The Behavior of Poly(amino acids) Containing L-Cysteine and Their Block Copolymers with Poly(ethylene glycol) on Gold Surfaces

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ABSTRACT: Poly(ethylene glycol) (PEG) is often used to biocompatibilize surfaces of implantable biomedical devices. Here, block copolymers consisting of PEG and L-cysteine-containing poly(amino acid)s (PAA's) were synthesized as polymeric multianchor systems for the covalent attachment to gold surfaces or surfaces decorated with gold nanoparticles. Amino-terminated PEG was used as macroinitiator in the ring-opening polymerization, (ROP), of respective amino acid *N*-carboxyanhydrides (NCA's) of L-cysteine (L-Cys), L-glutamate (L-Glu), and L-lysine (L-Lys). The resulting block copolymers formed either diblock copolymers, PEG-*b*-p(L-Glu$_x$-*co*-L-Cys$_y$) or triblock copolymers, PEG-*b*-p(L-Glu)$_x$-*b*-p(L-Cys)$_y$. The monomer feed ratio matches the actual copolymer composition, which, together with high yields and a low polydispersity, indicates that the NCA ROP follows a living mechanism. The L-Cys repeat units act as anchors to the gold surface or the gold nanoparticles and the L-Glu repeat units act as spacers for the reactive L-Cys units. Surface analysis by atomic force microscopy revealed that all block copolymers formed homogenous and pin-hole free surface coatings and the phase separation of mutually immiscible PEG and PAA blocks was observed. A different concept for the biocompatibilization of surfaces was followed when thiol-terminated p(L-Lys) homopolymer was first grafted to the surface and then covalently decorated with HOOC-CH$_2$-PEG-*b*-p(Bz-L-Glu) polymeric micelles. © 2013 Wiley Periodicals, Inc. J. Polym. Sci., Part A: Polym. Chem. **2014**, *52*, 248–257

KEYWORDS: block copolymers; poly(amino acids); poly(ethylene glycol); solid supports

INTRODUCTION The functionality and stability of biomedical devices depends on their interactions with the surrounding bioenvironment.[1–4] Therefore, the molecular architecture of the interface needs to be designed in such a way that it will promote biocompatibility. Gold layers or gold nanoparticles deposited onto the surface of biomedical devices may be exploited as an adhesion layer to which biocompatible polymers can be attached covalently by using the reactivity of thiol groups toward gold. The naturally occurring L-cysteine (L-Cys) carries a pendant thiol group, which readily forms covalent linkages to gold surfaces.[5] L-Cys can be homopolymerized to poly(L-cysteine), p(L-Cys) or copolymerized with other amino acids to form either block or random copolymers, thus conveying a reactivity toward gold to the resulting poly(amino acid)s (PAA's).[6–8]

In general, biocompatibility is achieved, when surfaces are rendered hydrophilic. Poly(ethylene glycol) (PEG) proved to be a superior biomaterial that is capable of conveying biocompatibility.[9,10] PEGylation is defined as the covalent attachment of PEG to proteins, drugs, polynucleotides, and other biopolymers, where it provides a stealth character to the resulting conjugates and their aggregates.[11–15] Equally, surfaces grafted with PEG will also exhibit biocompatibility.[16] When exposed to an aqueous environment, PEG coordinates with water molecules, thereby preventing direct interactions between proteins and the PEGylated surface or other PEGylated conjugates.[17,18]

When block copolymers are formed between PEG and p(L-Cys)-containing PAA's, the biocompatibility of the PEG is combined with the reactivity of p(L-Cys). Furthermore, copolymerizing L-Cys with other amino acid repeat units, as opposed to end-capping the polymer-chains with a single thiol group, generates multiple anchor sites along the copolymer chain that become available for the covalent attachment of the block copolymer to a surface. When used in biomedical applications, PAA's are inherently biocompatible and biodegradable as they consist of naturally occurring amino acids, and PEG with a sufficiently low-molecular weight will cleared via renal excretion.[19–24]

Additional Supporting Information may be found in the online version of this article.

This project was originally motivated by research into retinal prostheses which are implanted into the subretinal space and need to maintain their electrical activity while being biocompatible.[25] Implanting electronic devices into living organisms poses two challenges: (i) biocompatibility of the implant with the surrounding tissue and (ii) the edema caused by the surgical trauma. This study focuses on the synthesis and deposition behavior of block copolymers capable of conveying biocompatibility and also offers a path to decorating biocompatible polymer brushes with potential drug delivery systems that could carry drugs to alleviate the consequences of surgical trauma. It is inherently difficult to form dense polymer brushes on surfaces using a "grafting to" method. Only limited graft densities can be achieved by "grafting to" methods because of the concentration gradient that builds up by the already attached chains, which provides a strong kinetic impediment against additional grafting.[26,27] In addition, the large excluded volume that PEG exerts in an aqueous environment further hinders the formation of dense PEG brushes in "grafting to" procedures. Therefore, a multipoint attachment using an anchoring polymer that in itself is biocompatible was chosen here. Specifically, amino-terminated PEG was used as the macroinitiator for the ring-opening polymerization (ROP) of amino acid *N*-carboxyanhydrides (NCA's). L-Cys-containing PEGylated PAA's were synthesized, where the PAA block constitutes either a random copolymer of L-Cys and L-glutamate (L-Glu) or a block copolymer of the two. These block copolymers were coated onto gold surfaces or surfaces with gold nanoparticles. Furthermore, p(L-lysine), (p-(L-Lys)) terminated with a thiol group on one chain end was deposited onto gold surfaces and covalently decorated with polymeric micelles, which were also produced from PEGylated PAA's. The morphology of the resulting surfaces was investigated by atomic force microscopy (AFM).

EXPERIMENTAL

Materials

All chemicals were purchased from Sigma-Aldrich Chemicals St. Louis, MO and used as received, unless otherwise stated. Triphosgene was purchased from Tokyo Chemical Industry, Japan. (1S)-(-)-alpha-Pinene was purchased from Fisher Scientific, Waltham, MA. Alpha-carboxymethyl-ω-amino PEG, COOH-CH_2-PEG_{114}-NH_2 ($M_w = 5000$ g mol^{-1}) and α-methoxy-ω-amino PEG, CH_3-O-PEG_{114}-NH_2 ($M_w = 5000$ g mol^{-1}) were purchased from LaysanBio, Huntsville, AL. Gamma-benzyl-L-glutamate (Bz-L-Glu) was purchased from Bachem, Torrence, CA, and stored at room temperature and used as received, *S*-benzyloxycarbonyl-L-cysteine (Bz-O-L-Cys) was purchased from Sigma Aldrich Chemicals, St. Louis, MO and from Chengdu Unibiochem, China, and stored at -20 °C in a sealed container and ε-trifluoroacetyl-L-lysine (TFA-L-Lys) was purchased from Chem-Imex International, Wood Dale, IL and stored at room temperature. Acetonitrile was dried by reflux over CaH_2 under a dry nitrogen atmosphere and subsequently distilled prior to use. Dimethylformamide (DMF) was distilled under vacuum and over CaH_2. Methanolic potassium carbonate was prepared by the addition of 250 mg potassium carbonate to 40 mL of an aqueous methanol solution 9:1 (v/v) of H_2O: MeOH. Water was deionized with a Millipore Milli-Q system.

Instrumentation

All Proton Nuclear Magnetic Resonance, ^{1}H NMR, spectra were recorded on a Varian Unity Inova 500 (500 MHz) spectrometer equipped with a 5-mm triple resonance inverse detectable probe. DMSO-d_6, $CDCl_3$, and D_2O were used as solvents. Gel permeation chromatography (GPC) was performed on a Waters Breeze GPC system consisting of a 1515 isocratic pump, a TSK-gel α-*M* (particle size 13 μm, exclusion limit 1×10^7 g mol^{-1} for polystyrene in DMF), and a TSK-gel α-3000 (particle size 7 μm, exclusion limit 1×10^5 g mol^{-1} for polystyrene in DMF) (Tosoh Bioscience) column, and a 2414 refractive index detector under the following conditions: injection volume, 100 μL; flow rate, 0.5 mL min^{-1}; eluent, DMF; temperature, 50 °C, PS (purchased from Shodex) as the calibration standards. Dynamic light scattering (DLS) measurements on the polymeric aggregates using a 90Plus particle size analyzer (Brookhaven Instruments) were described previously.[28] Briefly, solutions for DLS measurements ranging in concentration from 0.25 to 5.0 g L^{-1} were obtained from a HOOC-CH_2-PEG-*b*-p(Bz-L-Glu)$_n$ stock solution. Five measurements of one min each were carried out and averaged.

X-ray scattering measurements were performed on an EMPYREAN diffractometer from PANanalytica, B.V., Almelo, The Netherlands in point focus geometry using a combination of crossed slits (0.2×1.0 mm^2) on the incident side and a programmable anti-scatter slit with a fixed divergence of 0.5° in front of a position sensitive PIXEL detector. The wavelength used was CuKα radiation ($\lambda = 0.154$ nm), monochromatized with Nickel filter. The measurements were carried out at room temperature over the range $2\theta = 15°$ to 30° in increments of 0.026°.

Surface morphologies were studied using an atomic force microscope NanoWizard (JPK Instruments, Germany) working in tapping mode with silicon cantilevers. Cantilevers were of type Arrow (NanoWorld, Switzerland) and had a resonance frequency of about 285 kHz and a force constant of about 42 N m^{-1}.

Gold-Coated Silicon Wafers

Silicon wafers were purchased from Silicon Valley Microelectronics, Inc. Gold coated wafers were obtained from Cornell University through the Boston Retinal Implant Project. They were prepared by depositing a 50 nm layer of titanium, followed by the deposition of a 50 nm layer of gold by electron beam evaporation in a Chemical Vapor Coating 6-pocket e-beam evaporator. Silicon wafers with Au nanoparticles with a diameter of 200–400 nm were kindly provided by Dr. Chengdu Liang of Oak Ridge National Laboratory. A previously described solution-based technique for the synthesis of gold nanoparticles was modified to allow for their synthesis on solid substrates.[29,30]

Synthesis of Protected NCAs

NCA of protected amino acids, were synthesized in dry ethyl acetate (EtOAc) in the presence of triphosgene. Respective amounts (0.05 or 0.1 g mL^{-1}) of γ-benzyl-L-glutamic acid (Bz-L-

TABLE 1 Summary of PEGylated PAA's and Their Properties

#	Polymer[a]	Actual PAA Composition by ^{1}H NMR	Yield (%)	M_n by NMR[b] g mol^{-1}	PDI by GPC
1	PEG-b-p(Bz-L-Glu)$_{50}$-*b*-p(Bz-O-L-Cys)$_{10}$	57/9	87	19,700	1.16
2	PEG-b-p(Bz-L-Glu)$_{100}$-*b*-p(Bz-O-L-Cys)$_{10}$	101/7	90	29,100	1.17
3	PEG-b-p(Bz-L-Glu)$_{100}$-*b*-p(Bz-O-L-Cys)$_{20}$	108/14	88	32,300	1.14
4	PEG-b-p(Bz-L-Glu$_{50}$-*co*-Bz-O-L-Cys$_{10}$)	50/8	91	18,000	1.26
5	PEG-b-p(Bz-L-Glu$_{100}$-*co*-Bz-O-L-Cys$_{10}$)	102/7	86	29,400	1.34
6	PEG-b-p(Bz-L-Glu$_{100}$-*co*-L-Bz-O-Cys$_{20}$)	100/19	89	31,600	1.33
7	p(TFA-L-Lys)$_{30}$-SH	29	87	9500[c]	1.27
8	HOOC-PEG-b-p(Bz-L-Glu)$_{12}$	10	78	7700	1.24
9	HOOC-PEG-b-p(Bz-O-L-Cys)$_{16}$	13	75	8500	1.26

[a] PEG: α-methoxy-ω-amino PEG and α-carboxlymethyl-ω-amino PEG, respectively, with a molecular weight of 5000 g mol^{-1} was used for all experiments. PDI: polydispersity index.

[b] M_n of block copolymers with the protected amino acid repeat units (p(Bz-*L*-Glu) and p(Bz-O-*L*-Cys)) as determined by ^{1}H-NMR spectroscopy.

[c] Synthesized as dimer from cysteamine.

Glu), ε-TFA-L-lysine (TFA-L-Lys), or S-benzyloxycarbonyl-L-cysteine (Bz-O-L-Cys) were suspended in freshly distilled EtOAc, 2.1 equiv. of α-pinene and 0.5 equiv. of triphosgene were added. The reaction mixture was heated at reflux (105–110 °C) until it became transparent, which took, depending on the α-amino acid, between 2.5 and 4.5 h. The mixture was cooled to room temperature, filtered, and the filtrate was concentrated under reduced pressure, and then crystallized in EtOAc/hexane. The obtained NCAs were recrystallized from a mixture of EtOAc/hexane cooled to 0 °C and then vacuum-dried. Yield: 86–93%. ^{1}H NMR for **Bz-O-L-Cys:** (DMSO-d_6, 500 MHz at 35 °C): δ/ ppm = 3.34 (2H, CH_2-S-CO), 4.74, (1H, CO-CH(CH$_2$)-NH), 5.29 (2H, -O-CH_2-Benzyl), 7.36 (5H, aromatic *H*), 9.1−9.24 (1H, -CO-CH-N*H*-). ^{1}H-NMR for TFA-**L-Lys:** (DMSO-d_6, 500 MHz at 35 °C): δ/ ppm = 1.36 (2H, CH-CH_2-CH$_2$), 1.49 (2H, CH-CH$_2$-CH_2-CH$_2$-), 1.73 (2H, CH-CH$_2$-CH$_2$-CH_2-CH$_2$-), 3.17 (2H, CH_2-NH-CO-CF$_3$), 4.43, (1H, CO-CH(CH$_2$)-NH), 9.08 (1H, N*H*-CO-CF$_3$), 9.40 (1H, CH-N*H*-CO). Lastly, ^{1}H-NMR for **Bz-L-Glu:** (DMSO-d_6, 500 MHz at 35 °C): δ/ ppm = 1.93 (2H, CH-CH_2-CH$_2$-CO), 2.04 (2H, (CH-CH$_2$-CH_2-CO), 4.45 (1H, CO-CH(CH$_2$)-NH), 5.09 (2H, -O-CH_2-C$_6$H$_5$), 7.36 (5H, CH$_2$-C$_6$H$_5$), 9.08 (1H, -CO-CH-N*H*-) (Supporting Information).

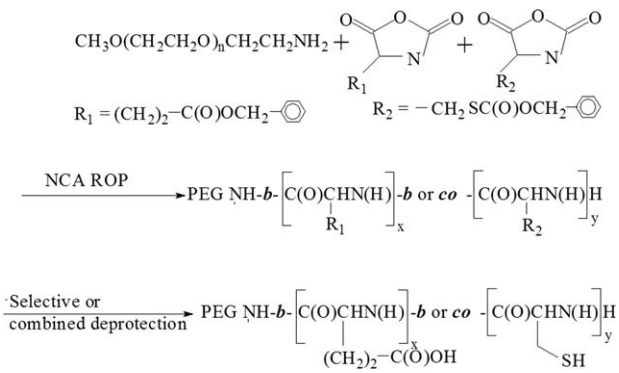
Synthesis of Methoxy-PEG-b-PAA Block Copolymers: (i) Methoxy-PEG-b-Poly(L-Glutamate-b-L-Cysteine), PEG-b-p(L-Glu)$_x$-b-p(L-Cys)$_y$ and (ii) Methoxy-PEG)-b-Poly(L-Glutamate-co-L-Cysteine), PEG-b-p(Glu$_x$-co-Cys$_y$)

PEGylated PAA triblock copolymers were obtained by ROP of the respective protected NCA's using CH$_3$-O-PEG-NH$_2$ with a molecular weight of 5000 g mol^{-1} as macroinitiator. The synthesis is exemplified for PEG-*b*-p(L-Glu)$_{100}$-*b*-p(L-Cys)$_{10}$: CH$_3$-O-PEG-NH$_2$ (3.5×10^{-5} mol) was dissolved in 5 mL of 0.2 M urea/DMF and added with a syringe to a solution of Bz-L-Glu-NCA (4.1×10^{-3} mol) in 0.2 M urea/DMF (5 mL) kept under a flow of dry argon. The polymerization was conducted at room temperature for 72 h. The formation of the diblock (PEG-*b*-p(Bz-L-Glu)$_{100}$) was determined to be complete when no more CO$_2$ evolved, at which point 4.1×10^{-4} mol of Bz-O-L-Cys-NCA was added. The polymerization was conducted similar to the method described by Wooley;[31] however, here the polymerization was performed under a blanket of Argon. Periodically, the pressure that built due to the CO$_2$ formation was checked via the bubbler and released via the Schlenk line. The polymerization continued for another 48 h, and the end point was again determined when CO$_2$ evolution had ceased. The PEG-*b*-p(Bz-L-Glu)$_{100}$-*b*-p(Bz-O-L-Cys)$_{10}$ triblock copolymer was isolated by precipitation into diethyl ether and purified by dialysis against distilled water for 3 d using a Spectra/Por cellulose ester (CE) membrane (Molecular weight Cut-off, (MWCO): 3500 g mol^{-1}) and isolated by freeze-drying. Yield: 90% ^{1}H NMR (DMSO-d$_6$, 500 MHz):δ/ppm = 3.6–3.7(PEG, 4H, -O-CH_2CH_2-), 2.81 (3H, CH_3-O-), 1.89 (2H, CH-CH_2-CH$_2$-COO-), 2.21 (2H, CH-CH$_2$-CH_2-COO-), 5.15 (2H, -O-CH_2-C$_6$H$_5$), 7.18 (5H, CH$_2$-C$_6$H$_5$-), 4.97 (2H, S-COO-CH_2- C$_6$H$_5$-), 3.83–4.19 (1H, -CO-CH(CH$_2$)-NH-), 7.95 (-CO-CH-N*H*-) see: Supporting Information.

Diblock copolymers in which the PAA block constitutes a random copolymer, PEG-*b*-p(Bz-L-Glu$_x$-*co*-Bz-O-L-Cys$_y$), were synthesized as described above, except that the Bz-L-Glu-NCA and Bz-O-L-Cys-NCA monomers were mixed prior to the addition of the CH$_3$-O-PEG-NH$_2$ macroinitiator. The polymerization was conducted for 4 d, and the yield was between 86 and 91%.

Synthesis of Cysteamine–Poly(ε-TFA-L-Lysine) (p(TFA-L-Lys)-SH)

The p(TFA-L-Lys)-SH homopolymer was obtained by ROP of TFA-L-Lys-NCA using cysteamine [S-(CH$_2$)$_2$-NH$_2$)]$_2$ as initiator. Cysteamine (6.5×10^{-5}mol) was dissolved in 5 mL of 0.2 M urea/DMF and added with a syringe to the TFA-L-Lys-NCA solution (2.5×10^{-3} mol) in 0.2 M urea/DMF (25 mL) kept under dry argon. The polymerization was conducted for 3 d at room temperature. The p(TFA-L-Lys)-SH polymer was isolated by precipitation into *n*-hexane. Next, the polymer was dissolved in *N*-methyl-2-pyrrolidone and dialyzed against



SCHEME 1 Synthesis of PEGylated PAA's using α-methoxy-ω-amino PEG as macroinitiator. Diblock copolymers with the following structure: PEG-*b*-p(L-Glu$_x$-*co*-L-Cys$_y$) are obtained when a mixture of the amino acid NCA's is polymerized, triblock copolymers with the following structure: PEG-*b*-p(L-Glu)$_x$-*b*-p(L-Cys)$_y$ are obtained when the amino acid NCA's are added successively.

water for 3 d using a Spectra/Por CE membrane (MWCO: 2000 g mol^{-1}) and isolated by freeze-drying. Yield: 87%. ^{1}H-NMR (DMSO-d$_6$, 500 MHz):δ/ppm = 3.40–3.57 (4H, S-C*H*$_2$-C*H*$_2$-N(H), 2.91 (2H, CH$_2$-C*H*$_2$-N(H), 1.42–1.73 (6H, CH-C*H*$_2$-C*H*$_2$C*H*$_2$-CH$_2$), 4.26 (1H, -CO-C*H*(CH$_2$)-NH-).

Synthesis of Carboxymethyl-PEG-b-(γ-Benzyl-L-Glutamate), HOOC-CH2-PEG-*b*-p(Bz-L-Glu)n

The HOOC-CH$_2$-PEG$_n$-*b*-p(Bz-L-Glu)$_n$ block copolymer was obtained by ROP of Bz-L-Glu-NCA using HOOC-CH$_2$-PEG-NH$_2$ with a molecular weight of 5000 g mol^{-1} as macroinitiator. HOOC-CH$_2$-PEG-NH$_2$ (3.5×10^{-5} mol) was dissolved in 5 mL of 0.2 M urea/DMF and added with a syringe to a solution of (Bz-L-Glu-NCA (5.5×10^{-4} mol) in 0.2 M urea/DMF (5 mL) kept under dry argon. The polymerization was conducted at room temperature for 48 h. The HOOC-PEG-*b*-p(Bz-L-Glu)$_n$ copolymer was isolated by precipitation into diethyl ether and purified by dialysis against distilled water for 3 d using a Spectra/Por CE membrane (MWCO: 3500 g mol^{-1}) and isolated by freeze-drying. ^{1}H NMR (DMSO-d$_6$, 500 MHz): δ/ppm = 1.8 (2H, CH-C*H*$_2$-), 2.35 (2H, CH-CH$_2$-C*H*$_2$-), 3.5 (PEG, 4H, -O-C*H*$_2$C*H*$_2$-), 3.9(1H, -CO-C*H*(CH$_2$)-NH-, 5.0 (2H, -O-C*H*$_2$-C$_6$H$_5$), 7.27(5H, CH$_2$-C$_6$*H*$_5$-), 8.1 (1H, -CO-CH-N*H*-).

Self-Assembly of HOOC-PEG$_n$-b-p(Bz-L-Glu)$_{12}$

Polymeric aggregates were formed when the block copolymer was dissolved directly in water as previously described.[24] Briefly, stock solutions (5.0 g L^{-1}) were prepared by stirring the copolymer for at least 12 h in distilled water. This stock solution was diluted to concentrations ranging between 0.25 and 2.5 g L^{-1}, the resulting solutions were filtered through a 0.45-μm Millex Millipore polyvinylidene fluoride (PVDF, Durapore) filter.

Deprotection of Cysteamine–Poly(ε-TFA-L-Lysine) (p(TFA-L-Lys)-SH) and Methoxy-PEG-b-PAA Block Copolymers

Between 100 and 500 mg of the polymer or copolymer, respectively, were suspended in a mixture of 9:1 (v/v) of

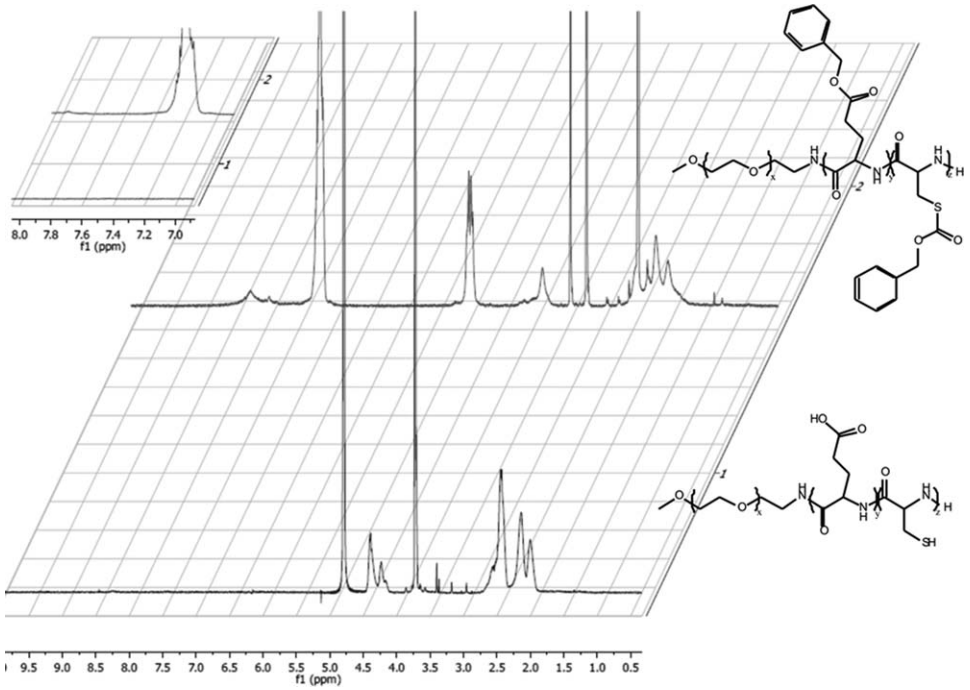


FIGURE 1 ^{1}H NMR spectrum of deprotected PEG-*b*-p(L-Glu$_{100}$-*co*-L-Cys$_{10}$) in the front, and protected PEG-*b*-p(Bz-L-Glu$_{100}$-*co*-Bz-O-L-Cys$_{10}$) in the back. Chemical shift differences are due to the use of D$_2$O used for the deprotected sample versus DMSO-d$_6$ for the protected sample. The inset shows the complete removal of the benzyl and the benzyloxylcarbonyl groups from the cysteine and glutamate repeat units. Additionally, all methylene protons associated with the protective groups are also no longer present in the spectrum in the front.

H_2O and MeOH with 250 mg K_2CO_3. The suspension was heated to reflux. During the course of the reaction, the initial suspension turned into a homogeneous solution, as deprotected PAA's are sufficiently hydrophilic to render the polymer or copolymer water-soluble. After 4–8 h, depending on the PAA chain length, the reaction mixture was cooled to room temperature and filtered. The filtrate was purified by dialysis against distilled water for 2 d using a Spectra/Por membrane (MWCO: 2000 g mol^{-1}), the resulting polymer was isolated by freeze-drying. In the course of this hydrolysis, protected p(TFA-*L*-Lys)-SH was converted into its deprotected derivative p(*L*-Lys)-SH, similarly, p(Bz-*L*-Glu) and p(Bz-O-*L*-Cys) were converted into p(*L*-Glu) and p(*L*-Cys).Yield: 90–95%.

Surface Coating

The gold surfaces were cleaned prior to use by dipping them in a piranha solution consisting of 70% concentrated sulfuric acid and 30% hydrogen peroxide, followed by copious rinsing with deionized water, and drying in a flow of nitrogen.

Immobilization of PEG-*b*-p(*L*-Glu-*b/co-L*-Cys) block copolymers onto Au-substrates and Au-nanoparticles present on silicon wafers

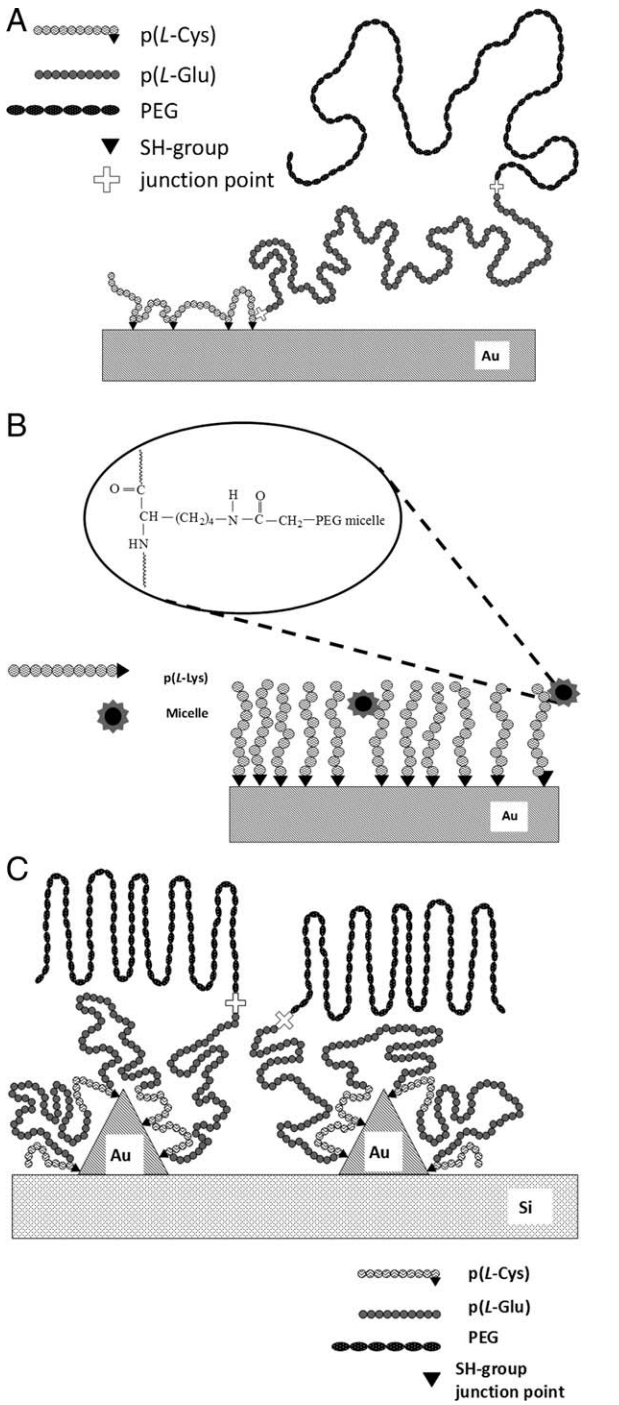
Deprotected PEGylated PAA's were dissolved in 1 mL of deionized H_2O to yield a 50 mM concentration and then deposited onto 1.0 × 1.0 cm squares cut from a gold-coated silicon wafer. The substrate was placed into a vial, and gently shaken at 150 rpm for approximately 12 h. The substrates were removed from the solution and first rinsed with copious amounts of distilled water, followed by a rinse with 70% ethanol. Coated substrates were dried in a stream of argon gas.

Preparation of an self-assembled p(*L*-Lys)-SH monolayer on gold substrate

The aqueous p(L-Lys)-SH solution (0.1 wt%) was prepared by direct dissolution of the polymer in distilled water and filtered through 0.45-μm Millipore PVDF filters prior to use. The gold-substrate, 1.0 × 1.0 cm squares cut from the Au-coated Silicon wafer, was incubated in the aqueous p(L-Lys)-SH solution for at least 12 h. The substrate was then removed, rinsed with water and 70% ethanol, and dried under a steam of argon.

Covalent attachment of HOOC-PEG-*b*-p(Bz-*L*-Glu)$_n$ aggregates to p(*L*-Lys), immobilized on Au-substrates

An aqueous solution of the HOOC-CH_2-PEG-b-p(Bz-L-Glu)n aggregates[32] in EDC/NHS (N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide/N-hydroxysuccinimide) (0.5%/1%/0.5% wt%, respectively) was freshly prepared at pH 6.5 and added to the p(L-Lys)-coated substrate to initiate the coupling reaction between the carboxyl groups that formed on the corona of the aggregates and the amino groups of the p(L-Lys) that extend from the substrate surface. The vials were gently shaken and after 24 h, the substrate was transferred to a clean empty vial rinsed with water to remove unreacted polymeric aggregates and dried under a steam of argon.[33]



SCHEME 2 (a) Shows the multipoint attachment of a PEGylated PAA chain to a gold surface. The PAA block consists either of a block copolymer of p(*L*-Glu)$_x$-*b*-p(*L*-Cys)$_y$ depicted here or a copolymer of p(*L*-Glu$_x$-*co*-*L*-Cys$_y$). (b) Shows the attachment of p(*L*-Lys)$_n$ oligomers to a gold surface via their terminal thiol group, followed by the covalent attachment of reactive polymeric micelles or aggregates, here: HOOC-CH_2-PEG-*b*-p(Bz-*L*-Glu)$_n$. (c) Shows the attachment of a PEGylated PAA chain to gold nanoparticles. The PAA block here consists of a random copolymer of p(*L*-Glu$_x$-*co*-*L*-Cys$_y$).

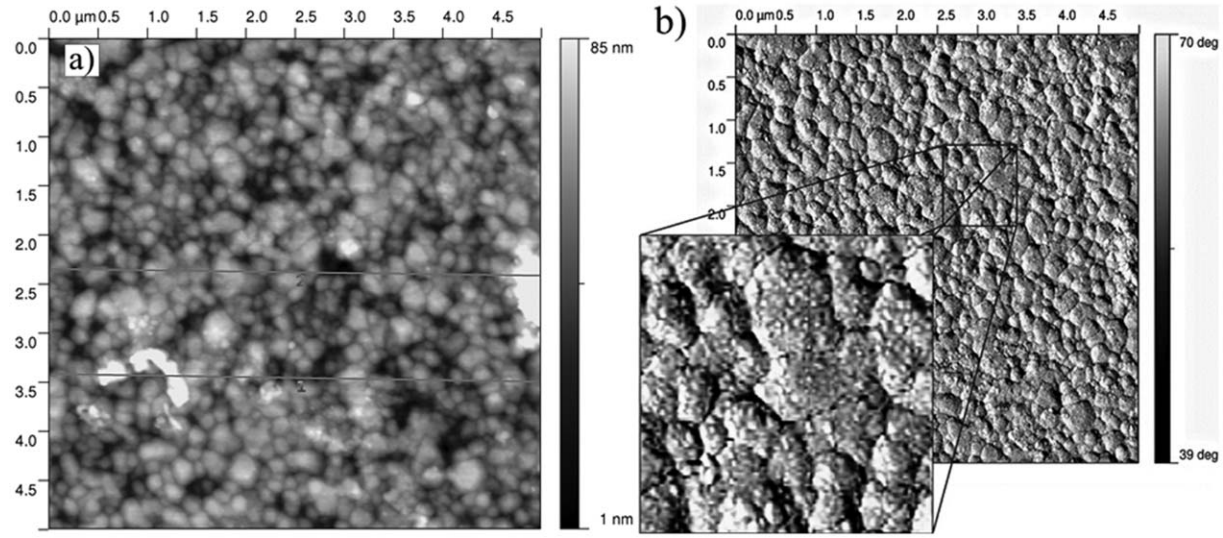


FIGURE 2 5 × 5-μm AFM images of a gold surface coated with PEG-b-p(L-Glu)$_{100}$-b-p(L-Cys)$_{20}$. (a) Height image and (b) Phase image with zoomed area.

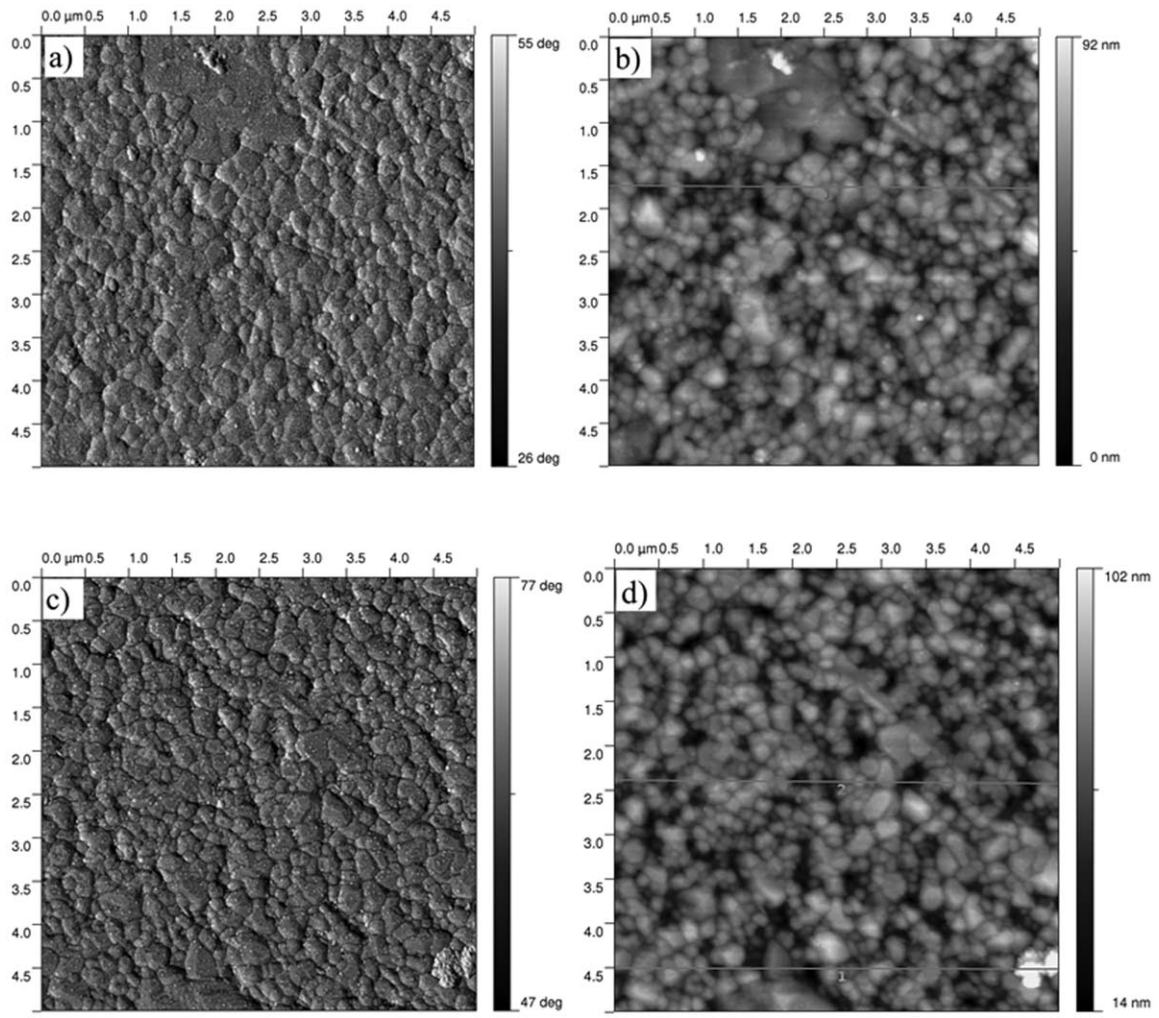


FIGURE 3 5 × 5-μm AFM image (left: phase image, right: height image) of a gold surface coated with PEG-b-p((L-Glu)$_{50}$-*co*-(L-Cys)$_{10}$) (a and b) and of PEG-b-p((L-Glu)$_{100}$-*co*-(L-Cys)$_{20}$) (c and d).

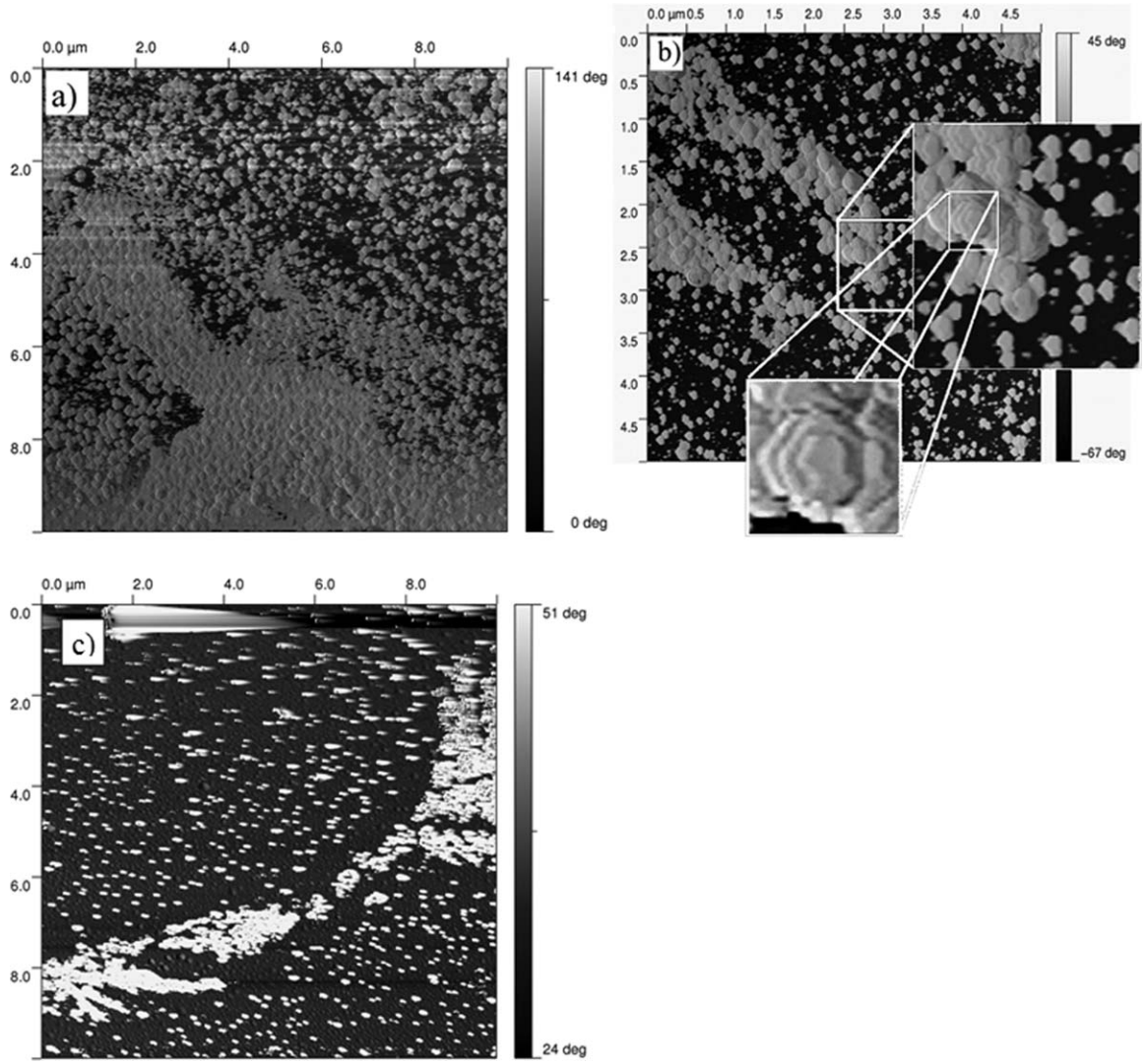


FIGURE 4 (a) 10 × 10-μm AFM phase image of gold nanoparticles with diameter ranging between 200 and 400 nm coated with PEG-b-p((*L*-Glu)$_{50}$-*co*-(*L*-Cys)$_{10}$). (b) 5 × 5-μm AFM phase image of PEG-b-p((*L*-Glu)$_{50}$-*co*-(*L*-Cys)$_{10}$). (c) For comparison, uncoated gold nanoparticles are shown in the 10 × 10-μm AFM phase image.

RESULTS AND DISCUSSION

Results of Polymer Synthesis

NCA's of amino acids were produced following established procedures based on the Fuchs-Farthing method.[34,35] All ROP's of amino acid NCA's reported here were conducted under anhydrous conditions at room temperature in the presence of urea. Previous research showed that urea efficiently suppresses the formation of secondary structures (β-sheets) between the nascent chains by breaking hydrogen bonds between polymer chains in the early stages of the polymerization.[36] Reaction times varied between 48 and 120 h, depending on PAA-block length and increased with increasing PAA block length. CO_2 was released periodically from the reaction mixture, and the polymerization was considered to be complete, when no more CO_2 developed within the reaction vessel.

Macroinitiators are facile for NCA ROP's[37] and PEGylated PAA block copolymers were synthesized by ROP of respective amino acid-NCA's using CH_3-O-PEG-NH_2 with a molecular weight of 5000 g mol^{-1} as macroinitiator. Diblock copolymers were synthesized when the amino acid NCA's were added as a mixture to the macroinitiator, yielding block copolymers with the PAA block consisting of a random PAA copolymer: PEG-*b*-p(Bz-*L*-Glu$_x$-*co*-Bz-O-*L*-Cys$_y$). Triblock copolymers were synthesized when the amino acid NCA's were added successively to the polymerization, with Bz-*L*-Glu-NCA added first, followed by Bz-O-*L*-Cys-NCA after the formation of PEG-b-p(Bz-*L*-Glu) was completed, thus yielding: PEG-*b*-p(Bz-*L*-Glu$_x$)-*b*-p(Bz-O-*L*-Cys$_y$). Completion of the first polymerization was determined by the lack of CO_2 development. Adding a second monomer, here Bz-O-*L*-Cys-NCA, to the polymerization mixture restarts the polymerization,

indicating that the chain end remained living. The analysis of the block copolymers (Table 1, experiments 1–6) shows that the actual polymer composition as determined by ^{1}H NMR spectroscopy matches the monomer feed ratio of the amino acid-NCA's used for polymerization. Copolymers were obtained in yields greater than 85%, their polydispersity ranged between 1.1 and 1.3, as determined by GPC. The reported molecular weights were determined by ^{1}H NMR spectroscopy and reflect the molecular weights of the copolymers with protected amino acid repeat units. The very good match between the monomer feed ratio and actual copolymer composition together with sufficiently high yields and a low polydispersity indicate that the NCA ROP follows a living mechanism, see Scheme 1, which is also in agreement with the findings of Gibson and Cameron.[38]

Homopolymers of TFA-L-Lys-NCA were synthesized using cysteamine as initiator to yield p(L-Lys)-S-S-p(L-Lys), Table 1, Experiment 7. The resulting homopolymer has 30 p(L-Lys) repeat units, when exposed to a gold surface, the disulfide bonds of cysteamine break, thereby producing p(L-Lys)-SH grafted chains with approximately 15 repeat units.

Homopolymers of p(TFA-L-Lys)-SH and PEGylated di- and triblock copolymers (PEG-*b*-p(Bz-L-Glu$_x$-*co*-Bz-O-L-Cys$_y$) and PEG-b-p(Bz-L-Glu$_x$)-*b*-p(Bz-O-L-Cys$_y$)) were deprotected so that their functional groups can undergo further chemical reactions. The progress of the deprotection reactions was followed spectroscopically, and deprotection reactions were considered complete when the protective groups were no longer detected by ^{1}H NMR analysis. The methanolic solution of K_2CO_3 used here removes benzyl and benzyloxycarbonyl groups simultaneously. The reaction times for the deprotection reactions varied between 4 and 8 h, and yields after purification ranged between 87 and 91%. Comparative ^{1}H NMR spectra for PEG-*b*-p(L-Glu$_{100}$-*co*-L-Cys$_{10}$) before and after the deprotection reactions are shown in Figure 1. Protons indicative of the aromatic ring and methylene groups are no longer present in the spectrum of the deprotected block copolymer.

Furthermore, diblock copolymers were synthesized using HOOC-CH_2-PEG-NH_2 with a molecular weight of 5000 g mol^{-1} as macroinitiator for the polymerization of Bz-L-Glu-NCA, and Bz-O-L-Lys-NCA, Table 1, Experiment 8, 9 yielding HOOC-CH_2-PEG-*b*-p(Bz-L-Glu)$_x$ and HOOC-CH_2-PEG-*b*-p(Bz-O-L-Cys)$_y$. Because the glutamate and cysteine building blocks in HOOC-CH_2-PEG$_n$-*b*-p(Bz-L-Glu)$_n$, and HOOC-CH_2-PEG-*b*-(Bz-O-L-Cys)$_y$ are protected by benzyl groups and benzyloxycarbonyl groups, respectively, the copolymers are highly amphiphilic and readily self-assemble in aqueous solution, forming polymeric aggregates with a hydrodynamic radius of about 65 nm obtained by unweighted intensity data of DLS.[28,39]

Block Copolymers on Solid Gold Surfaces

Two different concepts for the surface modification of solid substrates were investigated. Three PEGylated PAA triblock copolymers were selected, together with the p(L-Lys) grafted polymers to be decorated with polymeric micelles. Specifically, substrates

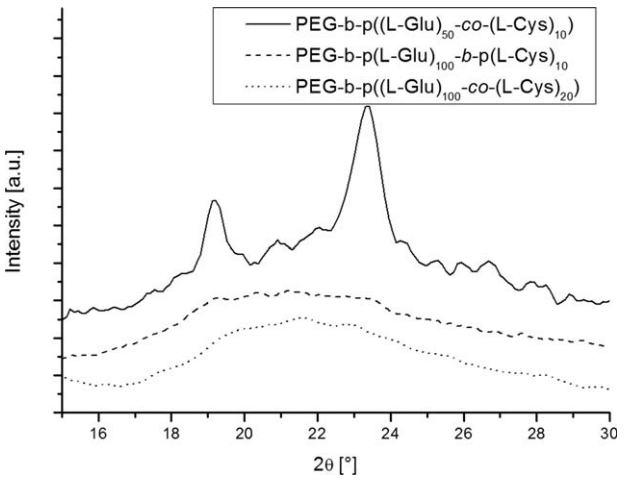


FIGURE 5 WAXS data of PEG-b-p((L-Glu)$_{50}$-*co*-(L-Cys)$_{10}$) (top), PEG-b-p(L-Glu)$_{100}$-*b*-p(L-Cys)$_{10}$ (middle), and PEG-b-p((L-Glu)$_{100}$-*co*-(L-Cys)$_{20}$) (bottom). The curves are shifted vertically for more clarity. When the PAA block has only a combined 60 repeat units, PEG is able to crystallize as indicated by the two Bragg reflections at 19.2 and 23.4°, but with higher amount of PAA, only amorphous halos are observed.

coated with gold or gold nanoparticles were modified via: (i) the terminal thiol group of the (L-Cys) repeat units in the PAA block of PEGylated PAA block copolymers were used for a multipoint attachment of the block copolymers, see Scheme 2(a). Here, the (L-Cys) building blocks formed either a random copolymer with (L-Glu) repeat units, yielding PEG-*b*-p(L-Glu-*co*-L-Cys), or a PAA diblock copolymer was formed, PEG-*b*-p(L-Glu)-*b*-p(L-Cys). (ii) Surfaces were modified first by grafting the surfaces with p(L-Lys)-SH and terminal amino groups in p(L-Lys) reacted with polymeric aggregates carrying carboxyl groups in their corona, HOOC-CH_2-PEG-*b*-p(Bz-L-Glu)$_n$, see Scheme 2(b). Here, covalent amide linkages were formed between the polymeric aggregate and the p(L-Lys) grafts present on the surface. This method is complementary to the previously described chemisorption between anionic surfaces and p(L-Lys)-*g*-PEG.[40]

As shown in Figure 2(a), a complete surface coverage was achieved when PEG-*b*-p(L-Glu)$_{100}$-*b*-p(L-Cys)$_{20}$ was coated onto the solid gold substrate. The surface layer is heterogeneous as a result of the phase separation of mutually immiscible blocks of PEG and PAA. The higher magnification image of the coating shown in Figure 2(b) reveals that in addition to the phase separation on a 500-nm level, small spherical domains in the size of a few nanometers are observed. It can be assumed that this additional phase separation is the result of the separation between the two PAA blocks. When the PAA block exists as a random copolymer, only the phase separation between the immiscible PEG and PAA blocks is observed, Figure 3. The small-scale separation as observed in Figure 2 is not observed when PAA exists as p((L-Glu)$_{100}$-*co*-(L-Cys)$_{20}$). When the molecular weight of the PAA block is reduced as in PEG-*b*-p((L-Glu)$_{50}$-*co*-(L-Cys)$_{10}$) uniform surface films were generated as well. The height images of Figure 3, clearly show a spherical morphology of the phase separated block

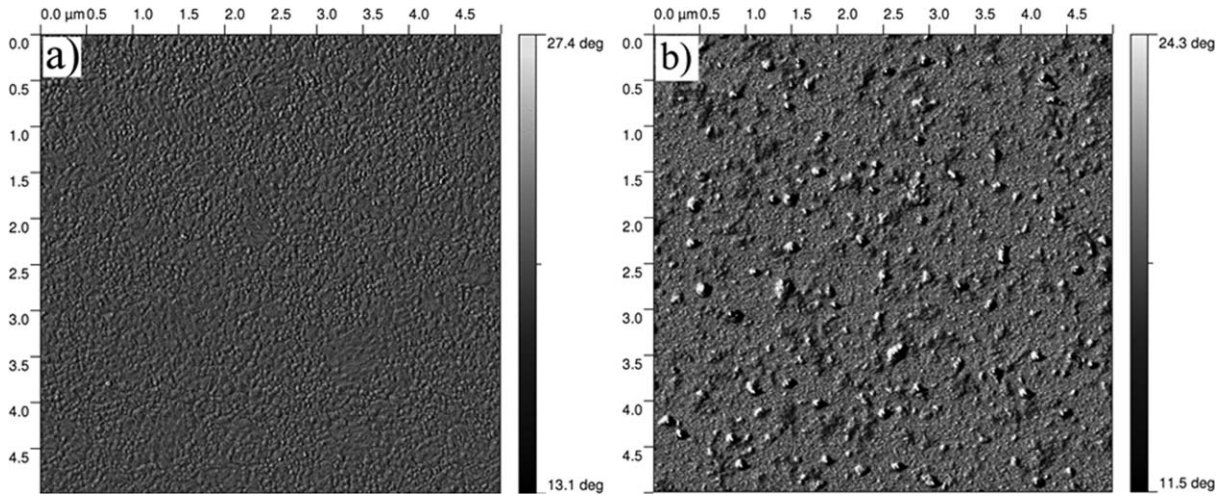


FIGURE 6 AFM phase image of (a) oligomeric p(L-Lys)$_{15}$ attached covalently to a gold surface by the terminal thiol group and (b) reactive micelles consisting of HOOC-CH$_2$-PEG-b-p(Bz-L-Glu)$_{13}$ covalently linked to the p(L-Lys) oligomeric surface layer.

copolymer. The more ordered phase separated morphology in PEG-*b*-p((L-Glu)$_{50}$-*co*-(L-Cys)$_{10}$) is caused by the lower molecular weight of the PAA block as compared to the previously discussed block copolymers (PEG-*b*-p(L-Glu)$_{100}$-*b*-p(L-Cys)$_{20}$).

Block Copolymers on Gold Nanoparticles

Scheme 2(c) depicts the coating of Au nanoparticles. L-Cys units adhere covalently to the nanoparticle. The PEG chains phase-separate and crystallize on top of the nanoparticles. Figure 4(a) shows that the block copolymer, here PEG-*b*-p((L-Glu)$_{50}$-*co*-p(L-Cys)$_{10}$) bound selectively to the gold nanoparticles, no polymer was deposited onto the intermittent silica surfaces. In areas where the polymer is highly enriched, larger polymer domains are formed that bridge the gold nanoparticles. Because the PAA block is covalently attached to gold nanoparticles, the PEG block must reside as the top layer of the polymer coating, which can be seen in Figure 4(b). This morphology is clearly the result of the crystallization of the PEG block and resembles screw dislocations.[41]

If this polymer coating is exposed to an aqueous environment, as it would be in a biological or biomedical application, the PEG block will coordinate with water and will form polymer brushes that reach into the aqueous phase. The WAXS data corroborate the results obtained by AFM as block copolymers with large PAA blocks show only amorphous halos for PEG-*b*-p(L-Glu)$_{100}$-*b*-p(L-Cys)$_{10}$ and PEG-*b*-p((L-Glu)$_{100}$-*co*-(L-Cys)$_{20}$). However, when the molecular weight in the PAA block is lower as in PEG-*b*-p((L-Glu)$_{50}$-*co*-p(L-Cys)$_{10}$), PEG is able to crystallize as indicated by the two Bragg reflections at 19.2 and 23.4°,[42,43] see Figure 5.

p(L-Lys)-Coated Surfaces Decorated with Polymeric Micelles

Figure 6(a) shows the coating of a gold surface with oligomeric p(L-Lys), attached covalently to the gold surface by the terminal thiol group, which is the result of initiating the polymerization with cysteamine. The phase separation in this case is the result of the ability of p(L-Lys) to crystallize. Reactive micelles consisting of HOOC-CH$_2$-PEG$_{114}$-b-p(Bz-L-Glu)$_{13}$ were covalently linked to the p(L-Lys) oligomers via condensation between the terminal carboxyl groups of the polymeric micelles and the amino functions on p(L-Lys). These micelles cannot be washed off, indicating their covalent attachment. The AFM image in Figure 6(b) shows micelle sizes of approximately 50 nm, attached to p(L-Lys).

CONCLUSIONS

PEGylated PAA block copolymers were synthesized with a narrow PDI, a good agreement between theoretical and actual chain length was achieved. It was successfully demonstrated that the thiol group of p(L-Cys) can be used to anchor the block copolymers to solid gold surfaces and also gold nanoparticles. The uniform coating of gold surfaces and the bridging between gold nanoparticles leading to rather uniform polymer layers indicate that anchoring PEGylated PAA's via L-Cys anchor sites is a viable approach to the coating of biomedically relevant surfaces.

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