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Quantification of aldehyde terminated heparin by SEC-MALLS-UV for the surface functionalization of polycaprolactone biomaterials

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34 **Abstract**

35 A straight forward strategy of heparin surface grafting employs a terminal reactive-aldehyde
36 group introduced through nitrous acid depolymerisation. An advanced method that allows
37 simultaneously monitoring of both heparin molar mass and monomer/aldehyde ratio by size
38 exclusion chromatography, multi-angle laser light scattering and UV-absorbance (SEC-
39 MALLS-UV) has been developed to improve upon heparin surface grafting. Advancements
40 over older methods allow quantitative characterization by direct (aldehyde absorbance) and
41 indirect (Schiff-based absorbance) evaluation of terminal functional aldehydes. The indirect
42 quantitation of functional aldehydes through labeling with aniline (and the formation of a
43 Schiff base) allows independent quantitation of both polymer mass and terminal functional
44 groups with the applicable UV mass extinction coefficients determined. The protocol was
45 subsequently used to synthesize an optimized heparin-aldehyde that had minimal
46 polydispersity ($PDI < 2$) and high reaction yields (yield $> 60\%$ by mass). The 8 kDa weight
47 averaged molar mass heparin-aldehyde was then grafted on polycaprolactone (PCL), a
48 common implant material. This optimized heparin-aldehyde retained its antithrombin activity,
49 assessed in freshly drawn blood or surface immobilized on PCL films. Anticoagulant activity
50 was equal to or better than the 24 kDa unmodified heparin it was fragmented from.

51

Introduction

Haemocompatibility is a major concern in clinical application of devices or biomaterials. The complexity of surface thrombosis of polymeric biomaterials restricts their use in blood environment. Hoffman et al's 1972 manuscript first described the covalent immobilization of heparin to construct a non-thrombogenic surface.[1] After nearly 40 years of research, heparin coating of surfaces still offers one of the best solutions for producing an anti-clotting surface.[2] The tethering of the heparin by a terminal functional group to the polymer (also referred to as end-point attachment) enables better preservation of its functional properties when compared to other techniques involving immobilization at multiple points.[3-5] The single point attachment allows heparin to protrude from the surface and interact with circulating molecules such as antithrombin.

Such attachment requires creating a reactive functional group at the terminus of the heparin chain for its subsequent covalent bonding to the surface. One well-recognized approach involves nitrous acid depolymerization to create heparin oligomers with reactive aldehyde groups. This has proven itself in the fabrication of heparin coated biomedical devices, such as the Carmeda® Bioactive Surface coating.[2, 6]

Heparin is composed of disaccharides containing L-iduronic acid and D-glucosamine residues [7] as seen in Figure 1. However, heterogeneity exists across the heparin polymer as the disaccharides undergo various chemical modifications producing a range of residues. In particular, 85-90% of D-glucosamine is N-sulphated and the rest are N-acetylated. The various residues react differently to nitrous acid treatment, which necessitates empirical optimization during acid depolymerization. The deamination and subsequent chain cleavage at pH 1.5 is highly specific at N-sulphated D-glucosamine residues whereas at pH 4.5 it is preferential for the unsubstituted D-glucosamine (~ 3% overall) and a minority of N-sulphated (5-8%).[7, 8]

FIGURE 1

The depolymerization products require monitoring for optimal oligomer chain lengths and MW sizes. If the reaction goes to completion the heparin will be broken down to disaccharides of approximately 1200 Da. However, the minimal effective antithrombogenic

unit of heparin consists of a pentasaccharide, where the minimal molecular weight is ~1500 to 3000 Da.[9] The pentasaccharide contains the minimum antithrombin sequence sufficient for interacting with and inhibiting Factor Xa, thus preventing thrombogenesis. Monitoring the degree of depolymerization to retain functionality was necessary for correlation to antithrombin activity.[10]

A number of methods have been utilized to monitor the resulting molecular weight of the heparin polymer. These include; MALDI-TOF,[11] gel electrophoresis,[12] ¹³C NMR spectroscopy,[13] and intrinsic viscosity.[14] A relatively new method towards characterizing heparin oligomers is size-exclusion chromatography coupled with multi-angle laser light scattering (SEC-MALLS). This provides a rapid, precise and accurate measurement of the molecular weight that does not rely on relative molar mass standards but retains the advantages of HPLC (i.e. automation and productivity). [15-17]

For the purpose of end-point immobilization, one also needs to quantitate the presence of the intact aldehyde group after acid depolymerization. The reactive aldehyde becomes a convenient method for subsequent grafting reactions. The aldehyde derivatization has previously been used to study heparin structure and sequence using highly sensitive reagents such as o-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride,[18] 2-aminoacridone,[19] and paranitrophenyl hydrazine.[20] While the above reagents work in limited situations for aldehyde characterization, they are poorly soluble, toxic, and relatively expensive. In contrast, aniline is soluble in both aqueous and organic solvents, has small steric profile, and quickly forms a strongly absorbing UV/vis chromophore at 369 nm that is within the optical window of most organic solvents employed for SEC. Aniline has previously been well characterized in aldehyde-derivatizing Schiff base reactions.[21-23]

In this manuscript we describe the use of multi-angle laser light scattering and size exclusion chromatography combined with online UV and refractive index detection (SEC-MALLS-UV) to quantitatively assess the heparin depolymerisation, oligomer mass, polydispersity, reaction yield, and heparin monomer/aldehyde ratios. The method was employed to monitor and optimize heparin depolymerisation to yield an 8 kDa oligomer that retained its antithrombotic activity. The optimized heparin-aldehyde was chemically grafted on polycaprolactone (PCL). Finally, the anticoagulant activity of heparin-aldehyde reductive amination-based surface immobilization was compared against multipoint covalent crosslinking of unmodified heparin.

113 **Experimental Section**

114 **Materials**

115 Heparin (sodium salt, ~25 kDa) was purchased from Yantai Dongcheng Biochemicals Co.
116 Ltd (China). Sulphuric acid, sodium nitrite, sodium cyanoborohydride, and HPLC-grade
117 aniline were purchased from Sigma-Aldrich Ltd. Inc. (Singapore). All chemicals and
118 materials were used as received.

119 **Heparin depolymerization by nitrous acid**

120 Heparin depolymerization was carried out at pH 1.5 and pH 4.0 as previously described.[10,
121 24] Briefly, freshly prepared nitrous acid was prepared by mixing in a fume hood 100 mL of
122 0.5 M sulfuric acid (for pH 1.5) or 28.5 mL 0.5M sulfuric acid (for pH 4.0) to a solution of
123 100 mL of 1M sodium nitrite or 71.4 mL of 5.5M sodium nitrite, respectively. Heparin (25
124 mg/mL dissolved in 18.3 MΩ water), a 5 mL aliquot, was added to 20 mL of nitrous acid
125 reagent (pH 1.5 or 4) that was cooled to 0°C or 25°C. Aliquots of 5 mL were taken at 0, 10,
126 20, and 45 min and subsequently neutralized with 1 M sodium hydroxide to yield a final pH
127 of 8.5. Neutralized aliquots were dialyzed (10 kDa MWCO) against 1 L deionized water
128 (diH₂O) for 12 h, with the water replaced every 3 h. Dialyzed samples were lyophilized and
129 stored at 4°C.

130 **Aniline derivatization of Heparin-aldehyde oligomers**

131 Aniline acetate reagent (10 µL of 10% v/v aniline in glacial acetic acid) was added to 90 µL
132 of the depolymerized heparin dissolved at 25 mg/mL lyophilized powder in diH₂O. After 1h
133 at RT, the samples were diluted to 0.5 mL and filtered (0.22 µm) into 1 mL glass HPLC vials
134 for SEC-MALLS-UV (size exclusion chromatography multi-angle laser light scattering-
135 ultraviolet) online characterization or they were subsequently processed for Schiff-base
136 reduction. Reduction of the Schiff-base was performed by adding 50 µL Coupling Buffer (50
137 mM NaBH₃CN in phosphate buffered saline) to the aniline acetate/depolymerized heparin
138 solution described above. The reaction mixture was incubated an additional 1h at RT, diluted
139 to 0.5 mL, filtered in HPLC vials, capped, and then analyzed by SEC-MALLS-UV.

140 **SEC-MALLS-UV quantitation of heparin and depolymerized heparin**

An Agilent 1100 series HPLC pump complete with degasser and PLGel aqueous 50 (Polymer Standards Service, Mainz, Germany) in 35°C thermostated oven was connected in-line with an Agilent 1100 UV/Vis detector, Wyatt MiniDawn 3-angle light scattering detector (light scattering calibration through HPLC grade toluene), and an Agilent 1100 refractive index detector. Elution buffer was 100 mM ammonium nitrate with 0.8% w/v sodium azide (pH 7.0) at a flow rate of 1.0 mL/min. Injection volumes were typically 50 µL. UV Extinction coefficients of aldehyde, phenyl Schiff-bases, and reduced phenyl Schiff-base (phenyl alkyl-amine) were quantified using glutaraldehyde, 1:1 meq ratio of mixed glutaraldehyde:aniline, and reduced glutaraldehyde:aniline, respectively at 280 and 369 nm on cuvette UV/Vis detector. Polymer mass was determined by the refractive index detector using a dn/dc of 0.13 for heparin, heparin-Schiff bases, and heparin-phenyl amine using the Wyatt ASTRA (version 5.19.1) software. The slight increase of mass from the aniline conjugation was negligible, but was factored into the dn/dc calculations (which derives the polymer mass) using the 'protein conjugate' template in the Wyatt Astra software. This software factors in the UV/Vis and dn/dc coefficients for both the polymer under analysis and the UV/Vis absorbing conjugate to derive the protein or polymer fraction by weight. Redundant MW analysis was performed by injecting polyethylene glycol standards of known MW to characterize the small molar mass oligosaccharides (< 3 kDa) that had minimal light scattering signals (Polymer Standards Service, Mainz, Germany). M_w and M_n values are generally regarded to be accurate within $\pm 10\%$ on properly calibrated SEC. Quantitation of polymer conjugate values (Table 1), heparin/aldehyde molar ratios, % reaction yield values were performed in triplicate.

Heparin surface functionalization of polycaprolactone (PCL)

Approximately 5g of PCL (Polycaprolactone) pellets were pressed into PCL films using a hydraulic hot-press (150 bar), at 70°C for 5 minutes. After cooling, the PCL film was sliced into 5x50 mm strips. The PCL strips were treated with 1N NaOH for 24h at 50°C, then washed with diH₂O and dried at room temperature for 24h. A portion of the alkaline treated PCL was subsequently treated with 40% w/v ethylenediamine/methanol solution for 1h at RT before they were soaked in 4°C PBS for 30 min, rinsed with additional PBS, and then vacuum dried overnight (PCL-NH₂ samples).

PCL films with a single point, end-terminal functional group immobilization (PCL-HEP samples) were synthesized with optimized heparin-aldehyde conditions (depolymerization parameters @ pH 4.0, 25°C for 20 min, with 10 kDa MWCO dialysis). PCL-NH₂ strips were immersed in optimized heparin-aldehyde (10 mg/mL) in Coupling Buffer (50 mM NaBH₃CN in phosphate buffered saline) for 2h at RT. Following the reaction, the films were soaked in 4°C PBS for 30 min, rinsed with addition PBS, and then vacuum dried overnight (PCL-HEP samples).

A control sample of electrostatically bound heparin-aldehyde was adsorbed on to alkaline treated PCL to yield PCL-AHA. Alkaline treated PCL strips were immersed in optimized heparin-aldehyde (10 mg/mL) for 2h at RT and then rinsed with diH₂O. Alternatively, unfractionated heparin (-NH₂ on GlcN disaccharides) was bound covalently to alkaline treated PCL (-COOH groups on PCL surface) through EDC/NHS coupling for a multipoint coupling comparison (PCL-MPH samples). Alkaline treated PCL strips were immersed in 50 mM MES buffer (pH 5.6) containing 50 mM EDC and NHS for 10 min at RT, before unfractionated heparin (10 mg/mL) was added. After 2 h, the samples were removed, soaked in 4°C PBS for 30 min, rinsed with additional PBS, and then vacuum dried overnight (PCL-MPH samples).

Thrombogenicity of optimized heparin-aldehyde

Anticoagulant activity of optimized heparin-aldehyde was first qualitatively assessed to ensure anti-thrombosis potential remained after acid depolymerization. Freshly procured Wister rat blood (6 mL, *Rattus norvegicus*) was added to 0.5 mL PBS buffer with the addition of 1) no heparin (neg. control), 2) unfractionated heparin (pos. control), or 3) optimized heparin-aldehyde (both heparin samples at a final conc. of 10 mg/mL) in 15 mL sterile polypropylene tube. Anticoagulant activity was visualized by the darkened red clot within a 6 cm petri dish (Figure 5A).

For quantitative analysis of solution based heparin activity a time course clotting assay[25, 26]. In brief, recalcified sodium citrate treated blood (30 µL) was mixed with 10 µL of either 10mg/mL heparin or heparin aldehyde in PBS with 10 µL of PBS alone as a control. The solutions were incubated at 37°C for pre-determined time points (5, 10, 40, 60 min). To finish the reaction, 5 mL of ddH₂O was added to the blood samples and incubated at 37°C for 5 min. The red hemoglobin stain in water was measured at 540nm using a UV-Vis spectrometer.

The greater the red colour generated, the lesser the thrombosis, since the released hemoglobin originated from erythrocytes separate from any clotting.

Anticoagulant and platelet adhesion of surface functionalized PCL strips

Anticoagulant activity of surface functionalized PCL was quantitatively assessed through surface activated clot formation and subsequent color density analysis (ImageJ Densitometry plugin [27]). This assay is based on the material surfaces ability to absorb plasma proteins leading to fibrin formation and erythrocyte adhesion through thrombin activated Factor XIII activity, firstly forming a red gel like coating which may procede to the darker red clot formation [28, 29], hence the colour intensity and area relate to the anticoagulant properties of the surface. High resolution images were recorded with preset aperture and exposure time. Platelet density from the 64 x 42 μm field of view images could be measured through grey scale density using the image J software, with four separate field of views taken for each film. The PCL strips (in triplicate) were placed in sterile disposable polymethylmethacrylate (PMMA) cuvettes and 1.5 mL of freshly drawn rat blood was quickly added and then covered. Thrombosis was allowed to continue for 30 min at RT, after which the PCL strips were removed and the excess blood was wicked away with Kimwipe tissues. The samples were air dried before photographic analysis. Statistical analysis was performed using t-Test; $* = p < 0.05$ and $** = p < 0.01$.

The *in vitro* thrombo-resistant properties of the PCL samples were determined by kinetic clotting assay similar to that described above. All the testing samples were equilibrated in normal saline water and placed in 12 well tissue culture plates prior to the assay. Fresh sodium citrate treated blood (20 μL) was added onto the sample films and controls (in triplicate) in an open atmosphere. The blood was recalcified with 10 μL of 0.2M CaCl_2 and incubated at 37°C for pre-determined time points (5, 10, 40, 60 min), at which point, 5ml of ddH₂O was added into each well and incubated at 37°C for 5 min. The red stained water was measured at 540nm using a UV-Vis spectrometer.

Platelet Adhesion was performed as described previously[30]. Briefly, fresh rabbit blood was mixed with 3.8 % (w/v) sodium citrate solution at a dilution ratio of 9:1, respectively. It was

then centrifuged at 700 rpm for 10 min to obtain platelet-rich plasma (PRP). The PRP was diluted with PBS in a 1:1 (v/v) ratio. Diluted (PRP 0.1 mL) was then introduced onto 1 sq. cm PCL samples and incubated at 37 °C for 1 h. After incubation, samples were rinsed with PBS and fixed with 3 % (v/v) glutaraldehyde/PBS overnight at 4 °C, and subjected to serial dehydration with 10%, 25%, 50%, 75%, 90% and 100% ethanol for 10 min each. Samples were dried, platinum coated, and observed under scanning electron microscopy . Platelet adhesion was determined by counting the total number of adherent platelets from representative SEM images at the 2000x magnification and normalized to pristine PCL.

X-ray photoelectron spectroscopy of PCL films

To verify the immobilization of the heparin groups on polycaprolactone pristine PCL, PCL-NH₂, and PCL-HEP (optimized heparin-aldehyde conditions at pH 4.0, 25°C for 20 min, with 10 kDa MWCO dialysis grafted onto PCL-NH₂) films were analyzed by X-ray photoelectron spectroscopy (VG ESCA Lab-220i XL XPS) with monochromatic Al K α (1486.71 eV) X-ray radiation (15 kV and 10 mA). The analysis area was 700 x 700 μ m with maximum analysis depth of ~4-8nm. Pass energies of 150 eV and 20 eV were used for the survey scans and high-resolution scans, respectively.

Results

Figure 1

λ_{max} and UV mass extinction coefficient for aldehyde and Schiff-base quantitation

Heparin was depolymerized in two reaction conditions at pH 1.5 and pH 4.0. The majority of the heparin monomers consist of disaccharides with one of three functional groups; ‘-H’, ‘-SO₃’, or an acetyl group, referred to the monomer abbreviations as GlcN, GlcNSO₃, or GlcNAc, respectively. At pH conditions of 1.5 and 4.0, heparin fragmentation is expected at GlcNSO₃ and GlcN disaccharides, respectively, as illustrated in Figure 1. Initial wavelengths scans of simple alkyl-aldehydes (i.e. glutaraldehyde, butanal) revealed a broad λ_{max} of 280 nm. We found that this could be used as a qualitative assessment of the aldehyde functional group, but was not ideal for quantitation for number of reasons; 1) Stabilized solvents such THF (w/BHT) have considerable absorbance at wavelengths < 300 nm and 2) direct mass extinction coefficients of aldehyde tend not to be linear, reproducible, or both. For example,

when glutaraldehyde was injected as a standard into the SEC-MALLS-UV, it was found to interact with the SEC column materials, resulting in uncharacteristically long elution times (> 15 min, data not shown). We proceeded to stabilize the heparin-aldehydes for extinction coefficient linearity and to prevent the terminal aldehydes from interacting with SEC column packing. Aniline was found to be a common laboratory reagent, soluble in both aqueous and organic phases, and has been well characterized towards rapid reactions with simple aldehydes[31]. Under the Schiff-base derivatization procedure, aniline was in high molar excess under acidic conditions, an environment where aldehyde reactions can be safely assumed to be driven to completion. The phenylimine Schiff base possesses a UV mass extinction coefficient ($\lambda_{\text{max}} = 369 \text{ nm}$) that is optimal for SEC analyses--large enough for nanogram sensitivity, but small enough to avoid saturation of the UV signal. This was an important consideration, especially concerning the relatively high amounts (compared to HPLC) of heparin oligomer that was needed for injection, to achieve signal to noise ratios required for quantitative evaluation for the three online detectors (MALLS, UV, and refractive index). For example, injections of 100-1000 μg of oligomer are common for small MW (<20 kDa) characterization by multiangle light scattering and refractive index detectors. Table 1 lists the parameters determined for the calculation of oligosaccharide molar masses and quantitation of the aniline Schiff-base.

Table 1

SEC-MALLS-UV characterization of depolymerized heparin and of aldehyde-derivatized Schiff bases by aniline.

Figure 1 displays the sequence of reactions, from nitrous acid depolymerization, Schiff-base derivatization with aniline and finally to Schiff base reduction with sodium cyanoborohydride. Figure 2A (refractive index detector, RI) and 2B (UV_{280} absorbance) shows the overlaid SEC-MALLS-UV data of the commercial porcine heparin as well as the depolymerized and fractionated heparin (at two reaction conditions) before Schiff-base derivatization. The reaction conditions were 10 min incubation at 25°C and at pH 1.5 and pH 4.0. The commercial porcine derived heparin had a broad peak, indicative of a large polydispersity, with a weight-averaged molar mass (M_w) of 24 kDa. Acid depolymerization of this sample at pH 1.5 and pH 4.0 had remarkably different SEC profiles. When cleavage occurred at the GlcN disaccharides (at pH 4.0), a dual peak elution profile was present—suggesting only 1-2

cleavages in the original porcine heparin polymer as seen in Figure 2A. This dual peak profiles comes about from cleavage near the polymer terminus, generating small molar mass fragments (weight-averaged molar mass, $M_w < 5$ kDa, peak retention volume > 9.5 mL) with a remaining larger molar mass fragment ($M_w > 10$ kDa, peak retention volume < 9.5 mL). At low pH, virtually non-specific cleavage occurs, since the majority of the disaccharides in heparin have the GlcNSO₃ functional group. To remove as much of the smaller fragments as possible, dialysis was performed at 10 kDa molecular weight cutoff, diluting the mono, di, tri-‘disaccharides’ etc. to negligible levels (henceforth ‘fractionated heparin’). This was done to concentrate the higher molecular weight oligomers that remained. While the RI signals, which are a direct measure of sample mass, display ~100 µg of fractionated heparin mass (in Figure 2A), the UV₂₈₀ signal was distinguishable for pH 1.5 fractionated heparin, but barely above the limit of quantitation (signal/noise = ~4) for pH 4.0 reaction conditions.

Figure 2

To demonstrate the improvement in sensitivity, the same fractionated heparin (pH 4.0, 10 min. incubation, 25°C depolymerization reaction conditions) and its terminal aldehyde group was derivatized with aniline to yield the phenyl-imine (henceforth Schiff-base). Figure 2C displays the UV₃₆₉ signals of the overlaid heparin-aldehyde (Figure 2C, black line) and the Schiff base (grey line), which was subsequently reduced to the heparin-aniline (dashed black line). For the three overlaid signals, injected mass was ~100 µg, with a representative plot displayed on the right axis (open circles). The UV₃₆₉ AUC (area under curve) signal was increased by over 2 orders of magnitude by converting the terminal aldehyde into the Schiff base. Treatment by sodium cyanoborohydride, a water soluble Schiff base/imine reducing agent, caused the AUC to fall by an order of magnitude. This provided sufficient evidence that the aldehyde was present, converted to Schiff base, and then subsequently reduced to the phenylaniline.

Optimized heparin-aldehyde M_w is prepared at pH 4.0, 25°C, and 20 min reaction time

Figure 3

The molar mass from the pH 1.5 depolymerized and fractionated heparin oligomers never exceeded 4 kDa as seen in Figure 3A. The quantitation of the aldehydes was compared to the molar amount of disaccharides present in heparin, since this is the smallest repeating unit or ‘monomer’ in heparin. In Figure 3A, the right axis displays the molar ratio of heparin disaccharides/aldehyde (always present on the terminal depolymerized heparin). If the heparin was completely depolymerized into its disaccharide building blocks, the ideal ratio would be 1:1. The molar mass differences were negligible in the nitrous acid incubation conditions of 10 or 20 minutes at either temperature tested. After 45 minutes in pH 1.5, regardless of temperature, no oligomer elution peaks were seen by the SEC characterization—the heparin had been completely depolymerized at this time point, and was diluted to negligible levels in the dialysis cleanup procedure. Taking the average of the four remaining conditions at pH 1.5, ~ 6 heparin disaccharide repeat units were intact per aldehyde terminus after depolymerization and dialysis.

The depolymerization at pH 4.0 allowed a milder cleavage mechanism, due to the scarcity of the disaccharides with the GlcN functional group. Lower temperatures and shorter incubations were thus more effective at controlling both molar mass and heparin/aldehyde ratios, as seen in Figure 3B. At 0°C, there was a retarded cleavage rate across the different incubation times, which eventually reached the same molar mass as the 25°C incubation—where cleavage was complete in 10 min or less. Here, weight-averaged molar mass (M_w) could be controlled by temperature and incubation time in the range of 14-20 kDa, although with substantial polydispersity. After 45 min for either temperature, the average heparin/aldehyde ratio was ~12. Polydispersity diminished at 0°C, but no changes were seen from 20 to 45 min at 25°C. Assuming that each disaccharide (on average) has a molar mass of ~ 570 Da, the aldehyde-derivatized heparin molar mass is ~8 kDa. This number agreed well with the number-averaged molar mass (M_n) of 7.5 kDa. Overall, in terms of optimal M_w , polydispersity, throughput, and reaction yield (see below), the best reactions conditions for heparin-aldehyde were at pH 4.0, 20 min reaction time at 25°C (henceforth ‘optimized heparin-aldehyde’). This optimized heparin-aldehyde will be the formulation considered towards the subsequent surface functionalization of PCL (PCL-AHA and PCL-HEP samples discussed below) and assessment of blood coagulation.

Reaction yields of depolymerized heparin followed by dialysis

After the heparin depolymerization, dialysis was employed to remove the smallest fragments and selectively concentrate the largest oligosaccharides. This had the desired effect, but at a cost of losing a percentage of the original heparin. Figure 3C compares the reaction yields of the twelve different reaction conditions. With the largest amount of cleavage attained at pH 1.5, a majority of the heparin was converted into fragments less than 2 kDa and dialyzed away due to the 10 kDa molecular weight cutoff (MWCO) dialysis membrane. As such, only ~25% of the original heparin remained at 10 and 20 min. An increased reaction yield was seen for the pH 4.0 reactions, where ~60% of the original heparin was retained. No statistical difference in reaction yields were seen between the 0°C and 25°C under the same pH conditions.

Surface functionalization of PCL strips with optimized heparin-aldehyde

Figure 4

The PCL strips underwent alkali treatment and aminolysis to introduce surface primary amine groups, as depicted in Figure 4. No significant differences were found for the PCL M_w after alkali treatment (data not shown). X-ray photoelectron spectroscopy (XPS) characterized presence of amine groups and the subsequent grafting of optimized heparin-aldehyde. XP spectra of C1s, O1s, N1s, and S2p are shown in Figure 4 and the data summarized in Table 2. As expected of organic polymer films, all survey spectra displayed carbon and oxygen in various ratios. C1s spectra show three contributions, C-C, C-O, and C=O at ~285, 287, and 289 eV, respectively.[32, 33] The O1s spectra, also shown in Figure 4, were fitted considering three contributions: at 532.2 and 533.7 eV, attributed to C=O and C-O, respectively and the third at 531.3 attributed to oxygen species in sulfates groups. Upon heparin grafting an intense peak of sulfur was recorded, suggesting the successful grafting or adsorption of heparin-aldehyde on the PCL-HEP sample. Simultaneously, the O1s contribution at 531.3 eV rising from oxygen species in sulfates groups increased consistently with S2p peak increase. The integration of the doublet 2p_{3/2} and 2p_{1/2} at 168.7 and 170 eV, respectively, allowed us to quantify 0.6% S on the surface. In contrast, only traces of sulfur, possibly arising from sulfates contamination, were detected on the PCL-NH₂ strips. Nitrogen/carbon (N/C), determined from both contributions in N1s peak (-NH₂ and -NH₃⁺, at 399.8 and 401.6 eV, respectively) and oxygen/carbon (O/C) ratios also increased as a consequence of heparin-aldehyde grafting. For both the PCL-NH₂ samples and PCL-HEP

383 samples, traces of sodium and chlorine were present, likely due to the phosphate buffered
384 saline rinses (data not shown).

Table 2

385
386 The molecular formula of a Heparin disaccharide is $C_{12}H_{19}NO_{20}S_3$. With the
387 depolymerization described in Figure 1, complete fragmentation at pH 1.5 would yield the
388 disaccharide $C_{12}H_{17}O_{18}S_2$. Therefore, the ratio O/S in the immobilized depolymerized heparin
389 will range between 7 and 9, depending on the degree of depolymerization. Based on this
390 reasoning, we calculated the % of oxygen arising from heparin in Table 2. O/C ratio in PCL
391 is ca. 0.30 and 0.31 in the PCL-NH₂ samples. We calculated O/C ratio for PCL-HEP by
392 subtracting O elements likely arising from heparin. The results were consistent with the
393 expected data (0.30-0.32) indicating that the overall molecular formula of heparin was
394 preserved on the PCL-HEP strips.

395
396 **Blood compatibility of PCL and surface functionalized heparin-aldehyde**

Figure 5

397
398 Qualitatively the heparin aldehyde was initially demonstrated to have noticeable
399 anticoagulant activity, similar to that of unmodified heparin when added to freshly drawn
400 blood, both of which considerably reduced clot formation after 30mins (Figure 5A image).

401 Quantitatively the colorimetric solution based clotting assay (Figure 5A graph) shows that
402 both heparin and heparin aldehyde prevent blood coagulation to a similar degree for the entire
403 60 minute time course.

404 Pristine polycaprolactone (PCL) was subsequently modified by alkaline treatment (PCL-Alk),
405 then with unmodified heparin through electrostatic adsorption (PCL-PeHEP) covalent bound
406 through multipoint amide crosslinking (PCL-MPH) and optimized heparin-aldehyde through
407 nonspecific electrostatic adsorption (PCL-AHA) or through a rapid end-terminal, single point
408 attachment with the aid of a reductive 'Coupling Buffer' (PCL-HEP). The samples were
409 tested over a 60 min time course also using the modified assay (Figure 5B)[25]. The pristine

PCL showed good early blood contacting qualities up to 40 mins, possibly due to the relative hydrophobicity of the surface. In comparison PCL-Alk being the most hydrophilic demonstrated the most prothrombotic profile. PCL-MPH, PCL-PeHEP and PCL-AHA all displayed antithrombotic qualities better than the PCL-Alk for the first 40mins. PCL-AHA provided a better surface than either PCL-PeHEP and PCL AHA even though increasing blood clotting was recorded for each of these treatments. However, the PCL-HEP produced the best antithrombotic surface, remaining consistent throughout the experiment with no indication of blood clotting for the entire 60 mins (Figure 5B)

A second experiment to assess surface antithrombotic activity by colormetric assessment of surface blood clotting was performed on samples PCL, PCL-MPH, PCL-AHA and PCL-HEP. These samples were immersed in freshly drawn recalcified citrated blood for 30 min, before surface initiated clot formation was assessed.[34, 35] PMMA cuvettes were chosen as the material has a long partial thromboplastin time and have been used to fabricate test cells for blood compatibility assay. The recalcified citrated blood did not clot on the surface of the PMMA cuvettes, instead the clots were generated from the surface of the PCL films in each sample. Results and representative surface clot photographs are displayed in Figure 5C. Unmodified heparin (PCL-MPH) displayed blood clotting density similar to pristine PCL. PCL surface functionalized with optimized heparin-aldehyde (PCL-AHA & PCL-HEP strips) exhibited an order of magnitude less clotting density compared to immobilized unmodified heparin. There was no significant difference in the method of surface immobilization--both had similar low magnitudes of clotting initiation and thrombus density. However, visual inspection of the photographs display that more intermittent clotting densities exist on the PCL-AHA (electrostatically bound) samples than on the PCL-HEP samples. As seen above, this suggests that PCL-AHA samples lacked complete surface coverage or the heparin-aldehyde became competitively displaced over time, as would be expected of nonspecifically bound heparin in a complex blood milieu. In contrast, the PCL-HEP samples displayed the lowest magnitude of thrombus density and had the most visually transparent surfaces. For comparison, the simplified surface chemistries of PCL, PCL-MPH, PCL-AHA, and PCL-HEP are displayed in Figure 5D.

Figure 6

Platelet adhesion and biomaterial surface activation is one of the primary initiating mechanisms of thrombosis.[36, 37] Figure 6A displays the SEM images of adherent platelets on pristine PCL and various heparin modified PCL surfaces. Pristine PCL surfaces are heavily concentrated with adhered, activated platelets, as revealed by their irregular shape and presence of numerous pseudopodia. The surface concentration of adhered platelets is much lower when heparin is carbodiimide-mediated covalently crosslinked to PCL (PCL-MPH). Electrostatically bound (optimized) heparin-aldehyde (PCL-AHA) displays similar activation as seen on pristine PCL (no statistical difference observed), likely due to the inflammatory action of aldehyde functional groups[38]. Compared to PCL, the surface concentration of adhered platelets was halved on heparin-aldehyde that is end-terminal crosslinked to PCL through reductive amination (PCL-HEP). A noticeable proportion of platelets are rounded (unactivated) in the PCL-HEP samples as compared to PCL and PCL-AHA films. As seen in Fig. 6B, PCL-HEP has significantly less platelet adhesion than both PCL and PCL-AHA.

455 Discussion

456 The use of static light scattering combined with size exclusion chromatography has been
457 described as the method of choice for determining heparin molecular weight in the absence of
458 a structurally similar calibrant[39, 40]. Since static light scattering molar mass measurement
459 is absolute, this method eliminates the need for calibration using molar mass standards, a
460 considerable advantage when working with chemically unique polymers, as structurally
461 similar standards are usually lacking. Other scenarios require absolute molar mass
462 measurement, especially when polymer-column adsorption interactions are likely (e.g.
463 aldehyde and ketone functional groups).[41-43]

464 A qualitative analysis at UV₂₈₀ allows direct detection of aldehydes, which may be beneficial
465 for separation and fractionation of heparin-aldehyde oligomers. A more sensitive quantitative
466 analysis employing aniline ensures effective quality control of the heparin prior to surface
467 functionalization. The quantitative analysis allows characterization of end-group functionality,
468 aldehyde/molar mass distribution ratios, assessment of side reactions, and heparin-aldehyde
469 half-life determination in various environments. Besides increasing sensitivity, other benefits
470 are noted, including long-term stabilization of the UV chromophore and preventing
471 aldehyde/SEC column interactions. The methodology also allows pre- and post-reduction
472 comparisons, each with unique UV absorbance spectra (phenylimine vs. phenylamine) to
473 quantify the aldehyde content, which may be useful for the reductive coupling of aldehyde-
474 amine functional groups.[44] Heparin fragmentation at pH 4 reaction conditions displayed
475 sufficient reactive aldehyde groups for use in surface immobilization with a molar ratio of
476 aldehyde to disaccharide heparin monomers of 12:1, whereas at pH 1.5 the ratio was greatly
477 reduced to approximately 6:1, after dialysis cleanup. By selective tuning of reaction
478 conditions combined with the proper choice of dialysis membranes, extended ranges of 1:1 to
479 20:1 heparin-aldehyde ratios could be envisioned. The height of the heparin brush border can
480 thus be regulated since a dodecasaccharide (12 mer or 6-disaccharides) has a length of 5 nm.
481 The antithrombotic properties of the heparin decreases with the shortening of the polymer
482 down to the pentasaccharide, for example, by reducing the chain to less than 18
483 oligosaccharides ($M_w = 5400$), antifactor IIa activity becomes undetectable [45].

484 Under optimized reaction conditions (pH 4, 25°C in 20 min or less), optimal molar masses of
485 ~8 kDa heparin (or less) were produced after dialysis. With cleavage of most GlcN residues,
486 no further MW degradation was seen after 20 min. The resultant MW of approximately 8

kDa M_w is large enough to have a high probability of including the pentasaccharide sequence responsible for binding antithrombin (approx. 1.5 to 3kDa) [9]. Heparin oligomers of 5 kDa have previously been shown to incorporate anticoagulant active pentasaccharide in 15 to 25% of the fragmented heparin oligomers.[46] Indeed, the optimized heparin-aldehyde (PCL-HEP) described above demonstrated superior whole blood, anticoagulant properties compared to a multipoint attached unmodified heparin (PCL-MPH, Fig. 5B and C) A higher platelet activation is observed due to the likely presence of unreacted aldehyde groups, The inhibition of platelet binding is a property associated with heparin ⁴, however it is not as directly linked to heparin as antithrombotic activity since antithrombosis has been observed without a proportional inhibition of platelet binding[47].The successful Carmeda bioactive surface was developed from studies carried out on surface tethering 8 kDa acid depolymerized heparin, then assessing its antithrombin activity.[48-51] Under optimized reaction conditions, we synthesized an 8 kDa heparin fragment in good yield under mild ambient conditions (aka optimized heparin-aldehyde). Prevention of blood clotting by the optimized heparin-aldehyde compared favorably to unmodified heparin when incubated in freshly drawn rat blood. Surface functionalized heparin, either through electrostatic adsorption or through covalent crosslinking yielded very different anticoagulation activity, when the optimized heparin-aldehyde (8 kDa, PCL-AHA or PCL-HEP samples) was compared to unmodified heparin (24 kDa, PCL-MPH samples). The 8 kDa heparin displayed an order of magnitude less blood clot density which was easily visually discerned in the photographs and statistically significant ($p < 0.01$, Figure 5C). However, no statistical significance was seen between heparin electrostatically or covalently bound to alkaline treated PCL. The XPS spectra showed the presence of heparin which suggests that the strategy of covalent binding succeeded explaining the relatively high anticoagulant activity observed. However, PCL-HEP samples displayed higher platelet activation than PCL-MPH, but less than PCL-AHA, suggesting that inflammatory aldehyde groups are still present after the reductive amination and the method of amination may need further improvement, and will be a focus of our future work. It was unexpected that optimized heparin-aldehyde had considerable amounts of electrostatic binding, as most of the cationic primary amines within the GlcN residues should have converted to terminal aldehyde groups under the acidic depolymerization reactions. We speculate that PCL-AHA still has a fair amount of electrostatic binding through electrical double layer interactions; heparin-aldehyde forms reversible hemiacetal linkages with the available hydroxyl linkages next to acidic carboxylic acids, or combination thereof. Similar aldehyde formation and subsequent hemiacetal formation has been observed in other

polysaccharide oxidations.[52, 53] Indeed, a more sophisticated investigation of blood proteins binding to a heparin functionalized surfaces favors hemiacetal formation as well, as it would still have an end-point covalent attachment. Gore et al. concluded that end point attachment of heparin yielded lower coagulation marker than any heparin that was bound by multipoint attachment.[54] Both mechanisms, however, would account for the slow release of heparin from physically adsorbed surfaces.[55, 56]

Some of the limitations in this work include assumptions in the stability and equilibrium of the aniline derived Schiff-base (see Figure 1). Although this equilibrium reaction has been known to strongly favor the reaction products, this may change under different conditions of pH, temperature, and buffer.[21-23] However, under the SEC buffer eluent, where there is no excess aniline and the reversible reaction may form small amounts of reactants if the elution times are large. Quantitating these small reactants isn't feasible, as additional chromophores can cause transimination reactions.[57] This reversibility may be an asset if the recovery of the heparin-aldehyde is necessary through the competitive addition of a small and volatile aldehyde (e.g. formaldehyde, acetaldehyde) that removes the aniline. Although this is mentioned for the reader's consideration, the Schiff base is regarded as stable under ambient physiological conditions. Also, it should be noted that optimized heparin-aldehyde presented throughout this manuscript was only optimized with respect to oligomer synthesis and was not optimized with respect to antithrombin activity, surface concentration, or method of surface functionalization, although many efforts by others have focused on these important areas.[58, 59] With the development of the previously described heparin-aldehyde quantitation assay, our future work will be focused on SEC-MALLS-UV based methods of characterizing heparin immobilized on PCL biomaterials, their mechanisms of binding, and residual amounts of aldehyde functional groups remaining.

Conclusion

Herein we have described a method to quantitate the formation of aldehyde-terminated heparin for use in the surface functionalization of biomedical devices. SEC-MALLS-UV based protocol facilitates assay development by detection of Schiff base on the terminal heparin-aldehyde functional group. The online analysis with light scattering, refractive index and UV detectors allows molar mass, concentrations of polymer and aldehyde containing

552 analytes to be quantified independently, simultaneously. The protocol was subsequently used
553 to synthesize an optimized heparin-aldehyde that had minimal polydispersity, high reaction
554 yield and retained antithrombin activity that was equal to or better than the unmodified
555 heparin it was synthesized from.

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707 Figure Captions

708 Figure 1. The heparin monomers typically consist of disaccharides that vary functionality (R-
709 group) by -H, SO₃, or acetyl groups—H and SO₃, have a specific method of cleavage that
710 can yield terminal aldehyde groups. i) Heparin is depolymerized by nitrous acid (HNO₂) to
711 form the heparin-aldehyde at the oligomer terminus position. ii) Aniline acetate reagent reacts
712 with the aldehyde to form the Schiff base iii) Sodium cyano-borohydride can be used to
713 reduce the Schiff-base to the phenylamine. GlcNSO₃ (N-sulfated-glucosamines), GlcN (N-
714 unsubstituted glucosamines), GlcNAc (N-acetylated-glucosamines).

715
716 Figure 2. Size exclusion chromatography of unfractionated heparin (black line) vs heparin
717 fractionated at pH 1.5 (dotted black line) and pH 4.0 (grey line) after 10 min incubation at
718 25°C. A) Differential refractive index detector and B) UV absorbance detector at 280 nm. C)
719 LEFT AXIS: UV absorbance detector at 369 nm comparing SEC profiles of heparin-aldehyde
720 (black line), heparin-Schiff base (grey line), and the reduced heparin phenylamine (black
721 dash). RIGHT AXIS: Refractive index spectra that is representative of the of a 100 µg
722 injection of optimized heparin-aldehyde.

723
724 Figure 3. A) Heparin depolymerization at pH 1.5 in nitrous acid, specifically targeting the
725 GlcNSO₃ containing disaccharides. Left axis: Weight-averaged molar mass (M_w , top of bar)
726 and number-averaged molar mass (M_n , bottom of bar) at two reaction temperatures, 0°C
727 (black bar) and 25°C (grey bar). Bar ‘lengths’ allow direct comparison of polydispersity
728 between formulations. M_w and M_n values are generally regarded to be accurate within ±10%.
729 Right axis: Molar ratio of heparin disaccharides (570 Da) to aldehydes at two reaction
730 temperatures, 0°C (black square) and 25°C (grey square). Aldehyde ratios were quantified by
731 UV/Vis absorption ($\lambda = 369$ nm) of their corresponding aniline Schiff-bases. B) Heparin
732 depolymerization at pH 4.0 in nitric acid, specifically targeting the GlcN containing
733 disaccharides. C) Percent reaction yield of oligosaccharides after nitric acid depolymerization
734 and dialysis fractionation using 10 kDa molecular-weight cut-off tubing.

735 Figure 4. Above) Reaction scheme of PCL strips surface functionalized with optimized
736 heparin-aldehyde, PCL-HEP, through the intermediate, PCL-NH₂. Below) PCL-NH₂ and
737 PCL-HEP samples analyzed by XPS with spectra peaks displayed for C1s, O1s, N1s, and S2p
738 and their decomposition. XPS data is summarized in Table 2.

739 Figure 5. A) Heparin Aldehyde anticoagulant properties quantitatively by colorimetric
740 analysis at 540nm of released hemoglobin from non-clotted erythrocytes in recalcified
741 citrated blood across a time course and qualitatively, the side image shows a comparison of
742 blood clotting in the presence of BOTTOM: PBS buffer ‘No Heparin’ (control), MIDDLE:
743 unmodified and unfractionated “heparin”, and RIGHT: optimized “heparin-aldehyde”. Size
744 of blood clot formed after 1 h incubation is estimated through clot surface area percentage
745 with respect to total petri dish area. B) Time course for coagulation of recalcified citrated
746 blood on PCL films with and without heparin treatments also by the colorimetric detection of
747 non clotted erythrocyte hemoglobin. C) Thrombosis formation on PCL films. PCL,
748 polycaprolactone film with no surface modifications was used as reference with a normalized
749 value of 1. PCL-MPH, surface functionalized PCL strips with covalently bound unmodified
750 heparin. PCL-AHA, optimized heparin-aldehyde (electrostatically bound) on PCL. PCL-
751 HEP, covalently crosslinked optimized heparin-aldehyde, terminal aldehyde attachment,
752 through Schiff base reduction. Significant differences compared with PCL and PCL-MPH
753 against PCL-AHA and PCL-HEP. D) Surface chemistry of pristine PCL, PCL-MPH, PCL-
754 AHA, and PCL-HEP.

Figure 6. A) Platelet adhesion on heparin-modified PCL relative to pristine PCL. PCL, pristine polycaprolactone, unmodified. PCL-AHA, optimized heparin-aldehyde (electrostatically bound) on PCL. PCL-HEP, covalently crosslinked optimized heparin-aldehyde, terminal aldehyde attachment, through Schiff base reduction. Significant differences compared with PCL. B) Representative SEM images of PCL and heparin-modified PCL strips after incubation with platelet-rich plasma.

Graphical Abstract Caption: Size exclusion chromatography protocol facilitates assay development by detection of aldehydes and Schiff base functional groups found on macromolecules in both aqueous and organic solvents. Online analysis with light scattering, refractive index and UV detectors allows molar mass, concentrations of polymer and aldehyde containing analytes to be quantified independently and simultaneously, with heparin-aldehyde employed as a model molecule for development.

L-iduronate-2-sulfate, N-sulfo-D-glucosamine-6-sulfate
($R=SO_3^-$) heparin disaccharide monomer

Figure 1

Figure 2

Figure 3

Figure 4

Figure 6

Heparin

SEC-MALL-UV

Elution volume