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Citation: Biointerphases **12**, 010501 (2017); doi: 10.1116/1.4978871
View online: http://dx.doi.org/10.1116/1.4978871
View Table of Contents: http://avs.scitation.org/toc/bip/12/1
Published by the American Vacuum Society

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(Received 9 December 2016; accepted 7 March 2017; published 21 March 2017)

In an effort to rationalize and optimize an antiapoptotic coating combining chondroitin sulfate (CS) and epidermal growth factor (EGF) for vascular applications, the authors here report the comparison of two grafting strategies aiming to display EGF in an oriented fashion on CS. For that purpose, the authors produced, purified, and characterized a chimeric protein corresponding to EGF that was N-terminally fused to a cysteine and a coil peptide. The chimera was covalently immobilized via its free thiol group or captured via coiled–coil interactions at the surface of a biosensor or on a chondroitin sulfate coating in multiwell plates, mimicking the coating that was previously developed by them for stent-graft surfaces. The interactions of grafted EGF with the soluble domain of its receptor or the impact of grafted EGF upon vascular smooth muscle survival in proapoptotic conditions indicated that the coiled–coil based tethering was the best approach to display EGF. These results, combined to direct enzyme-linked immunosorbent assay measurements, indicated that the coiled–coil tethering approach allowed increasing the amount of bioavailable EGF when compared to covalent coupling, rather than the total amount of grafted EGF, while using much lower concentrations of tagged EGF during incubation. © *2017 American Vacuum Society*. [http://dx.doi.org/10.1116/1.4978871]

I. INTRODUCTION

In the field of biomedical engineering, the development of smart materials promoting and sustaining tissue regeneration holds great promise for orthopedic, vascular, and neural applications. In that endeavor, the decoration of implant surfaces and scaffolds with peptides and proteins, especially adhesion peptides and growth factors, has been extensively studied to mimic nature's mechanisms of tissue growth and healing. Of interest, growth factor immobilization has been reported to preserve their biological activity in several cases while impeding cell membrane receptor internalization and degradation, thus sustaining their local action.[1–3] When compared to bolus delivery, growth factor immobilization may also limit systemic side-effects while diminishing the prohibiting costs associated with their short half-life, when infused.[4]

Researchers have initially investigated growth factor immobilization by adsorption because of the relative simplicity of the approach. However, adsorption does not allow to finely control protein orientation, which may mitigate protein interactions with cells and thus overall biological effect of proteins.[2,5,6] As an alternative, covalent binding of peptides and growth factors, on substrates harboring amino, carboxyl, and thiol groups, has been proposed.[2,7–12] In most cases, the reactive groups targeted by these chemistries are multiple within the proteins. They may be present in the vicinity or at the active site of the growth factor, thus impacting its activity when immobilized.[13] Genetic engineering may be used to add specificity to grafting.[4] First, the addition of amino acids providing a unique reactive group within the protein, such as a free thiol group on cysteine side-chain, can be used for site-specific covalent binding, as already reported for adhesion peptide[14] and vascular endothelial growth factor grafting.[15,16] Also, the addition of a short peptide tag on the protein of interest has already been reported to drive its specific adsorption.[17,18] At last, the addition of various tags that mediate interactions with specific biological partners have been proposed for affinity-based capture. Those include biotin interacting with streptavidin,[13] antibody Fc domain interacting with protein A/G,[19] proline-rich peptides interacting with SH3 domains,[20] heparin-binding domains to bind heparin,[21] collagen binding

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domains to bind collagen,[22] to name a few.[23] For both covalent and affinity-based immobilization, substrate should be inert to avoid nonspecific adsorption.[6,24–26] Furthermore, for a given growth factor, its amount and activity may also be influenced by the density, the length, and the flexibility of the chemical spacer used to hold it to the surface.[1]

Along with other research groups, we have exploited the naturally occurring coiled–coil structures that are found in proteins to develop an affinity-based capture system. This approach has indeed proved noteworthy for several tissue engineering and gene delivery applications by enabling the controlled grafting of growth factors,[27] antibodies,[28] as well as transcription factors[29] onto various polymer-based structures. More precisely, we have previously developed two *de novo* designed peptides (the Ecoil and the Kcoil), each composed of a distinct seven-amino acid motif (a heptad) that is repeated several times. On its own, each peptide is monomeric; when mixed, they specifically heterodimerize to form the canonical coiled–coil structure. We demonstrated the utility of this system to tether fully bioactive proteins in a specific, oriented and stable fashion on various surfaces and coatings, including polyethylene terephthalate,[30] polyethylenimine,[31] chondroitin sulfate (CS),[32] and carboxymethylated dextran.[26] In our strategy, one of the two 35-amino acid-long (five heptad-long) peptides (e.g., the Kcoil) is covalently bound to the surface via a unique engineered cysteine while the protein to be captured is expressed as a fusion protein containing the other coil peptide (e.g., the Ecoil). Capture is achieved via simple incubation. It has been demonstrated that the high stability of our coiled–coil structure was due to hydrophobic and ionic interactions, while the affinity and stability of the resulting complex can be controlled by varying the number of heptads of each coil peptide[33] or via peptide sequence variation.[34] All these features have made this complex an attractive tool for biotechnological and biomedical applications. For example, we successfully demonstrated the utility of the coiled–coil approach to tether the epidermal growth factor (EGF) being N-terminally tagged with the Ecoil peptide on a coating made of chondroitin sulfate that had been decorated with the Kcoil peptide.[32] This coating was shown to be promising to enhance the performance of stent-grafts employed for the endovascular treatment of abdominal aortic aneurysms or other vascular implants such as tissue-engineered vascular grafts. Notably, the oriented display of EGF was proven to give better results [better vascular smooth muscle cell (VSMC) survival] than a similar surface harboring amine-coupled EGF.[32] The superiority of the coiled–coil tethering approach versus the covalent amine coupling chemistry we tested could be potentially explained by (1) the distance between the growth factor and the surface being optimal in the case of the coiled–coil tethering approach and (2) the EGF presentation being random—thus suboptimal—when amine-coupled.

In an effort to rationalize and optimize our coating, more specifically to shed light on the key role of the coiled–coil, we here explore a novel strategy to graft EGF in an oriented and covalent fashion. This was achieved by designing a chimeric protein corresponding to EGF being N-terminally tagged with both a three-residue long cysteine extension and the 35-residue long Ecoil, namely, Cys-Ecoil-EGF. By grafting this protein using either the cysteine (for covalent thiol coupling) or the Ecoil (for noncovalent coiled–coil interactions), we were able to compare two oriented approaches only differing by the covalent versus noncovalent nature of grafting.

Altogether, three grafting strategies using Ecoil and Cys-Ecoil-EGF were assayed, as described in Fig. 1; their impact on protein performance was studied by surface plasmon resonance (SPR)-based biosensing, enzyme-linked immunosorbent assay (ELISA), as well as in an *in vitro* cell assay on chondroitin sulfate-coated surfaces.

II. EXPERIMENT

A. Chemicals and reagents

MilliQ water (18.2 MΩ cm; total organic compounds = 4 ppb) was generated using a Millipore Gradient A 10 purification system. Amicon Ultra-15 Centrifugal filter units 10 kDa were purchased from MilliporeSigma (Oakville, ON). FreeStyle™ F17 medium, L-glutamine, as well as geneticin were purchased from Invitrogen (Burlington, ON). Dulbecco's modified Eagle's medium (DMEM) was from GIBCO/BRL (Burlington, ON). Sodium chloride, chondroitin-4-sulfate, Dulbecco's phosphate buffered saline (modified PBS, without calcium chloride and magnesium chloride), fetal bovine serum (FBS), Kolliphor® P188, L-cysteine, acetic acid, anti-mouse-horseradish peroxidase conjugate, and sodium orthovanadate were purchased from Sigma-Aldrich Canada

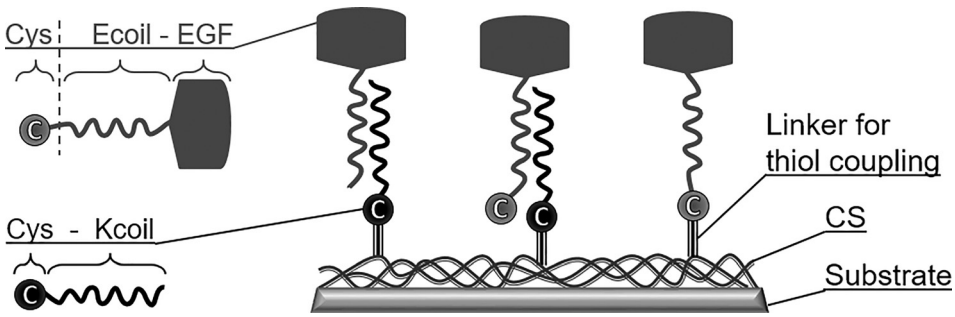

FIG. 1. Strategies for the oriented grafting of epidermal growth factor on CS. Noncovalent capture of (left) Ecoil-EGF and (middle) Cys-Ecoil-EGF via Ecoil/Kcoil coiled–coil interactions. (right) Covalent grafting of Cys-Ecoil-EGF via direct thiol coupling.

(Oakville, ON). CellBIND® flasks of 25 and 75 cm^2, 250 ml shake flasks and 96-well microplates were purchased from Corning, Inc. (Corning, NY). 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffered saline (HBS-EP), thiol coupling kit containing *N*-hydroxysuccinimide, ethyl-*N*′-(3-dimethyl-aminopropyl) carbodiimide hydrochloride, 2(2-pyridinyldithio) ethaneamine, and CM-5 sensor ships were obtained from GE Healthcare (Baie d'Urfe, Qc). 3-(2-pyridyldithio) propionyl hydrazide (PDPH), DMEM: F12 and pen-strep (penicillin–streptomycin) were purchased from ThermoFisher Scientific. Cysteine-tagged Kcoil peptide corresponding to the CGG-$(KVSALKE)_5$ sequence was synthesized in the peptide facility at University of Colorado, as described elsewhere.[33] The ectodomain of the EGF receptor fused to the Fc portion of an antibody (EGFRED-Fc) and the ELISA DuoSet kits for EGF quantification were purchased from R&D systems (R&D systems, Minneapolis, MN). TN1 peptone was purchased from Tekniscience (Terrebonne, Qc).

B. Cell culture

Cell culture was carried out in a humidified incubator at 37 °C and with 5% of CO_2. A human embryonic kidney (HEK) 293-6E cell line was used for recombinant protein expression of the Cys-Ecoil-EGF chimera. Cells were maintained in suspension in FreeStyle™ F17 medium supplemented with 4 mM of glutamine, 0.1% v/v of Kolliphor® P188, and 25 μg/mL of geneticin (G-418). Cell culture of human carcinoma cell line (A-431) was performed in T-flasks in Dulbecco's modified Eagle's medium supplemented with 10% of fetal bovine serum. VSMCs from rat (a7r5 cell line; ATCC, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium/nutrient mixture F-12 Ham's media (DMEM:F12) supplemented with 1% pen-strep and 10% fetal bovine serum. VSMCs were used between passages 13 and 15 for all the experiments.

C. Recombinant protein production

The Cys-Ecoil-EGF construct was designed as follows: MEFGLSWVFLVALLRGVQC*QVQECGG*EVSALEKEVSALEKEVSALEKEVSALEKEVSALEK**GGGGSGGGGSGGGGSELHHHHHHHHGT**MNSDSECPLSHDGYCLHDGVCMYIEA LDKYACNCVVGYIGERCQYRDLKWWELR.

That is, an N-terminal signal peptide (underlined) was followed by a short linker containing the cysteine residue (QVQECGG—italic). This was followed by five repeats of the Ecoil heptad—$(EVSALEK)_5$—that was separated from the EGF ligand by a $(G4S)_3$-$SEL(H)_8GT$ linker (bold). The corresponding cDNA sequence was flanked by EcoRI and BamHI sites on the 5′ and 3′ end, respectively. The cDNA encoding the fusion construct was optimized with human codons bias, synthesized by Genewiz (South Plainfield, NJ) and cloned into the pTT5 expression vector[35] between EcoRI and BamHI restriction sites.

For protein production, HEK293-6E cells were transiently transfected using 0.1% w/v plasmid and 0.14% w/v 25-kDa linear polyethyleneimine when the cell density reached 1.5 × 10^6 cell/ml, as previously described.[36] Post-transfection of 24 h, 0.5% w/v of TN1 peptone was added to the culture. The culture was stopped when mortality reached 20%, and centrifugation was performed for 20 min at 4500*g*. The supernatant was filtered and kept overnight at 4 °C. Cys-Ecoil-EGF was purified by ion metal affinity chromatography (IMAC): the supernatants containing the soluble protein was loaded on a Ni^{2+}-charged 5-ml His-trap HP column using an ÄKTA explorer 10 system (GE Healthcare, Bai d'Urfe, QC). The Cys-Ecoil-EGF protein was eluted by applying ten column volumes of PBS containing 500 mM of imidazole (10 min, flow rate of 5 ml/min). Imidazole excess was removed using Amicon Ultra-15 Centrifugal Filter Units (10 kDa, five cycles of 45 min at 4500*g*). Protein yield was determined using an Epoch™ Multivolume multisample Spectrophotomer System (BioTek Instruments). Protein expression monitoring during production and purification process was performed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) as follows: Samples were prepared using 3:1 ratio of Laemmli sample buffer supplemented with 5% v/v of ß-mercaptoethanol and were analyzed on 15% polyacrylamide gels. Coomassie brilliant blue R-250 was used for gel staining.

D. SPR-based assay

Real-time monitoring of the interactions between grafted Cys-Ecoil-EGF or Ecoil-EGF and the injected extracellular domain of the EGF receptor (EGFRED) fused to the Fc portion of an antibody (EGFRED-Fc) was performed using a Biacore T100 biosensor (25 °C). All experiments were carried out on CM5 sensorchips with HBS-EP as running buffer. EGFRED-Fc was injected at various concentrations (2, 5, 10, 20, and 50 nM) for 60 s at 100 μl/min over mock and Cys-Ecoil-EGF or Ecoil-EGF surfaces. The dissociation of the resulting complex was also monitored at the same flow rate for 120 s. Resulting sensorgrams were double-referenced prior analysis. Kinetic constants were determined assuming a simple 1:1 binding model using the software provided by the biosensor manufacturer.[36]

For Cys-Ecoil-EGF protein grafting on sensorchips, two distinct approaches were performed for the sake of comparison, as described in Fig. 1:

(1) By coiled–coil interactions: 70 RUs of Cys-Ecoil-EGF or Ecoil-EGF protein were captured by injecting them at 1 nM (100 μl/min) over 47 RUs of covalently immobilized cysteine-tagged Kcoil peptides via coiled–coil interactions (the Kcoil surface had been generated according to our already published protocol[37]).
(2) By thiol coupling procedure: 425 RUs of Cys-Ecoil-EGF protein were covalently attached through the thiol group of their unique cysteine to the biosensor surface by adapting the thiol-based covalent coupling procedure we used for Kcoil grafting (Cys-Ecoil-EGF was diluted in 10 mM acetate buffer, *p*H 5 at around 1 μM for coupling).

Note that in between EGFRED-Fc injections, guanidium hydrochloride (5 M) or acetate buffer (*p*H 5) with 1 M of NaCl were injected for the regeneration of the surfaces on which Cys-Ecoil-EGF was covalently bound or coiled–coil captured, respectively.

E. *In vitro* cell assay to evaluate soluble Cys-Ecoil-EGF bioactivity

A-431 cells were distributed in 48-well plate (250 μl/well; 10^6 cell/well in DMEM supplemented with 10% serum). After an overnight incubation at 37 °C, the cell monolayer was washed with PBS, and the complete medium was replaced by serum-free DMEM. After 3 h of serum starvation, purified Cys-Ecoil-EGF was added to the wells at final concentrations ranging from 0 to 100 nM for precisely 5 min. Then, cells were washed twice with PBS supplemented with sodium orthovanadate and harvested using lysis buffer prepared as previously described.[36] Cell lysates were collected, centrifuged for 20 min at 10 000*g* and supernatants were kept at 4 °C for subsequent analysis of EGF receptor autophosphorylation by Western blot, as already described.[36]

F. Cys-Ecoil-EGF grafting on chondroitin sulfate-coated surfaces

Carboxylated CellBIND 96-well plates were modified with carbohydrazide using standard carbodiimide chemistry, then chondroitin sulfate was grafted according to our previously described protocol.[38] Protein grafting was then performed on the chondroitin sulfate coating by adapting our previous protocol.[38] That is:

(1) For oriented capture of Cys-Ecoil-EGF protein, cysteine-tagged Kcoil was grafted to CS-coated surface using a thiol coupling to PDPH linker. Then, Cys-Ecoil-EGF was captured via Kcoil/Ecoil interactions.
(2) For covalent coupling of the Cys-Ecoil-EGF, the same covalent binding protocol was used by replacing the cysteine-tagged Kcoil with the Cys-Ecoil-EGF protein.

In each case, Cys-Ecoil-EGF quantification was performed by direct ELISA, including a 1-h incubation of biotinylated anti-EGF antibody, followed by 20-min incubation of horseradish peroxidase–streptavidin conjugate, and finally 10-min incubation of the substrate solution. Substrate oxidation was monitored for 10 min by repetitious absorbance readings at 630 nm. Absorbance slopes (unit: 10^{-3} absorbance unit/s) were compiled and are referred to as arbitrary units in the manuscript.

G. VSMC survival in serum-free medium

Surfaces decorated with Cys-Ecoil-EGF were tested in a VSMC survival assay in serum-free medium. Cells were seeded at 20 000 cell/well in 96-well plates in complete medium and then incubated at 37 °C, 5% of CO_2 for 24 h. Cells were rinsed two times with PBS in order to eliminate non and/or weakly adhered cells; then the complete medium was replaced by serum-free medium (DMEM:F12 with 1% of pen-strep) and was freshly replaced every two days for 5 days of culture. Cell density at j_0 (i.e., 24 h of adhesion) and j_5 (at the end of culture) was evaluated using a metabolic activity assay (i.e., Resazurin) as previously described.[32] Experiments were conducted in triplicates.

III. RESULTS AND DISCUSSION

A. Cys-Ecoil-EGF production and purification

A pTT5 expression plasmid was used for the production of an epidermal growth factor chimera bearing three tags: a cysteine extension and an Ecoil—$(EVSALEK)_5$ sequence—to enable capture through covalent thiol coupling and noncovalent coiled–coil interactions, respectively, as well as a $(His)_8$ tag for purification. All tags were added at the N-terminus of the human EGF sequence [see Fig. 2(a)], in accordance with our previous study on the influence of tag nature and position on the biological activity of the construct.[36] More specifically, the cysteine residue was positioned near the Ecoil, so that both coupling strategies would provide a comparable cross-linker length.

The double-tagged chimera, namely, Cys-Ecoil-EGF, was produced by transient transfection of HEK293-6E cells, using polyethyleneimine for plasmid DNA condensation, and purified by IMAC. Cys-Ecoil-EGF production and purification were assessed by SDS-PAGE under reducing conditions [Fig. 2(b)]. Purified Ecoil-EGF was used as control and detected around 18 kDa, despite its expected 12-kDa

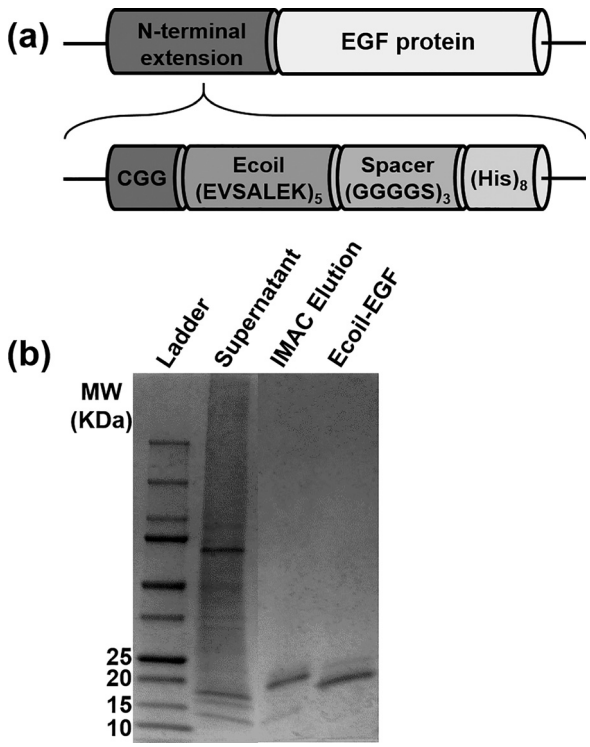

FIG. 2. Cys-Ecoil-EGF production and purification. (a) pTT5 expression plasmid for the production of the Cysteine-Ecoil-tagged hEGF. (b) SDS-PAGE analysis of Cys-Ecoil-EGF production and purification in reducing conditions after Coomassie Blue staining. Purified Ecoil-EGF was used as control.

molecular weight but in agreement with our previous reports.[36] The visible band in the cell culture supernatant indicated that the Cys-Ecoil-EGF chimera was well secreted in the medium, and analysis of IMAC elution indicated an efficient purification. The purified product was quantified by spectrophotometry and stored at −80 °C, then tested for biological activity *in vitro*.

B. Diffusible Cys-Ecoil-EGF bioactivity assay

A-431 cells overexpressing EGF receptors (EGFR) were amplified in multiwell-plates in DMEM with 10% FBS. When the cells were close to confluency, they were starved with serum-free medium for 3 h, then incubated for 5 min with concentrations of Cys-Ecoil-EGF ranging from 0.1 to 100 nM, or Ecoil-EGF as control. After cell lysis, EGFR phosphorylation levels were analyzed by Western blot using an antiphosphotyrosine antibody (Fig. 3). Receptor phosphorylation for the different concentrations of Cys-Ecoil-EGF was comparable to the levels observed for A-431 cells incubated with Ecoil-EGF, and in accordance with our previous report.[36] The absence of a visible band in the negative control (no growth factor) confirmed that the phosphorylation of the receptors we observed was indeed due to the biological activity of the EGF moiety of our construct. We already reported that the addition of the Ecoil tag at the N-terminus of EGF had no impact on its bioactivity in a diffusible state.[36] Here, we show that the further extension of the tag with a CGG sequence similarly did not induce any loss of biological activity either.

Altogether, a purified chimera of EGF was prepared with two additional moieties, that is, a free cysteine and an Ecoil tag while conserving the biological capabilities of the native growth factor in solution.

C. Immobilized Cys-Ecoil-EGF bioactivity assays

We then further assessed that both added moieties enabled the grafting of Cys-Ecoil-EGF on a substrate of interest, and evaluated whether the immobilized growth factor displayed biological activity. More specifically, we tested if the immobilization of EGF in an oriented and covalent fashion (via thiol bond formation) would be better suited than its oriented noncovalent capture (via coiled–coil interactions) for the generation of smart coatings to be applied, for instance, to stent-grafts or tissue engineered vascular grafts. Both grafting strategies were performed at the surface of an SPR biosensor as well as on cell culture wells on which our stent-graft coating made of chondroitin sulfate was recreated. On the one hand, the biosensing experiments allowed us to derive the apparent kinetic constants of binding between known quantities of immobilized EGF and its injected receptor ectodomain (EGFRED-Fc). On the other hand, EGF grafting in 96-well plates allowed assaying the ability of immobilized EGF to promote vascular smooth muscle cell survival in the absence of serum in an *in vitro* cell assay.

Results corresponding to SPR biosensing investigations are shown in Fig. 4 while related kinetic constants are reported in Table I. In a first set of experiments, our Cys-Ecoil-EGF construct was noncovalently captured on a biosensor surface where 47 RUs of the Kcoil peptide had been covalently immobilized; its binding to EGFRED-Fc was compared to that recorded when Ecoil-EGF chimera had

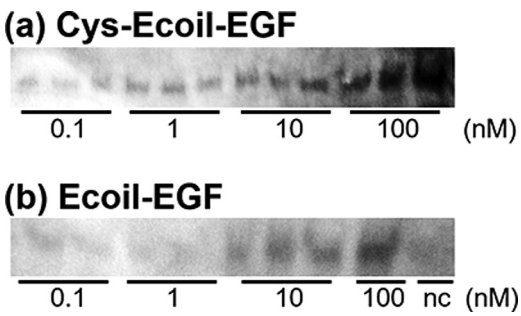

FIG. 3. Cys-Ecoil-EGF bioactivity in solution. Western blot analysis of EGF receptor phosphorylation in A431-cells, using an antiphosphotyrosine antibody, after a 5-min incubation with different concentrations of recombinant (a) Cys-Ecoil-EGF, or (b) Ecoil-EGF as positive control. Negative control with no growth factor is indicated by: nc.

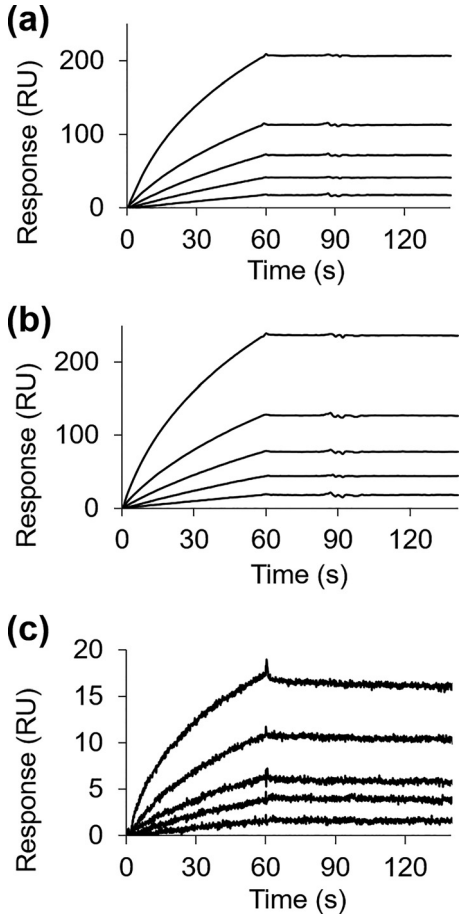

FIG. 4. Control-corrected sensorgrams corresponding to EGFRED-Fc injections over grafted (Cys-)Ecoil-EGF. EGFRED-Fc solutions of 2, 5, 10, 20, and 50 nM were injected over 70 RUs of (a) Ecoil-EGF or (b) Cys-Ecoil-EGF both captured at the biosensor surface by coiled–coil interactions, and (c) 425 RUs of Cys-Ecoil-EGF that had been covalently immobilized via thiol-based chemistry.

TABLE I. Kinetic and thermodynamic constants calculated for the EGFRED-Fc binding to the different surfaces displaying EGF moieties.

Grafting method	Protein	k_a (M^{-1} s^{-1})	k_d (s^{-1})	K_{Dapp} (pM)
Coiled–coil	Ecoil-EGF	$(5.49 \pm 0.06)\ 10^5$	$(3.4 \pm 0.8)\ 10^{-5}$	62 ± 15
Coiled–coil	Cys-Ecoil-EGF	$(5.19 \pm 0.04)\ 10^5$	$(2.4 \pm 0.7)\ 10^{-5}$	45 ± 15
Covalent bond	Cys-Ecoil-EGF	$(7.44 \pm 0.04)\ 10^5$	$(2.9 \pm 0.1)\ 10^{-4}$	388 ± 16

been captured via coiled–coil interactions. As shown in Figs. 4(a) and 4(b), when both chimeras were captured at similar levels (i.e., 70 RUs), injections of EGFRED-Fc at concentrations ranging from 2 to 50 nM resulted in sets of sensorgrams characterized by a similar amplitude (that is, around 200–250 RUs for the end of injection of the highest EGFRED-Fc concentration) and an almost null dissociation of the complexes upon buffer injection (thus a high stability of the ligand-receptor complex), as expected for a dimeric EGFRED-Fc (Ref. 36) rather than monomeric soluble EGFRED.[39]

The high similarity between these interactions was further confirmed when globally fitting both sets of sensorgrams. Indeed, calculated kinetic (see k_a and k_d in Table I), and thus apparent thermodynamic dissociation constants, were very similar ($K_{Dapp} = 45 \pm 15$ and 65 ± 15 pM for Cys-Ecoil-EGF and Ecoil-EGF, respectively, see Table I). Thus, these results confirmed that, within the chimera, the Cysteine tag does not significantly affect EGF bioactivity neither in solution (Fig. 3) nor when captured via coiled–coil interactions (Fig. 4 and Table I).

In stark contrast, when the Cys-Ecoil-EGF construct was immobilized to the sensor surface via thiol covalent coupling, a drastic reduction in the amplitude of the recorded signal was observed when the same EGFRED-Fc concentrations were injected [Fig. 4(c)]: a maximal amplitude of 17 RUs versus more than 235 RUs was observed, although 425 RUs vs 70 RUs of Cys-Ecoil-EGF had been grafted via thiol bond versus coiled–coil complex formation, respectively. These striking differences between both modes of grafting also affected the kinetic and thermodynamic constants of the interaction, as shown by an increase in k_d for the cysteine mediated immobilization of the Cys-Ecoil-EGF chimera [(29 vs 2.4) 10^{-5} s^{-1}, Table I]. It is important to note, however, that this lower apparent stability may result mainly from the low amount of EGF being available for binding. Indeed, even though a simple model of binding adequately depicted our SPR data, the apparent high stability of the EGFRED-Fc/EGF complexes is due to the dimeric nature of the injected EGFED-Fc. The dimeric EGFRED simultaneously binds to two EGF moieties, which creates an avidity situation that drastically affects the dissociation rate of the complex, as already observed with EGF binding to monomeric[39] versus dimeric[36] EGFRED. Thus, the density of bioavailable EGF directly affects the EGFRED-Fc ability to bind to two EGF moieties simultaneously, which in turn is reflected in both the amplitude of the signal (Fig. 4) and k_d value (Table I).

This SPR study was then complemented by investigating how cysteine- versus coiled–coil-mediated immobilization of EGF on chondroitin sulfate affected the levels of bioavailable EGF as well as its ability to promote VSMC survival. This was achieved by direct ELISA and in an *in vitro* cell assay, respectively. In that endeavor, chondroitin sulfate coatings were created in multiple-well plates as already performed by our team.[38] Thiol-based chemistry was then applied to covalently bind cysteine-tagged Kcoil or Cys-Ecoil-EGF via their unique cysteine on chondroitin sulfate coatings. For the direct immobilization of Cys-Ecoil-EGF via the formation of a covalent bond, EGF chimera concentrations from 1 nM to 1 μM were tested. In parallel, for coiled–coil-mediated tethering, wells were first incubated with 1 μM of Kcoil for peptide covalent coupling then incubated with 0.01–10 nM Cys-Ecoil-EGF. For both grafting strategies, the amounts of captured EGF were then estimated by a direct ELISA we had previously developed and validated.[38] As shown in Fig. 5(a), coiled–coil-mediated capture

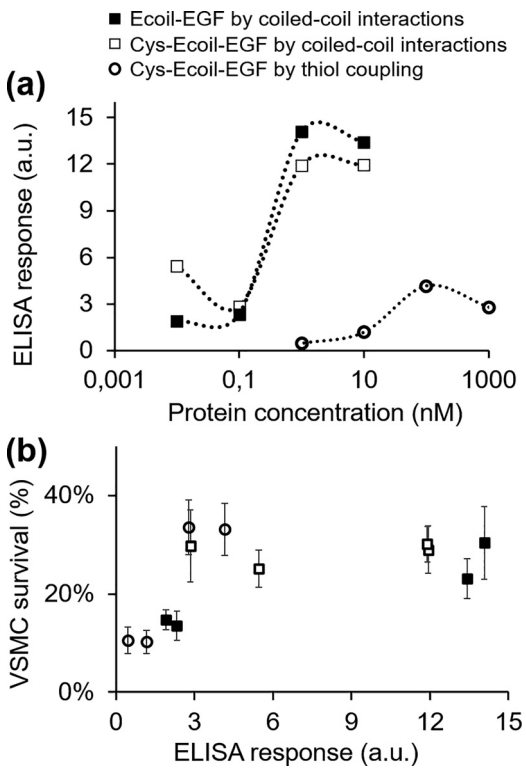

FIG. 5. Characterization of EGF grafting on chondroitin sulfate coating and its impact on VSMC survival in proapoptotic conditions. (a) Amounts of bioavailable grafted EGF were determined by direct ELISA for various concentrations of EGF chimera used during the grafting step (tethering via coiled–coil interactions or covalent coupling via thiol-based chemistry). (b) VSMC survival after 5 days in serum-free medium, relative to the cell number measured after adhesion. The data are plotted as a function of bioavailable EGF [as shown in panel (a)] for the three immobilization strategies.

was much more efficient than cysteine covalent coupling, since the incubation of Cys-Ecoil-EGF at 1 nM on Kcoil surface leads to the capture of 12 ELISA arbitrary units, whereas the best thiol coupling condition (i.e., 100 nM of Cys-Ecoil-EGF) led to only four ELISA arbitrary units. The same coatings were then evaluated in an *in vitro* cell assay. We previously demonstrated that a coating combining chondroitin sulfate and EGF promoted VSMC survival and resistance to apoptosis in serum-free conditions. The antiapoptotic effect was significantly improved on CS surfaces exposing oriented EGF (i.e., Ecoil-EGF tethered via coiled–coil interactions) compared to random EGF.[32]

The same assay was used here to test the effect of coiled–coil mediated versus covalently bound Cys-Ecoil-EGF. As shown in Fig. 5(b), increasing concentrations of incubated EGF increased VSMC survival for immobilized EGF quantities up to three ELISA arbitrary units. Cell survival then plateaued at a 30% value for higher quantities of grafted EGF. All eight conditions with an ELISA response higher than 3 A.U. were nonstatistically different, when compared to one another (independent two-sample t test, $p > 0.05$). Similarly, there was no statistical difference between the four conditions with an ELISA response lower than 3 A.U. ($p > 0.05$). However, when comparing one "low ELISA" condition with one "high ELISA" condition, we obtained, in all 32 cases, a statistically significant difference ($p < 0.05$). The VSMC survival was thus the same for equivalent quantities of bioavailable Cys-Ecoil-EGF, independently of the capture method, which is of salient interest.

Altogether, SPR measurements, direct ELISA and *in vitro* cell assay clearly demonstrate that the coiled–coil mediated tethering of Cys-Ecoil-EGF is a better strategy than its site-specific covalent immobilization for surface decoration. Indeed, capture resulting from low nanomolar solutions incubated on Kcoil surfaces gave similar outcomes for VSMC survival as surfaces resulting from covalent binding performed with 100-nM Cys-Ecoil-EGF concentrations [Fig. 5(b)].

The lower level of EGF we detected by ELISA after covalent immobilization can potentially be attributed to (1) a lower grafted amount, (2) EGF unfolding due to the covalent coupling procedure, and (3) a suboptimal display of the EGF moiety when thiol-bound versus coiled–coil-captured. However, the first two explanations are unlikely, for the following reasons. First, our SPR-based assays indicated that 425 RUs of covalently bound Cys-Ecoil-EGF resulted in less EGFRED-Fc accumulation than 70 RUs of the same protein captured via coiled–coil interactions (Fig. 4). Consequently, the differences in EGF levels we determined by ELISA are unlikely to be due to a lower EGF total amount within the coating. Second, the covalent coupling was performed at *p*H 5 (both in our SPR and *in vitro* cell assays). We have already demonstrated that a prolonged incubation of EGF at such a *p*H did not affect its ability to bind to its receptor.[26] Thus, the differences cannot be attributed to EGF unfolding during covalent coupling.

The correlation between ELISA level and VSMC survival, independent of the mode of grafting (Fig. 5), highly suggests that the EGF capture via coiled–coil interactions (noncovalent and oriented) enhances EGF display, making it more bioavailable (i.e., recognizable by ELISA antibodies as well as cell surface EGF receptors) than when bound by thiol coupling (covalent and oriented). Such an enhancement cannot be attributed to differences within the linker length, since the design of our capturing strategies results in the same theoretical distance between the surface and the growth factor (Fig. 1). Thus, it is more likely that the coiled–coil interaction itself is responsible for the observed differences in EGF bioavailability. Two main phenomena may explain this behavior: (1) coiled–coil formation buries the hydrophobic residues and neutralizes the negative charges of the Ecoil moiety, which may limit unwanted interactions between the Ecoil and the chains of chondroitin sulfate; (2) coiled–coil heterodimer is a more rigid secondary structure than Ecoil alone. The heterodimer formation may thus maintain EGF away from the surface, which would enhance its availability for signaling.

Altogether, the choice of an appropriate spacer/linker for biomolecule immobilization is crucial. No paramount rules may however be formulated in that endeavor. Although interesting work has been done on linkers for fusion protein design,[40] few reports include comparisons between different spacers for biomolecule immobilization. Spacer type and length were shown to be worth investigating for enzyme grafting on solid supports.[41,42] As for spacer flexibility, in good agreement with our results, Pallarola *et al.* found that rigid linkers facilitated the recognition of immobilized RGD, a short adhesion peptide, by their integrin biological partner.[43] A similar conclusion was drawn by Fiorilli and colleagues when performing dye coupling to surfaces using linkers harboring distinct flexibilities.[44]

IV. SUMMARY AND CONCLUSIONS

In order to improve our antiapoptotic chondroitin sulfate–EGF coating for endovascular implants, we have here investigated how the oriented covalent immobilization versus the oriented tethering (via coiled–coil interactions) of a chimeric EGF affected the performance of our coating. By combining an SPR-based biosensing approach, a direct ELISA, and an *in vitro* cell assay, we conclude that the coiled–coil oriented recruitment of EGF is superior to its covalent immobilization. This results from a better display of EGF rather than an increased total grafted amount.

ACKNOWLEDGMENTS

This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grant program, the Canada Research Chair on Protein-enhanced Biomaterials (G.D.C.), the Canadian Institutes of Health Research (CIHR) (CPG 127764), and the Natural Sciences and Engineering Research Council of Canada (NSERC) (PRCS 446571).

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