were critical point dried in liquid CO_2 (Critical Point Dryer, E3100, Quorum Technologies, UK). Finally, the samples were investigated under an environmental scanning electron microscope (ESEM FEI Quattro S, Thermo Fisher Scientific, FEI, Netherlands) in a high vacuum with 10 kV acceleration voltage.

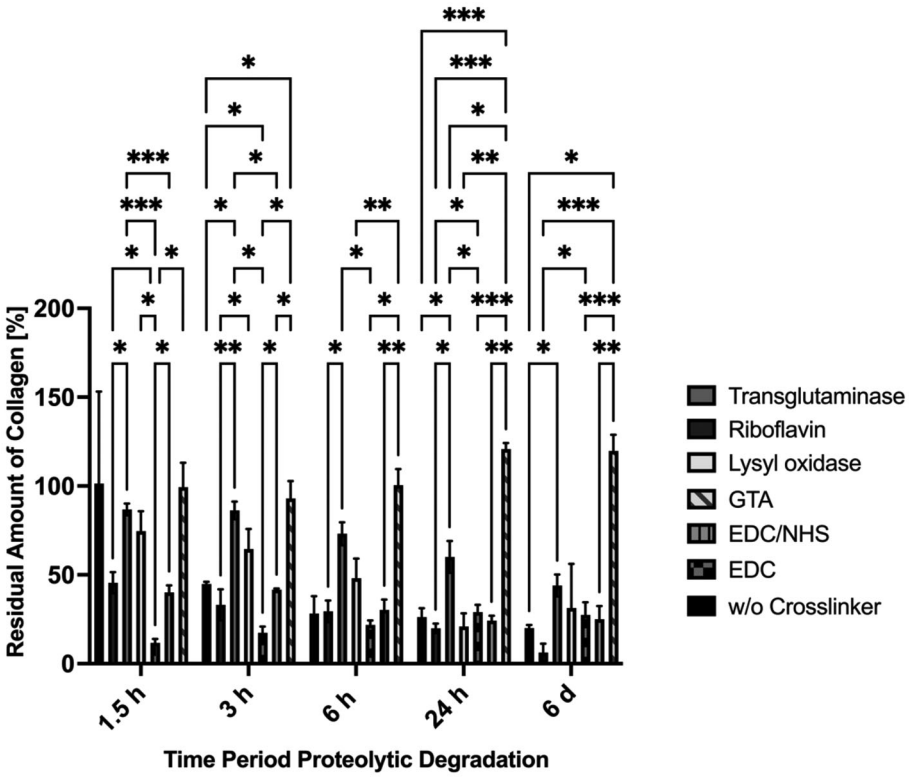


FIGURE 4 Collagenase digestion of collagen-modified samples. Differently crosslinked collagen was incubated with collagenase over a period of 6 days. At individual time points, the remaining amount of collagen on Ti6Al4V specimens was analyzed via sirius red staining. Statistical significance values are indicated as $p < .05$*, $p < .01$**, $p < .001$***, error bars show ± standard error of the mean ($n = 3$).

2.11 | Statistical and image analyses

Unless otherwise stated, all experiments were performed in triplicate ($n = 3$). The cell culture experiments were repeated in three independent experiments with at least three different MSC donors. Results are presented as mean ± standard deviation. In this study, one-way/two-way analysis of variance (ANOVA) with Tukey's post hoc test was applied with a significance level of $p < .05$, when not stated otherwise. Cells were visualized with a fluorescence microscope (Axio Imager 2; Carl Zeiss, Oberkochen, Germany) using ×40 and ×10 objectives. Images were recorded at room temperature and randomly selected areas of the samples were captured. Finally, images were analyzed using ImageJ.[29] All of the statistical analyses were determined with GraphPad Prism Version 8.0 (GraphPad Software, San Diego, USA).

3 | RESULTS

3.1 | Surface characterization

The AFM images clearly show the untreated TiO_2 sapphire (Figure 1A) and the presence of homogenously distributed APDS on functionalized sapphires (Figure 1B). The immobilization of APDS resulted in a grained structure and revealed an increased Sa value of 1.09 nm compared to those of untreated sapphires (Sa: 0.23 nm). The application of collagen revealed a uniform fibrous structure on the surface and an increasing Sa value to 1.42 nm (Figure 1C). In addition, the wettability of native Ti6Al4V and functionalized Ti6Al4V surfaces was determined by measurements of the water droplet contact angles (Figure 1D). The results of surface wettability testing showed that pure Ti6Al4V was located at 88.0 ± 0.6°, while sulfuric acidhydrogen

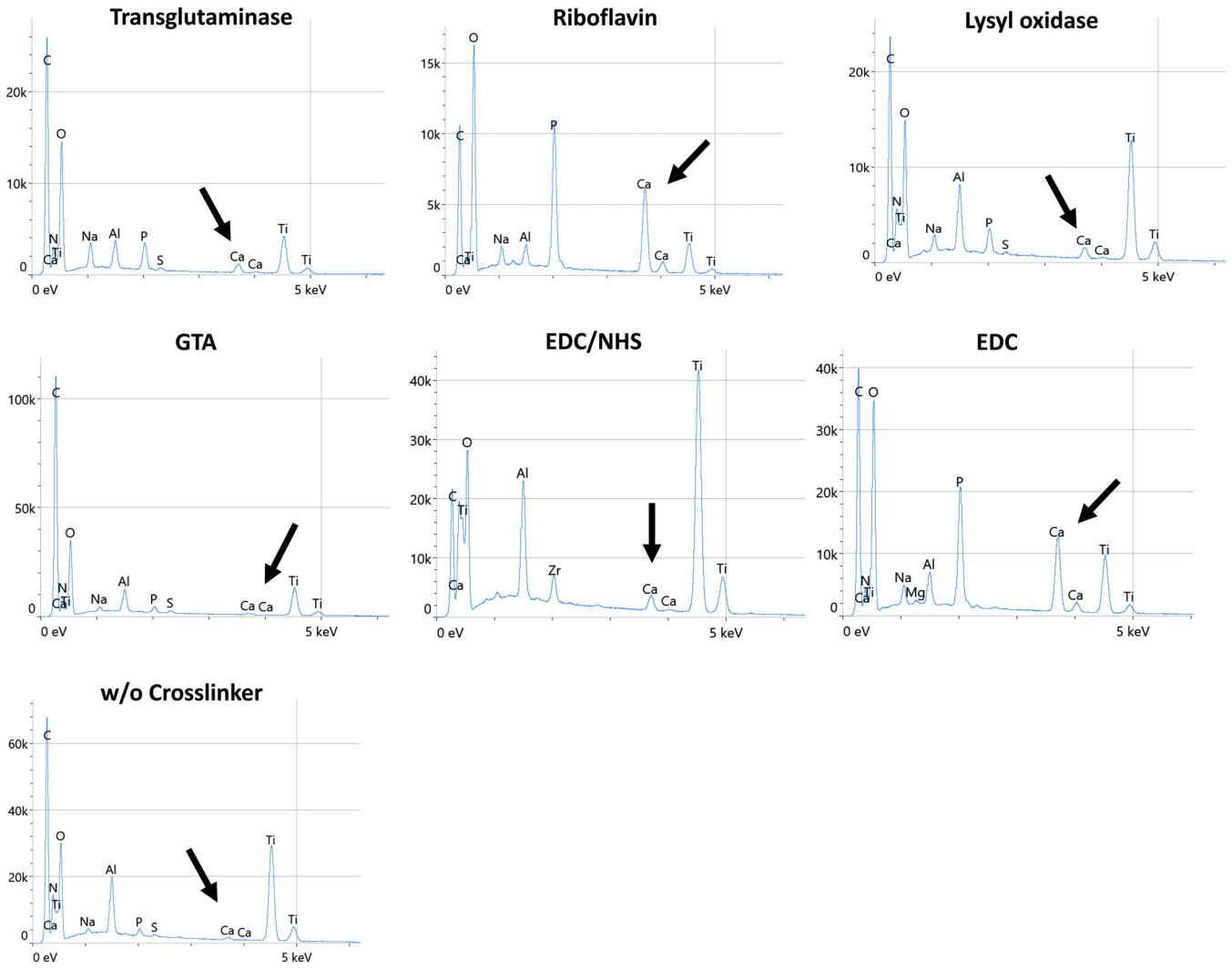


FIGURE 5 Energy dispersive X-ray analyses of the distribution of calcium after 21 days in human mesenchymal stem cells. hMSCs were cultivated over 21 days under mineralization conditions on various crosslinked collagen samples ($n = 3$). Calcium detection/peaks were highlighted with black arrows.

peroxide mixture treatment revealed a decrease of contact angle (78.4 ± 0.4°). Through the silanization procedure of the Ti6Al4V samples, a reduction of the contact angle to 63.3 ± 2.6° was observed. This result was even more intensified by applying collagen to the surfaces (43.6 ± 1.8°).

3.2 | Cell metabolic activity

The cell metabolic activity and proliferation was assessed using MTT assay. Increased levels of metabolic activity can be observed on specimens crosslinked with 2.5 mg/mL EDC, 1 mg/mL EDC/0.25 mg/mL NHS, and 0.2% of GTA, as indicated in Figure 2. Significant decreases in metabolic activity were shown by applying higher concentrations of GTA. In contrast, higher levels of EDC/NHS exhibited increased metabolic activity of hMSCs (Figure 2). Transglutaminase revealed the highest activity using 1:50 enzyme collagen ratio, whereas riboflavin showed the highest metabolic activity at 0.25% at 15 min under UV light. Furthermore, samples crosslinked with lysyl oxidase with a concentration of 1:25 exhibited the highest percentage of cell metabolic activity. These concentrations were selected for further experiments focusing on vinculin expression, osteogenic differentiation, and degradation stability.

3.3 | Immunostaining of adhesion-related protein

Cells cultivated on differently crosslinked collagen samples for 24 h showed a distinct actin network and stress fibers were clearly visible. Specifically, the expression of vinculin at the end of actin filaments was greatly increased on almost all surface conditions, excluding GTA crosslinked samples (Figure 3). Here, only poor activation of vinculin

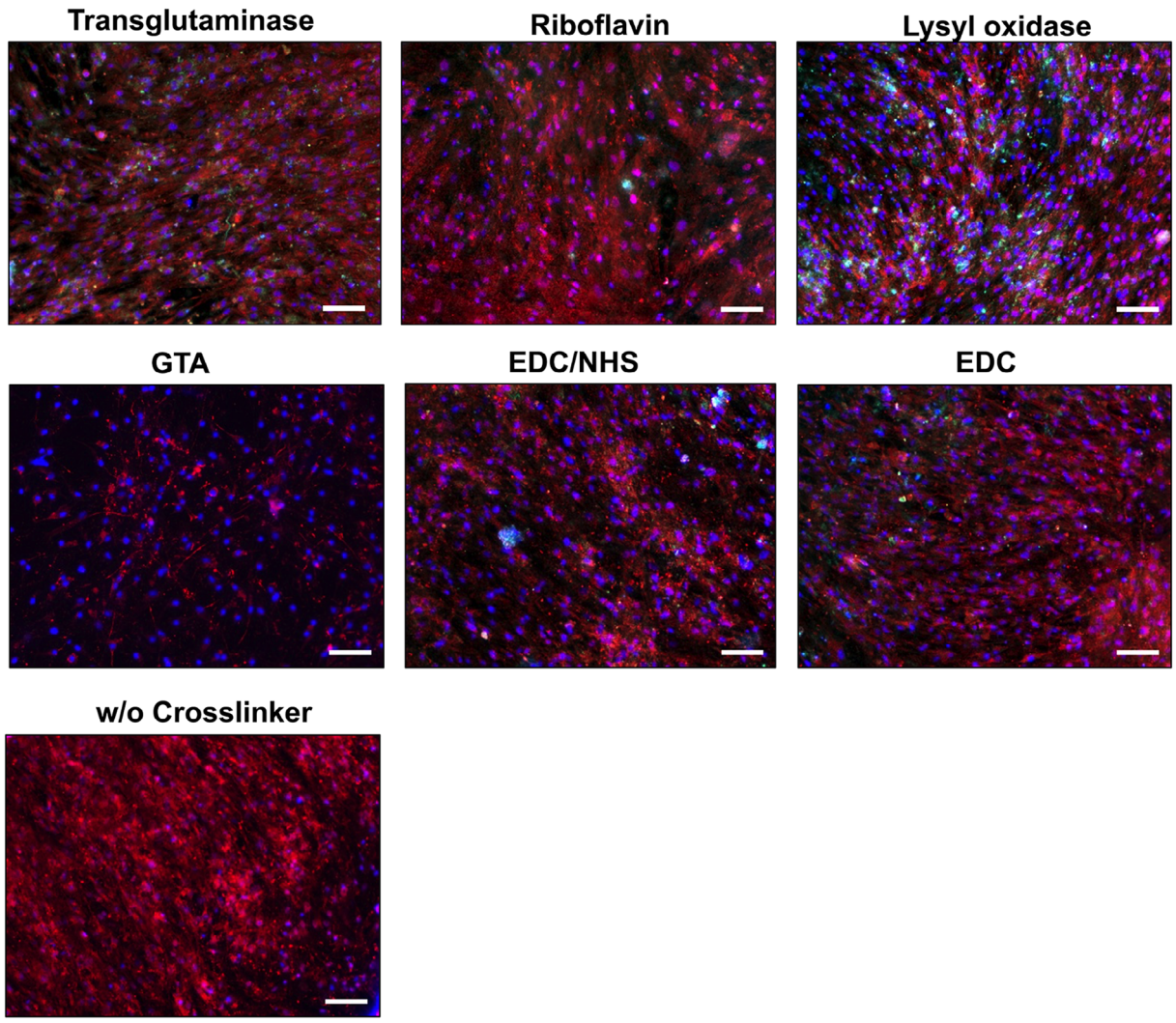


FIGURE 6 Immunofluorescence of human mesenchymal stem cells (hMSCs) on collagen-modified Ti6Al4V specimens using different crosslinkers. hMSCs were cultivated for 14 days under osteogenic differentiation conditions on the individual samples. The expression of RUNX2 was visualized with Alexa Fluor 555 (red), while ALP was stained with Alexa Fluor 488 (green). Cell nucleus was stained with DAPI (blue) ($n = 3$). Scale bars: 100 µm.

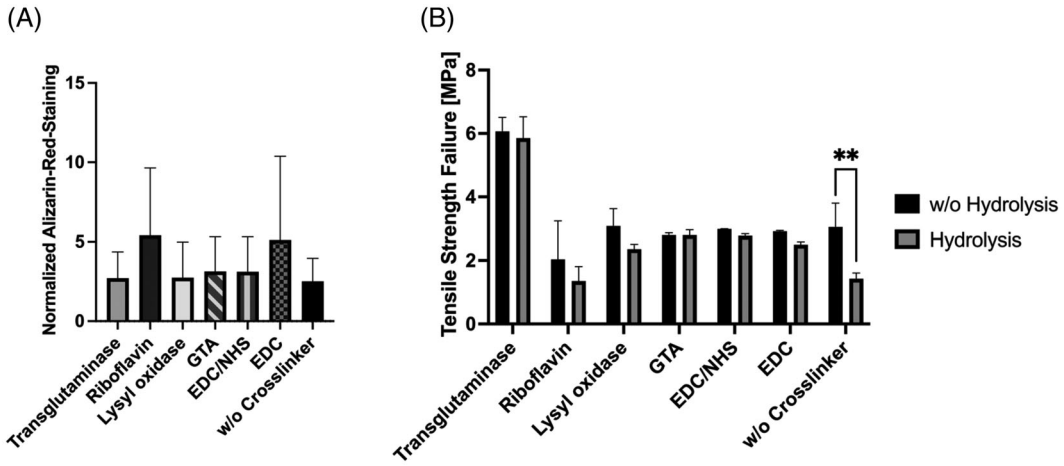


FIGURE 7 Alizarin red staining and tensile strength testing of various crosslinked titanium-collagen surfaces. (A) Human mesenchymal stem cells seeded on differently crosslinked collagen specimens were stained with Alizarin dye after 21 days of cultivation. (B) Furthermore, hydrolysis stability of the individual samples was tested via tensile strength testing. Statistical test was performed using Šídák's multiple comparisons test. Statistical significance values are indicated as $p < .01$**, error bars show ± standard error of the mean ($n = 3$).

was detectable on GTA-modified specimens. On these samples, hMSCs exhibited a predominately rounded shape and were rarely elongated.

3.4 | Degradation testing using collagenase

Collagenase degradation testing revealed that physically adsorbed collagen was almost completely digested (20% of collagen remained on the surface) after 6 days of incubation (Figure 4). A similar trend can be observed using riboflavin as crosslinker: Here, only 6.3% of collagen was left after collagenase treatment. Interestingly, the amount of collagen crosslinked with GTA increased over the period of incubation. After 1.5 h an amount of 99.3% was visualized, while after 6 days 119% of collagen was measured. The residual amount of collagen crosslinked with EDC was very low at every analysis time point (Figure 4). Looking at transglutaminase, a gradual decrease of collagenase was detected. Initially, 86.8% of collagen was measured after 1.5 h of degradation testing, while a 44% residual amount was observed after 6 days. Similarities can be observed using lsysl oxidase (Figure 4).

3.5 | Osteogenic differentiation of hMSCs

Samples crosslinked with riboflavin support the highest mineralization production, confirmed by calcium deposition in Figure 5. Furthermore, EDX analyses revealed a strong peak of calcium on EDC-modified titanium specimens. However, the lowest presence of calcium was detectable on physically adsorbed collagen and GTA-treated samples.

The expression of early osteogenic markers RUNX2 and ALP was analyzed after 14 days on various crosslinked collagen samples. It can be seen that RUNX2 was highly activated on all surface conditions, except on GTA-functionalized samples (Figure 6). The increased presence of ALP was visible on transglutaminase and lysyl oxidase modified titanium specimens.

Furthermore, Alizarin red staining showed highest mineralization activity using riboflavin and EDC as a crosslinker (Figure 7A). Nevertheless, high standard deviations can be also observed. Stem cells cultivated on all other crosslinkers exhibited almost the same mineralization ability. In order to test hydrolysis stability, a tensile strength test was performed. The graph in Figure 7B clearly showed that transglutaminase had the highest strength, even including the water-treated specimens. In contrast, riboflavin revealed the lowest strength levels in both categories, hydrolyzed and non-hydrolyzed specimens. Physically adsorbed collagen dropped significantly when samples were incubated in water. The other crosslinkers such as GTA, EDC/NHS, and EDC showed almost the same level of tensile strength.

4 | DISCUSSION

Collagen functionalization of biomaterials is a common tool in order to improve events like osseointegration. Through these modifications, bioinert materials such as titanium and its alloys biologically interact with the adjunct hard tissue. Due to the high degradation rate of natural collagen, crosslinking is essential to withstand proteolytics attacks and an aqueous environment. In this study, Ti6Al4V was bioactivated through a collagenous layer. Additionally, different crosslinkers, including enzymatic, chemical, and photochemical reagents were compared. Crosslinker efficiency was assessed in terms of collagenase resistance, hydrolysis stability, cell adhesion, and osteogenic differentiation behavior.

The most suitable results were achieved for EDC at 2.5 mg/mL and a combination of EDC/NHS with 1:0.25 mg/mL (Figure 2). This

was also confirmed by Müller et al, who also showed a decrease in cell proliferation and adhesion with higher EDC concentration.[4] Overall, all EDC and EDC/NHS concentrations tested in our study exhibited high metabolic cell activity (Figure 2). Immunofluorescence also revealed an increased expression of vinculin at the end of actin filaments when EDC and EDC/NHS were used (Figure 3). Nevertheless, some studies indicated that certain motifs within the collagen were rendered inaccessible by EDC/NHS in particular. However, it appears that the RGD motif or other important amino acid sequences were accessible to integrin receptors after crosslinking with EDC and EDC/NHS within collagen, leading to increased activity of the focal adhesion marker vinculin in hMSCs. The collagenase assay showed that EDC and EDC/NHS had a low remaining collagen content as soon as 1.5 h (Figure 4). After 6 h of incubation with collagenase only 27% and 25% was detected (Figure 4). Angele et al showed that a higher concentration of EDC (30 mg/mL) resisted degradation by collagenase, at least for equine collagen.[30] So the origin of the collagen seems to play an important role and depending on the species, the crosslinkers in the collagen seem to block some targets for collagenase. Within this study, bovine calf skin was used, so there may be less crosslinking than with equine collagens. When bovine collagen was used, however, the collagen content also dropped drastically after 6 days of exposure to enzymatic solution. Müller et al found a correlation between the density of amino groups on the titanium surface and the proteolytic resistance of the covalently immobilized collagen.[4] The higher the amino group concentration, the better the collagen could be immobilized on the surface and thus resist degradation.[4] Therefore, an additional ninhydrin assay could be performed to obtain accurate information about the density of the applied $-NH_2$ terminating APDS.

Sheperd et al reported that the crosslinker combination of EDC/NHS has a low degree of crosslinking to reagents such as GTA, for example.[31] However, they observed that the use of EDC/NHS increased the hydrophilicity of the surface, which was beneficial for the expression of fibronectin within the cell.[31] Takigawa et al were able to show in their study that the by-products of the degradation of GTA in later metabolic pathways resulted in increased cytotoxicity, although there is no evidence of carcinogenicity or mutagenicity with the use of GTA.[32] Within this study, it has been shown that as the concentration of GTA increased, the metabolic activity of hMSCs decreased significantly (Figure 2). Even the selection of a concentration that had shown high metabolic activity (0.2%) revealed a drastically decreased expression of vinculin in immunofluorescence compared to the other crosslinkers (Figure 3). Also, when the marker RUNX2 was analyzed, only a minor expression was observed and the activation of ALP could not be visualized (Figure 6). Interestingly, the degradation assay revealed that collagen content gradually increased over the incubation period of 6 days. Abke et al explained this by the equal staining of the collagenase and the remaining collagen.[33] The degradation of the crosslinked samples releases incorporated GTA by hydrolysis of the Schiff bases and makes it available for new covalent bonds.[33] Thus, the particular protein quantity most likely consists of a mixture of residual collagen and tethered collagenase.[33]

Riboflavin, that is, vitamin B2, has also been shown to be a biocompatible agent to achieve command crosslinking of collagen constructs with blue light. Riboflavin crosslinking of collagen is of particular interest due to the short processing times (15 min) required to observe significant improvements in mechanical properties (2.5-fold increase in elastic modulus). In this study, it was shown that the highest metabolic activity of hMSCs could be achieved by the application of 0.25% riboflavin and 15 min of UV light irradiation (Figure 2). Immunofluorescence showed that at the end of actin filaments of hMSCs a clear expression of vinculin on riboflavin crosslinked surfaces could be visualized. Furthermore, riboflavin has been used successfully for decades in patients with corneal ectasia.[34] Corneas have also been printed and crosslinked with riboflavin in the field of tissue engineering.[35] Tonndorf et al used the spider technique to produce collagen fibers.[36] Crosslinking with riboflavin significantly increased stiffness and thermal stability compared to non-crosslinked fibers.[36] Furthermore, Tonndorf et al showed in cell culture analyses that riboflavin-induced crosslinking achieved higher cytocompatibility for hMSCs compared to GTA crosslinking of collagen fibers.[36] In the present study, the hMSCs exhibited the highest accumulation of calcium on riboflavin crosslinked collagen surfaces, as shown in the EDX spectra (Figure 5). However, the collagenase assay on riboflavin crosslinked samples revealed a very low degradation stability and was thus below the values of adsorbed collagen (Figure 4). Priyadarshini et al demonstrated that the hydroxyproline content in the supernatant was significantly decreased in riboflavin crosslinked samples than in non-crosslinked samples.[37] Here, the samples were also incubated in collagenase solution for 48 h.[37] In addition, Laggner et al showed that after 120 min of collagenase digestion, an average of 50.5% and 80% of the crosslinked stroma in the 10- and 20-min riboflavin-collagen crosslinked groups remained undigested.[38] In contrast, the corneal stroma in the control group and the 5 min riboflavin-collagen group was completely digested after approximately 100 min.[38] These data suggest that the higher riboflavin dose along with UV exposure confers enzymatic resistance to corneal stroma.

Transglutaminases are a family of transferase enzymes that crosslink proteins by forming a bond between an ε-amine (lysine) and γ-carboxyl in glutamines. In a study of the mechanical properties of transglutaminase by Nair et al, the global mechanical properties such as tensile modulus and the breaking strength of transglutaminase-treated films were lower than those of non-crosslinked films of insoluble bovine dermal collagen type I, while elongation at break and plasticity increased.[18] Regardless, human dermal fibroblasts seeded on these substrates distributed well and adhered to the substrate, with low cytotoxicity and high cell proliferation.[18] This study found that the hMSCs on transglutaminase collagen crosslinked surfaces showed a high expression of vinculin and, moreover, in the collagenase assay 44% of collagen layers remained on the surface even after 6 days. Thus, transglutaminase was one of the best performers in terms of degradation stability. Furthermore, transglutaminase revealed the highest tensile strength testing compared to all other crosslinkers, even the hydrolysis-treated specimens (Figure 7B). It can be assumed that transglutaminase seems to be hydrolytically stable over 11 h.

Another enzymatic crosslinker, the lysyl oxidase, showed best metabolic activity using 1:25. At lower levels of lysyl oxiase (1:10), a decrease in hMSC metabolic activity can be observed (Figure 2). As can be seen in Figure 3, lysyl oxidase-collagen-specimens showed clear expression of vinculin at the end of actin filaments (Figure 3). Furthermore, analysis of early osteogenic markers revealed the highest expression of ALP using lysyl oxidase as crosslinking reagent (Figure 6). In degradation stability testing (Figure 4), a gradual decrease of the collagen amount remaining on the surface was detectable. In vivo, the lysyl oxidase catalyzes the first step of the covalent crosslinking of the ECM proteins collagen and elastin, which contribute to ECM stiffness and mechanical properties.[39] Here, a tensile strength test was performed and lysyl oxidase showed values in line with the majority of the crosslinkers used in this study (Figure 7B).

5 | CONCLUSION

In this study, different enzymatic, chemical, and photochemical crosslinkers were systematically evaluated in terms of degradation resistance, hydrolysis stability, metabolic activity, and osteogenic differentiation potential of hMSCs in vitro. Transglutaminase showed the highest enzymatic stability, tensile strength, and expression of vinculin and RUNX2 compared to the other established crosslinkers. Thus, transglutaminase holds great potential to facilitate an enhanced interaction with attached bone cells and thereby could potentially improve and accelerate osseointegration of a titanium-based bone implant in vivo.

AUTHOR CONTRIBUTIONS

Alena L. Palkowitz: Conceptualization, methodology, investigation, supervision, writing—original draft. **Sascha Rüger:** Investigation, writing—review and editing. **Maximilian Ziegler:** Investigation, writing—review and editing. **Eva Miriam Buhl:** Investigation, writing—review and editing. **Horst Fischer:** Conceptualization, methodology, resources, funding acquisition, supervision, writing—review and editing.

ACKNOWLEDGMENTS

The environmental scanning electron microscope FEI Quattro S was funded by the German Research Foundation (DFG)—495328185. The authors would like to thank Roswitha Davtalab for the isolation and cultivation of human mesenchymal stem cells. Furthermore, the authors thank Dr. Anke Aretz (Central Facility for Electron Microscopy, GFE, Aachen, Germany) for performing AFM analyses of the samples.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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How to cite this article: Palkowitz AL, Rüger S, Ziegler M, Buhl EM, Fischer H. Transglutaminase enables highly hydrolytically and proteolytically stable crosslinking of collagen on titanium surfaces and promotes osteogenic differentiation of human mesenchymal stem cells. *J Biomed Mater Res*. 2024;112(6):812-824. doi:10.1002/jbm.a.37661