Title: Thiol surface functionalisation via continuous phase plasma polymerisation of allyl mercaptan, with subsequent maleimide-linked conjugation of collagen.

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Title: Thiol surface functionalisation via continuous phase glow discharge polymerisation of allyl mercaptan, with subsequent maleimide-linked conjugation of collagen.

Abstract

Thiol groups can undergo a large variety of chemical reactions and are used in solution phase to conjugate many bioactive molecules. Previous research on solid substrates with continuous phase glow discharge polymerisation of thiol-containing monomers may have been compromised by oxidation. Thiol surface functionalisation via glow discharge polymerisation has been reported as requiring pulsing. Herein, continuous phase glow discharge polymerisation of allyl mercaptan (2-propene-1-thiol) was used to generate significant densities of thiol groups on a mixed macrodiol polyurethane and tantalum. Three general classes of chemistry are used to conjugate proteins to thiol groups, with maleimide linkers being used most commonly. Here the pH specificity of maleimide reactions was used effectively to conjugate surface-bound thiol groups to amine groups in collagen. XPS demonstrated surface-bound thiol groups without evidence of oxidation, along with the subsequent presence of maleimide and collagen. Glow discharge reactor parameters were optimised by testing the resistance of bound collagen to degradation by 8 M urea. The nature of the chemical bonding of collagen to surface thiol groups was effectively assessed by colourimetric assay (ELISA) of residual collagen after incubation in 8 M urea over eight days and after incubation with keratinocytes over fifteen days. The facile creation of useable solid-supported thiol groups via continuous phase glow discharge polymerisation of allyl mercaptan opens a route for attaching a vast array of bioactive molecules.

Keywords

Plasma chemistry, Thiol, Surface analysis, Biological activity, Protein engineering

Introduction

There is increasing interest in thiol-supported surfaces ¹ because thiol groups can undergo a vast number of reactions, providing a variety of routes for bioconjugation. Existing techniques for attaching thiols to surfaces include mercapto-silane self-assembly on silaceous and metal surfaces ², self-assembly on gold using thiol groups on both ends of the conjugating molecule ³, and the immobilisation of thiol containing conjugators on otherwise functionalised surfaces ^{4,5}. These approaches suffer from numerous problems including multi-step processes, substrate specificity, and the requirement for solution phase chemistry.

The previous use of continuous phase glow discharge polymerisation using thiol containing monomers led to surfaces showing poor structural retention, increased sulfonation and/or a loss of thiol functionality compared to their monomer precursors ⁶⁻⁸. Attempts to generate thiols on polyethylene with hydrogen sulfide glow discharge polymerisation led to a wide range of functional groups such as sulfides and episulfides ⁸. Of note, continuous phase glow discharge polymerisation of allyl mercaptan failed to generate useable thiol groups on silica, instead generating sulfoxide (S-O) moieties ⁶. However, the presence of oxide-containing moieties

suggested oxidation as the cause of failure to generate useable thiol groups. In addition, previous failures to generate thiol-supported surfaces may have been due to the use of saturated thiol-containing monomers. Saturated monomers, such as ethanethiol⁸, require relatively extreme glow discharge conditions to break saturated bonds, possibly extreme enough to destroy thiol functionalities. The aim of experimentation reported herein was to demonstrate the presence of useable surface thiol groups following functionalisation via continuous phase glow discharge polymerisation of allyl mercaptan and to demonstrate covalent conjugation of collagen to these thiol groups via a maleimide linker.

Considering the failure to generate useable surface supported thiol groups via continuous phase glow discharge polymerisation, a 2006 patent⁹ proposed that the generation of significant levels of thiol groups on surfaces required pulsed phase glow discharge together with an unsaturated monomer such as allyl mercaptan⁹. The characteristics of glow discharge polymerisation systems are important for applications and for process characterisation and optimisation¹⁰. Much research continues to be undertaken on characterising pulsed and continuous glow discharge polymerisation processes. Both types have advantages and disadvantages based on glow discharge characteristics, process windows, and physical restrictions. Pulsing the electric discharge on the microsecond timescale has been shown to improve structural retention of functional groups present in the parent monomer¹¹. Repetitive short bursts of glow discharge are theorised to control the number and lifetime of active functional groups created during the on-period, which then is followed by conventional reaction pathways (e.g. polymerisation) during the off-period.

Three general classes of chemistry are used to conjugate proteins to surface thiol groups¹²: 1) Iodoacetyl-containing reagents react with thiol groups in alkaline conditions (pH 7.2 to 9), resulting in stable thioether bonds. 2) Pyridyl disulfides react with thiol groups over a broad pH range to form disulfide bonds. 3) Maleic acid imide (maleimide) reactions are specific for thiol groups in the pH range of 6.5 - 7.5^{13,14}; at higher pH values, cross-reactivity with amine groups occurs.

Maleimides are the basis for most conjugation techniques to thiol groups. Double bonds in maleimide groups readily react with thiol groups to form stable carbon-sulfur bonds. Maleimides have been used previously to link thiol groups to bioactive molecules: N-ethylmaleimide on solid-supported surfaces¹, antibody-enzyme and hapten-carrier conjugates^{15,16}, and carrier proteins with thiol groups in solution¹⁷. The proposed reaction (Figure 1) for experimentation reported herein involves initial thiol surface functionalisation by continuous phase glow discharge polymerisation of allyl mercaptan (A), followed by addition of the maleimide-containing linker, Sulfo-SMCC (B), with subsequent addition of amine-containing protein (C), here represented by the amino acid glycine, with the result being conjugation of surface thiol groups to amine groups via Sulfo-SMCC (D).

Materials and Methods

Substrates

A mixed macrodiol polyurethane (Elast-Eon™)¹⁸⁻²⁵ (Kindly gifted by Biomerics, Salt Lake City, UT 84119, United States) was laser cut into 5 mm x 5 mm x 2 mm

blocks and into 8 mm diameter discs (0.15 mm thickness). The blocks were used for urea elution and X-ray photoelectron spectroscopy (XPS) studies, while the discs were used for keratinocyte studies (details below). Each block was designed to stand on one 2 mm x 5 mm edge, thereby exposing five faces to chemical modification. To enable orientation, each Elast-Eon™ disc was marked with an “L” on the surface opposite to the chemically treated side. Annealed 6 x 6 mm 99.9% pure tantalum squares were cut from 0.2 mm thick foil and polished on both sides to 0.05 Ra or better to standardise surface roughness (Goodfellow Ltd, Cambridge, United Kingdom; CFJ Metal Polishers, Oakleigh South, Australia; LaserXperts, Murwillumbah, Australia).

Cleaning

Elast-Eon™ specimens were sonicated in purified water with 2% phosphate-free detergent (Tween 20™, Sigma-Aldrich) for two 20 min periods, sonicated in purified water for two 10 min periods, sonicated in ethanol (Sigma-Aldrich) for two 20 min periods, sonicated in purified water for a further 10 min, and then dried in laminar-flow air at 20°C. Tantalum squares were passivated by immersion in 45% nitric acid (Sigma-Aldrich) at 20°C for 12 h, rinsed three times with purified water, and sonicated in purified water with Tween 20™ for two 20 min periods. Subsequently the squares were sonicated in purified water for three 10 min periods, sonicated in acetone (Sigma-Aldrich) for two 20 min periods, sonicated in purified water for 10 min, and then dried in air at 37 °C.

Glow discharge polymerisation

Glow discharge polymerisation of allyl mercaptan (~60%, Sigma-Aldrich, Product #06030) was performed in a custom-built reactor, described in detail elsewhere²⁶. The glow discharge was powered by a commercial generator (Rochester N.Y. HPG-2 High Frequency Plasma Generator). Downstream of the reactor were a MKS Baratron pressure sensor and a pumping system using a Jvac DDL 300 rotary high vacuum pump. A manual control valve (Leybold) was used to adjust pumping rates. Tantalum squares were placed flat on the lower electrode and treated on one side, turned over, and treated on the other side. Each Elast-Eon™ disc was treated on the surface opposite to the side marked with an "L." Elast-Eon™ blocks were placed on one edge, exposing five faces to glow discharge polymerisation. Care was taken to avoid oxygen contamination of specimens: Prior to glow discharge polymerisation, the reactor was evacuated via large and fine gauge conduits to a base pressure below 0.001 mbar. This pressure was maintained for periods of 30 - 60 min to evaporate any volatile agents in the system. A maximum leak rate of 0.01 mbar/minute was permitted. Subsequently, monomer was introduced to the system for 90 s, after which the vessel was evacuated again until a pressure of less than 0.001 mbar was obtained. For assessment of glow discharge chamber variables, allyl mercaptan monomer flow control and pump flow control valves were adjusted until a stable monomer pressure was obtained, followed by glow discharge initiation. After polymerisation, the reactor was evacuated to under 0.001 mbar before venting with air.

Maleimide attachment

To minimise oxidation, immediately following removal from the reactor, specimens were placed individually into wells in a 96-well plate containing 200 μ l maleimide solution, Sulfo-SMCC (Sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate, Thermo Fisher Scientific, Product #22322), diluted in pH 7.2 PBS (Thermo Fisher Scientific). Sulfo-SMCC is comprised of N-hydroxysuccinimide (NHS ester) and maleimide groups. NHS esters react with primary amines at pH 7 - 9 to form stable amide bonds and maleimides react with thiol groups at pH 6.5 - 7.5 to form stable thioether bonds. The Sulfo-SMCC concentration of 100 μ g/ml was in excess to saturate thiol binding sites. Specimens were oriented in wells such that five sides of the Elast-Eon™ blocks, both sides of the Elast-Eon™ discs, and both sides of the tantalum squares were exposed, for 2 - 4 h at room temperature. Specimens then were washed once with 0.05% Tween 20™ in PBS (pH 7.2) and then twice more with PBS (pH 7.2).

Optimisation of glow discharge polymerisation parameters

Broad ranges for glow discharge variables (pressure, power, time, frequency) of allyl mercaptan were based on previous research with ethanethiol^{27,28}. To optimise parameters for allyl mercaptan, after setting the glow discharge reactor as described above, monomer was introduced while varying pressure, duration, and power. To assess optimum exposure time, chamber pressure was set at 0.3 mbar, power at 20 W, frequency at 125 kHz, while time of exposure was varied between 0 and 120 s. To assess optimum power, chamber pressure was set at 0.3 mbar, exposure time was set at 60 s, frequency at 125 kHz, while power was varied between 2 and 40 W.

To assess optimum chamber pressure, exposure time was set at 60 s, power at 20 W, frequency at 125 kHz, while pressure was varied between 0 and 0.5 mbar. After maleimide attachment, specimens were incubated for 12 h, at room temperature, with an excess concentration (4 $\mu\text{g/ml}$) of collagen 1 (rat tail collagen, Sigma-Aldrich, Product #C7661) in PBS (pH = 7.4). Collagen 1 binding saturation had been noted previously from standard curves at a concentration of 2 $\mu\text{g/ml}$ (Supplementary figure 1). This was followed by one wash with 0.05% Tween 20™ in PBS (pH 7.4) and two washes with PBS (pH 7.4). To test the strength of collagen 1 bonding, specimens were incubated in 8 M urea (Sigma-Aldrich) in purified water for seven days, followed by ELISA to measure residual collagen 1. Three samples were used for each data point. Error bars represent one standard deviation either side of the mean, representing the 68% confidence interval.

Collagen 1 ELISA

The amount of collagen 1 on specimens was assessed by two-step ELISA. Prior to addition of primary antibody, specimens were washed with 0.05% Tween 20™ in PBS (pH 7.4), washed twice with PBS, and then blocked with 1% bovine serum albumin (BSA) ('BovoStar', Bovogen, Product #BSAS) in PBS (pH = 7.4) for 2 h. Mouse anti-collagen type 1 (Sigma-Aldrich, Product #C2456) was used as primary antibody, diluted 1:160000 in 1% BSA in PBS (pH = 7.4) and incubated for 2 h at room temperature. This was followed by three washes as previously described. Anti-mouse IgG, HRP-linked antibody (Cell Signaling Technology, Product #7076) was used as secondary antibody. It was diluted 1:8000 in 1% BSA in PBS (pH 7.4) and incubated for 2 h at room temperature. This was followed by 3 washes as previously

described. Specimens then were incubated in HRP substrate reagent (R&D Systems, Catalog #D999) for 25 min. Substrate reaction was stopped with 1 M sulfuric acid. Absorbance at 450 nm was measured in a Tecan Genios Pro microplate reader.

Urea elution of collagen 1

To assess bond strength, reactor parameters of 20 W, 0.3 mbar, 60 s, and 125 kHz were used, being within the optimal range determined previously for Elast-Eon™ and tantalum. Half of these specimens were incubated in Sulfo-SMCC solution and washed. Untreated, ppPropSH alone, and ppPropSH plus Sulfo-SMCC specimens then were incubated in a 4 µg/ml solution of collagen 1 (as previously described). Specimens then were incubated in 8 M urea for periods of up to 10 days. Two-step ELISA (as described above) was used to measure residual collagen 1. Three samples were used for each data point. Error bars represent one standard deviation either side of the mean, representing the 68% confidence interval.

Keratinocyte removal of collagen 1

Elast-Eon™ discs were functionalised using the optimised glow discharge parameters ascertained previously. Discs then were incubated in Sulfo-SMCC solution and washed. Specimens treated with ppPropSH plus Sulfo-SMCC, along with untreated specimens, then were incubated in a 4 µg/ml solution of collagen 1 and stored in PBS at 4°C. All specimens then were incubated with cell growth media (Gibco, Invitrogen, RPMI 1640 Medium, GlutaMAX™, Product # 61870) with 1%

penicillin and streptomycin and 10% foetal calf serum (Invitrogen, Product #10082147) at 37°C in room air, for 16 days, in a 48-well Costar plate. Immortalised keratinocytes (HaCaT) [Kindly gifted by Dr. Norbert Fusenig, Division of Differentiation and Carcinogenesis, German Cancer Research Centre, Heidelberg INF 280, 69120, Germany] were added to the cell-growth media in each well. HaCaT were added for periods of 5, 10, and 15 days, with all discs incubated for 16 days in media. Media changes were performed every five days. Incubated cells then were trypsinised (1% trypsin and 5 mM EDTA in PBS) for 30 min and washed three times with PBS. Two-step ELISA (as described above) was used to measure residual collagen 1. Three samples were used for each data point. Error bars represent one standard deviation either side of the mean, representing the 68% confidence interval.

X-ray photoelectron spectroscopy

XPS was performed on treated and untreated specimens, described in detail elsewhere²⁹. In brief, two spectrometers were used, either an AXIS-HSi or an AXIS-Nova (Kratos Analytical Inc., Manchester, UK), both with a monochromated Al K α source operating at a power of 180 W (12 kV x 15 mA), a hemispherical analyser operating in the fixed analyser transmission mode and the standard aperture (analysis area: 0.3 mm x 0.7 mm). The total pressure in the main vacuum chamber during analysis was typically between 10⁻⁹ and 10⁻⁸ mbar. Survey spectra were acquired at a pass energy of 160 eV. To obtain more detailed information about chemical structure, oxidation states etc., high resolution spectra were recorded from individual peaks at 40 eV pass energy (yielding a typical peak width for polymers of < 1.0 eV). Each specimen was analysed at an emission angle of 0° as measured

from the surface normal. Assuming typical values for the electron attenuation length of relevant photoelectrons the XPS analysis depth (from which 95 % of the detected signal originates) ranges between 5 and 10 nm for a flat surface.

All elements present were identified from survey spectra. The atomic concentrations of the detected elements were calculated using integral peak intensities and the sensitivity factors supplied by the manufacturer. Binding energies were referenced to the C 1s peak at 285 eV (aliphatic hydrocarbon). The accuracy associated with quantitative XPS is ca. 10% - 15%. Precision (ie. reproducibility) depends on the signal/noise ratio but is usually much better than 5%. The latter is relevant when comparing similar samples. Contributions to the carbon 1s signal from chemically different species were quantified by curve-fitting the C 1s high resolution spectra; Four significant fit components were used with the following tentative assignments³⁰: C(1) at 285.0 – 285.5 eV (C-C, C-H, C-S), C(2) at 286.3 - 286.7 eV (mainly C-O, possibly C-N), C(3) at around 288.0 eV (C=O, O-C-O, N-C=O) and C(4) at approx. 289.5 eV (O-C=O, N-C(O)-O). Two different spots were examined on each three samples, giving a total of 24 data sets. Data is presented as tables with mean values (+/- deviation) of 6 measurements per treatment.

Results

Optimisation of glow discharge reactor parameters

Collagen 1 binding to Elast-Eon™ was optimal with pressure at 0.1 - 0.3 mbar, power at 10 - 40 W, and time at 20 - 60 s (Figure 2), while binding to tantalum was optimal with pressure at 0.2 - 0.5 mbar, power at 5 - 20 W, and time at 60 s (Supplementary figure 2).

Urea elution of collagen 1 from Elast-Eon™

Collagen 1 was eluted completely from untreated Elast-Eon™ samples after less than one day. A significant increase in collagen 1 attachment to Elast-Eon™ was seen after ppPropSH treatment, with 44% of initially bound collagen 1 being retained at one day. A further increase in collagen 1 bonding strength was noted with use of the maleimide linker, Sulfo-SMCC, with retention at one day being 90%. These differences appeared even more pronounced at five days, with residual collagen 1 on ppPropSH specimens falling slightly to 32%, while residual collagen 1 on ppPropSH plus Sulfo-SMCC linked specimens persisted at 88% (Figure 3).

Urea elution of collagen 1 from tantalum

From day four onwards, the bond strength differences conferred by each of the surface treatments became evident, with stepwise increases in retention of collagen 1. At six days, residual collagen 1 on untreated tantalum was 3%, while on ppPropSH treated tantalum it was 9%, and on specimens treated with ppPropSH along with Sulfo-SMCC it was 29% (Figure 4).

Keratinocyte removal of collagen 1 from Elast-Eon™

Collagen 1 conjugated via Sulfo-SMCC to ppPropSH treated Elast-Eon™ was significantly more resistant to degradation by keratinocytes: after five days 21% of adsorbed collagen 1 remained, compared to 85% on specimens treated with ppPropSH and Sulfo-SMCC. These differences persisted at ten days (14% and 65%) and fifteen days (10% and 28%) (Figure 5).

XPS

On untreated Elast-Eon™, atomic concentrations of oxygen, nitrogen, carbon, silicon, C(2) (ether) and C(4) (urethane) were consistent with results reported elsewhere for Elast-Eon™²⁰ (Table 1): the nitrogen peak at 400.2 eV was consistent with –N-COO and N-O bonds (urethane nitrogen)³¹, the peak at 287 eV was consistent with polyether groups, and the peak at 290 eV was consistent with polyurethane (Figure 6). On untreated tantalum, tantalum oxides were present, along with small amounts of contaminants (C, Si) (Table 2).

The primary XPS region for sulfur species is the S 2p between 161 eV – 169 eV. After treatment with ppPropSH, on both Elast-Eon™ and tantalum, a strong sulfur peak appeared at 164 eV, consistent with the presence of thiol groups^{32,33} (Figure 7). Negligible change at 167 - 169 eV indicated that oxidised thiol groups

(thiosulfonates and thiol-sulfonates) were absent. On Elast-Eon™ attenuated Elast-Eon™ substrate signal (Si, N) still was detected following ppPropSH treatment (Table 1). On tantalum substrate, O and Si were suppressed and Ta was not detected following ppPropSH treatment (Table 2).

Elast-Eon™ and tantalum treated with ppPropSH and then with Sulfo-SMCC revealed a slight increase in O and N (Tables 1 and 2), consistent with the presence of Sulfo-SMCC. The weak increase in intensity of the C 1s spectrum between 288 and 290 eV was consistent with imide and ester groups in Sulfo-SMCC (Figure 6), and the slight increase in oxidised sulfur, represented by the slight increase in intensity between 167-169 eV (Figure 7), indicated the presence of SO₃ groups.

Following incubation in collagen 1, increased intensity of N and O signals were observed, specifically an increase in the C(3) component of the C 1s spectrum (increased intensity centred on 288.5 eV – Figure 6), being characteristic for amide and hydroxyl bonds in proteins). These changes were more obvious on Elast-Eon™ than on tantalum.

Discussion

Continuous phase glow discharge polymerisation of the unsaturated monomer, allyl mercaptan, led to effective thiol functionalisation of an emblematic polymer (Elast-Eon™) and metal (tantalum). Oxidation, choice of monomer, and the presence of impurities have been implicated in previous failures to generate thiol-supported surfaces. Following glow discharge polymerisation of allyl mercaptan, XPS revealed peaks at 164 eV on Elast-Eon™ and tantalum, consistent with the presence of thiol groups. There was no evidence of oxidation (thiosulfinates and thiolsulfonates). On Elast-Eon™, some substrate signal was detected following ppPropSH treatment, indicating that the ppPropSH layer was thin. On tantalum, the lack of a Ta signal following ppPropSH treatment suggested a film thicker than 20 - 30 nm. Allyl mercaptan contains carbon double bonds, which are more reactive, permitting the use of less aggressive conditions. Glow discharge pulsing, the subject of a 2006 patent⁹, was not necessary for effective thiol-functionalisation, probably because of the unsaturated nature of the bonds within allyl mercaptan. Impurities were implicated in a previous failure to generate useable thiol groups with continuous phase glow discharge polymerisation of 80% purity allyl mercaptan⁶. Per the supplier (Sigma Aldrich) of the ~60% purity allyl mercaptan used in the experiments reported herein, the remaining ~40% consisted primarily of allyl disulfide and allyl sulfide, both of which are unsaturated thiol-containing molecules and therefore likely to be similarly polymerisable to allyl mercaptan; these “impurities” are likely to have contributed to effective surface thiol functionalisation. XPS changes consistent with the addition of maleimide were detected following the

addition of Sulfo-SMCC (imide and ester groups, as well as SO₃ groups). XPS clearly demonstrated the presence of collagen on functionalised and Sulfo-SMCC treated Elast-Eon™ surfaces, less obviously on the Ta substrate: increases in N and O concentrations, along with an increase in the amide signal, are typical for proteins.

Quantitative XPS has been quoted as having an uncertainty of 10-15%³⁴ and involves significant conjecture about underlying chemical changes and reactivity³⁵. For these reasons, chemical derivatisation is commonly added to solid-supported functional group analyses, using tag molecules with measurable activity. For example, a maleimide-based label, N-ethylmaleimide, was used previously to quantify thiol groups colourimetrically¹. However, chemical derivatisation on surfaces, particularly on plasma-treated surfaces, is also associated with significant problems such as selectivity and non-specific adsorption. The ELISA technique reported herein proved to be an effective colourimetric method for directly measuring bound collagen and indirectly assaying surface thiol groups. Subtle incremental increases in collagen resistance to degradation by urea proved to be measurable and allowed optimisation of glow discharge reactor parameters, with increased degradation resistance correlating with increased bond strength. Optimal reactor parameters were shown to be 125 kHz, 10 - 20 W, 0.2 - 0.3 mbar, and 60 s, for both Elast-Eon™ and tantalum. These parameters were similar to ranges described elsewhere for pulsed glow discharge polymerisation of thiol-containing monomers^{9,36}. Collagen ELISA as a method for optimising substrate-bound thiol groups may have benefits over other chemical derivatisation techniques: it serves the dual purpose of measuring the density of the protein in question, as well as retained

protein function. Protein conjugation and activity depends on many surface properties, including wettability, surface charge and a specific pKa³⁷ which may differ from the optimum parameters for the binding of other maleimide-based colourimetric markers such as N-ethylmaleimide. Collagen ELISA following incubation in 8 M urea showed a stepwise increase in attachment of collagen to substrate: the addition of ppPropSH leading to electrostatic and/or ionic bonding and the addition of Sulfo-SMCC to ppPropSH-treated surfaces leading to covalent conjugation. Many cells, including keratinocytes, produce collagenolytic enzymes³⁸. The demonstrated resistance of covalently conjugated collagen to degradation by keratinocytes suggests a possible route for improving the longevity of tissue-implanted materials.

Three general classes of chemistry are used to conjugate proteins to thiol groups, with maleimide linkers being used most commonly. Here the pH specificity of maleimide reactions was used effectively to conjugate surface-bound thiol groups to amine groups in collagen. Thiol groups are highly versatile. Their facile creation on surfaces via continuous phase glow discharge polymerisation of allyl mercaptan opens a route for attaching a vast array of bioactive molecules.

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Figures

Figure 1: Conjugation of amine groups to thiol-supported surfaces via maleimide linkers.

Figure 2: Optimisation of reactor parameters for Elast-Eon™. Three samples were used for each data point. Error bars represent one standard deviation either side of the mean, representing the 68% confidence interval.

Figure 3: Elution of collagen 1 from Elast-Eon™. Three samples were used for each data point. Error bars represent one standard deviation either side of the mean, representing the 68% confidence interval.

Figure 4: Elution of collagen 1 from tantalum. Three samples were used for each data point. Error bars represent one standard deviation either side of the mean, representing the 68% confidence interval.

Figure 5: Keratinocyte degradation of collagen 1 on Elast-Eon™. Three samples were used for each data point. Error bars represent one standard deviation either side of the mean, representing the 68% confidence interval.

Figure 6: XPS C1 s spectra for Elast-Eon™.

- Following the addition of Sulfo-SMCC, the weak increase in intensity between 288-290 eV was consistent with imide and ester groups in Sulfo-SMCC.

- Following the addition of collagen, an increase in N and O was observed, specifically increased intensity in the C(3) component (centred on 288.5 eV), being characteristic for amide and hydroxyl bonds in proteins.

Figure 7: XPS S2 p spectra for Elast-Eon™.

- Following ppPropSH, the strong sulfur peak at 164 eV was consistent with thiol groups.
- Negligible change at 167-169 eV indicated the absence of oxidised thiol groups.
- Following the addition of Sulfo-SMCC, the slight increase in intensity between 167-169 eV indicated the presence of SO₃ groups.

Supplementary figure 1: Standard curves of collagen 1 conjugated to Elast-Eon™ and tantalum, showing saturation at 2 µg/ml.

Supplementary figure 2: Optimisation of reactor parameters for tantalum.

Tables

Table 0-1: XPS surface chemical compositions of Elast-Eon™ specimens, expressed as atomic ratios X/C (atomic concentration of element/species X relative to the total atomic concentration of carbon).

Table 0-2: XPS surface chemical compositions of tantalum specimens, expressed as atomic ratios X/C (atomic concentration of element/species X relative to the total atomic concentration of carbon).

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Conjugation of amine groups to thiol-supported surfaces via maleimide linkers.

39x42mm (600 x 600 DPI)

A

Optimisation of reactor parameters for Elast-Eon™. Three samples were used for each data point. Error bars represent one standard deviation either side of the mean, representing the 68% confidence interval.

67x94mm (600 x 600 DPI)

Elution of collagen 1 from Elast-Eon™. Three samples were used for each data point. Error bars represent one standard deviation either side of the mean, representing the 68% confidence interval.

42x24mm (600 x 600 DPI)

Accepte

B Tantalum

Elution of collagen 1 from tantalum. Three samples were used for each data point. Error bars represent one standard deviation either side of the mean, representing the 68% confidence interval.

52x38mm (600 x 600 DPI)

Accep

Keratinocyte degradation of collagen 1 on Elast-Eon™. Three samples were used for each data point. Error bars represent one standard deviation either side of the mean, representing the 68% confidence interval.

34x17mm (600 x 600 DPI)

Accepted

XPS C1 s spectra for Elast-Eon™.!! + Following the addition of Sulfo-SMCC, the weak increase in intensity between 288-290 eV was consistent with imide and ester groups in Sulfo-SMCC.!! + Following the addition of collagen, an increase in N and O was observed, specifically increased intensity in the C(3) component (centred on 288.5 eV), being characteristic for amide and hydroxyl bonds in proteins.

388x308mm (300 x 300 DPI)

ACCE

XPS S2 p spectra for Elast-Eon™.!! + Following ppPropSH treatment, the strong sulfur peak at 164 eV was consistent with thiol groups.!! + Negligible change at 167-169 eV indicated the absence of oxidised thiol groups.!! + Following the addition of Sulfo-SMCC, the slight increase in intensity between 167-169 eV indicated the presence of SO₃ groups.

389x308mm (300 x 300 DPI)

Acce

Elast-Eon™

Tantalum

Supplementary figure 1: Standard curves of collagen 1 conjugated to Elast-Eon™ and tantalum, showing saturation at 2 µg/ml.

66x65mm (600 x 600 DPI)

AC

Supplementary figure 2: Optimisation of reactor parameters for tantalum.

64x104mm (600 x 600 DPI)

	Untreated Elast-Eon™		Elast-Eon™ + ppPropSH		Elast-Eon™ + ppPropSH + Sulfo-SMCC™		Elast-Eon™ + ppPropSH + Sulfo-SMCC™ + Collagen 1		Peak Assignments
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
O	0.336	0.001	0.138	0.016	0.160	0.012	0.208	0.021	
C (1)	0.722	0.006	0.846	0.018	0.813	0.007	0.749	0.013	C-C, C-H, C-S
C (2)	0.207	0.005	0.130	0.014	0.141	0.009	0.178	0.012	C-O, C-N
C (3)	0.006	0.001	0.020	0.004	0.025	0.003	0.062	0.005	C=O, O-C-O, N-C=O
C (4)	0.057	0.001	0.004	0.001	0.021	0.002	0.011	0.002	O-C=O, N-C(O)-O
N	0.048	0.002	0.020	0.002	0.029	0.003	0.066	0.004	
Si	0.209	0.001	0.057	0.008	0.052	0.012	0.064	0.017	
S	0		0.132	0.013	0.114	0.008	0.089	0.008	

Accepted Article

	Untreated Ta		Ta + ppPropSH		Ta + ppPropSH + Sulmo-SMCC™		Ta + ppPropSH + Sulfo-SMCC™ + Collagen 1		Peak Assignments
	Mean	Dev.	Mean	Dev.	Mean	Dev.	Mean	Dev.	
O	1.597	0.046	0.107	0.002	0.160	0.017	0.182	0.004	
C (1)	0.766	0.009	0.869	0.007	0.767	0.014	0.751	0.003	C-C, C-H, C-S
C (2)	0.148	0.006	0.092	0.006	0.159	0.008	0.164	0	C-O, C-N
C (3)	0.010	0.004	0.032	0.001	0.033	0.002	0.044	0.004	C=O, O-C-O, N-C=O
C (4)	0.076	0.001	0.007	0	0.041	0.004	0.040	0.002	O-C=O, N-C(O)-O
N	0		0.008	0.002	0.044	0.002	0.055	0.004	
Si	0.081	0.013	0.004	0.001	0.007	0.001	0.010	0	
S	0		0.272	0.006	0.157	0.001	0.161	0.007	
Ta	0.516	0.015	0		0		0		

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Title:

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Date:

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