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Citation for published version (APA):

DOI:
10.1021/acsapm.9b00334

Document status and date:
Published: 09/08/2019

Document Version:
Publisher’s PDF, also known as Version of Record (includes final page, issue and volume numbers)

Please check the document version of this publication:
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A Supramolecular Platform for the Introduction of Fc-Fusion Bioactive Proteins on Biomaterial Surfaces

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Abstract: Bioorthogonal chemistry is an excellent method for functionalization of biomaterials with bioactive molecules, as it allows for decoupling of material processing and bioactivation. Here, we report on a modular system created by means of tetrazine/trans-cyclooctene (Tz/TCO) click chemistry undergoing an inverse electron demand Diels−Alder cycloaddition. A reactive supramolecular surface based on ureido-pyrimidinones (UPy) is generated via a UPy-Tz additive, in order to introduce a versatile TCO-protein G conjugate for immobilization of Fc-fusion proteins. As a model bioactive protein, we introduced Fc-Jagged1, a Notch ligand, to induce Notch signaling activity on the material. Interestingly, HEK293 FLN1 cells expressing the Notch1 receptor were repelled by films modified with TCO-protein G but adhered and spread on functionalized electrospun meshes. This indicates that the material processing method influences the biocompatibility of the postmodification. Notch signaling activity was upregulated 5.6-fold with respect to inactive controls on electrospun materials modified with TCO-protein G/Fc-Jagged1. Furthermore, downstream effects of Notch signaling were detected on the gene level in vascular smooth muscle cells expressing the Notch3 receptor. Taken together, our results demonstrate the successful use of a modular supramolecular system for the postprocessing modification of solid materials with functional proteins.

Keywords: supramolecular biomaterial, protein G, Jagged1, click-chemistry, supramolecular additive

Introduction: Biofunctionalization of biomaterials for application in medical devices often undergo treatments requiring organic solvents, a high temperature, pressure, or voltage (e.g., solution or melt electrospinning, 3D printing, injection molding). The decoration of biomaterials with functional proteins such as growth factors, cytokines, and signaling molecules can be poorly compatible with such processing techniques. Therefore, bioorthogonal conjugation chemistries have been implemented for the postprocessing modification of biomaterial surfaces. A family of reactions referred to as “click-chemistry” has gained popularity among material scientists because of its hallmark features of fast kinetics, high yields, easily removable harmless byproducts, high selectivity without a catalyst, and insensitivity to water or oxygen. Examples of applications of click-chemistry in biomedical sciences are reported using photo-activated thiocysteamine and oxime and hydrazones formation via reaction of aldehydes/
ketones with respectively alkoxyamines or hydrazides,\textsuperscript{8,9} strain promoted azide–alkyne cycloadditions (SPAAC),\textsuperscript{10–12} and inverse electron demand Diels–Alder cycloadditions (iEDDAC).\textsuperscript{13–17} The latter class comprises the reaction occurring between a tetrazine (Tz) and a strained \textit{trans}-cyclooctene (TCO). Reported rate constants for this reaction are as high as $3.3 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ (in H$_2$O at 25 °C), thereby making the Tz/TCO conjugation the fastest bioorthogonal reaction known so far.\textsuperscript{18,19} This class of reactions has been found to be very suitable for targeting biomolecules such as proteins and antibodies in complex environments. iEDDAC chemistry has been used for the labeling of antibodies with nanoparticles and radioactive probes for cell targeting,\textsuperscript{20,21} rapid \textit{in vivo} protein conjugation,\textsuperscript{22} or \textit{in vivo} fluorescent labeling of antibodies.\textsuperscript{23}

Recombinant protein G presents two immunoglobulin (IgG)-binding domains. The major binding site in IgG is located in the Fc domain of the antibody.\textsuperscript{24} Protein G binds strongly all human IgG subclasses with a $K_d \sim 2 \times 10^{-10}$ M,\textsuperscript{25} thereby finding several applications as an affinity matrix for purification and detection of antibodies. The Fc-binding properties of protein G have been exploited for the immobilization of Fc-fusion proteins,\textsuperscript{26,27} including Notch ligands Dll1, Dll4, and Jagged1.\textsuperscript{28–30} As a model Fc-fusion bioactive protein, we chose the Notch ligand Jagged1. Jagged1-mediated Notch signaling has been recently proposed as a target for cardiovascular regenerative medicine as the Notch signaling pathway is a powerful orchestrator of development, homeostasis, and wound healing of such tissues.\textsuperscript{31,32} Jagged1-functionalized materials based on poly(ethylene glycol) (PEG) have been proposed as artificial niches for stem cells, which find applications in myocardial regeneration\textsuperscript{33,34} and pancreatic islet cell immunoprotection\textsuperscript{35} and for elucidating cell-matrix interactions.

Scheme 1. Chemical Structures of the Molecules Used in This Study: Bifunctional Poly(\(\varepsilon\)-caprolactone)-Based Polymer (UPy-PCL), UPy-Functionalized Tetrazine Additive for Post-Modification (UPy-Tz), Product of the iEDDAC Reaction between UPy-Tz and TCO-Modified Protein G (TCO-OEG$_4$-Protein G, TCO-pG, Green); Concept of Step-Wise Modification of the Supramolecular UPy-PCL Surface with Fc-Fusion Protein: Formulation of Supramolecular UPy-PCL/UPy-Tz System (Top), TCO/Tz Reaction at the Surface (Middle), Site-Specific Binding of Fc-Fusion Protein on TCO-pG (Bottom)
interactions.36,37 Furthermore, Notch signaling has been found to have varied roles in cancer,18,39 with the Notch-Jagged signaling representing a target for studying the origin of metastasis.40

Recently, we combined the iEDDAC click reaction with the dynamic traits of a supramolecular material system based on ureido-pyrimidinone (UPy).41 Materials based on the strong, directional interactions between hydrogen-bonding UPy groups represent an excellent framework for ECM-mimicking biomaterials.32–44 These supramolecular systems exhibit both macromolecular properties of thermoplastic elastomers and the dynamic character that can be found in highly complex biological systems. In solid UPy-polymers, lateral stacking of UPy-dimers and flanking urea units leads to the formation of a nanoscale fibrous assembly.45 Cell-instructive additives (e.g., peptides or anti fouling molecules) can be robustly incorporated into the pristine material by means of matching supramolecular moieties to generate modular and easily processable materials for regenerative medicine applications.46–49 In a recent study, supramolecular UPy-poly(ε-caprolactone) (UPy-PCL) was modified with a UPy-conjugated Tz (UPy-Tz) additive.41 We were able to detect fluorescent TCO-modified enhanced yellow fluorescent protein (TCO-EYFP) immobilized on a three-dimensional fiber mesh fabricated with electrospinning, reporting the first evidence of successful decoupling of material processing and functionalization on a UPy-based modular system. The functionalization of UPy-PCL with reactive UPy-Tz additives allowed the introduction of anti fouling coatings on the material surface to reduce protein and cell adhesion.50 Nevertheless, a biological read-out that proves the efficacy of the post-modification approach in immobilizing proteins has not been provided yet. Here, for the first time, we report the activity in a biological context of a supramolecular UPy-based material loaded with a TCO-modified protein, namely, protein G (Scheme 1). Recombinant protein G is modified with TCO-moieties, and the resulting TCO-protein G variant (TCO-pG) is characterized to verify its structural integrity and cytotoxicity. UPy-PCL containing UPy-Tz is processed into cast films and electrospun fibers, and the biocompatibility, functionality, and bioactivity of the materials are investigated in comparison with materials created by random adsorption of unmodified recombinant protein G (pG).

**Materials and Methods**

**Materials.** UPy-PCL (Mw ≈ 2700 g/mol) and UPy-Tz (MW = 1190 g/mol) were synthesized by SyMO-Chem BV (Eindhoven, NL) as previously described.37 TCO-OEG-NHS ester (equatorial isomer) was obtained from Jena Bioscience (Jena, DE). 1,1,1,3,3,3-Hexafluoroisopropanol (HFIP) was obtained by FluoroChem (Hayfield, UK). Other chemicals were obtained from Sigma-Aldrich and used without further purification, unless otherwise specified.

**Protein G Conjugation and Analysis by LC-QTOF-MS.** Recombinant protein G (ThermoFisher, 21 444 g/mol; pG) in a 1:1 mixture of water/glycerol v/v was reacted with 10 equiv of TCO-OEG-NHS ester at 20 °C and 550 rpm for 1 h. The reaction was repeated three times, for a total of 30 equiv of TCO-OEG-NHS ester. Afterward, the mixture was purified using 10 000 MWCO Amicon filters by diluting the reaction mixture with phosphate buffered saline (PBS) solution at pH 7.4 and centrifuging at 13 400 rpm for 5 min. The conjugated protein remained in the filter and was obtained by inverted spinning and analyzed using a Waters Xevo G2 Quadrupole Time of Flight (QToF) Liquid Chromatograph–Mass Spectrometer equipped with an Agilent Polaris C18A reverse phase column (ID 2.0 mm, length 100 mm). Proteins were flowed (0.3 mL/min) over the column using a 15 vol % to 75 vol % water/acetonitrile gradient with 0.1 vol % formic acid prior to analysis in positive mode in the mass spectrometer. Deconvolution of the m/z spectra was done using the MaxENT1 algorithm in the MassLynx software.

**Preparation of Drop Cast Films.** For the preparation of drop cast films, 5 mol vol % of UPy-Tz was with the dry UPy-PCL from stock solutions in HFIP, and the amount of solvent was adjusted to reach a final UPy-PCL concentration of 50 mg/mL. Drop casting was performed by distributing 50 μL of solution on 14 mm Ø glass coverslip. Afterward, the films were dried overnight in a vacuum at 40 °C. For cell culture experiments, the films were sterilized with UV light for 10 min.

**Electrospinning.** Electrospinning was carried out on climate EC-CLI electrospinning equipment from IME Technologies (Waalre, NL). A 300 mg/mL solution of UPy-PCL with the addition of 5 mol vol % UPy-Tz was dissolved in an 8:2 mixture of CHCl₃/HFIP v/v overnight before loading in a syringe equipped with a 19G flat-tipped nozzle. The solution was fed at a constant flow rate of 55 μL/min with a voltage of 13 kV and a tip-collector distance of 15 cm. Electrospun fibers were collected on a rotating (100 rpm) cylindrical collector covered in aluminum foil. After electrospinning, the meshes were dried overnight in vacuum. For cell culture experiments, samples were sterilized with UV light for 10 min.

**Scanning Electron Microscopy (SEM).** Scanning electron microscopy (SEM) was performed using an FEI Quanta 600 and Xt Microscope Control software. Mesh samples were mounted on a metal stub by using double sided carbon tape. The samples were visualized under a low vacuum with an accelerating voltage of 10 kV and a working distance of 10 mm. The fiber diameters were determined from multiple high magnification images using ImageJ software.

**Circular Dichroism (CD).** The secondary structures of recombinant pG and TCO-pG were evaluated with CD spectroscopy using a JASCO J-815 spectrometer and a quartz cuvette with 1 cm path length. Spectra were collected between 200 and 300 nm at room temperature. Data are expressed as molar residual ellipticity (MRE), which is calculated from the measured ellipticity using the equation:

\[
\text{MRE} = \frac{\theta \times m}{c \times l \times n_i}
\]

where \(\theta\) is the ellipticity in millidegrees, \(m\) is the molecular weight in grams per mole, \(c\) is the protein concentration in milligrams per milliliter, \(l\) is the path length of the cuvette in centimeters, and \(n_i\) is the number of amino acids in the molecule.

**Water Contact Angle Measurements.** Water contact angle (WCA) measurements on drop cast films (n = 5) were performed on an OCA 30 system from Dataphysics using SCA20 software. A 5 μL drop of deionized water was placed on the films, and images were captured 10 s after placement of the water drop. Water contact angles were determined from the recorded images.

**Activation of Notch Signaling by Immobilized Ligands.** For induction of Notch signaling with immobilized extracellular domain of the Jagged1 ligand, UPy-PCL drop cast films or electrospun meshes containing 3 mg vol % UPy-Tz were incubated with a 50 μg/mL solution of TCO-pG in PBS at room temperature for 1 h. As a control for specifically adsorbed protein, substrates were incubated with a 50 μg/mL solution of unconjugated recombinant pG in PBS at room temperature for 1 h. After coating, substrates were washed three times with PBS and further blocked with 10 mg/mL of Bovine Serum Albumin (BSA) in PBS for 2 h. As recombinant protein G lacks albumin-binding domains, this blocking step prevents aspecific adsorption of Fc-ligands at a later stage to surfaces that are not covered with protein G (conjugates). The blocked surfaces were washed with PBS and incubated with recombinant Fc-Jagged1 chimera (R&D systems) or only Immunoglobulin G (IgG) and Fc fragments (Jackson ImmunoResearch) at concentrations of 1 μg/mL in BSA 1 mg/mL in PBS for 3 h. After washing, cells were immediately seeded on the coated surfaces.52 Inhibition was performed by the addition of 10 μM N-[N-(3,5-difluorophenace-
(S)-β-Alanyl-S-phenylglycine t-butyl ester (DAPT) to the culture medium from stock solutions in dimethyl sulfoxide (DMSO). For comparison, all other groups were treated with the same amount of DMSO.

Protein Quantification. The amount of pG or TCO-pG deposited on either cast films or electrospun meshes was quantified by QuantiPro micro-BCA assay (Sigma-Aldrich) by directly incubating the protein-functionalized materials with the kit’s working solution at 37 °C for 2 h, followed by absorbance measurements at 562 nm. The quantification of immobilized Fjagged1 on electrospun meshes (n = 3) was carried out using a similar procedure as described for protein G. The absolute absorbance of meshes coated with pG/BSA or TCO-pG/BSA was subtracted from the absorbance of samples bioactivated by incubation with an FcJagged1 solution at 1 μg/mL.

Cell Culture. Human embryonic kidney (HEK) 293T cells stably expressing full-length Notch1 (HEK293 FLN1) were cultured in complete medium consisting of Dulbecco’s Modified Eagle Medium (DMEM, Gibco) supplemented with 5 vol % heat inactivated fetal bovine serum (HI-FBS) (Invitrogen) and 1 vol % penicillin–streptomycin solution (Invitrogen), at 37 °C and 5 vol % CO2 in a humidified atmosphere. HEK293 FLN1 were cultured on tissue culture treated polystyrene under puromycin selection (1 μg/mL), passaged at 80 vol % confluence, and seeded at a concentration of 1.0 × 10^5 cells/cm² on drop cast films or electrospun meshes.

Primary human coronary artery smooth muscle cells (HCASMC, Lonza) were purchased and placed at passage 3, and experiments were carried out with cells at passage 5 or 6. Cells were cultured in 231 basal medium (Gibco) supplemented with 5 vol % smooth muscle growth supplement (SMGS, Gibco) and 1 vol % penicillin–streptomycin solution (Invitrogen), at 37 °C and 5 vol % CO2 in a humidified atmosphere on cell culture treated polystyrene. As a control for expression of α-smooth muscle actin in the differentiated state, the basal medium was supplemented with 1 vol % smooth muscle differentiation supplement (SMDS, Gibco) instead of SMGS. HCASMC was passaged at 80 vol % confluence and seeded at a concentration of 5.0 × 10^3 cells/cm².

Cytotoxicity, Cell Viability Assay. Cytotoxicity of different concentrations of TCO-pG in solution for the HEK293 FLN1 cell line was assessed using an LDH Cytotoxicity Assay kit (Pierce) following the manufacturer’s instructions. Cell viability was assessed

Figure 1. Characterization of products of conjugation of pG with TCO-OEG₄-NHS ester. Chromatogram (A) and deconvoluted mass spectrum (insert) of recombinant protein G used for conjugation reaction. Chromatogram (B) and deconvoluted mass spectrum (insert) of the 1 to 5 times TCO-conjugated product. (C) CD spectrum reporting mean residual ellipticity (MRE) of conjugated (solid line) and unconjugated (dashed line) protein G, (D) relative LDH activity of HEK293 FLN1 cells exposed to pG, TCO-OEG₄-NHS ester, and TCO-pG in solution for 24 h.
as metabolic activity on different materials using an XTT in vitro Toxicology Assay kit (Sigma-Aldrich) following the manufacturer’s instructions.

**Luciferase Reporter Assay.** Notch signaling activity in HEK293 FLN1 cells was measured by transfecting cells with a 12xCSL-Luciferase reporter construct\(^5\) (250 ng per 10\(^5\) cells) 1 day before seeding. Poly(ethylene imine) (PEI) was used as a transfection vector in a 2:1 ratio with the DNA construct. Luminescence intensity was detected after 48 h from seeding with a Luciferase Assay Kit (Promega) following the manufacturer’s instruction (\(n \geq 6\)). Luminescence intensity was normalized for cell amount, which was measured with the CyQuant Assay kit (ThermoFisher) following the manufacturer’s instruction.

**Gene Expression Analyses.** For gene expression analyses, HCASMC was cultured on the different substrates (\(n = 6\)) for 48 h. Total RNA was isolated using the RNeasy isolation kit (Qiagen). cDNA was synthesized with 150 ng of RNA per sample using the M-MLV Reverse Transcriptase enzyme/kit (Bio-Rad). cDNA samples were subjected to quantitative polymerase chain reaction (qPCR) using iQ SYBR Green Supermix (Bio-Rad) and the Bio-Rad IQ5 detection system (Version 1.6). mRNA expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a reference gene and expressed as a fold increase with respect to the control group. Primer sequences are available in the Supporting Information.

**Fluorescence Microscopy.** Cells were first washed with PBS, fixed in 3.7 vol % formaldehyde (Merck) for 10 min, washed with PBS, and permeabilized with 0.5 vol % Triton X 100 (Merck) for 15 min. Cells were then incubated with phallolidin-Atto488 (Sigma-Aldrich) in the dark for 45 min, followed by incubation with DAPI (0.1 \(\mu\)g/mL) in PBS for 5 min. Finally, samples were washed and mounted on cover glasses with Mowiol (Sigma). Samples were imaged with a Zeiss Axiovert 200 M epifluorescence microscope.

**Statistical Analyses.** Data are expressed as average ± standard deviation. Statistically significant differences were determined using a nonparametric Kruskall–Wallis test followed by Dunn’s posthoc test. Asterisks in the figures indicate significant differences as follows: *\(p < 0.05\), **\(p < 0.01\), and ***\(p < 0.001\). All statistical analyses were performed using GraphPad Prism 5 Software (GraphPad Software, Inc.).

### RESULTS AND DISCUSSION

**Synthesis and Characterization of Reactive TCO-OEG\(_4\)-Protein G Conjugate.** The protein G-mediated immobilization of Fc-tagged Jagged1 has been demonstrated to be beneficial compared to the random adsorption of the ligand on cell culture substrates.\(^5\)\(^4\) The difference is attributed to a better orientation of the Fc-ligand on protein G with respect to uncontrolled adsorption. Therefore, a Tz-reactive TCO-functionalized variant of protein G was obtained by reacting the recombinant protein with a TCO-OEG\(_4\)-NHS ester. The reaction conditions for TCO conjugation of recombinant protein G (Figure 1A) were optimized with the aim of minimizing unconjugated protein, in order to exclude the influence of randomly adsorbed protein on the following experiments. In a precedent study, EYFP was reacted with 3 equiv of TCO-OEG\(_4\)-NHS ester. The reaction conditions for TCO conjugation of recombinant protein G (Figure 1A) were optimized with the aim of minimizing unconjugated protein, in order to exclude the influence of randomly adsorbed protein on the following experiments. In a precedent study, EYFP was reacted with 3 equiv of TCO-OEG\(_4\)-NHS ester. The reaction conditions for TCO conjugation of recombinant protein G (Figure 1A) were optimized with the aim of minimizing unconjugated protein, in order to exclude the influence of randomly adsorbed protein on the following experiments. In a precedent study, EYFP was reacted with 3 equiv of TCO-OEG\(_4\)-NHS ester. The reaction conditions for TCO conjugation of recombinant protein G (Figure 1A) were optimized with the aim of minimizing unconjugated protein, in order to exclude the influence of randomly adsorbed protein on the following experiments. In a precedent study, EYFP was reacted with 3 equiv of TCO-OEG\(_4\)-NHS ester.
moieties was obtained, and no unconjugated protein could be detected anymore (Figure 1B).

The modified protein G was characterized by means of CD spectroscopy in order to compare signals coming from unmodified pG and TCO-pG. The shape of the two traces is found to be comparable, and it is consistent with the spectra reported in the literature (Figure 1C), indicating that the modification did not affect the folding state of the protein.\(^5\) In order to study the cytotoxicity of TCO-pG, a HEK293 FLN1 cell line expressing Notch1 receptors was chosen in view of the upcoming Notch signaling activity studies. TCO-pG was found to have no or low cytotoxicity for HEK293 FLN1 cells at a concentration up to 10 \(\mu\)M, with cell viability higher than 80 vol % (Figure 1D). Cell viability decreased to about 60 vol % when the TCO-pG reached concentrations of 100 \(\mu\)M, although it is unlikely that such an elevated concentration is found in the applied context.

**Cell Response to pG or TCO-pG Functionalized Films.**

Drop cast films of UPy-PCL with 5 mol vol % UPy-Tz were employed as a simple model to test cell response to materials in which protein G was incorporated either via adsorption (pG) or specific iEDDAc reaction (TCO-pG). First, the WCA of functionalized materials was measured to ascertain whether the short oligo(ethylene glycol) spacer present in TCO-pG might affect the material hydrophilicity and therefore cell behavior. The WCA decreased upon protein incorporation from 70° ± 1° for UPy-PCL/UPy-Tz to 67° ± 3° and 63° ± 4° for UPy-PCL/UPy-Tz with pG and TCO-pG, respectively (Figure 2A). The measured WCA values are consistent with previously reported values for comparable systems,\(^4\) and they are not likely to affect the cell response on bioactive films.

HEK293 FLN1 cells adhered on films with specifically adsorbed pG, while they were repelled by surfaces on which TCO-pG was reacted with the Tz moieties present at the surface (Figure 2B). As the viability of HEK293 FLN1 cells that did not adhere is not different from the viability of cells adhered on control UPy-PCL/UPy-Tz film or on randomly adsorbed protein G (Figure 2C), toxicity effects of surfaces modified with iEDDAc possibly being the cause of the unfavorable cell response toward materials functionalized with reactive TCO-pG can be excluded. After protein immobilization via either adsorption or “click” reaction, the films were functionalized with the Fc-Jagged1 ligand (or Fc fragment only as a negative control) to measure Notch signaling activity. Consistently with the observed cell response, cells stimulated on materials with adsorbed protein G/Fc-Jagged1 exhibited a significant 2.8-fold increase in Notch activity, while no significant effect was detected on materials with TCO-pG/Fc-Jagged1 (Figure 2D).

To discriminate between the effects of the iEDDAc reaction and the TCO-modified protein G on cell adhesion, we observed the behavior of HEK293 FLN1 cells and CASMC, a good model for cardiovascular applications, on a film of UPy-PCL without any UPy-Tz that have been incubated for 1 h with either pG or TCO-pG. For both cell types, the response in terms of adhesion and morphology is consistent with the one observed in the presence of UPy-Tz. On UPy-PCL and UPy-PCL/pG films, the cells formed healthy confluent monolayers, while they formed few clustered aggregates on UPy-PCL with adsorbed TCO-pG (Figure S1). In light of this, it is concluded that the negative cell response to TCO-pG, either reacted or adsorbed, might be due to conformational changes of the protein in contact with the relatively hydrophobic material, which might lead to the exposure of cell-repellent portions of pG. Although the CD spectroscopy characterization did not reveal structural changes of TCO-pG in solution with respect to pG (Figure 1C), we cannot exclude that the TCO-modification could have affected the stability of the protein’s folding state when in the proximity of the material. Although the CD spectroscopy characterization in solution did not reveal structural changes of TCO-pG with respect to pG (Figure 1C), this measurement is performed in solution only, and it is not indicative of the interaction of the protein with the material surface during adsorption. We cannot exclude that the stability of the protein’s folding state in the proximity of the solid material surface is compromised because of the presence of TCO moieties. WCA measurements were performed to get insights on the material surface characteristics after functionalization. The introduction of TCO-pG on the material’s surface decreases the average WCA of 7° ± 4° only (Figure 2A), and this value is almost identical to the WCA decrease caused by pG adsorption, which instead supports cell-adhesion. Therefore, we are unable to make a connection between surface hydrophilicity and cell-repulsion based on the available data.

**Cell Response on Functionalized Electrospun Meshes.** The proposed material modification was translated from a two-dimensional film to a porous fibrous mesh prepared with electrospinning, as this is a more relevant structure for applications such as scaffolds for soft tissue engineering. A
solution of UPy-PCL with 5 mol vol % UPy-Tz was electrospun into meshes with an average fiber diameter of 2.3 ± 0.7 μm (Figure 3A). The reaction of TCO-pG and coating of the unmodified pG were carried out as described for the cast film. Quantification of the amount of protein G deposited on the electrospun meshes revealed that the iEDDAC reaction of TCO-pG on the material surface leads to a 7.2-fold increase in the amount of protein presented on the scaffold with respect to the amount of pG present after a specific adsorption (Figure 3B). Therefore, it can be concluded that the immobilization of pG via TCO-Tz chemistry allows loading of the pristine material with a wider range of bioactive protein concentrations than mere adsorption. Protein quantification was attempted for cast films as well, but the amount of protein G turned out to be below the detection limit of the assay (data not shown). The difference can likely be attributed to the large surface area that characterizes porous electrospun meshes compared to two-dimensional films. A higher amount of immobilized protein G is hypothesized to cause a higher amount of immobilized Fc-fusion protein. To test this hypothesis, the amount of FcJagged1 bound to electrospun scaffolds functionalized with either pG or TCO-pG was quantified as well. The results show an average 9.6-fold increase in FcJagged1 loading (Figure S2). Although the increase is not statistically significant due to the large spread in the values, the data suggest that the iEDDAC-mediated functionalization might yield a higher Fc-fusion protein loading on the scaffold.

The HEK293 FLN1 cells’ response notably improved on the electrospun substrates with respect to cast films. Cells were able to adhere on UPy-PCL/UPy-Tz meshes with protein G either adsorbed or immobilized via specific iEDDAC reaction, although they showed slight clustering behavior on the latter (Figure 4A), indicating that the cells still prefer cell−cell contact instead of cell-material contact. Nevertheless, cell metabolic activity on the protein-loaded material was found to be similar to that on the pristine UPy-PCL/UPy-Tz (Figure 4B), indicating that HEK293 FLN1 cells do not suffer cytotoxic effects of the protein-modified material. After introduction of the bioactive Fc-Jagged1 ligand on the material, activation of Notch signaling in HEK293 FLN1 cells was detected on both substrates. The immobilized Fc-Jagged1 ligand elicited a 2.9-fold and 5.7-fold increase in

Figure 4. (A) Fluorescence micrographs of HEK293 FLN1 cells cultured on UPy-PCL/UPy-Tz electrospun meshes coated with pG (left) or reacted with TCO-pG (right). F-actin (green), nuclei (blue). Scale bar is 50 μm. (B) Metabolic activity of HEK293 FLN1 cells cultured on electrospun UPy-PCL/UPy-Tz with adsorbed pG or reacted TCO-pG. (C) Notch signaling activity of HEK293 FLN1 cells cultured on UPy-PCL/UPy-Tz electrospun meshes coated with pG or reacted with TCO-pG. Data are normalized to the negative control (Fc-fragment only). Inhibition is carried out with 10 μM DAPT; all the other conditions are treated with the same amount of DMSO.
The mechanism of action of the Notch signaling pathway involves a number of inter- and intracellular processes that occur after ligand–receptor binding, such as endocytosis, glycosylation, and feedback regulation of ligand and receptor levels. These processes are essential for the translation of the signal into a functional response of the cell; therefore we found it relevant to investigate whether the complexity of the biological process could be recapitulated on the bioactive material, besides the ligand–receptor binding phenomena demonstrated in Figure 4C. Primary human vascular smooth muscle cells endogenously expressing the Notch3 receptor were cultured on UPy-PCL/UPy-Tz electrospun meshes modified with TCO-pG/Fc-Jagged1 to track the expression of genes regulating transcription downstream of NICD translocation to the nucleus, namely, HEY1 and HES1, and genes related to the regulation of ligand and receptor levels, namely, JAG1 and NOTCH3. Gene expression analyses reveal upregulation of expression levels of all the mentioned genes with respect to control samples presenting the TCO-pG/Fc fragment only. Consistently, the addition of DAPT, a γ-secretase inhibitor, decreases expression levels for all the investigated genes (Figure 5). Interestingly, the expression intensity of all the investigated genes on TCO-pG/Fc-Jagged1 functionalized substrates is higher than on meshes coated with pG/Fc-Jagged1. This might be due to a higher amount of Fc-Jagged1 immobilized on the material surface, as it is shown in Figure 3B that performing the iEDDAC reaction allowed the immobilization of more protein G with respect to random absorption.

Figure 5. Relative expression of Notch target genes (HEY1, HES1) and lateral positive feedback target genes (JAG1, NOTCH3) on vascular smooth muscle cells cultured on electrospun UPy-PCL/UPy-Tz, coated with pG (right group) or reacted with TCO-pG (left group). Substrates were functionalized with Fc-Jagged1 and used without (black bars) and with (white bars) inhibition with DAPT. Data are normalized to the scaffold functionalized with the Fc fragment only (dashed line; dotted lines represent the standard error of the control).

In this study, the ability of TCO-pG to bind and orient Fc-fusion ligands was tested by performing bioactivation of the surface with FcJagged1, a Notch ligand. The results in Figure 3B and Figure S2 show that the reactive immobilization results in a 7.2-fold higher loading of TCO-pG, and a (not significant) 9.6-fold increase in FcJagged1 binding. Nonetheless, no statistical difference in Notch signaling intensity was detected between chemical immobilization and physical adsorption (Figures 4C and 5), even though it is known that Notch signaling activity increases linearly with the concentration of surface-bound FcJagged1 in this cell model. One explanation for this might be that the covalently bound TCO-pG does not provide the correct orientation of the Fc-fusion ligand. Improvements on this aspect could be done by developing site-directed protein modification chemistry, thereby providing control on the targeted residues. Regardless, the iEDDAC-mediated protein immobilization offers the advantage of a covalent anchoring of the protein to the material, thus providing stable functionalization in complex environments such as body fluids. Here, a physically adsorbed protein is susceptible to displacement from the surface by other proteins with larger affinity for the material surface.

Taken together, our results represent the first evidence of successful postprocessing bioactivation of a supramolecular UPy-based solid material by means of click chemistry for the immobilization of Fc-fusion ligands. A large number of cytokines, enzymes, and receptor extra-cellular domains are commercially available as Fc-fusion proteins due to advances in the techniques for their large scale production. Furthermore, up to date, nine Fc-fusion proteins have been approved as drugs by the FDA, and a large number are currently in clinical trials since the Fc domain is often used to prolong the half-life of their conjugate counterpart in vivo or enhance their therapeutic efficacy. Besides Fc-fusion proteins, the system described in this work can be potentially used for binding antibodies, which are frequently used in pharmacology to target receptors and cellular processes with high specificity.

In light of this, it is expected that the development of
substrates postmodified to incorporate Fc-tagged protein and antibodies will find broad applicability in therapeutic strategies that require processed biomaterials.

**CONCLUSIONS**

A versatile and modular platform for introduction of bioactive Fc-fusion proteins on supramolecular solid materials based on UPy was developed via immobilization on Fc-binding protein G. TCO modification of recombinant protein G allowed functionalization of UPy-PCL/UPy-Tz surfaces via a fast iEDDAC reaction. The introduction of TCO-pG on cast or electrospun surfaces turned out to affect the biocompatibility of the material in a processing-dependent manner. Films of UPy-PCL/UPy-Tz functionalized with TCO-pG turned out to be cell-repellent, while mere adsorption of protein G did not compromise cell adhesion. Instead, UPy-PCL/UPy-Tz fibers created by electrospinning supported cell adhesion, viability, and functionality. Bioactivation of supramolecular substrates was carried out by incorporation of Fc-Jagged1, a Notch ligand of interest in tissue regeneration strategies, on the Fc-binding material surface. The Fc-fusion ligand retained its functionality on TCO-pG modified electrospun materials, indicating that complex cellular processes such as Notch signaling can be recapitulated on the modified material in cell models expressing Notch1 and Notch3 receptors. In conclusion, a convenient strategy based on bioorthogonal click-chemistry is found to be suitable to modify supramolecular material surfaces. We envision that the system described in this work potentially has broad applications in the display of bioactive proteins, such as Fc-fusion proteins and therapeutic antibodies, onto processed solid materials.

**ASSOCIATED CONTENT**

 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsapm.9b00334.

List of primers used for gene expression; fluorescence micrographs of HEK293 FLN1 and CASMC on UPy-PCL cast films with adsorbed pG or TCO-pG; quantification of FcJagged1 immobilization on pG or TCO-pG functionalized electrospun meshes (PDF)

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Notes

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

Carlijn Bouten is acknowledged for valuable discussions and Maaike Schotman for assistance with CD measurements. The ICMS Animation Studio is acknowledged for the design of some of the cartoons. This work was funded by the European Research Council (FP7/2007-2013) Grant Agreement 604514 and ERC Grant Agreement 308045 and the Ministry of Education, Culture, and Science (Gravity Program 024.001.03).

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