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Preserved bioactivity and tunable release of a SDF1-GPVI bi-specific protein using photo-crosslinked PEGda hydrogels

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ABSTRACT

Chemokine-induced stem cell recruitment is a promising strategy for post myocardial infarction treatment. Injection of stromal cell-derived factor 1 (SDF1) has been shown to attract bone marrow-derived progenitor cells (BMPCs) from the blood that have the potential to differentiate into cardiovascular cells, which support angiogenesis, enabling the improvement of myocardial function. SDF1-GPVI bi-specific protein contains a glycoprotein VI (GPVI)-domain that serves as an anchor for collagen type I (Col I) and III, which are exposed in the wall of injured vasculature. In this study, we generated a cytocompatible hydrogel via photo-crosslinking of poly(ethylene glycol) diacrylate that serves as a reservoir for SDF1-GPVI. Controlled and sustained release of SDF1-GPVI was demonstrated over a period of 7 days. Release features were modifiable depending on the degree of the crosslinking density. Functionality of the GPVI-domain was investigated using a GPVI-binding ELISA to Col I. Activity of the SDF1-domain was tested for its CXCR4 binding potential. Preserved functionality of SDF1-GPVI bi-specific protein after photo-crosslinking and controllable release was successfully demonstrated *in vitro* supporting the implementation of this drug delivery system as a powerful tool for therapeutic protein delivery in the treatment of cardiovascular ischemic disease.

1. Introduction

Myocardial infarction (MI) and its subsequent long term effects, including chronic ischemic heart disease, account for the greatest cause of morbidity and mortality in developed countries [1]. Myocardial necrosis after ischemic conditions leads to alterations in cardiac extracellular matrix (ECM) composition [2,3]. These alterations are initiated by a strong inflammatory response triggered by cardiomyocyte death. During the inflammatory phase of the reparative response after MI, macrophages scavenge dead cells from wounded tissue. The following proliferative phase in myocardial repair is characterized by the repopulation of the infarcted area by myofibroblasts, which produce large amounts of ECM proteins including collagens. Crosslinking of these collagens takes place during the maturation phase leading to scar formation, which completes the reparative response [3]. Alterations in cardiac ECM composition changes the ventricular geometry [3], which is followed by a volume overload and subsequently leads to the elongation of cardiomyocytes and thinning of the ventricular wall [4]. Additionally, matrix metalloproteinase-activated degeneration of collagen crosslinks leads to further dilation of the ventricle [4]. Subsequent impairment of cardiac diastolic and systolic function progressively reduces the cardiac output, which ultimately leads to chronic heart failure (HF) [4]. Based on the increasing socio-economic burden of ischemic cardiovascular disease, research has expanded rapidly in the field of cardiovascular regenerative medicine over the last few years and several therapeutic strategies have

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emerged. However, a therapeutic strategy to reconstitute cardiac muscle in patients is still an unmet goal and organ transplantation remains the only option to treat severe HF [5].

One strategy to enhance cardiac function post MI is the application of adult stem cells such as bone marrow-derived mesenchymal progenitor cells (BMPCs) via injection [1,6]. BMPCs have been identified as an eligible cell type for a cardiovascular stem cell-based therapy, although their potential to differentiate into all cell types of the cardiovascular lineage including cardiomyocytes is controversially discussed [7]. It is hypothesized that improvement of cardiac function after BMPC injection is due to paracrine effects such as secretion of cytokines and proangiogenic growth factors [8]. Cardiac patches have been used to replace damaged myocardium with natural heart-derived substrates that support that cardiac contractile function [9]. Furthermore, cardiac patches have been applied successfully to enable a localized delivery of adult stem cells and improved stem cell engraftment into the heart [8,10] as these patches provide structural cues that enhance stem cell viability during the delivery process [11]. Another relevant application for cardiac patches is the localized and controlled release of therapeutic proteins such as vascular endothelial growth factor [12]. Preservation of cardiac contractile function is partly achieved by a reduction in infarct size and an enhanced capillary density [13], which in turn has been achieved by the delivery of angiogenic growth factors [12] or chemokines [14] via hydrogels. Among these, poly(ethylene glycol) (PEG)-based hydrogels have been identified as excellent drug delivery systems [15,16]. Their hydrophilicity and low protein binding characteristics enable an effective release of incorporated drugs and support the maintenance of protein bioactivity [15]. One promising chemokine to induce neovascularization is stromal cell-derived factor 1 (SDF1). It binds to the C-X-C chemokine receptor type 4 (CXCR4), also known as fusin or CD184, which is expressed on the surface of hematopoietic and cardiovascular progenitor cells [17]. CXCR4-expressing cells are thought to play a crucial role in angiogenesis post MI [18,19]. Therefore, several studies aimed on the utilization of SDF1 through a number of delivery mechanisms including adenoviral gene delivery [20], intra-myocardial protein injection [21] or via protein-releasing hydrogels [14,22,23]. Although the prolonged presence of pure SDF1 has been shown to result in improved cardiac function after MI in animal models [18,21,24], optimal delivery features allowing a sustained release of bioactive SDF1 remains to be developed. To reach an accumulation of SDF1 in the affected area, Ziegler et al. designed a recombinant bi-specific protein, which contains a soluble form of the platelet collagen receptor glycoprotein VI (GPVI) [25] in addition to the SDF1 domain (SDF1-GPVI) [13]. In the pathophysiology of MI, collagens and other ECM proteins from the sub-endothelial matrix are exposed to the vessel lumen of coronary arteries most often after the disruption of an atherosclerotic plaque [26]. GPVI binds to the collagen triple helix, which is comprised of the repetitive amino acids triplet glycine-proline-hydroxyproline, in all three collagen chains. Most notably, these residues are most frequent at the N-termini of collagen types I (Col I; α1) and III (α1) and the C-terminal domain of Col I (α1). Col I and III are highly present in the sub-endothelium of vessel walls [27], thus serving as docking sites for bi-functional proteins containing a GPVI domain [13,28] and subsequently lead to accumulation of SDF1-GPVI at the site of vascular injury (Fig. 1). The effect of the bi-specific protein SDF1-GPVI after direct injection has been shown for post-MI treatment in a mouse MI model [13,29]; however, we hypothesize that a sustained delivery of SDF1-GPVI may hold great potential to further improve cardiac function. Hence, the aim of this study was to generate a drug releasing hydrogel based on photo-crosslinked PEG diacrylate (PEGda), which serves as a reservoir for the SDF1-GPVI protein. The potential of this hydrogel to provide a sustained and tunable release of SDF1-GPVI as well as preserving the bioactivity of the protein was assessed *in vitro*.

2. Materials and methods

2.1. Cell culture

T17b murine endothelial progenitor cells (mEPCs) were kindly provided by Dr. A.K. Hatzopoulos (Department of Cell and Developmental Biology, Vanderbilt University, USA) and cultured as previously described [30,31] using Dulbecco's Modified Eagle Medium (DMEM) high glucose (BE12-741F, Lonza, Verviers, Belgium) containing 20% fetal calf serum (FCS; 10270, Life Technologies, Paisley, UK), 100 U/ml penicillin and 100 μg/ml streptomycin (15140, Life Technologies), 1% non-essential amino acids (11140, Life Technologies) and 0.1 mM β-Mercaptoethanol (28625, Serva, Heidelberg, Germany). Cells were grown to 80% confluence.

Human endothelial colony forming cells (ECFCs; 00189423, Lonza, Basel, Switzerland) were cultured in Endothelial Cell Growth Medium (ECGM; C-22010, Promocell, Heidelberg, Germany), supplemented with ECGM supplement mix (C-39215, Promocell), 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 1% Glutamin (25030, Life Technologies). Medium was exchanged every 2 days and cells were passaged when reaching a confluence of 70–80%.

2.2. Hydrogel fabrication

Hydrogels were prepared by dissolving 0.15 g/ml PEGda (average M_n 6000; 701963, Sigma Aldrich, Taufkirchen, Germany) in Dulbecco's Phosphate Buffered Saline (DPBS; 14190, Life Technologies). The photoinitiator Irgacure 2959 (I 2959; 2-

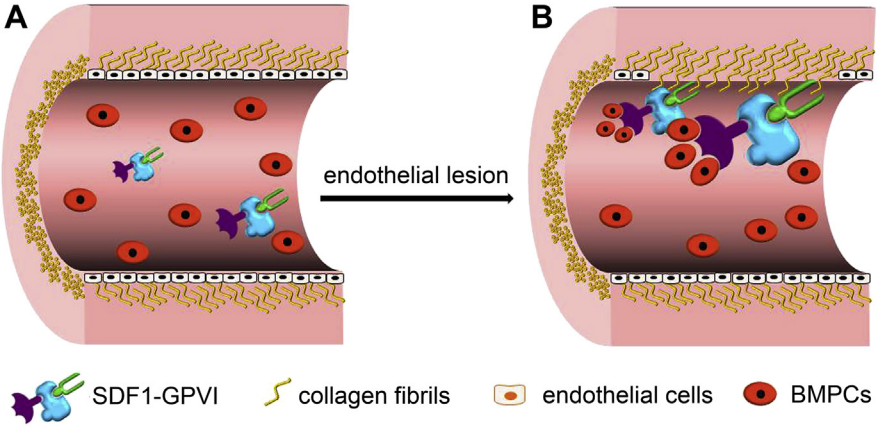

Fig. 1. Effect of the bi-specific protein SDF1-GPVI. Schematic of a blood vessel (A) before and (B) after endothelial lesion. At the area of endothelial injury, SDF1-GPVI binds to collagen types I and III via its GPVI-domain (green) and recruits BMPCs from the circulating blood with the SDF1-domain (purple). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone, 410896, Sigma Aldrich) was dissolved in 70% ethanol (Merck, Darmstadt, Germany). The concentration of I 2959 used for hydrogel fabrication was 750 μg/ml unless stated otherwise. For release and functionality assays, the polymer solution was blended either with bovine serum albumin (BSA; A9576, Sigma Aldrich) or SDF1-GPVI at the provided concentrations. The solution was crosslinked at a wavelength of 365 nm with a UV light intensity of 5 mW/cm^2 using an UV crosslinker (60-BLX-365, PEQLAB, Erlangen, Germany). Hydrogel stability was measured by studying weight loss over 4 weeks. Hydrogels were incubated in DPBS at 37 °C and 5% CO_2 and weighed at defined time points.

2.3. MTT assay

Hydrogels without incorporated protein were prepared as described above ($n = 3$) and washed with DPBS to prevent the gels from drying. For cytotoxicity studies, 5×10^4 mEPCs were seeded onto the hydrogel. To allow a proper cell attachment, mEPCs were incubated on the hydrogel surface for 30 min at 37 °C and 5% CO_2. Subsequently, mEPC medium was added into each well until the hydrogel was covered. mEPCs cultured in 12-well plates at the same density and medium volume served as controls. After 4 days of incubation, viable cells were visualized via a Thiazolyl Blue Tetrazolium Bromide (MTT) solution (M2128, Sigma Aldrich).

2.4. Release kinetics

Hydrogels with photoinitiator concentrations of 750, 1000 and 5000 μg/ml were prepared as described above ($n = 3$). In addition, 3200 μg/ml BSA was introduced prior to the UV gelation process. After crosslinking, the hydrogels were washed and incubated with DPBS at 37 °C and 5% CO_2 in order to enable BSA release. Supernatants were removed at 0, 2, 4, 12, 24, 48, 168 and 816 h and frozen immediately at −80 °C. To quantify the released protein, a Bradford assay was performed according to the manufacturer's protocol (Roti®-Nanoquant K880.2, Carl Roth, Karlsruhe, Germany). After 5 min incubation at room temperature, the absorption was measured at 595 nm using an Infinite® M 200 Pro reader (TECAN, Groedig, Austria).

2.5. SDF1-GPVI release

The release of SDF1-GPVI was assessed using hydrogels with a surface area of 1 ± 0.1 cm^2 ($n = 3$). Hydrogels were prepared by adding either 50 μg/ml or 100 μg/ml of the protein to the PEGda solution. After washing, hydrogels were incubated with DPBS over 7 days at 37 °C and 5% CO_2. The supernatant was collected from each hydrogel after 48, 96 and 168 h. To quantify the SDF1-GPVI concentration in the supernatant, a human IgG ELISA assay (human IgG ELISA kit, 0801182, Immunotek™, Buffalo, USA) was performed according to the manufacturer's protocol. This ELISA kit, which detects human IgG, was determined suitable as the protein SDF1-GPVI exhibits a human IgG2 domain. Absorption was measured using the Infinite® M200 Pro reader.

2.6. Immunofluorescence staining of a human artery vessel wall

Carotid arteries were obtained from patients undergoing bypass surgery. These studies were in accordance with institutional guidelines and were approved by the Ethics Committees of the Eberhard-Karls-University Tübingen and the Landesärztekammer Baden-Württemberg (IRB #694/2012BO1 and F-2011-068). Human arteries were harvested and processed as previously described [32]. Tissue sections of human artery vessel walls were deparaffinized and rehydrated. After antigen retrieval in a microwave oven for 8 min in 10 mM Tris, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.05% Tween 20 (pH 9.0) and 10 mM citrate solution in PBS (pH 6.0), slides were processed as described before [33] and stained using a primary pan-laminin antibody (1:50; ab11575, Abcam, Cambridge, UK). Following secondary antibody incubation using an Alexa Fluor® 594-conjugated goat anti-rabbit IgG (1:250; A-11037, Life Technologies), slides were exposed to DAPI solution (124653, Merck) and mounted in ProLong® Gold antifade reagent (P36394, Life Technologies). Fluorescence images were acquired using an Axio Observer microscope (Carl Zeiss, Göttingen, Germany).

2.7. Functionality of GPVI binding

An ELISA assay was performed to characterize the GPVI binding of released SDF1-GPVI to Col I ($n = 3$). SDF1-GPVI, solubilized in DPBS, was biotinylated via incubation with 0.5 M EZ Link Sulfo NHS LC Biotin (21335, Pierce, Rockford, USA) for 1 h at 27.5 °C. Biotinylation was stopped using 10 mM Trizma Base (T1503, Sigma Aldrich) at pH 9.5. Well plates were coated with 10 μg/ml Col I, which was isolated from bovine skin as previously published [34]. Control wells were coated using 10 μg/ml murine laminin-111 (354232, BD Biosciences, Bredford, USA) or 10 μg/ml Albumin Fraction V BSA (A13910500, Applichem, Darmstadt, Germany) at 4 °C over night. Plates were washed with Tris-Buffered Saline (TBS) and incubated with a 1% BSA solution for 90 min to block unspecific binding sites. SDF1-GPVI was diluted in TBS containing 5% BSA. To enable protein binding to Col I, BSA or laminin-111, plates were incubated with the desired concentration of SDF1-GPVI over 90 min. After washing with 4-(2-hydroxythyl)-1-piperazineethanesulfonic acid buffer, the protein was fixed with a 2.5% glutaraldehyde solution (ZC881439041, Merck) for 10 min. Plates were rinsed with TBS followed by an incubation period of 90 min with an ExtrAvidin® antibody (1:1000; E2636, Sigma Aldrich). The substrate 4-Nitrophenylphosphat disodium salt hexahydrate (N9389, Sigma Aldrich) was added to visualize protein binding. Time of incubation ranged between 5 and 25 min to reach reasonable binding signals. The reaction was stopped by addition of 1.5 M NaOH (A1551, AppliChem) and absorption was measured using an ELISA plate reader (Synergy™ HT Multi-Mode Microplate Reader, BioTek, Bad Friedrichshall, Germany). Incubation was performed at room temperature unless stated otherwise.

2.8. Staining of the CXCR4 receptor on ECFCs

Human ECFCs were seeded onto glass chamber slides (177402, Thomas Scientific, Swedesboro, USA) coated with 0.01% gelatin (G1890, Sigma Aldrich) at a density of 5×10^4 cells per chamber and cultured over night at 37 °C and 5% CO_2. After 16 h, cells attached to the glass slides, which were then washed with DPBS and fixed using 4% paraformaldehyde (11762.01000, Morphisto, Frankfurt, Germany) for 10 min at room temperature. After DPBS washing, unspecific binding sites were blocked by incubation with goat IgG-containing blocking buffer for 30 min. A CXCR4 antibody (1:100, ab2074, Abcam) was incubated over night at 4 °C. The secondary anti-rabbit IgG antibody (1:250, Alexa Fluor 488®, A-11034, Invitrogen) was incubated for 25 min in the dark. After cells were washed with DPBS and exposed to DAPI, the slides were mounted using ProLong® Gold antifade reagent. Fluorescence images were obtained using an Axio Observer microscope.

2.9. SDF1 binding to the CXCR4 receptor

ECFCs were seeded onto the gelatin-coated chamber slides with a density of 1.7×10^5 cells per ml. After an incubation period of 16 h, ECFCs were rinsed with DPBS and unspecific binding sites were blocked by incubation with goat IgG containing blocking solution for 30 min. Supernatants containing 240 ng released SDF1-GPVI collected from the hydrogels ($n = 9$) were added to each chamber. 240 ng of fresh, non-released SDF1-GPVI served as positive control, whereas the negative control was treated with DPBS only. After incubation, cells were washed and subsequently fixed with 4% paraformaldehyde for 10 min. A FITC-conjugated human IgG antibody (1:50; 109-095-098, Jackson Immuno Research, West Grove, USA) was used to determine protein binding. After 90 min incubation in the dark, cells were washed again and exposed to DAPI for 15 min. Images were acquired using the Axio Observer microscope system and fluorescence expression was quantified as gray value intensity (GVI) per cell using ImageJ software (National Institutes of Health, Bethesda, USA).

2.10. Data analysis

All data are presented as means ± standard deviations. Statistical significance was determined by one-way analysis of variance (ANOVA) using Origin software (OriginPro 8G, Northhampton, UK). $p \leq 0.05$ was defined as statistically significant.

3. Results

3.1. Generation of a hydrolytically stable non-cytotoxic PEGda hydrogel

UV crosslinking was employed to generate a solid and transparent PEGda hydrogel (Fig. 2A–C). Swelling properties and hydrolytic stability of the PEGda hydrogels generated using 750 μg/ml I 2959 were assessed in a weight loss study (Fig. 2D). PEGda hydrogels showed a significant increase in weight within the first 2 h (0 h: 454 ± 24 mg versus 2 h: 557 ± 28 mg; $p = 0.0009$), followed by a constant value over the observed time of 816 h in total. After a period of 4 weeks, no significant weight loss of PEGda hydrogels occurred, indicating a 4-week hydrolytic stability. Cell viability and cytocompatibility of the hydrogel was assessed after 4 days utilizing an MTT assay (Fig. 2E,F). The blue color indicated that mEPCs, which were seeded onto the hydrogel, were able to metabolize the MTT. Therefore, the hydrogel had no cytotoxic impact on the cells under the used experimental conditions.

3.2. Protein release from the hydrogel

In order to identify the suitable parameters for hydrogel fabrication, BSA was used as a control protein, which was encapsulated in PEGda hydrogels fabricated using different concentrations of I 2959. A Bradford assay was performed to monitor BSA release from the hydrogels with an average surface area of

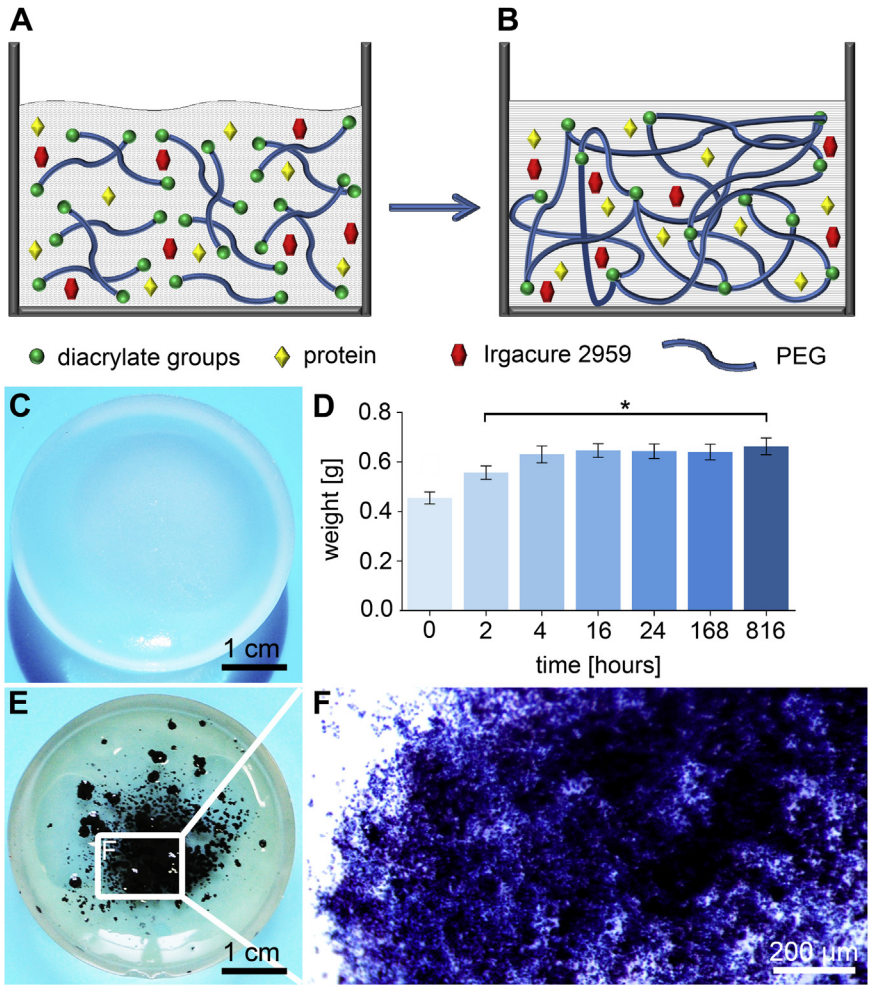

Fig. 2. Schematic of hydrogel fabrication: (A) aqueous polymer-I 2959 solution prior to gelation and (B) the solid hydrogel after UV-crosslinking. (C) Photograph of a solid PEGda hydrogel. (D) Swelling properties of the PEGda hydrogel. *$p < 0.05$ 2, 4, 8, 16, 24, 168 and 816 h versus 0 h (E, F) MTT-assay of seeded murine endothelial progenitor cells on PEGda hydrogels. Blue color indicates metabolizing, viable cells. Image (F) represents a higher magnification of the boxed section highlighted in image (E). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

947 ± 25 cm^2 over 168 h (Fig. 3A). The cumulative protein concentration of the supernatant was determined after 0, 2, 4, 8, 12, 24, 48 and 168 h. An initial burst was noted, followed by a sustained BSA release over 7 days. Furthermore, different release profiles emerged depending on the concentration of I 2959. A decrease in I 2959 concentration resulted in an increased BSA release. The cumulative release of BSA after 168 h ranged from 16.8 ± 11.3 μg/ml BSA (1.9% of the amount incorporated) using 5000 μg/ml of I 2959, 127.5 ± 9.7 μg/ml BSA (14.4% of the amount incorporated) using 1000 μg/ml of I 2959, to 156.1 ± 9.7 μg/ml (17.7% of the amount incorporated) when 750 μg/ml of I 2959 were used. The highest amount of protein release after 168 h was obtained using 750 μg/ml I 2959. Therefore, this concentration was defined as suitable for our further experiments.

A human IgG ELISA assay kit was used to determine the cumulative SDF1-GPVI concentration in the collected supernatants after 48, 96 and 168 h. Release profiles of SDF1-GPVI showed a slow release with cumulative release concentrations yielding 0.13 ± 0.04 μg/ml for hydrogels that were prepared using 3.75 μg SDF1-GPVI and 0.34 ± 0.04 μg/ml using 7.5 μg SDF1-GPVI respectively after 168 h (Fig. 3B). The release profile of 3.75 μg SDF1-GPVI showed a decrease of 0.001 μg/ml in protein concentration between 96 and 168 h, resulting in 3.5% cumulative release of the total protein amount used for hydrogel preparation. The addition of 7.5 μg SDF1-GPVI prior to hydrogel formation lead to a constant increase in released SDF1-GPVI, with a total amount of 4.5% after 168 h. Furthermore, release profiles of hydrogels containing 7.5 μg SDF1-GPVI depicted significantly higher concentrations of released protein at all three time points ($p < 0.05$, 7.5 μg versus 3.75 μg SDF1-GPVI).

3.3. GPVI binding capacity after hydrogel release

A GPVI binding assay was performed to verify proper diffusion out of the polymer network and to prove unaffected collagen-binding affinity of the GPVI domain via targeted binding to Col I after the UV gelation process (Fig. 4). Laminins are glycoproteins that are highly present in the basement membrane of human artery vessel walls [35] (Fig. 4A,B). Similarly to BSA, laminin-111 did not bind SDF1-GPVI and hence served as negative control in the testing of the GPVI domain's bioactivity and specificity towards Col I (Fig. 4C,D). Binding signals of biotinylated SDF1-GPVI on Col I and BSA were compared and reached statistical significance at a concentration of 1.25 μg/ml SDF1-GPVI ($p = 0.006$; SDF1-GPVI binding to Col I versus BSA) (Fig. 4C). When compared to laminin-111, GPVI binding to Col I reached statistical significance at a concentration of 0.625 μg/ml SDF1-GPVI ($p = 0.019$, Col I versus laminin-111) indicating a lower background signal on

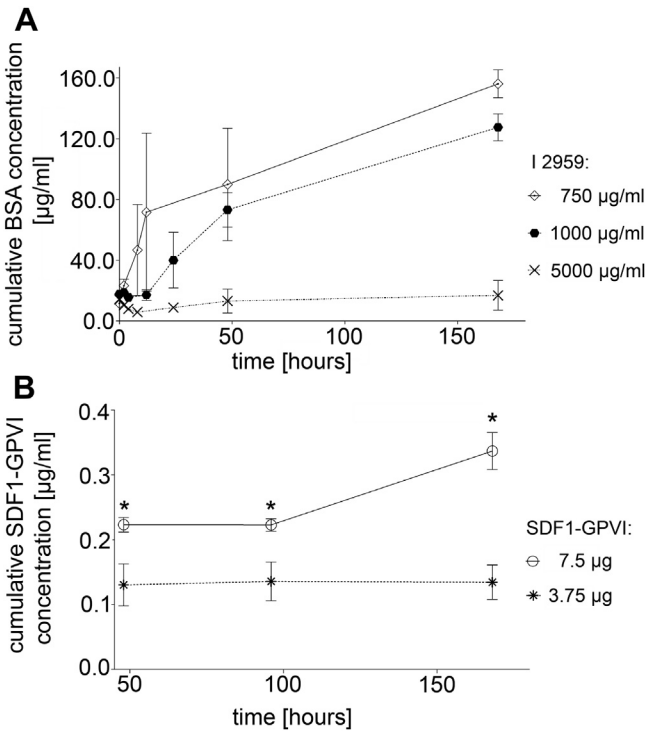

Fig. 3. Release profiles of BSA and SDF1-GPVI. (A) Bradford assay of released BSA from PEGda hydrogels with varying I 2959 (750, 1000, 5000 µg/ml) concentrations. For these experiments a total amount of 1600 µg BSA was integrated. (B) A human IgG-ELISA was employed to determine the amount of released SDF1-GPVI via its IgG domain. The release profiles depend on the loading amount of SDF1-GPVI (3.75 or 7.5 µg/ml). The I 2959 concentration is 750 µg/ml. A sustained protein release is observable using an incorporated amount of 7.5 µg SDF1-GPVI. $^{*}p < 0.05$, 7.5 µg versus 3.5 µg SDF1-GPVI.

laminin-111 than on BSA (Fig. 4C). Moreover, SDF1-GPVI released from the PEGda hydrogels after 2 days showed significantly higher binding signals to Col I when compared to laminin-111 (Col I 1.55 ± 0.07 versus laminin-111 1.07 ± 0.05; $p = 2.5 \times E^{-17}$) (Fig. 4D). Binding signals to Col I remained significantly higher after a period of 6 days (Col I 1.20 ± 0.09 versus laminin-111 0.92 ± 0.07; $p = 0.000001$); however, overall reduced binding signal intensities were observed from day 2 to day 6, indicating an increasing loss of GPVI binding capacity over time (Fig. 4D).

3.4. SDF1-GPVI exhibits a functional SDF1 domain after hydrogel release

CXCR4-expressing ECFCs (Fig. 5A,B) were utilized to determine the SDF1 domain binding potential of released SDF1-GPVI. The IgG2 domain of SDF1-GPVI served as antigen epitope for binding of the detection antibody, enabling verification of protein binding to the cells (Fig. 5C–E). Fluorescence expression was quantified as GVI per cell, which allowed the comparison of the binding signals of fresh and released SDF1-GPVI (Fig. 5F). Significantly higher fluorescence intensities were identified in the released SDF1-GPVI samples when compared to the negative controls (0.12 ± 0.01 versus 0.09 ± 0.02; $p = 0.0003$). No significant difference was detected between the released SDF1-GPVI samples and the positive controls (freshly supplemented SDF1-GPVI). This result was highly reproducible (Suppl. Fig. 1) and indicates that neither the hydrogel fabrication procedure nor the diffusion out of hydrogels significantly impact protein functionality.

4. Discussion

It has been previously reported that only 5–10% of injected cells survive and can integrate within the myocardium due to the harsh infarct environment post MI [36]. Ziegler et al. demonstrated that the recombinant bi-specific protein SDF1-GPVI is able to improve cardiac function after transient myocardial ischemia in a mouse model. In detail, homing of BMPCs to the site of infarction and subsequent neovascularization was enhanced, resulting in an improved ejection fraction in comparison to the control group that was treated with the Fc-fragment of immunoglobulin G2 (FcIgG2) of SDF1-GPVI [13]. An accumulation of CXCR4-positive cells was observed after 5 days in the infarct zone of the SDF1-GPVI-treated animals; however, neither the presence of SDF1-GPVI nor CXCR4-expressing cells were detectable after 28 days [13].

The quality of SDF1-induced BMPC homing is highly dependent on the level of CXCR4 surface expression on the BMPCs [37]. Previous studies demonstrated that SDF1 expression is up-regulated in

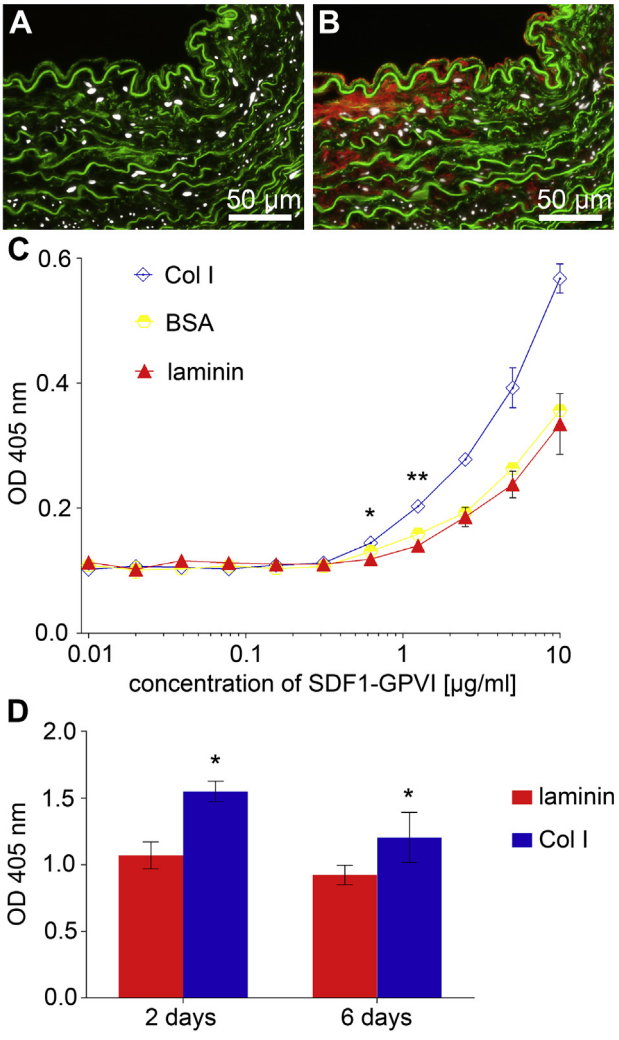

Fig. 4. (A, B) Immunofluorescence staining of an adult human artery vessel wall: (A) negative control; (B) laminins are immunostained in red, elastin autofluorescence is shown in green, cell nuclei are depicted in white. (C) An ExtraAvidin® coupled ELISA using biotinylated SDF1-GPVI shows the different binding affinities of GPVI to Col I, BSA and laminin-111. $^{*}p < 0.05$, Col I versus laminin-111, $^{**}p < 0.05$, Col I versus BSA. (D) GPVI binding signal intensity of the released SDF1-GPVI to Col I. Laminin-111 serves as negative control. $^{*}p < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

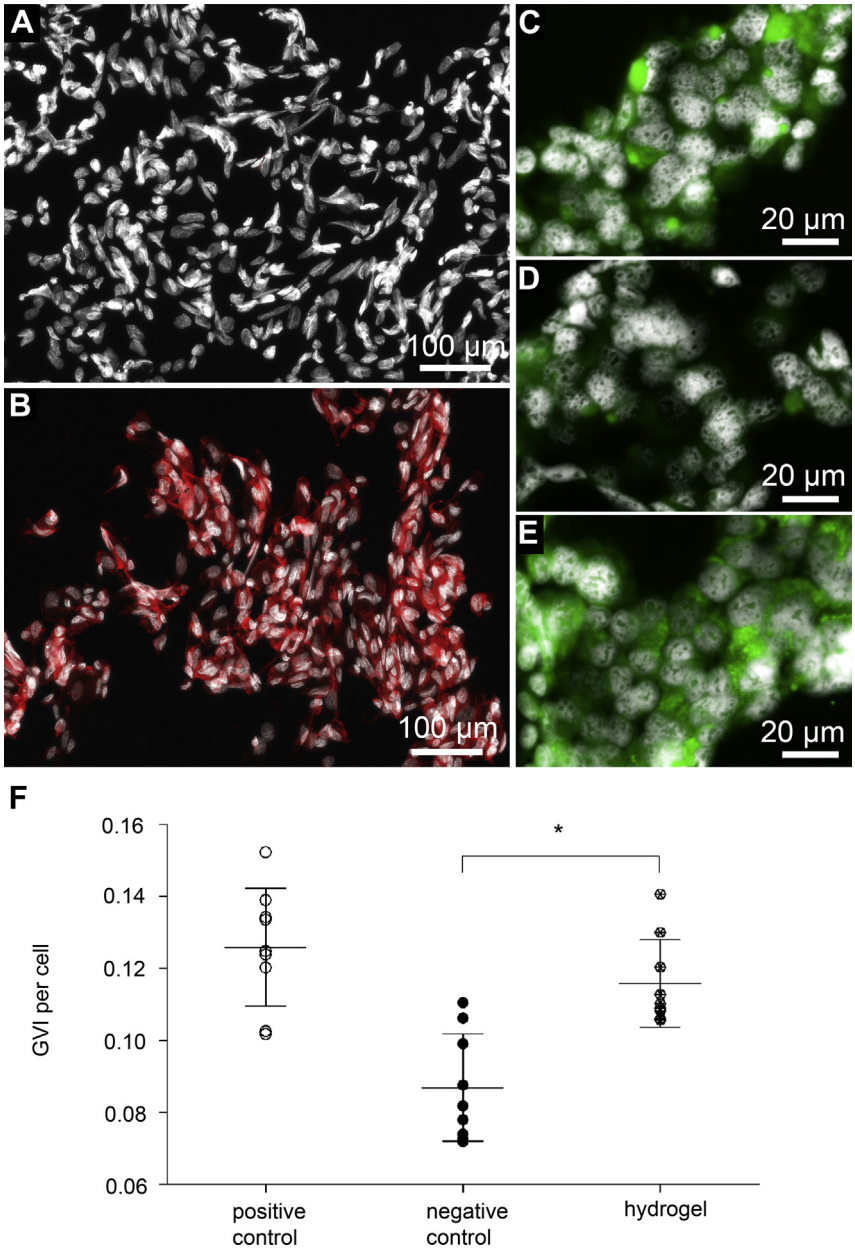

Fig. 5. (A, B) Immunofluorescence staining of ECFCs: (A) IgG control; (B) CXCR4 is highly expressed (red). Cell nuclei are stained with DAPI (white). (C–E) SDF1-domain binding affinity to CXCR4 of ECFCs (green): (C) positive control using fresh SDF1-GPVI, (D) incubation with DPBS as negative control and (E) released SDF1-GPVI. Cell nuclei are displayed in white. (F) Corresponding semi-quantifications are depicted as gray value intensities (GVI). $^{*}p < 0.01$ released SDF1-GPVI versus negative control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the myocardium rapidly after MI [38], but CXCR4 expression of BMPCs reaches its peak 4–7 days in delay [37]. Additionally, SDF1 expression in the myocardium declines 4–7 days after MI [38]. Therefore, a prolonged presence of SDF1 in the myocardium may enhance BMPC homing to the site of MI. Moreover, the therapeutic effect of chemokines as homing factors is mainly dependent on a concentration gradient. Effective BMPC recruitment via SDF1 can be achieved by a sustained delivery from hydrogels that supports the prolonged formation of a concentration gradient [24]. In the present study, we aimed to generate a PEGda hydrogel that can serve as a reservoir for SDF1-GPVI bi-specific protein, which can potentially allow a controlled and sustained release of SDF1-GPVI, therefore enabling the maintenance of a concentration gradient over a prolonged time period. Similar to our results, several studies have demonstrated the release of SDF1 from hydrogels with an initial burst release followed by a slower sustained release over 7 [14,22,24] or 21 days [39]. As higher dosages of SDF1 have been identified to be crucial for BMPC mobilization from the bone marrow [40], this burst release is advantageous. The fast release of proteins after the hydrogel has been placed into release medium is possibly caused by the entrapment of large amounts of encapsulated protein on the surface of the hydrogel during the fabrication process [41]. In our study, different BSA and SDF1-GPVI release profiles can be explained by the differences in molecular weight (BSA ~66 kDa versus SDF1-GPVI ~130 kDa) in combination with a relatively low incorporated protein concentration of 3200 µg/ml BSA and either 50 or 100 µg/ml SDF1-GPVI. It has been previously reported that a reduction in protein concentration in combination

with an increase in drug molecular weight can result in a decelerated drug release due to reduced diffusion kinetics [42]. Moreover, the average surface area of the SDF1-GPVI containing hydrogels was 1% of the surface area of BSA-loaded hydrogels. This could further suggest a decline in the release of SDF1-GPVI as it has been reported that smaller surface areas lead to a delayed release [42], which is consistent with our results. The release profile of hydrogels loaded with 3.5 μg SDF1-GPVI showed no sustained release over 7 days indicating that the protein loading concentration is too low for the desired application. However, a constantly increasing release of SDF1-GPVI could be observed from the hydrogels loaded with 7.5 μg SDF1-GPVI with supernatant concentrations of 0.22 ± 0.01 μg/ml after 96 h and 0.34 ± 0.03 μg/ml after 168 h. Previous *in vitro* studies demonstrated that BMPC migration is most effective at concentrations ranging from 0.05 to 0.25 μg/ml SDF1 [39]. Therefore we hypothesize that the concentrations of SDF1-GPVI in the supernatant of PEGda hydrogels loaded with 7.5 μg SDF1-GPVI are sufficient for BMPC homing during the observed time frame.

The percentage of released protein is also influenced by the concentration of the incorporated protein. We observed that by incorporating low protein concentrations, the cumulative release after 168 h was 3.6% for 50 μg/ml and 4.5% for 100 μg/ml SDF1-GPVI. Mechanisms of protein immobilization to the hydrogel, which occur during the crosslinking process, include free radical-induced protein conjugation or the reaction of acrylate groups with lysine groups of the proteins via Michael-type addition [43]. Photoinitiator concentration and acrylate groups define the amount of immobilized protein. Maintaining these concentrations results in an immobilized protein amount that is constant irrespective of the incorporated protein concentration [15]. Furthermore, the immobilized protein molecular weight plays a crucial role in its diffusion kinetics, as drug diffusion decelerates at increasing molecular weight [44]. Consistent with previous reports [15], we verified that protein diffusion could be decelerated by increasing the concentration of I 2959. This effect is due to a higher crosslinking density and mass-swelling ratio of the hydrogel resulting in a slower protein release [15] that enables the adaptation of the release profile to patient-tailored parameters. We further identified a concentration of 750 μg/ml I 2959 as the minimal concentration necessary for the formation of a solid gel, while allowing the fastest drug release. The photoinitiator concentration used for the crosslinking of PEGda is a critical parameter. Previous studies have shown that it correlates with the amount of free radicals generated during UV crosslinking [45,46]. In turn the free radicals attack proteins [39,46–48], nucleic acids [48,49] and cell membranes [50] and subsequently lead to a reduced protein bioactivity and cytotoxic effects. The cytotoxicity of I 2959 has been discussed intensively as some studies demonstrated that it is a suitable photoinitiator for the fabrication of cell-encapsulating hydrogels [45]. Others reported that I 2959 depicts cytotoxic effects dependent on the cell type [50]. Furthermore, acrylate groups have been identified as a source of potential cytotoxicity [51]. MEPCs cultured on the surface of the photo-crosslinked PEGda hydrogels generated in this study were viable after a culture period of 4 days. This might also be due to the relatively low concentration of 750 μg/ml I 2959 which was used to photo-crosslink PEGda chains. Higher photoinitiator concentrations were described as cytotoxic [52].

Several studies have demonstrated a reduction in protein bioactivity after release from photo-crosslinked hydrogels [39,46,48]. Whereas the exposure to low UV light intensities showed no significant impairment of protein quality [48], high intensity UV light exposure of 100 mW/cm^2 has been reported to negatively impact protein quality via photo-oxidation and the formation of protein aggregates [46]. PEGda hydrogels used in this study were crosslinked using the minimal UV light intensity of 5 mW/cm^2 for solid gel formation in combination with I 2959, which contributed to a preserved protein bioactivity. We conclude that using the minimal concentration of I 2959 and UV light exposure is advantageous in order to preserve protein bioactivity.

Triggered by UV light irradiation, free radicals can be formed during the dissociation of I 2959. Their insufficient selectivity leads to modifications of amino acids, which in turn might destroy the protein structure by disruption of peptide bonds and the addition of crosslinks between proteins [48]. We observed that released SDF1-GPVI bound specifically to Col I, which indicates a preserved GPVI functionality. However, a decline in GPVI binding signal intensity could be observed for released SDF1-GPVI from day 2 until day 6. As the estimated half-life of SDF1-GPVI is 48 h [13], a fraction of released SDF1-GPVI might have lost its binding capacity to Col I after 6 days. The SDF1 domain showed specific binding to CXCR4-positive cells without a culture time-dependent impaired binding intensity when compared to fresh SDF1-GPVI.

The combination of parameters used for the fabrication of the PEGda hydrogels was chosen with regard to minimal free radical and UV light exposure, which may explain the preserved bioactivity of the bi-specific protein. Furthermore, the acrylate groups of PEGda support protein bioactivity maintenance [46]. This positive effect can be attributed to the conversion of free radicals into less reactive and destructive species by acrylate groups and their function as free radical scavengers [46,48]. In this study, PEGda with an average molecular weight of 6 kDa was employed resulting in a relatively high polymer solution viscosity. Viscous polymer solutions act as cages that protect incorporated proteins from free radical attacks [46]. Thus, the stimulation of BMPCs via G-CSF administration in combination with the release of functional SDF1-GPVI from the generated PEGda hydrogel may result in enhanced rates of integrated BMPCs in the myocardium. This strategy is an interesting alternative approach to the currently used systemic injection of SDF1-GPVI and should be investigated with an *in vivo* MI model.

5. Conclusion

In this study, a cytocompatible photo-crosslinked PEGda hydrogel enabled a sustained release of the bi-specific protein SDF1-GPVI *in vitro*. Protein release profiles were tunable by the protein loading dose and the degree of crosslinking. Binding of the GPVI domain to Col I as well as specific binding of the SDF1 domain to CXCR4-positive cells after SDF1-GPVI release was successfully demonstrated. We determined that the bioactivity of the hydrogel-released SDF1-GPVI was comparable to fresh SDF1-GPVI, indicating that no loss of functionality occurred during the protein encapsulation via UV light. The PEGda hydrogels designed in this study can serve as suitable encapsulating systems for bi-specific proteins and could be used for the release of therapeutics. The design of an effective SDF1-GPVI release system might support angiogenesis in injured myocardium, while restoring cardiac function and thus serve as an attractive alternative for the therapeutic treatment of ischemic cardiovascular disease.

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Appendix A. Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.biomaterials.2014.04.116.

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