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Exposure of the lysine in the gamma chain dodecapeptide of human fibrinogen is not enhanced by adsorption to poly(ethylene terephthalate) as measured by biotinylation and mass spectrometry

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Abstract

Conformational changes in adsorbed fibrinogen may enhance the exposure of platelet adhesive sites that are inaccessible in solution. To test this hypothesis, mass spectrometric methods were developed to quantify chemical modification of lysine residues following adsorption of fibrinogen to biomaterials. The quantitative method used an internal standard consisting of isotope-labeled fibrinogen secreted by human HepG2 cells in culture. Lysine residues in the internal standard were partially reacted with NHS-biotin. For the experimental samples, normal human fibrinogen was adsorbed to polyethylene terephthalate (PET) particles. The adsorbed fibrinogen was reacted with NHS-biotin and then eluted from the particles. Constant amounts of internal standard were added to sample fibrinogen and analyzed by liquid chromatography/tandem mass spectrometry. Biotinylation of the lysine residue in the platelet-adhesive gamma chain dodecapeptide (GCDP) was quantified by comparison to the internal standard. Approximately 80% of the GCDP peptides were biotinylated when fibrinogen was reacted with NHS-biotin in solution, or adsorbed onto PET. These results are generally consistent with previous antibody binding studies and suggest that other regions of fibrinogen may be crucial in promoting platelet adhesion to materials. The results do not directly address but are consistent with the hypothesis that only activated platelets adhere to adsorbed fibrinogen.

Keywords

fibrinogen; protein adsorption; mass spectrometry; conformational change; gamma chain dodecapeptide; biomaterial; polyethylene terephthalate

Introduction

Chemical labeling of proteins followed by mass spectrometry is frequently used to map residues in proteins.¹ These procedures should be amenable to the mapping of the exposed domains in adsorbed proteins. However, mass spectrometry often yields semi-quantitative or

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Conflict of interest

No benefit of any kind will be received either directly or indirectly by the author(s).

‘all-or-nothing’ results. With adsorbed proteins, a wide range of conformational states and orientations may be expected. Thus, quantitative mass spectrometry is desirable to observe subtle yet biologically important changes in domain exposure between the solution state and the adsorbed state. We have worked extensively to develop practical quantitative mass spectrometric methods with high sensitivity.^{2,3} Here, we present methods that have been further developed and applied to the study of conformational changes in fibrinogen upon adsorption to a polymer surface.

When materials are exposed to concentrated protein solutions, high rates of adsorption result in extensive crowding of proteins on the surface. The close proximity of neighboring proteins limits their ability to unfold/denature on the surface. Lower solution concentrations decrease the rate of adsorption and allow more time for protein conformational changes. The result is that, after extended periods of time, less protein is present on surfaces if adsorbed from low concentration solutions due to the larger surface area occupied by the spread proteins. This phenomenon has been observed using radiolabeled proteins,⁴ optical measurements of surface protein density,^{5–7} sum frequency generation,⁸ circular dichroism,⁹ and directly by AFM^{10,11}. Mass spectrometric methods such as ToF-SIMS can also detect large scale conformational changes in adsorbed proteins,¹² while other methods may detect changes in exposure at specific sites in proteins.^{13,14}

In solution, platelets aggregate upon the binding of the $\alpha_{IIb}\beta_3$ integrins to the fibrinogen gamma chain dodecapeptide (GCDP) ($\gamma 400-411$).¹⁵ Although other fibrinogen domains such as $\gamma 365-383$ may also contribute to this process, the A $\alpha 572-575$ RGDS or A $\alpha 95-97$ RGD are not required.^{15,16} The situation is less clear following adsorption of fibrinogen to surfaces. Platelets incubated with a cocktail of activation inhibitors adhere to adsorbed fibrinogen, but not to other adsorbed proteins.¹⁷ Platelets also spread on enzymatically digested fibrinogen fragments adsorbed to surfaces, but only those that contained the gamma chain dodecapeptide (GCDP) or the RGDS site.¹⁷ Others have suggested that a subset of activated platelets is always present in a given preparation, and these adhere to adsorbed fibrinogen.^{18,19} Binding of monoclonal antibodies directed against a portion of the GCDP suggests that the domain becomes more accessible upon adsorption to a surface,²⁰ although an antibody directed against the GCDP itself shows much less of an effect, if any.²¹

We hypothesized that the accessibility of the lysine in the GCDP would be enhanced by adsorption from solutions containing low concentrations of fibrinogen. To test this hypothesis, we previously used qualitative MALDI-TOF mass spectrometry to study biotinylation of fibrinogen adsorbed to poly(ethylene terephthalate) (PET) particles. Biotinylation of the GCDP itself was difficult to observe by MALDI-TOF, but peptides in the gamma chain adjacent to the GCDP were present only when fibrinogen was adsorbed from low concentration solutions.¹³ In the current study, we developed techniques for quantitative mass spectrometry to determine the extent of biotinylation of the GCDP as a function of solution fibrinogen concentrations, using nano-HPLC with electrospray ionization and an ion trap mass spectrometer.

Methods

Cell Culture

HepG2 cells were cultured in Eagle’s minimum essential medium (MEM) containing 2 mM glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 U/L penicillin, 100 U/L streptomycin, and 10% fetal bovine serum (FBS) at 37°C. When the cells were 80–90% confluent, they were passaged into roller bottles containing 160 mL of leucine-free MEM (Wash. U. Tissue Culture Support Center) with 10% FBS (dialyzed to remove amino acids) and 105 $\mu\text{g/mL}$ $^{13}\text{C}_6$ - ^{15}N -leucine (Sigma-Aldrich). After two days, medium was

replaced with 100 mL of serum-free, leucine-free MEM containing 1 μ M all-trans retinoic acid (dissolved in dimethyl sulfoxide at 0.15 μ g/mL), $^{13}\text{C}_6$ - ^{15}N -leucine (105 μ g/mL; Sigma-Aldrich), 1 μ g/mL aprotinin (to inhibit plasmin) and a primary hepatocyte supplement (Lonza). The retinoic acid was added to enhance production of fibrinogen.²² Medium was collected every 48 h and protease inhibitor was added (Complete, Mini Protease Inhibitor Cocktail Tablets; Roche, 1 tablet/10mL medium, about 10 tablets). The medium was then concentrated to 5 mL and buffer exchanged with D-PBS pH 7.4 (Pierce) using ultrafiltration centrifuge tubes (Sartorius). The medium was then stored at -80°C . The concentration of fibrinogen in the medium was determined by ELISA (AssayMax Human Fibrinogen ELISA Kit) following manufacturer's protocols.

Fibrinogen Purification and Preparation of Isotope Labeled Internal Standard

Isotope-labeled fibrinogen was immunoprecipitated with a polyclonal anti-human fibrinogen antibody (IgG, Dako) that was attached to CNBr-activated Sepharose 4B beads (GE Healthcare). Antibody beads were prepared according to manufacturer's protocol and stored at 4°C in 0.1 M NaHCO_3 and 0.5 M NaCl. The fibrinogen solution was incubated with the antibody beads overnight at 4°C and then rinsed 3x with D-PBS. Bound fibrinogen was then incubated with 10 mg/mL of sulfo-NHS-biotin (Fisher) for 5 min in D-PBS. Excess biotin was removed and the partially biotinylated, isotope-labeled fibrinogen was resuspended in SDS sample buffer (10% glycerol, 62.5 mM Tris-HCL, pH 6.8, 2% SDS, 0.01 mg/ml bromophenol blue) and stored at -80°C .

PET particle fabrication

PET particles were fabricated by dissolving sheets of PET in dichloromethane with 5% (v/v) trifluoroacetic acid. Each sample was filtered through 0.45 μm PTFE filter (Fisher) into 1 L of DI water while stirring. Emulsions were stirred overnight and PET particles were removed by filtration. The particles were stored under vacuum until use. Surface area was measured by BET.

Protein Adsorption and Surface Biotinylation

PET particles (150 mg) were placed in a 1.5 mL silicone-coated polypropylene Eppendorf tube and washed 3x in ethanol and then 3x in D-PBS. Disposable polystyrene columns (Fisher) with 2 mL bed volumes were filled with the PET particles in D-PBS and allowed to settle for 30 min. The column was washed with D-PBS (3 mL), followed by 3 mL of 0.0005–2 mg/mL human fibrinogen (Sigma) in PBS. The PET surfaces were incubated with the fibrinogen solutions for 2 h at 37°C and then washed 3x with 5 mL of PBS. The PBS washes were collected in 1 mL samples for analysis with a spectrophotometer at 280 nm to monitor the decrease in protein concentration and to ensure that non-adsorbed fibrinogen was removed by the washing steps. A 10 mg/mL solution of sulfo-NHS-Biotin in PBS was added to the column for 5 min. D-PBS (5 mL) was then flowed through the column to remove excess biotin solution. Elution buffer (4% SDS, 40 mM Tris, 8 M urea, pH 8.5) was incubated in the column overnight at 37°C . The adsorbed fibrinogen was eluted from the column with 1 mL of elution buffer. An additional 1 mL of elution buffer was added to the column and incubated for 1 h prior to elution. Protein was collected and concentrated using ultrafiltration centrifuge tubes. For biotinylation of fibrinogen in solution, 0.05 mg/mL fibrinogen was incubated in 10 mg/mL Sulfo-NHS-biotin for 5 min. The fibrinogen was concentrated and desalted using ultrafiltration centrifuge tubes as above and resuspended in SDS sample buffer.

Testing the column washing protocol

A 2 mg/mL fibrinogen solution was incubated with a 10 mg/mL solution of DyLight 488 NHS ester (Pierce) at a 400:1 molar ratio of fibrinogen:dye for 16 h in the dark at room temperature. Unbound dye was removed by dialysis. PET particles (150 mg) were washed and packed into polystyrene columns as described above. All flow-through, washes, and elutes were collected and assessed on a fluorometer (CytoFluor Multi-Well Plate Reader, PerSeptive Biosystems).

SDS-PAGE

Each sample was purified by SDS-PAGE using Tris-HCl linear gradient 4–15% gels (Pierce). For each sample, a lane of internal standard was also run. The concentration of the internal standard in each lane was constant for a given batch of internal standard. The amount of internal standard per lane was chosen to yield a strong signal by mass spectrometry (about 2 µg or 5.9 pmol of fibrinogen per lane). The amount of sample fibrinogen to add to each lane was determined by running a first gel containing a range of dilutions of the sample. By densitometry of Sypro Ruby stained gels, the amount of sample fibrinogen was calculated to yield approximately 1:1 (sample fibrinogen):(internal standard fibrinogen) (Supplemental Figures S1 & S2).

Trypsin digestion and mass spectrometry

The gamma chain bands were excised from Sypro Ruby stained gels in a sterile cabinet to prevent contamination with dust, with the bands visualized by a black light. One sample band was mixed with one internal standard band in a 1.5 mL Eppendorf tube. The samples were alkylated with 100 mM iodoacetimide. In-gel trypsin digestions were performed overnight at 37°C with sequence grade trypsin (Princeton Separations). The samples were then dried in an Eppendorf Vacufuge. Trypsin-digested peptides were dissolved in 30 µL of 5% acetonitrile/0.1% formic acid (FA). The peptide solutions (5 µL) were injected into a nanoflow LC system (Eksigent) mounted on a Picoview nanoelectrospray head (New Objective, Woburn, MA) attached to an ion trap mass spectrometer (LTQ, ThermoFinnigan). The peptides in the mixture were separated at a flow rate of 250 nL/min. A gradient of 15% acetonitrile/0.1% FA to 50% acetonitrile/0.1% FA was run over 60 min, with peptides separated on a 75 µm × 15 cm column custom packed with 5 µm C18AQ Magic Resin (Michrom Bioresources, Inc.). The LTQ was operated in positive ion mode using a capillary voltage of 12 V, a capillary temperature equal to room temperature, and a spray voltage of 1.4 kV. Data were collected in full scan MS and ion specific MS/MS mode and peptides were identified using SILT mass.³

Data Analysis

The mass spectrometric data were analyzed to determine the relative amount of sample peptide to internal standard peptide. The parent ion intensities (MS1) were integrated over the elution peak at the molecular weights of the peptide of interest. The integrations were performed automatically using a custom processing method in Xcalibur (ThermoFinnigan), and all assignments were manually verified.

Results

Quantification scheme

The workflow of the quantification scheme is illustrated in Figure 1. We produced isotope-labeled human fibrinogen in liver-derived cells cultured in medium containing isotope-labeled leucine to serve as an internal standard. The amount of fibrinogen secreted by the HepG2 cells was relatively low, but in roller bottle culture, supernatant concentrations as

high as about 50 µg/mL were obtained. The isotope-labeled fibrinogen was purified from the rest of the proteins in the medium by immunoprecipitation. The protein was then biotin-labeled on the immunoprecipitation resin and removed from the resin using SDS-PAGE sample buffer. SDS-PAGE was used to estimate the concentration of the internal standard fibrinogen in the eluate. Typically, one round of immunoprecipitation yielded a quantity of internal standard sufficient for the analysis of 5–15 samples. Sample fibrinogen from the experiments (about 2 µg) was purified by SDS-PAGE alongside about 2 µg of internal standard fibrinogen. The bands corresponding to the gamma chain were excised. One band of internal standard fibrinogen was added to the same tube as one band of sample fibrinogen. The combined bands were reacted with iodoacetamide to block cysteine residues and then trypsinized. Resulting tryptic peptides were dissolved and injected in the mass spectrometer.

The main tryptic peptide of interest in this study was LTIGEGQQHHLGGAK, which contains seven residues of the GCDP. The GCDP existed as LTIGEGQQHHLGGAK(biotin)QAGDV when biotinylated, due to the inability of trypsin to cleave after biotinylated lysine.²³ To determine the ratio of sample fibrinogen to internal standard fibrinogen, we also analyzed the peptide VELEDWNGR from the gamma chain of fibrinogen ('control peptide'). This peptide is between two arginines with no intervening lysines, and thus should not be affected by biotinylation. Measuring the ratio of (isotope-labeled VELEDWNGR):(non-isotope-labeled VELEDWNGR) yielded a measure of the ratio of internal standard fibrinogen to sample fibrinogen. This ratio is symbolized here as '(I/S)' (symbols summarized in Table 1).

Nature of mass spectrometric results

Representative chromatograms are shown in Figure 2 for the three peptides of interest, i.e. the control peptide, unbiotinylated GCDP and biotinylated GCDP. For each peptide, the top chromatogram shows the MS1 elution peak of the non-isotope-labeled peptide derived from sample fibrinogen. The second chromatogram shows the MS1 elution peak for the isotope-labeled peptide derived from the internal standard. The ratio of internal standard to sample (I/S) may be estimated by dividing the area under the curve in Figure 2A(ii) by the area under the curve in Figure 2A(i). The ratio of unbiotinylated peptide in the sample to that in the internal standard (r_{ub}^{MS}) is the ratio of the area under the curve in Figure 2B(i) to the area under the curve in Figure 2B(ii). The ratio of biotinylated peptide in the sample to that in the internal standard (r_b^{MS}) is the ratio of the area under the curve in Figure 2C(i) to the area under the curve in Figure 2C(ii).

Precision of mass spectrometric quantification by MS1

To test the reproducibility of the approach, we produced standard curves using serial dilutions of biotinylated, non-isotope-labeled fibrinogen and a constant amount of biotinylated isotope-labeled fibrinogen. The amount of each type of fibrinogen was estimated by densitometry of fibrinogen bands in Sypro Ruby-stained SDS-PAGE gels. The percent isotope labeling of the control peptide, unbiotinylated GCDP and biotinylated GCDP was measured by mass spectrometry. The intensities of parent ions in the MS 1 elution peak gave close to the expected results (Figure 3). The results showed that the method was generally powered sufficiently to distinguish differences in concentration between 2–2.5 fold dilutions of the control peptide with $n=3$ (see Supplementary Table S1). With the biotinylated GCDP and unbiotinylated GCDP, a 4–5 fold dilution was generally needed to show a statistically significant difference with $n=3$. The higher precision with the control peptide was likely due to the higher intensity of the signal, due to the fact that the GCDP peptide is split into unbiotinylated and biotinylated forms. Additionally, the

unbiotinylated and biotinylated GCDP tended to be in both doubly ($z = +2$) and triply ($z = +3$) charged forms, with the latter being more intense. We have previously demonstrated that the peaks with the highest intensities give the most accurate signal,³ and thus the $z = +3$ MS1 peaks were used to quantify the amounts of the unbiotinylated and biotinylated GCDP peptides. For the control peptide, the $z = +2$ peak was much more intense than the $z = +3$ peak. Thus, the $z = +2$ peak was used for analysis of the control peptide.

Data analysis

It is likely that the internal standard peptides will not be 100% isotope-labeled. This can be observed simply by injecting the internal standard by itself and measuring the ratio of isotope-labeled to unlabeled control peptide (see Fig. 4A for workflow). We measured approximately 98% isotope-labeled control peptide when internal standard was injected by itself. This means that the probability (p) that an individual leucine in the internal standard fibrinogen was isotope-labeled was 98%.

This probability ' p ' may be used to calculate the amount of the internal standard in 'light' and 'heavy' forms. The control peptide (VELEDWNGR) contains one leucine and thus p is the fraction of 'heavy' control peptide in the internal standard. The GCDP has two leucines, so assuming random incorporation of isotope-labeled leucine, the fraction of internal standard GCDP that is doubly isotope-labeled is p^2 . The fraction of internal standard GCDP that is not isotope-labeled at either leucine is $(1-p)^2$. Any internal standard GCDP in the 'light' form will contaminate the signal from the sample GCDP. The concentration of 'light' unbiotinylated GCDP derived from the internal standard will be $f_{IS}^{ub} [I](1-p)^2$, where f_{IS}^{ub} is the fraction of the internal standard GCDP that is unbiotinylated and $[I]$ is the concentration of internal standard in the injection.

The ratio that is measured by mass spectrometry of (unbiotinylated sample GCDP): (unbiotinylated internal standard GCDP) is thus:

$$r_{ub}^{MS} = \frac{f_{smp}^{ub} [smp] + f_{IS}^{ub} [I](1-p)^2}{f_{IS}^{ub} [I]p^2} \quad [1]$$

where f_{smp}^{ub} is the fraction of the sample GCDP that is unbiotinylated, and $[smp]$ is the concentration of fibrinogen in the sample. The ratio measured by mass spectrometry of (biotinylated sample GCDP): (biotinylated internal standard GCDP) is:

$$r_b^{MS} = \frac{(1 - f_{smp}^{ub}) [smp] + (1 - f_{IS}^{ub}) [I](1-p)^2}{(1 - f_{IS}^{ub}) [I]p^2} \quad [2]$$

because $f_{smp}^{ub} + f_{smp}^b = 1$, where f_{smp}^b is the fraction of sample that is biotinylated. These equations may be simplified by dividing the numerator and denominator by $[smp]$. This introduces the quantity (I/S), the mol ratio of internal standard fibrinogen to sample fibrinogen, i.e. $(I/S) = [I]/[smp]$. Thus, the equation [1] may be re-arranged as:

$$f_{smp}^{ub} = f_{IS}^{ub} (I/S) (r_{ub}^{MS} p^2 - (1-p)^2) \quad [3]$$

and equation [2] may be written as:

$$f_{smp}^{ub} = \left(1 - \left(1 - f_{IS}^{ub}\right) \left(I/S\right) \left(r_{ub}^{MS} p^2 - (1 - p)^2\right)\right) \quad [4]$$

The system is overdetermined, so either equation [3] or [4] may be used to calculate f_{smp}^{ub} . The quantity (I/S) is measured for each sample via the control peptide (VELEDWNGR).

Each batch of internal standard was reacted with 10 mg/mL NHS-biotin for 5 min while still on the immunoprecipitation resin. The ratio f_{IS}^{ub} (the fraction of biotinylated GCDP in the internal standard) was measured independently for each batch of internal standard using a method suggested by equation [3]. If internal standard is simply diluted with non-isotope-labeled, completely unbiotinylated fibrinogen (i.e. normal human fibrinogen), then f_{smp}^{ub} is equal to 1 and equation [3] may be solved for f_{IS}^{ub} , with (I/S) and p measured as described above, and r_{ub}^{MS} measured by mass spectrometry. Figure 4B shows the workflow and the expected results for dilution of the biotinylated internal standard with normal fibrinogen. Using this dilution method, we found that f_{IS}^{ub} was about 0.2, meaning that the GCDP in the internal standard was about 80% biotinylated.

Mass spectrometric measurements of three biological replicates of fibrinogen biotinylated for 5 min in solution at 0.05 mg/mL showed less deviation when the mass spec data were analyzed using equation [3] compared to equation [4] (Figure 5). These are three repeated measurements from three biological samples and should show very little variation. Equation [3] uses only mass spec data from the unbiotinylated GCDP, while equation [4] uses only mass spec data from the biotinylated GCDP. The source of lack of precision with the biotinylated GCDP is unknown, but a co-eluting peak was often, but not always present with the biotinylated, isotope-labeled GCDP (Supplemental Figure S3). In the subsequent analysis, f_{smp}^{ub} was calculated using equation [3] exclusively due to the higher precision of the results obtained. Note that the ratio of r_b^{MS} to r_{ub}^{MS} could be used as a simple measure of biotinylation. However, this ratio of ratios relies on high quality data for both the unbiotinylated and biotinylated GCDP for accuracy.

Extent of biotinylation of adsorbed fibrinogen GCDP

Figure 6 shows results from ten independent experiments analyzed using equation [3]. Fibrinogen was adsorbed to PET across a range of solution fibrinogen concentrations and then biotinylated on the surface for 5 min. For comparison, fibrinogen in solution at 0.05 mg/mL was also biotinylated for 5 min. For nine of the ten experiments, values of f_{smp}^{ub} were usually within the range of 0.1–0.35. The results overall indicated that the lysine on the GCDP was easily accessible to NHS-biotin in solution, on the immunoprecipitation resin and when adsorbed onto surfaces. Furthermore, no dependence on the solution concentration of fibrinogen was apparent for the surface-biotinylated samples. Thus, the data indicate that the GCDP lysine is no more or less accessible to NHS-biotin when adsorbed to surfaces under conditions that we have previously shown affect the spread area of adsorbed fibrinogen.¹³ One outlier experiment (far right of Figure 6) showed much lower levels of biotinylation (higher f_{smp}^{ub}). This result may have been due to the use of older, somewhat inactivated NHS-biotin. Regardless, no difference was observed between 0.05 and 2 mg/mL fibrinogen, reinforcing the other results. We conclude that the accessibility of the lysine in the gamma chain dodecapeptide is not affected by spreading of fibrinogen onto PET, at least

to the small molecule probe used in this study and with the current limits on quantitative mass spectrometry.

The likelihood of a Type II error was assessed. If adsorption were to increase the exposure of the GCDP lysine, the amount of unbiotinylated GCDP would be expected to decrease.

Solution phase labeling resulted in a mean f_{smp}^{ub} of 0.236 ± 0.056 . With this standard deviation, a 2.2-fold decrease in the concentration of unbiotinylated peptide would be required to demonstrate a difference with $n = 4$ and a statistical power $1 - \beta = 0.8$. This corresponds to $f_{smp}^{ub} = 0.12$. The actual values for adsorbed fibrinogen are shown in Table 2. For the outlier experiment with a lesser degree of biotinylation (f_{smp}^{ub} in the range of 0.8) with $n = 3$, fibrinogen adsorbed from a 2 mg/mL solution had $f_{smp}^{ub} = 0.808 \pm 0.033$. The experiment was sufficiently powered to detect a difference if fibrinogen adsorbed from a 0.05 mg/mL solution had a $f_{smp}^{ub} = 0.65$ (a 2.2 fold decrease), assuming a standard deviation of 0.07. The actual value was $f_{smp}^{ub} = 0.855 \pm 0.065$.

We also addressed two potential questions about the methods. Supplemental Figure S4 examines the effectiveness of the washing protocol to remove non-adsorbed fibrinogen. Non-adsorbed fibrinogen could remain in the column and the overall measurement would reflect some degree of solution-phase labeling. To characterize the washing protocols, fibrinogen was fluorescently labeled and adsorbed to PET particles as in a typical experiment. The amount of fibrinogen in subsequent washes was measured. Three washes were found to be sufficient to reduce fibrinogen to levels that were difficult to measure. Three washes were thus used in all experiments. Additionally, the biotinylation protocol itself required more washes, so any biotinylated non-adsorbed fibrinogen would likely be washed from the column prior to elution with SDS. A second potential question is why we might expect to see such vastly different behavior between 2 mg/mL fibrinogen solutions and, for example, 0.1 mg/mL. Supplemental Figure S5 shows that the surface area of PET particles, measured by BET, was such that 0.1 mg/mL solutions were not sufficient to fill a complete monolayer (monolayer coverage in the range between the dotted line, as measured in reference 19), while the 2 mg/mL solutions were in excess of a monolayer.

Discussion

It has been debated if conformational changes in the adsorbed state enhance the accessibility of the adhesive sites in fibrinogen to platelet receptors. Salzman demonstrated that despite similar quantities of fibrinogen adsorption, platelet reactivity increased with increasing carbon chain length on different poly(alkyl methacrylate) surfaces. The ability of anti-fibrinogen antibodies to bind to adsorbed fibrinogen was highly correlated to platelet adhesion,²⁴ suggesting that the *native* structure of fibrinogen was needed for platelet binding to adsorbed fibrinogen. Later, Savage and Ruggeri found that a cocktail of platelet inhibitors did not prevent platelet adhesion to adsorbed fibrinogen.¹⁷ Fibrinogen fragments that contained the GCDP but not the A α 572–575 RGDS site supported some adhesion of unactivated platelets, but not to the same degree as intact fibrinogen. A fragment that contained only the A α 95–97 RGD site did not support unactivated platelet adhesion. These results suggested a role for both the GCDP and the A α 572–575 RGDS site in unactivated platelet adhesion. These sites are not recognized by unactivated platelets in solution, such that conformational changes would be necessary to promote unactivated platelet adhesion, which is the opposite of the conclusions of Salzman. However, Bonnefoy et al. countered that platelets that adhered to adsorbed fibrinogen might actually have been due to a small number of activated platelets present despite the presence of multiple inhibitors.^{18,19}

Monoclonal antibody binding has been used extensively to detect conformational changes in fibrinogen upon adsorption. For the GCDP, the most commonly used antibodies are 4-2 (antigen: γ 392–406; i.e. the peptide called ‘unbiotinylated GCDP’ in this study) and 4A5 (antigen: γ 402–411; most of the GCDP). Both of these antigens contain the lysine that was interrogated in this study (γ 406). The two antibodies were shown to increase their binding to fibrinogen adsorbed from 1% plasma over the course of about 3 days of residence time on a surface, although the increase in binding of 4A5 was small compared to that of 4-2.²¹ A later study showed that total bound 4A5 did not change over time, but the affinity of this antibody for adsorbed fibrinogen decreased over time.²⁵ Others working with 4A5 and fibrinogen adsorbed to different materials also found little change in antibody binding over the course of 2 h.²⁶

Further study by Moskowitz et al. with fibrinogen adsorbed on polystyrene showed that as the solution concentration of fibrinogen increased, binding of the 4-2 antibody to surface-bound fibrinogen increased monotonically up to a saturated level. The 4-2 antibody bound poorly to fibrinogen in solution, so the surface likely caused enhanced exposure of the antigen,²⁰ a conclusion also reached by Salzman²⁷ and Ugarova²⁸. Siedlecki performed AFM with tips coated with 4-2 antibody, showing that the antibody became less likely to bind with extended residence time of fibrinogen on mica.²⁹ Fibrinogen adsorbed onto hydrophilic glass did not bind antibody 4-2, but the antibody did bind to polystyrene-adsorbed fibrinogen, suggesting a role for less hydrophilic surfaces in the exposure of the GCDP.³⁰

Antibody binding thus suggests that the GCDP, or at least γ 392–406, becomes exposed upon adsorbing to surfaces. In a previous study, we used MALDI-TOF to map the exposure of lysines in adsorbed fibrinogen.¹³ We found that peaks with m/z corresponding to the biotinylated GCDP were rarely observed. However, peptides corresponding to γ 370–395 were frequently found to be biotinylated if fibrinogen was adsorbed from 0.1 and 0.5 mg/mL, but never if adsorbed from 1 mg/mL solution, or if biotinylation was performed in solution. This was consistent with previous findings that the 4-2 antibody (antigen: γ 392–406) shows a larger increase in binding upon adsorption than 4A5 (antigen: γ 402–411). The current study demonstrated that the GCDP does not show greater exposure upon adsorption, at least with the small biotin probe and within the limits of current mass spectrometric technology. Being charged and at the C-terminus of the gamma chain, the lysine γ 406 might be relatively exposed in solution. Indeed, the crystal structure of human fibrinogen obtained by Doolittle in 2009 does not contain γ 396–411 because this domain is too unstructured.^{31–33}

The current study was powered to detect greater than a 2.2 fold increase in biotinylation. The possibility exists that a smaller fraction of fibrinogen molecules undergo conformational changes leading to platelet adhesion. However, fibrinogen adsorbed from 0.1, 0.05 and 0.0005 mg/mL solutions should have been sub-monolayer, and previous AFM studies suggest nearly ubiquitous conformational changes at sub-monolayer conditions.¹¹ Future studies will surely benefit from advances in mass spectrometric quantification.

Whereas other mass spectrometric methods detect global changes in protein conformation,¹² the current technique is the ability to detect conformational changes at specific sites. Future studies should focus on conformational changes in other regions of fibrinogen, although new internal standards and techniques will need to be tailored to each tryptic peptide. For example, the sequence adjacent to the GCDP may exhibit more changes in accessibility to NHS-biotin (WYSMKKTTMKIIPFNR, γ 376–391). However, the peptide presents some challenges. Biotinylation will produce a range of products due to the presence of three lysines between adjacent arginines. The sequence also does not contain a leucine for

isotope-labeling, but isoleucine, phenylalanine, lysine or threonine are possible alternatives as these are also essential amino acids. The A α 572–575 RGDS site is also of interest, but also does not contain nearby leucines. Alternative protein modifications may also be explored, for example Latour and colleagues have recently used a tryptophan-modifying reagent to detect changes in conformation in adsorbed lysozyme and glucose oxidase.¹⁴ Tryptophan is not present near the RGDS site in fibrinogen, but is present in γ 376–391. Combined with advancements in mass spectrometry and large scale production of isotope-labeled fibrinogen, the structure of adsorbed fibrinogen could be extensively mapped using these techniques.

Conclusions

Quantitative proteomic methods were used to study protein conformational changes upon adsorption. Isotope-labeled internal standards of human fibrinogen were produced in cell culture. Standard curves suggested relatively high precision using the integrated MS1 elution peaks. Our results strongly suggested that the GCDP lysine was not more accessible upon adsorption, at least to this small molecule probe and within the current limits of quantitative mass spectrometry.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Sinz A. Chemical cross-linking and mass spectrometry for mapping three-dimensional structures of proteins and protein complexes. *J Mass Spectrom.* 2003; 38(12):1225–37. [PubMed: 14696200]
2. Bateman RJ, Munsell LY, Morris JC, Swarm R, Yarasheski KE, Holtzman DM. Human amyloid-beta synthesis and clearance rates as measured in cerebrospinal fluid in vivo. *Nat Med.* 2006; 12(7):856–61. [PubMed: 16799555]
3. Elbert DL, Mawuenyega KG, Scott EA, Wildsmith KR, Bateman RJ. Stable isotope labeling tandem mass spectrometry (SILT): integration with peptide identification and extension to data-dependent scans. *J Proteome Res.* 2008; 7(10):4546–56. [PubMed: 18774841]
4. Tsai WB, Grunkemeier JM, Horbett TA. Human plasma fibrinogen adsorption and platelet adhesion to polystyrene. *J Biomed Mater Res.* 1999; 44(2):130–9. [PubMed: 10397913]
5. Ramsden JJ. Concentration scaling of protein deposition kinetics. *Physical Review Letters.* 1993; 71(2):295–298. [PubMed: 10054913]
6. Wertz CF, Santore MM. Effect of surface hydrophobicity on adsorption and relaxation kinetics of albumin and fibrinogen: Single-species and competitive behavior. *Langmuir.* 2001; 17(10):3006–3016.
7. Wertz CF, Santore MM. Adsorption and relaxation kinetics of albumin and fibrinogen on hydrophobic surfaces: Single-species and competitive behavior. *Langmuir.* 1999; 15(26):8884–8894.
8. Clarke ML, Wang J, Chen Z. Conformational changes of fibrinogen after adsorption. *J Phys Chem B Condens Matter Mater Surf Interfaces Biophys.* 2005; 109(46):22027–35. [PubMed: 16853860]
9. Hylton DM, Shalaby SW, Latour RA Jr. Direct correlation between adsorption-induced changes in protein structure and platelet adhesion. *J Biomed Mater Res A.* 2005; 73(3):349–58. [PubMed: 15834930]

10. Sit PS, Marchant RE. Surface-dependent conformations of human fibrinogen observed by atomic force microscopy under aqueous conditions. *Thrombosis and Haemostasis*. 1999; 82(3):1053–1060. [PubMed: 10494763]
11. Agnihotri A, Siedlecki CA. Time-dependent conformational changes in fibrinogen measured by atomic force microscopy. *Langmuir*. 2004; 20(20):8846–8852. [PubMed: 15379516]
12. Xia N, Castner DG. Preserving the structure of adsorbed protein films for time-of-flight secondary ion mass spectrometry analysis. *J Biomed Mater Res A*. 2003; 67(1):179–90. [PubMed: 14517875]
13. Scott EA, Elbert DL. Mass spectrometric mapping of fibrinogen conformations at poly(ethylene terephthalate) interfaces. *Biomaterials*. 2007; 28(27):3904–17. [PubMed: 17582492]
14. Fears KP, Sivaraman B, Powell GL, Wu Y, Latour RA. Probing the Conformation and Orientation of Adsorbed Enzymes Using Side-Chain Modification. *Langmuir*. 2009; 25(16):9319–9327. [PubMed: 19610641]
15. Farrell DH, Thiagarajan P, Chung DW, Davie EW. Role of fibrinogen alpha and gamma chain sites in platelet aggregation. *Proc Natl Acad Sci U S A*. 1992; 89(22):10729–32. [PubMed: 1438269]
16. Podolnikova NP, Yakubenko VP, Volkov GL, Plow EF, Ugarova TP. Identification of a novel binding site for platelet integrins alpha(IIb)beta(3) (GPIIb/IIIa) and alpha(5)beta(1) in the gamma C-domain of fibrinogen. *Journal of Biological Chemistry*. 2003; 278:32251–32258. [PubMed: 12799374]
17. Savage B, Ruggeri ZM. Selective recognition of adhesive sites in surface-bound fibrinogen by glycoprotein IIb-IIIa on nonactivated platelets. *J Biol Chem*. 1991; 266(17):11227–33. [PubMed: 2040630]
18. Bonnefoy A, Liu Q, Legrand C, Frojmovic MM. Efficiency of Platelet Adhesion to Fibrinogen Depends on both Cell Activation and Flow. *Biophysical Journal*. 2000; 78(6):2834–2843. [PubMed: 10827966]
19. Bonnefoy A, Liu QD, Jerome WG, Legrand C, Frojmovic MM. Platelets in suspension require preactivation to adhere to immobilized fibrinogen. *Fibrinogen*. 2001; 936:459–463.
20. Moskowitz KA, Kudryk B, Collier BS. Fibrinogen coating density affects the conformation of immobilized fibrinogen: Implications for platelet adhesion and spreading. *Thrombosis and Haemostasis*. 1998; 79(4):824–831. [PubMed: 9569199]
21. Horbett TA, Lew KR. Residence Time Effects on Monoclonal-Antibody Binding to Adsorbed Fibrinogen. *Journal of Biomaterials Science-Polymer Edition*. 1994; 6(1):15–33. [PubMed: 7947470]
22. Nicodeme E, Nicaud M, Issandou M. Retinoids stimulate fibrinogen production both in vitro (hepatocytes) and in vivo. Induction requires activation of the retinoid X receptor. *Arterioscler Thromb Vasc Biol*. 1995; 15(10):1660–7. [PubMed: 7583541]
23. Azim-Zadeh O, Hillebrecht A, Linne U, Marahiel MA, Klebe G, Lingelbach K, Nyalwidhe J. Use of Biotin Derivatives to Probe Conformational Changes in Proteins. *Journal of Biological Chemistry*. 2007; 282(30):21609–21617. [PubMed: 17545162]
24. Lindon JN, McManama G, Kushner L, Merrill EW, Salzman EW. Does the conformation of adsorbed fibrinogen dictate platelet interactions with artificial surfaces? *Blood*. 1986; 68(2):355–362. [PubMed: 3730606]
25. Grunkemeier J, Wan C, Horbett T. Changes in binding affinity of a monoclonal antibody to a platelet binding domain of fibrinogen adsorbed to biomaterials. *Journal of Biomaterials Science-Polymer Edition*. 1996; 8(3):189–209. [PubMed: 8996693]
26. Balasubramanian V, Grusin NK, Bucher RW, Turitto VT, Slack SM. Residence-time dependent changes in fibrinogen adsorbed to polymeric biomaterials. *Journal of Biomedical Materials Research*. 1999; 44(3):253–260. [PubMed: 10397927]
27. Shiba E, Lindon JN, Kushner L, Matsueda GR, Hawiger J, Kloczewiak M, Kudryk B, Salzman EW. Antibody-detectable changes in fibrinogen adsorption affecting platelet activation on polymer surfaces. *Am J Physiol*. 1991; 260(5 Pt 1):C965–74. [PubMed: 2035620]

28. Lishko VK, Kudryk B, Yakubenko VP, Yee VC, Ugarova TP. Regulated unmasking of the cryptic binding site for integrin $\alpha(M)\beta(2)$ in the gamma C-domain of fibrinogen. *Biochemistry*. 2002; 41(43):12942–12951. [PubMed: 12390020]
29. Soman P, Rice Z, Siedlecki CA. Measuring the time-dependent functional activity of adsorbed fibrinogen by atomic force microscopy. *Langmuir*. 2008; 24(16):8801–8806. [PubMed: 18616311]
30. Tunc S, Maitz MF, Steiner G, Vazquez L, Pham MT, Salzer R. In situ conformational analysis of fibrinogen adsorbed on Si surfaces. *Colloids and Surfaces B: Biointerfaces*. 2005; 42(3–4):219–225.
31. Kollman JM, Pandi L, Sawaya MR, Riley M, Doolittle RF. Crystal Structure of Human Fibrinogen. *Biochemistry*. 2009; 48(18):3877–3886. [PubMed: 19296670]
32. Pandi L, Kollman JM, Lopez-Lira F, Burrows JM, Riley M, Doolittle RF. Two Families of Synthetic Peptides That Enhance Fibrin Turbidity and Delay Fibrinolysis by Different Mechanisms. *Biochemistry*. 2009; 48(30):7201–7208. [PubMed: 19588915]
33. Bowley SR, Okumura N, Lord ST. Impaired Protofibril Formation in Fibrinogen N308K Is Due to Altered D:D and A:a Interactions. *Biochemistry*. 2009; 48(36):8656–8663. [PubMed: 19650644]

Figure 1.
Work flow for quantifying surface biotinylation of fibrinogen.

A Control peptide: Human fibrinogen γ 274–282

B Unbiotinylated GCDP: Human fibrinogen γ 392-406

C Biotinylated GCDP: Human fibrinogen γ 392-411

Figure 2.

(A) Chromatograms of MS1 elution peaks of the doubly charge control peptide (VELEDWNGR). (i) intensities of native control peptide at 559.1–560.1 amu of native peptide, (ii) intensities of isotope-labeled control peptide at 562.1–563.1 amu (B) Chromatograms of MS1 elution peaks of the triply charged unbiotinylated gamma chain dodecapeptide (GCDP). (i) intensities at 515.4–516.4 amu of native peptide. (ii) intensities of isotope-labeled peptide at 519.4–520.4 amu. (C) Chromatograms of MS1 elution peaks of the triply charged biotinylated gamma chain dodecapeptide (GCDP). (i) intensities at 747.9–748.9 amu of native peptide, (ii) intensities of isotope-labeled peptide at 751.8–752.8 amu. Area under the curve is listed for each peak.

Figure 3. Standard curves at various ratios of (isotope-labeled internal standard fibrinogen):(sample fibrinogen). $\blacklozenge$ = Control peptide (VELEDWNGR), $\circ$ = unbiotinylated GCDP, X = biotinylated GCDP. The line of perfect of correlation is shown.

Workflow for characterization of each batch of internal standard

A Determine extent of isotope-labeling

- 1) Internal standard (i.e. isotope-labeled fibrinogen) made in HepG2 cells with $^{13}\text{C}_6$, ^{15}N -leucine (abbreviated 'L(^{13}C)') will not be 100% isotope-labeled.
- 2) To determine this quantity, inject internal standard alone into the mass spectrometer.
- 3) Observe the fraction of control peptide that is labeled by comparing VEL(^{13}C)EDWNGR ('heavy') intensity to VELEDWNGR ('light').
- 4) This fraction is p , the probability that any leucine in the internal standard fibrinogen is isotope-labeled.
- 5) Use p to calculate the fractions of different forms of GCDP in the internal standard as:

$$(1-p)^2 \text{ LTIGEGQQHHLGGAKQAGDV}$$

$$2p(1-p) \begin{cases} \text{LTIGEGQQHHL}^{(13}\text{C})\text{GGAKQAGDV} \\ \text{L}^{(13}\text{C})\text{TIGEGQQHHLGGAKQAGDV} \end{cases}$$

$$p^2 \text{ L}^{(13}\text{C})\text{TIGEGQQHHL}^{(13}\text{C})\text{GGAKQAGDV}$$

- 6) Correct for amount of 'light' peptides in measurements of experimental samples.

B Determine extent of biotinylation

- 1) Dilute internal standard (i.e. isotope-labeled fibrinogen) with different concentrations of unbiotinylated (i.e. normal) fibrinogen
- 2) Use the control peptide (VELEDWNGR) to determine (I/S), the ratio of internal standard fibrinogen to normal fibrinogen
- 3) Measure the percent of unbiotinylated GCDP that is isotope labeled by mass spec (ordinate of figure below)
- 4) Calculate the fraction of the internal standard GCDP that is unbiotinylated (f_{IS}^{ub})

Figure 4.

Workflow for characterization of the extent of isotope-labeling and degree of biotinylation of each batch of the internal standard.

Figure 5.

Precision of values for f_{sample}^{ub} after biotinylation of fibrinogen in solution at a 0.05 mg/mL. Data are repeated mass spec measurements of three biological replicates. Analysis was by equations [3] or [4] (see text). Equation 3 yielded more precise values for f_{sample}^{ub} and was used in subsequent analyses.

Figure 6.

(A) Mass spectrometric measurements of the unbiotinylated GCDP were analyzed by equation [3] for surface and solution-biotinylated samples. Similar degrees of biotinylation were found for all conditions, suggesting that the GCDP lysine is not more exposed when adsorbed from low concentration solutions. On the far right, an outlier experiment is shown in which biotinylation was much lower than in other experiments, perhaps due to older NHS-biotin. Regardless of the source of lower biotinylation, the concentration of fibrinogen in solution had no effect on extent of biotinylation, consistent with the other findings.

Table 1

Parameters used in data analysis

Parameter	Description	Measured or calculated?
f_{IS}^{MS}	Fraction of control peptide (VELEDWNGR) that is isotope labeled.	Measured as shown in Figure 2A (expressed as a fraction instead of a ratio).
r_{ub}^{MS}	The ratio unbiotinylated GCDP in the sample relative to the internal standard.	Measured as shown in Figure 2B.
r_b^{MS}	The ratio of biotinylated GCDP in the sample relative to the internal standard.	Measured as shown in Figure 2C.
p	The probability that a leucine in a protein expressed by HepG2 cells is isotope labeled.	A property of each batch of internal standard, calculated from f_{IS}^{MS} when only internal standard is injected into the mass spec, described in Figure 4A.
[I]	The concentration of internal standard (i.e. isotope-labeled fibrinogen) in a mass spec injection.	Not measured
[samp]	The concentration of sample fibrinogen in a mass spec injection.	Not measured
(I/S)	The ratio [IS]/[samp]	Calculated from p (property of a batch of internal standard) and f_{IS}^{MS} (measured in a mass spec run using VELEDWNGR peptide).
f_{IS}^{ub}	Fraction of the internal standard GCDP that is unbiotinylated.	A property of each batch of internal standard, measured as shown in Figure 4B by diluting internal standard with normal fibrinogen.
f_{samp}^{ub}	Fraction of the sample GCDP that is unbiotinylated; the goal of the study is to calculate this quantity.	Calculated using parameters above in equation 3 and/or 4.
f_{samp}^b	Fraction of the sample GCDP that is biotinylated.	Should equal $1 - f_{samp}^{ub}$.

Note: Superscript 'MS' indicates a quantity measured by mass spec. Subscript 'IS' indicates 'internal standard'. Subscript 'samp' indicates 'sample'. 'ub' = unbiotinylated. 'b' = biotinylated.

Table 2

Summary of results in Figure 6

Fibrinogen solution concentration (mg/mL)		f_{samp}^{ub}	n
Solution	0.05	0.236 ± 0.056	10
Surface	2	0.294 ± 0.15	6
	0.1	0.234 ± 0.057	5
	0.05	0.360 ± 0.12	5
	0.0005	0.243 ± 0.034	3