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Research Article

Assembly and Study of Mesenchymal Stem Cells on Controlled Chemistry Surfaces

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Abstract

The cell responds to the physical constraints exerted by its environment by a set of mechanisms grouped under the term of mechanotransduction. These processes involve the molecules involved in cell adhesion, the cytoskeleton and the nucleus. These environmental constraints, whether related to the rigidity of the support, to its topography or to the nature of its surface chemistry, will modulate the cellular morphology and impact the behavior of the cell. In order to study this influence of the support, we have seeded bone marrow mesenchymal stem cells from a primary culture of a 24 years old individual on virgin mica surfaces or treated homogeneously with natural molecules (fibronectin and the cyclic RGD peptide) or with polyelectrolyte multilayers (five cycles of Chitosan / PAA or Chitosan / PSS). We then studied the morphology, proliferation and differentiation of these cells after 12 days of culture. As a result, bone marrow mesenchymal stem cells adhere to all surfaces, whether treated or not, and although they are less spread on virgin surfaces, they adopt a fibroblastic type morphology similar to their physiological phenotype. Their percentage of confluence varies significantly depending on the surface treatment used. Indeed the maximum confluence was observed for the surfaces grafted with fibronectin (93.25 ± 2.75%) whereas the surfaces treated with the polyelectrolyte multilayers have much lower confluence percentages (61.00 ± 4.08% for the chitosan / PAA couple) and 54.75 ± 1.75% for the Chitosan / PSS couple), mainly due to cell latency at the beginning of culture. Finally, cells cultured on our surfaces do not respond to any of the three Oil Red O, Alcian Blue or Alizarin Red S stains, suggesting a lack of differentiation in the adipogenic, chondrogenic or osteogenic pathways induced by these surfaces. Thus, the control of the support chemistry alone does not allow control of cell differentiation.

Introduction

The current demand for transplant is then far outpacing the supply. To overcome this organ shortage, alternative treatments were developed, by reconsidering the fact that instead of transplanting a whole organ, autologous implant can be developed and implanted in the patient as a living and functional organ. In other words, donor tissue is harvested and dissociated into individual cells, and the cells are attached and cultured onto a proper substrate that is ultimately implanted at the desired site of the functioning tissue [1]. Thanks to the cell cultures advances, many isolated cell populations can be expanded in vitro which require a very small number of donor cells to prepare such implants. However, it was proved that isolated cells cannot form new tissues by themselves [1,2]. Most primary organ cells are anchorage-dependent and require specific environments that include a specific supporting material for their development. Therefore the success of any cell transplantation therapy relies on the development of suitable substrates for both in vitro and in vivo tissue culture. Currently, these substrates, mainly in the form of tissue engineering scaffolds, prove less than ideal for applications, not only because they lack mechanical

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strength, but they also suffer from a lack of interconnection channels, i.e. the lack of the perfect knowledge and control (concerning the artificial scaffold) of the chemical group organization at the surface prevent the complete use of the current developed scaffold.

The native environment of the stem cells is the extracellular matrix it is a 3D scaffold comprising an insoluble aggregate of several highly organized, multifunctional proteins and glycoamineglycans. The physiological response of the cells to the external stimuli is completely driven by the chemical and physical nature of the environment, they will first integrate it, after interpreting it, they will generate a physiological response to the external environment [2,3]. It is then obvious, that the migration, the proliferation, the differentiation and the apoptosis of the cell depend completely to their environment, and the combined influence of the liquid media and the ECM structure (in term of geometry and chemistry).

Since the end of the eighties many efforts have been developed in the development of surfaces that can interact with living systems (bacteria, cells, stem cells) [4]. Indeed the development of tools for nanotechnology which allowed the creation and the characterization of surfaces from the macro- to nano-metric scale enabled the development of new investigation to better understand life processes at the nano-scale [5]. It is then not surprising that a new interdisciplinary research area was developed, the so-called Nanobiotechnology. The combination of studies, from numerical to experimental, enlightened that living systems, display fascinating behavior, as they can integrate, respond, communicate to each other in response to their environment. And even a change at the nanometric level can induce a change in cells shape, development and also the communication between each other [4,6].

Stem cells are defined as undifferentiated biological cells that under certain conditions can differentiate into characteristic cells and also can divide in order to produce more stem cells. The cells withdraw can be realized from different sources, as adipose tissue from lipid cells through liposuction, from blood or bone marrow typically from femur or iliac crest, the latter being the most characterized and therefor the most common in literature [7]. One of the most important roles of stem cells is that they are reservoirs of reparative cells ability, as they can differentiate and act in case of a wound signal or disease condition. However, there are some limitations in order to study this precise role in reparative tissue, mostly due to the lack of useful cell-specific markers. Nevertheless, stem cells have the advantage of the easiness in withdraw, isolation and further expansion in cell cultures over many generations keeping at the same time their capacity to differentiate [8].

In one hand, Mesenchymal stem cells depending on their shape (round or flattened) vary more in adipocytes or osteoblast when there are exposed to a medium containing differentiation factors of those two types of cells. In a second hand, a chemical controlled support represents a support on which the cells can be deposited and interact. The support, or substrate can be functionalized by molecules can be natural or synthetic to enhance its interaction with the stem cells. As the cells will bind to the substrates via their own specific sites, it is then an interesting point of view to control surface chemistry for a better comprehension of the connection role between the plasma membrane and the support in cellular performance. Indeed, the cellular behavior is influenced by the substrate depending on the binding ability of the adhesion molecules. In the other hand, the influence of the support geometry was quickly studied, thanks to the development of new method to create nano-structure at the surfaces as shown by Hsu et al. [9] and by Cheng et al. [10]. The shape of the surface, in term of nano-structures was shown to plan an important role on stem further evolution, as it influences together the cellular shape. Zhang et al. [11] showed that cells cultivated on large islands spread and present specific markers of the osteoblastic line, whereas those who are cultivated on small islands that have a preferential orientation in adypocitairy way. Moreover, when cells are maintained in a round shape but the cytoskeleton was submitted to low tension, their multipotenty can be conserved in longer time. The cellular shape, as presented above, modifies the cytoskeleton organization which drives the one or the other way of differentiation. Those two combined parameters orient the cellular behavior and can predetermine the cellular path.

Few other groups develop polymers substrates. Lee et al. could demonstrate that issues of human mesenchymal cells from the bone marrow cultivated on fibronectin have bigger tendency to orient towards the adipogenic route compared to a classic culture. In the same way, the cells cultivated on collagen have oriented more easily towards the neurogenic route, and they demonstrate that cells adopted a rounded morphology most of the time have a tendency for adipogenesis markers, while cells that have a spread and extended morphology show higher neurogenesis markers [12]. Thus, the nature of ligand has an impact on the cellular behavior. Yamato et al. [13] realized cell cultures on a poly (N-isopropylacrylamide) (PNIPAM) layer whose properties are similar to those of polystyrene surfaces utilized in traditional cell culture but who acquires hydrophilic properties and hydrates as the temperature decreases (below the so-called LCST for Lower Critical Solution Temperature), leading to cells detachment. In the same way, Aggarwal et al. [14] determined that a surface modification by alternating multilayer pH responsive polyelectrolytes (chitosan and heparin) allow the construction of surfaces on which the cells could grow and develop. Their results presented a difference of thickness and hydration depending on the pH at which the layers were deposited, suggesting that it is possible to influence the cellular compatibility of polyelectrolytes multilayers by varying the physical and chemical parameters in the moment of structure elaboration. Others groups showed that the cells cultivated on a certain support have different behavior depending on the type of support and the ligand density grafted at its surface [15]. A very interesting work was published recently by Kilian et al. [16] where they showed that with the same surface chemistry, different stem cells differentiations could be obtained and showed that controlling the shape of a substrate can drive the stem cell differentiation.

According to the literature, it was noticed that the physical and chemical properties of surfaces are important for the stem cells growth and based on those results, the question that appear to us, if we fix geometry, what would be the influence of the chemistry on those substrates? In this work will then develop a surface functionalization process in order to allow grafting of proteins as fibronectin (FN) and/or peptides as cyclic RGD (cycRGD) by using self-assembly of silane monolayer. Afterwards, the grafting of FN and cycRGD will be conducted on homogeneous surfaces and followed by assembly of stem cells The main objective of the study is to develop surfaces capable of promoting and understanding the influence of chemistry and geometry of the surface in other words the impact of chemical controlled surfaces and chemical and geometrical controlled surfaces on adhesion, proliferation and differentiation of stem cells. Cell morphology has a direct influence on the control of differentiation, so it is possible to condition the cell fate by modulating cell morphology, either by topographical constraints (geometric support) or the nature of the surface ligands (chemistry support).

As in a part of this work, we will prepare surfaces chemically controlled by Gas phase silanization. Silane self-assembled monolayers (SAMs) can be used to create anchoring groups at the surface of the both substrates. This method can be used either on homogeneous and

on patterned substrates, as shown by Pallandre et al. [17] SAMs are molecular assemblies formed spontaneously on surfaces by adsorption and are organized into more or less large ordered domains [18,19]. In some cases molecules that form the monolayer do not interact strongly with the substrate. This is the case for instance of the two-dimensional supramolecular networks [20]. In other cases the molecules possess a head group that has a strong affinity to the substrate and anchors the molecule to it [17]. Self-assembled monolayers are ordered molecular aggregations that are formed adventitious by the absorption of a surfactant with a specific affinity of its head group to a substrate [21]. This underlying process makes SAMs intrinsically manufacturable, hence technologically attractive for surface engineering [22]. The most popular combinations of self-assembling molecules and substrates are represented by alkylsilanes on silicon, as White sides [23] mentions in his report and mainly thiols on gold [22]. One important advantage of the silane is that the latter terminated monolayers show a higher physical and chemical stability compared to thiol terminated monolayer. SAMs can serve as models for studying membrane properties of cells and organelles and cell attachment on surfaces [23]. SAMs are also useful because each adsorbate molecule can be tailored to attract the tail groups can be modified after the assembly of the SAMs, so they are ideal candidates for the surface modification of our substrates.

Materials and Methods

Materials: All the chemical reagents were purchased from Sigma-Aldrich. All the reagents were used as received.

Characterization techniques

Contact angle measurements were performed on a Digidrop (GBX) device using calibrated water droplets. Three measurements are made in triplicate for each surface and the average of the three values was considered.

The following techniques X-Ray Refletometry (XRR), X-Ray Photoelectron Spectroscopy (XPS), Atomic Force Microscopy (AFM) were combined and were made in order to certify the obtaining of a silane monolayer and a hydrolysis without degrafting the silane.

Synthetic Methods

Synthesis of natural homogeneous surfaces

Gas phase silanization: The assembly of the reactor used in our experiment is composed of pyrex glass and its capacity is about 0.7 dm^3. Its supplier is the company Techlab (France). Before starting the experiment, the reactor was cleaned with a piranha solution (50-50 in volume scale of hydrogen peroxide and sulfuric acid) during 20 minutes. Afterwards it was rinsed with de-ionized water Mili-Q five or six times. The last rinse was with ethanol. Following the reactor was connected with a pump in order to eliminate any possible gas and water. It was kept under pumping and heating at 100ºC for 24 hours.

Sample preparation: The prepared pieces, wafers of silicium (1 cm^2), were cleaned as well as the reactor with piranha solution as described above also during 20 minutes. The only difference is that these were rinsed only with water, without ethanol. They were very carefully dried at high temperature and next they were placed inside the reactor for a complete drying for more than 24 hours, meanwhile the reactor was reaching the 100ºC temperature. The used silane is 10 – (carboxymethoxy) decyldimethylchlorosilane. It was injected 0.2 ml of it following 24 hours of vaporization. After the silaniazation the samples were submitted to soxhlet extraction (acetone bath) to detach the overflow of silane adsorbed on the surfaces. The reactor was provisionally cleaned three times with chloroform, rinsed with ethanol and dried at room temperature.

Hydrolysis of ester: For the ester hydrolysis it was prepared 200 ml of hydrochloric acid solution 0.1N in a beaker previously cleaned with piranha solution. The hydrolysis was realized for only two of the samples at different temperatures during one hour; one sample was submitted at 50°C and the other one was left at room temperature for 2 hours.

Grafting of cyclic RGD and Fibronectine: Silicium and mica surfaces (1 cm^2) were used. They were previously cleaned by piranha bath for 30 minutes in order to eliminate contaminants.

In order to graft the interest natural molecules (namely the fibronectine (FN) and the cyclic RGD peptide (cycRGD) the surfaces were modified to add anchor groups. For this a reason the esters groups were hydrolyzed to creat COOH groups. The later will allow bringing into reaction the surfaces with FN or cycRGD with help from EDC/NHS (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide/3-(Maleimido) propionic acid N-hydroxy succinimide ester) couple. In order to do that it was utilized a 2 steps procedure:

a) First step consist in grafting the 10 – (carboxymethoxy) decyldimethylchlorosilane, by gas phase silanization. More precisely, the surfaces were cleaned by piranha and then they were brought in contact with silane vapors in a vacuum chamber under argon atmosphere. The exposure was made during 24 hours. Next, a soxhlet cleaning with pure acetone was made also during 24 hours with the final purpose to eliminate silane residues and other contaminants.

b) Second step consists in grafting the fibronectin and the cyclic RGD peptide. The surfaces were first activated, an aqueous solution of NHS (0.05 M) and EDC (0.2 M) for 3 hours, followed by rinsing in Milli-Q water. Further, the surfaces were brought in contact during 3 hours at ambient temperature with a solution of purified fibronectin in PBS or purified cycRGD in PBS, rinsed in purified PBS and then dried.

Synthesis of artificial homogeneous surfaces

All surfaces and glassware used are already thoroughly cleaned in a pirranha bath to remove contaminants and all washes are done in mili-Q water. Two pairs of polyelectrolytes (chitosan / poly (sodium 4-styrenesulfonate) and chitosan / polyacrylic acid) were deposited on the surfaces of mica and silicon.

5, 10, 15 and 20 cycles were carried out, each consisting of a deposit of chitosan (a cationic polyelectrolyte), followed by a deposition of poly (sodium 4-styrenesulphonate) denoted PSS (a strong anionic polyelectrolyte) or of acid polyacrylic PAA (a weak anionic polyelectrolyte) so that the outer layer is anionic.

Each solution was made at a concentration of 10-2 mol / l in a final volume of 50 ml. The chitosan solution is carried out at 5% by volume of acetic acid. All solutions are equilibrated to a pH of between 6 and 6.5 using solutions of HCl (0.5M) and NaOH (0.5M).

The surfaces were then dried and oven dried for 24 hours at 137 degrees to remove acetic acid residues.

Biological Characterization

Cellular culture: All cell culture manipulations were performed under sterile conditions under a laminar flow hood in a clean room. The cultures were incubated in an oven at 37°C. under a 5% CO_2 atmosphere. Two cell types were used: a $Caco_2$ colonic carcinoma cell line and a

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primary culture of human bone marrow mesenchymal stem cells from a 24-year-old individual. Stem cell maintenance was performed with trypLE® Express solution (Gibco 12604-013). For the maintenance of $Caco_2$, trypsin (25200056 Gibco) and sterile phosphate buffer (PBS, D8537 Sigma) were used. The counts were performed using a Vicell device (Beckman Coulter).

The stem cells were inoculated at the third pass (P3) in 6-well plates (NunclonTM Delta Surface, Cat No. 140675, Thermo Fisher Scientific, Denmark) at a density of 2000 cells per square centimeter on mica surfaces placed on a plate. agarose bed, or directly downhole and in αMEM medium (M4526 Sigma) supplemented with 10% decomplemented fetal calf serum, 4mM glutamine, 1% 'AntibioticAntimycotic' solution (A5955 Sigma: solution 100X stock containing 10000 units of penicillin, 10 mg of streptomycin, and 25 µg of amphotericin per milliliter), 5 µL of FGF2 at 0.1 mg / mL. The medium was replaced every third day by fresh medium.

After 12 days of culture, the cells having reached confluence, the surface-free (control) wells were treated for 17 days with adipogenic specific differentiation medium, or StemPro® osteogenic specific medium containing 1% of "Antibiotic-Antimycotic" solution. The chondrogenic differentiation controls were inoculated in 24-well plates (Falcon ™ Multiwell 24 well, Cat # 353047, Becton Dickinson, USA) at a density of 3.5 x 10 5 cells per square centimeter and immediately treated with chondrogenic differentiation medium. StemPro® for 17 days. The medium was replaced every third day by fresh medium.

The colon carcinoma cell line ($Caco_2$) was also used to test the experiment protocol prior to seeding stem cells. These cells were cultured on glass slides in 6-well plates previously lined with agarose. The culture medium used was DMEM (D5671 Sigma) supplemented with 10% decomplemented fetal calf serum, 4 mM Glutamine and 1% "Antibiotic-Antimycotic" solution. The medium was replaced every third day by fresh medium.

Cellular confluence estimation: After 12 days of culture, the confluence rates were determined manually by the grid method. The culture photographs were standardized and contrasted with the GIMP 2.8 software and a grid of 408 squares was applied. The percentage of confluence for each photograph was calculated by determining the percentage of grid occupancy by the cells. The percentage of confluence for each condition was obtained by averaging the percentages of confluence of 10 photographs.

Differentiation test: After removal of the culture medium, the surfaces and the cell mats were rinsed twice with 1X sterile PBS. Under a fume hood, 400 µL of a 4% formaldehyde solution was deposited at the bottom of the wells and the plates were left for 30 minutes at room temperature. For the wells treated with the adipogenic medium, 60% isopropanol was used instead of formaldehyde (400 µL for 2 minutes). The fixative was then removed and the wells rinsed three times with distilled water. 400 µL of Oil Red O solution (1%), Alcian Blue (1%) prepared in a solution of 0.1 N HCl, or Alizarin Red S (2%) at pH 4.2 were then deposited on the bottom wells to reveal lipid droplets, proteoglycans and calcium, respectively. The dyes were left in the wells for 3 minutes before being removed and the surfaces were rinsed again three times with distilled water. The Alcian Blue was rinsed beforehand with 0.1 N HCl solution. The photographs were made under an inverted microscope using the blue filter.

Results and Discussion

Surface characterization

Contact angles: To rapidly ensure the surface modification success, contact angle measurements were performed. The contact angles for silanized surfaces grafted with fibronectin and the cyclic RGD peptide and also silicon surfaces. All the results are gathered in Table 1.

Table 1: Values of the contact angles of the silanized surfaces.

Sample	Water contact angle 1		Water contact angle 2	
	First experiment	After 3 minutes	First experiment	After 3 minutes
Non-hydrolyzed silane	122.5	125.8	122.5	125.8
	121	122	121	122
hydrolyzed silane (At ambient temperature)	80.8	71.5	80.8	71.5
	82.6	71.2	82.6	70.5
hydrolyzed silane (50°C)	74.7	60.8	74.1	60.8
	72.9	54	72.4	54
Silicone surface	Untreated	Fibronectin	Cyclic RGD	
	23±0.53°	39.62±1.65°	26.82±0.81°	

The decreasing of contact angle with hydrolysis confirm the creation of O=C-OH groups at the surface of the silicon wafer. The contact angle valu obtained, i.e 122° for the ester terminated silane and 60°-70° for the caroboxylic acid terminated silane (the value is completely dependent to the condition of hydrolysis) are in good agreement with Hughes-Chinkhota results [24]. Commonly, hydrophobic surfaces display a higher contact angle with water, up to 90°. In their results, they notify the hydrophobic nature of silane with ester end-groups and the fact that its decrease is related to hydrolysis of the ester groups. This suggests thus that acid hydrolysis reaction was successful for the creation of acid groups at the surface of our substrates. Moreover, by increasing the water drop contact time on the surfaces angle decreases, which confirm an interaction between the chemical groups grafted at the surface of the silicon substrates, indeed the carboxylic acid groups are able to interact with water by acide/base interactions contrary to the ester groups that are inert.

The Si-O-Si bond is sensitive to acidic media, that's the reason why we have selected 2 conditions, at low acid concentration, but at two different temperatures (room temperature and at 50°C), both substrates were put in contact with the acid solution during 2 hours. To check the influence of the hydrolysis condition on the silane monolayer, we also performed water contact angle measurements for its rapid results. As expected we noticed no change in the no treated surface and a visibly increasing of hydrophilicity in the other two cases. In regards to the water contact angle results, the lowest angle was observed for the substrates that were hydrolyzed at 50°C, which confirm that at this conditions, we have created higher amount of OH-C=O groups at the surface of our substrates. Moreover, we have observed 20° difference in only 3 minutes after the deposition of the water drop, which showed that the surface is much more reactive.

The results of the contact angles of the functionalized surfaces by the polyelectolyte multilayer method, using two couples of chitosan / PAA and chitosan / PSS polyelectolytes and varying the number of layers, are shown in Table 2.

Table 2: values of the contact angles of the polyelectrolyte deposition surfaces of different cycles.

Couple Number of cycles	chitosan / PAA	chitosan / PSS
5	55.76	68.2
10	34.96	48.98
15	70.02	55.88
20	63.99	43.78

The contact angles of the surfaces having undergone 5, 10, 15 and 20 cycles of polyelectrolyte deposition for the two pairs are all less than 90°. These surfaces therefore have more apolar profiles than the untreated surfaces (23°), which are greater than those of the silanized surfaces grafted with the natural molecules (fibronectin and cyclic RGD) which reveal a slight decrease in hydrophilicity of the surfaces. So, these treatments have all affected the polar profile of silicon, which suggests that our surfaces remain sufficiently hydrophilic to allow cell adhesion.

XRR: The silane used contains 10 carbons, taking into account that the length of the C-C-C bond is about 2.5 Angstrom, we expected a thickness of about 1.5 nm.

Silanization in the gas phase was conducted at different temperatures, 100° C; 80° C, 70° C. Once measured, all the layers have a thickness of about 3 nm, which means that a bilayer has been grafted onto the surface and not a monolayer. We then decided to reduce the temperature again to 50°C, and the results of XRR confirm a monolayer thickness (a thickness of about 2 nm was measured). So, we finally implemented silanization in the gas phase for the grafting of a monolayer of 10- (carboxymethoxy) decyldimethylchlorosilane (Figure 1).

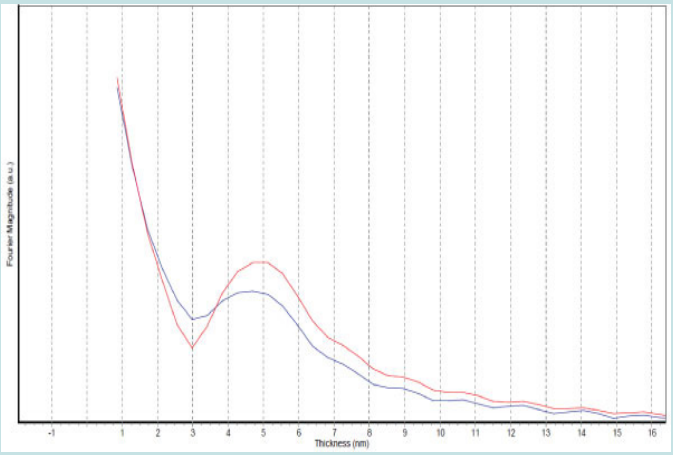

Figure 1: Thickness of the silane monolayers implemented by silanization in the gas phase measured by XRR.

XPS: C 1s core-level spectrum (Figure 2) of 10 – (carboxymethoxy) decyldimethylchlorosilane grafted monolayer exhibits three main components. The main peak centered at 284.8 eV is attributed to aromatic carbon atoms. The two other components appearing around 286.4 and 289 eV are characteristic of aliphatic carbons bound to oxygen [25]. The first one originates from C O species (C O and C OR groups) and the second one from the double O C O species (carboxylic acid and ester groups). The C 1s spectra of 10 – (carboxymethoxy) decyldimethylchlorosilane after the alcaline hydrolysis, exhibits three also three main components but the peak realated to C=O and O-C=O bond is much more prononced as it can be seen in the spectra at the top of the Figure 2 of sample 2.

Once the NHS grafted at the surface, (Figure 3) the C1s spectra remains similar to the one of hydrolyzed silane surface (Figure 3 sample 3). But, by analyzing the nitrogen their respective binding energy area, spectra characteristic nitrogen bound to an aliphatic carbon for NHS derivatized silane surface (Figure 4 sample 3) and were obtained.

The C 1s core-level spectra of Cyc-RGD and fibronectine grafted silane samples exhibit significant changes (Figure 3 sample 4 and sample 5 respectively). One main component is still centered at 284.8 eV, which is the fingerprint of aliphatic carbon atoms the two peaks shifted to

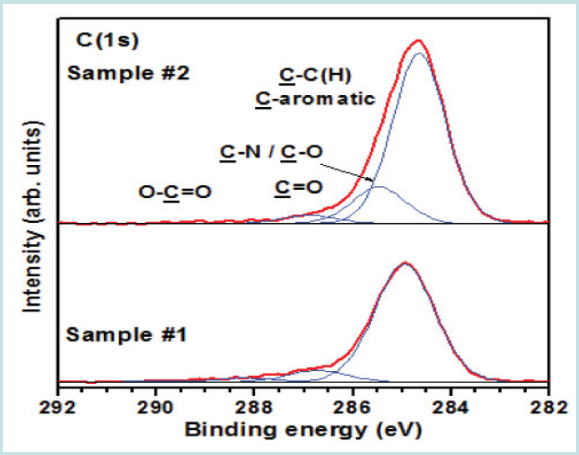

Figure 2: C1s spectra of the ester-silane (sample 1) and carcoxylic acid silane monolayers (Sample 2).

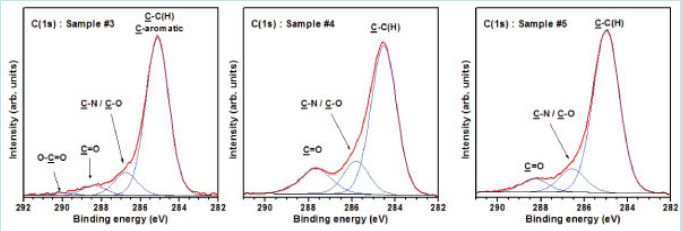

Figure 3: C1s spectra of the NHS-silane (sample 3) and surface after the grafting of cycRGD (Sample 4), surface after the grafting of fibronectine (Sample 5).

appear around 285.9 and 287.8 eV are much more intense. These two last peaks are characteristic of aliphatic carbons bound to nitrogen (C-N) and of aliphatic carbons involved in an amide group (O-C=O), respectively. Moreover, a component centered at 399.8 eV characteristic of nitrogen involved in an amide group (N-C=O) appears in the N1s core level photoemission spectra of those samples (Figure 4, sample 4 and 5). These C 1s and N 1s XP spectra are those expected for cycRGD and Fibronectine. It thus obvious that the process we have developed allow the graftinf of Fibronectine and cycRGD at the surface of oxydized silicon surfaces.

AFM: Atomic force microscopy (AFM) images were recorded in order to obtain onformation on the homogeneity of the modifid surfaces since unegrafted patches on the layer would be extremely detrimental for the further biological application of the surfaces. The topographical images of the silanes modified by with EDC/NHS indicate that the layer is homogeneous (white dots are related to salts that are deposited during the reaction.

Those salt can be esaily removed when the surface is imerged in PBS solutionn as they are more soluble) (Figure 5A). Once the cycRGD is grafted (see Figure 5B), the surface still homogeneous. As the cycRGD is a a molecule, no important modification of the surface was expected. Once the fibronectine was grafted, the surface topography shiw new features, as it can be seen in Figure 5C and D. Moreover a proteine organization coul be clearly seen in the small scan image depicted in Figure 5D.

Biological study

Testing the experimental protocol: Cells from bowel carcinoma ($Caco_2$) were first cultivated on glass surfaces and observed. They normally adhered and proliferated over the degreased glass with ethanol

96% (our surfaces were degreased by piranha mix) and traditional culture supports. On the other hand the adhesion was inhibited by the agarose layer at 1% (Figure 6).

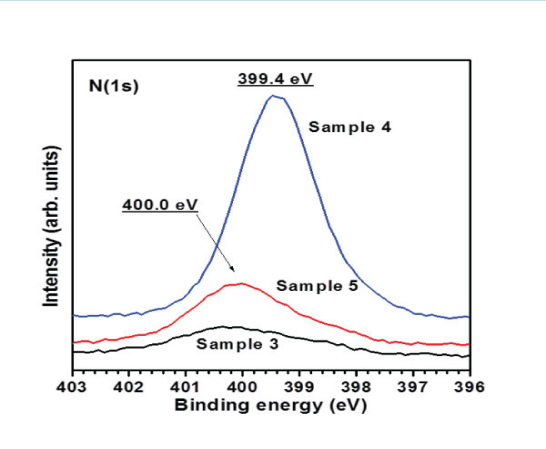

Figure 4: N1s spectra of NHS-silane (sample 3) and surface after the grafting of cycRGD (Sample 4), surface after the grafting of fibronectine (Sample 5).

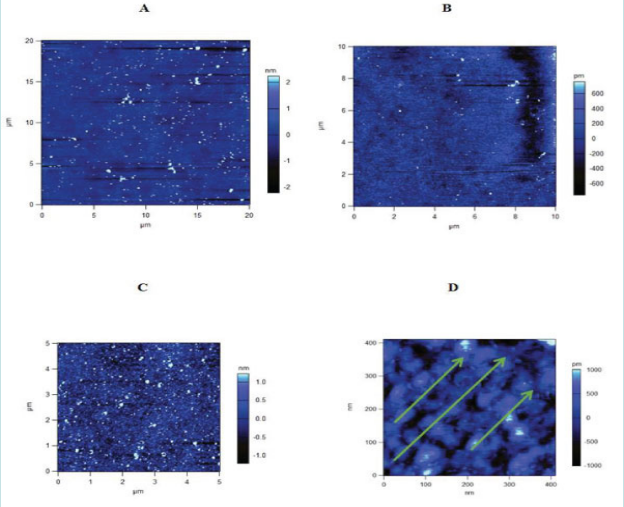

Figure 5: AFM images showing silanized and modified surfaces. (A) Silane + EDC / NHS, (B) Silane + EDC / NHS + cycRGD, Silane + EDC / NHS + fibronectin (large scale), Silane + EDC / NHS + fibronectin (small scale).

Cellular confluence estimation

Cells grown on fibronectin-grafted surfaces gave the best results with an average confluence percentage of 93.25 ± 2.75%. In addition, these cells are the only ones to have a higher confluency rate than controls. Other conditions do not exceed 80% confluence. The cells cultured on the surfaces grafted with the cyclic RGD peptide of fibronectin have a confluence percentage of 57.45 ± 6.45%. The cells grown on the surfaces modified with five layers of the Chitosan / polyacrylic acid pair have a confluence percentage of 61.00 ± 4.08%. Finally, the cells grown on the surfaces modified with five layers of the Chitosan / Poly pair (sodium 4-styrenesulfonate) have a confluence percentage of 54.75 ± 1.75% (Figure 7). It is important to note that the cells grown on the polyelectrolyte multilayers experienced a lag time during the first three days of culture after seeding.

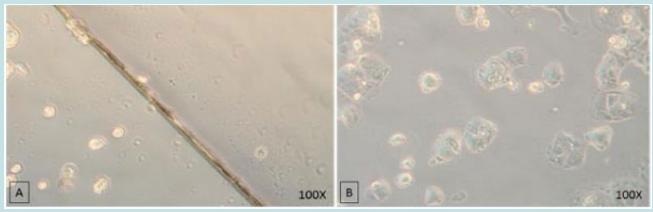

Figure 6: Protocol validation; The agarose inhibits the cell adhesion (A). They normally adhere on the degreased glass surface (B).

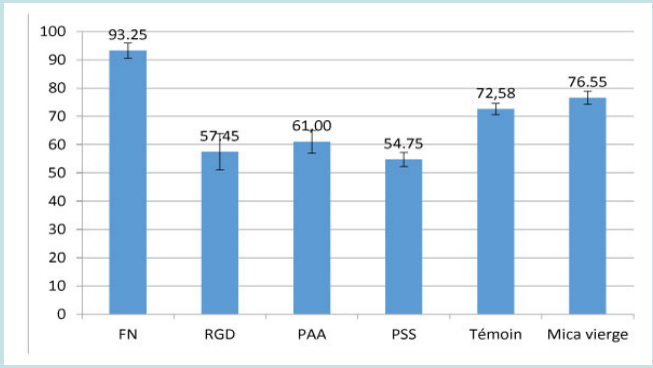

Figure 7: Confluence percentage after 12 days of stem cells.

Morphologic Comparison

The cells grown on the virgin mica surfaces have an elongated fibroblastic morphology, similar to that of the cells of the control condition (Figure 8A and B), with the difference that they appear to have more difficulty spreading over the support and tend to retain a phenotype as a needle.

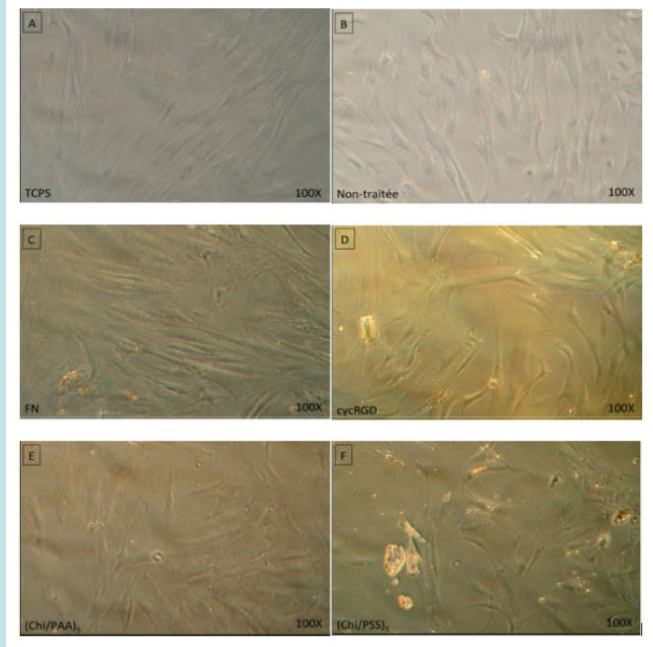

Figure 8: Morphological comparison of cells grown on surfaces with different treatments.

The cells cultured on the modified surfaces, whether they are fibronectin or cyclic RGD peptide-grafted surfaces, or polyelectrolyte-deposited surfaces, have a very clear and spread fibroblastic morphology (Figure 8C to 64F). These cells take up more space than those sown on virgin mica surfaces, their morphology being closer to that of the cells under control conditions.

Differentiation test

The coloration of treated surfaces with Red Oil O, Alcian Blue and Alizarin Red S had no effect on the samples. In fact, unlike the control wells, these surfaces that were grafted with natural molecules or were treated with polyelectrolytes don't show any coloration. The typical morphology of adipocytes, chondrocytes and osteoblasts are not visible which suggest a stem cells differentiation absence on these surfaces and for this kind of treatment (Figure 9).

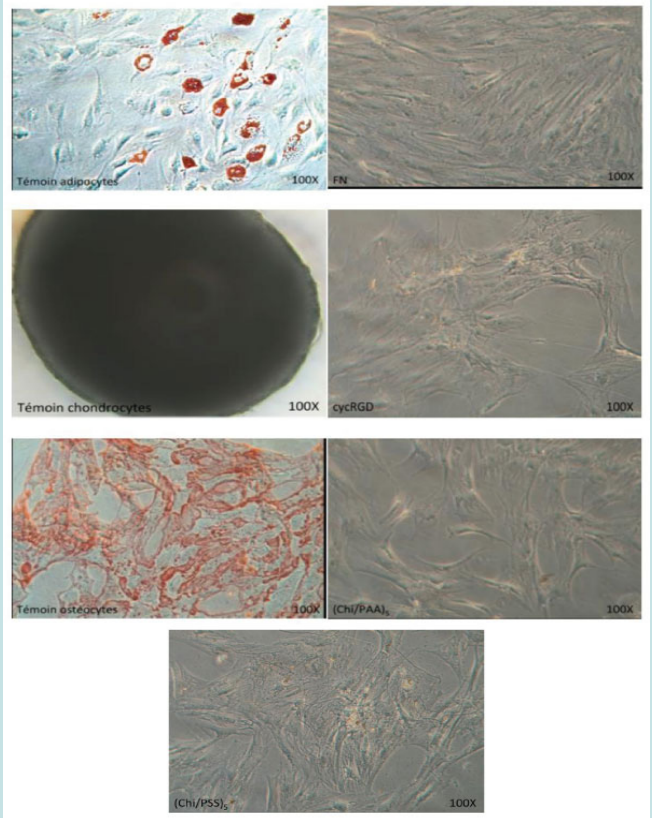

Figure 9: The various differentiation tests via the staining of surfaces treated with Oil Red O, Alcian Blue and Alizarin Red S.

Discussion

In physiological condition, the bone marrow mesenchymal stem cells have a fibroblastic type morphology, that is to say an all-length shape, and spread on their support. This phenotype is found on cells cultured on the TCPS and on treated mica surfaces. The relatively small spread of cells on virgin mica can be explained by conditions less favorable to adhesion. Despite a hydrophilic profile and although allowing strict adhesion and cell proliferation, this untreated surface does not provide a perfectly satisfactory cell environment.

The cells cultured on fibronectin and the cyclic RGD peptide for their part have access to binding sites on which they adhere in physiological conditions, favoring their binding to the support and their spreading in all directions. The mesenchymal stem cells seeded on fibronectin appear to have proliferated better than in all other conditions. In addition, they proliferated faster than on conventional culture media, demonstrating the effectiveness of surface treatment with this protein. The surfaces grafted with the cyclic RGD peptide were not seeded in a very homogeneous manner, which may justify the lowest percentage of confluence obtained by the method of the grids compared to surfaces grafted with fibronectin.

The 3-day lag time of the cells seeded on the surfaces treated with the polyelectrolyte multilayers may be responsible for the difference in confluency at 12 days with the other treatments. During this time, the cells maintained a needle morphology, similar to that of cells grown on virgin mica, and did not split. This latency corresponds to a period of adaptation of the cells to their support. Once adapted, the cells spread out and proliferated normally. The more apolar profile of these surfaces may explain this latency, the cells being able to adhere to the supports but not under optimal growth conditions. These results can be directly related to the deposition protocol, the pH of the various polyelectrolytes having an influence on the final structure of the multilayers as mentioned by Aggarwal et al [14].

The flat surfaces did not induce differentiation of the bone marrow mesenchymal stem cells after 12 days of culture, at which time the cells seeded on the fibronectin reached their maximum confluence. These results, coupled with the morphological study of cells indicate that surface treatments alone are not sufficient to induce differentiation of these cells. This conclusion is contrary to the results of Lee et al [12] who obtained spontaneous differentiation of bone marrow mesenchymal stem cells from 10 to 20% on acrylamide hydrogels grafted with fibronectin. It is important to note the difference in stiffness between these hydrogels and our mica surfaces. On the other hand, if the control cells subjected to the differentiation media are involved in the adipogenic and osteogenic pathways under the sole effect of the differentiating factors, the chondrogenic differentiation has in turn required seeding at a very high density. Only in the presence of a chondrogenic differentiation medium and at this very high density did the cells reorganize into micro masses. This lack of differentiation may mean that the proliferative pathway of these cells is maintained by the surfaces, which would be particularly true for those transplanted with fibronectin. In a complementary manner, this could imply a maintenance of the stem properties of these cells.

The study of treated mica surfaces provided us with essential information on the behavior of mesenchymal bone marrow stem cells on homogeneous supports. If these polyelectrolytes could prove very interesting especially for the easy and controlled detachment of stem cells without enzymatic action, fibronectin seems to be by far the most effective molecule we currently have. The thorough characterization of the cell profile after culture on these surfaces must be seriously considered to confirm or invalidate a maintenance of the stem properties. The next step of this project will be the study of controlled chemistry and geometry surfaces in order to compare their potential impact on stem cell differentiation with the results obtained in this part (on chemically controlled surfaces).

Conclusion and Perspective

As has been shown in the literature review, all primary organ cells are anchorage-dependent, which requires specific environments including a specific carrier material for their development. During this part of the work, we succeed in putting in place a gas-phase silanization of oxidized silicon surfaces as well as for mica surfaces. A monolayer of silane was successfully obtained, which allowed the grafting of biological molecules such as cycRGD and fibronectin. The combination of different characterization techniques allowed us to confirm that the molecules are

grafted homogeneously. Finally, when the surfaces have been subjected to stem cells, we have found that even if these surfaces do not allow the differentiation of the stem cells, they are cytocompatible, i.e, they enable the stem cells growth and high confluence rate was obtained for the fibronectine derived surfaces.

This work paves the way for a more generic project that will combine surface chemistry and surface geometry to determine parameters that may play a major role in stem cell development and / or differentiation.

References

1. RP Lanza, RS Langer, J Vacanti, Éd. Principles of tissue engineering. 4th ed. Amsterdam: Academic Press, an imprint of Elsevier. 2014.
2. CA Parent. A Cell's Sense of Direction. Science. 1999; 284: 765-770.
3. PP Girard, EA Cavalcanti-Adam, R Kemkemer, JP Spatz. Cellular chemomechanics at interfaces: sensing, integration and response. Soft Matter. 2007; 3: 307.
4. CA Custódio, J F Mano. Cell Surface Engineering to Control Cellular Interactions. Chem Nano Mat. 2016; 2; 376-384.
5. AC Lima, JF Mano. Micro-/nano-structured superhydrophobic surfaces in the biomedical field: part I: basic concepts and biomimetic approaches. Nanomed. 2015; 10; 103-119.
6. NM Alves, I Pashkuleva, RL Reis, JF Mano. Controlling Cell Behavior Through the Design of Polymer Surfaces. Small. 2010; 6: 2208-2220.
7. A Uccelli, L Moretta, V Pistoia. Mesenchymal stem cells in health and disease. Nat Rev Immunol. 2008; 8: 726-736.
8. FP Barry, JM Murphy. Mesenchymal stem cells: clinical applications and biological characterization. Int J Biochem Cell Biol. 2004; 36: 568-584.
9. S Hsu, CY Chen, PS Lu, CS Lai, CJ Chen. Oriented Schwann cell growth on microgrooved surfaces. Biotechnol Bioeng. 2005; 92: 579-588.
10. ZA Cheng, OF Zouani, K Glinel, AM Jonas, MC Durrieu. Bioactive Chemical Nanopatterns Impact Human Mesenchymal Stem Cell Fate. Nano Lett. 2013; 13: 3923-3929.
11. D Zhang, KA Kilian. The effect of mesenchymal stem cell shape on the maintenance of multipotency. Biomaterials. 2013; 34: 3962-3969.
12. J Lee, AA Abdeen, D Zhang, KA Kilian. Directing stem cell fate on hydrogel substrates by controlling cell geometry, matrix mechanics and adhesion ligand composition. Biomaterials. 2013; 34: 8140-8148.
13. M Yamato, C Konno, M Utsumi, A Kikuchi, T Okano. Thermally responsive polymer-grafted surfaces facilitate patterned cell seeding and co-culture. Biomaterials. 2002; 23: 561-567.
14. N Aggarwal, N Altgärde, S Svedhem, G Michanetzis, Y Missirlis, T Groth. Tuning Cell Adhesion and Growth on Biomimetic Polyelectrolyte Multilayers by Variation of pH During Layer-by-Layer Assembly: Tuning Cell Adhesion and Growth on Biomimetic Polyelectrolyte. Macromol Biosci. 2013; 13: 1327-1338.
15. RG Flemming, CJ Murphy, GA Abrams, SL Goodman, PF Nealey. Effects of synthetic micro- and nano-structured surfaces on cell behavior. Biomaterials. 1999; 20: 573-588.
16. KA Kilian, B Bugarija, BT Lahn, M Mrksich. Geometric cues for directing the differentiation of mesenchymal stem cells. Proc Natl Acad Sci. 2010; 107: 4872-4877.
17. A Pallandre, K Glinel, AM Jonas, B Nysten. Binary Nanopatterned Surfaces Prepared from Silane Monolayers. Nano Lett. 2004; 4: 365-371.
18. JC Love, LA Estroff, JK Kriebel, RG Nuzzo, GM Whitesides. Self-Assembled Monolayers of Thiolates on Metals as a Form of Nanotechnology. Chem Rev. 2005; 105: 1103-1170.
19. SM Barlow, R Raval. Complex organic molecules at metal surfaces: bonding, organisation and chirality. Surf Sci Rep. 2003; 50: 201-341.
20. JA Elemans, S Lei, S De Feyter. Molecular and Supramolecular Networks on Surfaces: From Two-Dimensional Crystal Engineering to Reactivity. Angew Chem Int Ed. 2009; 48: 7298-7332.
21. F Schreiber. Structure and growth of self-assembling monolayers. Prog Surf Sci. 2000; 65: 151-257.
22. A Ulman. Formation and Structure of Self-Assembled Monolayers. Chem Rev. 1996; 96: 1533-1554.
23. SR Wasserman, YT Tao, GM Whitesides. Structure and reactivity of alkylsiloxane monolayers formed by reaction of alkyltrichlorosilanes on silicon substrates. Langmuir. 1989; 5: 1074-1087.
24. CN Hughes-Chinkhota, Malathi Bandab, Joseph M Smolinskia, Robert A Thomasb, Dayton M Petiboneb, James D Tucker, et al. Oligonucleotide immobilization using 10-(carbomethoxy)decyl-dimethylchlorosilane for mRNA isolation and cDNA synthesis on a microfluidic chip. Sens Actuators B Chem. 2011; 155: 437-445.
25. R Chen, JA Hunt, S Fawcett, RD'sa, R Akhtar, JM Curran. The optimization and production of stable homogeneous amine enriched surfaces with characterized nanotopographical properties for enhanced osteoinduction of mesenchymal stem cells: optimization and production of stable homogeneous amine enriched surfaces. J Biomed Mater Res A. 2018; 106: 1862-187.