HYALURONAN-BASED PERICELLULAR MATRIX: SUBSTRATE ELECTROSTATIC CHARGES AND EARLY CELL ADHESION EVENTS

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Abstract

Cells are surrounded by a hyaluronan-rich coat called ‘pericellular matrix’ (PCM), mainly constituted by hyaluronan, a long-chain linear polysaccharide which is secreted and resorbed by the cell, depending on its activity. Cell attachment to a surface is mediated by PCM before integrins and focal adhesions are involved. As hyaluronan is known to bear a negative charge at physiological pH, the relevance of its electrical properties in driving the early cell adhesion steps has been studied, exploring how PCM mediates cell adhesion to charged surfaces, such as polyelectrolyte multilayer (PEM) films. Poly(ethylene imine) (PEI) and poly(sodium 4-styrene sulphonate) (PSS), assembled as PEI/PSS and PEI/PSS/PEI layers, were used. The nanoscale morphology of such layers was analysed by atomic force microscopy, and the detailed surface structure was analysed by X-ray photoemission spectroscopy. PCM-coated and PCM-depleted MG63 osteoblast-like cells were used, and cell density, morphology and adhesive structures were analysed during early steps of cell attachment to the PEM surfaces (1-6 h). The present study demonstrates that the pericellular matrix is involved in cell adhesion to material surfaces, and its arrangement depends on the cell interaction with the surface. Moreover, the PCM/surface interaction is not simply driven by electrostatic effects, as the cell response may be affected by specific chemical groups at the material surface. In the development of biomimetic surfaces promoting cell adhesion and function, the role of this unrecognised outer cell structure has to be taken into account.

Keywords: Pericellular matrix; hyaluronan; cell adhesion; polyelectrolyte multilayers.

Introduction

Cell adhesion to extracellular matrix or foreign substrates is mediated by a hyaluronan-based coat, the so-called pericellular matrix (PCM), with defined morphological, biochemical, and biomechanical features (Zimmerman et al., 2002; Lee et al., 1993; Knudson and Knudson, 1993). This hydrophilic layer, featuring anti-adhesive properties, interposes between the plasma membrane and the nearby surfaces (Entwistle et al., 1996).

Hyaluronic acid is a non-sulphated glycosaminoglycan, composed of D-glucuronic acid and D-glucosamine, that is negatively charged under physiological conditions. Hyaluronan synthesis and deposition change depending on cell activities, such as cell growth, confluence, mitosis, or detachment from a substrate, and is modulated by calcium or lactate concentration, pO₂, viral transformation, and serum (Stern, 2003).

All living cells are coated with this polysaccharide-rich layer of widely varying thickness, ranging between a few tenths of a µm and several µm. The large size of the PCM before integrin-mediated focal adhesions are effective (Cohen et al., 2004). In a time-frame of ms following such interaction, cell adherence to substrates occurs, in turn followed by cytoskeletal reorganisation and activation of signalling cascades that are responsible for cell spreading, migration, proliferation, differentiation, and survival (Bigerelle and Anselme, 2005). PCM is therefore likely to regulate cell adhesion to any surface, including biomaterials and tissue engineering scaffolds, before the formation of integrin-mediated focal adhesion complexes.

At a nanoscale level, an additional role of PCM is the interplay of electrical charges of hyaluronan chains with those present on substrate surfaces, where protonation/de-protonation of specific functional groups are present (Finke et al., 2007). In this paper, the response of cells, with and without PCM, to biomaterials with a controlled chemical structure and electrical charge was analysed in order to explore the relevance of the hyaluronan-based PCM and its electrical properties in cell adhesion. To this purpose, self-assembling films of polyelectrolyte multilayers (PEM) were chosen to evaluate cell reactions to a well-defined charged surface. PEM are built-up using a layer-by-layer deposition method based on electrostatic interactions, with the assembly of polyanion/polycation constituting a layer pair. The tunability of PEM provides
a unique opportunity to mimic the complex in vivo extracellular matrix environment. In this study cationic poly(ethylene imine) (PEI) and anionic poly(sodium 4-styrene sulphonate) (PSS) have been used. PEI may exert some cytotoxicity when used at high concentration, such as 5 mg/mL, but at lower concentration, i.e. 2 mg/mL, adhesion and proliferation of cells is undisturbed (Tryoen-Tóth et al., 2002; Brunot et al., 2007; Niepel et al., 2011). PSS was found to be biologically accepted when used at 1 mg/mL or 5 mg/mL (Ting et al., 2010; Tryoen-Tóth et al., 2002). Based on these previous reports, we selected low concentrations that have been described to be non-toxic.

In this study, MG63 osteoblast-like cells were seeded on two polyelectrolyte films with opposite electric charge to further define the role of PCM in the early phases of cell adhesion to synthetic substrates, with particular reference to the role of electrostatic interactions at the nanoscale level.

Materials and Methods

Polyelectrolyte multilayers

Polyelectrolyte solutions were prepared as follows: cationic poly(ethylene imine) (PEI, MW 750,000) and anionic poly(sodium 4-styrene sulphonate) (PSS, MW 70,000) were purchased from Sigma, and dissolved in ultrapure Millipore water. Solutions of PEI (pKa(app) ~8.8) and PSS (pKa(app) ~2.1) were prepared in Millipore water at a concentration of 1 mg/mL and pH 8.8 and 6.4, respectively. Simple immersion in the PEI or PSS solutions for 15 min were enough to achieve a complete layer of each polyelectrolyte: PEM were built-up on glass chamber slides by alternating PEI and PSS layer deposition to get a ~3.0 nm thick PEI-PSS film with an anionic surface, and a 4.5 nm thick PEI-PSS-PEI film with a cationic surface. Before polyelectrolyte deposition, glass surfaces were irradiated with UV-O3 for 30 min at atmospheric pressure in a Jelight Instruments apparatus (λpeak of 185 and 254 nm) to remove any carbon moiety, washed extensively with ultrapure water and dried with blown nitrogen. For clarity, the cell-facing outer layer is used throughout the text as a shorthand for multilayers, with PEI indicating the PEI/PSS/PEI multilayer, and PSS for the PEI/PSS multilayer.

AFM analysis

Atomic force microscopy (AFM) was applied using a Nanoscope IIIA-MultiMode AFM (Digital Instruments, Santa Barbara, CA, USA) with a “J scanner” in tapping mode under ambient conditions. The force was maintained at the lowest possible value by continuous adjusting the set point during imaging. Images were recorded using 0.005-0.02 Ω·m phosphorous (n)-doped silicon tips mounted on cantilevers with a nominal force constant of 40 N/m and a resonance frequency of 300 kHz.

XPS analysis

Angular-dependent X-ray photoelectron spectroscopy (AD-XPS) with a small spot apparatus (Axis-Ultra, Kratos Analytical Ltd, Manchester, UK), equipped with hemispherical analyser, was used to acquire both compositional survey and detailed scans.

The XPS measurements have been performed in Angular Dependent mode. That is, by exploiting the change in the depth analysed by changing the angle of photoelectron take-off. Setting the take-off angles at 0° (normal incidence, sampling depth ~10 nm) and 70° (surface-enhanced incidence, sampling depth ~3.4 nm) with respect to the normal axis to the sample surface, the concentration from the bulk to the outer film surface can be measured (Briggs and Seah, 1990). Thus, the estimated thickness are 9.4 nm at 0° and 4.7 nm at 70° take-off angles (θ), respectively (Popat et al., 2004). In order to avoid any damage to the sample during the data acquisition, the X-ray source (Al Kα,1) was used at a reduced power of 15 kV and 10 mA, with a ≤ 1.33 x 10⁶ Pa pressure. All binding energies were referenced to the C 1s neutral carbon peak at 284.6 eV (Suzuki et al., 1988). The Shirley-type background was subtracted from each spectrum. The peak fitting analysis was performed using the XPS-PEAK41 software and Gaussian curves, with constant full width at half-maximum for all the components of a given peak.

Preparation of control substrates

Human fibronectin (Sigma-Aldrich, Milan, Italy) was prepared at 0.1 mg/mL in MilliQ water and kept at room temperature for 30 min. Human type I collagen (Sigma) was dissolved at 1 mg/mL in 0.1 M acetic acid (pH 3) at room temperature for 60 min. Both proteins were diluted to 5 μg/mL in phosphate-buffered saline (PBS), and used to coat the glass plates (Nalgene Nunc, Roskilde, Denmark) by overnight incubation at 4 °C.

Cell culture and seeding

MG63 cells were grown in Iscove’s Modified Dulbecco’s Medium (IMDM, Invitrogen, Carlsbad, CA) supplemented with 10% foetal bovine serum (FBS, Mascia Brunelli, Milan, Italy), 2 mM L-glutamine, 100 U/mL penicillin and 0.1 mg/mL streptomycin (Invitrogen) in a humidified 5% CO2 atmosphere at 37 °C.

Cells from 80% confluent cultures were collected with 0.5% trypsin-EDTA (Invitrogen) and seeded on PEM, Permanox® and fibronectin or type I collagen at a density of 3.5x10⁴ per cm². To remove the pericellular matrix, MG63 cells were treated with 5 U/mL Streptomyces hyaluronidase (Sigma) in complete medium for 15 min and then plated on the different substrates with 2 U/mL hyaluronidase-added medium, to avoid new hyaluronan secretion. For clarity, the prefix ‘hyal’ is used onward for cells deprived of pericellular matrix.

Cell transfection with GFP

MG63 cells were cultured with antibiotic-free medium for 24 h, then transfected using Neon™ Transfection System Kit (Invitrogen). Briefly, 0.5 μg of plasmid DNA with green fluorescent protein (GFP) were added to 5x10⁶ cells resuspended in buffer. Following cell microporation (three pulses 1300V/10 ms, Microporator MP-100, Digital Bio Technology, Seoul, Korea), the cell suspension was seeded in a 6-well plate. After 48 h GFP-positive cells were detached and used in the experiment.
Erythrocyte exclusion test
Sheep red blood cells (Sigma), $1 \times 10^7$, were added to MG63 cells at 3 h from seeding on 10 % FBS-coated glass coverslips, and time-lapse microscopy applied after 5 min.

Hyaluronan labelling
Following detachment using trypsin-EDTA, $2.5 \times 10^4$ GFP-transfected cells/cm² were seeded on the different substrates. After 30 min, GFP-positive cells were incubated with 2 μg/mL biotinylated HA-binding protein (bHABP) (Seikagaku Corporation, Tokyo, Japan) for 2 h at 37 °C, washed with PBS, stained using 5 μg/mL Streptavidin-Alexafluor 586 (Molecular Probes, Invitrogen) for 30 min at 37 °C, and immediately observed by confocal microscopy (Eclipse E600, Nikon, Tokyo, Japan). Images of the pericellular matrix (red) around GFP-positive cells (green) were acquired using a 60x objective and a high-resolution digital camera. bHABP-stained sample for each condition was treated with hyaluronidase, and the disappearance of the red layer taken as a confirmation.

Cell number and spreading
The cells were seeded in duplicate on the different substrates, and non-adherent cells were removed with PBS. After 1, 3 and 6 h, adherent cells were fixed with 3 % (w/v) paraformaldehyde plus 2 % saccharose in PBS, permeabilised using 0.5 % Triton X-100 in HEPES buffer, and incubated with 0.5 % μg/mL phalloidin-TRITC (Sigma) for 45 min in the dark, to stain cytoskeletal f-actin. Then cell nuclei were stained with Hoechst 33342, 10 min in the dark (Sigma). After three washes with PBS, the specimens were mounted in glycerol/PBS and observed with a fluorescence microscope (Nikon Eclipse E800, Nikon). The number of adherent cells was obtained by counting the nuclei in six non-overlapping fields with a 20x objective. To assess cell spreading, six images (20x magnification) were taken on each sample and analysed using a dedicated software (LUCIA Measurement, version 4.60, Nikon Instruments, Tokyo, Japan). Changes of colour intensity based upon the fluorescence signal per pixel were detected, and the fluorescent area on the total area visualised (135,000 mm² at 20x magnification) was calculated. The cell spreading was quantified by dividing the red fluorescent area/total area ratio for the number of cells.

Fluorescence microscopy of adhesive proteins
After 6 h from seeding on PEM and control substrates, MG63 cells were fixed and permeabilised as described above. Following incubation with 0.5 % bovine serum albumin (BSA) in PBS with 10 % FBS for 15 min at room temperature to block unspecific binding sites, the adhesive proteins were stained using a 1:400 mouse anti-vinculin (Sigma), 2 μg/mL rabbit anti-paxillin, and 20 μg/mL mouse anti-actin antibodies, respectively, and observed with a fluorescence microscope (Nikon Eclipse E800, Nikon). Changes of colour intensity based upon the fluorescence signal per pixel were detected, and the fluorescent area on the total area visualised (135,000 mm² at 20x magnification) was calculated. The cell spreading was quantified by dividing the red fluorescent area/total area ratio for the number of cells.

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**Fig. 1.** AFM micrographs of different PEM films. Topography of (a) the PEI/PSS bilayer and (b) the PEI/PSS/PEI multilayers.
anti-integrin β1 antibodies (Santa Cruz Biotechnology, Inc., Heidelberg, Germany) in PBS + 0.2 % BSA and a fluorescein–conjugated secondary antibody. Cytoskeletal f-actin was stained as described above. Fluorescent images were acquired by confocal laser scanning microscopy.

**Western blot analysis**

Cells were seeded in 60 mm-plate coated with the different substrates, and after 6 h whole-cell proteins were extracted by cold lysis. Cells were washed twice with cold PBS, scraped off the culture dishes, and resuspended in RIPA (radio-immunoprecipitation assay) buffer supplemented with a protease-inhibitor cocktail (Roche, Milan, Italy). After 30 min incubation at 4 °C, the samples were centrifuged at 14,000 rpm for 20 min at 4 °C, and the supernatant containing the protein extract was analysed by standard SDS-PAGE, transferred onto nitrocellulose membranes, and probed with the appropriate antibodies (FAK, phospho-FAK (Tyr 576/577); alpha-tubulin, Santa Cruz Biotechnology). The bound primary antibodies were detected using an appropriate horseradish peroxidase-conjugated secondary antibody (anti-rabbit IgG or anti-mouse IgG, peroxidase-linked; Amersham, Little Chalfont, UK) and the relevant band was visualised using a chemiluminescence detection kit (ECL, GE Healthcare, Milan, Italy). The experiment was repeated twice.

**Results**

**Surface characterisation of polyelectrolyte films**

**Morphology at the nanoscale**

The PEI/PSS bilayers and the PEI/PSS/PEI multilayers were built by the layer-by-layer deposition method (see Materials and Methods). Fig. 1 shows the nanoscale morphology of the film surfaces as obtained by AFM in tapping mode.

Both films exhibited a quite uniform substrate coverage. In fact, the PEI/PSS surface showed a uniform distribution of large PEM granules with Rq = 0.64 ±0.15 nm, whereas the PEI/PSS/PEI surface consisted of a granular but smoother film, with Rq = 0.39 ±0.04 nm. Therefore, according to the literature, the very low values of Rq measured are not expected to significantly affect the adhesion and spreading of cells.

**Surface chemical structure: XPS quantitative results**

Table 1 reports the atomic percentage of sulphur and nitrogen in the two PEM films, respectively derived from the areas of the S 2p peak, assumed as the marker of the PSS layer, and N 1s, assumed as the marker of the PEI layers. The data have been evaluated both for bulk-like and surface-enhanced conditions.

The comparison between the quantitative data reported in Table 1 for 0° and 70° XPS take-off angle for PEI/PSS film shows a marked increase (about 1.8 factor) at the surface of S signal, due to the -SO₃⁻ groups of PSS, while the N signal (from the amine groups of PEI) increases only by about 1.3 times, confirming that the surface of the film is predominantly formed by the PSS layer. The signal from PEI is still relevant, due to the fact that under surface-enhanced mode the sampling depth is still about 3.4 nm, therefore sampling the whole ~3 nm-thick PEM film.

Likewise, the data reported in Table 1 for the PEI/PSS/PEI film show a huge increase of the N 1s peak (~1.6), and a slight decrease of the S 2p signal in surface-enhanced mode, indicating that the film surface is constituted by a PEI layer. In this case the sampling depth in surface-enhanced mode, ~3.4 nm, is slightly lower than the PEM film thickness (~4.5 nm).

**Surface chemical structure: XPS peak shape analysis**

Further hints on the structure of the films are derived from the analysis of the shape of the C 1s and N 1s peaks (Fig. 2).
Taking into account that the XPS sampling depth is higher than the film thickness for both PEI/PSS and PEI/PSS/PEI films, the XPS peaks recorded are a convolution of the signals originating from the whole film.

Thus, the C 1s peaks for both PEI/PSS and PEI/PSS/PEI films resulted from a convolution of the two components due to PSS, i.e., component 1 (~285.0 ±0.2 eV) and 2 (~286.7 ±0.2 eV), assigned to phenyl ring carbons and methylene groups, and to C-SO$_3^-$ groups, respectively (Santos et al., 2001), and the single component 3 (at 285.7 ±0.2 eV) due to PEI, and assigned to the C-NH, C-NH$_2$, C-N groups in the PEI chains (Finšgar et al., 2009).

Accordingly, the C 1s peak shape for PEI/PSS films is dominated by components 1 and 2, typical of the outer PSS layer, with a smaller contribution from the underlying PEI layer, while the C 1s peak in PEI/PSS/PEI films is dominated by component 3, characteristic of the outer PEI layers.

The N chemical state supported the above assignments, and provided further information on the chemical nature of the film surfaces, using the chemical state of the PEI film nitrogen as a marker of the interactions with PSS. Indeed, for the PEI/PSS films the N 1s peak is formed by two components of similar intensity, respectively due to quaternary nitrogen groups (~402.2 ±0.2 eV binding energy (BE)), and to the backbone amine nitrogen (400.0 ±0.2 eV BE) (Finšgar et al., 2009). On the other hand, for the PEI/PSS/PEI films the N 1s peak was essentially formed by the component due to the amine groups (assigned to chain inside the PEI layer, not directly interacting with PSS chains), with a small quaternary nitrogen component, due to PEI chains at the interface with the PSS layer, where the positively charged quaternary nitrogen groups act as counter-ions partially neutralising the negatively charged sulphonic groups.

Thus, we conclude that for PEI/PSS/PEI films the layer exposed to the biological medium is a homogeneous PEI layer covering the surface. At variance with this, for the PEI/PSS films, two potential structures are suggested: a layered structure with PSS lying onto PEI, with quaternary nitrogen at the very interface and an underlying layer of neutral PEI chains, or alternatively, an underlying PEI layer with a partially mixed layer of PSS and PEI.

**Biological studies**

**Peri-cellular matrix evidentiation**

The pericellular matrix of MG63 cells was seen using the erythrocyte exclusion assay. MG63 secreted a consistent pericellular matrix, seen as a red blood cell-free, clear, halo-like area surrounding the cell (Fig. 3a), which was removed after hyal-treatment (Fig. 3b). By time-lapse video microscopy, the kinetics of hyaluronan-coat formation around the cells was captured. Indeed, PCM formation is a rapid process; as already reported by Evanko et al. (1999), the cells synthesise a thick coat, and then resorb it in a few minutes (data not shown).

By confocal microscopy the pericellular matrix of the GFP-positive MG63 cells on PEI substrates was seen as a thick red border around the cells, which at 3 h keep a round shape (Fig. 4a). Following treatment with hyaluronidase, the cells were completely devoid of hyaluronan (Fig. 4b), and this has the effect of reducing the number of attached cells. On PSS films the cells tend to spread and elongate, showing a ‘granular’, irregular border of red hyaluronan. Their PCM was totally removed by hyaluronidase (Fig. 4c,d), with no evident effect on cell shape or number.
On Permanox®, many cells were round, with a thick and homogeneous pericellular matrix, quite similar to that observed on PEI (Fig. 4e). Instead, the coat of MG63 cells seeded on fibronectin was similar to that observed for PSS: hyaluronan was only on a few spread cells and mainly distributed along the flanks of the cells (Fig. 4g). On collagen, some cells showed a strong continuous layer, while others had an irregular pericellular matrix (Fig. 4i).

Cell adhesion and spreading
The adhesion of MG63 cells to PEM and the other substrates was evaluated after 1, 3 and 6 h. Many cells adhered to PSS and PEI after 1 h, and no change was evident when cells were treated with hyaluronidase (Fig. 5). At 3 and 6 h from seeding, on PSS the cell density was only slightly lower with and without PCM, whereas on PEI the number of adherent cells was significantly lower.
reduced compared to the first time-point. Interestingly, the cells treated with hyal were less able to adhere to PEI with respect to untreated cells, with a significant difference at 6 h (untreated vs. hyal: $p = 0.0495$).

The highest cell adhesion was found on fibronectin, with or without hyal treatment, and the number of adherent cells increased over time (Fig. 5). As with PSS, many cells attached to collagen I at 1 h, with a small decrease observed at longer time-points. The slight decrease in the cell number on PSS and type I collagen at 3 h vs. 1 h is probably due to a slower adhesion process: many cells try to interact with the surface at 1 h, but not all the initial weak interactions mature to a stable adhesion. No difference was recorded with or without PCM. In contrast, on Permanox® the cells adhered weakly at 1 h, but after 3 h the number of adherent cells was increased. Cells pre-treated with hyaluronidase adhered less compared to untreated cells, as on PEI, and the difference became significant after 6 h ($p < 0.05$ untreated vs. hyal).

By quantitative analysis of cell spreading, the cell area was shown to increase over time on all substrates, with the exception of PEI (Fig. 6). On PSS and fibronectin, the cells showed a well spread morphology already after 1 h, while on the other substrates the cells exhibited a roundish shape and covered only small areas. At later time-points, on collagen and Permanox®, the cells began to spread, showing a fully spread morphology at 6 h, while the cells on PEI kept a rounded shape with no increase in the area covered (Fig. 6).
No remarkable differences were reported after hyaluronan treatment compared to normal conditions for any of the substrates, but a tendency towards lower values of spreading was detected on PSS and PEI after 6 h.

Finally, cells cultured on PEI and PSS showed a different rate of migration by time-lapse microscopy: the cells on PEI were nearly immobilised on the surface, whereas cells on PSS were actively moving; such behaviour did not change after pericellular matrix removal (not shown).

**Western blot analysis**

The effect of the different substrates on the expression and activity of focal adhesion kinase (FAK) protein was evaluated by western blot. FAK is a 125 kDa cytoplasmic tyrosine kinase widely expressed in the focal adhesion complex. The phosphorylated FAK activates several downstream signalling molecules implicated in integrin-mediated signalling pathways. These in turn are involved in cell adhesion, migration, and proliferation (Mitra *et al.*, 2005). As shown in Fig. 7, total and phospho-FAK levels were similar in both conditions for fibronectin, while a reduction was induced by PCM removal on the other substrates, especially for PEI, where FAK expression was strongly decreased. In Fig. 7, the ratio of phospho-FAK to total FAK band density, normalised on tubulin, is represented (two separate experiments). Activated FAK was high on fibronectin, while a two-fold decrease in FAK

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**Fig. 6.** Evaluation of cell spreading on the substrates. MG63 were cultured on the different substrates for 1, 3 and 6 h. The spreading was evaluated after staining cytoskeletal f-actin with phalloidin-TRITC. The area covered by cells increases over time on all substrates except on PEI, where cells maintain a round shape. Results are reported as mean ± SEM of the fluorescent area of single cells. Black bars, untreated cells; striped bars, hyaluronidase-treated cells.
activity was recorded on PEI, as compared to fibronectin. FAK activity on collagen and PSS was similar, and higher compared to PEI.

Cytoskeleton and focal contacts
The organisation of focal contacts on different substrates was analysed at 6 h after seeding, using immunocytochemical recognition of integrin β1, paxillin and vinculin, and rhodamine-phalloidin for the cytoskeletal actin.

On PSS, MG63 cells were elongated, with actin stress-fibres tethered into focal adhesion contacts, where vinculin and paxillin were clearly visible and co-localised with actin. Integrin β1 was present as a cytoplasmic pool (Fig. 8a,c,e). When cells were pre-treated with hyaluronidase, only few focal contacts formed. At the cell periphery paxillin was absent and vinculin diminished; moreover, no stress-fibres were seen (Fig. 8d,f).

Cells seeded on PEI were round in shape, and actin was seen as a cortical layer at the cell edge, filopodia and tips, without stress-fibres. The adhesion molecules did not organise into focal contacts, and were located centrally in the cell body. No change was detected when the pericellular matrix was removed (Fig. 9).

On Permanox®, the cells were spread and actin was organised in fibres but, as on PEI, focal contacts were essentially missing, and vinculin was detected only in a few cells (Fig. 10e). This suggests the organisation of late focal complexes, which do not mature into focal adhesions. Removal of the pericellular matrix changed cell morphology and organisation, as many cells exhibited a roundish shape, with an undefined cytoskeletal pattern. Compared to untreated cells, no change in the adhesion molecules was appreciated (Fig. 10b,d,f).

Cells on fibronectin were well spread, elongated or polygonal in shape, with several focal adhesions. At the focal adhesion sites the adhesive molecules were strongly stained, and vinculin clearly co-localised with actin (Fig. 11a,c,e). The cells without PCM were quite similar to untreated cells (Fig. 11b,d,f).

The focal contacts were also well organised on collagen-coated chambers. Paxillin and vinculin staining was prominent at the cell periphery, where they co-localise with actin. Actin stress fibres were also observed (Fig. 12c,e), while integrin β1 was localised within the cytoplasm (Fig. 12a). Cells treated with hyaluronidase did not change their morphology. However, stress fibres were not organised, and paxillin was strongly reduced (Fig. 12b,d,f).

Discussion
In this paper, we analysed the role of PCM in the interaction of osteoblast-like cells with polyelectrolyte multilayers in order to investigate the early phases of cell attachment to a biomaterial substrate.

It is generally accepted that cells respond to a variety of surface-related features, such as chemistry, topography, wettability and stiffness, and integrate the resulting input to select behavioural pathways. Indeed, the early events of cell-substrate interaction, including changes in cell shape and cytoskeletal organisation, modulate short and long-term events, such as migration, proliferation, secretion and differentiation.

We used MG63 osteoblast-like cells as a model of PCM-coated cells. The thickness of the hyaluronan layer was indirectly detected by the erythrocyte exclusion assay, and a live-imaging technique using a glycosaminoglycan-specific fluorescent dye. To test if cells actually used negatively-charged PCM when adhering to a surface, cell morphology and adhesive structures during cell attachment, i.e. at 1, 3 and 6 h, were investigated. Cell shape and cytoskeletal organisation are good candidates to evaluate cell adhesion, since they are causally related to both long-term cell behaviour and substratum structure (Cretel et al., 2008). In this study, we assumed that electrostatic forces would drive the early interaction of cells with synthetic substrates if the negatively charged hyaluronan-based PCM produces different cell behaviour respectively on a positively- or negatively-charged PEM surface. Likewise, the electrostatic interaction should affect cell behaviour if the charged hyaluronan-based PCM is efficiently removed. Two polyelectrolyte multilayers were constructed by the layer-by-layer method, giving a positively charged PEI and negatively charged PSS as terminal cell-facing layer. PEI is largely used as an initial layer for PEM deposition, thanks to its ability to provide a uniform anchoring network for the consecutive layers’ formation (Kolasińska et al., 2007). Minor cell toxicity has been reported for PEI (Brunot et
Fig. 8. Confocal image of MG63 cells on PSS, labelled for actin (red) and adhesion molecules (green), without (a, c, e) or with (b, d, f) hyaluronidase treatment. Adhesion molecules include integrin β1 (a, b), paxillin (c, d) and vinculin (e, f). Cells show focal contacts with actin stress fibres, vinculin and paxillin clearly seen. After treatment with hyaluronidase the adhesion sites are few, without actin fibres and paxillin. Scale bar = 20 μm.
Fig. 9. Confocal image of MG63 cells on PEI, labelled for actin (red) and adhesion molecules (green), without (a, c, e) or with (b, d, f) hyaluronidase treatment. Integrin β1 (a, b), paxillin (c, d) and vinculin (e, f). Actin is running at the cell edge and adhesion molecules are not organised in focal contacts. Pericellular matrix removal has no effect. Scale bar = 20 μm.
Fig. 10. Confocal image of MG63 cells on Permanox®, labelled for actin (red) and adhesion molecules (green), without (a, c, e) or with (b, d, f) hyaluronidase treatment. Integrin β1 (a, b), paxillin (c, d) and vinculin (e, f). Actin fibres and vinculin form late focal complexes, while no focal contacts are observed. After treatment with hyaluronidase the cytoskeleton is disrupted. Scale bar = 20 μm.
Fig. 11. Confocal image of MG63 cells on fibronectin, labelled for actin (red) and adhesion molecules (green), without (a, c, e) or with (b, d, f) hyaluronidase treatment. Integrin β1 (a, b), paxillin (c, d) and vinculin (e, f). Cells form highly organised focal adhesions, with vinculin-actin co-localisation. After treatment with hyaluronidase no change is observed. Scale bar = 20 μm.
Fig. 12. Confocal image of MG63 cells on type I collagen, labelled for actin (red) and adhesion molecules (green), without (a, c, e) or with (b, d, f) hyaluronidase treatment. Integrin β1 (a, b), paxillin (c, d) and vinculin (e, f). Focal contacts are seen, similar to those observed on PSS. After treatment with hyaluronidase the number of adhesion sites is reduced, with no stress fibres and organised paxillin. Scale bar = 20 μm.
partly dependent on the working concentration (Tryoen-Toth et al., 2002) and the cell type. PSS is similarly compatible and currently used as a potassium-binding resin in hyperkalaemia (Inaba et al., 2000).

Cells are highly mechanosensitive, and the assembly and disassembly of matrix adhesions are regulated in a dynamic fashion in response to cell signals and the exogenous tension provided by matrix rigidity/substrate stiffness (Berrier and Yamada, 2007). In our study, PEI and PSS films were quite thin (~3-5 μm), and the resulting stiffness was negligible in terms of cell response: if cells ‘sense’ the effective stiffness of rigid objects that are not in direct cellular contact, as shown by other authors (Buxboim et al., 2010), in our experimental system the MG63 cells ‘sensed’ the stiff glass substrate underlying both PEM. Therefore any change of cell behaviour would be due to surface chemistry and/or charge.

The absence of movement of PEI-seeded cells, as seen by time-lapse microscopy, and the thick PCM layer adherent to the cell profile, observed by confocal microscopy at 3 h, suggested that PEI did not favour cell adhesion, despite its positive charge. MG63 cells on PEI showed a PCM similar to that observed a few minutes after seeding or just before detachment from a substrate, as in pre-mitotic cells, which show a dense accumulation of the pericellular matrix bordering the cell just before detachment and rounding (Cohen et al., 2003; Evanko et al., 1999). Indeed, it has been shown that if cell spreading is restricted, more round cells enter into apoptosis in comparison to spread cells (Re et al., 1994).

We observed a tendency of PEI adherent cells to detach, particularly if deprived of their PCM. This phenomenon might be due to initial electrostatic PCM-PEI interactions, without further development of stable adhesion phenomena. Moreover, high levels of FAK protein expression did not correspond to an increased activity, and this expression was drastically reduced following PCM removal. Indeed, FAK phosphorylation, which mediates cell migration/proliferation, is sensitive to surface chemistry (Keselowsky et al., 2006). The ‘mixed’ composition of the PSS-terminated PEM, with some PEI-areas, likely to reduce the electrical charge on PSS, does not affect the pro-adhesive ability of the sulphonic groups. This definitely indicates that the behaviour of MG63 cells on PSS- and on PEI-terminated PEM cannot be accounted for in terms of elementary electrostatic interactions.

In conclusion, we provided evidence for the role of the charged hyaluronan-based PCM in mediating interactions between cells and synthetic surfaces and regulating cell adhesion to charged PEM surfaces. Unexpectedly, osteoblast-like cells did not adhere to PEI, despite the electrostatic interaction between the negatively charged hyaluronan molecules and the positively charged groups on PEI. Similarly, negatively charged PSS surfaces exhibited a pro-adhesive activity, in spite of the expected repulsive interactions with negatively charged hyaluronan-based PCM. Surface chemistry therefore prevails over total surface charge in inducing cell adhesion to PEM, and confirms the wide potentiality of thin PEM films for the design of engineered ECM, where charge cues, chemical groups and nanoscale structure induce phenotypic cell modulation (Newcomer et al., 2011).
Cell interaction with the external environment appears to be effectively mediated by PCM, playing as a sensor transducer triggering signalling events before integrin involvement (Cohen et al., 2006). Such a “remote mechanosensitivity” model, through which cells “sense” the external matrix from a distance of a few µm and responds to it by activating adhesion and spreading, independently of the simple interaction of negative-to-positive forces, opens novel perspectives on the future design of surfaces for biomedical application.

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References


Discussion with Reviewer

Reviewer II: Do the authors think that PCM/material interactions could be important in stem cell differentiation regulation as seen with the role of adhesions in response to stiffness, chemistry and topography?

Authors: We think that PCM could play a role in stem cell differentiation, as this process is strongly dependent on bridging between the cell surface and substrate with subsequent ‘mechanotransduction’.

In a recent paper on MSC response to substrate stiffness, a first phase of cell adhesion where ‘.the cell adheres passively and starts to sense the substrate’ is reported (Tam et al., 2012). In our opinion, this ‘passive adhesion’ step is coincident with the PCM/surface phase of interaction, which could not be as passive as it appears. Indeed, this biological structure is able to escape some simple theoretical rules, such as positive-to-negative electrostatic interaction, as shown in the present work. Likewise, PCM could modulate other cell activities.

A recent paper by Darzynkiewicz and Balazs (2012) shows that the expression of hyaluronan (HA) on the cell surface correlates with the differentiation status of stem and progenitor cells, with the highest HA expression observed on the surface of the most primitive (Lin-) stem cells, and a progressive decline seen to be concurrent with differentiation.

Finally, Mathieu and Loboa (2012) have found that ‘...osteogenic differentiation is prevalent in MSCs with a stiff, spread actin cytoskeleton and greater numbers of focal adhesions, while adipogenic or chondrogenic differentiation are encouraged when MSCs have a spherical morphology associated with a dispersed actin cytoskeleton with few focal adhesions’. Since from our study and others, PCM apparently modulates the formation of adhesive structures, it may be included in the mechanisms/structures that stand upstream to stem cell differentiation process.

In conclusion, PCM/substrate interaction may be a determinant in mesenchymal stem cells biology, as the cell employs PCM to ‘contact’ the surface, then the material surface features dictate the PCM organisation, which in turn affects cytoskeletal actin, adhesive structures and cell spreading, required for subsequent cell migration/differentiation.

Additional References

Mathieu PS, Loboa EG (2012) Cytoskeletal and focal adhesion influences on mesenchymal stem cell shape, mechanical properties, and differentiation down osteogenic, adipogenic, and chondrogenic pathways. Tissue Eng Part B 8: 437-444.