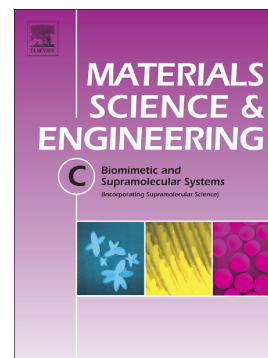


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Multilayer Platform to Model the Bioactivity of Hyaluronic Acid in Gastric Cancer

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ABSTRACT

Hyaluronic acid (HA) has a key role in cancer progression. The HA's molecular weight (M_w) is altered in this pathological state: increased concentration of shorter fragments due to the overexpressed hyaluronidases and ROS. Aiming to mimic this microenvironment, we developed a Layer-by-Layer (LbL) platform presenting HA of different M_w s, namely 6.4, 752 and 1500kDa, to study the influence of HA M_w on the formation of focal adhesion sites (FAs), and the involvement of paxillin and CD44 in this process. High paxillin expression and formation of FAs, via CD44, is observed for MKN45 cells seeded on LbLs presenting HA 6.4kDa, with the activation of the ERK1/2 pathway, responsible for cell motility and tumour progression. In contrast, activation of p38 pathway, usually related with cancer latency, is observed for cells seeded on LbLs with high M_w HA, *i.e.* 1500kDa. Overall, we demonstrate the suitability of the developed platform to study cancer invasiveness.

Keywords: Hyaluronic acid, QCM-D, cancer invasiveness, molecular weights, ECM model

1. INTRODUCTION

The communication between cells and their microenvironment is crucial for the regulation of cellular functions in either normal or diseased states [7, 41]. An example, is the formation of cancerous tissue that is usually accompanied by an abnormal cellular metabolic activity and an altered stroma. In specific types of cancers, *e.g.* gastric cancer, an accumulation of hyaluronic acid (HA) in the tumour microenvironment and surrounding stroma is observed [16, 27]. HA is a negatively charged glycosaminoglycan, whose amount is tightly regulated by the interplay of HA synthetases (HAS) and hyaluronidases (HYAL). Three different transmembrane enzymes, HAS1, HAS2 and HAS3 generate HA of different molecular weight (M_w), *i.e.* between 100 and 2000, >2000 and between 100 and 200 kDa, respectively. [56] After synthesis, HYALs are responsible for the additional modification of the size of HA [12, 49]. This process is extremely important, as most of the HA bioactivities are size-dependent, *e.g.* only short HA fragments are angiogenic inducers which are associated with cancer cell migration and tumour aggressiveness [30]. The expression of HYALs and HASs have been shown to be altered in tumours resulting in specific HA profiles characterized by overexpression of HA and enrichment on short oligomers [12, 35].

These alterations affect the cellular activity via interactions of HA with cell surface receptors [13]. Previously, it was demonstrated that the HA M_w s affects its interaction with CD44 (the main HA cell surface receptor) [2] and the subsequent signalling cascades, that lead to different expression of cortactin and the formation of cell membrane protrusions [4]. The CD44 glycoprotein function, such as HA binding, is enhanced in cancer through the altered glycosylation of CD44 [33]. CD44 is involved in the activation of different signalling cascades that can alter cell proliferation rate, promote/inhibit cell migration and remodelling, among other biological outcomes [45]. It has a key role in the activation of the complex FAK-Src through interaction with Src tyrosine kinase and triggers the activation of several other protein kinase cascades that lead to the phosphorylation of proteins associated with the (re)organization of the cytoskeleton, such as paxillin [22, 32, 50]. Paxillin is a main component in the regulation of the assembly, disassembly and signalling of focal adhesions (FAs) [29]. The (dis)assembly of FAs is a dynamic process required for cell motility and migration [39].

In this context, we hypothesise that the HA's M_w influences the activation of FAs and the motility and aggressiveness of cancer cells. HA of different M_w s, namely 6.4, 752 and 1500 kDa, were incorporated into Layer-by-Layer (LbL) constructs (using poly-L-lysine, PLL, as polycation) and the interactions of these substrates with MKN45 - a gastric cancer cell line with a high expression of CD44,

was evaluated. The developed LbL systems have the advantage of presenting HA in an extracellular matrix (ECM)-relevant way, *i.e.* with restricted mobility but still able to reorganize and bind specifically to other biomolecules, as previously demonstrated [4].

2. MATERIALS AND METHODS

2.1. Materials. All chemicals were used as received without further purification. Poly-L-lysine hydrobromide (PLL; M_w 30-70kDa), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) $\geq 98.0\%$ and N-hydroxysuccinimide (NHS) were obtained from Sigma-Aldrich (Portugal). Hyaluronic acid (HA; M_w 6.4kDa, 752kDa and 1500kDa) was purchased from Lifecore (USA). Phalloidin-tetramethylrhodamine B isothiocyanate (phalloidin-TRITC), 4,6-diamidino-2-phenylindole, dilactate (DAPI) from Sigma-Aldrich (Portugal) and the rabbit monoclonal [Y113] to paxillin from Abcam (Cambridge, UK) were used for immunostaining. The secondary antibody, Alexa Fluor® 488 donkey anti-rabbit IgG, was obtained from Life Technologies (Canada). Anti-CD44 antibody [KM201] (ab25340) from Abcam (Cambridge, UK) was used for CD44 blocking.

2.2. Assembly of polyelectrolytes multilayers. The substrates used in this study were gold coated glasses ($1 \times 1 \text{ cm}^2$). The LbL build-up was performed as previously described.[1] Briefly, the substrates were first immersed into a polycation solution (PLL, 0.5 mg.mL^{-1} in 0.15 M NaCl) for 15min, then removed, washed (0.15 M NaCl , $\text{pH} \approx 6.0-6.5$) and immersed into the polyanion solution (HA, 1 mg.mL^{-1} in 0.150 M NaCl) supplemented with a crosslinking agent (EDC, 0.400 M and NHS, 0.100 M) for 15min. This procedure was repeated until a 10 layers system (PLL-HA)₅ was obtained (n represents the M_w of HA, *i.e.* n=6.4, 752, 1500kDa). The so-coated substrates were rinsed with water (to remove the excess of salt), dried at room temperature and characterized or used in further studies.

2.3. Culture, expansion and seeding of gastric cancer cell lines. Human gastric cancer cell line MKN45 were cultured in RPMI-1640 medium (Sigma-Aldrich, Portugal) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Biochrom AG, Germany) and 1% antibiotic/antimycotic solution (ATB) (final concentration of penicillin 100units/mL and streptomycin 100 mg.mL^{-1} ; Gibco, UK). Cells were cultured in a $5\% \text{ CO}_2$ incubator at 37°C . Before the *in vitro* studies, the (PLL-HA)₅ coated substrates were sterilized by exposing them to UV light for 30min. Confluent MKN45 were harvested

and seeded on (PLL-HAN)₅ substrates at a density of 11000cells/cm² using RPMI-1640-10% FBS-1% ATB medium. Bare gold-coated glasses were processed as the other samples and used as controls.

2.4. Characterization of MKN45 Cell Adhesion by Real Time Quartz Crystal Microbalance with Dissipation (QCM-D). (PLL-HAN)₅ were deposited on commercially available gold-coated QCM-D crystals (Biolin Scientific, Sweden) using a spin-coater. Briefly, 100 μ L of each polyelectrolyte solution was allowed to adsorb on the quartz crystal for 15min. Between layers adsorption, a spin coating was performed during 10s at 3000rpm to remove the excess of the solution and the unbound materials, with an additional wash spinning using 0.15M NaCl solution. The coated sensors were placed in the QCM-D flow chamber and a stable baseline was acquired by flowing the RPMI medium at flow rate of 50 μ L.min⁻¹. The MKN45 cells (0.400 $\times$ 10⁶ cells/mL) were then flowed over the substrates at rate of 50 μ L.min⁻¹ for 1h. Then, the flow was stopped for 1h50min to allow cell attachment. Finally, the sensors were rinsed with RPMI to remove unbound cells. at flow rate of 100 μ L.min⁻¹. The resonance frequency shift, Δf , and the dissipative shift, ΔD , were recorded at several harmonics (n=3, 5, 7, 9, 11 and 13). The obtained data are presented as $\Delta D/\Delta f$ (3rd overtone) plots. For the CD44 blocking experiment, the MKN45 cells were incubated with anti-CD44 antibody for 1h, followed by centrifugation and washing prior to their seeding into the QCM-D.

2.5. Cell morphology, distribution and protein expression. After the period of cell culture, the samples were washed twice with PBS, fixed in 10% neutral buffered formalin for 30min at 4°C, permeabilized with 1% Triton X-100 in PBS for 30min at 4°C, and blocked with 3% BSA in PBS for 30min at room temperature. The paxillin expression was evaluated by anti-paxillin monoclonal antibody [Y113] (AB32084, 1:250 in 1% w/v BSA/PBS), followed by anti-rabbit Alexafluor-488 (1:500 in 1% w/v BSA/PBS). A phalloidin-TRITC conjugate was used (1:200 in PBS for 30min) to assess cytoskeleton organization. Nuclei were counter-stained with DAPI (1mg.mL⁻¹ in 1% BSA in PBS for 30min). The cells were observed under confocal laser scanning microscope (TCS SP8, Leica, Germany). Scanning electron microscopy (SEM) was used to evaluate cell spreading and morphology. The (PLL-HAN)₅ samples with the cultured MKN45 cells were washed with PBS, fixed with 10% formalin and dehydrated using ethanol of increasing concentrations (50%, 70%, 90% and 100%). The samples were coated with 0.6nm thick platinum coating and examined at an accelerating voltage of 3.00kV in a Zeiss Auriga Compact scanning electron microscope. The cells' filopodia length and number were measured

from at least 10 isolated cells ($n=10$) using the SEM pictures. The quantitative image analysis was performed using ImageJ analysis software (Version 2.0.0-rc-68/1.52c).

2.6. Cell migration by Live imaging. MKN45 were seeded as described above on (PLL-HAn)₅ samples and allowed to attach for 6h. Live monitoring of cell migration was performed at 37°C in an inverted microscope (Zeiss Axio Observer, Germany) equipped with a temperature and CO₂ control device (5% CO₂). Time-lapse images (40X) were captured every 5min using the Zen software. Cells were followed for 16h. Image stacks were analysed with ImageJ analysis software (Version 2.0.0-rc-65/1.52c) using manual tracking.

2.7. Protein array. Cell lysates were subjected to Proteome Profiler™ using the Human Phospho-MAPK Array kit. MKN45 cells were cultured in the QCM-D chambers, following the procedure mentioned before. After the dynamic cell seeding, the sensors were washed with PBS and treated with the Lysis Buffer 6, provided on the array kit, to disrupt the cells and extract the protein lysate. The protein analysis and quantification were determined using the Bio-Rad Protein Assay kit, following the manufacturer instructions.

2.8. Statistical Analysis. The normality of the data was evaluated using Shapiro–Wilk test ($p<0.05$). When the data did not follow a normal distribution an initial Kruskal–Wallis test was executed followed by Mann-Whitney test. In all the cases, a significant variation was considered for $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***).

3. RESULTS AND DISCUSSION

3.1. Build-up of LbL comprising hyaluronic acid of different sizes and cell adhesion monitored by real-time QCM-D

The stacking of oppositely charged polyelectrolytes (PEs) through LbL assembly is a well-established methodology to coat surfaces [18] or generate self-standing membranes [54]. The process comprises the alternate deposition of polycations and polyanions on a substrate that leads to the formation of a multilayer film maintained through electrostatic interactions between the polyions (**Figure 1**) [10]. Under physiological conditions (ionic strength of 0.15M NaCl, neutral pH), poly-L-lysine (PLL, strong PE – **Figure 1A**) and HA (weak PE - **Figure 1B**) generate dynamic systems where the PEs are able to

diffuse between layers. This dynamic process depends on the PE size (M_w) as well as on the addition of crosslinking agents: two factors that influence the film growth and modulate the properties of the generated assemblies [2, 10, 40]. In our case, the different layers are crosslinked through carbodiimide chemistry to ensure the stability of the systems under physiological conditions. From a molecular mobility perspective, this system is confined but yet enough adaptive not to compromise HA ability to interact with proteins, such as CD44 [2].

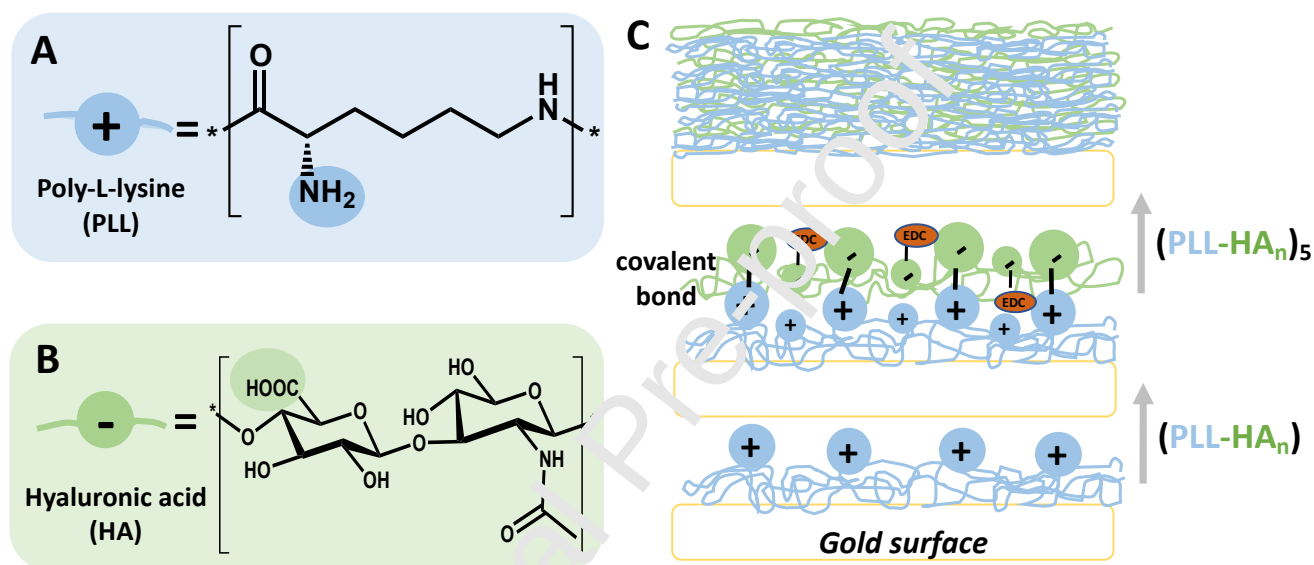


Figure 1. Chemical structure of the polycation, PLL (A) and polyanion, HA (B) used for the construction of the LbL films. (C) Schematic presentation of $(\text{PLL-HA})_5$ assembly on gold-coated glasses, showing the EDC/NHS-mediated crosslinking.

CD44 is the main HA receptor and is overexpressed in different types of cancers [15, 45]. This receptor is selected as a pathologically relevant target to evaluate the efficiency of the generated assemblies as models to study the impact of HA M_w s in the response of cancer cells. The gastric cancer cell line MKN45 overexpress CD44 and are sensitive to HA M_w [4, 20]. We, therefore, followed *in situ* the interactions of MKN45 cells with the LbL substrates using Quartz Crystal Microbalance with Dissipation (QCM-D). The QCM-D is an acoustic technique [3, 5, 17] that measures frequency shifts (Δf) of an oscillating piezoelectric quartz crystal in response to the hydrodynamic adsorbed mass and the energy dissipation (ΔD) of this mass, which gives an indication of its viscoelastic properties [9, 43, 48].

These characteristics make the QCM-D a valuable tool to evaluate the build-up of the LbL assemblies and their properties as a function of the HA's M_w s, as we previously demonstrated [2]. In addition, the QCM-D allows to follow in real time structural changes in the LbL system, that are induced upon cell adhesion (**Figure 2**). The changes in Δf and ΔD registered *in situ* after the injection of cells demonstrated three different stages: 1) seeding of the cells; 2) cell attachment, spreading and LbL/ECM remodelling; and 3) detachment of the loosely attached cells (**Figure S1**).

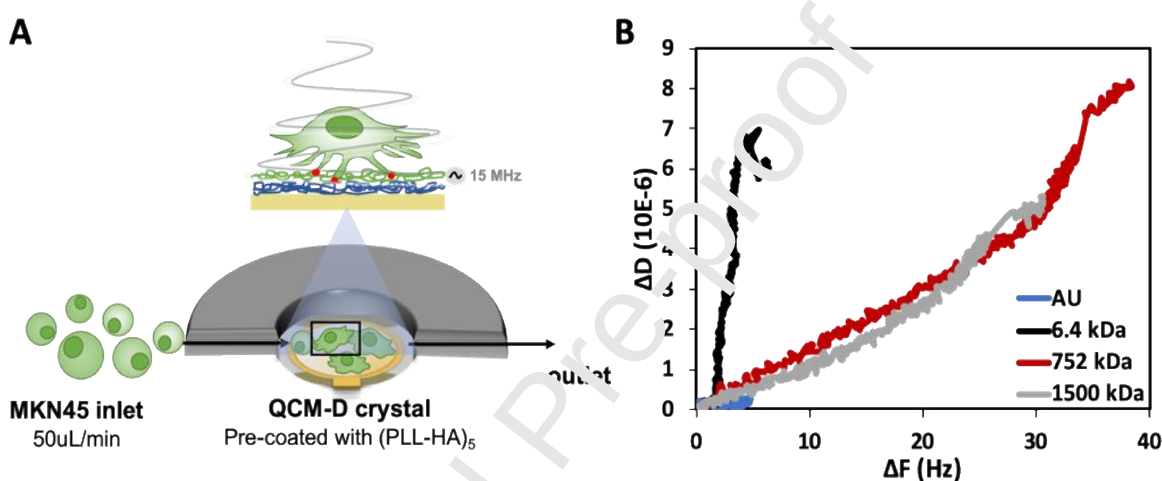


Figure 2. (A) Schematic presentation of QCM-D setup in the presence of cells and (B) representative $\Delta D/\Delta f$ plots (3th overtone) showing changes during the interaction between the MKN45 cells and the (PLL-HA)₅ surfaces, during 2h30 of cells culture.

The correlation between Δf and the adsorbed mass is not straightforward when cells are used as sensory input because they are relatively big for the used frequency: QCM can detect changes within a layer of apx. 250nm (at most) for the used fundamental frequency (5MHz) [38, 44]. Therefore, cells represent an acoustically “thick” layer with thickness (nearly 10 μ m) much larger than the maximum penetration depth of the acoustic wave [51]. Because the cell body is above the detection limit of the QCM-D shear wave, Δf is not indicative for the number of cells deposited on the sensor. However, specific interactions taking place on the sensor surface within the detection limit (*i.e.* adsorption of proteins secreted by the cells, receptor-ligand interactions, etc.) can be detected but only provide qualitative information about the dynamics of cells’ adhesion [17, 44]. ΔD can corroborate Δf signal as it

provides data for the viscoelastic properties of the mass deposited on the quartz sensor and thus, indication about cell adhesion strength, formation of FAs and cytoskeleton assembly [37].

The deposited LbL films have a thickness between 40-80nm depending on the HA M_w (thicker LbL films are build-up from HA with higher M_w s), as previously shown and characterized in detail [2]. Considering the limitation in the penetration depth of the acoustic shear waves, the QCM-D data can provide information about the following specific events occurring upon the injection of cells: 1) the rearrangements/modification of the viscoelastic properties of the LbL films induced by the MKN45 cells; 2) formation of contact points between the cells and the multilayer surface; 3) secretion of proteins by the MKN45 cells. $\Delta D/\Delta f$ plots show the direct relation of the deposited mass and its mechanical properties [5]. Analysis of the $\Delta D/\Delta f$ plots (**Figure 2B**) showed that MKN45 cells seeded on (PLL-HA_{6.4})₅ induce an abrupt increase in ΔD with no significant changes in Δf , while MKN45 cells in contact with the multilayers comprising the HAs of higher M_w s, *i.e.* (PLL-HA₇₅₂)₅ and (PLL-HA₁₅₀₀)₅, induce an exponential increase of the Δf and ΔD . The negligible response observed for cells seeded on bare gold is related with the lack of a viscoelastic layer underlying the adherent MKN45 (ΔD) and secreted ECM (Δf) for the studied timeframe and the poor MKN45 cells adhesion onto the gold sensors. The results obtained for the cells in contact with LbL with short HA (*i.e.* 6.4kDa) can be associated with the formation of FAs and further reorganization of the substrate layers [5]. MKN45 cells on (PLL-HA₇₅₂)₅ and (PLL-HA₁₅₀₀)₅ behave differently: during the injection of the cell suspension and adhesion stage (I and II in **Figure S1**) the deposited mass and the energy dissipation increase linearly. These changes are an indication of the secretion of proteins by the cells (mass increase) and/or gradual remodelling of the underlying LbL system [28, 51].

It is known that the viscoelastic and mechanical properties of the underlying layers is able to modulate the adhesion and behavior of cells [21]. Based on this knowledge and considering the distinct stiffness of the LbL films (*i.e.* stiffer films are generated with HA_{6.4}) [2], we tested the generated LbL systems with a different gastric cancer cell line, *i.e.* AGS, that is known to overexpress CD44 but in the cytoplasm (with an expectable lower response to exogenous HA), in contrast to the MKN45 that expresses this receptor at the membrane [4]. In this case, the results showed a different trend on the $\Delta D/\Delta f$ plots: the LbL films generated with HA_{6.4} and HA₇₅₂ showed a gradual increase on ΔD and Δf , with the occurrence of a plateau over time (**Figure S2**), a profile characteristic of the deposition of proteins and surface remodelling. In contrast, the LbL film generated with HA₁₅₀₀ showed a response similar to the one observed for the MKN45 cells, which we associate to the unspecific cells-HA₁₅₀₀

interaction or due to the higher thickness of LbL film (up to 80nm) reducing the sensitivity on the events that occur at the surface of the LbL films. In addition, the observed response on bare gold is attributed to the higher adhesion of AGS cells and the secretion of ECM proteins. These results show that the MKN45 response to the different LbL films is highly driven by the biochemical characteristics of the HA, *i.e.* its M_w , and not only due to mechano-transduction processes.

3.2. Morphology of MKN45 cells seeded on (PLL-HA)₅ under dynamic and static conditions

We evaluated the impact of the HA M_w on the morphology of MKN45 cells adhered on the (PLL-HA)₅ samples. Cells were seeded either in the QCM-D chamber (dynamic cell seeding: 1h of constant flow rate, followed by 1h30min under static conditions) or under standard static conditions during 3 and 6h. These culture conditions were chosen based on our previous studies demonstrating an amplification of cellular response when dynamic culture conditions are used [5]. Moreover, the constant flow during the QCM-D cells' seeding, resembles the physiological dynamic conditions where the invasive cells forms an intermediate metastatic state known as circulating tumour cells (CTC) [34]. In addition, under standard culture conditions the cells are not able to adhere to the surfaces in less than 3h, limiting the direct correlation between the culture time in dynamic and static conditions.

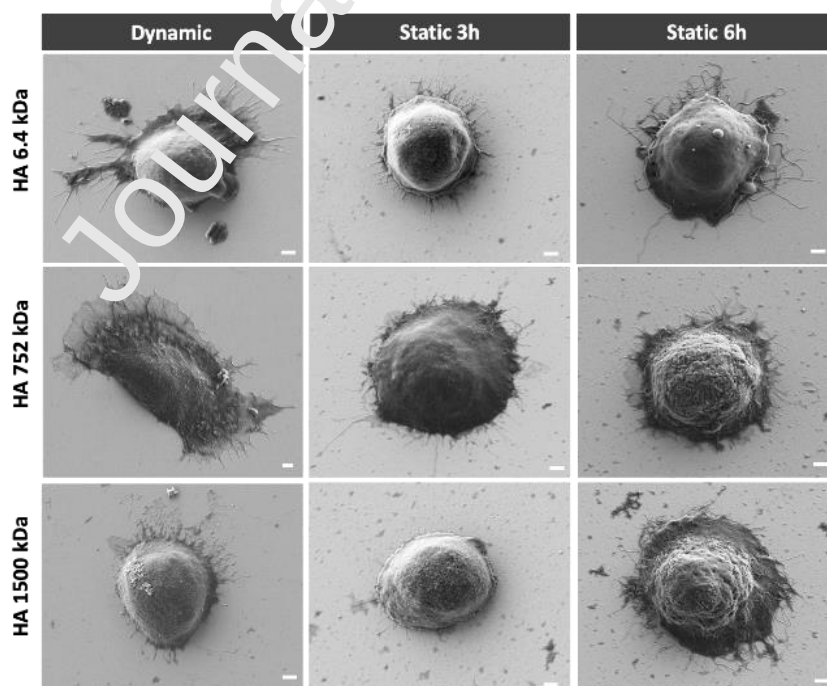


Figure 3. Representative SEM images of MKN45 cells cultured under dynamic (50 $\mu\text{L}/\text{min}$ flow for 2h30 in QCM-D chambers) and static (3 and 6h) conditions on $(\text{PLL-HA})_5$ samples. Scale bar is equal to 1 μm . SEM images of the cells seeded on bare gold are presented in the supplementary information (**Figure S3**).

We observed different cellular morphology in response to the used conditions and LbLs (**Figure 3**): most cells have a round shape but the type of protrusive structures at the leading edge of the cells, their number and size were different. Lamellipodia are sheet-like projections on the leading edge of the cell. They are highly dynamic and promote cell migration through formation of protrusions driven by actin polymerisation. Filopodia are thin, long and actin rich cytoplasmic protrusions, which are used by the cells to probe their microenvironment [11, 31]. We observed formation of both filopodia and lamellipodia for all cells seeded under dynamic conditions independently of the M_w of HA. Two types of filopodia are visible: ones growing from the cell body and others growing from lamellipodia. The number of the filopodia and their length are similar for cells cultured on $(\text{PLL-HA})_5$ samples comprising HA of the same M_w under dynamic and 6h of static cell culture (**Figure S4**). The M_w of the HA present in the LbL films significantly influenced the cellular response at any of the tested culture conditions: HA of 6.4kDa stimulate formation of filopodia, especially the ones growing from lamellipodia (**Figure 4**).

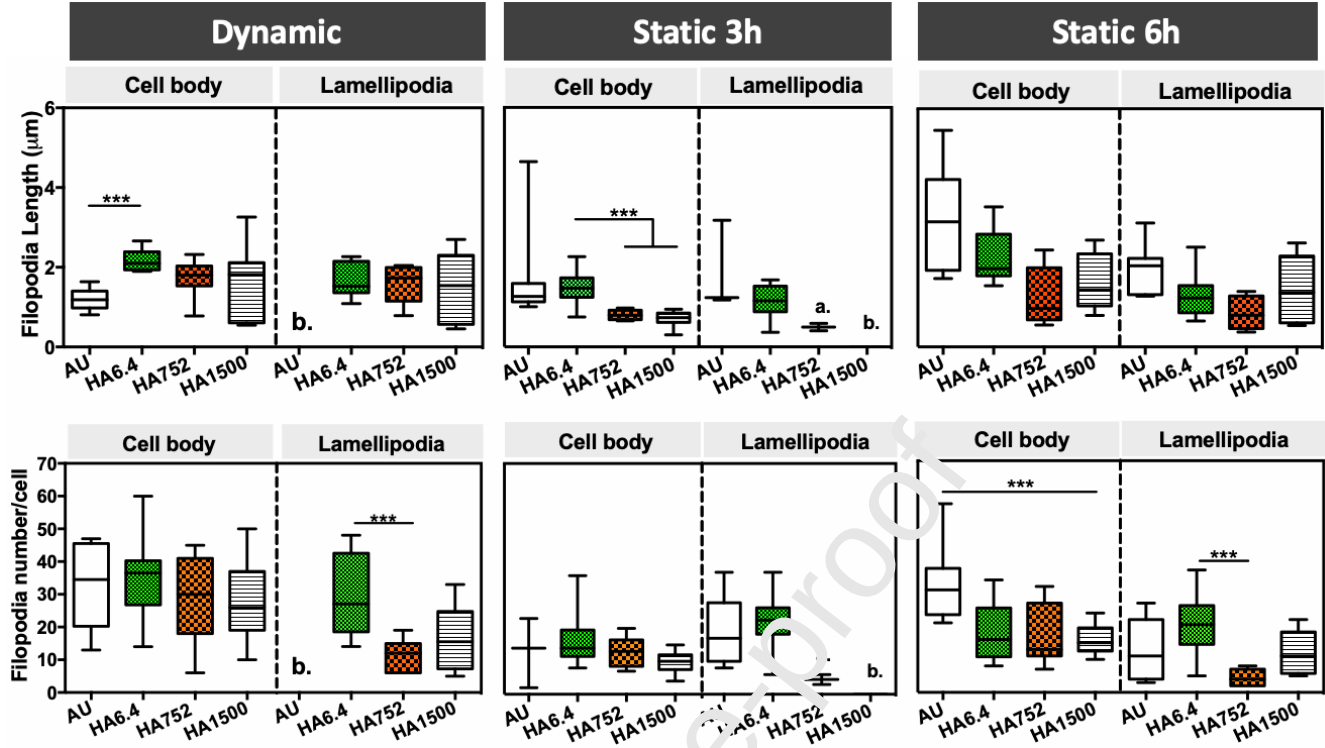


Figure 4. Quantitative analysis of (A) filopodia length and (B) filopodia number of MKN45 cultured at dynamic and static conditions for 3 and for 6h. Significant differences are marked for $p < 0.001$ (***). Samples with less than three measurements of filopodia lengths or number ($n < 3$) are marked with (a.) and without lamellipodia ($n = 0$) marked with (b.).

As mentioned above, both filopodia and lamellipodia are cellular features associated with cell migration. However, in contrast to lamellipodia, filopodia cannot drive cellular migration by itself [23]. Therefore, our results (Figures 3 and 4) suggest that cells seeded on (PLL-HA_{6.4})₅ have higher motility as compared with the cells cultured in contact with HA of 752 and 1500kDa.

3.3. Cell motility depends on the HA size

Recently, we reported the influence of HA M_w on the motility of MKN45 cells cultured on one bilayer (PLL-HA_n)₁ system: we observed shorter cellular tracks for samples with HA of 6.4 kDa. Cellular motility increased on samples comprising HA of higher M_w due to the binding and clustering of CD44 receptors leading to the formation of invadopodia [4]. Herein, we used thicker LbL systems, composed by 5 bilayers of (PLL-HA_n)₅, where the HA is presented in a more complex way (higher entanglement),

being distributed in the LbL network due to known ability of HA to diffuse throughout the layers during the build-up of the LbL films [2]. This way, it is possible to mimic the entanglement of HA in the ECM and the confined, but still available, binding sites to interact with the cells. In addition, the viscoelastic (*e.g.* hydration) and topographical properties of the ten layers' LbL films, resembles better the physiological microenvironment, where cells can sense the different stiffness and depth of the surface, throughout the formation of protrusions, and transduce the sensing of these environment into different biological responses [14]. Surprisingly, the opposite trend was observed for the motility of MKN45 cells seeded on these thicker systems, *i.e.* MKN45 cells in contact with (PLL-HA_{6.4})₅ have longer displacement tracks, while cells cultured on (PLL-HA₇₅₂)₅ and (PLL-HA₁₅₀₀)₅ have shorter displacements (**Figure 5**). Moreover, pronounced morphological changes are visible for the motile cells on (PLL-HA_{6.4})₅ samples (**Figure 5** – yellow/green arrows).

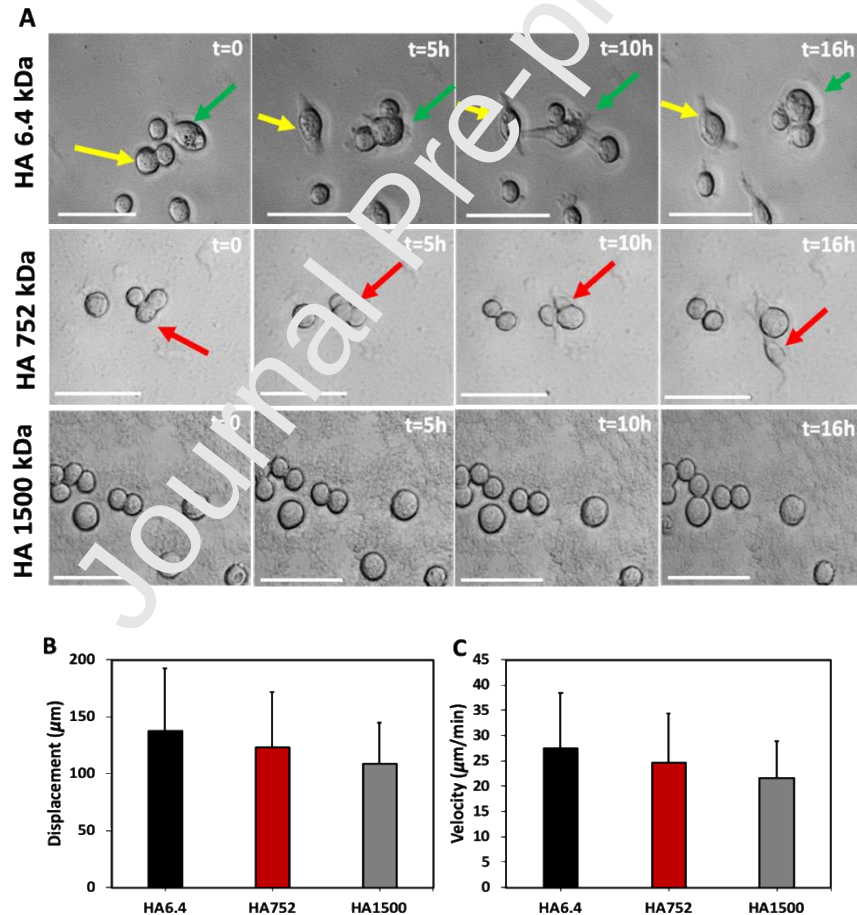


Figure 5. (A) Time-lapse images of MKN45 cells seeded on the different (PLL-HA_n)₅ LbL films after 6h of cell culture. The images were captured every 5min, up to 16h (arrows with the same colour point to the same cell).

Scale bars correspond to 50 μ m. **(B)** Displacement and **(C)** velocity of the cells (n=10 of 2 replicas) calculated from the time-lapse imaging (statistically non-significant).

Cellular adhesion and migration on the (PLL-HA)₅ LbL films can be regulated by biochemical signals, as well as by mechano-transduction - a phenomenon associated with the mechano-sensing ability of the CD44 receptors [25, 42]. The increasing of the layers' number results in the formation of softer constructs especially in the case of LbLs comprising long HA chains, *i.e.* (PLL-HA₇₅₂)₅ and (PLL-HA₁₅₀₀)₅ [46]. These results indicate that the observed response of MKN45 cells seeded on (PLL-HA_{6.4})₅ samples (higher cellular adhesion, **Figure S5**, migration and cytoskeleton rearrangement) is driven by both mechano- and biochemical cues, mediated by CD44 cell surface receptors [8, 30].

3.4. Influence of HA M_w on the activation of focal adhesion sites

To better understand the impact of the HA's M_w on cell adhesion and migration it was analysed the expression of paxillin at the FAs and in the stress fibres. Paxillin is a molecular adaptor – it contains binding sites for different proteins and thus is crucial in the activation of various pathways (by facilitating the binding of different proteins), their signalling specificity and their crosstalk with other pathways. Upon cellular adhesion, paxillin is recruited to the FAs where is involved in the activation of intracellular signalling cascades that lead to the reorganization of FAs and actin fibres. The tyrosine phosphorylation of paxillin is important in regulating FAs dynamics, through their assembly and disassembly, leading to cell adhesion and migration [6, 24, 29]. Herein, it is evaluated the expression of paxillin by MKN45 cells in contact with the (PLL-HA)₅ LbL films (**Figure 6**). Our results demonstrated a correlation between the paxillin expression and the M_w of HA: cells attached to the LbL film presenting HA of 6.4 kDa express more paxillin at all studied conditions and this expression is reduced with the increment of the HA's M_w s (**Figure 6**). Of note, paxillin was mainly located in the cytoplasm for cells cultured under static conditions and on the cellular membrane when dynamic cell culture was used instead. Formation of FAs is observed for MKN45 cells cultured on (PLL-HA_{6.4})₅ either under dynamic, on the membrane, or static (6h) conditions, on peripheral filopodia (**Figure 6**, white arrows).

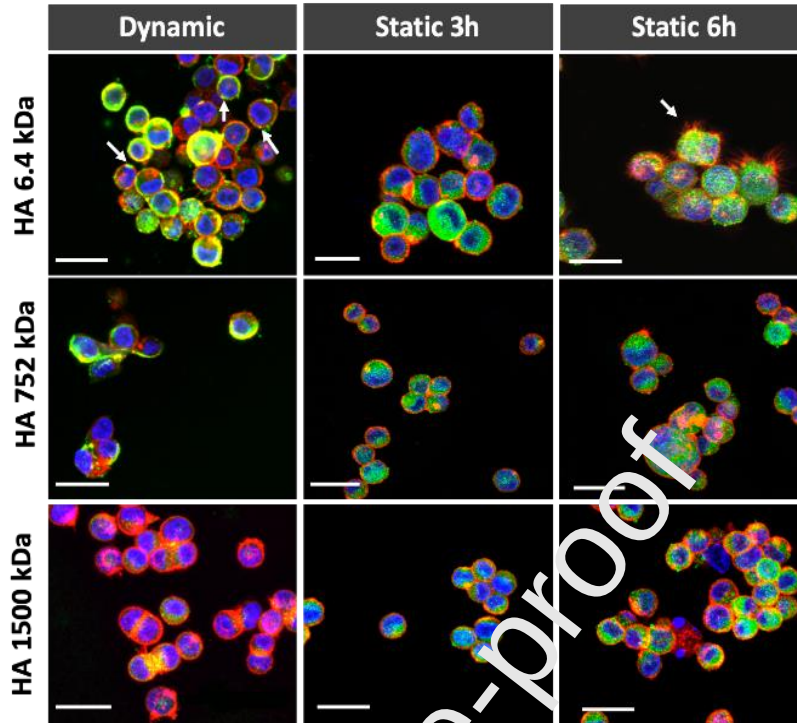


Figure 6. Paxillin expression (green) of MKN45 cells cultured on (PLL-HA)_n samples (n = 6.4, 752 and 1500kDa) under dynamic (QCM-D) and static conditions (3 and 6h). Scale bars correspond to 25µm. Actin is stained in red and nuclei in blue. These results are in good agreement with the cell motility data (**Figure 5**): cells seeded on (PLL-HA_{6.4})₅ samples are more motile and express more paxillin. White arrows indicate the formation of FAs. Paxillin staining of cells seeded on bare gold are presented on **Figure S6**.

3.5. HA mediated activation of mitogen-activated protein kinases (MAPKs)

The observed correlation between the size of HA and cellular motility, FAs formation and paxillin expression suggests that cellular mechanisms responsible for cancer invasiveness and aggressiveness can be also altered as a function of HA size. Therefore, it is analysed the phosphorylation of three major families of mitogen-activated protein kinases (MAPKs): the ERK1/2 and JNK1-3 kinases, the p38 isoforms $\alpha/\beta/\delta/\gamma$, and additional key signalling molecules, such as Akt, GSK-3, p70 S6 kinase, TOR, p53 and CREB. The MAPK signalling pathways can be activated by different cellular receptors, proteins, growth factors or/and hormones. The activated pathways are fundamental in regulating processes such proliferation, differentiation, migration, stress response and apoptosis.

We analysed the protein lysate from the MKN45 cells seeded under dynamic conditions because this culture is closer to the *in vivo* environment where both biochemical signalling and mechano-

transduction determine cellular response. The Phospho-MAPK array kit was used to analyse the lysates from cells cultured on (PLL-HA_{6.4})₅ and (PLL-HA₁₅₀₀)₅ because of the distinct behaviour that it is observed for cells cultured on these samples (**Figures 7 and S7**).

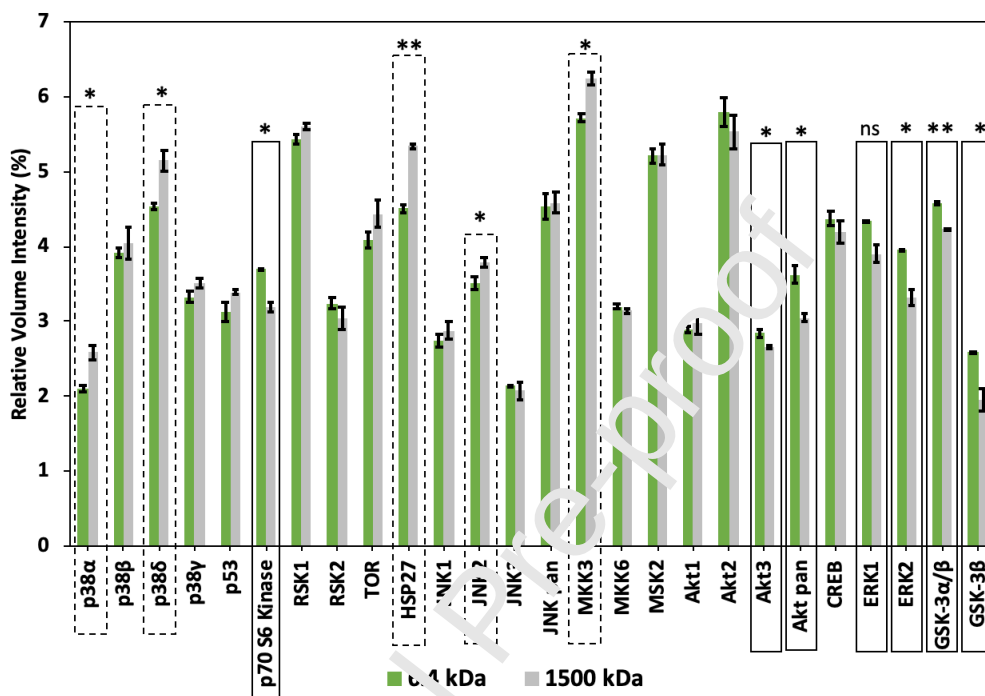


Figure 7. Analysis of the Phospho-MAPK protein array for MKN45 cell seeded under dynamic conditions (QCM-D), on the LbL films presenting HA of 6.4kDa and 1500kDa. Phosphorylated proteins activated in response to HA of 6.4kDa are marked with solid black line and to HA of 1500kDa are marked with dashed black line. Significant differences are marked for $p < 0.05$ (*) and $p < 0.01$ (**). Results showing a tendency but without statistically differences are highlighted and marked with *ns*.

The activation of two distinct mutually inhibitory signalling cascades in response to different sizes of HA, is observed. MKN45 cells attached to (PLL-HA_{6.4})₅ have higher levels of ERK1/2 and GSK-3α/β phosphorylation, while cells seeded on (PLL-HA₁₅₀₀)₅ have upregulated p38α/δ, HSP27 and MKK3 phosphorylation. The relationship between the phosphorylated proteins of p38-MAPK family are known: MKK3 is responsible for the phosphorylation of p38α and p38δ, but not p38β while p38α is responsible for the activation of HSP27 [19, 26]. These results confirmed the hypothesis that HA of different M_w induce the activation of different pathways: short HA activates the ERK1/2 downstream

cascade that regulates cell differentiation and spreading, whereas HA of high M_w is related to p38 phosphorylation considered to be stress activator, regulating cellular apoptosis (**Figure 8A**) [52]. These results are consistent with the ones showing cell adhesion, migration and paxillin expression. The role of low HA M_w on triggering ERK1/2 pathway, through the activation of FAK-Src signalling is enabled by the phosphorylation of paxillin [6, 53]. In addition, the migration and invasion of tumorigenic cells through the phosphorylation of focal adhesion kinases, requires the participation of GSK-3 β in many cases modulated by the Akt pathway [55]. The crosstalk between these two pathways on tumour cell behaviour is described: high p38 MAPK activity in combination with low ERK1/2 pathway action is associated to tumour cell latency [47], which can be correlated to the results obtained for cells seeded on the samples presenting HA of 1500 kDa.

However, this data does not clearly identify the mechanism by which HA induces paxillin phosphorylation. As mentioned before, CD44 is one of the main receptors for HA, and it is known to induce phosphorylation of the complex Src/FAK leading to the formation FA complexes. To evaluate if the HA/CD44 interaction participates on the recruitment of paxillin to the FA sites, the CD44 receptors were blocked and paxillin expression evaluated under dynamic conditions (**Figure 8B**).

Blocking CD44 affected tremendously the expression of paxillin by the cells cultured on the (PLL-HA_{6.4})₅ LbL films (**Figure 8B**): in this case MKN45 cells express less paxillin that is mostly localised in the cytoplasm (**Figure 8B** vs **Figure 6**). Paxillin expression does not change significantly with the blocking of CD44 for cells in contact with samples presenting HA of 752kDa and 1500kDa, (**Figure 6** vs **Figure 8**). Of note, the blocking of the CD44 receptors of cells cultured on bare gold does not alter significantly the paxillin expression (**Figure S8**). In this case, *i.e.* when HA is not present on the surface, cell adhesion and spreading is not mediated by CD44 but via different mechanisms, *e.g.* integrin-mediated ones, where proteins such as Src, focal adhesion kinases (FAK) and paxillin are recruited to form the FA complexes [36].

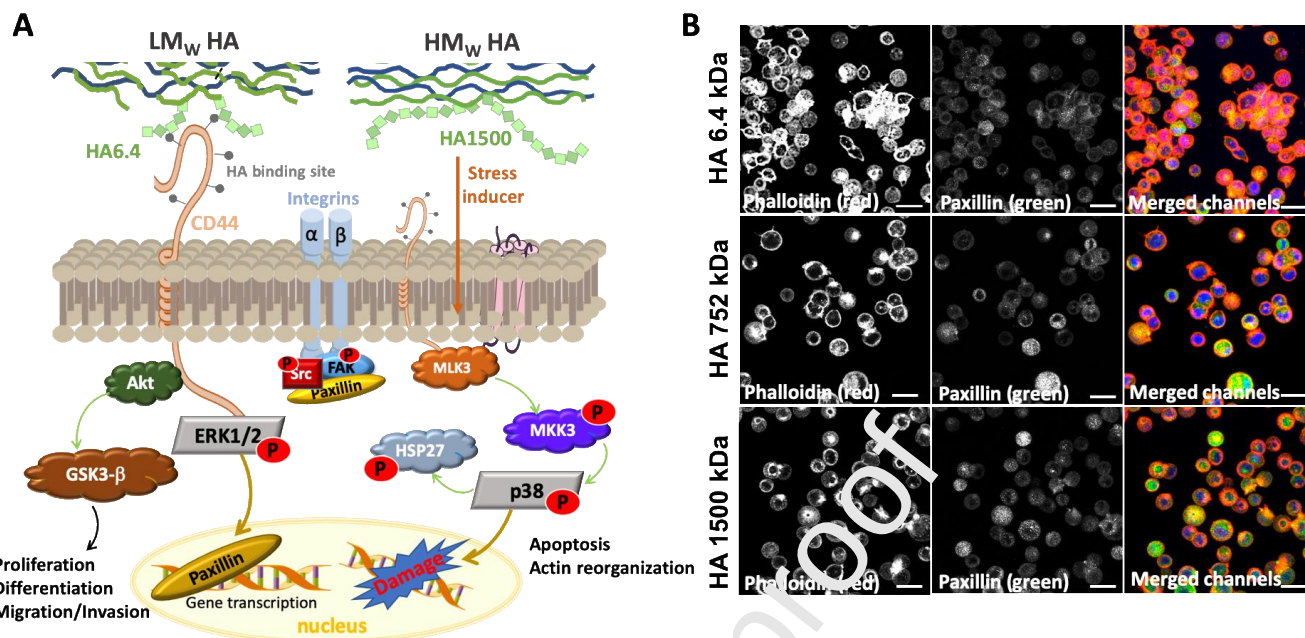


Figure 8. (A) Schematic representation of the proposed mechanism of the signalling cascades activated by HA of different sizes: low M_w (6.4kDa) and high M_w (1500kDa). (B) Effect of CD44 blocking on paxillin expression (actin in red, paxillin in green and nuclei in blue) for MKN45 cells seeded on (PLL-HA)_n samples (n=6.4, 752 and 1500kDa) under dynamic conditions. Scale bars correspond to 25μm.

4. Conclusion

We developed a feasible and versatile platform that can incorporate HA of different M_w s without compromising its recognition by specific cellular receptors, *i.e.* its bioactivity. We demonstrated that the M_w of HA influences dramatically the response of gastric cancer cells: short HA oligomers (6.4 kDa) induce the formation of FAs and cell migration via the activation of the MAPK-ERK signalling cascade, which is responsible for the invasiveness and aggressiveness of gastric cancers. In contrast, surfaces presenting higher M_w HA (*i.e.* 752 kDa and 1500 kDa) inhibits cell migration, thus triggering tumour cell latency through activation of the p38 signalling cascade and inhibition of the ERK1/2 pathway. The obtained results contribute to the understanding of HA involvement in tumour progression and allow the development of therapeutic approaches based on HA that target specific pathways. In particular, our findings encourage the use of HA of short chains as valid targets in the development of therapies for gastric cancers that overexpress CD44.

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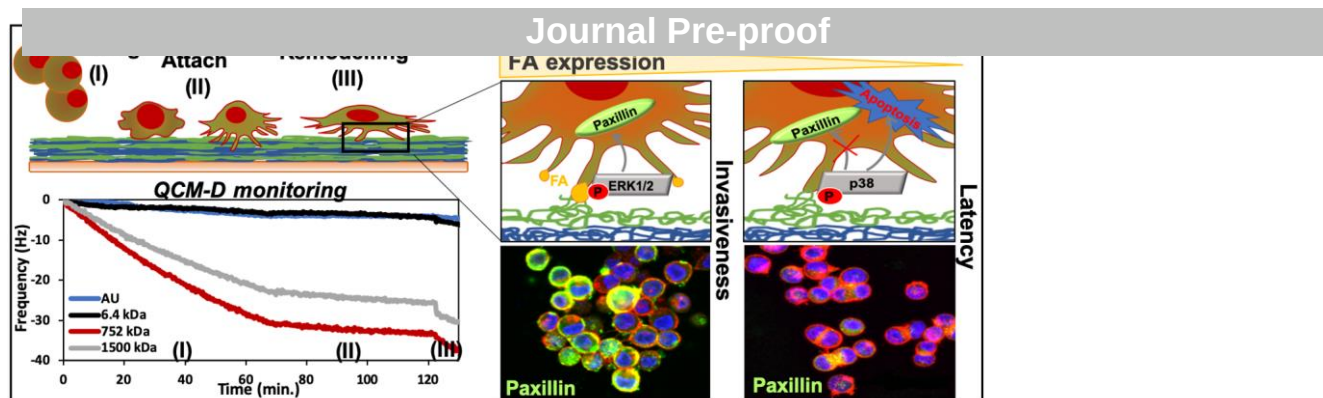
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Journal Pre-proof

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Graphical abstract

- Using QCM-D we were able to follow in real-time the rearrangement of the surface layers of the (PLL-HA)₅ LbL film upon adhesion of cancer cells.
- The HA of 6.4 kDa stimulates the formation of cancer cell protrusions, namely filopodia and lamellipodia.
- The HA M_w s influences the formation of focal adhesion sites, with the activation of paxillin in the presence of HA of 6.4 kDa.
- HA of 6.4 kDa activates the ERK1/2 pathway responsible for cells migration whereas HA of 1500 kDa inhibits cancer cells invasiveness.