Multifunctional poly(2-ethyl-2-oxazoline) copolymers containing dithiolane and pentafluorophenyl esters as effective reactive linkers for gold surface coatings

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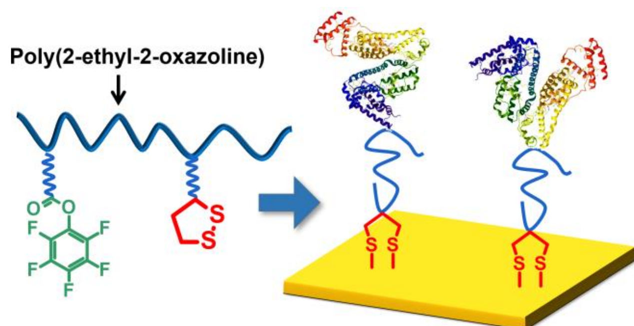
Abstract

Surface functionalization with biological macromolecules is an important task for the development of sensor materials, whereby the interaction with other biological material should be suppressed. In this work, we developed a novel multifunctional poly(2-ethyl-2-oxazoline)-dithiolane conjugate as a versatile linker for gold surface immobilization of amine-containing biomolecules, containing poly(2-ethyl-2-oxazoline) as antifouling polymer, dithiolane for surface immobilization and activated esters for protein conjugation. First, a well-defined carboxylic acid containing copoly(2-ethyl-2-oxazoline) was synthesized by cationic ring-opening copolymerization of 2-ethyl-2-oxazoline with a methyl ester-containing 2-oxazoline monomer, followed by post-polymerization modifications. The side-chain carboxylic groups were then converted to amine-reactive pentafluorophenyl (PFP) ester groups. Part of the PFP groups was used for the attachment of dithiolane moiety, which can efficiently bind to gold surfaces. The final copolymer contained 1.4 mol% of dithiolane groups and 4.5 mol% of PFP groups. The copolymer structure was confirmed by several analytical techniques, including NMR spectroscopy and size-exclusion chromatography. The kinetics of the PFP ester aminolysis and hydrolysis demonstrated significantly faster amidation compared to hydrolysis, essential for subsequent protein conjugation. Successful coating of gold surfaces with the polymer was confirmed by spectroscopic ellipsometry, showing a polymer brush

thickness of 4.77 nm. Subsequent modification of the coated surfaces was achieved with bovine serum albumin as a model protein. This study introduces a novel reactive polymer linker for gold surface functionalization and offers a versatile polymer platform for various applications, including biosensing and surface functionalization.

TOC graphic



1. Introduction

In recent decades, the functionalization of surfaces with biologically active molecules emerged as an important research area, fueling advancements in fields such as material sciences and bioengineering.[1, 2] The attachment of biomolecules to surfaces through multifunctional polymer linkers received significant attention due to its potential to create tailor-made coatings with enhanced biocompatibility, bioactivity, and stability.[3-5] This approach enables the precise modulation of surface properties and creates new paths toward the development of advanced biomedical devices such as biosensors.[6, 7]

Immobilization of proteins (e.g., antibodies) to surfaces coated by biologically inert polymers represents an appealing strategy in biosensor development, as the polymer can serve as both a reactive linker for protein attachment as well as to protect the surface from undesirable interactions with biological matter.[8, 9] In general, two approaches can be used to attach proteins on surfaces using polymer linkers. The coating of surfaces with pre-formed polymer-protein conjugates offers a higher level of control over the chemical structure of the polymer-protein conjugates.[10] On the other hand, this method leads often to relatively low coating efficiency due to the bulkiness of the

conjugates. This drawback can be generally resolved using the second immobilization method, which relies on the functionalization of surfaces with non-fouling polymers containing reactive handles followed by covalent attachment of proteins.[11]

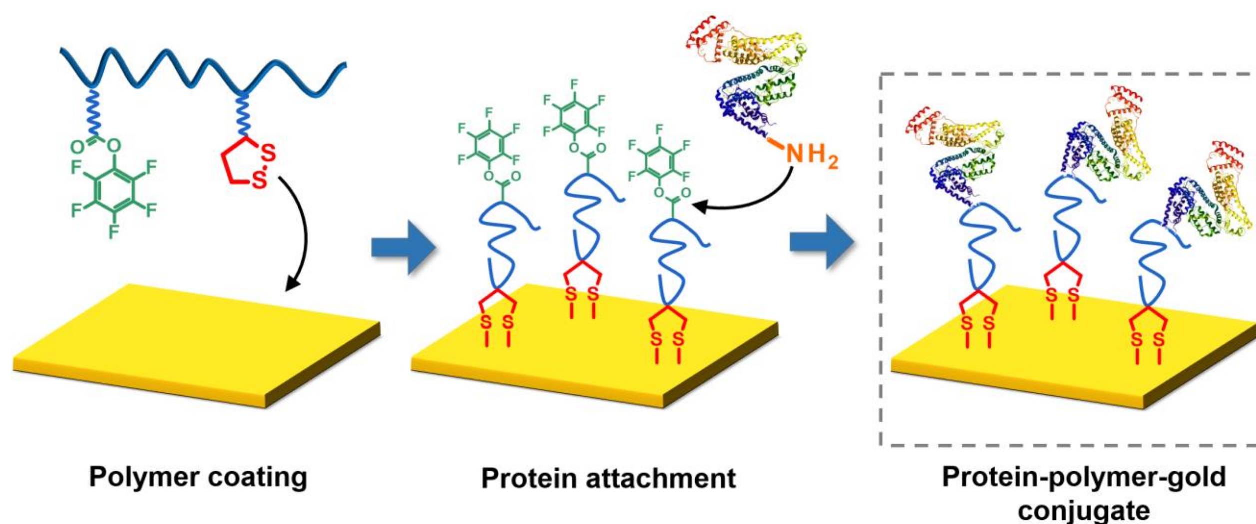
Several bioconjugation reactions have been developed for protein conjugation,[12] including primary amine amidations,[13-15] thiol-maleimide reactions,[16] or more selective "click" reactions.[17] The attachment of proteins to surfaces via their lysine-originated primary amines represents by far the most common polymer conjugation technique, known for its versatility and robustness.[18] On the other hand, a certain loss of protein activity is often observed due to the possible active site blockage.[19] For amine conjugation, polymers containing carboxylic acid groups are generally used, which are activated as *N*-hydroxysuccinimide ester (NHS)[20] or pentafluorophenyl ester (PFP).[21] Compared to the frequently used NHS-conjugation strategy, PFP-based conjugations offer superior hydrolytic stability that leads to higher conjugation yields in aqueous media.[22] Several anti-fouling polymers have been used for protein-surface immobilization,[23] such as polyethylene glycol (PEG),[24] poly(*N*-(2-hydroxypropyl)methacrylamide) (PHPMA)[25] or poly(oligoethylene glycol acrylate) (POEGA).[26]

Poly(2-oxazoline)s (PAOx) represent an emerging class of polymers that are particularly suitable for surface immobilization of proteins due to their biocompatibility,[27, 28] synthetic versatility,[29, 30] and flexibility.[31] In particular, coating of surfaces with water-soluble poly(2-methyl-2-oxazoline) (PMeOx) and poly(2-ethyl-2-oxazoline) (PEtOx) leads to biocompatible surfaces with very low fouling from blood plasma proteins, outperforming analogous surfaces coated with PEG.[32, 33] Furthermore, the synthetic versatility of PAOx paves the way to well-defined multifunctional polymers with orthogonal functional groups at both chain ends as well as in side chains.[34, 35] In this sense, PEtOx with amine-reactive PFP-ester side chains were recently synthesized and readily modified by several low-molar mass amines.[36]

Despite the potential of PAOx, reports on the application of PAOx linkers for protein-surface attachment are sparse. As an example, Jordan and co-workers employed PEtOx bottlebrush polymers to attach the green fluorescent protein to a nanopatterned diamond surface as a step toward the development of diamond-based nanosensors.[37] In another report, Krause et al used peptide crosslinked PEtOx hydrogels as a sensor material for the detection of proteases with a

quartz crystal microbalance.[38] Besides these studies on relatively complicated systems, there are, to the best of our knowledge, no reports on protein conjugation to surfaces via the PAOx platform.

In this work, we describe for the first time the synthesis of amine-reactive gold surfaces based on biocompatible PEtOx brushes (**Scheme 1**). First, the multifunctional copolymer linker was synthesized, containing a 1,2-dithiolane side chain group for gold surface attachment as well as a pentafluorophenyl ester side chain group for protein attachment. The copolymer structure was confirmed by ^{1}H NMR spectroscopy and size-exclusion chromatography (SEC). The copolymer was then coated onto a gold surface, forming a polymer brush monolayer, as confirmed by spectroscopic ellipsometry and X-ray photoelectron spectroscopy (XPS). Finally, the polymer-coated surface was modified with bovine serum albumin (BSA) as a model protein demonstrating the potential of this novel polymer linker. In the future, this strategy can be employed for the construction of advanced biosensors.

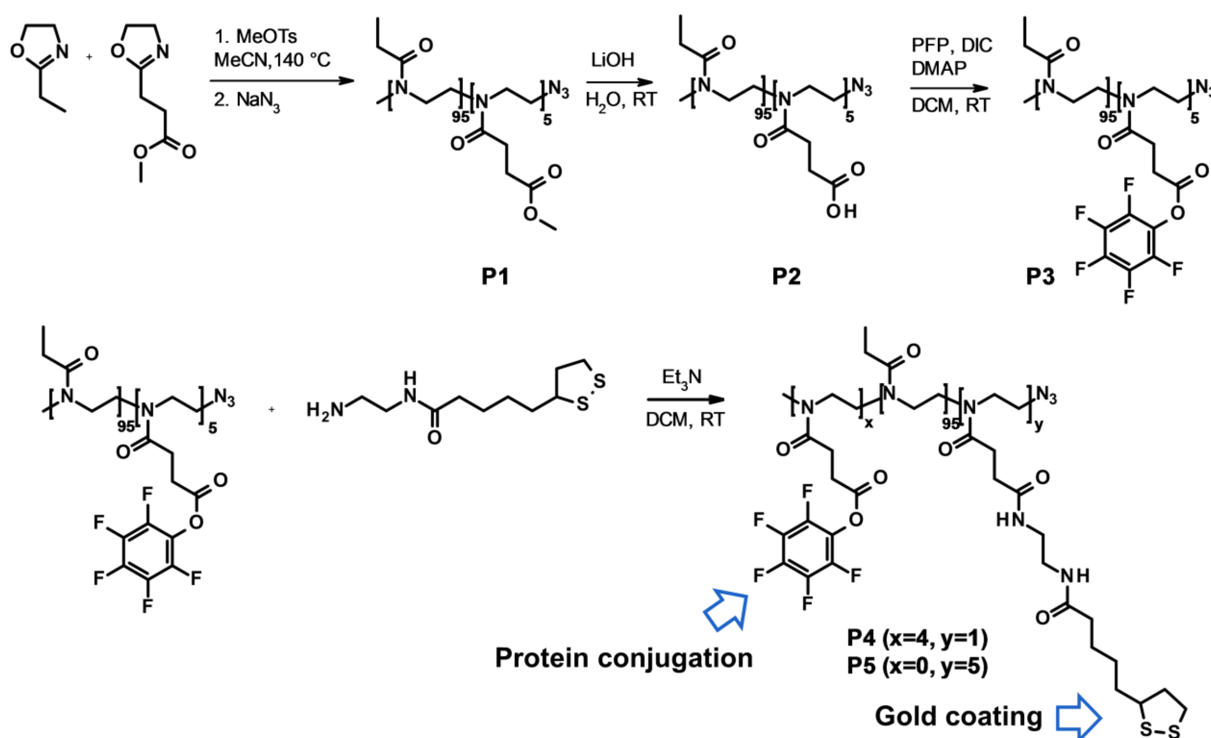


Scheme 1. Cartoon illustrating the coating process, showing attachment of biocompatible polymer linker to the gold surface via dithiolane reactive groups followed by immobilization of proteins via amide bonds.

Results and Discussion

The general approaches used in the synthesis of polymer conjugates are depicted in **Scheme 2**. In the first step, the methyl ester groups containing poly(2-ethyl-2-oxazoline) (PEtOx) copolymers

were prepared by cationic ring-opening copolymerization (CROP) of 2-ethyl-2-oxazoline (EtOx) with 2-methoxycarboxypropyl-2-oxazoline (MestOx) using methyl *p*-toluenesulfonate (MeOTs) as the initiator. The polymerization was terminated with sodium azide, introducing an azide end group for further functionalization. In this step, a high degree of chain-end functionality was desired, as the incomplete azide termination would lead to the introduction of terminal hydroxyl groups (by termination with adventitious water), causing polymer crosslinking in the later esterification step.[36] The prepared copolymer **P1** contained 6 mol% of MestOx units and was well-defined, with low dispersity (*Đ)* of 1.07 and M_n ~ 10 kDa. The methyl ester groups were then hydrolyzed to free carboxylic acids using lithium hydroxide to obtain copolymer **P2** as we previously developed as an easy method.[39] The success of the hydrolysis was confirmed by ^{1}H NMR spectroscopy from the disappearance of the methyl ester singlet peak at δ = 3.7 ppm. The free carboxylic acids were further used to introduce amine-reactive PFP ester groups through Steglich esterification of pentafluorophenol, using DIC as the coupling agent and DMAP as the cocatalyst as we recently reported.[36] The obtained polymer conjugate **P3** was well defined with a *Đ* of 1.08.



Scheme 2. Schematic representation of the synthesis of amine-reactive poly(2-ethyl-2-oxazoline)-dithiolane polymer conjugates.

Table 1. Characteristics of the synthesized copolymers

Polymer	M_n (kDa)[a]	$Đ$[a]	Dithiolane content (mol. %)[b]	PFP content (mol. %)[b]
P1	10.4	1.07	-	-
P2	10.1	1.06	-	-
P3	10.9	1.08	0	5.9
P4	11.6	1.12	1.4	4.5
P5	13.1	1.17	5.9	0

[a]Determined by SEC in DMAc [b] Determined by ^{1}H-NMR spectroscopy.

In the next step, the polymer was decorated with the gold-binding dithiolane moieties. As the commercial dithiolane-containing lipoic acid was not suitable for polymer modification, it was first modified with ethylenediamine, using carbonyldiimidazole (CDI) as an amide bond coupling agent.[40] The obtained *N*-lipoyl(ethylenediamine) was used directly for reaction with **P3**, yielding PEtOx-dithiolane functional polymers. The chemical composition of the copolymers can then be easily adjusted by selecting the desired PFP/dithiolane ratio. Using this method, we prepared a copolymer containing both PFP and dithiolane groups (**P4**), which was further employed as a hydrophilic linker for the polymer-protein conjugation. Furthermore, using an excess of *N*-lipoyl(ethylenediamine) resulted in the full amidation of the PFP groups, leading to conjugate **P5** that only has lipoyl side chains and does not have remaining PFP groups. The size exclusion chromatography of both PEtOx-dithiolane conjugates revealed minor signs of polymer coupling (presumably due to the slight contamination of *N*-lipoyl(ethylenediamine) with unreacted ethylenediamine); however, their dispersity remained reasonably low ($Đ < 1.2$, **Table 1**). The structure of the copolymers was confirmed by ^{1}H NMR spectroscopy (**Figure 1**) by the presence of peaks originating from alpha-methylene protons adjacent to the PFP group ($\delta = 3.05$ ppm) and

peaks of the lipoic moiety (δ = 2.2 ppm and δ = 3.3 ppm). As the targeted DP of copolymers is 100, there are on average 1.4 dithiolane groups per P4 chain (**Table 1**).

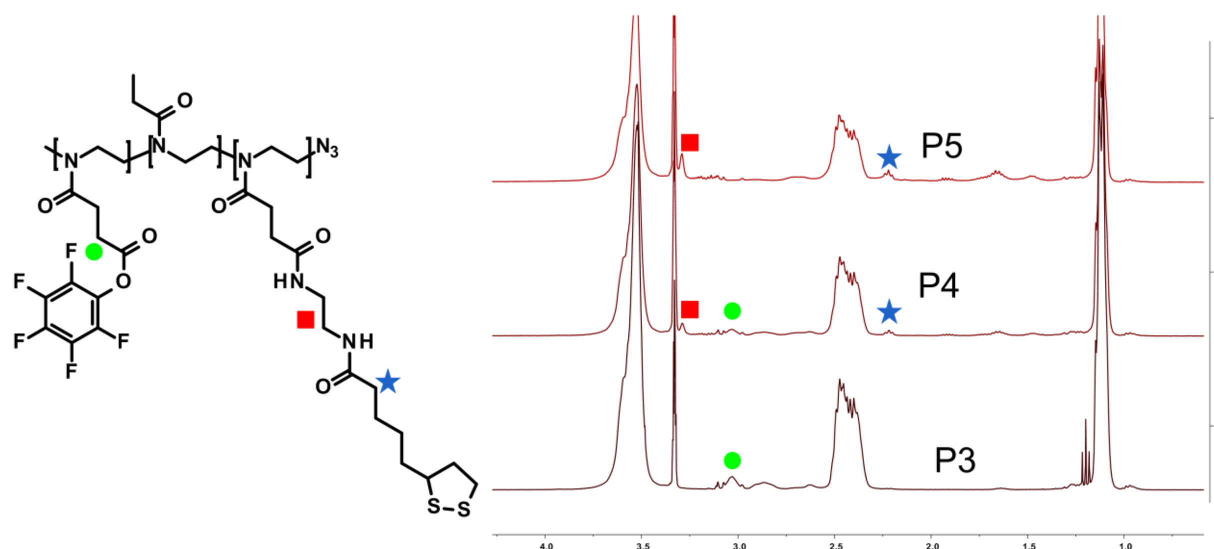


Figure 1. ^{1}H NMR spectra of copolymers **P3-P5** in CD_3OD.

To be suitable as a robust linker for the gold-binding of proteins, the reaction of the PFP side chains with a protein amine should be as fast as possible. On the other hand, the reactive groups should be relatively stable towards hydrolysis, as this hampers the conjugation yield and leads to carboxylic acid side chains that will be negatively charged in physiological conditions. The fast hydrolysis is the main drawback of the most commonly used NHS ester compounds.[41] The aminolysis and hydrolysis kinetics of polymer **P4** were investigated in PBS by UV-VIS spectroscopy, as both reactions generated yellow pentafluorophenolate ions with an absorption maximum of 267 nm (**Figure 2**). The ethanolamine conjugation reaction occurred fast and reached complete conversion within 5 min. However, the hydrolysis of **P4** in pure PBS was much slower, with a half-life of 120 minutes and 95 % conversion after 600 min. This reactivity difference provides a sufficient timeframe for selective surface functionalization reactions with minimal hydrolysis.

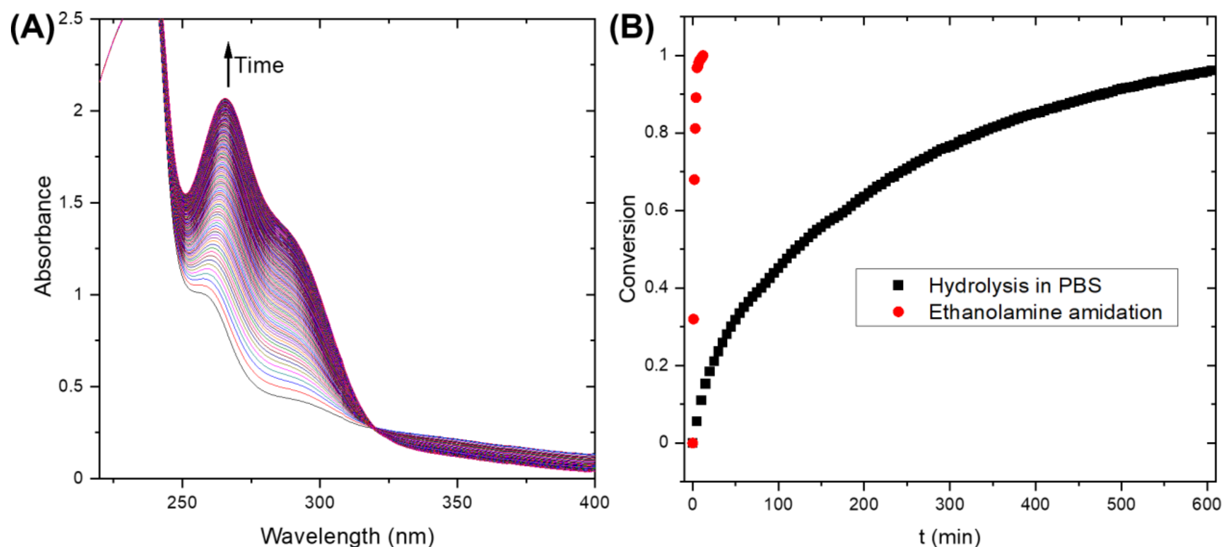


Figure 2. Hydrolysis of **P4** (c = 1 mg mL^{-1}) in PBS buffer (pH = 7.4) at 25 °C as monitored by UV-VIS spectrometry (A). Kinetics of **P4** hydrolysis and amidation with ethanolamine (B).

The copolymer **P4** was used for coating gold surfaces by the drop-drying technique[42] to obtain polymer brush **P4@Au**. The polymer was dissolved in acetonitrile, and transferred on a gold surface, and the solvent was allowed to evaporate at room temperature. This step was followed by thorough washing and drying. The remaining polymer surface thickness was measured by spectroscopic ellipsometry revealing a thickness of 4.77 nm. This thickness corresponds to a grafting density of 0.3 chains/nm, suggesting the brush architecture of the copolymers in the coated surface. This polymer brush thickness should be sufficient to possibly reduce the fouling from blood plasma but thin enough to ensure the interactions between the gold surface and attached proteins in the envisaged biosensing applications. Furthermore, the successful coating was confirmed by XPS. The C_{1s} high-resolution spectrum revealed the typical bands for C-N bonds, as well as amide bonds (**Figure 3**). The peaks corresponding to the C-F, C-S, and O-C=O bonds were not observed in the C_{1s} spectrum as they correspond to only less represented PFP, respectively dithiolane moieties in the polymer. The elemental composition of the surface calculated from the XPS survey spectrum, represented by values of O/C and N/C ratios, matched that of the calculated composition of the bulk **P4** copolymer. Furthermore, the XPS analysis proved the presence of F atoms in the P4-coated surface. The XPS-based fluorine content was 0.42 %, suggesting that part of the PFP groups was "hidden" closer to the gold surface.

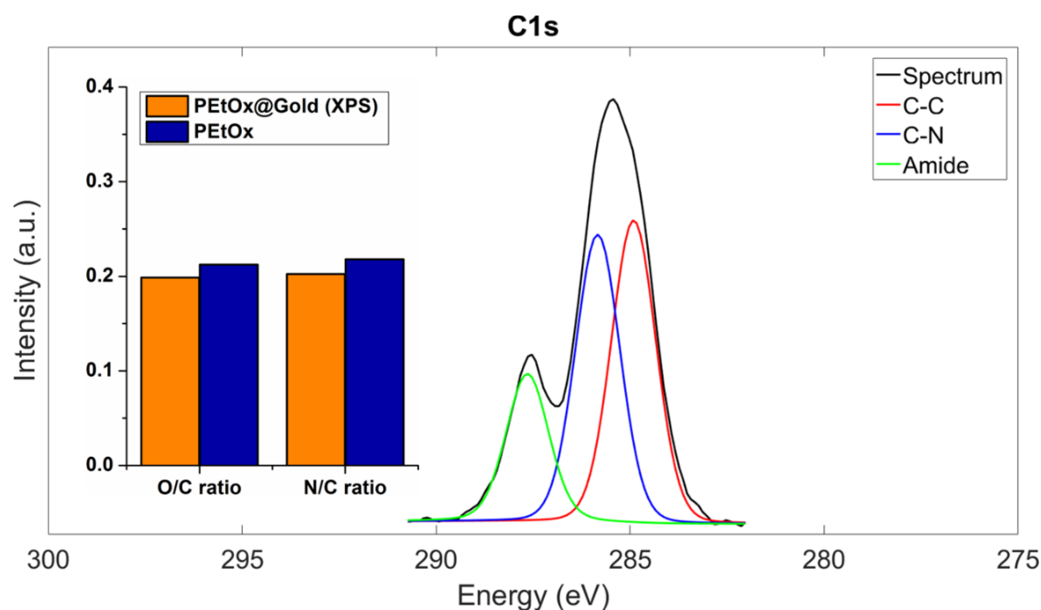


Figure 3. High-resolution C_{1s} XPS of P4-coated gold surface P4@Au. The inset graph shows the comparison of the elemental composition of the P4-coated gold surface and bulk P4.

The polymer-coated surface **P4@Au** was then modified with BSA as a model protein. The protein was dissolved in PBS (pH = 7.4) and applied to the polymer-coated gold surface. After incubation at room temperature, the excess unreacted BSA was washed with water and the remaining coating was dried. The functionalization was followed by spectroscopic ellipsometry by measuring the surface coating thickness upon modification (**Figure 4**). Within three hours, the thickness of the surface increased by nearly 2 nm, confirming the successful functionalization with BSA. In a control experiment, modification of the **P5** polymer-gold surface (without PFP groups) with BSA led to a negligible increase in spectroscopic thickness, which can be attributed to non-specific interactions of the protein with the polymer surface. This suggests the anti-fouling properties of the PEtOx-lipoic acid coating.

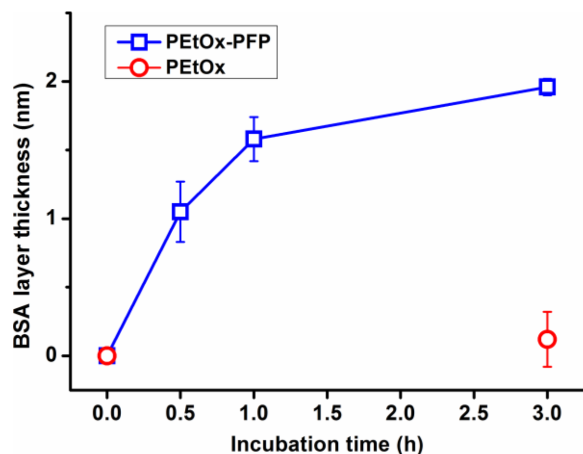


Figure 4. Increase in ellipsometry thickness of the polymer-coated gold surfaces upon incubation with BSA in PBS (pH = 7.4).

Finally, the static water contact angle values of the coated surfaces were measured. As the PEtOx-based copolymers are hydrophilic and water-soluble, such measurements provide further insights into the surface coating (**Figure 5**). After coating the gold surface with **P4**, the hydrophilic character of the polymer caused a significant drop in the surface water contact angle. After modification with BSA, the slightly more hydrophobic character of the protein (compared to PEtOx) resulted in a slight increase in the water contact angle. On the other hand, removing the remaining PFP groups with an excess of aminoethanol led to a more hydrophilic surface, indicating that the PFP-ester-containing polymer coating can not only be used for protein conjugation to the surface but also for modification of the surface properties.

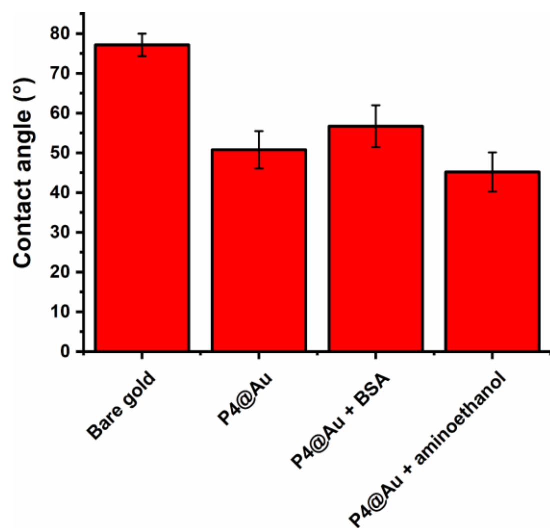


Figure 5. Static water contact angles of the reference gold substrate (bare gold) and the gold surfaces coated with P4 before and after modification with BSA and aminoethanol.

Materials and Methods

Materials

Acetonitrile (Sigma-Aldrich) was dried in a solvent purification system (J. C. Meyer). 2-Ethyl-2-oxazoline (EtOx; Aldrich) was distilled over barium oxide and stored under argon. 2-Methoxycarboxypropyl-2-oxazoline (MestOx) was synthesized as previously described [43] and stored under argon. Methyl tosylate (MeOTs) was distilled and stored under argon. All other chemicals were purchased from Sigma-Aldrich or Tokyo Chemical Institute and used as received.

Instrumentation

Polymerizations were performed in a capped vial in a microwave reactor (Biotage Initiator Sixty equipped with an IR temperature sensor) under temperature control. Size-exclusion chromatography (SEC) was performed using an Agilent 1260-series HPLC system equipped with a 1260 online degasser, a 1260 ISO-pump, a 1260 automatic liquid sampler (ALS), a thermostated column compartment (TCC) at 50 °C equipped with two PLgel 5 μm mixed-D columns and a precolumn in series, a 1260 diode array detector (DAD), and a 1260 refractive index detector (RID) using *N,N*-dimethylacetamide (DMAc) containing 50 mM of LiCl (flow rate of 0.5 mL min^{-1}) as a mobile phase. Molar masses were calculated against poly(2-ethyl-2-oxazoline) standards (made in house). ^1H NMR spectra were recorded in CD_3OD on a Bruker Avance 400 MHz spectrometer. UV–vis analysis was performed using Varian Cary 100 Bio UV-vis spectrophotometer equipped with a Cary temperature and stir control.

Synthesis of PEtOx-PMestOx-N_3 (P1)

A solution of EtOx (7.4 g, 75.2 mmol), MestOx (754 mg, 4.8 mmol), MeOTs (120.6 μL, 0.80 mmol) in acetonitrile with an initial total monomer concentration of 4 M was sealed in a microwave vial under argon atmosphere. The polymerization mixture was heated to 140 °C for 14 min under microwave irradiation and subsequently cooled to ambient temperature. Solid sodium azide (3 eq. of initial MeOTs amount) was added under argon and the mixture was stirred at ambient temperature overnight. The solution was then concentrated under reduced pressure, and the residue was dissolved in dichloromethane (DCM). The solution was filtered through a short pad of alumina, and the polymer was precipitated in diethyl ether.

Synthesis of PEtOx-P(COOH)-N_3 (P2)

A solution of LiOH (15 eq. relative to the methyl ester groups amount) in distilled water (20 mL) was added to the solution of **P1** (3 g) in distilled water (40 mL), and the resulting mixture was stirred at room temperature. After 2 h, the mixture was acidified to pH ~ 3, transferred to the dialysis tubing (molecular weight cut-off 1 kDa), and dialyzed against distilled water for two days, followed by the recovery of the polymer by freeze-drying.

Synthesis of PEtOx-P(PFP)-N_3 (P3)

The obtained copolymer **P2** having pendant carboxylic acid functions (1 g) was dissolved together with pentafluorophenol (1.5 eq. relative to the methyl ester groups amount) in DCM (10 mL) and cooled to -18 °C. A solution of *N,N′*-diisopropylcarbodiimide (DIC, 1.5 eq. relative to the carboxyl groups) and *N*,*N*-dimethylaminopyridine (catalytic amount) in DCM (10 mL) was then added, and the resulting mixture was stirred at room temperature overnight, followed by filtration through a short pad of alumina, precipitation in cold diethyl ether, and drying in vacuo.

***N*-lipoyl(ethylenediamine)**[40]

Lipoic acid (3 g, 14.5 mmol) and *N,N′*-carbonyl diimidazole (2.59 g, 16.0 mmol) were dissolved in DCM (50 mL) and stirred at room temperature under an argon atmosphere for 2 h. The resulting solution was added dropwise to ethylenediamine (10 mL) in DCM (50 mL), followed by stirring at room temperature overnight under an argon atmosphere. After the reaction was completed, the solution was washed with brine (100 mL) and Na_2CO_3 (1 M, 150 mL). The organic phase was dried with anhydrous magnesium sulfate and filtered. As the lipoyl(ethylenediamine) becomes hardly soluble in organic solvents once evaporated, a calculated aliquot of the DCM solution was used directly in the next step without further evaporation/purification. The concentration of *N*-lipoyl(ethylenediamine) in this stock solution was determined by evaporating an aliquot of the stock solution in a pre-weighed flask.

Synthesis of PEtOx-dithiolane conjugates (P4, P5)

Polymer **P3** (1 g) was dissolved in DMF (3 mL) and added to a solution of *N*-lipoyl(ethylenediamine) (0.3 eq. of the PFP groups) and triethylamine (0.4 eq. of the PFP groups) in DCM and stirred at room temperature for 48 h. Afterward, the mixture was precipitated in diethyl

ether and dried in vacuo. The crude copolymer **P4** was dissolved in DCM and filtered through a short pad of alumina, precipitated in diethyl ether, and dried in vacuo. The PFP-free copolymer P5 was prepared in a similar manner using an excess of *N*-lipoyl(ethylenediamine) (1.5 eq. of the original PFP groups) and triethylamine (2 eq. of the PFP groups).

Coating of gold surfaces with P4 and P5

The polymer solution in acetonitrile (c_{pol} = 10 mg mL^{-1}, 1 mL) was transferred onto a gold-coated wafer (2.5 cm x 2.5 cm) using a micropipette. The solvent was allowed to evaporate at room temperature (~15 min) freely. Then, the coated wafer was repeatedly washed with acetonitrile, distilled water, isopropanol and dried in an argon flow.

BSA functionalization

A solution of BSA in phosphate buffer-saline (PBS, c_{BSA} = 5 mg mL^{-1}, 1 mL, pH = 7.4) was transferred to the polymer-coated gold wafer surface using a micropipette. The system was kept at room temperature for different times (30 min, 1 h, and 3 h), followed by washing with distilled water and drying in the flow of argon.

Characterization of polymer-coated surfaces

Spectroscopic ellipsometry of coated surfaces was performed using a J.A. Woollam M-2000X spectroscopic ellipsometer to measure the dry thickness of the polymer coatings. Ellipsometric data were obtained in air at room temperature in the wavelength range λ = 245–1000 nm at angles of incidence of 60, 65, and 70°. The data were fitted with CompleteEASE software using a multilayer model. The thickness and refractive index of the layers were obtained from simultaneous fitting of the obtained ellipsometric data using Cauchy dispersion functions. The thicknesses are reported for 3 points on the surface as mean ± standard deviation.

XPS analysis was performed using a PHI 5000 Versaprobe II spectrometer with a monochromatic Al K_α X-ray source (hν = 1486.6 eV) operating at 23.5 W. A vacuum of $<10^{-6}$ Pa was obtained for all measurements. Survey scans and high-resolution spectra (C1s) were recorded with pass energies of 187.85 eV (eV step = 0.8 eV) and 23.50 eV (eV step = 0.1 eV), respectively, with a take-off angle of 45° and a spot diameter of 100 μm. Four points were measured per sample. Elemental analysis was performed using a Shirley background and employing the relative sensitivity factors

as supplied by the manufacturer (Multipak (v9.6.1) software). All samples were stored in vacuum before analysis.

Static water contact angle (WCA) analysis was performed using a Krüss Easy Drop system at room temperature. Per polymer sample, four to seven water droplets of 2.0 μL of deionized water were placed on different positions of the sample surface. Measurements were performed in triplicates.

Conclusions

In summary, we developed a robust platform for the attachment of amine-containing biomolecules to solid surfaces via a biocompatible poly(2-oxazoline) linker. First, a well-defined multifunctional PEtOx was synthesized, containing orthogonal dithiolane groups for gold surface attachment, as well as pentafluorophenyl ester groups for amine conjugations. On top of that, a third functionality (azide) was introduced to the chain end for possible future bioconjugations via "click" chemistry, which was not explored in the current work. The kinetics of the polymer aminolysis and hydrolysis were assessed, demonstrating a rapid amine conjugation and much slower hydrolysis, enabling selective amidation of the PFP-esters. The copolymers were then coated onto gold surfaces, forming a brush monolayer architecture, as confirmed by spectroscopic ellipsometry. The successful modification of these coated surfaces with BSA showcased their potential for biosensing applications. This study not only contributes to the field of polymer chemistry and surface modification but also offers a promising avenue for the development of functionalized gold-coated surfaces with potential applications across various disciplines, which will be further explored in our ongoing work.

Author contributions

The manuscript was written through the contributions of all authors. All authors have approved the final version of the manuscript.

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Notes

R.H. is one of the founders of Avroxa BVBA that commercializes poly(2-oxazoline)s as Ultroxa®. The other authors have no conflicts to declare.

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