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# Matrix Metalloproteinase 9 (MMP-9) Mediated Release of MMP-9 Resistant Stromal Cell-Derived Factor 1α (SDF-1α) from Surface Modified Polymer Films

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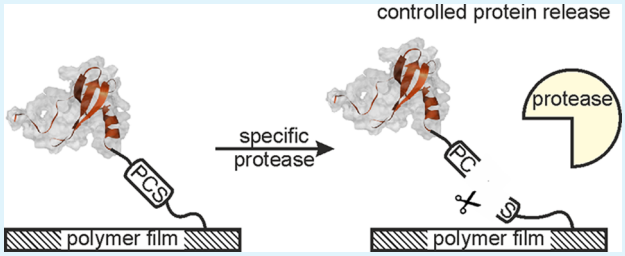
Supporting Information

**ABSTRACT:** Preparation of smart materials by coatings of established surfaces with biomolecules will lead to the next generation of functionalized biomaterials. Rejection of implants is still a major problem in medical applications but masking the implant material with protein coatings is a promising approach. These layers not only disguise the material but also equip it with a certain biological function. The anti-inflammatory chemokine stromal cell-derived factor 1α (SDF-1α) is well suited to take over this function, because it efficiently attracts stem cells and promotes their differentiation and proliferation. At least the initial stem cell homing requires the formation of a concentration gradient. Thus, a reliable and robust release mechanism of SDF-1α from the material is essential. Several proteases, most notably matrix metalloproteinases, are upregulated during inflammation, which, in principle, can be exploited for a tightly controlled release of SDF-1α. Herein, we present the covalent immobilization of M-[S4V]-SDF-1α on novel biodegradable polymer films, which consist of heterobifunctional poly(ethylene glycol) and oligolactide-based functionalized macromers. A peptidic linker with a trimeric matrix metalloproteinase 9 (MMP-9) cleavage site (MCS) was used as connection and the linkage between the three components was achieved by combination of expressed protein ligation and Cu(I) catalyzed azide/alkyne cycloaddition. The MCS was used for MMP-9 mediated release of M-[S4V]-SDF-1α from the biomaterial and the released SDF-1α derivative was biologically active and induced strong cell migration, which demonstrates the great potential of this system.




**KEYWORDS:** *Stromal cell-derived factor 1α, cross-linked macromer, immobilization, matrix metalloproteinase 9, release, tissue regeneration*

## INTRODUCTION

In today's medicinal applications, a number of novel biomaterials have been developed beside established materials like titanium, silicone or apatite. These "smart" biomaterials are used as transplants but are also applied in emergency medicine to cover skin burns. In the design of modern biomaterials, two important aspects have to be considered, which include (1) biodegradability and (2) recognition by the host immune system. Biodegradable polymers can be of natural origin or derived from extracellular matrix components, like collagen[1] or Matrigel.[2] Another possibility is the usage of synthetic materials, such as poly(lactic acid) (PLA) or poly(lactic-*co*-glycolic acid) (PLGA),[3] which become easily metabolized and possess a well-defined structure and physico-chemical properties. Furthermore, the use of natural and synthetic materials decreases the risk of adverse immune reactions.[4,5] PLA, however, lacks a suitably high and defined density of functional groups for effective surface modification. Using a macromer based approach, we were able to incorporate heterobifunctional PEG upon film cross-copolymerization and activate the hydroxy-terminus of PEG for subsequent surface modification.

Scientists have tried to incorporate beneficial proteins like growth factors and/or chemokines[6,7] to equip these surfaces with additional biological functions. After injury, it is very important that the time of hypoxia is kept as short as possible. A key mediator of this process is hypoxia-inducible factor 1α (HIF-1α)[8] and besides the regulation of genes related to oxygen transport and energy metabolism, HIF-1α also induces the transcription of growth factors and cytokines[9] as well as chemokines.[10] One of the most promising chemokines is stromal cell-derived factor 1α (SDF-1α, also called CXCL12). SDF-1α belongs to the family of CXC chemokines and

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mediates its effects via two G-protein coupled receptors, called CXCR4 and CXCR7.[11] Although CXCR4 acts as classical GPCR with G-protein mediated signal transduction,[12] the role of the CXCR7 is controversially discussed in literature.[13] It is probably able to form a functional receptor as a heterodimer with the CXCR4[14] but it is also described as a scavenging receptor to modify local SDF-1$\alpha$ concentrations.[15] All these receptor mediated effects lead to the SDF-1$\alpha$ specific cellular responses including homing of progenitor and stem cells toward the injured tissue[16] as well as promoting angiogenesis[17] and neovascularization.[18] Thus, SDF-1$\alpha$ coated biomaterials would facilitate wound healing of injured tissue and also skin regeneration through coverage of the injured area would be conceivable.

A disadvantage of employing chemokines like SDF-1$\alpha$ is their tight regulation in space and time. Many different chemokines are expressed, released and degraded during every phase of inflammation and successful wound healing depends on these balanced phase-specific chemokine patterns.[19] Furthermore, chemokine activity is interlinked to the formation of stable concentration gradients, which corroborates the need of a specific release mechanism that unleashes the protein within a beneficial time frame. The general idea of such a controlled system is illustrated in Figure 1.

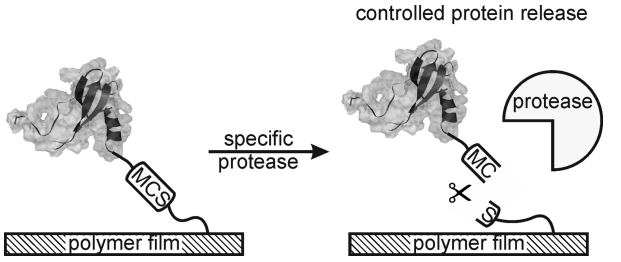


**Figure 1.** Illustration of a conceptual idea of a controlled protein release from a biomaterial/polymer film. A chemokine is extended by a peptide including a multimeric protease cleavage site (MCS) and immobilized on a polymer film. Upon activation of a protease, cleavage of the chemokine leads to a concentration gradient, which is necessary to induce specific biologic reactions.

During inflammation, an extracellular matrix breakdown occurs, which is necessary for the invasion of cells.[19] This entire process is regulated by different proteases, whereby matrix metalloproteinase 9 (MMP-9) is one of the most important and best characterized representatives. However, MMP-9 also inactivates SDF-1$\alpha$ by N-terminal processing,[20] which leads to a shortened SDF-1$\alpha$ that has been reported to be a potent antagonist.[21] In 2007, a mutant was identified ([S4V]-SDF-1$\alpha$), which is resistant against MMP-9 action, but still possesses chemotactic activity.[22] With this taken into account, MMP-9 is the perfect protease to release SDF-1$\alpha$ at the correct time point.

Herein, we describe the development of an elegant and robust strategy to immobilize and release the MMP-9 resistant SDF-1$\alpha$ analog M-[S4V]-SDF-1$\alpha$ on/from novel azided cross-linked copolymer films (biodegradable PEG/macromer films). The macromer concept allows for high versatility by cross-copolymerization with varying content and chemical functionality of heterobifunctional PEG methacrylates. SDF-1$\alpha$ is covalently immobilized on this polymeric substrate by a linker peptide containing an optimized trimeric MMP-9 cleavage site (MCS, full sequence is given in Figure 4D). MMP-9 will be activated by physiological mechanisms, will recognize the MCS, cleave the linkage and release M-[S4V]-SDF-1$\alpha$ from the surface to mediate its regenerative effects. The integration of all three components was achieved by a combination of expressed protein ligation (EPL) and Cu(I) catalyzed azide/alkyne cycloaddition (CuAAC). This novel bioactive film could be used as coating of established materials or in direct applications for skin and wound healing.

## EXPERIMENTAL SECTION

**Macromer Synthesis.** Three-armed oligolactide-based macromers were synthesized by oligomerization of lactide, starting from a trivalent alcohol followed by methacrylation. In a typical batch, 15 g of trimethylolpropane ethoxylate (450 Da) was weighted and 14.4 g of dilactide was added to oligomerize an average of two lactic acid units per arm of the trivalent alcohol. To this end, the reaction flask was placed on a magnetic stirrer and heated to 150 °C under constant nitrogen flow. After 15 min, 90 $\mu$L of tin(II) 2-ethylhexanoate was added to catalyze the ring-opening polymerization. After 15 min, the nitrogen flow was stopped, the flask was sealed and the reaction was left to proceed overnight. The reaction mixture was allowed to cool under nitrogen flow, and 200 mL of freshly distilled anhydrous tetrahydrofuran (THF) was added. This solution was cooled to −14 °C and 21 mL of triethylamine was added. Methacryloyl chloride (12.2 mL) was added dropwise at a rate to maintain the temperature below −10 °C. When the full volume was added, the reaction was allowed to proceed overnight. Triethylamine salts were removed from the reaction mixture by centrifugation. The supernatant was concentrated using a rotary evaporator and precipitated in a sodium bicarbonate buffer. The precipitate was again dissolved in acetone and precipitated in water. The viscous product was dried using reduced pressure in a rotoevaporator.

**Copolymerization and Activation to Achieve Azided Polymer Films.** Polymer films were cross-copolymerized on silanized (see the Supporting Information) glass supports by copolymerization of the macromer and a monomethacrylated poly(ethylene glycol) derivative ("PEG anchor", 2000 g/mol). Macromer and PEG anchors were dissolved in pure DMSO and the reaction was started by addition of the initiator azobisisobutyronitrile (AIBN) to yield a concentration of 4.5% (mol initiator/mol methacrylate). Twenty microliters of the readily mixed polymerization solution was transferred onto circular mirror-finished V2A steel discs (18 mm diameter; Hoppe Präzisionstechnik GmbH) and spread with the silanized glass discs. This setup was then subjected to a controlled heating regimen of 10 min at 85 °C and 30 min at 70 °C in nitrogen atmosphere. The setup was cooled to room temperature and the polymer film-coated glass discs were carefully lifted off the steel discs. The glass-supported polymer films were rinsed in acetone and immersed in deionized water for 15 min before being dried at reduced pressure for at least 6 h. These polymerized films (2.54 $cm^2$ area) had a molar PEG anchor content of 15%.

Hydroxyl groups of the PEG/macromer films were derivatized in a three-step procedure, as illustrated in Figure 2. First, the groups were carboxylated with 0.1 M succinic anhydride in THF overnight at room temperature. After the carboxylated films were washed in THF, they were immersed in 0.1/0.05 M *N,N′*-dicyclohexyl-carbodiimide/*N*-hydroxysuccinimide in anhydrous THF for 5.5 h at room temperature. Modified films were thoroughly rinsed in dichloromethane *p.a.* and quickly transferred into chilled Teflon wells (custom-made). In the last step, each film was quickly covered with a 4 °C solution of 1.0 mM 11-azido-3,6,9-trioxaundecan-1-amine in dry DMF. The wells were sealed and the reaction was continued overnight at 4 °C on an orbital shaker. After completion, solutions were aspirated and the modified films were sequentially rinsed in DMF and water and finally dried with compressed air. The films were stored in nitrogen-purged containers at 4 °C until further processing.

Carboxylated films without activation and azide modification were utilized as adsorption controls during the immobilization experiments.

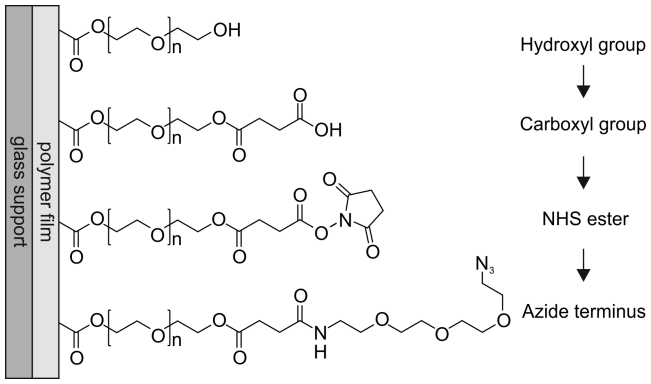


**Figure 2.** Three-step concept of polymer film modification: Carboxylation of hydroxyl groups, NHS activation, azide−amine linker immobilization.

**Film Characterization.** The films' surface hydrophilicity was assessed by static water contact angle measurements (Krüss G10). The contact angles of 8 $\mu$L droplets of Milli-Q water were determined at 0.5, 2 and 10 min after droplet deposition (3 films, 3 droplets/film: $n$ = 9). For further characterization, see the Supporting Information.

**Peptide Synthesis.** The peptide Cys−Gly(Pro−Leu−Ser−Leu−Arg−Ser)$_3$−Ahx−Glu−Lys−Ahx−Pra−$NH_2$ (MCS peptide; Ahx: 6-aminohexanoic acid, Pra: propargylglycine) was synthesized by fully automated solid phase peptide synthesis (SPPS) on a SyroII peptide synthesizer (Multisyntech) using the Fmoc/*t*Bu strategy. A detailed protocol is reported elsewhere.[23] Further details and analytical data are presented in the Supporting Information.

**M-[S4V]-SDF-1$\alpha$ Expression, Purification, Expressed Protein Ligation and Refolding.** Protein expression, expressed protein ligation and refolding was performed as described previously[24,25] with minor modifications. The protein thioester was generated by using the established IMPACT system and conjugated to the MCS peptide by expressed protein ligation[26] via the N-terminal cysteine of the peptide. All experimental details and analytics are presented in Figure 4 and in the Supporting Information.

**MMP-9 Digestion of M-[S4V]-SDF-1$\alpha$-MCS in Solution.** Recombinant human MMP-9 was supplied from R&D systems (# 911-MP-010). MMP-9 activation was performed by incubation of 1−10 $\mu$L MMP-9 (100 $\mu$g/mL) with *p*-aminophenylmercuric acetate (1 mM final concentration) for 24 h at 37 °C, as stated in the manufacturers protocol. Refolded M-[S4V]-SDF-1$\alpha$-MCS was diluted to 10 $\mu$M (100 $\mu$L) in MMP-9 activation buffer (50 mM Tris, 10 mM $CaCl_2$, 150 mM NaCl, 0.05% Brij-35 (w/v), pH 7.5) and the digestion was started by addition of 20 ng activated MMP-9. Samples were taken every 30 min and the digestion was monitored by Tricine-SDS-PAGE using a 16% acrylamide gel as described by Schägger.[27] This method is most useful to analyze small proteins. Furthermore, all samples were desalted by applying ZipTip pipet tips and analyzed by MALDI-ToF-MS on an Ultraflex III (Bruker Daltonics) using dihydroxybenzoic acid or $\alpha$-cyanocinnamic acid as matrix substances to ensure the correctness of the mass.

**M-[S4V]-SDF-1$\alpha$-MCS Immobilization by Cu(I) Catalyzed Azide/Alkyne Cycloaddition.** Refolded M-[S4V]-SDF-1$\alpha$-MCS was immobilized on azided PEG/macromer films and carboxylated PEG/macromer films as negative control. Glass slides with the films on top were placed into a 12-well plate and equilibrated in 1 mL of immobilization buffer (20 mM HEPES, 0.5 M NaCl, pH 8.0). 0.1 eq $CuSO_4$, 0.2 eq tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine and 0.3 eq sodium ascorbate were preincubated in immobilization buffer for 30 min at 37 °C. The ligation product M-[S4V]-SDF-1$\alpha$-MCS (1 eq, 2 $\mu$M) and all reagents were combined, added to the equilibrated films (500 $\mu$L each) and the immobilization reaction was performed overnight at 4 °C by gently shaking. The next day, the supernatant was removed and each film was washed three times with immobilization buffer for 5 min.

**Control of the Immobilization and Enzymatic Release by ELISA.** The M-[S4V]-SDF-1$\alpha$-MCS loaded films were removed from the glass slides, transferred to Eppendorf tubes, preincubated with 500 $\mu$L MMP-9 activation buffer for 15 min and the protein release was started by addition of 100 ng activated MMP-9. The reaction was performed overnight at 37 °C. The supernatant was removed, and the films were washed three times for 5 min with TBS-T (50 mM Tris, 150 mM NaCl, 0.5% Tween-20, pH 7.6).

MMP-9 treated and untreated PEG/macromer films were incubated with 5% BSA in TBS-T at room temperature. After 1 h, the solution was replaced by rabbit-anti-SDF-1$\alpha$ antibody (abcam, 1:1000 in TBS-T) and films were incubated for 1 h at room temperature. After removal of the primary antibody, the films were washed three times for 5 min with TBS-T. The secondary antibody goat-anti-rabbit-HRP (Santa Cruz Biotechnology; 1:5000 in TBS-T) was added and the solution was incubated for 1 h at room temperature. After washing with TBS-T, 500 $\mu$L of 3,3′,5,5′-Tetramethylbenzidine (TMB) was added and after 1 min, 500 $\mu$L of 0.25 M HCl was used to stop the reaction. The absorbance of the solution was measured at 450 nm using a microplate reader (TECAN Group Ltd.). All data were normalized to the control and analyzed with GraphPad Prism 5 v.5.03. Significance was determined by one-way ANOVA analysis and subsequent Bonferroni's and Tukey's multiple comparison test.[28]

**Cell Culture.** COS-7 cells (African green monkey kidney fibroblast-like cell line) were purchased from ATCC (CRL-1651). The cells were cultivated in standard Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose and L-glutamine supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), 100 units/mL penicillin and 100 mg/mL streptomycin. They were maintained at 37 °C in a humidified atmosphere of 5% $CO_2$ and were grown to confluence prior to use.

**Signal Transduction Assay.** The chimeric G-protein $G_{\alpha\Delta6qi4myr}$ was kindly provided by Evi Kostenis.[29] For inositol phosphate ($IP_x$) accumulation assays, the cells were seeded into 48-well plates (45.000 cells/well) and transiently cotransfected with 0.32 $\mu$g of CXCR4 plasmid DNA and 0.08 $\mu$g of $G_{\alpha\Delta6qi4myr}$ plasmid DNA using Metafectene. The assay was performed as described previously.[30] In brief, cells were stimulated with protein solution in concentration ranges of $10^{-6}$ to $10^{-11}$ M for 2 h at 37 °C. IP levels were determined by anion exchange chromatography and data were analyzed with GraphPad Prism 5 v.5.03.

**Cell Migration Experiments.** Cell migration experiments were performed in 96-well Boyden chambers (HTS Transwell plates, 5 $\mu$m pore size, Corning) as reported earlier.[31] Jurkat cells used in this assay were kindly provided by Manja Kamprad (Institute of Clinical Immunology and Transfusion Medicine, Universität Leipzig). In brief, Jurkat cells, which express the CXCR4 endogenously, were cultivated in RPMI 1640 medium (+10% FCS and 1% glutamine). Cells were spun down by centrifugation and resuspended to (4−6) × $10^5$ cells/mL in chemotaxis medium (RPMI 1640 + 2% FCS). All SDF-1$\alpha$ variants were diluted to concentrations between 5 and 500 nM and 250 $\mu$L of the appropriate dilution was filled into the lower chamber. The upper chamber was filled with 100 $\mu$L cell suspension and the plates were incubated for 2 h at 37 °C in a humidified atmosphere of 5% $CO_2$. Afterward, 200 $\mu$L was removed from the lower chamber and diluted 1:5 with PBS. Living cells were stained with fluoresceine diacetate (10 ng/mL final) and counted by flow cytometry (Partec). A sample without SDF-1$\alpha$ was used to determine basal migration (0%) and 100% migration is set by the number of deployed cells per well.

When M-[S4V]-SDF-1$\alpha$-MCS loaded films were used instead of soluble SDF-1 $\alpha$ variants, the system was adopted to a 12-well format. Chemotaxis medium was supplemented with 2 mM $CaCl_2$ to achieve optimal conditions for MMP-9 activity. Millicell hanging cell culture inserts (5 $\mu$m pore diameter, Millipore) were used as the upper chamber. Glass slides with M-[S4V]-SDF-1$\alpha$-MCS loaded films were placed into a new 12-well plate and 1.5 mL of chemotaxis medium was added. The inserts were placed on top of the appropriate wells and filled with 400 $\mu$L of cell suspension including (1−2) × $10^6$ cells/mL. SDF-1$\alpha$ release was induced by addition of 400 ng of activated MMP-9 to the lower chamber. The migration was performed for 4 h at 37 °C

in a humidified atmosphere of 5% $CO_2$. Afterward, inserts were removed and the assay was continued as described above. Pure medium was used to obtain basal cell migration and 50 nM soluble M-SDF-1$\alpha$ served as the positive control. All experiments were performed in duplicates and repeated for at least three times. Data were analyzed with GraphPad Prism 5 v.5.03 and significance was determined by one-way ANOVA analysis followed by Bonferroni's and Tukey's multiple comparison test.[28]

## RESULTS AND DISCUSSION

**Film Synthesis and Characterization.** The biomaterial used in this study is a novel cross-copolymerized film composed of a three armed biodegradable lactide-based macromer and 15% (mol/mol) PEG-2000-monomethacrylate. Films were fabricated by thermally initiated radical polymerization. Macromers consisted of a trimethylolpropane ethoxylate core (450 Da), which was modified by a ring-opening reaction with an average of two lactic acid groups per arm and finally methacrylated to introduce reactive sites for polymerization. This macromer was cross-copolymerized with PEG-monomethacrylate on silanized glass supports to obtain films with free hydroxyl groups that are covalently attached to the glass supports. Via the hydroxyl groups, the films were first carboxylated and then azided (Figure 2). Carboxylated films without azidation served as controls for physical adsorption of the SDF-1$\alpha$ variants.

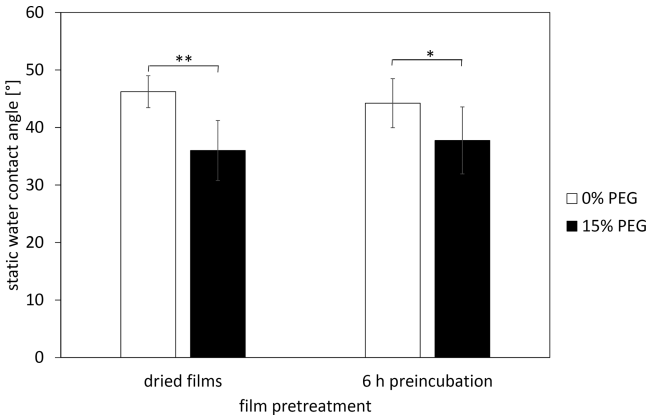


**Figure 3.** Static water contact angles of unmodified polymer films without (0%) versus with 15% PEG anchor 10 min after droplet deposition on the films. Angles were recorded before and after a 6 h preincubation in phosphate buffer (PBS). Whiskers represent standard deviations and significance was determined by performing Tukey's multiple comparison test, ** $p < 0.01$, * $p < 0.05$.

The lactide component is biodegradable, which ensures final metabolism of the material and clearance from the body.[32,33] Upon cross-polymerization, these macromers form monoliths with compressive moduli of approximately 20 MPa. The copolymerization with PEG-monomethacrylate decreases the compressive modulus. Altogether, these macromers can be used for the fabrication of free-standing polymeric devices with elastic moduli in the kPa to MPa range, depending on the device geometry, chemical composition and porosity. Surface hydrophilicity was determined by static water contact angle measurements of macromer films. The fabricated films with 15% PEG 2000 show contact angles lower than those of macromer films without the PEG modification (Figure 3). This difference was maintained after a 6 h preincubation period in phosphate buffered saline (PBS). In this time frame, initial protein adsorption and cell adhesion are assumed to take place. A final measurement after 24 h of preincubation in PBS leads to no further significant decrease in contact angles of the macromer films with or without PEG. Contact angles in the range of 30 to 50° classify these surfaces as hydrophilic. Moreover, they are likely to reduce unspecific protein adsorption via surface available PEG in the macromer films.[34] The hydroxyl end of the heterobifunctional PEG molecules can be further derivatized to result in a functionalized biomaterial, as shown here with azide groups. The chemistry and amount of PEG in the films can be adjusted to adapt surface hydrophilicity and degree of surface modification to desired values, which thereby reduces immunogenicity.[35]

**Recombinant Expression, Expressed Protein Ligation and Refolding.** Lactide-based materials have been successfully applied in several tissues like brain,[36] heart[37] or bone,[38,39] which illustrates the versatility of lactide-based materials. SDF-

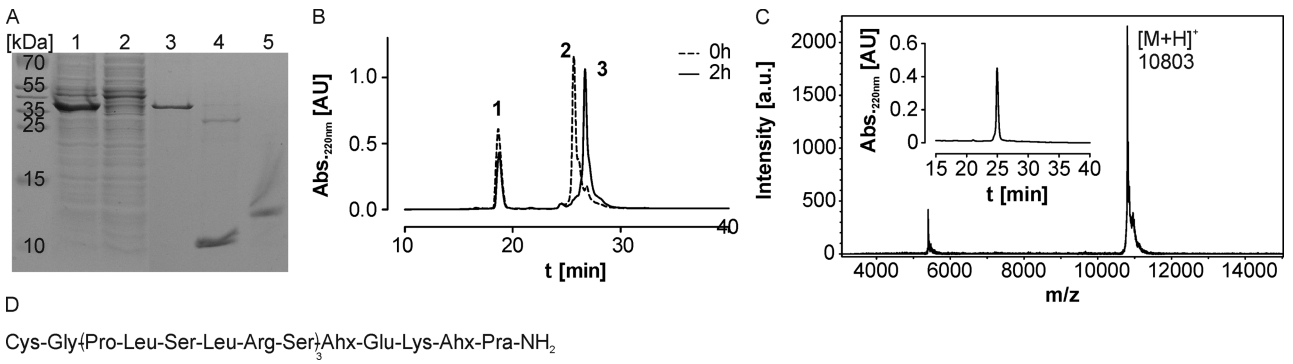


**Figure 4.** Protein expression and purification, expressed protein ligation and refolding of M-[S4V]-SDF-1$\alpha$-MCS. (A) Tricine-SDS-PAGE. Lane 1, cell lysis 6 h after IPTG induced protein expression; lane 2, lysis supernatant; lane 3, pooled solubilizates; lane 4, elution after purification and thiol induced tag cleavage (M-[S4V]-SDF-1$\alpha$-thioester); lane 5, purified ligation product (M-[S4V]-SDF-1$\alpha$-MCS). (B) Documentation of the expressed protein ligation of M-[S4V]-SDF-1$\alpha$-thioester and MCS peptide. The reaction was followed by RP-HPLC using a linear gradient system water/acetonitrile from 20 to 60% ACN in 40 min. Peak 1 (18.7 min) corresponds to the MCS peptide, peak 2 (25.7 min) represents the M-[S4V]-SDF-1$\alpha$-thioester and peak 3 (26.7 min) corresponds to the ligation product M-[S4V]-SDF-1$\alpha$-MCS. (C) Analytical data of refolded M-[S4V]-SDF-1$\alpha$-MCS. MALDI-ToF-MS of the purified protein ($M_{calc}$ = 10 802 Da), inset: RP-HPLC using a linear gradient system water/acetonitrile from 20 to 60% ACN in 40 min, a single peak was detected at 24.9 min. (D) Peptide sequence of the MCS peptide (1) used in this work (Ahx: 6-aminohexanoic acid, Pra: propargylglycine); MMP-9 is intended to cleave between serine and leucine.

1α seems to be an excellent coating for such biomaterials because it also mediates anti-inflammatory effects in brain, heart and bone.[40−42] Furthermore, it is able to home progenitor and stem cells and to promote angiogenesis and neovascularization.[16−18] Thereby, these PEG/macromer films could also serve as wound cover in skin injuries. All SDF-1α variants used in this work, were recombinantly expressed in *Escherichia coli*, as described previously[24,25] using the established IMPACT system. Notably, the initial methionine, which is not present in the mature human protein, is not cleaved by *E. coli*. Analytical data of peptide synthesis, protein expression, expressed protein ligation and protein refolding are summarized in Figures S1 (Supporting Information) and 4.

**MMP-9 Mediated Processing of M-[S4V]-SDF-1α-MCS and its Specific Release after Immobilization.** SDF-1α activity is regulated by many proteases, namely matrix metalloproteinases 2 and 9 (MMP-2, MMP-9),[20] dipeptidyl-peptidase 4 (DPP-4),[43] neutrophile elastase,[44] cathepsin G[45] and carboxypeptidase N and M (CPN and CPM).[46,47] These versatile degradation mechanisms demonstrate the complex regulation of SDF-1α. Segers et al. developed S-[S4V]-SDF-1α[22] in which the Ser4 to Val mutation eliminates the natural MMP-2 and MMP-9 cleavage site while the additional N-terminal serine residue mediates DPP-4 resistance. In principle, any other additional amino acid destroys the DPP-4 cleavage site, because it is only able to cleave off dipeptides with the sequence XP or XA (X = any amino acid).[48] Addition of an N-terminal amino acid to SDF-1α would result in the sequence XK, which is not accepted by DPP-4. This was confirmed by another study using V-[S4V]-SDF-1α.[31] Herein, the initial methionine was not cleaved, which should mediate the same effect as the serine or valine described by and Segers et al. and Baumann et al.[22,31] Furthermore, the exchange at position four was adopted and this M-[S4V]-SDF-1α was modified C-terminally with a peptide consisting of an optimized trimeric MMP-9 cleavage site (MCS) observed by Turk et al.[49] Notably, the cleavage sites of enzymes from the MMP family seem to be similar. Especially, MMP-2 and MMP-7 are able to recognize and cleave the same peptide sequences than MMP-9.[49] As mentioned before, MMP-2 is also included in the regulation of SDF-1α. Therefore, one would expect a synergistic effect in terms of the presented idea of a controlled SDF-1α release.

The M-[S4V]-SDF-1α-MCS construct was digested by addition of 20 ng of activated MMP-9, and a fast cleavage of the C-terminal part at the desired positions is demonstrated (Figure 5). Every 30 min, samples were taken and the resulting products were analyzed. Figure 5A visualizes a close to complete digestion after 1.5 h at 37 °C. After 2 h, the digestion was stopped and the final product was characterized by MALDI-ToF-MS (Figure 5B). The sample at 0 h shows the intact M-[S4V]-SDF-1α-MCS ($M_{calc}$ = 10 802 Da). After 2 h, only the expected product M-[S4V]SDF-1α-CGPLS ($M_{calc}$ = 8559 Da) and a corresponding matrix adduct (M + DHB, $M_{calc}$ = 8713 Da) were observed. In all additional samples, only intermediates (digestion after the second or third MMP-9 cleavage site) were detected (data not shown). This suggests a very fast recognition of the cleavage sequence by MMP-9 and further corroborate that the Ser4 to Val mutation of SDF-1α developed by Segers et al.[22] is responsible for the MMP-9 resistance.

Because SDF-1α essentially needs a concentration gradient to induce cell migration, it is indispensable to build up a SDF-1α presenting surface, which also ensures its release.

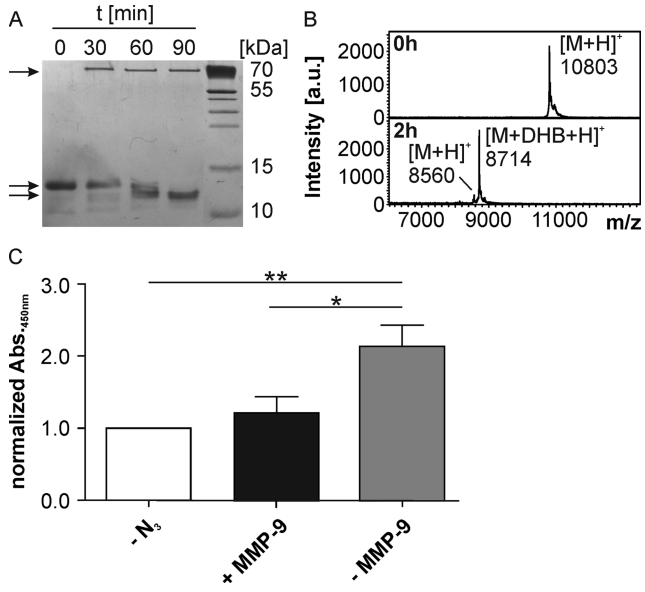


**Figure 5.** MMP-9 mediated digestion of M-[S4V]-SDF-1α-MCS in solution and release after immobilization. (A) Tricine-SDS-PAGE of samples taken after 0−90 min. Upper arrow indicates activated MMP-9. The two arrows below indicate the starting material M-[S4V]-SDF-1α-MCS (middle arrow) and the resulting product M-[S4V]-SDF-1α-CGPLS (lower arrow). In the samples taken at 30 and 60 min, intermediates also are observable. (B) MALDI-ToF-MS of undigested (upper panel) and MMP-9 digested M-[S4V]-SDF-1α-MCS (lower panel) after 2 h. M-[S4V]-SDF-1α-MCS ($M_{calc}$ = 10 802 Da), M-[S4V]-SDF-1α-CGPLS ($M_{calc}$ = 8559 Da). Noteworthy, the signal of the product is just barely present, but one signal at 8714 Da is observed, additionally. The mass difference is related to a matrix adduct of dihydroxybenzoic acid (DHB, $M$ = 154 Da). Other matrix substances showed only the expected product, but the quality of the spectra were significantly decreased. (C) ELISA after immobilization of M-[S4V]-SDF-1α-MCS and treatment with MMP-9. - $N_3$: COOH modified films incubated with M-[S4V]-SDF-1α-MCS demonstrating unspecific adsorption of M-[S4V]-SDF-1α-MCS. + MMP-9: Azide modified films incubated with M-[S4V]-SDF-1α-MCS and treated with MMP-9 after immobilization. - MMP-9: Azide modified films incubated with M-[S4V]-SDF-1α-MCS demonstrating the specifically immobilized M-[S4V]-SDF-1α-MCS. Data represent the average of three independent experiments shown as mean ± standard deviation. All data were normalized to the control and analyzed with GraphPad Prism 5 v.5.03. Significance was determined by performing Bonferroni's and Tukey's multiple comparison test, ** $p < 0.01$, * $p < 0.05$.

Therefore, M-[S4V]-SDF-1α-MCS was immobilized by using the alkyne group of propargylglycine on azided PEG/macromer films by Cu(I) catalyzed azide/alkyne cycloaddition (CuAAC).[50,51] The reaction was performed using the mild $CuSO_4$/sodium ascorbate system[50] at 4 °C overnight. After immobilization, the supernatant was removed and the films were washed and finally transferred to Eppendorf tubes. MMP-9 was added, and the solution was incubated overnight at 37 °C. COOH modified films were used as controls to investigate unspecific adsorption of M-[S4V]-SDF-1α-MCS. Furthermore, the immobilization and release was controlled by films containing immobilized M-[S4V]-SDF-1α-MCS where no MMP-9 was added.

After incubation with MMP-9, all films were analyzed by ELISA to determine the relative SDF-1α content compared to the negative control (COOH modified films; Figure 5C). A significant increase of the signal was observed for specifically

immobilized M-[S4V]-SDF-1$\alpha$-MCS by CuAAC (-MMP-9, $p < 0.01$). The signal decreased significantly (+ MMP-9, $p < 0.05$) after incubation with MMP-9 indicating efficient release of M-[S4V]-SDF-1$\alpha$-CGPLS from the PEG/macromer films. In total, a physiologically relevant mechanism was employed to ensure SDF-1$\alpha$ release at a specific time during inflammation.

**Biological Activity of MMP-9 Digested M-[S4V]-SDF-1$\alpha$-MCS.** All SDF-1$\alpha$ variants were tested for biological activity on CXCR4 using an inositol phosphate ($IP_x$) accumulation assay and cell migration experiments. At first, COS-7 cells were transiently cotransfected with CXCR4/$G_{\alpha\Delta 6qi4myr}$ and all SDF-1$\alpha$ variants were analyzed regarding to the influence of their mutations/modifications. Recombinantly expressed M-SDF-1$\alpha$ was taken along all experiments as positive control and for normalization. All data are summarized in Table 1.

**Table 1. Summary of Biological Activity of SDF-1$\alpha$ Variants Investigated by $IP_x$ Accumulation Experiments after CXCR4 Stimulation[a]**

| SDF-1$\alpha$ variant | $EC_{50}$ value [nM] | $pEC_{50}$ ± SEM | efficacy [%] | $n^b$ |
|---|---|---|---|---|
| M-SDF-1$\alpha$ | 3.0 | 8.52 ± 0.12 | 103.2 ± 3.8 | 9 |
| M-[S4V]-SDF-1$\alpha$ | 14.0 | 7.85 ± 0.07 | 99.4 ± 2.7 | 3 |
| M-[S4V]-SDF-1$\alpha$-MCS | 26.2 | 7.58 ± 0.23 | 70.0 ± 6.1 | 4 |
| M-[S4V]-SDF-1$\alpha$-CGPLS | 5.5 | 8.25 ± 0.23 | 47.0 ± 4.2 | 4 |

[a]Each experiment was performed in duplicates and the maximum activity of M-SDF-1$\alpha$ was set to 100% and used for normalization. [b]$n$ = number of independent experiments.

The $EC_{50}$ value of M-SDF-1$\alpha$ was determined to be 3.0 nM, which matches to values reported in literature.[31] The generated M-[S4V]-SDF-1$\alpha$-CGPLS is biologically active on CXCR4 and in comparison to M-SDF-1$\alpha$ a similar $EC_{50}$ value was obtained (3.0 vs 5.5 nM, respectively; Table 1). All other variants showed slightly increased $EC_{50}$ values of 14.0 nM for M-[S4V]-SDF-1$\alpha$ and 26.2 nM for M-[S4V]-SDF-1$\alpha$-MCS. Interestingly, C-terminal modifications seem to have an influence on receptor activation. The variants M-[S4V]-SDF-1$\alpha$-MCS and M-[S4V]-SDF-1$\alpha$-CGPLS could not fully activate the CXCR4 with efficacies of around 70% and 47%, respectively. It is known that SDF-1$\alpha$ forms dimers under certain conditions[52,53] and the dimeric form seems to differ in its CXCR4 activation profile.[54] In case of both, M-[S4V]-SDF-1$\alpha$-MCS and M-[S4V]-SDF-1$\alpha$-CGPLS, an additional cysteine was introduced, which could support possible dimers by disulfide bridging.

**Cell Migration Capacity of MMP-9 Processed and Film Released M-[S4V]-SDF-1$\alpha$-CGPLS.** Next, cell migration experiments were performed with Jurkat cells as model system. These immortalized T lymphocytes express the CXCR4 receptor endogenously. Again, M-SDF-1$\alpha$ was used as a control. As shown in Figure 6A, all relevant variants induce a similar migration profile and all curves possessed a bell-like shape with a maximum migration rate between 60% and 70%. The highest migration rate for M-SDF-1$\alpha$ and M-[S4V]-SDF-1$\alpha$-CGPLS was observed between 50 and 100 nM. In the case of M-[S4V]-SDF-1$\alpha$, a slightly right shifted profile was detectable with a maximum between 100 and 250 nM. Noteworthy, in all performed experiments, no difference between M-[S4V]-SDF-1$\alpha$-CGPLS and wild-type M-SDF-1$\alpha$ could be observed.

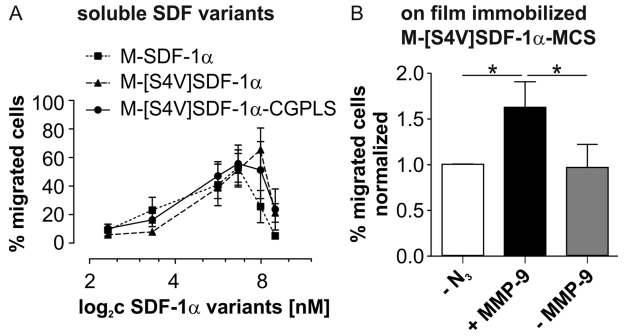


**Figure 6.** SDF-1$\alpha$ induced Jurkat cell migration. (A) Concentration dependent migration behavior of Jurkat cells incubated with different soluble SDF-1$\alpha$ variants using a 96-well Boyden chamber assay system. Dotted line represents M-SDF-1$\alpha$, which was used as the positive control. The dashed line indicates the cell migration behavior, which was induced by M-[S4V]-SDF-1$\alpha$ and the solid line represents C-terminally modified M-[S4V]-SDF-1$\alpha$-CGPLS (product of M-[S4V]-SDF-1$\alpha$-MCS after MMP-9 digestion). Data represent mean ± standard error of the mean of three independent experiments. (B) Migration behavior of Jurkat cells induced by immobilized/released M-[S4V]-SDF-1$\alpha$-MCS under different conditions. - $N_3$: COOH modified polymer films incubated with M-[S4V]-SDF-1$\alpha$-MCS. Thereby, only unspecific binding is possible. + MMP-9: $N_3$ modified polymer films after immobilization of M-[S4V]-SDF-1$\alpha$-MCS. At the beginning of the migration assay MMP-9 was added to mediate the specific release, which enables the formation of a concentration gradient. - MMP-9: $N_3$ modified polymer films after immobilization of M-[S4V]-SDF-1$\alpha$-MCS. No MMP-9 was added to ensure that the migration effect was induced by MMP-9. Data represent mean ± standard deviation of at least three independent experiments. All data were normalized to the control and analyzed with GraphPad Prism 5 v.5.03. Significance was determined by performing Bonferroni's and Tukey's multiple comparison test, * $p < 0.05$.

Taken together, all tested SDF-1$\alpha$ variants are biologically active in the nanomolar range, with minor differences between the individual analogs.

As shown above, immobilization and MMP-9 mediated release of SDF-1$\alpha$ from azide functionalized PEG/macromer films was successful (Figure 5C). Thereby, the protocols of the release and migration experiments were combined and optimized to evaluate whether film-released M-[S4V]-SDF-1$\alpha$-CGPLS is suitable to induce proper cell migration. The migration time was extended to 4 h and the amount of MMP-9/mL was increased to release the highest possible amount within the optimal conditions for the cells. Films without the terminal azide (- $N_3$; no specific immobilization) were used as the negative control and films with immobilized M-[S4V]-SDF-1$\alpha$-MCS but without MMP-9 in the medium (- MMP-9; no MCS cleavage) were used to specify the observed effect. Figure 6B illustrates a significant difference ($p < 0.05$) between both controls and the MMP-9 mediated release of M-[S4V]-SDF-1$\alpha$-CGPLS (+ MMP-9). The Jurkat migration rate is 1.6 times higher than that of the control without the terminal azide, which was used for normalization. The significant increase in cell migration confirmed our concept of the targeted release of SDF-1$\alpha$ from the PEG/macromer films and further revealed a proof of principle of the initially introduced system (Figure 1).

Herein, rapid release was intended but, if the cleavage site is not optimal for a distinct protease, the cleavage will be slower and a more continuous systems will be achieved to create long-term effects.

## CONCLUSION

In conclusion, a novel polymeric film could be synthesized, which is biodegradable and prevents random protein adhesion. Further biological properties were included by site specific immobilization of the chemokine SDF-1$\alpha$. The design of an additional C-terminal MMP-9 cleavage site allowed an adjustable release from this polymer film, which is physiologically relevant and could bring SDF-1$\alpha$ into play in a beneficial time frame after injury. With this approach, we could combine the advances of a novel biomaterial and increase its functionality. Furthermore, the introduced M-[S4V]-SDF-1$\alpha$ analog is equally potent in chemotaxis as the natural protein, but it is less affected from degradation by proteases. This combination could be interesting for medicinal applications after skin injury and could improve wound healing and tissue regeneration after surgery. Furthermore, this approach is flexible and robust and the transfer to alternative systems (other materials, proteins or protease cleavage sites) can be easily performed

## ASSOCIATED CONTENT

### Supporting Information

A detailed description of the experimental setup for glass support silanization, film characterization, peptide synthesis as well as recombinant protein expression, purification and refolding is given as Supporting Information. Furthermore, characterization of the synthesized peptide and the process of expressed protein ligation and final characterization of M-[S4V]-SDF-1-MCS is presented. This material is available free of charge via the Internet at http://pubs.acs.org.

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The paper was written through contributions of all authors. All authors have given approval to the final version of the paper.

### Notes

The authors declare no competing financial interest.

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## ABBREVIATIONS

MMP-9, matrix metalloproteinase 9
SDF-1$\alpha$, stromal cell-derived factor 1$\alpha$
CXCR4/7, CXC chemokine receptor 4/7

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