

## Bioactivation of 3D Cell-imprinted Polydimethylsiloxane Surface by Bone Proteins Nanocoating for Osteogenic Differentiation

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### Abstract

Physical and chemical parameters that mimic the physiological niche of the human body have an influence on stem cell fate by creating directional signals to cells. Micro/nano cell patterned polydimethylsiloxane (PDMS) substrates, due to their ability to mimic the physiological niche, have been widely used in surface modifications. Integration of other factors such as the biochemical coating on the surface can achieve more similar microenvironmental conditions and promote stem cell differentiation to the target cell line. Herein, we investigated the effect of physical topography, chemical functionalization by acid bone lysate (ABL) nanocoating, and the combined functionalization of bone proteins nanocoated surface and topographically edited surface. We have prepared four distinguishing surfaces: plain PDMS, physically modified PDMS by 3D cell topography pattern, chemically modified PDMS with bone proteins nanocoating, and chemically modified nano 3D cell-imprinted PDMS by bone proteins (ABL). Characterization of ABL was carried out by Bradford staining and SDS page analysis followed by the MTT assay for evaluation of cell viability on ABL coated PDMS. Moreover, FESEM and Profilometry for determination of optimal coating thickness were utilized and the best coating concentration was identified and selected. The binding and retention of ABL to PDMS were confirmed by FTIR and BCA analysis. Sessile drop static water contact angle measurements on substrates have shown that the combined chemical functionalization and nano 3D cell-imprinting on PDMS surface leads to better surface wettability by 68% compared to plain PDMS. Eventually, the results of ALP measurement, Alizarin red S staining, Immunofluorescence staining, and real-time PCR have shown that nano 3D cell-imprinted PDMS surface functionalized by extracted bone proteins, ABL, is able to guide the fate of ADSCs toward osteogenic differentiation. Eventually, chemical modification of the cell-imprinted PDMS substrate by bone proteins extraction, not only improved the cell adhesion and proliferation but also contributed to the topographical effect itself and caused a significantly synergistic influence on the process of osteogenic differentiation.

### **1** Introduction

Cells can translate their morphology and substrate chemistry into a fate decision. One of the most influential microenvironments on the stem cell fates is extracellular matrix (ECM)[1-3]. ECM, due to its unique chemical, mechanical and physical properties, provides the appropriate conditions for the cells vital activities such as adhesion, migration, proliferation, and differentiation. [4]. Additionally, during embryonic development, various factors such as morphological features and mechanical loadings can also influence the cell fate. [5]. Once stem cells leave their niche, they lose their developmental potential immediately and limiting their application in stem cell therapy [6]. Despite widespread scientific attempts to create an effective, dependable, and cost-effective ECM, achieving these features has not been reached. Due to the hierarchal structure of the ECM, the basic geometries that have been used in studies up to now, are not able to mimic the natural structure of ECM sufficiently. In fact, creating an appropriate topography for each cell type requires a lot of trial and error [7].

Paying attention to the factors affecting cell retention after transplantation and balancing the cell fate can help to establish a robust method of cell therapy[8-10]. Moreover, physical features has a great influence on the stem cell proliferation, migration, and differentiation through mimicking the natural niche for each special cell[11-14]. Accordingly, a culture substrate that mimics the surface topography and shape of the natural ECM can be used to stimulate stem cell development [15-19]. Each distinguish pattern might be able to guide the stem cells to a distinct lineage [20]. Using nanopit topographies, for example, have been demonstrated to drive stem cell fates toward osteogenesis[13, 21, 22], while nano ridge/groove patterns encourage neurogenic differentiation of these cells[23-26].

Beside patterns, topographical feature dimensions are another important characteristic of the culture substrate[11, 27]. For instance, topographies with micro scale dimension have been demonstrated to stimulate cytoskeleton formation and differentiation[28, 29]; on the other hand, patterns with nano scale dimension are known to generate interconnected cell-cell interactive networks[30-32]. Several studies have employed the cell-imprinting strategy for the stem cell differentiation. In this method, specific patterns of target cells were used as the cell culture substrate. Their results approved that the topographical features can promote cell activity and lineage specification on their own[33-35]. The cell-imprinting method has been used to differentiate stem cells towards chondrocytes[33], tenocytes[35], keratinocytes[34], and osteocytes[36] which has opened up a new line to the field of physical stem cell differentiation. Moreover, in another study, differentiation of mesenchymal stem cells toward myoblast was confirmed by applying the myoblast-imprinted substrates [37].

Another important property for determining the fate of stem cells is the chemical composition and growth factors in the tissue. By preparing the natural compounds on the surface of stem cell culture, closer conditions can be provided for stem cell differentiation to distinctive cell lines. Bone induction and differentiation are the most important factors in bone tissue engineering which are guided by both background (microenvironment/extracellular matrix) and biomechanics (physical forces). Biomaterials such as collagens, hydroxyapatite, proteoglycans, and cell adhesion glycoproteins, including fibronectins and laminin showed an awesome effect on bone induction and differentiation[38]. On the other hand, demineralized bone matrix (DBM) has been widely used for bone regeneration since Uris's discovery of bone morphogenetic proteins (BMPs) in the 1960s [39]. However, other studies showed that the inorganic mineral[40] and growth factors innate to the bone, specially TGF-β are removed during the demineralization process. Aiming to preserve total bone protein, acid bone lysate (ABL) isolated from rat long bones was used. In the previous study, proteomic analysis of ABL and whole-genome microarray demonstrated the presence of 394 proteins of bone-derived including TGF-B1 that independent of their harvested source and also multiple clusters including ribosomal protein and collagens. In addition, their results showed that ABL activates the TGF-β target genes interleukin 11, proteoglycan 4, and NADPH oxidase 4 [41]. Although the exact role of growth factors in ABL is not clear still, TGF-β1 knows as one of the factors that induce migration of mesenchymal stem cells [42, 43] and targets osteoclasts [44]. As a result, due to preserving a large number of bone growth factors, ABL was assumed as an appropriate selection for simulation of bone niche.

In this study, we used ADSCs to identify the performance of nanocoated 3D cell culture by bone proteins. To investigate the effect of chemical modification and surface topography on ADSCs differentiation to osteoblast, a culture surface was designed by Polydimethylsiloxane (PDMS). PDMS substrate was made by 3D cell-imprinting topography method then coated with a nanometric layer of ABL solution through 3-Aminopropyltriethoxysilane (APTES) and glutaraldehyde (GA) as cross-linker. This system was employed to direct osteogenic differentiation of ADSCs based on chemical and physical approaches. Preparing cell-imprinted substrate needs osteoblast cells which obtained from long bones of neonatal rats[45]. Long bones osteoblast applied at current paper due to provide higher numbers of cells than calvarial and is relatively easy to do, so making it ideal for experimental purposes. Finally, we have prepared different surfaces by physically and chemically surface modification, then we evaluated the synergistic effect of ABL nanocoating on the 3D cell-imprinted topography for the osteogenic differentiation.

## 2 Materials And Methods

# 2.1 Cell isolation, expansion & characterization

## 2.1.1 Osteoblast cells isolation

All the experiments were approved by the ethics committee of the Pasteur Institute of Iran, and all methods were performed in accordance with the relevant guidelines and regulations. The study was carried out in compliance with the ARRIVE guidelines. Osteoblast cells were isolated from long bones (i.e., humerus, radius, metacarpus, femur, tibia, fibula, and metatarsus) of Three-days neonatal rats. Rats sterilize with 70% ethanol and cut off head and body using large scissors. The limbs from the body were cut by sharp scissors then the skin and soft tissue were scraped. Cut off the epiphysis and bone fragments were immersed in sterilized PBS to remove any residual soft tissue. Afterwards, they were incubated in trypsin (0.25% in sterilized PBS, 1 ml per animal) for 15 min at 37°C. The fragments were washed in DMEM (Gibco, Switzerland) and were incubated in collagenase type II (2% in sterilized PBS, 1 ml) for 30 min at 37°C two times after discarding trypsin solution. DMEM was added to the bone fragments solution and was centrifuged (at 1500 g, 5 min) two times. Obtained cell suspension were cultured in proprietary bone culture medium contains DMEM supplemented with ascorbate (50  $\mu$ g/mL), dexamethasone (10 nM) and  $\beta$ -Glycerol phosphate (4 mM) and 10% fetal bovine serum (FBS, Gibco) including penicillin (100 IU/mL)-streptomycin (100  $\mu$ g/mL) (Sigma, United States) [45]. Five days post cell culture, the cells reached 90% confluence at passage zero which is suitable for the cell-imprinted pattern.

## 2.1.2 Characterization of isolated osteoblasts

At first, crystal violet staining was used to study the shape, population, and precision of live osteoblast cells isolation. The isolated cells were characterized using alizarin red S and osteocalcin immunofluorescence staining. For Alizarin red S and crystal violet staining, cells after 4 days were fixed in 4% glutaraldehyde (GA) solution for 20 min at 25°C. Then followed by washing with sterilized water, cells were stained by adding freshly Alizarin Red S (Sigma) solution, pH 4.2 for 10 min and crystal violet for 30

min at 25°C. Finally, cells were washed with phosphate-buffered saline (PBS) solution and were photographed by an optical microscope (BEL, INV2, Italy). For osteocalcin immunofluorescence staining of isolated cells, presented protocol at the immunofluorescence staining part was carried out.

## 2.1.3 Adipose-derived stem cells isolation

Adipose-derived stem cells (ADSCs) were taken from one rat and allogeneic were used. The ADSCs were isolated from bilateral inguinal fat pads of healthy rats (Wistar albino, male, 250). First, adipose tissues were washed three times in phosphate-buffered solution (PBS) with 3% Penicillin/Streptomycin (Sigma), then, they were cut into 1-2 mm slices and were digested in 0.01 mg/ mL Collagenase Type I (Sigma, United States) at 37°C for 2 hours. The solution was passed through a 75 µm filter to remove undigested tissue, followed by neutralization of the enzyme with DMEM (Gibco) containing 10% FBS; Finally, they were centrifuged at 1300 rpm for 5 min in order to separate the cellular pellets. The obtained solution consist of ADSCs was cultured in DMEM/Ham's F12 supplemented with 10% FBS (Gibco) and penicillin/streptomycin (100 IU/ml and 100 µg/ml, Sigma, United States) and was incubated at 37°C in a 5% CO<sub>2</sub> incubator [46]. After 72 hours, non-adherent cells were discarded, and the medium was changed every three days. The ADSCs at the third passage were used for differentiation evaluation.

## 2.1.4 Fabrication of Cell-Imprinted substrates

Polydimethylsiloxane (PDMS, SYLGARD 184, RTV, Dow Corning, USA) was used for the fabrication of cellimprinted substrates. The silicone resin and the curing agent were mixed in a 10:1 (w/w) ratio according to the manufacturer's instruction. Extracted osteoblast cells from neonatal rat bone with a 90% confluency were fixed using a 4% GA solution for one hour and were washed with deionized water. The resin-curing agent mixture was poured on the fixed cells and was retained at 37°C for 48 hours to transfer the cell pattern into the PDMS. The cured silicone rubber was then peeled from the fixed cells, followed by washing in a 1 M NaOH solution to remove the remaining cells/debris and other chemicals from the substrates. The mass of resin-curing and the curing time was the same for all the samples[35].

## 2.1.5 Isolation and characterization of ABL

Adult male rat long bones are used to create ABL following the removal of the soft tissue. Then, the bone ends were removed, and bone marrow was flushed with phosphate-buffered saline (PBS) (Figure 3-A). Clean bone fragments were grinded in a mortar and pestle with the average size<2 mm and were washed with Dulbecco's modified Eagle medium (DMEM) supplemented with antibiotics (Invitrogen Corporation, Carlsbad, CA, USA). Ten grams of grinded bone were incubated with 50 ml of 0.1 N HCL (20% weight/volume) for 16 hours at 25°C while being stirred. Then harvested solution was centrifuged at 1200 RPM for 10 min, pH neutralized, and after the second centrifugation 1200 RPM for 10 min obtained solution which is named ABL, was filtered by a 0.2 µm syringe filter and stored at -20°C. Before each experiment stocks were thawed and used immediately [47]. The protein concentration of ABL was quantitated by Bradford protein assay and verified by means of UV-Vis spectrophotometry (biophotometer 6131, Eppendorf) at 595 nm. A standard sample was prepared with bovine serum albumin (BSA) as a control for this experiment [48]. To prove the existence of a complex of proteins with

different molecular weights in ABL solution, the sodium dodecyl sulphate polyacrylamide (SDS-PAGE) gel electrophoresis with 12% acrylamide gradient gels was applied. The samples were run by reducing method and Coomassie brilliant blue R 250 was used for gel staining. The gel pictures were taken by Gel Doc imaging system (Bio-Rad, Italy).

# 2.2 PDMS surface modification for ABL binding

PDMS substrates, with and without cell-imprinted pattern, were treated by argon plasma for 3 min at the pressure of 0.3 mbar followed by their immersion in 10% 3-Aminopropyl triethoxysilane (APTES) (Sigma-Aldrich, USA) at 50°C for 2 hours. After removing the APTES solution and washing twice with nuclease-free water, the samples were stored in a desiccator over 4% GA (Sigma-Aldrich, USA) solution as a crosslinker overnight. Thereafter, the samples were washed thrice with nuclease-free water, sterilized at ethanol 70%, and were placed under UV light for 45 min. Eventually, the samples were incubated with ABL solution and followed storage at 4°C overnight, ABL solution was removed and were washed with nuclease-free water [49]. Schematic presentation of the surface modification of PDMS is depicted in Figure 1.

# 2.3 Determination of optimal ABL concentration 2.3.1 Field emission scanning electron microscopy

In order to select the proper thickness of ABL on the cell-imprinted PDMS, field emission scanning electron microscopy (FESEM, Zeiss, SUPRA TM 40) was used. Due to the presence of pits on the PDMS surface which results in a high error in thickness determination, a silicon wafer was used as a model sample for thickness determination. The clean silicon wafers were incubated with 100%, 70%, 30%, and 10% of the initial concentration of ABL solution. To capture the FESEM, silicon wafer substrates were cut cross-sectionally and a 5 nm platinum layer was coated on the studied surface by sputtering. Finally, surfaces were observed by FESEM microscopy.

# 2.3.2 Profilometry

The thickness of the ABL layer coated on the silicon wafer as a model sample was measured using a contact surface profilometer equipped with a diamond stylus tip (Taylor-Hobson, Form Talysurf 120L). The silicon wafers were incubated with 100%, 70%, 30%, and 10% of the initial concentration of ABL solution. To determine the thickness of the coating layer, a part of the samples was covered by parafilm tape to create a border between the coated and uncoated areas. Then, the difference between the two parts was measured.

# 2.4 Cell viability of chemically-modified surface by MTT assay

To evaluate the effect of chemical modification of PDMS by ABL coating on cell viability, the MTT (3-[4,5dimethythiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma) assay was performed. ABL at optimum concentration obtained from FESEM and profilometer was coated on the PDMS substrate as described in the chemical surface modification method. Before cell seeding, plain PDMS substrate was immersed in 70% ethanol for 30 min, and dried under a laminar hood followed by UV light irradiation for 45 min. The MG-63 cells with a density of 10<sup>4</sup> cells/well were seeded on the sterilized samples and tissue culture plate as the control and incubated for 3, 5, and 7 days. At all-time points the MTT solution at a concentration of 0.5 mg /ml was added to each well, then cells were stored in the incubator for 4 hours at 37°C. After the formation of formazan crystal, the medium was removed, and the crystals were dissolved in isopropanol. The plate was placed in the orbital shaker for 15 min to enhance the dissolution process. Optical density was measured by Elisa reader (ELX800 Universal Microplate Reader, BIO-TEK Instruments, USA) at 570 nm. Finally, Equation (I) was used to calculation of the cell viability, compared to control. The evaluation was repeated 3 times.

Equation 1

 $\%Viability = \frac{meanODofsample}{meanODofcontrol}x100$ 

# 2.5 Characterization of ABL coated substrate

# 2.5.1 Chemical characterization of the coatings

PDMS modification steps were scanned over a scanning region of 400–4000 cm-1, using the attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectroscopy (Shimadzu IRPrestige-21), and the characteristic peaks of infrared transmission spectra were recorded. Spectra of Plain PDMS, plasma-treated PDMS+APTES+GA, and plasma-treated PDMS+APTES+GA+ABL were recorded to investigate surface changes after each grafting step.

## 2.5.2 Hydrophilicity measurement by contact angle

Water (deionized) droplet contact angle (CA) measurement was performed on the Plain PDMS, physically modified PDMS by cell topography pattern (PDMS-Phys), chemically modified PDMS with APTES+GA+ABL (PDMS-Chem), and chemically modified PDMS consist of cell topography pattern by APTES+GA+ABL (PDMS-Phys-Chem) surfaces to analyze hydrophilicity of the physically and chemically modified/unmodified substrates after 14 and 21 days, using the static sessile drop method (Krüss, Germany).

## 2.5.3 Stability of surface proteins

In order to evaluate the ABL's proteins attachment and stability of proteins retention on the PDMS-Chem and PDMS-Phys-Chem substrates, a micro-BCA protein assay kit (Thermo Scientific, USA) was used. Proteins retention on the Plain PDMS was measured as control substrate to compare with the amount of attached and remained proteins on physically and chemically modified substrates. In such for PDMS-Chem and PDMS-Phys-Chem, ABL (12 µg/mL) was coated on the PDMS substrates as described in the chemical surface modification method and Plain PDMS as a control group incubated with ABL solution at the same concentration and stored at 4°C overnight. The prepared substrates were stored under cell incubation conditions. This assay was evaluated on days 0, 14, and 21 after protein attachment. At the specific time points, the samples (4 cm<sup>2</sup>) were treated with 0.05% Tween 20 (Sigma-Aldrich, USA) for 30 min and they were washed twice with nuclease-free water to remove non-adherent proteins. Finally, the number of proteins retained on the surfaces was measured according to the standard protocol of the kit. The absorbance of samples was measured at 562 nm with Multiskan Spectrum microplate reader (Thermo Scientific, Singapore). For all substrates, the percentage ratio of attached and retained proteins to the initial concentration was calculated at all-time points.

# 2.5.4 Cell seeding on fabricated PDMS

Fabricated PDMS substrates were sterilized before stem cell seeding. The PDMS substrates were immersed in 70% ethanol for 30 min, and dried under a laminar hood followed by UV light irradiation for 40 min. The sterilized substrates were fitted into the 6-well culture plates. ADSCs ( $1 \times 10^4$  cells per cm<sup>2</sup> in 100 µL of culture medium) at passage three were seeded on five different points of 6-well culture plate on the following samples and were eventually incubated at 37°C: Plain PDMS, PDMS-Phys, PDMS-Chem, and PDMS-Phys-Chem. After 5 hours, 1 mL of fresh culture medium DMEM/Ham's F12 (3:1 ratio) and 10% (v/v) FBS were added to cover the whole surface of the substrates. Culture plates were incubated at 37°C with 5% CO<sub>2</sub> for 21 days, and half of the medium was replaced with fresh medium every 2–3 days. Schematic presentation of preparing the PDMS-Phys-Chem substrate and isolation of ADSCs was shown in Figure 2.

# 2.6 Comparison of different substrates on cell viability and osteogenic differentiation

## 2.6.1 Alkaline phosphatase assay

ALP activity was investigated as one of the osteogenic differentiation factors. ADSCs were cultured on the 6-well polystyrene Plate, Plain PDMS, PDMS-Phys, PDMS-Chem and PDMS-Phys-Chem substrates (3 × 10<sup>3</sup> cells per well) in DMEM/Ham's F12 consist of 10% FBS. After 14 and 21 days, the medium was collected and analyzed by auto-analyzer (Hitachi 917, Germany) and alkaline phosphatase kit (Pars Azmoon, Iran) according to its standard protocol based on the DGKC method. The absorbance was read at 405 nm.

## 2.6.2 Crystal violet and alizarin red S staining

The ADSCs (5 × 10<sup>3</sup>) were seeded into the 6-well polystyrene Plate, Plain PDMS, PDMS-Phys, PDMS-Chem, and PDMS-Phys-Chem substrates for 14 and 21 days. After 14 days, the cells were fixed with 4% glutaraldehyde solution for 24 hours. Analysis of viable cells was evaluated by the crystal violet staining method. Fixed cells were stained with 1% crystal violet in 50% methanol for 10 min at 25°C, then washed with distilled water. Viable cells cultured on different substrates were observed under an optical microscope (BEL, INV2, Italy).

The calcium deposited from the cells as a result of osteogenic differentiation was examined using alizarin red S staining on fixed cells. After 14 and 21 days, cells were washed three times with PBS, fixed with 4% paraformaldehyde in PBS (pH 7.4) for 15 min, incubated with alizarin red S (pH 4.2; Sigma-Aldrich) for 30 min at 25°C and washed three times with distilled water then the samples were visualized under an optical microscope (BEL, INV2, Italy). For quantification of extracellular matrix mineralization by alizarin red S staining, cells were incubated with 10% acetic for 30 minutes at 25°C. Afterward, supernatants were collected from all substrates and heated at 85°C for 15 minutes, cooled with ice for 5 minutes, and centrifuged at 20,000 g for 15 minutes. Supernatants were collected for all samples by a microplate reader.

## 2.6.3 Immunofluorescence staining

Immunofluorescent (IF) staining was done for ADSCs (5 × 10<sup>3</sup>cells in 6-well size plate) cultured on a Plate, Plain PDMS, PDMS-Phys, PDMS-Chem, and PDMS-Phys-Chem substrates after 21 days. The cells on the substrates were fixed by immersion in 4% formaldehyde in PBS for 15 min followed by washing with cold PBS 3 times, 5 min each. To permeabilize cell membranes, samples were incubated in 0.25% Triton X-100 in PBS for 10 min followed by washing samples with PBS for 5 min, and this step was repeated 3 times. To block nonspecific labeling protein, the samples were incubated with BSA 1% for 30 min at 25°C. Substrates consisting of fixed cells were incubated in osteocalcin (OCN) primary antibody (1:200 in PBS, Rat polyclonal anti-osteocalcin, Abcam, USA) overnight at 4° C followed by washing with PBS as described in the previous steps. Afterward, samples were incubated for 1 hour with secondary antibody (1: 100 dilutions with PBS, Abcam, USA) FITC-conjugated in the dark at 25°C and washed as described previously. Finally, Hoechst (Sigma-Aldrich, St. Louis, USA) was added to nuclear staining and the samples were visualized by fluorescence microscope (Zeiss LSM 510 Meta).

# 2.6.4 Real-Time PCR

The expression of bone-specific genes was assessed for ADSCs cultured on Plate, Plain PDMS, PDMS-Phys, PDMS-Chem, and PDMS-Phys-Chem substrates after 21 days using real-time PCR. Total RNA of cells was extracted using RNx -plus solution (Qiagen, Germany). Then DNA was removed from solutions and the concentration of RNA samples was quantified by measuring the absorbance at 260 using a spectrophotometer (NanoDrop 1000, ThermoScientific, USA). Eventually, the complementary DNA (cDNA) was synthesized according to the manufacturer's instructions of the cDNA synthesis kit (Qiagen, United States). The StepOne<sup>™</sup> real-time PCR system (ABI) was used for PCR assay. Each reaction was contained 20 µl SYBR PCR Master Mix, 4 µl cDNA, 4.4 µl of RNAse-free water, and 0.8 µl of each primer. The primer's sequences are listed in Table 1. Amplification conditions for the PCR were adjusted at an initial denaturation at 95°C for 2 min followed by 40 cycles at 95°C for 5 sec, 60°C for 25 sec. The Collagen type 1, Osteocalcin, and RUNX2 as target genes and GAPDH as the housekeeping gene were studied. Finally, to analyze differences in Ct values, the relative fold change method  $(2^{-\Delta\Delta CT})$  was used. Experiments were performed twice, by three technical replicates per sample. Moreover, gene expression of osteoblast was measured as a positive control and compared with all samples. The sequences of primers used in real-time PCR are summarized in Table 1.

Sequences of primers used in real-time PCR		
Gene	Forward primer	Reverse primer
GAPDH	TCAAGGCTGAGAACGGGAA	TGGGTGGCAGTGATGGCA
Collagen I	CGATGGCTGCACGAGTCA	GGTTCAGTTTGGGTTGCTTGTC
Osteocalcin	CAGCGAGGTAGTGAAGAGACC	TCTGGAGTTTATTTGGGAGCAG
RUNX2	GGTCAGATGCAGGCGGCCC	TACGTGTGGTAGCGCGTGGC

## 2.7 Statistical analyses

Statistical analysis was performed using the Origin program. Data statistically were analyzed using oneway analysis of ANOVA, followed by Tukey multiple comparison tests to determine the statistical significance. The probability level at which differences were considered significant was P<0.05.

## **3 Results And Discussion**

## 3.1 Characterization of isolated osteoblasts

Figure 3-A shows the isolated bone from a neonatal rat that was used for osteoblast cell extraction. The signed section was used for the cell isolation. Crystal violet and alizarin red S staining of isolated cells from neonatal rat bone confirmed the proper isolation of osteoblast cells. Figure 3-B shows the crystal violet staining of isolated osteoblast after five days of culture and it confirms that the cells were alive before they were fixed. Alizarin red S staining of isolated osteoblast compared to ADSCs as negative control are shown in Figure 3-C. The intensity of alizarin red S staining at plate view and microscopic images indicates the high purity of osteoblast cell isolated osteoblast cells. Figure 3-D shows the IF staining of isolated osteoblast cells which osteocalcin stained with green color and the nuclei stained with blue color. The merged picture indicates the nuclei embedded within osteocalcin proteins and it confirmed the identity of the isolated cells as osteoblast.

## 3.2 Characterization of ABL

In this work, ABL was utilized for chemical surface modification of PDMS substrate to investigate its effect on stem cell differentiation. For this purpose, ABL was isolated from rat long bones (Figure 4-A). The concentration of total proteins content of ABL was calculated by Bradford assay to be 40 µg/µl. SDS-

PAGE assay was used to prove the existence of a complex of proteins with different molecular weights in ABL solution and is shown in Figure 4-B. The appearance of the wide range of bands in the SDS-PAGE gel indicates the presence of a diversity of proteins and growth factors in the ABL solution. Strauss et. al. [47] has previously performed proteomic analysis on ABL which has revealed 394 proteins including TGF-B1 (1.3 ± 0.2 ng/ml). Their results indicated the presence of ribosomal protein in ABL solution, in addition to collagens and TGF-B1.

## 3.3 Determining the optimum concentration of ABL based on coating thickness and cell viability

After the chemical surface modification of PDMS substrates by plasma, APTES, and GA, the ABL solution at rate 100%, 70%, 30%, and 10% of initial concentration was coated on the PDMS substrates. The coated PDMS substrates were further analyzed by FESEM and profilometer to measure the obtained protein thickness on PDMS. Figure 5. Shows the FESEM pictures of protein coating on the PDMS surface. As expected, the maximum thickness of about 375 nm is obtained by applying 100% ABL solution on modified PDMS. Going a diluted solution of 70%, the thickness does not vary significantly and in fact, we have reached a thickness of approximately 240 nm. Decreasing the ABL solution concentration to 30% and 10% has resulted in approximately 45 nm, and 36 nm thick ABL coating, respectively.

Furthermore, to confirm the results obtained by FESEM, the thickness of the ABL coating at different concentrations were measured by profilometer and the results are summarized in Table 2. These results are in line with the FESEM observations, and the thickness values are in the same order of magnitude. Amongst obtained protein coatings, 50 nm thickness, obtained by 30% of ABL concentration, offers us the optimum thickness. In fact, 50 nm thickness allows us to successfully bind ABL proteins to the substrate without fully covering the cell-imprinted patterns, a fundamental contributor to the cell's fate. In other words, 50 nm ABL coating not only offers an effective protein coating for ADSCs sensing (chemical effect) but also allows the cell-imprinted topography to contribute to the cells fate (physical effect). On the other hand, 100% and 70% of ABL fill the cell pattern and make that topographic effect ineffective. Therefore, in this study, the concentration of 30% is selected to further investigation on surface characterization and ADSCs differentiation.

Concentration rate of ABL solution	Coating thickness
100%	374±10 nm
70%	231±10 nm
30%	50±10 nm
10%	45±10 nm

Results of ABL coating thickness profile measurements.
Table 2

Furthermore, to evaluate the impact of chemical modification of PDMS on the viability of the cells, the MTT assay was measured. Figure 6. shows the cell viability of MG-63 cells after 3, 5, and 7 days of direct contact with plain PDMS and 30% ABL coated PDMS (chemically modified marked as PDMS-ABL in Figure 6). The standard cell culture plate was used as a control. Clearly, the plain PDMS substrate shows poor cell attachment and cell viability due to PDMS surface hydrophobicity compared to the cell culture plate. While there is no significant difference between cell viability of the culture plate and the chemically modified PDMS after 5 and 7 days of culture. The results also confirm the cytocompatibility of the ABL-coated PDMS surface.

## 3.4 Characterization of modified PDMS surface with ABL

In order to observe the attached functional groups after chemical surface modification of PDMS, FTIR spectrophotometry analysis was carried out. For this purpose, ATR-FTIR spectra of Plain PDMS, plasmatreated PDMS+APTES+GA, and plasma-treated PDMS+APTES+GA+ABL substrates were recorded and are shown in Figure 7. For the Plain PDMS, the absorption band appears at 2960 cm<sup>-1</sup> which is attributed to the typical peak of the C-H methyl stretch, while the absorption band at 1254 cm<sup>-1</sup> is related to the Si- $CH_3$ . Moreover, the strong bands for the wavenumber between 1000–1100 cm<sup>-1</sup> are related to the asymmetric stretching vibration of Si-O bonds. In fact, the pick at 1008 cm<sup>-1</sup> is associated with the Si-O-Si asymmetric stretching vibration, while 1054 cm<sup>-1</sup> is associated with the vibration of the Si-O bond in Si-O-C, and the peak at 798 cm<sup>-1</sup> is attributed to the Si-CH<sub>3</sub> bending vibration in Plain PDMS [50, 51]. After the APTES and GA conjugation, a new small broad peak appeared around 1559 cm<sup>-1</sup>, which is attributed to the formation of amine bicarbonate salt (-HN<sub>3</sub>(HCO<sub>3</sub>)) [50]. In fact, the appearance of the absorption band around 1624 cm<sup>-1</sup> confirms the N-H presence due to APTES treatment. Emerging of a small peak around 1662 cm<sup>-1</sup> is related to the vibration of the imine bond (C=N) due to GA crosslinking [52]. Eventually, after coating PDMS with the ABL solution, the absorption bands of proteins emerged at 3300–3500 region cm<sup>-1</sup> which are related to the N-H vibration of peptide groups. The characteristic peaks of collagen functional groups appeared around 3370 cm<sup>-1</sup> and 1630 cm<sup>-1</sup> which are associated with the O-H and N-H stretching vibration of collagen. Moreover, the peaks that appeared around 1558 cm<sup>-1</sup> and 1472 cm<sup>-1</sup> could be related to the peptide carbonyl group (-C=O) stretching vibration of amide I and N-H bending of amide II respectively [53, 54] which possibly exist due to the presence of other proteins and growth factors in the ABL solution. These results confirmed that applying the mentioned chemical surface modification method, the collagen and other ECM proteins of the ABL solution were successfully attached to the PDMS substrate.

Another important factor related to chemical substrate improvement is surface wettability. In this regard, the surface static contact angle measurements of Plain PDMS and modified PDMS surfaces (PDMS-Phys, PDMS-Chem, PDMS-Phys-Chem) were evaluated by the sessile drop method and were repeated after 7 days, and 14 days post-treatment. Each measurement was carried out in three different points of the substrate and its mean values are shown in Figure 8-A. Schematic presentation of contact angle

measurements on different substrates and time points are shown in Figure 8-B. Clearly, the Plain PDMS surface is hydrophobic with a contact angle of 109.3± 2.14°. Physical and chemical surface modifications show a significant reduction in contact angles. The mean contact angles for the topographically modified PDMS substrate (PDMS-Phys) was measured to be CA=88.89± 3.54° while after chemical modifications (PDMS-Chem), it was reduced to CA=47.21± 2.82° meaning a transition from hydrophobic to hydrophilic surface. This reduction of water contact angle was even more profound when both topographical and chemical modifications were performed on PDMS (PDMS-Phys-Chem) reaching CA=41.30± 2.23°. These results confirm the significant success of chemical modification of the PDMS by ABL in increasing substrate wettability toward hydrophilic regions (<70°) for PDMS-Chem and PDMS-Phys-Chem substrates. Such increase in wettability is based on the formation of amine functional groups on the PDMS surface, which in turn creates hydrogen bonds with water. Kuddannaya et.al has previously reported similar results for reduction of contact angle after chemical modification of the PDMS by APTES and/or GA [49]. These results also justify the higher cell viability of chemically modified PDMS observed in Figure 6.

Moreover, the water contact angle for PDMS surface after treatment by plasma, APTES, and GA was measured. Despite the significant effect of silanization on the reduction of water contact angle, hydrophilicity was lower than the surfaces with ABL coating at day 0 (CA=63.61± 2.37°). Furthermore, results indicate that the chemical modification of cell-imprinted substrate (PDMS-Phys-Chem) compared to chemical modification of flat substrate (PDMS-Chem) had a greater reduction in the contact angle degree due to higher surface roughness in the cell-imprinted substrate. Similar results have been observed by Sharma et. al.[55]. The same trend of surface roughness effect was observed for PDMS-Phys and Plain PDMS substrates; however, both remained at the hydrophobic regions (~ 100°).

Furthermore, water contact angle degrees appear to increase over time for chemically modified PDMS surface (PDMS-Chem and PDMS-Phys-Chem) while these values remain constant for the cell-imprinted and plain PDMS surface. The CA for PDMS-Chem and PDMS-Phys-Chem has increased by only 22% and 21%, respectively even after 7 days post-treatment. This is due to the decomposition of functional groups on the PDMS surface over time.

ABL's proteins attachment and retention on their surfaces were analyzed by micro BCA and was compared with plain PDMS as a control, and is shown in Figure 8-C. The figure shows the percentage ratio of retained proteins to the initial concentration on the PDMS-Chem, and PDMS-Phys-Chem substrates at days 0, 14, and 21. There was no protein attachment on the Plain PDMS substrate. While the percentage ratio of attached and remained proteins on the PDMS-Chem substrate on days 0, 14, and 21 compared to initial concentration were obtained to be 38.2%, 29.45%, and 26.34%, respectively. This enhances on attachment and retention of proteins was even more profound for PDMS-Phys-Chem and were measured to be 43.8%, 33.8%, and 27.2% on days 0, 14, and 21. The first conclusion for these results is the lack of ability for protein attachment on the Plain PDMS. In fact, the amount of attached and remained proteins on chemically modified substrates indicates the importance of stable covalent binding between proteins and substrates. As in previous studies, it has been shown that the modification of

PDMS surface with APTES and GA as cross-linker due to the formation of functional groups (-NH<sub>2</sub> or -COOH), prepared the conditions for effective covalent binding of proteins into the substrate. Akther et. al, has previously shown that APTES can be used as a silane connector for binding biomolecules to PDMS substrate [56]. Nonetheless, the result shows a reduction in surface-bound proteins over time that could be related to the degradation of protein under incubation conditions. The best result, however, is observed for both physically and chemically modified substrates (PDMS-Phys-Chem). This means the combination of cell-imprinted substrate and stable covalent bonding is an appropriate approach to maintain protein binding to the PDMS substrate. Moreover, hydrophilicity of the surface is one of the effective factors for the better absorption and increase of the retention of proteins on the PDMS substrate. As previously observed in Figure 8-A, the hydrophilicity was decreased over time and therefore, it can lead to lower protein retention. Furthermore, based on hydrophilicity results, the maximum hydrophilicity is related to PDMS-Phys-Chem substrate, which shows higher protein bonding and retention. Therefore, from the FTIR, contact angle, and micro BCA analysis it could be concluded that the combined nano 3D cell-imprinted substrate and nanocoating bone proteins on the surface (PDMS-Phys-Chem) offers the best surface wettability and protein attachment, and its stability over time is improved. In turn, these results could lead to improved cell adhesion, cell proliferation, and differentiation which is further investigated.

# 3.5 Effect of substrate modification on cell adhesion and proliferation

Cell adhesion and proliferation on the Plain PDMS, PDMS-Phys, PDMS-Chem, and PDMS-Phys-Chem substrates was evaluated by crystal violet staining and are shown in Figure 9. Comparing the number of stained cells on each substrate, cell adhesion and proliferation have significantly improved after surface treatments. The improvement for PDMS-Phys could be attributed to the effect of the cell's topographic pattern leading to increased surface roughness. As previously reported, the presence of APTES caused more cell adhesion and proliferation on chemically modified PDMS substrate due to their better surface wettability[56]. Therefore, there is no surprise to observe better cell attachment and proliferation on PDMS-Chem substrate. A similar trend is observed for the combined cell-imprinted PDMS surface and nanocoated bone proteins (PDMS-Phys-Chem). In this case, the combination of the topographical effect of cell-imprinted surface, and the presence of APTES and ABL lead to a synergic effect responsible for the highest number of cell adhesion and proliferation.

# 3.6 Investigation of osteogenic differentiation potency of physical and chemical modifications

In order to evaluate the potency of different substrates to guide cells towards osteogenic differentiation, ALP activity was investigated. ALP activity, as an early marker of osteoblast differentiation[57], was measured for cells cultured on Plate, Plain PDMS, PDMS-Phys, PDMS-Chem, and PDMS-Phys-Chem after 14 and 21 days and it is shown in Figure 10. The results show that Plain PDMS offers higher ALP activity compared to the culture plate. This activity increases significantly after surface topographical changes on PDMS-Phys (*p* <0.001). Similar results on increasing the ALP activity by cell-imprinted substrate have been reported in a previous study by Kamguyan..et al [36]. Chemical modification of substrate with ABL nanocoating leads to higher ALP activity, and eventually, the combined cell-topographic effect and ABL coating lead to the highest ALP activity on PDMS-Phys-Chem substrate after 14 days. However, after 21 days, ALP activity has no significant difference between PDMS-Chem and PDMS-Phys-Chem.

Another marker toward the late stage of osteoblast differentiation indicating a bone formation is the release of calcium from the cells[58]. Therefore, Alizarin red S staining of cultured cells was used on the Plate, Plain PDMS, PDMS-Phys, PDMS-Chem, and PDMS-Phys-Chem substrates after 14 and 21 days, and its microscopic pictures are shown in Figure 11-A. A gradual trend of increased Alizarin red S staining intensity is observed for PDMS-Phys, PDMS-Chem, and PDMS-Phys-Chem respectively. The increase in alizarin red S staining intensity is an indication of higher efficacy of substrate for osteogenic differentiation which in this case belong to PDMS-Phys-Chem substrate. The quantification of Alizarin red S staining was measured and is shown in Figure 11-B. Based on these results; topographical changes based on 3D nano cell-imprinting on PDMS substrate lead to significantly higher osteogenic differentiation 14 days after cells were cultured (p< 0.001). The chemical modification of substrate by nanocoating bone proteins (PDMS-Chem) appears to be more effective than cell-imprinting effect in improving osteoblast differentiation. Finally, the combined cell-imprinting and bone proteins nanocoating leads to maximum calcium nodule formation and matrix mineralization and consequently the highest level of cell differentiation after 14 days. A slight increase in the number of calcified nodules was observed after 21 days for Plain PDMS substrate compared with Plate. Over time, reaching 21 days after cell culture, the intensity of Alizarin red S staining increases in all substrates. However, there is no significant difference between PDMS-Chem and PDMS-Phys-Chem anymore. In fact, these results are in line with our previous observation of ALP activity on the chemically modified substrates.

The osteogenic bone markers synthesized during osteogenic maturation of ADSCs cultured on different substrates were evaluated by the immunofluorescence (IF) staining of osteocalcin (OCN) after 21 days and is shown in Figure 12. The nuclei were blue-stained with Hoechst embedded within the green-stained osteocalcin proteins by FITC fluorescence. The production of OCN due to osteogenic maturation by the cells cultured was higher for PDMS-Phys-Chem substrate compared to other groups, which revealed the cooperation of physical and chemical modification on increasing OCN protein expression. The intensity of OCN staining on the PDMS-Phys and PDMS-Chem substrates showed higher protein expression compared with the Plate and Plain PDMS groups, which demonstrate that both physical and chemical modification without cooperation has a significant effect on the expression of OCN. Higher osteocalcin protein expression is related to the osteoblast topography on the PDMS-Phys substrate and is attributed to the presence of bone proteins (ABL) for the chemically modified PDMS substrate (PDMS-Chem). Moreover, in the Plate and Plain PDMS groups, the intensity of green fluorescence was less than all modified substrates. This trend has previously been observed for alizarin red S staining results, the lowest number of nucleus between all substrates belongs to Plain PDMS due to the low tendency of the cells to attach to the unmodified PDMS surface. This is understandable considering the hydrophobic surface of Plain PDMS. On the other hand, the intensity of the blue color, which represented the number of adherent

cells on the Plate substrate was higher than all PDMS substrates. However, green-stained osteocalcin, which confirms the osteogenic differentiation of ADSCs was not indicated. Overall, the results indicate that the combined nano 3D cell-imprinting and bone proteins nanocoating (PDMS-Phys-Chem) not only offers better cell adhesion and proliferation but it also increases the OCN expression, representing improved osteogenic differentiation.

To quantitatively evaluate bone-specific gene expression by the ADSCs seeded on the Plate, Plain PDMS, PDMS-Phys, PDMS-Chem, and PDMS-Phys-Chem substrates, the real-time PCR was applied. Gene expression of osteoblast was measured as a positive control. The expression of RUNX2, Collagen I, and Osteocalcin genes after 21 days has been illustrated in Figure 13. Gene expression of ADSCs cultured on the standard tissue culture plate was considered as a negative control and all data were presented according to their results. Based on the results for all substrates, expression of Col1 showed a downregulation while RUNX2 and OCN showed an upregulation in comparison to the negative control. This fold change trend was similar to the gene expression of osteoblast as a positive control. Improvement of osteogenic differentiation on the Plain PDMS surface compared with the Plate substrate was indicated in previous studies. This enhancement could be related to the mechanical properties of substrates. The Young's modulus for PDMS substrate (at a ratio of 10:1 curing agent) was measured to be 2.6 MPa [59] which is two orders of magnitude higher than the Young's modulus of cross-linked collagens of osteoids (25-40 kPa) in natural bone ECM [12], but it offers closer stiffness since the Elastic Modulus of tissue culture plate reaches 3 GPa, five orders of magnitude higher stiffness [60]. As previously proven, the cells sense the substrate's elastic or viscoelastic properties [12, 61]. In the present study, ADSCs on Plain PDMS express 8-fold greater OCN and near 4-fold greater RUNX2 in comparison with the Plate substrate. The higher number of fold changes for PDMS-Phys is attributed to the nano topographic cell pattern on the PDMS substrate which sends signals to guide the stem cells' osteogenic differentiation. The signaling takes place because stem cells get trapped in an osteoblast shape and dimensional template. Similar findings were reported that in the absence of chemical and mechanical signals, topographical patterns on the cell culture substrate could direct stem cell differentiation[33, 36]. Moreover, as we expected for the PDMS-Chem substrate, based on ALP assay, Alizarin red S staining, and IF staining results, the real-time PCR results showed a similar trend in bone-specific gene expression with positive control group. This improvement is related to the nanocoating layer of bone proteins on the PDMS surface which chemically mimics the bone niche; therefore, the chemical signals due to the bioactivation of PDMS surface are sensed by ADSCs and guide its fate towards osteogenic differentiation. In the case of PDMS-Phys-Chem, the fold change of RUNX2 gene was calculated similarly to the positive control, and no significant differences were observed while the Col1 gene expression was lower than osteoblast. Furthermore, the OCN gene expression as a late osteogenic marker and one of the major players for the maturation of mineral species and modulates osteogenic differentiation, was increased in all groups. This increase for PDMS-Phys-Chem was significantly more than all other substrates (p<0.001). The fold change of OCN gene expression for PDMS-Phys-Chem was calculated 22fold compared with undifferentiated ADSCs, likewise, it was calculated 79-fold for osteoblast. In addition, the expression of OCN for PDMS-Chem and PDMS-Phys were calculated 12-fold and 13-fold respectively

compared with the negative control. Hence, it can be concluded that simultaneous applying of nanocoating of bone proteins on the topographical cell pattern caused a synergistic effect on bone-specific gene expression.

Increases in bone-specific gene expression on the substrates coated by ABL could be related to the presence of bone-specific proteins and growth factors such as TGF-B1 in ABL solution. In support of this speculation, several researches have previously shown that ABL activates the TGF-β target genes: interleukin 11, proteoglycan 4, and NADPH oxidase 4 [47, 62, 63]. This is particularly important because these genes play an important role in bone regeneration [47, 62, 63]. However, the amount and activity of BMPs in ABL have not yet been determined and HCl used in ABL extraction could potentially deactivate pH-sensitive growth factors[64]. In addition, the activated signaling pathway due to TGF-B1 has an important effect on the activation of a large number of target genes such as Collagen I, and they are effective on the proliferation and osteogenic differentiation of MSCs [65]. Therefore, it is expected to observe a rise of bone-specific gene expression for substrates coated with ABL due to the presence of a wide range of proteins including growth factors, especially active TGF-B1. On the other hand, TGF-B activity and consequently, cell response after ABL adsorption to the surface, was proven by a bioassay in another study published by Strauss et al. In their study, the effect of TGF-B activity was indicated on the expression of target genes that are involved in bone regeneration and genes that are responsible for regenerating the extracellular matrix[41]. Therefore, it can be concluded that ABL coating on the PDMS surface could lead to elevated chemical signaling and mimic the osteoconductive ECM of osteoblasts due to its agents increasing the expression of the bone-specific genes. Finally, our findings approved that ABL nanocoating with an appropriate thickness on top of osteoblast imprinted PDMS enhances the osteogenic differentiation of ADSCs.

### **4** Conclusion

In this work, we have utilized an approach comprising both physical and chemical modifications of PDMS substrate mimicking the physiological niche of natural cells to guide Adipose-derived Stem Cells' fate. We have investigated the effect of topography by creating a nano 3D cell-imprinting on PDMS substrate. Additionally, we have successfully extracted ABL bone proteins from rat's long bone, and have utilized it for chemical treatment of PDMS substrate. GA, APTES, as cross linker, and ABL solutions with an initial percentage of 100%, 70%, 30%, and 10% were applied on plasma-treated PDMS substrate. The optimal ABL concentration for chemical surface treatment of PDMS was selected after FESEM and profilometry analysis of the coating on the surface. It was shown that 30% ABL solution leads to 50 nm-thick coating on PDMS substrate (PDMS-chem), and cell-imprinted PDMS surface (PDMS-Phys-Chem) for further studies. The cytocompatibility of the ABL-coated substrate was confirmed by measuring the cell viability of MG63 cell line on it compared with Plain PDMS and culture plate.

the emergence of proteins absorption bands at 3300-3500 wavenumber ranges attributed to the N-H vibration of peptide groups.

Sessile drop static water contact angle measurements on all substrates showed an increased wettability after treatments. The improved wettability for physically modified PDMS (PDMS-Phys) was minimal while a clear transition from hydrophobic to hydrophilic surface was observed when Plain PDMS surface (CA=109.3°) was chemically treated (CA=47.21° for PDMS-Chem, and CA=47.21° for PDMS-Phys-Chem). Repeated contact angle measurements over time have shown a general trend of increasing static contact angle for all substrates. Proteins attachment and retention significantly improved for chemically treated surfaces especially combined ABL nanocoated on cell-imprinted PDMS substrate (PDMS-Phys-Chem). In turns, our results indicate an enhanced ADSCs' attachment and proliferation after treatment indicating the most promising result for the combined physically and chemically modified surface (PDMS-Phys-Chem).

Moreover, the amount of released alkaline phosphatase and calcium nodule formation of all substrates after 14 and 21 days of culture was evaluated by ALP assay and Alizarin red S staining. ALP activity on the PDMS-Phys-Chem substrate significantly was more than all groups on day. A similar trend was observed on day 21, however did not show a significant difference with PDMS-Chem. The result of Alizarin red S staining intensity for PDMS-Phys-Chem showed maximum calcium nodule formation and consequently the highest level of cell differentiation after 14 days. Eventually, the potency of physical and chemical surface modification at the level of gene expression and protein expression by real-time PCR and IF staining studied. The results approved a synergistic effect of simultaneous applying of nanocoating of bone proteins and topographical cell pattern. Overall, we have introduced a new cell culture substrate based on 3D cell-imprinting and proteins nanocoating in order to mimic the cell topography and ECM. We have shown the effect of biophysical and biochemical surface modification and their contribution to these factors on the PDMS substrates. nanocoating of osteoblast cell-imprinted PDMS with specific bone proteins caused a significant increase in differentiation factors. It means that bioactivation of PDMS surface consists of cell topography template not only improved cell adhesion but also led to specific differentiation pathways. The results show the synergistic effect of physical and chemical surface modification on the osteogenic differentiation of ADSCs. In this study, for the first time, the micro/nano cell-imprinted surface modified with nanocoating layer of specific proteins content and a special platform taking into account the topographically and biochemically characteristics was made. The proposed method can be applied to construct the characteristic biomimetic substrates in order to stem cells differentiation to other mature cells as well.

### Declarations

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#### Figures



Schematic presentation of PDMS surface modification by APTES, GA, and ABL.



Schematic presentation of the isolated ADSCs cultured on the chemically modified PDMS substrate consists of osteoblast cell-imprinting topography.



(A) Extracted long bone from neonatal rat, (B) Crystal violet staining of the isolated osteoblast cells after 5 days (original magnification ×100). (C) Microscopic image (original magnification ×100) and plate view of Alizarin red S staining of isolated osteoblast cells versus ADSCs cells as a negative control. (D) Immunofluorescence staining of the isolated osteoblast cells cultured on a plate by FITC-conjugated antibody for osteocalcin labeling and Hoechst staining for cell nucleus.



(A) Harvested long bones from the adult rat for ABL extraction and (B) Quantification of ABL's proteins by SDS-PAGE. The test was repeated twice to obtain reliable results. The gel pictures were cropped and full-length gels are presented in Supplementary Figure S 1.



The cross-sectional FESEM micrographs of (A) 100%, (B) 70%, (C) 30% and (D) 10% of initial concentration of ABL.



Cell viability of MG-63 cell line on the plain PDMS and chemically modified PDMS substrate by ABL coating (PDMS-ABL). The standard cell culture plate was used as a control group. The data are shown as the mean  $\pm$  SD. \*\*\*p value of <0.001 between two groups. p-value of  $\geq$ 0.05, indicates a statistically non-significant (ns) result.



FTIR-ATR spectra of Plain PDMS, plasma-treated PDMS+APTES+GA, and plasma-treated PDMS+APTES+GA+ABL at the wavenumber between 500 cm<sup>-1</sup> to 4500 cm<sup>-1</sup>.



(A) Surface hydrophilicity analysis of PDMS surfaces as a function of time. Plain PDMS compared with treated groups; PDMS-Phys (cell-imprinted PDMS), PDMS-Chem (flat PDMS with APTES+ GA+ABL), and PDMS-Phys-Chem (cell-imprinted PDMS with APTES+ GA+ABL) at day 0, 7, and 14 after surface modification. Error bars indicate the standard deviation of the means n=3 samples. (B) Schematic of contact angle for a water drop placed on different surfaces. (C) Micro BCA assay for calculation amount of attached and retained proteins on the Plain PDMS, PDMS-Chem, and PDMS-Phys-Chem substrates on 0, 14, and 21 days. The graph is represented as the mean  $\pm$  SD. \**p*-value of <0.05, \*\**p*-value of <0.01, and \*\*\**p* value of <0.001 between two groups. Ø represents zero measurement on the plain PDMS.



Cell attachment and proliferation of ADSCs on the different PDMS substrates were assessed by crystal violet staining after 14 days.



#### Figure 10

ALP activity of ADSCs cultured on the Plate, Plain PDMS, PDMS-Phys, PDMS-Chem, and PDMS-Phys-Chem after 14 and 21 days. All data are normalized regard to the optical densities of cells on each substrate and reported as means ± standard deviation (n=5). \**p*-value of <0.05, \*\**p*-value of <0.01, and \*\*\**p*-value of <0.001 between two groups. *p* value of  $\geq$ 0.05, indicates a statistically non-significant (ns) result.



(A) Microscopic images of alizarin red S staining on different substrates after 14 and 21 days of cell seeding. (B) Quantitative analysis of Alizarin red S staining of ADSCs grown on different PDMS substrates compared to Plate group after 14 and 21 days. The optical density measured at 405 nm and the results are presented as mean  $\pm$ standard error of the mean of three independent experiments. \*p < 0.05, \*\*p < 0.01, and \*\*\*p < 0.001.



Immunofluorescence staining of ADSCs cultured on a Plate, Plain PDMS, PDMS-Phys, PDMS-Chem, PDMS-Phys-Chem substrates after 21 days. Hoechst staining for cell nucleus and FITC-conjugated antibody for osteocalcin (OCN) labeling were applied.



Gene expression of (A) RUNX2, (B) Collagen I, and (C) Osteocalcin in ADSCs cultured on the Plate (as a negative control), Plain, PDMS-Phys, PDMS-Chem, and PDMS-Phys-Chem substrates after 21 days. (\*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001, p value of  $\geq 0.05$ , indicates a statistically non-significant (ns) result, n = 3)

### **Supplementary Files**

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