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Ultralow protein adsorbing coatings from clickable PEG nanogel solutions: Benefits of attachment under salt-induced phase separation conditions and comparison with PEG/albumin nanogel coatings

Casey D. Donahoe[§], Thomas L. Cohen[†], Wenlu Li[‡], Peter K. Nguyen[§], John D. Fortner[‡], Robi D. Mitra^{§,†}, and Donald L. Elbert^{§,*}

[§]Department of Biomedical Engineering, Washington University in St. Louis, Campus Box 1097, 1 Brookings Drive, St. Louis, MO 63130, United States

[†]Department of Genetics, Washington University in St. Louis, Campus Box 8510, 4444 Forest Park Boulevard, St. Louis, MO 63108, United States

[‡]Department of Energy, Environmental, & Chemical Engineering, Washington University in St. Louis, 1 Brookings Drive, St. Louis, MO 63130, United States

Abstract

Clickable nanogel solutions were synthesized by using the copper catalyzed azide/alkyne cycloaddition (CuAAC) to partially polymerize solutions of azide and alkyne functionalized poly(ethylene glycol) (PEG) monomers. Coatings were fabricated using a second click reaction: a UV thiol-yne attachment of the nanogel solutions to mercaptosilanated glass. Because the CuAAC reaction was effectively halted by the addition of a copper-chelator, we were able to prevent bulk gelation and limit the coating thickness to a single monolayer of nanogels in the absence of the solution reaction. This enabled the inclusion of kosmotropic salts, which caused the PEG to phase-separate and nearly double the nanogel packing density, as confirmed by Quartz Crystal Microbalance with Dissipation (QCM-D). Protein adsorption was analyzed by single molecule counting with total internal reflection fluorescence (TIRF) microscopy and cell adhesion assays. Coatings formed from the phase-separated clickable nanogel solutions attached with salt adsorbed significantly less fibrinogen than other 100% PEG coatings tested, as well as poly-L-lysine-*g*-PEG (PLL-*g*-PEG) coatings. However, PEG/albumin nanogel coatings still outperformed the best 100% PEG clickable nanogel coatings. Additional surface crosslinking of the clickable nanogel coating in the presence of copper further reduced levels of fibrinogen adsorption closer to those of PEG/albumin nanogel coatings. However, this step negatively impacted long-term resistance to cell adhesion and dramatically altered the morphology of the coating by atomic force microscopy (AFM). The main benefit of the click strategy is that the partially polymerized solutions are stable almost indefinitely, allowing attachment in the phase-separated state without danger of bulk gelation, and thus, producing the best performing 100% PEG coating that we have studied to date.

*Corresponding Author: elbert@biomed.wustl.edu.

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Supporting Information. Synthesis of PEG-azide and PEG-cyclooctyne, SEC profiles, TIRF images, micrographs of VS:BSA and VS:Am nanogel coatings with surface crosslinking in 1.5 M salt, cell adhesion to nanogel coatings after 5 seedings, and cell adhesion to clickable nanogel coatings attached via SPAAC. This material is available free of charge via the Internet at <http://pubs.acs.org>.

INTRODUCTION

While materials including polyoxazolines¹ and betaines² have garnered significant attention as biocompatible coatings, PEG is still widely used for the surface modification of materials requiring “inert” or specifically defined interactions with biological environments. Strategies for incorporating PEG have evolved from covalent grafting of linear or star shaped chains^{3, 4} and adsorption of block copolymers⁵ to surface initiated polymerization of patterned brush coatings⁶ or microscopically thin hydrogels,⁷ and deposition of preformed hydrogel particles.^{8–11} However, advances have not kept pace with the demands of implantable biomaterials as well as *in vitro* diagnostics^{9, 12} both of which remain limited by the lowest levels of protein adsorption achievable.^{13, 14}

PEG coatings derived from microgel or nanogel solutions allow convenient means to create dense yet thin hydrogel coatings (< 100 nm), to enhance areal coverage on surfaces with sparse covalent attachment sites, and to create modular surface assemblies. Most often microgel solutions are fabricated using free radical polymerization with PEG-diacrylates,^{10, 15, 16} although examples formed from addition polymerization of macromers has been well reviewed.¹⁷ Microgel size is typically determined by the surfactant added to produce aqueous emulsions^{10, 18} or inverse emulsions,^{11, 19} although solutions of nanogels or small microgels have been fabricated without the use of surfactants or organic solvents all together.^{8, 20} Relatively fewer examples exist of microgel solutions applied as surface coatings compared to their applications as drug delivery vehicles. Libera et al. has developed several examples of such coatings including PEG-amine microgels crosslinked *in situ* in the dry state with an electron beam²¹ and PEG-diacrylate/acrylic acid microgel solutions electrostatically deposited on PLL.¹¹ Lyon et al. also has created various microgel compositions of PEG and pNIPAm to allow thermal collapse of the microgels.¹⁵ These microgel solutions have been attached to amino-silanated glass via EDC coupling or electrostatic interactions after centrifugation or spin coating.^{10, 22} In a unique approach Norde et al. studied protein adsorption and bacterial adhesion to a coating of PEG-coated silicon nanoparticles that were electrostatically deposited and crosslinked to each other with PEG-diacrylate to provide a more mechanically robust coating.¹⁶

We previously demonstrated multiple rounds of resistance to *in vitro* fibrinogen-promoted cell adhesion of thin coatings produced from PEG nanogel solutions⁸ and quantified lower numbers of individually adsorbed proteins when compared to other popular surface coatings, including covalently coupled monolayers of: bovine serum albumin (BSA), BSA/gelatin, dextran, linear polyacrylamide, glucose, IgG, linear PEG, and multi-arm PEG.^{12, 23} Interestingly, a hybrid nanogel coating of PEG crosslinked with BSA, outperformed a 100% PEG nanogel coating, although a monolayer of eight-arm PEG was shown to be more repellent than a monolayer of BSA.¹² While the mechanism behind this phenomenon remains unknown, elimination of the albumin component remains desirable for implantable biomaterials due to possible immunogenic responses²⁴ and gradual degradation of the protein. Here we illustrate methods for the attachment of clickable 100% PEG nanogel solutions that significantly improve the coating's resistance to protein adsorption, as measured by single molecule counting.

Numerous chemistries have been employed to expand the versatility of PEG end group functionalities and conjugation strategies. ‘Click’ chemistry has become immensely popular for aqueous bio-conjugations due to the high yields that are quickly achievable under mild physiological conditions.²⁵ Because these reactions tend to be highly specific and exclude native biomolecules, they may be incorporated orthogonally with various other reactive groups allowing construction of more sophisticated and highly functionalized biocompatible materials.²⁶ Despite toxicity concerns for using copper in biological applications, CuAAC

remains the most popular click reaction, and PEG polymers are frequently modified with these functional groups.²⁷ Alternatively, a variety of cyclooctynes have been designed by Bertozzi et al. and implemented in strain-promoted azide/alkyne cycloadditions (SPAAC) in order to obviate the need for a copper catalyst.²⁸ Additionally, photo-initiated thiol-ene and thiol-yne reactions between thiols and either alkenes or alkynes have garnered substantial attention.^{29, 30}

These click chemistries have been widely explored for fabricating PEG hydrogels,^{31, 32} most notably by Anseth et al. and Bowman et al.^{33–36} Hydrogels have been photopatterned using both photonic reduction of the copper catalyst to produce CuAAC hydrogels^{33, 37} and photonic initiation of thiol-ene reactions.^{35, 36} While click chemistry has been used to create or more commonly to functionalize materials below the macroscopic scale, we have found only one example where it was used in crosslinking PEG to fabricate microgel solutions, which were crosslinked within mesoporous-silica templates.³⁸ Surfaces have also been endowed with clickable moieties by grafting clickable PEGs to silicon or glass substrates or by chemically modifying PEG surfaces after deposition.^{39–41} SPAAC moieties have also been applied to cellular surfaces, without the concern of copper toxicity.⁴² Deposition of azide-terminated alkanethiolates onto gold surfaces allows for the creation of clickable self assembled monolayers (SAMs).^{43, 44} Alternatively, thin coatings including a polyelectrolyte film⁴⁵ and PEG hydrogel³² have been crosslinked via click reactions on top of silicon or glass surfaces. Click chemistry has been used for the covalent attachment of PEG coatings to the underlying surface by using substrates such as: alkyne or thiol functionalized silanes and resins,^{32, 46, 47} azide functionalized epoxy resins,⁴⁸ or a unique cyclooctyne functionalized epoxy silane.⁴⁹

In this study we exploited separate, sequential click reactions for the crosslinking of PEG as well as its attachment to glass surfaces to form thin hydrogel coatings. Using CuAAC we found that we could rapidly generate nanogel solutions from multi-armed PEG-azide and PEG-alkyne monomers and then easily halt the polymerization with addition of a copper-chelating agent to prevent bulk gelation (Scheme 1A). The ability to halt nanogel growth and solution reaction is a unique feature of this system that allows for stable long-term storage, limitless reaction times on surfaces, and monolayer coverage with the hydrogel coating. Nanogel solutions were mixed with a photoinitiator and attached to mercaptosilanated glass using the UV thiol-yne reaction (Scheme 1B). We quantified protein adsorption to this clickable nanogel coating using TIRF microscopy, which enabled single molecule (i.e. single protein) counting, but determined that hybrid PEG/albumin nanogel coatings still greatly outperformed the 100% PEG nanogel coatings attached in PBS. However, by attaching the clickable nanogel coatings in the phase-separated state using high concentrations of a kosmotropic salt, we were able to double the packing density (Scheme 1C) and drastically reduce protein adsorption. Attaching linear PEG under phase separation, or “cloud point”, conditions has been well known to successfully increase grafting density though similar results were not obtained with star PEG.^{50, 51} We also studied the effects of crosslinking density within the nanogel coating by surface crosslinking residual end groups after attachment (Scheme 2A), which could likewise be performed under phase separation conditions with the addition of salt (Scheme 2B). These modifications produced a surface that was comparable the PEG/albumin coatings by TIRF. However, AFM revealed drastic changes in coating morphology when coatings were further crosslinked and long-term resistance to cell adhesion was lost. Thus, the best 100% PEG coating that we have studied to date is a clickable nanogel coating attached with salt in the phase-separated state. Other 100% PEG nanogel solutions cannot be conjugated in the phase-separated state due to bulk polymerization, indicating a potentially important orthogonality of copper-catalyzed click chemistry.

EXPERIMENTAL METHODS

Materials

Unless otherwise specified, all reagents were purchased from Sigma Aldrich (Saint Louis, MO, USA). Four- and eight-arm PEG-OH MW 10,000 (Creative PEGWorks, Winston Salem, NC, USA), four-arm PEG-alkyne MW 10,000 (Creative PEGWorks), PEG-dithiol MW 3,400 (Creative PEGWorks), and dibenzocyclooctyne (DBCO-acid, Click Chemistry Tools, Scottsdale, AZ, USA) were purchased commercially. Eight-arm PEG-vinyl sulfone (PEG-VS) and eight-arm PEG-amine were synthesized as described previously,⁸ and four-arm PEG-azide and four-arm PEG-cyclooctyne were synthesized as described in the supporting information all from PEG-OH MW 10,000. PLL-*g*-PEG was synthesized in a previous study from PLL MW 375,000 and monomethoxy PEG MW 5,000 at a 7:1 grafting ratio.⁵²

Nanogel Solution Syntheses

Clickable nanogel solutions were synthesized from four-arm PEG-azide and four-arm PEG-alkyne. Each was dissolved to a concentration of 200 mg/mL in a solution of phosphate buffered saline (PBS) already containing 50 mg/mL sodium L-ascorbate and filtered with 0.22 μ m syringe filters. PEG solutions were mixed at equimolar ratios of azides to alkynes, and copper(II) sulfate (10 mg/mL in water) was added at 0.004 equivalents per azide/alkyne pair (4 μ L per 1 mL of PEG) then immediately vortexed. The mixture was aliquoted into 1.5 mL microcentrifuge tubes and rotated at 40 r.p.m. at room temperature until a nanogel size of 100 nm was surpassed as measured by dynamic light scattering (DLS) in a manner described previously.⁸ The reaction was halted by adding 10 equivalents of ethylenediaminetetraacetic acid (EDTA; 0.1 M in water) per copper (16 μ L per 1 mL of nanogel solution) and vortexed immediately. Nanogel solutions were allowed to mix for an additional hour and then frozen at -80°C . Nanogel solutions crosslinked via Michael addition chemistry were synthesized from PEG-VS and either BSA or PEG-amine as described before and diluted to 100 mg/mL to slow the solution reaction before freezing at -80°C .⁸

Size Exclusion Chromatography (SEC)

Molecular weight distributions were approximated by SEC using a 300×8 mm gel filtration column packed with $5\mu\text{m}$ particles with 150 Å pore size (Keystone Scientific Inc., Bellefonte, PA, USA). A chelated clickable nanogel solution (200 mg/mL), its precursor PEG-alkyne solution (100 mg/mL) including sodium ascorbate (50 mg/mL), and various solution standards of different MW linear PEGs dissolved in PBS were each injected (10 μ L) into a mobile phase of 0.5 M dibasic sodium phosphate in water flowing at 1 mL/min. The column flow through was analyzed with a UV detector at 200 nm and the peaks were integrated using XCalibur software between time points specified by the peak retention times of the linear PEG standards.

Nanogel Coatings

All nanogel coatings were attached to 12 mm round glass coverslips (Ted Pella Inc., Redding, CA, USA) or, for TIRF, 24×40 mm rectangular coverslips (Fisher Scientific, Waltham, MA, USA) which were mercaptosilanated as described before.⁸ Briefly, coverslips were washed with water, ethanol, and acetone then immersed in 5% (v/v) solutions of (3-mercaptopropyl)trimethoxy silane (MPTS) in acetone for 1 h at room temperature. Coverslips were washed with acetone and cured for 25 min at 110°C before being stored under vacuum. Volumes of clickable nanogel solutions were mixed 1:1:1 with the photoinitiator 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropionone (Irgacure-2959 or

I-2959; 0.15 mg/mL in water) and either PBS or 1.5 M sodium sulfate in PBS. Nanogel solutions without sodium sulfate were incubated on top of the glass surface and under a UV flood lamp (365 nm, 100 W) for 20 min to allow surface attachment by thiol-yne reaction. Alternatively, the coverslips were inverted on top of 100 μ L droplets of nanogel solutions which did include sodium sulfate, sandwiching the mixture with an upper PEG-rich phase between the mercaptosilane above and a hydrophobic glass dish below (treated with Sigmacote). After 5 min to allow complete phase separation, these coverslips were exposed to the same UV treatment. Michael addition nanogel solutions were incubated on top of the coverslips at 37°C for 1 h (PEG-VS/BSA) or 40 min (PEG-VS/PEG-amine) and unreacted vinyl sulfone groups capped at 37°C with either 100 mg/mL PEG-dithiol in PBS for 30 min for TIRF or 50 mg/mL BSA or PEG-amine overnight for cell adhesion studies. All coverslips were washed thoroughly with PBS. Clickable nanogel solutions were also attached to glass without UV. Briefly, glass was silanated with (3-chloropropyl)trimethoxy silane as with MPTS above, then subjected to halide-azide exchange by incubating the coverslips with 5 mg/mL sodium azide in dimethylformamide (DMF) for 48 h at room temperature under gentle agitation.⁵³ The azidosilanated coverslips were then incubated with solutions of 200 mg/mL four-arm PEG-cyclooctyne in PBS for 30 min at 37°C and washed. Clickable nanogel solutions were finally incubated with the cyclooctyne functionalized surfaces for another 30 min at 37°C, attaching via SPAAC. Also, non-silanated coverslips were incubated with 2 mg/mL PLL-*g*-PEG in PBS (0.22 μ m filtered) for 1 h at room temperature,⁵² as a standard for TIRF and cell adhesion.

TIRF

Single molecule counting of adsorbed proteins was performed with TIRF microscopy in custom flow cells which attach to the silanated coverslips to form ~50 μ L channels. Nanogel coatings were fabricated *in situ* as described above, with flow cells being inverted during the UV incubation of clickable nanogel solutions attached with salt. For each experiment, a fresh solution of bovine fibrinogen was fluorescently labeled overnight at room temperature with Cy5 dye (GE Healthcare Biosciences, Pittsburgh, PA). The fibrinogen was dissolved at 2.5 mg/mL in 0.1 M sodium carbonate buffer (pH 9.3) and mixed with 171x molar excess of Cy5 (10 mg/mL in dimethyl sulfoxide; DMSO). Unreacted dye was removed by dialysis. Nanogel coated flow cells were washed with 1 mL of PBS and incubated with the Cy5-labeled fibrinogen for 1 h in the dark at room temperature. The flow cells were washed again and imaged as described before.¹² Briefly, the surfaces were excited with a 640 nm, 40 mW laser and imaged with a Nikon TE-2000 inverted microscope (Nikon, Melville, NY). Five 263.6 $\times$ 263.6 μ m images of each surface were processed with MetaMorph and Matlab, using the average count for each surface. Protein counts were normalized against standards in each experiment to account for variations in fibrinogen concentration and labeling efficiency. Statistical significance ($p < 0.05$) was calculated in Matlab using ANOVA with a post-hoc Tukey-Kramer test.

QCM-D

Mass deposition was measured on 5 MHz quartz crystal sensors with 50 nm silicon dioxide coatings (QSX-303, Q-Sense, Sweden). Sensors were cleaned prior to each experiment with 10 min treatment in a UV-ozone chamber, 30 min incubation in 2% sodium dodecyl sulfate (SDS), thorough rinsing with deionized water, drying under pure nitrogen, and 10 min more of UV-ozone treatment. Cleaned sensors were silanated and immediately placed in the chambers of a Q-Sense E4 (Q-Sense). PBS was flowed over the sensors at 0.1 mL/min for at least 1 h at 22°C and frequency (F) and dissipation (D) baselines were recorded. The sensors were removed and functionalized as described above, incubating nanogel solutions without salt on top of the sensors and nanogel solutions with salt beneath the inverted sensors during UV treatment. Because the sensors are not transparent, UV light was applied from below in

the case of the latter. The nanogel coated sensors were returned to the Q-Sense E4 and PBS was flowed over the sensors for 2 h and baselines recorded. The baselines from before and after coating application were stitched together to measure changes in F and D. Clickable nanogel coatings attached without salt remained under PBS within the Q-Sense E4 and underwent no surface crosslinking. Alternatively, clickable nanogel coatings attached with salt were surface crosslinked by changing to a flow of 1.5 M sodium sulfate in PBS for 5 min and then 1.5 M sodium sulfate, 2 mM copper(II) sulfate, and 50 mg/mL sodium ascorbate in PBS. The temperature was raised to 37° C for all sensors and incubated for 1 h. The clickable nanogel coating attached with salt was washed with a flow of 0.1 M EDTA in PBS for 1 h at 22°C and then PBS for another 1 h. Changes in mass were modeled with Q-Tools software (version 3.0.16.555, Q-Sense) from F and D changes between the 22 °C PBS baselines at 0.1 mL/min flow surrounding each step. The third, fifth, seventh, ninth, and eleventh overtones were input into a single-layer Voigt (viscoelastic) model with constant fluid density (1000 kg/m³) and viscosity (1.0 × 10⁻³ kg/(m·s)) assumed.

AFM

Nanogel coatings were fabricated on round glass coverslips per normal. Surfaces were kept hydrated and examined under PBS in tapping mode at 1.0 Hz scanning rates using a Veeco Nanoman Scanning Probe Microscope (Veeco Instruments, Plainview, NY) and MESP tips (Bruker AFM Probes, Camarillo, CA, USA) with nominal spring constants of 1 N/m and nominal resonant frequencies of 75 kHz.

Cell Adhesion

Nanogel coatings were fabricated on round glass coverslips per normal and incubated with 2.5 mg/mL fibrinogen for 2 h at 37°C then washed. 3T3 mouse fibroblasts were seeded on the surfaces at 100,000 cells/cm² in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA, USA) and 1% Antibiotic-Antimycotic (ABAM; Life Technologies) and imaged the next day after gentle washing. Surfaces were reseeded every two days until cell adhesion overwhelmed the surface or until 9 seedings. Cell adhesion could alternatively be promoted by conjugating a cysteine-containing cell adhesion peptide, RGD (Seq: Ac-GCGYGRGDSPG-NH₂; GenScript, Piscataway, NJ, USA), to the nanogel coating using a UV thiol-yne reaction. Briefly, 200 nmol of RGD (MW 1066.12) in 200 µL of PBS was incubated on the nanogel surface and subjected to UV irradiation for 20 min, allowing covalent reaction between the nanogel solution alkynes and cysteine thiols. Percentage of surface coverage by adhered cells was approximated for some images using ImageJ software.

RESULTS AND DISCUSSION

Clickable nanogel solutions were successfully created by mixing solutions of four-arm PEG-azide and four-arm PEG-alkyne dissolved in PBS with high amounts of sodium ascorbate and adding small volumes of highly concentrated copper. The rate of growth was effectively modulated with copper concentration (Figure 1), which allowed faster production of nanogel solutions than possible with the Michael type addition.⁸ These kinetics resemble those achieved by Hu et al.⁵⁴ To allow sufficient time for DLS measurements, we chose a concentration of 0.004 equivalents per azide/alkyne for most syntheses resulting in reaction times of approximately 2 h to reach 100 nm. Chelation with 10x EDTA stopped the growth in nanogel size and in fact, when solutions were remeasured 1 h later showed slight decreases in hydrodynamic diameter (e.g. from 111.7 to 95.0 nm). The reduction in size might stem from eliminating the pseudo-crosslinks formed by copper complexes between PEG-alkynes⁵⁵ or increased hydrophilicity of the nanogels in the presence of copper,

resulting in more swelling of the nanogel before chelation. We verified the capacity of copper(II) sulfate to complex multiple alkynes by adding a high concentration of copper, 0.5 equivalents, to a 20% (w/v) solution of PEG-alkyne dissolved in PBS with 50 mg/mL sodium ascorbate to form a solid hydrogel. This gel was dissolved when incubated in 0.1 M EDTA overnight. Nanogel formation was further corroborated by the large shift in molecular weight distribution observed when comparing SEC profiles of PEG-alkyne to that of a 120 nm nanogel solution (Figure S1). The shift was quantified by integrating the areas between multiple time points established by linear PEG benchmarks of various molecular weights and showed large increases in fractions larger than PEG 35,000 and PEG 100,000 and a marked decrease in the fraction smaller than PEG 8,000 (Table 1). This demonstrates the presence of nanogels in these partially polymerized solutions, and in much greater proportions than expected from Flory-Stockmeyer statistics.⁸

The ability of the clickable nanogel coatings attached to mercaptosilanated glass to resist protein adsorption was quantified using TIRF microscopy to count individually adsorbed, fluorescently labeled proteins (Figure 2). The irradiation time of 20 min used to attach the coatings was selected as long enough to crosslink 100 μ L bulk hydrogels of 50 mg/mL four-arm PEG-alkyne with equimolar amounts of PEG-dithiol, but short enough to minimize evaporation induced from the lamp heat. Controls including Michael addition nanogel solutions of PEG-VS crosslinked with either BSA (VS:BSA) or PEG-amine (VS:Am) were also evaluated. Cy5-labeled fibrinogen was used as a model protein relevant to blood contacting biomaterials, but we found that 1 h incubations at physiological concentration (2.5 mg/mL) saturated even the best coatings, such that adsorbed proteins could not be individually resolved and counted. Adsorption to most nanogel coatings was quantified using a 1:100 dilution of the physiological concentration (Figure 2A), while adsorption to several less resistant coatings was quantified using a 1:1000 dilution (Figure 2B). Previously, VS:BSA nanogel coatings were shown more resistant to IgG adsorption¹² and cell adhesion⁸ than VS:Am nanogel coatings. Here, we confirmed the extremely low amount of fibrinogen adsorption on the VS:BSA nanogel coatings by TIRF and compared the resistance to the commonly used non-covalent coating PLL-*g*-PEG. While PLL-*g*-PEG has performed well at resisting protein adsorption as measured by relatively insensitive measurements such as Optical Waveguide Lightmode Spectroscopy (OWLS), here we found that PLL-*g*-PEG adsorbed too much fibrinogen to quantify by TIRF (Figure 2A). Furthermore, although the VS:Am nanogel coating adsorbed more than 100x the number of fibrinogen molecules as that of the VS:BSA nanogel coating, the VS:Am coating was still superior to PLL-*g*-PEG. Clickable nanogel coatings attached in PBS (i.e. not phase-separated) could not be quantified at the standard dilution of 1:100 of fibrinogen, similar to PLL-*g*-PEG. At a 1:1000 dilution of fibrinogen, clickable nanogel coatings attached in PBS adsorbed more than 2x the number of proteins as the VS:Am nanogel coatings (Figure 2B). The two-fold difference between the clickable and VS:Am nanogel coatings was unexpected as both are composed of 10 kDa PEG monomers crosslinked by different chemistries into similarly sized nanogel solutions. This discrepancy may be due to differences in the nanogel architecture, considering the VS:Am nanogel solutions utilize 8-arm PEGs and the clickable nanogel solutions utilize 4-arm PEGs. Differences in arm length, mesh sizes, or density of unreacted chains might account for this result, but the mechanism remains unknown. Also, potential effects of positively charged amine groups within the VS:Am nanogel coating on resisting fibrinogen adsorption cannot be discounted.

We then tested the resistance to fibrinogen adsorption of clickable nanogel solutions attached with salt in the phase-separated state. When the clickable nanogel solutions were mixed with sodium sulfate to a final concentration of 500 mM, they became cloudy and rapidly phase-separated within minutes to produce a densely packed PEG-rich phase atop the solution. Glass flow cells were inverted to juxtapose the mercaptosilanated surface to the

PEG-rich phase during attachment of the clickable nanogel solution, resulting in a 10 fold reduction in protein adsorption (Figure 2A). Clickable nanogel solutions attached with salt in the phase-separated state were superior to VS:Am nanogel coatings but inferior to VS:BSA coatings (Figure 2A). VS:BSA and VS:Am nanogel solutions cannot be attached in the same manner without resulting in visibly thick hydrogel films. The Michael addition reaction between vinyl sulfones and amines occurs at a non-negligible rate at the higher PEG concentrations that develop after phase separation and the nanogels rapidly crosslink into microspheres and larger aggregates.⁵⁶

The other processing step studied via TIRF was the surface crosslinking of residual PEG chains left unreacted after nanogel formation and surface attachment. Surface crosslinking was carried out by incubating the clickable nanogel coatings in PBS spiked with sodium ascorbate and copper for 1 h at 37°C. In attempting to stitch the nanogel coatings into more densely packed hydrogel films, surfaces were also incubated in 1.5 M salt to induce phase separation along with sodium ascorbate and various concentrations of copper to promote graduated extents of crosslinking. Surface crosslinking with 2 mM copper in either PBS or 1.5 M salt provided modest improvements in resisting protein adsorption. Incubation in salt without any copper had no significant effect on overall protein adsorption but had larger variability than seen otherwise. Addition of higher amounts of copper (8 mM) eradicated any substantial resistance to protein adsorption. These saturated protein counts most likely originate from damage to the surface caused by stress introduced by the additional crosslinking. Large areas available for protein adsorption seem to be visible under TIRF microscopy when compared to fluorescent images of other nanogel coatings (Figure S2). Surface crosslinking was replicated in VS:BSA and VS:Am nanogel coatings by incubating the coatings in PBS or 1.5 M salt overnight at 37°C. These incubations provided some extended opportunity for the slower Michael type crosslinking reaction, before vinyl sulfones were capped per usual. While crosslinking (i.e. incubation) in PBS provided no notable changes, crosslinking in 1.5 M salt slightly worsened the VS:Am nanogel coating and obliterated the resistance of the VS:BSA nanogel coating. The VS:BSA and VS:Am coatings were visibly damaged by the phase separation process (Figure S3), providing evidence for the potentially damaging effects of internally crosslinking such thin surface coatings.

To be certain that the observed differences in protein adsorption were not a product of the variability in nanogel size, we produced three stocks of different VS:BSA nanogel sizes and two stocks of different clickable nanogel sizes from the same precursor solutions of each type. Coatings produced from all sizes of the VS:BSA nanogels (53–102 nm) still outperformed coatings produced from all sizes of clickable nanogels attached with salt without surface crosslinking (84–128 nm) by similar margins (Figure 3). The data seem to suggest that all the nanogel sizes examined are above a threshold necessary to achieve maximal surface coverage on the silanated glass. However, nanogel formation remains critical in achieving low protein adsorption as evidenced by previous comparisons of VS:BSA nanogel solutions to monolayers of eight-arm PEG-VS.¹²

We hypothesized that the drastic improvement in protein resistance of the clickable nanogel coatings when attached with salt was due to increased PEG concentrations in the phase-separated state. We expected that collapsed nanogels would attach at higher packing densities than in the swollen state. Indeed, QCM-D found that the nanogel coatings attached with salt had approximately twice the increase in surface density as the nanogel coatings attached without salt (Table 2). The clickable nanogel coatings were attached *ex situ* under UV and QCM-D baselines measured from before and after coating the sensors were stitched together to model changes in mass. We measured the error that could be expected from removing these sensors during analysis by replicating the procedure with sensors that were

left unmodified then returned to PBS flow. The magnitude of error ($244 \pm 214 \text{ ng/cm}^2$; $2.44 \pm 2.14 \text{ nm}$ of water) was found to be negligible relative to the masses of the nanogel coatings measured. The equivalent thickness of water provides a maximum estimate for the coating thickness, as we assume the nanogel coatings, comprised of unknown proportions of PEG and water, to be perhaps slightly denser than water. VS:BSA nanogel coatings were previously determined to be 1001.2 kg/cm^3 using a combination of QCM-D and OWLS.⁸ As nanogel packing density will affect the mass measurement and this calculation of average thickness, the clickable nanogel coating attached without salt (50.3 nm) appears to be packed less densely than the VS:BSA nanogel coating (75.2 nm, previously reported⁸). However, if the clickable nanogel coating is attached with salt the average thickness increases (106.4 nm) beyond that of the VS:BSA nanogel coating. Increased packing density clearly improved the clickable nanogel coating's resistance to protein adsorption measured by TIRF, but could not alone account for the low levels found on VS:BSA nanogel coatings. Clickable nanogel coatings attached with salt seem to approach maximal packing density as the average thickness is near the approximate 100 nm diameter of the nanogels.

The clickable nanogel coating attached with salt was further used to study *in situ* the effects of surface crosslinking in 1.5 M salt with 2 mM copper. The very slight fluctuation in mass observed during this step (80 ng/cm^2) was actually less than that observed on the clickable nanogel coating attached without salt, which remained incubated in PBS but was subjected to the same temperature of 37°C during this step (510 ng/cm^2). This confirms that the nanogel coating was well washed after attachment and that subsequent addition of copper did not attach any residual PEG in solution but only crosslinked the existing hydrogel structure.

Multiple nanogel coatings were analyzed with tapping mode AFM under PBS to assess topological differences that arise from the conditions explored (Figure 4). Phase profiles were examined in addition to height profiles, revealing changes in material properties along the surface. Images of clickable nanogel coatings became distinctly less resolute and more difficult to obtain when attached with salt (Figure 4A vs. Figure 4B). Although not all the nanogels of this coating are individually resolvable, AFM did not detect any deep gaps in surface coverage as evidenced by the relatively small height changes measured (less than 5 nm). It is more likely that the nanogels are packed so tightly together that the boundaries of these soft particles are actually intermeshed, impeding the ability of AFM to detect their edges. On the other hand, extended overnight surface crosslinking in 1.5 M salt with 1 mM copper strongly improved resolution of the clickable nanogel coating attached with salt (Figure 4C), enabling imaging on smaller spatial scales (Figure 4D). The phase profile clearly shows a tightly packed coating of spherical nanogels. These surfaces also exhibit curious circular patches of raised nanogels that were especially well resolved by AFM. We can assume that nanogel coatings attached with salt but without surface crosslinking are packed just as tightly since the surface attachment step is identical. Nanogels of all coatings measure reasonably close to 100 nm. Some shrinking in the final nanogel size might be expected with surface crosslinking in 1.5 M salt. However, the high packing density of these coatings may allow some reduction in nanogel size without compromising surface coverage. Otherwise, interstitial gaps may begin to appear between the nanogels as they shrink. This might explain why surface crosslinking in 1.5 M salt did not garner significant improvements in the VS:BSA or VS:Am nanogel coatings. Interstitial gaps resulting from nanogel shrinkage or stress-induced surface damage may prove to be quite detrimental as they may allow adsorption of proteins that are smaller than fibrinogen within the gaps.

Non-specific cell adhesion was examined as a corroborative, although less sensitive method of qualifying protein adsorption, as cells attach to artificial surfaces via layers of adsorbed proteins. Surfaces were, therefore, first incubated for 2 h with 2.5 mg/mL fibrinogen to more

quickly elicit any possible cell adhesion responses. We studied most of the same conditions investigated with TIRF with all surface crosslinking being performed overnight (Figure 5). Additionally, controls of a PLL-*g*-PEG coating, a common non-fouling surface, and an RGD-conjugated clickable nanogel coating attached with salt without surface crosslinking were seeded. Despite its inferior resistance to protein adsorption as measured by TIRF, the PLL-*g*-PEG coating completely resisted cell adhesion. Although the lack of correlation between results is surprising, other examples exist of PEG surfaces with relatively high protein adsorption that also resist cell adhesion.⁵⁷ Adhesion of well-spread cells to the RGD-conjugated coating demonstrates the effectiveness of the UV thiol-yne chemistry for the attachment of cysteine-containing peptides and demonstrates a lack of toxicity from the leaching of any residual copper. Again the VS:BSA nanogel coatings performed very well with no significant cell adhesion after 1 round of seeding. However, both the VS:Am nanogel coating and the clickable nanogel coating attached with salt without surface crosslinking appeared equally cell free, despite the disparities seen with TIRF. The clickable nanogel coatings attached without salt performed noticeably worse, as expected, but displayed some degree of resistance to cell adhesion. Subtle long-term increases in cell adhesion were observed on the clickable nanogel coatings attached with salt without surface crosslinking versus the VS:BSA and VS:Am nanogel coatings without surface crosslinking, as seen after 5 rounds of cell seeding (Figure S4).

The surface most drastically affected by surface crosslinking was the clickable nanogel coating attached with salt. Surface crosslinking greatly increased cell adhesion on both clickable nanogel surfaces crosslinked with copper in PBS and 1.5 M salt, in contrast to the improvements observed with TIRF. The coatings crosslinked with copper in PBS especially demonstrated an immediate and complete deterioration of its ability to resist cell adhesion, with a complete monolayer of well spread cells forming after 1 seeding. Surface crosslinking within these coatings may have an exaggerated effect because the higher packing density achieved by attachment with salt should allow for more crosslinking opportunities and potentially less damage to the surface. It is difficult to determine why the TIRF and cell adhesion results conflict, but is important to note that other proteins found in the media serum that are much smaller in size than fibrinogen may contribute to cell adhesion, e.g. vitronectin. While we did not study the mechanical properties of our coatings, stiffer (more crosslinked) hydrogels do not necessarily enhance cell adhesion,⁵⁸ and so increased stiffness from surface crosslinking would not necessarily account for our observations. Importantly, the cell adhesion results agreed with TIRF on the superiority of clickable nanogel coatings attached with salt versus those attached without salt if the surface crosslinking step was omitted.

Surface crosslinking of the VS:BSA nanogel coating in 1.5 M salt led to adhesion of large cell aggregates, consistent with the increase in protein adsorption measured by TIRF. The VS:Am nanogel coating with surface crosslinking in 1.5 M salt, on the other hand, initially maintained its resistance for several rounds of cell seeding but then rapidly deteriorated after 5 rounds of seeding. The VS:BSA and VS:Am nanogel coatings with surface crosslinking in PBS maintained similar resistance to cell adhesion as their counterparts with no surface crosslinking until about 9 rounds of seeding, when subtle declines were observed (Figure 6). However, the extent of surface crosslinking that actually occurred overnight without salt-induced phase separation may be mild. The VS:BSA nanogel coating has been shown previously to resist fibroblast adhesion for up to 3 weeks.⁸

Few other studies on biocompatible surface coatings attempt to examine long-term cell adhesion. Most often, surface coatings are evaluated for long-term stability by patterning cells onto selectively adhesive regions of the surface and monitoring the long-term pattern integrity, which is deteriorated as cells migrate onto and firmly adhere to the protein

resistant regions. Study to study comparisons are difficult as cell type, cell seeding density, media content, substrate material, and the various strategies for attaching the chemically similar polymers may affect results. The dimensions, shapes, and ligands used to promote adhesion in patterned cell studies add additional sources of variability, making studies that compare multiple types of coatings valuable. Still several of the best examples of PEG coatings demonstrate superb resistance to fibroblast adhesion for multiple weeks similar to our results, but not necessarily for multiple months. Patterned oligo(ethylene glycol) terminated SAMs began to collapse after 1 week,⁵⁹ PLL-*g*-PEG grafted at high temperatures maintained pattern integrity for at least 36 days,⁵¹ and PEG-functionalized polymer brush coatings resisted twice-a-week cell seedings for 3 weeks after which gradual increases in adhesion were observed.⁶⁰

Finally, we also demonstrated that clickable nanogel solutions could be attached in a UV-free process to cyclooctyne functionalized azidosilanated glass, exhibiting comparable resistance to cell adhesion after 1 seeding (Figure S5). However, for unknown reasons, these clickable nanogel coatings produced saturated counts on TIRF (data not shown).

CONCLUSION

We have shown click chemistry to be a useful tool in the facile synthesis and attachment of 100% PEG nanogel coatings. Particularly, fabrication of nanogel solutions by CuAAC enabled attachment to surfaces in the phase-separated state without risk of bulk gelation. The increased nanogel surface density resulted in greatly improved resistance to protein adsorption by TIRF. The additional step of surface crosslinking was also easily performed with CuAAC and showed that higher crosslinking densities provide modest improvements in resistance to fibrinogen adsorption, but greatly negatively impacted resistance to cell adhesion. These clickable nanogel coatings represent the best coatings of 100% PEG that we have examined for short-term protein resistance by TIRF and provide new insights into structural properties of coatings that affect the adsorption of proteins. The use of clickable functionalities further eliminates any non-specific conjugations to biomolecules that might occur through either reaction with insufficiently capped vinyl sulfone groups or electrostatic interactions with amine groups. However, the results further establish that PEG/albumin nanogel solutions are superior for producing protein resistant coatings, suggesting synergy between the PEG and albumin in resisting protein adsorption and cell adhesion. Lack of long-term resistance to cell adhesion currently precludes these particular PEG nanogel coatings and similar surface coatings from *in vivo* applications, including biosensors and medical implants. However, as we have demonstrated significantly lower levels of protein adsorption compared to PEG-*g*-PLL and other previously studied coatings, the nanogel coatings seem to comprise superior surfaces for *in vitro* biological diagnostics or other applications requiring strong short-term resistance to protein adsorption.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Konradi R, Acikgoz C, Textor M. Polyoxazolines for nonfouling surface coatings - a direct comparison to the gold standard PEG. *Macromol Rapid Commun.* 2012; 33(19):1663–76. [PubMed: 22996913]
2. Inoue Y, Ishihara K. Reduction of protein adsorption on well-characterized polymer brush layers with varying chemical structures. *Colloids Surf B Biointerfaces.* 2010; 81:350–357. [PubMed: 20705439]
3. Jo S, Park K. Surface modification using silanated poly(ethylene glycol)s. *Biomaterials.* 2000; 21(6):605–16. [PubMed: 10701461]
4. Groll J, Ademovic Z, Ameringer T, Klee D, Moeller M. Comparison of coatings from reactive star shaped PEG-stat-PPG prepolymers and grafted linear PEG for biological and medical applications. *Biomacromolecules.* 2005; 6(2):956–62. [PubMed: 15762665]
5. Lee JH, Kopecek J, Andrade JD. Protein-resistant surfaces prepared by PEO-containing block copolymer surfactants. *J Biomed Mater Res.* 1989; 23(3):351–68. [PubMed: 2715159]
6. Ahmad SA, Leggett GJ, Hucknall A, Chilkoti A. Micro- and nanostructured poly[oligo(ethylene glycol)methacrylate] brushes grown from photopatterned halogen initiators by atom transfer radical polymerization. *Biointerphases.* 2011; 6(1):8–15. [PubMed: 21428690]
7. Kizilel S, Sawardecker E, Teymour F, Perez-Luna VH. Sequential formation of covalently bonded hydrogel multilayers through surface initiated photopolymerization. *Biomaterials.* 2006; 27(8): 1209–15. [PubMed: 16157371]
8. Scott EA, Nichols MD, Cordova LH, George BJ, Jun YS, Elbert DL. Protein adsorption and cell adhesion on nanoscale bioactive coatings formed from poly(ethylene glycol) and albumin microgels. *Biomaterials.* 2008; 29(34):4481–93. [PubMed: 18771802]
9. Saaem I, Papasotiropoulos V, Wang T, Soteropoulos P, Libera M. Hydrogel-based protein nanoarrays. *J Nanosci Nanotechnol.* 2007; 7(8):2623–32. [PubMed: 17685276]
10. South AB, Whitmire RE, Garcia AJ, Lyon LA. Centrifugal deposition of microgels for the rapid assembly of nonfouling thin films. *ACS Appl Mater Interfaces.* 2009; 1(12):2747–54. [PubMed: 20356152]
11. Wang Q, Uzunoglu E, Wu Y, Libera M. Self-assembled poly(ethylene glycol)-co-acrylic acid microgels to inhibit bacterial colonization of synthetic surfaces. *ACS Appl Mater Interfaces.* 2012; 4(5):2498–506. [PubMed: 22519439]
12. Tessler LA, Donahoe CD, Garcia DJ, Jun YS, Elbert DL, Mitra RD. Nanogel surface coatings for improved single-molecule imaging substrates. *J R Soc Interface.* 2011; 8(63):1400–8. [PubMed: 21325313]
13. Vogler EA. Protein adsorption in three dimensions. *Biomaterials.* 2012; 33(5):1201–37. [PubMed: 22088888]
14. Shard AG, Tomlins PE. Biocompatibility and the efficacy of medical implants. *Regen Med.* 2006; 1(6):789–800. [PubMed: 17465760]
15. Bridges AW, Whitmire RE, Singh N, Templeman KL, Babensee JE, Lyon LA, Garcia AJ. Chronic inflammatory responses to microgel-based implant coatings. *J Biomed Mater Res A.* 2010; 94(1): 252–8. [PubMed: 20166218]
16. Holmes PF, Currie EP, Thies JC, van der Mei HC, Busscher HJ, Norde W. Surface-modified nanoparticles as a new, versatile, and mechanically robust nonadhesive coating: suppression of protein adsorption and bacterial adhesion. *J Biomed Mater Res A.* 2009; 91(3):824–33. [PubMed: 19051305]
17. Albrecht K, Moeller M, Groll J. Nano- and Microgels Through Addition Reactions of Functional Oligomer and Polymers. *Advances in Polymer Science.* 2011; 234:65–93.
18. Wu X, El Ghzaoui A, Li S. Aggregates and hydrogels prepared by self-assembly of amphiphilic copolymers with surfactants. *J Colloid Interface Sci.* 2012; 374(1):127–34. [PubMed: 22402181]
19. Mizrahi B, Irusta S, McKenna M, Stefanescu C, Yedidion L, Myint M, Langer R, Kohane DS. Microgels for efficient protein purification. *Adv Mater.* 2011; 23(36):H258–62. [PubMed: 21805505]

20. Kettel MJ, Hildebrandt H, Schaefer K, Moeller M, Groll J. Tenside-free Preparation of Nanogels with High Functional beta-Cyclodextrin Content. *ACS Nano*. 2012; 6(9):8087–93. [PubMed: 22862562]
21. Hong Y, Krsko P, Libera M. Protein surface patterning using nanoscale PEG hydrogels. *Langmuir*. 2004; 20(25):11123–6. [PubMed: 15568866]
22. Nolan CM, Reyes CD, Debord JD, Garcia AJ, Lyon LA. Phase transition behavior, protein adsorption, and cell adhesion resistance of poly(ethylene glycol) cross-linked microgel particles. *Biomacromolecules*. 2005; 6(4):2032–9. [PubMed: 16004442]
23. Tessler LA, Reifenger JG, Mitra RD. Protein quantification in complex mixtures by solid phase single-molecule counting. *Anal Chem*. 2009; 81(17):7141–8. [PubMed: 19601620]
24. Balasse E, Odot J, Gatouillat G, Andry MC, Madoulet C. Enhanced immune response induced by BSA loaded in hydroxyethylstarch microparticles. *Int J Pharm*. 2008; 353(1–2):131–8. [PubMed: 18162346]
25. Kolb HC, Finn MG, Sharpless KB. Click Chemistry: Diverse Chemical Function from a Few Good Reactions. *Angew Chem Int Ed*. 2001; 40:2004–2021.
26. Lim RK, Lin Q. Bioorthogonal chemistry: recent progress and future directions. *Chem Commun (Camb)*. 2010; 46(10):1589–600. [PubMed: 20177591]
27. Hiki S, Kataoka K. Versatile and selective synthesis of “click chemistry” compatible heterobifunctional poly(ethylene glycol)s possessing azide and alkyne functionalities. *Bioconjug Chem*. 2010; 21(2):248–54. [PubMed: 20078027]
28. Jewett JC, Sletten EM, Bertozzi CR. Rapid Cu-free click chemistry with readily synthesized biarylazacyclooctynones. *J Am Chem Soc*. 2010; 132(11):3688–90. [PubMed: 20187640]
29. Hoogenboom R. Thiol-yne chemistry: a powerful tool for creating highly functional materials. *Angew Chem Int Ed Engl*. 2010; 49(20):3415–7. [PubMed: 20394091]
30. Hoyle CE, Lowe AB, Bowman CN. Thiol-click chemistry: a multifaceted toolbox for small molecule and polymer synthesis. *Chem Soc Rev*. 2010; 39(4):1355–87. [PubMed: 20309491]
31. Malkoch M, Vestberg R, Gupta N, Mespouille L, Dubois P, Mason AF, Hedrick JL, Liao Q, Frank CW, Kingsbury K, Hawker CJ. Synthesis of well-defined hydrogel networks using click chemistry. *Chem Commun (Camb)*. 2006; (26):2774–6. [PubMed: 17009459]
32. Lundberg P, Bruin A, Klijnsma JW, Nystrom AM, Johansson M, Malkoch M, Hult A. Poly(ethylene glycol)-based thiol-ene hydrogel coatings-curing chemistry, aqueous stability, and potential marine antifouling applications. *ACS Appl Mater Interfaces*. 2010; 2(3):903–12. [PubMed: 20356297]
33. Adzima BJ, Tao Y, Kloxin CJ, DeForest CA, Anseth KS, Bowman CN. Spatial and temporal control of the alkyne-azide cycloaddition by photoinitiated Cu(II) reduction. *Nat Chem*. 2011; 3(3):256–59. [PubMed: 21336334]
34. Aimetti AA, Machen AJ, Anseth KS. Poly(ethylene glycol) hydrogels formed by thiol-ene photopolymerization for enzyme-responsive protein delivery. *Biomaterials*. 2009; 30(30):6048–54. [PubMed: 19674784]
35. DeForest CA, Polizzotti BD, Anseth KS. Sequential click reactions for synthesizing and patterning three-dimensional cell microenvironments. *Nat Mater*. 2009; 8(8):659–64. [PubMed: 19543279]
36. Fairbanks BD, Schwartz MP, Halevi AE, Nuttelman CR, Bowman CN, Anseth KS. A Versatile Synthetic Extracellular Matrix Mimic via Thiol-Norbornene Photopolymerization. *Advanced Materials*. 2009; 21:5005–5010.
37. Chen RT, Marchesan S, Evans RA, Styan KE, Such GK, Postma A, McLean KM, Muir BW, Caruso F. Photoinitiated alkyne-azide click and radical cross-linking reactions for the patterning of PEG hydrogels. *Biomacromolecules*. 2012; 13(3):889–95. [PubMed: 22332589]
38. Yap HP, Johnston APR, Such GK, Yan Y, Caruso F. Click-Engineered, Bioresponsive Drug Loaded PEG Spheres. *Advanced Materials*. 2009; 21:4384–4352.
39. Wendeln C, Rinnen S, Schulz C, Arlinghaus HF, Ravoo BJ. Photochemical microcontact printing by thiol-ene and thiol-yne click chemistry. *Langmuir*. 2010; 26(20):15966–71. [PubMed: 20857903]

40. Sun XL, Stabler CL, Cazalis CS, Chaikof EL. Carbohydrate and protein immobilization onto solid surfaces by sequential Diels-Alder and azide-alkyne cycloadditions. *Bioconjug Chem*. 2006; 17(1):52–7. [PubMed: 16417251]
41. Liu X, Zheng HN, Ma YZ, Yan Q, Xiao SJ. Microwave irradiated click reactions on silicon surfaces via derivatization of covalently grafted poly(PEGMA) brushes. *J Colloid Interface Sci*. 2011; 358:116–122. [PubMed: 21440900]
42. Krishnamurthy VR, Wilson JT, Cui W, Song X, Lasanajak Y, Cummings RD, Chaikof EL. Chemoselective immobilization of peptides on abiotic and cell surfaces at controlled densities. *Langmuir*. 2010; 26(11):7675–8. [PubMed: 20450194]
43. Hudalla GA, Murphy WL. Using “click” chemistry to prepare SAM substrates to study stem cell adhesion. *Langmuir*. 2009; 25(10):5737–46. [PubMed: 19326875]
44. Hudalla GA, Murphy WL. Immobilization of peptides with distinct biological activities onto stem cell culture substrates using orthogonal chemistries. *Langmuir*. 2010; 26(9):6449–56. [PubMed: 20353153]
45. Such GK, Quinn JF, Quinn A, Tjinto E, Caruso F. Assembly of ultrathin polymer multilayer films by click chemistry. *J Am Chem Soc*. 2006; 128(29):9318–9. [PubMed: 16848452]
46. Ostaci RV, Damiron D, Capponi S, Vignaud G, Leger L, Grohens Y, Drockenmuller, E. Polymer brushes grafted to “passivated” silicon substrates using click chemistry. *Langmuir*. 2008; 24(6):2732–9. [PubMed: 18247640]
47. Ostaci RV, Damiron D, Grohens Y, Leger L, Drockenmuller E. Click chemistry grafting of poly(ethylene glycol) brushes to alkyne-functionalized pseudobrushes. *Langmuir*. 2010; 26(2):1304–10. [PubMed: 19785428]
48. Durmaz YY, Sangermano M, Yagci Y. Surface Modification of UV-Cured Epoxy Resins by Click Chemistry. *J Polym Sci Pol Chem*. 2010; 48:2862–2868.
49. Kuzmin A, Poloukhine A, Wolfert MA, Popik VV. Surface functionalization using catalyst-free azide-alkyne cycloaddition. *Bioconjug Chem*. 2010; 21(11):2076–85. [PubMed: 20964340]
50. Irvine DJ, Mayes AM, Satija SK, Barker JG, Sofia-Allgor SJ, Griffith LG. Comparison of tethered star and linear poly(ethylene oxide) for control of biomaterials surface properties. *J Biomed Mater Res*. 1998; 40(3):498–509. [PubMed: 9570083]
51. Ogaki R, Zoffmann Andersen O, Jensen GV, Kolind K, Kraft DC, Pedersen JS, Foss M. Temperature-Induced Ultradense PEG Polyelectrolyte Surface Grafting Provides Effective Long-Term Bioresistance against Mammalian Cells, Serum, and Whole Blood. *Biomacromolecules*. 2012; 13(11):3668–77. [PubMed: 23082770]
52. Elbert DL, Hubbell JA. Self-assembly and steric stabilization at heterogeneous, biological surfaces using adsorbing block copolymers. *Chem Biol*. 1998; 5(3):177–83. [PubMed: 9545428]
53. Johnson JA, Lu YY, Burts AO, Lim YH, Finn MG, Koberstein JT, Turro NJ, Tirrell DA, Grubbs RH. Core-Clickable PEG-Branch-Azide Bivalent-Bottle-Brush Polymers by ROMP: Grafting-Through and Clicking-To. *J Am Chem Soc*. 2011; 133(3):559–66. [PubMed: 21142161]
54. Hu X, Li D, Zhou F, Gao C. Biological hydrogel synthesized from hyaluronic acid, gelatin and chondroitin sulfate by click chemistry. *Acta Biomater*. 2011; 7(4):1618–26. [PubMed: 21145437]
55. Rodionov VO, Fokin VV, Finn MG. Mechanism of the ligand-free CuI-catalyzed azide-alkyne cycloaddition reaction. *Angew Chem Int Ed Engl*. 2005; 44(15):2210–5. [PubMed: 15693051]
56. Nichols MD, Scott EA, Elbert DL. Factors affecting size and swelling of poly(ethylene glycol) microspheres formed in aqueous sodium sulfate solutions without surfactants. *Biomaterials*. 2009; 30(29):5283–91. [PubMed: 19615738]
57. George PA, Donose BC, Cooper-White JJ. Self-assembling polystyrene-block-poly(ethylene oxide) copolymer surface coatings: resistance to protein and cell adhesion. *Biomaterials*. 2009; 30(13):2449–56. [PubMed: 19201020]
58. Turturro MV, Sokic S, Larson JC, Papavasiliou G. Effective tuning of ligand incorporation and mechanical properties in visible light photopolymerized poly(ethylene glycol) diacrylate hydrogels dictates cell adhesion and proliferation. *Biomed Mater*. 2013; 8(2):025001. [PubMed: 23343533]
59. Luk YY, Kato M, Mrksich M. Self-assembled monolayers of alkanethiolates presenting mannitol groups are inert to protein adsorption and cell attachment. *Langmuir*. 2000; 16:9604–9608.

60. Fan X, Lin L, Messersmith PB. Cell fouling resistance of polymer brushes grafted from ti substrates by surface-initiated polymerization: effect of ethylene glycol side chain length. *Biomacromolecules*. 2006; 7(8):2443–8. [PubMed: 16903694]

Figure 1. Small PEG gels (25 μ L) were formed with a fixed 18% (w/v) PEG concentration and various equivalents of copper. Gelation time was measured to the nearest minute as when the solution became too viscous to pipette. Data is presented as mean $\pm$ standard deviation (n=3).

Figure 2.

Cy5-labeled fibrinogen molecules were counted using TIRF on PLL-*g*-PEG (orange) coatings as well as VS:BSA (red), VS:Am (blue), and clickable (green) nanogel coatings. Nanogel coatings were studied across a matrix of conditions including attachment with salt and surface crosslinking of the coating. Surface crosslinking was achieved in the VS:BSA and VS:Am nanogel coatings by incubating overnight in PBS or 1.5 M salt before capping the reactive groups and by incubating the clickable nanogel coatings in PBS or 1.5 M salt spiked with sodium ascorbate and various amounts of copper for 1 h. Protein counts were normalized against standard nanogel coatings of: (A) VS:BSA or (B) VS:Am. Fibrinogen was incubated for 1 h at a (A) 1:100 dilution or (B) 1:1000 dilution of physiological concentration (2.5 mg/mL) and oversaturated counts were marked using ($\wedge$). Statistical significance ($p < 0.05$) was determined for (A) and (B) separately on unsaturated counts relative to the conditions referenced in the final row with (*, **, #). Data is presented as mean $\pm$ standard deviation.

Figure 3.

Counts of Cy5-labeled fibrinogen adsorbed at 0.25 mg/mL were compared across coatings produced from three different sizes of VS:BSA nanogels (red) and two different sizes of clickable nanogels attached with salt without surface crosslinking (green). The large differences in nanogel size do not account for the consistently lower levels of protein adsorption seen on VS:BSA coatings. Data is presented as mean $\pm$ standard deviation (n=2).

Figure 4.

Images from AFM show height (top) and phase (bottom) profiles for (A) clickable nanogel coatings attached without salt with no surface crosslinking, (B) clickable nanogel coatings attached with salt with no surface crosslinking and (C, D) clickable nanogel coatings attached with salt with surface crosslinking overnight in 1.5 M salt with 1 mM copper. Scale bars all represent 500 nm. The images in (D) are higher spatial resolution images of the sample in (C). The color scale represents 5 nm for the height profiles of (A, B), 20 nm for the height profiles of (C, D), and 30° for all phase profiles.

Figure 5.

Cell adhesion of 3T3 fibroblasts was examined one day after the first seeding at 100,000 cells/cm² on several surfaces that were incubated with 2.5 mg/mL fibrinogen for 2 h at 37°C. Surface crosslinking was allowed overnight on some VS:BSA and VS:Am nanogel coatings before capping and implemented with 2 mM copper overnight on some clickable nanogel coatings both at 37°C. Additionally, control surfaces of PLL-*g*-PEG adsorbed to glass, and clickable nanogel solutions attached with salt with no surface crosslinking and conjugated to RGD via a UV thiol-yne reaction were examined. Approximate percentage of surface coverage by adhered cells is indicated in the insets. The scale bar represents 1 mm.

	No surface crosslinking	Surface crosslinking in PBS	Surface crosslinking in 1.5 M salt
VS:BSA
VS:Am
Clickable attached without salt
Clickable attached with salt

Figure 6.

Cell adhesion of 3T3 fibroblasts was examined one day after the ninth seeding at 100,000 cells/cm² on several surfaces that were initially incubated with 2.5 mg/mL fibrinogen for 2 h at 37°C. Surface crosslinking had been allowed overnight on some VS:BSA and VS:Am nanogel coatings before capping and implemented with 2 mM copper overnight on some clickable nanogel coatings both at 37°C. Approximate percentage of surface coverage by adhered cells is indicated in the insets. The scale bar represents 1 mm.

Scheme 1.
Strategies for synthesis and attachment of clickable nanogel solutions

Scheme 2.
Strategies for surface crosslinking of nanogel coatings

Table 1
Molecular weight distributions

The molecular weight distribution as determined by SEC of a clickable nanogel solution is markedly skewed towards higher weights than that of PEG-alkyne. Percentages of UV detectable content are delineated by linear PEG benchmarks.

Linear PEG MW	PEG-alkyne (MW 10,000)	Nanogel (r= 120 nm)
> 100,000	9.22%	32.43%
35,000 – 100,000	2.07%	20.60%
8,000 – 35,000	41.55%	42.09%
< 8,000	47.17%	4.87%

Table 2
Mass measurements of clickable nanogel coatings

Changes in mass (and their equivalent thicknesses for masses of water) were measured with QCM-D for two types of clickable nanogel coatings, including one attached without salt and one attached with salt. The coating attached with salt underwent surface crosslinking in 1.5 M salt with 2 mM copper. The coating attached without salt did not undergo surface crosslinking but was incubated in PBS and raised to 37°C as well during this step.

	Clickable nanogel coating attached without salt, no surface crosslinking		Clickable nanogel coating attached with salt, surface crosslinking in 1.5 M salt	
	Change in Mass (ng/cm ²)	Equivalent Thickness of Water (nm)	Change in Mass (ng/cm ²)	Equivalent Thickness of Water (nm)
<i>Nanogel coating</i>	5,030	50.3	10,640	106.4
<i>Surface crosslinking step (or 37°C sham incubation)</i>	(510)	(5.1)	80	0.8