

Method for estimating protein binding capacity of polymeric systems

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Abstract

Composite biomaterials made from synthetic and protein-based polymers are extensively researched in tissue engineering. To successfully fabricate a protein-polymer composite, it is critical to understand how strongly the protein binds to the synthetic polymer, which occurs through protein adsorption. Currently, there is no cost-effective and simple method for characterizing this interfacial binding. To characterize this interfacial binding, we introduce a simple three-step method that involves: 1) synthetic polymer surface characterisation, 2) a quick, inexpensive and robust novel immuno-based assay that uses protein extraction compounds to characterize protein binding strength followed by 3) an in vitro 2D model of cell culture to confirm the results of the immuno-based assay. Fibrinogen, precursor of fibrin, was adsorbed (test protein) on three different polymeric surfaces: silicone, poly(acrylic acid)-coated silicone and poly(allylamine)-coated silicone. Polystyrene surface was used as a reference. Characterisation of the different surfaces revealed different chemistry and roughness. The novel immuno-based assay showed significantly stronger binding of fibrinogen to both poly(acrylic acid) and poly(allylamine) coated silicone. Finally, cell studies showed that the strength of the interaction between the protein and the polymer had an effect on cell growth. This novel immuno-based assay is a valuable tool in developing composite biomaterials of synthetic and protein-based polymers with the potential to be applied in other fields of research where protein adsorption onto surfaces plays an important role.

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1. Introduction

Tissue engineering aims at restoring structure and function of tissues after severe trauma or disease by developing tissue constructs in vitro that are later implanted in the patient [1]. To develop these constructs, a biomaterial that acts as a scaffold for cells and growth factors is needed [1]. Currently there are a

variety of biomaterials, including natural (e.g. fibrin, collagen, chitosan) and synthetic polymers (e.g. polyesters, silicone). All of these biomaterials have advantages as well as some drawbacks. Therefore, a combination of two or more of them is often used to develop new biomaterials known as composites [2,3]. Combination of natural protein-based polymers such as fibrin (excellent biocompatibility and bioactivity) with synthetic ones (versatility and mechanical strength) is a popular choice used in tissue engineering for a wide variety of tissues [4–7]. To develop these composite biomaterials it is very important to understand at the molecular level protein adsorption, including the interfacial binding strength, between the protein and the synthetic polymer surface.

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Proteins are interfacially active molecules as they spontaneously accumulate at interfaces through a process known as adsorption [8,9]. This process can be due to long-ranged and strong electrostatic attraction between a charged interface and the oppositely charged amino-acids of the side chains of the protein, leading to a significant free energy change that favours the adsorption process [10]. In other instances, this process is driven by the marginal structural stability of the protein, which is influenced by hydrophobic interactions, hydrogen bonds, Coulomb and van der Waals interactions. The interface can compete with some of these interactions thereby unfolding the protein structure to minimize the total free energy of the system, resulting in a surface-induced protein-denaturation [11]. Techniques currently available for determination of these protein interactions on polymeric materials include surface plasmon resonance, in situ ellipsometry, quartz crystal microbalance, total internal reflection fluorescence spectroscopy and attenuated total reflectance – Fourier transform infrared spectroscopy. However, these techniques are highly specialized and often time consuming and expensive [12–14]. Therefore, within the fields of tissue engineering and biomaterials and with the purpose of developing novel composites of protein and synthetic polymers there is a need for a simple, quick and cost-effective method for characterizing the interfacial binding between a synthetic polymeric material and the protein of interest.

We describe here a simple three-step method based on a quick, sensitive and robust immuno-based assay to determine the interfacial binding strength of proteins on synthetic polymeric surfaces. The model protein for this study was fibrinogen (fbg), the precursor of fibrin, which is well known for its strong adsorption properties on solid surfaces and has often been used as a test protein in similar studies [15,16]. The choice of synthetic polymer was silicone (Sil) because it is widely used in the biomedical field [17–19]. Sil was coated with acrylic acid and allylamine monomers using plasma polymerisation [20] to create different Sil surfaces. Fbg was adsorbed on the different surfaces and the interfacial binding pattern and strength were analysed using an immuno-based assay that uses high concentration of protein extraction compounds which, to the best of our knowledge, has never been done before. Finally, a simple in vitro 2D model of cell culture to confirm the results of the immuno-based assay was performed.

2. Materials and methods

2.1. Plasma polymerization

Medical grade silicone sheets (0.12 mm thickness) were derivatized with acrylic acid (AcA) or allylamine (AIA) monomers by plasma polymerization. Silicone sheets without derivatization (Sil) were used as reference (Altrika Ltd., UK) (Table 1). AcA and AIA were purchased from Aldrich (UK). The base pressure in the reactor was maintained at 3.0×10^{-3} mbar and the plasma was sustained by radio-frequency (13.56 MHz) signal generator. AcA polymerization was carried out in a production reactor and the AIA polymerization in the research reactor at Altrika Ltd., UK. AcA and AIA were polymerized

Table 1
Grouping of polymers used along with the plasma polymerized substrate coating.

Polymer	Coating	Abbreviation	Diameter (mm)
Silicone	–	Sil	5
Silicone	Poly(acrylic acid)	Sil-AcA	5
Silicone	Poly(allylamine)	Sil-AIA	5

at a plasma power of 2 W and 3 W respectively with a total flow rate of 2.0 standard cubic centimetres per minute (sccm). The polymerization process was carried out at a pressure of 4.0×10^{-2} mbar and the deposition time was 20 min. Monomers were allowed to flow for further 20 min after turning off the plasma to reduce the uptake of atmospheric oxygen when exposed to the laboratory atmosphere [21]. Presence of the polymer was confirmed by X-ray photoelectron spectroscopy at Altrika Ltd., UK as part of their routine quality control analysis (results not shown).

2.2. Scanning electron microscopy (SEM)

All surfaces (5 mm discs) non-coated and coated with fbg (150 μ g/ml for 1 h at 37 °C) were characterized by SEM. Sil discs were mounted on stubs, gold sputtered coated (Agar Auto Sputter Coater, Agar Scientific, UK) and observed (FEI Inspect F, Oxford Instruments, UK).

2.3. Atomic force microscopy (AFM)

The surface roughness of the Sil discs was characterized with NTEGRA Probe NanoLaboratory (Nt-MDT Co.) microscope. Noncontact silicon Golden 01 (NSG01) cantilever was used for imaging (1.6 \times 3.4 mm and thickness 0.3 mm) in scanning measuring head configuration. The surface topographical images of the discs obtained via AFM were analysed using the Nova image (version 1.0.26) processing software (Nt-MDT Co.). Surface roughness (Ra) was calculated for all surfaces in duplicates keeping the parameters for measurements identical.

2.4. Antibodies

Polyclonal antibodies [anti-fibrinogen polyclonal antibody produced in goat (α Fb-Gpc) and anti-goat IgG alkaline phosphatase conjugate (α G-AP)] were obtained from Sigma–Aldrich (St. Louis, MO, USA) and diluted in blocking buffer (BB) [1% bovine serum albumin (BSA, A9647, Sigma–Aldrich, UK) in PBS].

2.5. Immuno-based assay

The binding of fbg on Sil discs and polystyrene (PS) well surface was measured using a novel immuno-based assay. Bovine fbg was obtained from Sigma–Aldrich (F8630, UK) and was reconstituted to a stock concentration of 5 mg/ml in PBS. Prior to binding, all available binding sites in the 96 well ELISA plate (655061, Greiner Hi-binding 96 well ELISA plate, UK) were

blocked via incubation with BB (1 h, 37 °C, 100 µl/well). After blocking, all wells were washed twice with PBS (100 µl) and discs were placed at the bottom of the wells. PS wells used as reference were not pre-blocked (Table 2).

Fbg (100 µl) was adsorbed over the range 0–250 µg/ml on PS wells and Sil discs placed at the bottom of the wells for an 1 h at 37 °C in triplicate and the excess protein was washed off by rinsing with PBS (100 µl). Care was taken to prevent the discs from falling off during the washes. After fbg adsorption, all wells (with and without discs) were further blocked with BB (100 µl/well) for 1 h at 37 °C followed by another PBS wash step (100 µl). Next, the fbg adsorbed wells (with and without discs) were incubated (1 h, 37 °C) first with αFb-Gpc (1:2000 dilution in BB) and then with αG-AP (1:1000 dilution in BB). Between each antibody incubation, the wells were washed with 200 µl of wash buffer [Tris–NaCl, 0.1% BSA, 10 mM NaN₃ and 0.1% Triton X-100 (30632 GN, BDH chemicals)]. The phosphatase activity was detected in the dark using para-nitro-phenylphosphate (1 mg/ml in 0.1 M diethanolamine) as substrate before stopping with 100 µl of 0.1 M EDTA (pH 9.6, 100935V, BDH chemicals, UK). For precision, 100 µl of the solution from all wells containing discs was pipetted into fresh wells without any discs and the absorbance read at 405 nm (Biorad 550 96 well plate spectrophotometer, UK).

2.6. Binding strength assay

Protein extraction compounds were used as a tool to compare the binding strength of fbg to the different derivatized silicone discs. All the wells were pre-blocked with BB (100 µl) for 30 min at 37 °C prior to placing the discs. Fbg (150 µg/ml) was adsorbed (100 µl) onto the different Sil discs for 1 h at 37 °C and washed twice with PBS (100 µl). All compounds (Table 3) were prepared in distilled water and incubated (100 µl) with the protein coated discs for 15 min and washed twice with 100 µl of PBS. Further to this, discs were incubated with αFb-Gpc (100 µl) and the steps were continued as described in section 2.5. Control discs were not treated with protein extraction compounds.

2.7. Human dermal fibroblasts

Primary human dermal fibroblasts (HDF) were established from routine surgical excisions of normal skin, obtained with informed consent and local ethics committee approval. 1 × 1 mm pieces of dissected skin were cultured dermal side down in T25 tissue culture flasks (8 per flask), in 3 ml of Dulbecco's Modified Eagle Medium (DMEM, 31885-023, Gibco, UK) supplemented

Table 2
Different BSA blocking parameters for silicone discs and polystyrene wells.

Groups	Abbreviation	Pre-blocking with BSA
Silicone	Sil	Yes
Silicone + Poly(acrylic acid)	Sil-AcA	Yes
Silicone + Poly(allylamine)	Sil-ALA	Yes
Polystyrene well	PS	No

Table 3
Protein extraction compounds used during the study at different concentrations.

Compound	Concentration	Company
Decon-90	1%, 2%, 5% vol/vol	Decon Labs, UK
Sodium dodecyl sulphate (SDS)	0.25%, 0.5%, 1%, 2% wt/vol	Sigma, UK
Glycine	0.025 M, 0.05 M, 0.1 M	Sigma, UK
Pluronic® L101 (L101)	1% vol/vol	Basf, UK
Tween-20	0.03%, 1%, 2% vol/vol	Sigma, UK
Pluronic® F-127 (F-127)	1% wt/vol	Sigma, UK
Urea	2 M, 4 M, 6 M, 8 M	Sigma, UK

with 10% fetal bovine serum (FBS, 10270-106, Gibco, UK), 100 U/ml penicillin and streptomycin (15140-122, Gibco, UK) and 200 µM L-glutamine (25030-024, Gibco, UK) at 37 °C with 5% CO₂. Medium was changed twice per week. Adherent HDF egress cultures are typically established within 3 weeks [22]. Cells were used at passage 5.

2.8. Cell seeding

For cell viability, 5 × 10⁴ HDFs were seeded on 13 mm diameter borosilicate glass coverslips (631-0150, VWR International, UK) in 12 well plates and 8 mm diameter Sil discs, coated/non-coated with fbg, in 24 well plates (n = 3 per coverslip/Sil disc). For SEM and confocal microscopy, 7 × 10³ HDFs were seeded per coverslip/Sil disc (n = 2). After 30 min incubation at 37 °C with 5% CO₂, 2 ml of supplemented DMEM were added per well and plates cultured at 37 °C with 5% CO₂. Medium was changed every 3 days.

2.9. Cell viability by alamarBlue® assay

On days 2, 4 and 7 culture discs were transferred to fresh wells so only the cell viability of cells growing on the discs surface would be measured. 1 ml of 10% alamarBlue® (DAL1025, Invitrogen™, UK) stock diluted into phenol free supplemented DMEM (11,880, Gibco, UK) was added per well and incubated at 37 °C with 5% CO₂ for 3 h. For each sample, 1 ml was transferred to a cuvette (FB55147, Fisher Scientific, UK) and following the manufacturer's instructions, Absorbance was measured at 570 nm against air using a M550 double beam UV/visible spectrophotometer (Camspec, UK). Absorbance at 600 nm of phenol free DMEM was subtracted from sample values [22].

2.10. SEM of seeded discs

After 2 days of culture, specimens were fixed in 2.5% glutaraldehyde (Agar Scientific, UK) overnight, washed with 0.1 M sodium cacodylate buffer (Agar Scientific, UK) and post-fixed in 1% osmium tetroxide (Sigma–Aldrich, UK) in cacodylate buffer for 1 h; then washed in cacodylate buffer, dehydrated through a graded series of industrial methylated spirit (20–60%) and ethanol (70–100%), equilibrated in 100% ethanol and left to dry overnight. Finally, specimens were mounted on stubs; gold sputtered coated (Agar Auto Sputter Coater, Agar Scientific, UK) and observed (FEI Inspect F, Oxford Instruments, UK).

2.11. Immunostaining and confocal microscopy of seeded discs

After 2 days of seeding, specimens were fixed in 4% paraformaldehyde overnight. Fixed specimens were washed twice with PBS, permeabilised with 2 drops of 0.5% Triton X-100 in PBS for 5 min at room temperature, washed 3 times with PBS and incubated in block buffer (0.5% BSA in PBS, pH 7.4) for 30 min at room temperature. Block buffer was drained into tissue paper and samples were incubated with green phalloidin (Alexa Fluor® 488 phalloidin, A12379, Invitrogen, USA; 1:100 in block buffer) for 1 h at room temperature inside a dark humidified chamber, washed 5× in wash buffer (0.1% Triton X-100 and 0.1% BSA/PBS, pH 7.4), then once in PBS and once in distilled water. Samples were transferred to slides with 1 drop of Vecta Mount™ (H-5000, Vector, USA) and viewed under a confocal laser microscope (LEICA DMIRE2, Leica, Germany).

2.12. Statistics

SigmaStat 3.5 software was used. Comparisons between groups were made using one-way ANOVA. A p-value < 0.05 was considered a significant result.

3. Results

3.1. SEM and AFM

SEM analysis of the different surfaces (Fig. 1) showed that derivatized Sil surfaces presented a rougher topography than

non-derivatized Sil ones. SEM characterization suggested fbg deposition on all surfaces. The roughness parameter Ra was calculated by AFM. Ra is one of the primary parameters used to quantify surface texture and is defined as the relative roughness of an area [23]. The topographical images for the different Sil discs were obtained at two different locations and the average Ra values were calculated. Fig. 2 presents one image of each Sil surface obtained at a particular location. On visual observation, it can be clearly observed that the Sil-AcA and Sil-AIA surfaces are rougher than native Sil: the mean roughness increased from 75.498 nm for native Sil to 254.393 nm and 242.030 nm for Sil-AcA and Sil-AIA respectively. The surface of native Sil is relatively flat and smooth however, for both the plasma polymerized surfaces nano-scaled protrusions with irregular shapes, different sizes and specific distribution can be seen which could be due to different preparation processes.

3.2. Immuno-based assay

The next part of the study involved understanding binding of fbg to Sil discs and comparing the results with PS control, which was used as reference due to its established binding properties, attributed to the presence of carboxyl (COOH) and hydroxyl (OH) groups making the surface hydrophilic and improving its adsorption properties. A key requirement of this assay was to ensure adsorbed fbg bound to the Sil discs and not to the surrounding surface. This was achieved by blocking the wells with BB before placing the Sil discs in the wells. Fbg adsorption on blocked wells gave an absorbance below 0.2 suggesting

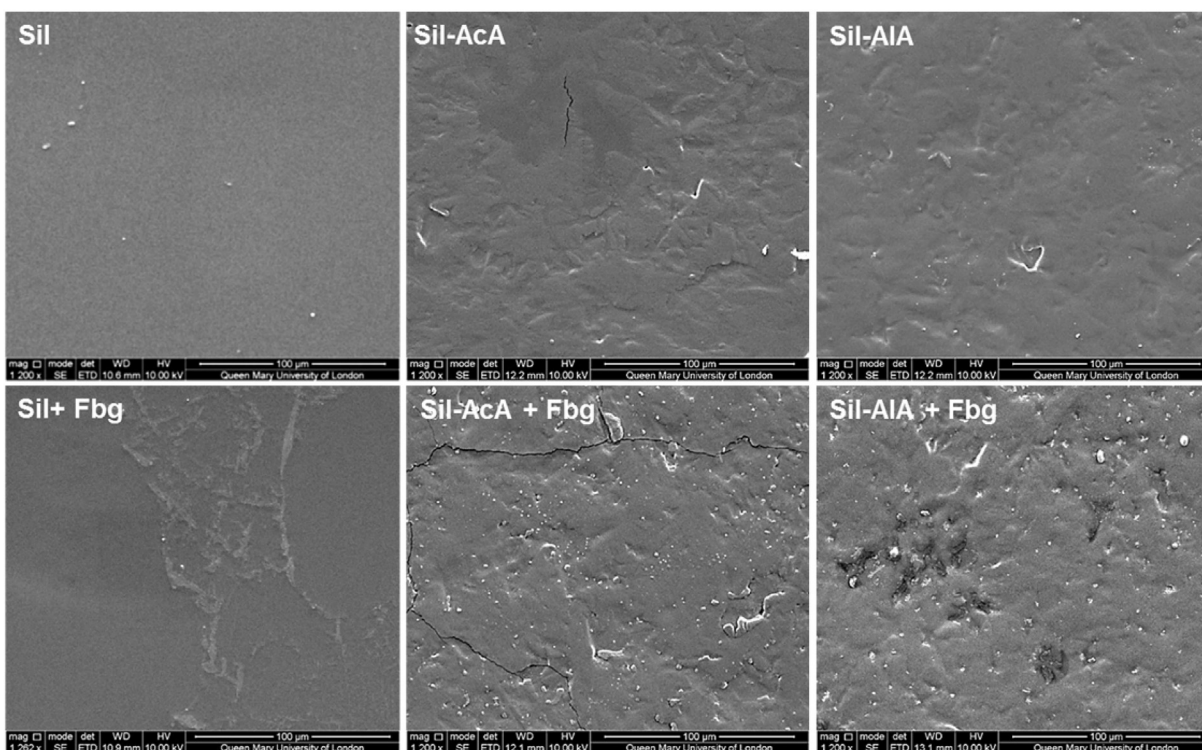


Fig. 1. SEM images of the different surfaces coated and non-coated with fibrinogen.

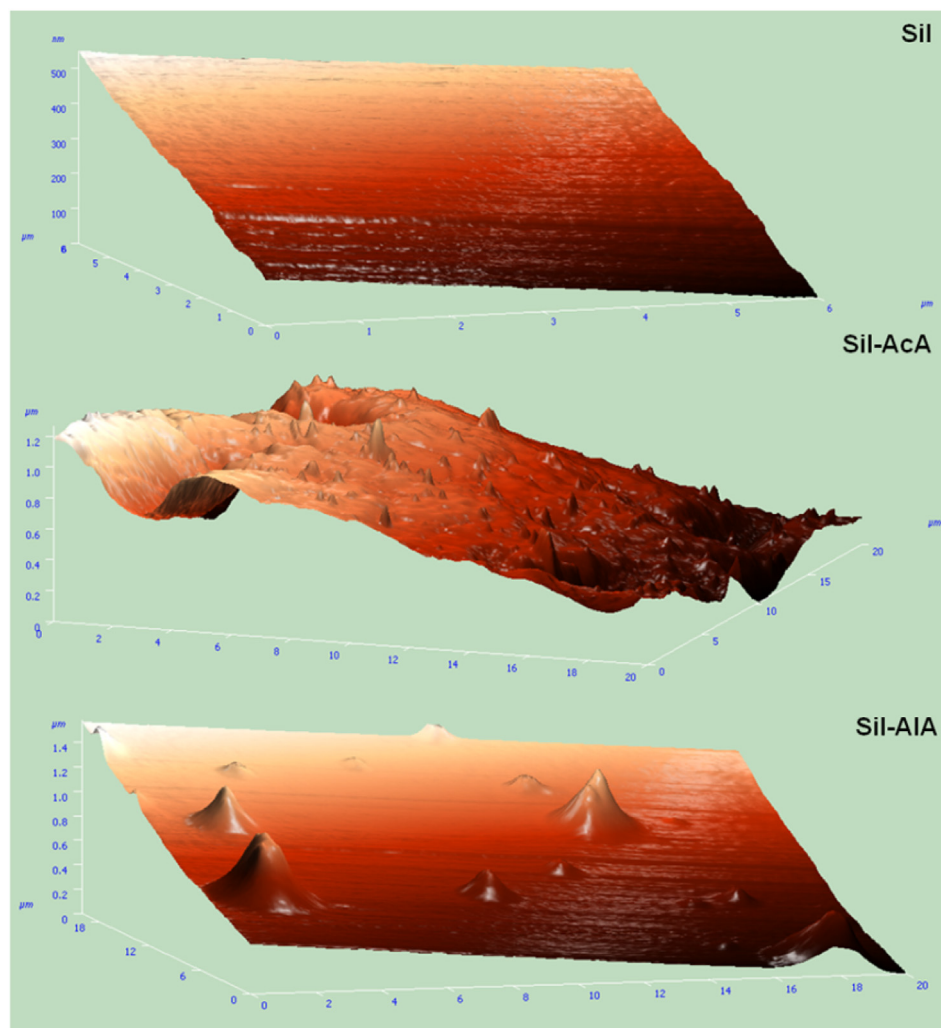


Fig. 2. Surface roughness images, obtained by AFM, of the different surfaces used in this study.

maximum blocking and confirming that protein adsorption would be only on the discs (Fig. 3).

To characterize protein binding, fbg was added over the range 0–250 $\mu\text{g/ml}$. The general protein binding trend observed for PS control and Sil discs was that with the increase in fbg concentration there was a linear increase in absorbance at 405 nm which after a particular concentration saturated and levelled out (Fig. 4). The saturation point was calculated to be the last point after which the absorbance decreased or remained equal. The binding curves of PS and Sil-AIA saturated at a concentration of 50 $\mu\text{g/ml}$ whereas for Sil-AcA the saturation was observed at 150 $\mu\text{g/ml}$. Considerable variations for fbg binding were detected for native Sil and according to the criteria selected above, the saturation point was considered to be 50 $\mu\text{g/ml}$.

Theoretically from the above data, the protein adsorption per area (area of silicone discs is 19.625 mm^2 while area of PS well is 66.915 mm^2) was calculated to be 0.075 $\mu\text{g/mm}^2$ for PS, 0.255 $\mu\text{g/mm}^2$ for Sil and Sil-AIA, and 0.764 $\mu\text{g/mm}^2$ for Sil-AcA. These results established roughly the amount of fbg bound on the various Sil surfaces but did not shed light on the binding strength.

3.3. Binding strength of fbg to different silicone surfaces

To compare the robustness of the interaction between the protein and the different Sil surfaces, protein extraction compounds capable of removing proteins from these surfaces were investigated. The binding strength between the fbg and Sil surfaces was established by measuring the reduction in absorbance (on the addition of the compounds) which was related to fbg removal from Sil surfaces. A variety of anionic (SDS) and non-ionic surfactants (Tween-20, F-127 and L101), the surface active cleaning agent Decon-90, the protein denaturant Urea and the protein precipitating agent Glycine (Table 3) were tested. Initially, the efficacy of these compounds against fbg coated Sil and Sil-AcA discs was studied. Once a suitable compound was selected, its effects were tested on all different Sil groups (Sil, Sil-AcA, and Sil-AIA).

Overall performance for some of the compounds tested (Decon-90, Glycine, Urea and SDS) was not satisfactory as very little or no difference in binding strength was seen between Sil and Sil-AcA surfaces (Fig. 5). However, in the presence of non-ionic surfactants (Tween-20, F-127 and L101) a different trend

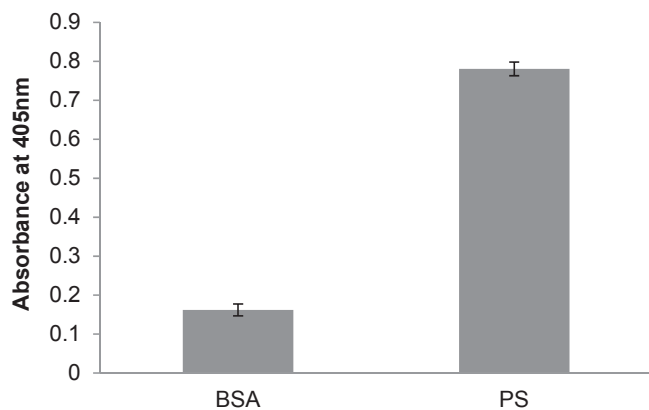


Fig. 3. Immuno-based assay to optimize fibrinogen adsorption onto non-blocked polystyrene (PS) wells without any disc. 1% blocking buffer was used as a negative control. Graph shows mean values \pm SEM.

was observed (Fig. 5). A significant decrease (Table 4) in protein binding was seen when these surfactants were used at a concentration of 1% on Sil discs. In contrast, the protein bound to the Sil-AcA discs did not show much variation in absorbance, exhibiting stronger binding strength.

Observing the above results, it was decided to continue using non-ionic surfactants for the rest of the assay. Due to its availability and cost-effectiveness, Tween-20 was tested on all the different Sil discs at varying concentrations (Fig. 5). Results suggested 46.76% reduction in binding of fbg to Sil-AcA, 71.89% to Sil-AIA and 82.93% to native Sil in the presence of

Tween-20 (2% vol/vol). Therefore, it was concluded that fbg bound with greater affinity to Sil-AcA surfaces followed by Sil-AIA and with the least affinity to native Sil.

3.4. Cell viability by alamarBlue[®] assay

The aim of the following cell in vitro assays was to validate the above results and study the biocompatibility of the different surfaces. All Sil discs with/without fbg showed higher metabolic activity than the control samples (HDFs on coverslips, an established in vitro model of 2D culture of fibroblasts' monolayers [22]) proving that it is possible for cells to survive and proliferate on the different surfaces used in this study (Fig. 6). Significant increase in cell viabilities was observed in Sil-AcA on day 7 compared to uncoated Sil. Similarly, significant results were observed on day 4 and 7 on fbg coated Sil-AcA in comparison to fbg coated Sil, suggesting that strongly bound fbg is a better substrate for cell viability and proliferation. These results confirmed the superiority of Sil-AcA (coated with and without fbg) to native Sil (coated with and without fbg) clearly suggesting the advantages of plasma polymerizing Sil surfaces and the importance of the binding strength of fbg for cell proliferation.

3.5. SEM and confocal microscopy

Cells were observed to form a monolayer on all surfaces. However, on fbg coated and non-coated Sil discs the monolayer was observed to detach from areas of the surface (Figs. 7 and 8). This detaching monolayer was composed of viable cells as

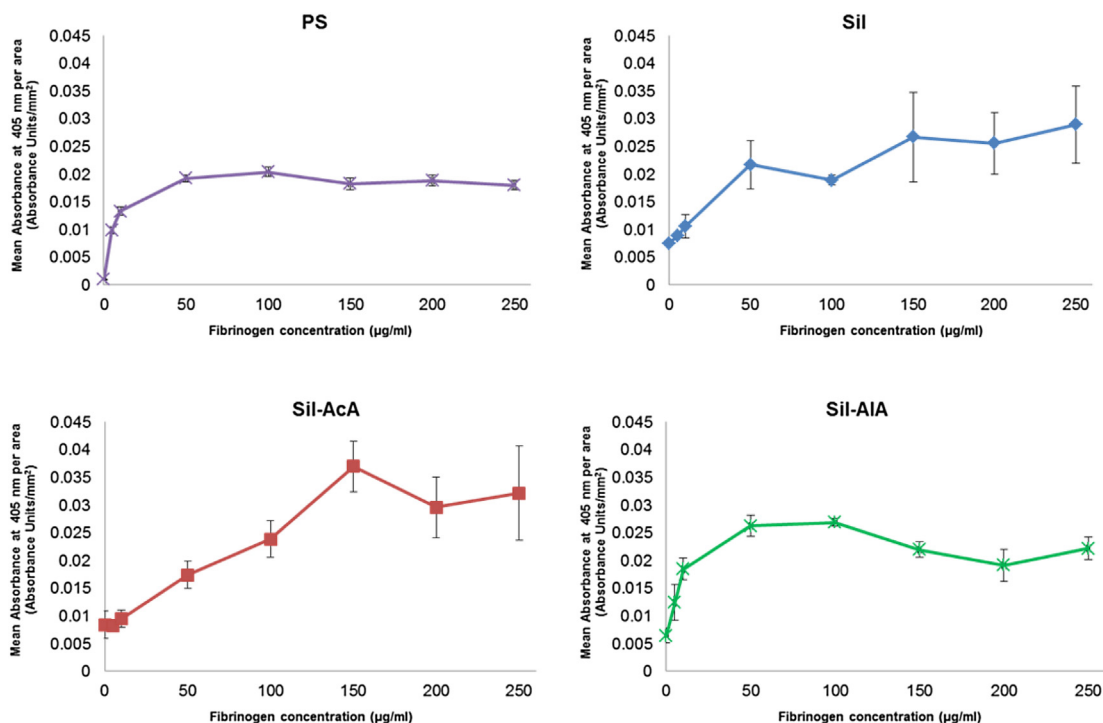


Fig. 4. Immuno-based assay to characterise fibrinogen adsorption onto native silicone (Sil), poly(acrylic acid) coated silicone (Sil-AcA) and poly(allylamine) coated silicone (Sil-AIA). Non-blocked polystyrene (PS) wells without any discs were used as reference. Graph shows mean values \pm SEM.

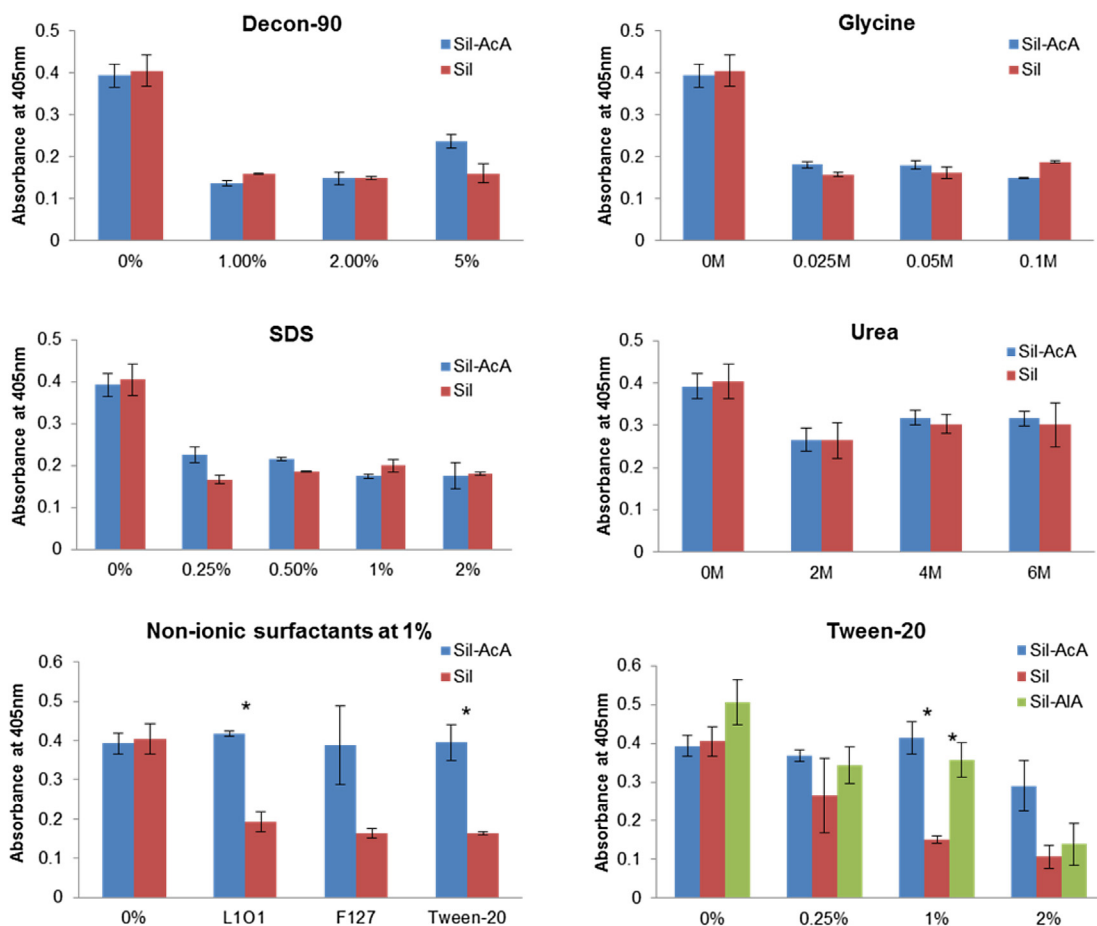


Fig. 5. Immuno-based assay using protein extraction compounds to compare the binding strength of fibrinogen to the different surfaces. Compounds used were: Decon-90 (1%, 2%, and 5% vol/vol); Glycine (0.1, 0.025 and 0.05 M); SDS (0.25%, 0.5%, 1% and 2% wt/vol); Urea (2, 4 and 6 M); non-ionic surfactants (L101, F-127 and Tween-20, 1% vol/vol); and Tween-20 (0.25%, 1% and 2% vol/vol). Graphs show mean values \pm SEM. *shows statistical significance (see Table 4 for p-values) for the non-ionic surfactants.

demonstrated by the alamarBlue[®] assay, a phenomenon that has been reported before not only for fibroblasts but also for other cell types [24,25]. Fig. 8 shows a small semi-detached cell monolayer in addition to a cell with weak surface attachment for Sil surfaces. Cells did not form clusters or detaching monolayers when in contact with Sil-AcA and Sil-AIA surfaces suggesting that native Sil surfaces require surface modifications to improve cell attachment. On comparing Sil-AcA and Sil-AIA (Fig. 8), cells were more spread out with longer filaments and attachment points when in contact with Sil-AcA surfaces, both coated and non-coated with fbg, further stressing on the superior binding properties of poly(acrylic acid) substrate. Similar findings have been reported with various cell types and it has been proved that

cells show higher affinity to substrates coated with poly(acrylic acid) [26]. Although no differences between cell adhesion and spreading were observed between Sil-AcA and Sil-AcA coated with fbg (Figs. 7 and 8), more growth on fbg coated Sil-AcA discs was seen than on Sil-AcA discs without fbg (Fig. 6) which can be attributed to the presence of fbg, providing a suitable substrate for cell growth.

4. Discussion

When developing novel composites of synthetic and protein-based polymers for tissue engineering applications it is essential to understand the protein-polymer interfacial binding, which occurs through protein adsorption. However, it is complex to characterize this interaction [12] and there is need for developing a robust and standardized assay for routine use [27]. In the present study we propose a sensitive, reproducible and rapid novel immuno-assay to measure surface bound proteins (specific antibody recognition system) and compare the protein binding strength using a range of protein extraction compounds. The method proposed in this study was: 1) to characterize the

Table 4
p-Values for non-ionic surfactants. *Shows statistical significance ($p < 0.05$).

Comparison	Tween-20 (0.25%)	Tween-20 (1%)	Tween-20 (2%)	F-127 (1%)	L101 (1%)
Sil vs Sil-AcA	0.348	*0.007/0.004	0.062	0.091	*0.001
Sil vs Sil-AIA	0.509	*0.010	0.627	–	–

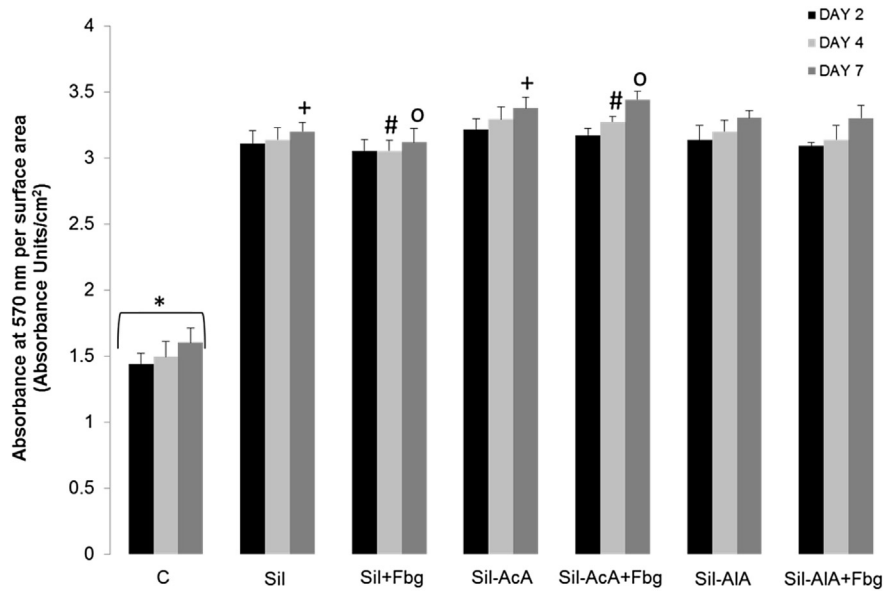


Fig. 6. Cell viability by alamarBlue[®] assay of HDFs cultured on coverslips (C) and the different silicone surfaces coated or not coated with fibrinogen. alamarBlue[®] activity was normalised per surface area. Graph shows mean values \pm standard deviation. C was significantly lower at all time points than the different silicone surfaces (* $p < 0.001$); significantly higher activity per surface area after 7 days of culture was measured for Sil-AcA discs compared with Sil discs (+ $p = 0.047$); higher activities per surface area for Sil-AcA + Fbg samples compared to Sil + Fbg at days 4 (# $p = 0.012$) and 7 (° $p = 0.010$) of culture were observed.

topography of the different Sil surfaces used; 2) to characterize the amount of protein bound on the different surfaces and compare the binding strength using protein extraction compounds; 3) confirmatory in vitro 2D model of cell culture to validate the immuno-based assay results. Fig. 9 summarizes the method proposed with a scheme of the immuno-based assay, the novel part of the method.

Topography of Sil surfaces was analysed using SEM and AFM to observe the microscopic differences after plasma polymerization. As expected, significant topographical changes were observed: the roughness of Sil surfaces increased with plasma treatment to give a characteristic hill–valley structure. These results are commonly observed and reported in various scientific literature and have been related to the mechanism of polymerization initiated by the radicals generated at polymer surfaces [28,29].

The proposed immuno-based assay on the different Sil surfaces showed a sharp increase in protein binding at lower concentrations. On further increasing the concentration, the protein binding reached a limiting point suggesting complete saturation. The literature describes this as an ideal adsorption isotherm which provides information about the protein - surface affinity [30]. On observing the binding curve and calculating the saturation points, the amount of fbg bound was quantified. Results were expressed for Sil discs and PS control in terms of protein bound per mm² by calculating the total area of the disc (πr^2) and well ($h * 2\pi r + \pi r^2$) respectively. It was concluded that Sil-AcA bound the highest amount of fbg in comparison to the other surfaces.

In this study the protein-surface interaction at the molecular level remained unknown and protein extraction solutions were used to investigate the protein binding strength.

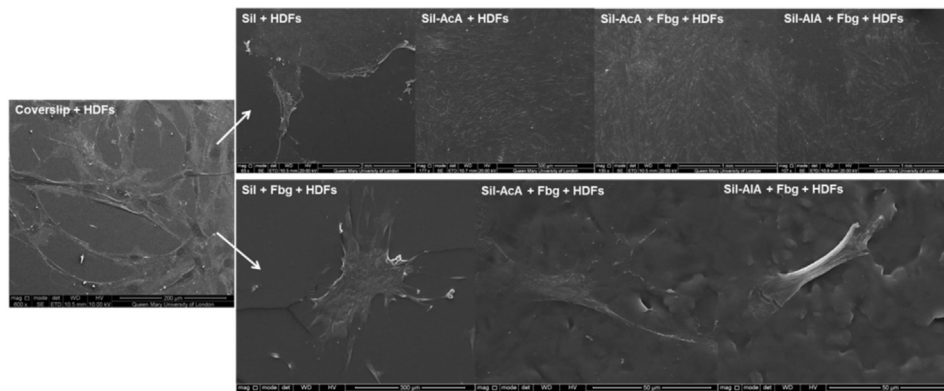


Fig. 7. SEM images of HDFs cultures (day 2) on coverslips (control) and the different silicone surfaces coated or not coated with fibrinogen.

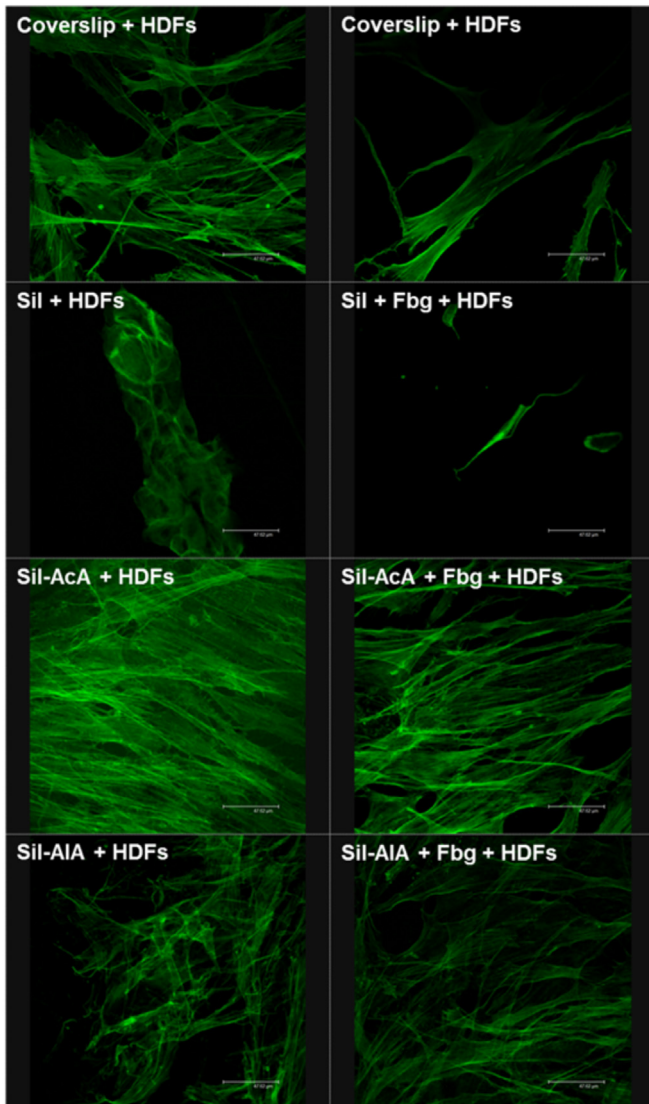


Fig. 8. Confocal microscopy images of HDFs cultures (day 2) on coverslips (control) and the different silicone surfaces coated or not coated with fibrinogen.

Surfactants are compounds that lower the surface tension of a liquid and are often used to remove proteins bound from surfaces of food processing equipment and from porous/non-porous polymer membranes [31]. Therefore, the anionic surfactant SDS and the non-ionic surfactants Pluronic® L101, Pluronic® F-127 and Tween-20 were investigated in this study along with the protein denaturant Urea, the surface active cleaning agent Decon-90 and the protein precipitating agent Glycine. Our results suggested a considerable drop in fbg absorbance at all concentrations in the presence of anionic surfactants and denaturant detergents, but little variation between Sil and Sil-AcA. It is an established fact that lower concentrations of anionic surfactants have similar protein denaturing effect as higher concentrations of Urea, disrupting the covalent bonds of proteins [32,33]. Decon-90 is a complex emulsion of anionic and non-ionic surfactants used as surface cleansing agent in the laboratory and is known for its strong denaturing properties. Saturated or nearly saturated solutions

of aliphatic aminoacids such as glycine have been extensively investigated as precipitating agents of plasma proteins such as fibrinogen [34]. Thus, all these compounds (SDS, Glycine, Decon-90 and Urea) had a denaturing effect on fbg and therefore a drop in absorbance was observed in the presence of these compounds at all concentrations. On the addition of non-ionic surfactants (F-127, L101 and Tween-20) to fbg coated Sil discs, a different trend was observed. With increasing concentration of Tween-20 a significant amount of fbg was removed from native Sil surfaces in comparison to polymerized Sil surfaces, suggesting stronger interaction between fbg and polymerized Sil surfaces (Fig. 5). Non-ionic surfactants like Tween-20 and Pluronic® are known to reduce protein adsorption and also prevent aggregation, precipitation or denaturation of the protein [35]. The reduction in absorbance for native Sil in comparison to plasma polymerized Sil was expected. This can be attributed to the addition of the desired monomers on the Sil surfaces by plasma polymerization providing active sites for stronger protein polymer binding [28]. Alternatively, native Sil surfaces lack functional groups and are relatively inert which could be one of the major reasons for weaker fbg attachment to Sil surface as observed in the above results. On comparing binding of fbg to the plasma polymerized surfaces in the presence of surfactants, fbg attachment to Sil-AcA surface seemed stronger than to Sil-AIA surface. This can co-relate to the high concentration of $-COOH$ groups on Sil-AcA surface providing higher surface area for protein immobilization [36]. Further to this, AcA coating allows proteins to remain in the native state providing a protective barrier (hindering denaturation process) and thereby increasing the protein and polymer interaction [37]. This theory confirms the results reported here for plasma polymerized surfaces.

Cell viability assay confirmed the biocompatibility of all surfaces and superiority of Sil-AcA (coated with and without fbg) to native Sil (coated with and without fbg) clearly suggesting the advantages of plasma polymerizing Sil surfaces. Moreover, results from the alamarBlue® assay suggested the binding strength of fibrinogen has an effect on cell proliferation. Similarly, microscopic results suggested better attachment of fbg on the polymerized surfaces.

The novel immuno-based assay described works on the principle of antibody conjugate binding to fbg adsorbed on Sil surfaces and uses the surface extraction capabilities of surfactants to measure fbg interfacial binding strength. It has a minimum requirement of resources (fbg used 25 μ g and Sil discs were 5 mm in diameter), therefore making it inexpensive. Advantageously, it is highly sensitive and can be easily combined into daily laboratory procedures. It can be applied to compare a range of polymeric surfaces to test their biocompatibility and binding strength of different proteins. However, the assay will be less efficient if the protein adsorption on the polymeric surfaces changes the protein conformation making it difficult to be recognized by the antibody conjugate system. The assay presented improves and simplifies current methods, making it a valuable tool in developing new biomaterials for clinical applications in different fields of tissue engineering. Moreover, we believe it has the potential to be used in other fields of research where protein adsorption plays an important role, such as in the fibrotic response to

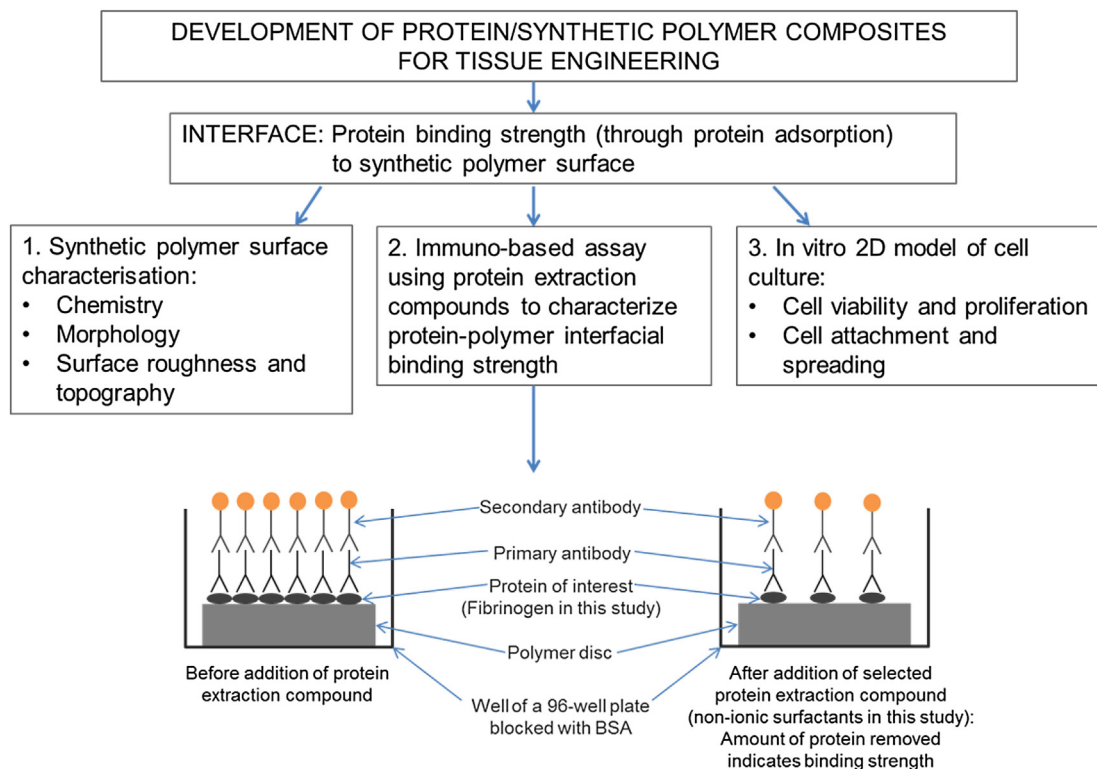


Fig. 9. Summary of the method proposed in this paper to characterize the interface of protein-synthetic polymer composites, including a schematic diagram of the novel immuno-based assay using protein extraction compounds, and more specifically non-ionic surfactants, to characterise interfacial protein binding strength.

implanted biomaterials [38], antibacterial activity of biomaterials [39] or characterisation of biofilm matrixes [40].

5. Conclusion

The immuno-based assay described can be used to determine interfacial protein binding strength on polymeric surfaces using protein extraction compounds as a tool, which to the best of our knowledge, has never been done before. It has the potential to be applied to solid materials of different formats and sizes including 3D shapes. Using this method we quantified the amount of fbg bound on Sil surfaces and proved that fbg bound with a stronger affinity to Sil-AcA surface. This helped to conclude that Sil surfaces with AcA coating can be used as suitable choice to manufacture fbg – Sil-AcA composite biomaterials for tissue engineering purposes. We are currently developing a fbg – Sil-AcA composite scaffold based on these results and further testing the application of this assay onto other polymeric surfaces as well as 3D constructs.

Conflict of interests

The authors have no conflict of interest to declare.

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