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Gelmi, A; Higgins, M J.; and Wallace, Gordon G., "Resolving sub-molecular binding and electrical switching mechanisms of single proteins at electroactive conducting polymers" (2013). *Faculty of Science, Medicine and Health - Papers: part A*. 147.
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Abstract

Polymer-based electrodes for interfacing biological tissues are becoming increasingly sophisticated. Their many functions place them at the cross-roads of electromaterials, biomaterials, and drug-delivery systems. For conducting polymers, the mechanism of conductivity requires doping with anionic molecules such as extracellular matrix molecules, a process that distinguishes them as biomaterials and provides a means to control interactions at the cellular-electrode interface. However, due to their complex structure, directly observing the selective binding of target molecules or proteins has so far eluded researchers. This situation is compounded by the polymer's ability to adopt different electronic states that alter the polymer-dopant interactions. Here, the ability to resolve sub-molecular binding specificity between sulfate and carboxyl groups of dopants and heparin binding domains of human plasma fibronectin is demonstrated. The interaction exploits a form of biological 'charge complementarity' to enable specificity. When an electrical signal is applied to the polymer, the specific interaction is switched to a non-specific, high-affinity binding state that can be reversibly controlled using electrochemical processes. Both the specific and non-specific interactions are integral for controlling protein conformation and dynamics. These details, which represent the first direct measurement of biomolecular recognition between a single protein and any type of organic conductor, give new molecular insight into controlling cellular interactions on these polymer surfaces.

Keywords

electrical, switching, mechanisms, single, proteins, electroactive, conducting, polymers, sub, resolving, molecular, binding

Disciplines

Medicine and Health Sciences | Social and Behavioral Sciences

Publication Details

Gelmi, A., Higgins, M. J. & Wallace, G. G. (2013). Resolving sub-molecular binding and electrical switching mechanisms of single proteins at electroactive conducting polymers. *Small*, 9 (3), 393-401.

Resolving sub-molecular binding and electrical switching mechanisms of single proteins at electroactive conducting polymers

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Received Date:

Title Running Head: Protein–organic conducting polymer interactions

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Polymer based electrodes for interfacing biological tissues are becoming increasingly sophisticated. Their many functions place them at the cross-roads of electromaterials, biomaterials and drug delivery systems. For conducting polymers, the mechanism of conductivity requires doping with anionic molecules such as extracellular matrix molecules, a process that distinguishes them as biomaterials and provides a means to control interactions at the cellular-electrode interface. However, due to their complex structure, directly observing the selective binding of target molecules or proteins has so far eluded researchers - this situation is compounded by the polymer's ability to adopt different electronic states that alter the polymer-dopant interactions. Here, we demonstrate the ability to resolve sub-molecular binding specificity between sulfate and carboxyl groups of dopants and heparin binding domains of human plasma fibronectin. The interaction exploits a form of biological 'charge complementarity' to enable specificity. When an electrical signal is applied to the polymer, the specific interaction is switched to a non-specific, high affinity binding state that can be reversibly controlled using electrochemical processes. Both the specific and non-specific interactions are integral for controlling protein conformation and dynamics – these details, which represent the first *direct measurement* of biomolecular recognition between a single protein and any type of organic conductor, give new molecular insight into controlling cellular interactions on these polymer surfaces.

Keywords: Conducting Polymer, Polypyrrole, Atomic Force Microscopy, Protein Adhesion, Fibronectin

Introduction

Organic conducting polymers (OCP) permit conductive pathways along their conjugated backbone and are breaking new grounds in applications such as implantable electrodes for neural recording¹ or stimulation^{2,3}, tissue regenerative medicine^{4, 5}, biosensing^{6, 7}, and electronic cell culture systems⁸. They are easily applied as coatings on metal electrodes to increase charge storage capacity, lower impedance and reduce the risk of toxic electrochemical reactions⁹. During growth of the polymer, anionic molecules (dopants), which can be drugs or other biomolecules, are incorporated to counter balance charge generated on the polymer backbone. Electrochemical reduction of the polymer to induce charge neutrality initiates the release of dopants; a reversible process that is utilised in drug delivery applications^{10, 11}. Alternatively, large polyelectrolyte dopants such as glycosaminoglycans (GAGs) (e.g. heparin sulfate, chondroitin sulfate, hyaluronic acid and dextran sulfate) remain entrapped in the polymer and are used to present anchorage sites for chemical functionalization¹² or bioactive groups^{13,14}, enhance biocompatibility¹⁵, or bioselectivity¹⁶, and control cellular interactions at the electrode surface¹⁷.

Selective binding of target molecules by recognition elements (e.g. dopants) of the polymer is critical for biosensor, immunosensor and bioseparation applications; however non-specific binding remains an ongoing issue for obtaining unequivocal electrical readouts. Similarly for cell-based applications, controlling specific protein interactions at the polymer surface is becoming a key strategy. For example, the electrically-dependent conformation of proteins is thought to underlie the ability to control cell adhesion¹⁸ and migration¹⁹. By looking at the ensemble conformation of fibronectin (FN) protein using fluorescence techniques, it is proposed that the proteins electrically 'switch' between a folded and unfold state to control the cell interaction²⁰. However, the molecular details of such protein interactions are not known and as yet no direct measurement for probing single protein molecule binding or conformation on an organic conducting polymer, or any type of organic conductor (e.g. carbon nanotube, and graphene), under electrical control has been demonstrated; understanding the dynamics and physics of single protein interactions will be a major step forward in this field of organic bioelectronics. For this reason, we covalently linked FN to an Atomic Force Microscope (AFM) tip and used force measurements to demonstrate the first direct measurement of specific protein adhesion to an organic conducting polymer, polypyrrole, doped with the GAGs, chondroitin sulphate (CS), hyaluronic acid (HA) and dextran sulfate (DS), as a function of an applied voltage. The GAG dopants can make up to 50% of the polymer composition and not all their anionic groups participate in charge neutralization of the polymer. For example, free sulfonate and carboxyl groups are anticipated to be involved in surface interactions defined by classical GAG-protein adhesion and recognition²¹, which is important for regulating cellular activities, including cell adhesion, extracellular matrix modelling and fibrillogenesis²².

Results and Discussion

Characterization of Protein Functionalized Tip

Human plasma fibronectin (FN) was covalently coupled to the AFM tip using a common procedure involving glutaraldehyde as cross-linking agent (Figure 1A and see materials and methods). Fluorescent images of AFM tips labelled with primary anti-fibronectin rabbit antibody before (Figure 1B, left image) and after (Figure 1B, right image) indicated that the functionalization procedure resulted in an FN coating on the chip, along the cantilever and at the tip end. Force measurements were performed after each functionalization step to assess the involvement of the introduced functional groups (i.e. -OH , NH_3^+ and C=O) and final coupling of the FN on the interaction between the tip and conducting polymer (Figure 1C). For these initial measurements, only PPy films doped with CS (PPy/CS) were assessed. Plasma treated silicon nitride tip (SiN_3) bearing -OH groups, which are hydrophilic and negatively charged at neutral pH, showed no adhesion to (PPy/CS). In contrast, 3-EDSPA treated tips terminated with protonated NH_3^+ groups at neutral pH showed an electrostatic double layer force that is attractive upon approach followed by direct tip-surface adhesion of 2.0 ± 0.14 pN during retraction of the tip from the polymer surface. This attractive interaction with the positively charged tip indicates the polymer surface is negatively charged and further supports the presence of anionic, sulfate groups of the CS. Similarly to the plasma treated tips, the glutaraldehyde (GAH) functionalized tips bearing reactive carbonyl groups are hydrophilic though showed a small adhesion of 406 ± 35 pN with the polymer surface. The interaction of the FN functionalized tip showed longer range interactions, consisting of multiple adhesion peaks, that are characteristic for this type of protein interaction observed using AFM^{23, 24}. These initial force measurements were thus useful in providing general information on the polymer surface chemistry (e.g. surface charge) and, importantly, confirming the FN interaction with the polymer, which is discussed in further detail below.

AFM Force Spectroscopy of Fibronectin – Organic Conducting Polymer Interactions

Figure 2A shows a schematic of the protein and chemical structures of the FN and polymer, respectively, and experimental setup that enables AFM force measurements of the FN-polymer/dopant interactions to be investigated as a function of an applied potential. During the force spectroscopy measurements, the FN-functionalized probe is brought into contact with the polymer electrode to initiate binding and then FN-surface adhesion forces acting on the probe are detected as it is withdrawn. Several types of FN-polymer interactions are detected for non-electrically stimulated polymers (Figure 2B), including no adhesion, 'non-specific' FN binding, and desorption (plateau) forces. The most prevalent FN interaction (42% of force

curves, $n = 900$) is that related to the extension of the bound protein until final detachment from the surface (Figure 2B, top and middle force curve), which is schematically shown in Figure 2C to describe the corresponding interactions in the force curves. The initial part of this interaction is referred to as ‘non-specific’ adhesion (Figure 2B and C; i) and related to bulk adhesive interactions of the FN functionalized tip involving inter- and intra-protein interactions and detachment of several proteins from the surface. If a single FN protein remains tethered between the tip and surface, force-induced unfolding of domains can occur (27% of force curves, $n = 900$) (Fig. 2B; peaks prior to and regions ii and iii) (Figure 2C; ii and iii) and is identified by successive rupture peaks with spacings of 13.1 ± 0.4 nm (mean $\pm$ s.e.m., $n = 137$) and 28.8 ± 0.7 nm (mean $\pm$ s.e.m., $n = 107$) correlated to the length of unfolded intermediates or FNIII domains^{23, 25, 26}, respectively (Figure 2D). Forces of 147.1 ± 8.6 pN (mean $\pm$ s.e.m., pulling-rate 500 nm/sec, $n = 198$) required for domain unfolding are also characteristic of single molecule FN interactions. Successive rupture peaks also occur due to detachment of multiple FN–polymer binding sites wherein the first binding site detaches (Figure 2B; peak prior to region iv) (Figure 2C iv), followed by extension of the protein (Figure 2B; iv), until detachment of the second and final binding site (Figure 2B and C; final peak). These binding events can occur in the absence of domain unfolding (Figure 2B; v) and have peak spacings of 58.9 ± 3.0 nm (mean $\pm$ s.e.m., $n = 67$) (Figure 2D), which are greater than the lengths of unfolded FN domains and therefore distinguish detachment of the protein from the surface. This peak spacing of 58.9 ± 3.0 nm also suggests that binding is most probable at sites on the protein separated by this distance or, alternately, the existence of specific binding sites on the protein. The detachment of the protein can also proceed less commonly via ‘non-specific’ desorption forces that show a constant force independent of the extension length, i.e. plateau forces (Figure 2B; vi in bottom curve). Plateau forces are commonly observed for polyelectrolyte chain desorption from a surface, much like a polymer chain being ‘peeled’ off the surface, and arise due to dependencies on the dissociation rate of repeating polymer chain-surface contacts relative to the extension rate²⁷ and presence of an oppositely charged surface²⁸.

To elucidate interactions involving detachment (or effective binding) sites of the protein, we focused on analysing individual force profiles representing the unfolding and/or extension of FN, as described above in Figure 2B (top and middle curves). More specifically, the analysis involved subtracting the distances of all peak spacings, including those associated with unfolding and protein–surface detachment, from the distance at the final peak. This gives what we refer to as the “corrected binding distance” (CBD), representing the extended length of the FN, tethered between the tip and surface, just prior to undergoing domain unfolding and/or surface detachment (Figure 2C; far right schematic). The CBD is thus governed by the distance between the two attachment positions (dashed lines) of the protein to the tip and polymer;

this being firstly, the covalent attachment of the protein to the probe and secondly, the non-covalent protein-surface binding site that is either the first of multiple binding sites to detach, as shown in Figure 2C, or the sole (and final point of detachment) when no other binding sites are present.

Probability density functions of the CBD show a primary peak distribution value ranging from 59 - 66 nm for all polymers, with a secondary peak distribution value ranging from 116 – 119 nm for CS and DS polymers and possibly PTS and HA (Figure 3A). Minor peak CBD distribution values also appear at 171 nm and 166 nm for CS and PTS, respectively (Figure 3A). The significance of these CBD distributions is that, even though FN binding to the tip is generally expected to be random via gluteraldehyde linkages with nucleophilic amine groups at the N-terminus and on free lysine residues, their observation indicates that both the tip and polymer must bind to specific sites on the FN. Furthermore, the presence of CBD values at 166-171 nm corresponds to the length of ≈ 160 -175 nm for fully stretched plasma FN^{29, 30, 31}. To obtain these values, it would require that the FN be attached to an N-terminus and interact with the polymer via the opposing N-terminus. This binding configuration, which presumably allows the entirety of the extended FN molecule to freely interact, is likely to facilitate binding at additional sites separated by the distance of 58.9 ± 3.0 nm (mean $\pm$ s.e.m., $n = 67$) previously observed in the rupture spacings (Figure 2D). Repeated sampling of the 3 different peak CBD distribution values within sets of force curves ($n = 25$) from individual AFM tip experiments ($n = 36$) further supports the detection of multiple binding sites along a single FN molecule instead of multiple interacting FN molecules having 3 different specific binding configurations and each with an extended length differing by ≈ 60 nm. These observations suggest that FN preferentially has a fixed attachment to the tip in the vicinity of an N-terminus with binding occurring at specific sites along its length.

Based on the above observations, we use the known FN structure and dimensions of the FNI, FNII, FNIII domains based on X-ray crystallography studies³² to construct coordinate map for identifying positions on the protein involved in binding to the polymer (Figure 3B). The map gives a calculated FN length of 158.8 nm by taking into account the size of each individual domain and absence of variable splicing regions for plasma FN. The preferred N-terminus binding position of the tip is made the zero reference point in order to identify the binding locations. The extent of net positive charge of each FN domain³³, which generally underlies their binding affinity to sulfate sites on GAGs³⁴, is also specified. When cross-referencing the peak distribution values of the CBD (black asterix) to their locations on the model, we observe that they superimpose to well-known Heparin (Hep) binding domains (Figure 3B; black highlighted domains),

including a particular sequence of the HepII FNIII₁₂₋₁₄ near the COOH-terminus, HepIII FNIII₄₋₆ and HepI FNI₁₋₅. Interestingly, the compact conformation of FN is stabilized by intermolecular bonds at the positions of these three domains³⁵ but can be disrupted by interacting surface groups, particularly hydrophilic and negatively charged surfaces, causing the protein to adopt a more unfolded conformation^{36,37}. We suggest that the binding of this particular sequence of Hep domains is presumably facilitated by their close association and thus collective presentation to the polymer surface during the force spectroscopy measurements. The main CBD distribution at 59-66 nm indicates most probable binding occurs at the HepII domain and accordingly this is recognized as the highest affinity GAG binding site due to the presence of discontinuous positively charged amino acids in FNIII₁₃₋₁₄ domains that form a cationic cradle³⁸. Standard deviations of the CBD distributions associated with the HepII domain reflect the binding specificity at this position (Figure 3B; horizontal arrows above HepII region), which is highest for CS followed by DS and then HA and PTS. A higher affinity and/or specificity of binding for CS is also evident in single molecule FN-polymer unbinding forces that are significantly higher for CS (164 ± 10.1 pN, $n = 61$) compared to DS (115 ± 11.0 pN, $n = 41$), HA (108 ± 8.5 pN, $n = 30$) and PTS (124 ± 12.5 pN, $n = 66$) (Fig. 3C). Along with heparin, CS is a main competitor in FN binding with important binding determinants located to the HepII FNIII₁₃₋₁₄ domains³⁹. Heparin-FN interactions have been mimicked using synthetic polysaccharides like DS⁴⁰, while for the non-sulfated HA it is uncertain if binding on its own is possible or requires the presence of mediating proteoglycans⁴¹. Many of these early studies involve binding of FN fragments whereas our approach using intact FN shows that all polymers, including those with HA, are capable of specific binding. Even for small molecular weight PTS bearing a single sulfate group per molecule, its distribution at the polymer surface is sufficient to enable specific binding of the HepII domain.

Effect of Electrical Stimulation on Fibronectin Binding

In contrast to measurements on gold electrodes that show no adhesion dependence on the applied potential (Figure 4A), FN adhesion on the polymers significantly increases by $\approx 5 - 10$ times at positive potentials and then decreases as the applied potential is reversed (Figure 4B). This is highlighted in force profiles of the gold electrode (Figure 4A) and CS doped polymer (Figure 4B) acquired at applied potentials of +0.4 V, 0 V and -0.4 V during the cyclic voltammograms. The reversibility of adhesion during cycling of the potential between +0.4 V and -0.4 V is conveyed in graphs that are analogous to cyclic voltammograms (solid grey curve) but plot the maximum adhesion force (black dashed curve) as a function of voltage (Figure 5). Combining force and cyclic voltammetry measurements has recently been demonstrated for DNA interactions on gold electrodes and referred to as 'Roburograms' (Robur is the Latin for 'force')⁴². All

the polymers showed a similar response, in particular, a dramatic reversible adhesion between 0.2-0.4 V at scan rates of 50 mV/sec. Although the interaction of multiple FN molecules is likely, the increase in adhesion does not scale with (n) possible number of interacting proteins given that a single compact FN molecule of $\approx 20 \times 15$ nm is comparable to the interaction area of the probe. Compared to the gold, the polymer electrode micro- and nanotopography and porosity significantly increase the charge storage capacity, as evidenced by a significant increase in the area of the cyclic voltammograms (Figure 5, c.f. gold and polymer). A related increase in surface charge density is most likely responsible for a strong columbic attraction with the majority of negatively charged FN domains that effectively “pins-down” the protein. Furthermore, the outward diffusion of charge balancing cations occupying anionic sites on the dopant during the applied potential is rate limiting. This renders the anionic sites unavailable for neutralization of positive charges generated on the polymer backbone – thus giving rise to a high positive surface charge density. When cycling at slower scan rates of 5mV/sec, as demonstrated for CS doped polymers, the ion diffusion is conversely not rate-limited and charge neutralization of the polymer can proceed to negate attraction of the FN (Figure 5).

Molecular Switching of Fibronectin Binding & Conformation via Electrochemical Control

Based on the above picture emerging of the FN interactions, we propose a model that provides molecular insight into the switching mechanisms of single molecule FN-conducting polymer interactions, particularly as the protein alters binding affinity and conformation in response to equilibrium and non-equilibrium redox states of the polymer under electrical control (Figure 6). The involvement of heparin domains in binding to the non-electrically stimulated polymers is explained by a ‘bio-specific’ interaction whereby cationic sites of the protein and anionic sites of the dopant undergo charge polarization at small separation distances (Figure 6A). The implication for the subsequent formation of discrete bonds at heparin domains, coupled to the flexibility of the protein’s extended conformation, is that local structural changes are induced to actively present cell binding (RGD) domains⁴³. This binding configuration can be electrochemically switched to induce non-specific binding (Figure 6B); a reversible process that is expected to significantly alter the protein conformation. Oxidation of the polymer results in a dramatic increase in binding strength; however the flexibility of the protein is diminished (Figure 6B). During reduction of the polymer, the polymer backbone is uncharged and the protein is presumably able to interact with free anionic groups in a similar manner to the non-stimulated polymer (Figure 6C). While others have focused on the FN conformation^{18, 19, 20}, our study uniquely quantifies the FN-polymer interfacial forces that play a

critical role in force-dependent signal transduction processes such as cellular forces exerted on FN through integrins to regulate cell adhesion and migration.

Conclusions

The study demonstrates a new approach by using AFM to *directly* measure biomolecular recognition of single molecules under electrical control that is also valuable in its application to other organic conductors (e.g. graphene and carbon nanotubes), semiconductors and metals. The approach will be of significant interest in the areas of the cell-electromaterial interface, organic bioelectronics, electrophysiology and implantable electrodes (e.g. cochlear implant), particularly as researchers endeavour to fabricate organic electrodes that make better electrical 'connections' to living cells and tissues. It opens up the possibility of assessing a range of other biomolecules (e.g. growth factors) whose redox-controlled surface binding and subsequent release is implicated as an important strategy for temporally controlling nerve, muscle and stem cell growth and survival. It is also likely to burgeon in the immense field of electrically-based biosensing that is heavily reliant upon biomolecular recognition at electrodes.

Acknowledgements: This work has been supported by the Australian Research Council under the Australian Research Fellowship and DP110104359 (Dr Michael Higgins) and ARC Federation Fellowship of Prof. Gordon Wallace. We also greatly acknowledge the Australian National Fabrication Facility (ANFF) for providing Atomic Force Microscopy instrumentation.

Methods:

The pyrrole monomer was obtained from Merck and distilled prior to use. The chemicals used as the dopants were the sodium salts of *para*-toluenesulfonic acid (pTS), hyaluronic acid (HA), dextran sulfate (DS) and chondroitin sulfate A (CS). CS and DS were obtained from Sigma, pTS from Merck and HA from Fluka. All solutions were prepared with deionised Milli-Q water (18.2M Ω). Gold coated mylar was firstly prepared by cutting into strips of 0.5 cm by 2 cm area and then cleaned with methanol and Milli-Q water. An aqueous monomer solution of 0.2 M pyrrole and 2 mg/mL of the counter-ion dopant was degassed in N₂ for 10 min prior to polymerisation of the polymers. Polypyrrole (PPy) films were grown galvanostatically at a current density of 0.25 mA/cm² for 10 min in the aqueous monomer solution using an eDAQ EA161 potentiostat and recorder to monitor the polymerization charge. Polymer growth was performed in a standard 3-electrode electrochemical cell with the gold coated mylar as the working electrode, a platinum mesh counter electrode and Ag/AgCl reference electrode. After growth, the films were washed with Milli-Q water, gently dried with N₂ gas and placed in petri dishes until use.

The functionalization chemicals 3-ethoxydimethylsilylamine propyl (3-EDSPA) and gluteraldehyde (GAH) were obtained from Sigma Aldrich. Human plasma fibronectin (FN) was obtained from Sigma Aldrich. Phosphate buffer saline (PBS) was prepared at pH 7 in Milli-Q water (18.2M Ω). The tip is functionalized using an aminosilization method to covalently bind the protein to the tip. Silicon nitride tips are used for this method due to the availability of silicon oxide groups on the surface. The tips were initially cleaned with a plasma cleaner to remove any impurities or functionalized groups on the surface. Once cleaned the tips were immediately functionalized to minimise contaminants on the surface. The tips were placed into the EDSPA solution at room temperature for 1 h. The tips were then removed, washed consecutively with toluene, then PBS solution. The tips were then immersed in the GAH solution for 1 h, and then rinsed with PBS solution. The tips were finally immersed in the FN solution for 1 h, then rinsed and refrigerated in PBS solution until use. To visualize functionalization of the tips, FN functionalized AFM probes were prepared for antibody binding using Alexa488 goat anti-rabbit IgG (Invitrogen) and primary anti-fibronectin rabbit antibody (ICN Biomedicals Inc). The tips were blocked in PBS with 10% donkey serum at room temperature for 1 hr then incubated in primary anti-fibronectin (rabbit) antibody in PBS with 10% donkey serum at 37 °C. The tips were rinsed twice in PBS, then incubated in a secondary antibody (Alexa488 goat anti-rabbit IgG) in PBS with 10% donkey serum. The tips were then rinsed twice with PBS and imaging using the Zeiss Axio Epifluorence microscope.

Electrochemical–AFM (EC-AFM) was implemented by positioning a 2-dimensional electrochemical cell on the sample stage of an MFP-3D Asylum Research AFM (Santa Barbara, CA). The electrochemical cell was made out of a Teflon block that consisted of a u-shaped fluid well etched around a platform. A PPy

polymer film was glued onto the platform and acted as the working electrode. A platinum mesh counter electrode was positioned in the fluid well and a silver wire pseudo-reference electrode was placed alongside the polymer film. Electrochemical measurements were controlled using an eDAQ EA161 potentiostat/recorder and carried out in PBS buffer solution, which was injected into the fluid well to immerse the three electrodes. Force spectroscopy measurements were firstly performed on non-electrically stimulated films with PBS as the electrolyte. 5 consecutive force curves were collected at one x-y position with a total of 5 different positions across the film being sampled. This was repeated using 3 different functionalized tips on the same film. For each of the differently doped films (i.e. CS, DS, HA, PTS), a total of 3 film samples were measured. Force curve parameters included a 500 nm ramp distance, 0.5 Hz scan rate, 1 sec dwell time at the surface and trigger force of 1nN. Force curves were then acquired during electrical stimulation of the films under the EC-AFM. Polymer films were electrically stimulated by applying cyclic voltammetry from -400 to +400 mV with either scan rates of 50mV/sec or 5mV/sec for 5 cycles. The z-piezo and deflection signal were outputted to the eDAQ EA161 potentiostat/recorder and recorded simultaneously with the current and voltage. Analysis of the force curves were carried out using the Asylum AFM software in IGOR PRO (Wavemetrics)

Figure Legends:

Figure 1. A) Schematic of procedure for protein functionalization of AFM tip. **B)** Fluorescence image of fluorescently labelled AFM tip before (left image) and after (right image) protein functionalization. **C)** AFM force curves performed on PPy/CS after each functionalization step, including plasma treatment (SiN_3), amino-salinization (3-EDSPA), glutaraldehyde treatment (GAH) and final coupling of the protein (FN).

Figure 2. A) Schematic diagram of AFM tip functionalized with FN interacting with a conducting polymer electrode (blue), polypyrrole (PPy), comprising a conjugated backbone (black chemical structure) with entrapped dopants (green) such as chondroitin sulphate (CS), hyaluronic acid (HA) or dextran sulfate (DS). FN is a protein dimer comprising two identical monomers linker by a pair of C-terminal disulfide bonds. Each monomer contains three types of repeating domains, types FNI (orange rectangle), FNII (blue diamond), FNIII (red ovals) and an N-terminal. Force spectroscopy measurements were performed as a function of an applied voltage using electrochemical–AFM. The PPy-electrode operated as the working electrode in 3-electrode electrochemical cell, including auxiliary and reference electrodes, positioned under the AFM. **B)** AFM force curves for the interaction of FN with non-electrically stimulated PPy/CS showing the trace (orange) and retrace (blue) curves. The peak at (i) corresponds to initial detachment of the tip and fibronectin molecules from the surface. The two subsequent peaks (1st and 2nd dashed lines) and their spacing of 27.1 nm (ii) and 28.5 nm (iii) correspond to the sequential unfolding of FNIII modules (~ 75 amino acid residues). A peak spacing of 47.8 nm at point (iv) is greater than that for FN unfolding and correlates with multiple detachment of FN-polymer binding sites. The first binding site detaches (3rd dashed line) followed by extension of the protein until the second and final site detaches (4th dashed line). Such binding events can occur in the absence of domain unfolding (point v in bottom curve). **C)** Corresponding schematic depicting the different interactions in top curve in (A). (i) non-specific adhesion peak; (ii) unfolding of 1st domain (red circle); (iii) unfolding of 2nd domain (red circle); (iv) detachment of 1st

FN-polymer binding site (green triangle); (final peak) detachment of 2nd and final FN-polymer binding site (green triangle). For analysis of the corrected binding distance (CBD), the “added” distances gained during extension of the protein (i.e. peak spacings due to unfolding/multiple detachment sites) are subtracted from the final peak distance. The CBD (vertical arrow) therefore represents the extended length of the FN prior to unfolding or surface detachment and is effectively the same situation as that depicted prior to (ii).

D) Probability density functions of distances between successive rupture peaks in force profiles from all polymers. Red curves are individual gaussian fits and automated multipeak fitting (blue curves) (IGOR PRO, Wavemetrics). Peak distributions at 13.1 ± 0.4 nm (mean $\pm$ s.e.m., $n = 137$) and 28.8 ± 0.7 nm (mean $\pm$ s.e.m., $n = 107$) have previously been attributed to intermediate and fully unfolded lengths of FNIII domains, respectively. Higher peak value distributions at 58.9 ± 3.0 nm (mean $\pm$ s.e.m., $n = 67$) correspond to distances between multiple protein-polymer binding sites.

Figure 3. A) Probability density functions of corrected binding distance (CBD) for CS ($n = 121$), DS ($n = 77$), HA ($n = 65$), PTS ($n = 112$) doped polymers. Light grey curves are individual gaussian fits and automated multipeak fitting (solid dark curves) (IGOR PRO, Wavemetrics). The peak CBD distribution values labelled in blue are: (CS) 59.0 ± 9.3 nm (mean $\pm$ s.d., $n = 70$), 116.0 ± 11.7 nm (mean $\pm$ s.d., $n = 27$), 171.0 ± 6.6 nm (mean $\pm$ s.d., $n = 13$); (DS) 64.3 ± 13.6 nm (mean $\pm$ s.d., $n = 51$), 116.7 ± 3.8 nm (mean $\pm$ s.d., $n = 16$); (HA) 65.5 ± 18.0 nm (mean $\pm$ s.d., $n = 38$), 119.1 ± 6.3 nm (mean $\pm$ s.d., $n = 20$); (PTS) 59.9 ± 14.4 nm (mean $\pm$ s.d., $n = 69$), 116.0 ± 8.5 nm (mean $\pm$ s.d., $n = 22$), 166.0 ± 8.6 nm (mean $\pm$ s.d., $n = 14$). **B)** Model of extended FN showing calculated length of 158.8 nm based on x-ray crystallography dimension of FNI (2.5 nm, rectangles), FNII (0.7 nm, diamonds) and FNIII (3.2 nm, ovals), where 0 nm corresponds to the attachment position of FN to the AFM tip. The heparin binding domains, HepII_{FN12-14} (position = 59.1 – 68.7 nm), HepIII_{FN4-6} (position = 115.7– 125.3 nm) and HepI_{FN1-5} (position = 143.2– 158.8 nm) are coloured in black. The peak CBD distribution values labelled in (A) and listed above are marked at their respective positions on the protein with a black asterisk. Standard deviations calculated from the full width half

maximum (FWHM) (where $\text{FWHM} = 2\sqrt{2\ln 2}\sigma$) of peak CBD distribution values of the $\text{HepII}_{\text{FN12-14}}$ domain for each dopant are given by the length of the horizontal arrows. **C)** Single molecule FN-polymer unbinding forces for each polymer. The values are taken from the force value of the final peak (detachment of the polymer) and represent the primary peak distribution of probability density functions.

Figure 4. Representative force curves for **(A)** gold electrode and **(B)** PPY/CS polymer taken at 400 mV, 0 mV and -400 mV from a cyclic voltammogram with a scan rate of 50mV/sec.

Figure 5. Maximum adhesion force (black dashed curve) versus voltage and corresponding cyclic voltammograms (solid grey curve) for the gold electrode and PPy polymer films doped with CS, DS, HA and pTS. The adhesion force values (black circles) represent an average from individual force curves collected at each time point during 3 CV cycles performed at a scan rate of 50mV/sec. The bottom right graph is the same measurement performed for the PPy/CS CS polymer at a slower scan rate of 5 mV/sec.

A. Bio-specific “charge complementarity”

B. Non-specific “electrochemically induced”

C. Neutralised backbone

Figure 6. A) Non-Electrically Stimulated Polymer: The model translates the AFM observations to the expected binding interaction of a single FN molecule (only one FN monomer is shown) given the involvement of multiple heparin domains (blue domains). The ability of multiple binding via the different heparin domains indicates that the FN is more likely to adopt an extended conformation, which is depicted in the model based on previous representations of unfolded FN adsorbed onto negatively charged surfaces⁴⁴. This configuration is considered to be more bioactive due to the presentation of RGD regions. Because FN is an amphoteric protein, repulsion of the predominately negatively charged protein with sulfate or carboxyl groups (green charges) of the GAGs can revert to attractive forces due to preferential alignment of the cationic heparin domains (blue highlighted). When the protein is at a very small separation distance from the polymer, the charges interact as discrete entities rather than part of an overall net charge and subsequent columbic attraction of individual ion pairs governs the protein-surface binding, as opposed to the net protein interaction. This type of bio-specific interaction is referred to as

“charge complementarity”⁴⁵. **B) Electrically Stimulated Polymer:** The application of a positive voltage induces charge (black positive charge) on the polymer backbone causes a strong columbic attraction of the negatively charged FN domains. At higher scan rates (50 mV/sec), outward diffusion of charge balancing cations (C+) occupying anionic sites on the immobile GAGs is rate limiting. This renders anionic sites on the dopant unavailable for charge neutralization of the positive charges on the polymer and protein adhesion is present. At lower scan rates (5mV/sec), outward cation diffusion (black arrows) is not rate-limited and charge neutralization of the polymer proceeds to decrease protein adhesion. **C)** During the application of negative potentials, the polymer backbone is uncharged and free sulfate or carboxyl groups are expected to similarly interact with the protein, as described above in **(A)**. The mechanism is reversible (red arrow) between **(B)** and **(C)**.

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