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Early-stage detection of VE-cadherin during endothelial differentiation of human mesenchymal stem cells using SPR biosensor

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ABSTRACT

Surface plasmon resonance (SPR) biosensors are most commonly applied for real-time dynamic analysis and measurement of interactions in bio-molecular studies and cell–surface analysis without the need for labeling processes. Up to present, SPR application in stem cell biology and biomedical sciences was underused. Herein, a very simple and sensitive method was developed to evaluate human mesenchymal stem cells trans-differentiation to endothelial lineage of over a period of 14 days based on VE-cadherin biomarker. The SPR signals increased with the increase of the amount of VE-cadherin expression on the cell surface during cell differentiation process. The method was able to detect ≈27 cells per mm^2. No significant effect was observed on the cell viability during the cell attachment to the surface of immune-reactive biochips and during the SPR analysis. Using this highly sensitive SPR method, it was possible to sense the early stage of endothelial differentiation on day 3 in label-free form, whereas flow cytometry and fluorescent microscopy methods were found unable to detect the cell differentiation at the same time. Therefore, the proposed method can rapidly and accurately detect cell differentiation in live cells and label-free manner without any need of cell breakage and has great potential for both diagnostic and experimental approaches.

1. Introduction

Mesenchymal-to-endothelial transition is an essential phenomenon during tissue regeneration, leading to an increased vascular density in target tissues (Oswald et al., 2004). Human amniotic mesenchymal SCs (hAMSCs) have been demonstrated to offer great applications in cell-based therapies, and regenerative medicine and display differentiation potential to various tissue (Van Der Merwe, 2001; Vo-Dinh and Cullum, 2000). In biomedical science, the early detection of cell differentiation could be useful for predicting the reconstitution of target tissue. In addition, considerable less is known regarding molecular mechanisms through the differentiation processes (Nikolova-Krstevski et al., 2008). Thus, development of highly sensitive and accurate biosensor approaches for detecting SCs differentiation, particularly in the earliest steps, is more crucial than ever.

Different approaches could be used for the monitoring of SCs differentiation process. It has been shown that various cell specific markers and proteins such as Von Willebrand factor (vWF), vascular endothelial-cadherin (VE-cadherin), vascular endothelial growth factor receptor-1 (VEGFR-1/flt-1) and −2 (VEGFR-2/KDR) are induced during endothelial maturation of progenitor cells (Oswald et al., 2004; Pusztaszeri et al., 2006). Therefore, it is possible that these markers and proteins are used for the cell differentiation process.

VE-Cadherin is a transmembrane protein that elicit reciprocal junction of endothelial cells and plays a specific role in the preservation and renewal of endothelium integrity (Gorlatov and Medved, 2002; Gulino et al., 1998). In normal conditions, VE-cadherin efficiently augments cell-to-cell physical integrity in luminal surface of blood vessels. This protein is also the major determinant of blood vascular integrity is concurrently abolished by the elimination or aberrant function of VE-cadherin expression between tumor cell juxtaposed to endothelial cell (Lewalle et al., 1997). Following down-regulation or loss of VE-cadherin, the expansion of various tumors is facilitated to distant sites. Accordingly, some authors have acclaimed that monitor-

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ing the VE-cadherin expression can serve as a helpful tool in the investigating tumor growth and expansion (Corada et al., 2002; Labelle et al., 2008). Commensurate with this statement, it is noteworthy that VE-cadherin is an important cell surface marker to be monitored either in normal or abnormal conditions, targeting the dynamics of endothelial lineage in different context.

In general, for SCs differentiation capacity, various conventional in vitro proteomic and genomic assays, including immunocytochemistry, flow cytometry, western blotting and real-time PCR assays are commonly used (Fu and Kraitchman, 2010; Khalilzadeh et al., 2016; Panetta et al., 2009). Most of these analytical methods that are based on labeling process, are time-consuming and expensive. For instance, labeling agents such as fluorochromes (Challen et al., 2009), MRI agents (Kraitchman et al., 2003) and radionuclides (Cao et al., 2006) possess poor tissue penetration, high background and a short half-life.

After nearly three decades, surface plasmon resonance (SPR) technology was marketed and released as an appropriate biosensor for studying the biomolecular and cell–surface interactions in the sensitive, real-time and label-free form (Abadian et al., 2014; Karlsson et al., 1991; Liedberg et al., 1993; Lofas, 1995; Pockrand et al., 1978; Schasfoort et al., 2013; Zeidan et al., 2015). In SPR biosensors, any mass changing on the gold surface can be introduced in the form of response unit (RU) signals by a transducer (Van Der Merwe, 2001). SPR is also routinely used for the measurement of minute concentrations with the sensitivity to refractive index changes (10^{-6} refractive index units=RIUs) in near of sensor surface (Davis and Wilson, 2000; Rich and Myszka, 2000).

The most typical range application of SPR method was devoted to the evaluation of kinetics, thermodynamic parameters and the affinity constant of ligand–analyte interactions. In this case, the interaction of drugs with albumin, RNA and DNA and also antigen-antibody interactions have been studied by SPR based biosensors (Banères-Roquet et al., 2009; Fathi et al., 2016; Katsamba et al., 2006; Mariani et al., 2013; Mohseni et al., 2016; Sharifi et al., 2017). Nevertheless, very little research has been allocated for the development of biosensors for the detection of cellular dynamics via SPR technique. Given that SPR having the depth of evanescent wave around 300–400 nm, the use of this technology is limited for analyzing micrometer-sized particles, notably cells. However, qualitative and cell surface analysis can still be carried out, but detailed quantitative evaluations like kinetic and thermodynamic tests need to be set up in these conditions (Cuerrier et al., 2008). Up to present time, cell fixation on chip and cell injection on the antibody array of chip are two most common types of research design that have been applied for cell analysis and study by SPR technique (Cortès et al., 2011; Etayash et al., 2015; Kuo et al., 2011; Stojanović et al., 2014; Tyagi et al., 2015; Viitala et al., 2013). The fixation of non-adherent and adherent cells on a chip is a suitable policy for the study of cellular activity and morphology occurring in cells (Etayash et al., 2015; Rezabakhsh et al., 2017; Robelek and Wegener, 2010; Yanase et al., 2007).

In the current study, a label-free SPR method was developed and validated for the monitoring of the early-stage differentiation of hAMSCs into endothelial-like phenotype in term of VE-cadherin (or CD144 expression) over a period of 14 days and the results were compared with those obtained from the flow cytometric analysis and immunofluorescence imaging methods. To the best of our knowledge, this is the first application of SPR technique for the study of mesenchymal stem cells differentiation into endothelial cells assessed by VE-cadherin.

2. Experimental

2.1. Materials and reagents for SPR studies

Mouse anti-human VE-cadherin (CD144) (Cat no: MAB938), Alexa Fluor®488-conjugated mouse anti-human VE-Cadherin (Cat no: 53-1449-41) and PE-conjugated goat anti-mouse (Cat no: ab7002) antibodies were prepared from R & D system, eBioscience and Abcam companies respectively. Pure gold chip was obtained from Bionavis Company (Finland). All other chemicals were obtained from Sigma-Aldrich (Steinheim, Germany) and were of analytical-reagent grade and used without any purification.

2.2. Cell lines culturing and expansion

Human Umbilical Vein Endothelial Cells (HUVECs, NCBI code: C554) which were used as a positive control and human liver carcinoma cell line (HepG2, NCBI code: C158) used as a negative group were purchased from National Cell Bank of Iran. The hAMSC line (Cat No: C10680) was obtained from Iranian Biological Resource Center. Three different cell media were used in the culture of different cell lines: high glucose-Dulbecco's modified Eagle's (DMEM/HG, Gibco) for the HUVECs, Roswell Park Memorial Institute (RPMI, Gibco) for the HepG2 and low glucose-DMEM (DMEM/LG, Gibco) for hAMSCs. All media were enriched with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin (Biosera). The cells were kept at 37 °C in a humidified atmosphere with 5% CO_2 incubator. Cells were detached by 0.25% Trypsin-EDTA (Gibco) solution when they reached 70–80% confluence.

2.3. Endothelial differentiation of hAMSC

To induce endothelial differentiation, hAMSCs were maintained in Endothelial Cell Growth media M-199 supplemented with EGM-2 cocktail (Cat No: C-22010, Promocell) and 2% fetal calf serum (FCS, Promocell) for 14 days. The medium was replenished every 2–3 days. Differentiation of hAMSCs into endothelial-like phenotype was studied by SPR biosensor method and also with flow cytometry analysis and immunofluorescence microscopy on days 1, 2, 3, 5, 7 and 14. The morphological changes in relation to the endothelial acquisition were monitored through the experiment.

2.4. SPR instrument and technique

A multi-parameter SPR device (MP-SPR Navi 210A, BioNavis Ltd, Tampere, Finland) with gold chips (BioNavis Ltd, Finland) was used to examine antibody-cell affinity interactions. All Au-sensors were made of 240 mm^2 BK7-glass with a coating of a 50 nm gold layer. The chips had a Cr layer underneath as the adhesive metal layer. The cell analysis was performed in the fixed angle way at 670 nm wavelength at 30 °C. Prior to the SPR experiments, the whole flow path was washed and filled with the related running buffer in any section. The cells were injected into apparatus with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) solution. The flow chamber has dimensions of 2 mm×5 mm×100 μm (width×length×height) and all cell injection was performed at flow rate of 50 μl/ml.

2.4.1. Cleaning the bare gold sensor surface

The ammonia/hydrogen peroxide solution was used for cleaning and removing the impurity on the bare gold surface. To get the more accurate data, the cleaning step is essential prior to immobilization step. Briefly, each Au-sensor slide was immersed in the 30 ml boiling solution of 30% ammonia (NH_4OH), 30% hydrogen peroxide (H_2O_2) and double distilled water (DDW) at the ratio of 1:1:5 for 15 min at 90 °C. Then, the sensors were removed from the solution, washed several times with Milli-Q-water and pure ethanol and dried under a stream of N_2 gas.

2.4.2. Preparation of self-assembled monolayer (SAM) by 11-mercaptoundecanoic acid (MUA)

The cleaned and bare gold chips were submerged in the mixed

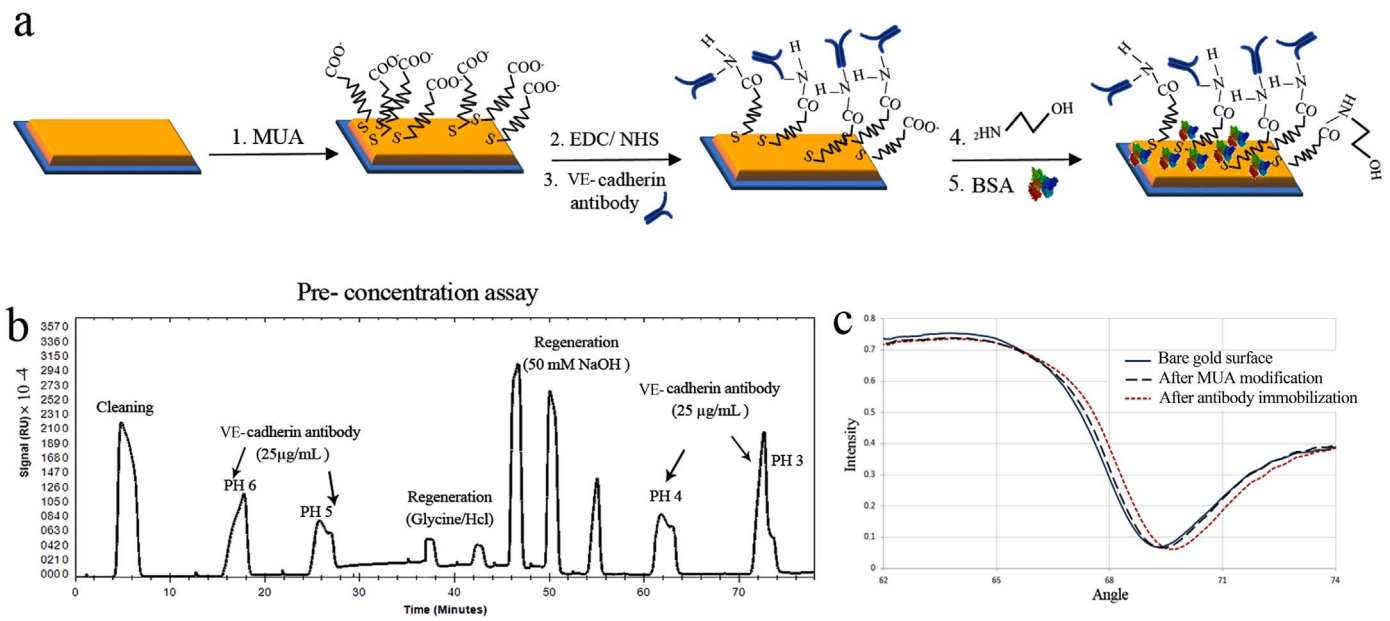

Fig. 1. A schematic illustration of random immobilization of VE-cadherin antibody (**a-1**). Self-assembled monolayer (SAM) was generated on the bare gold surface after 24 h of treatment with 11-Mercaptoundecanoic acid (MUA) (**a-2**). Activating the negatively charged of carboxy groups with EDC/NHS (**a-3**). The covalent immobilization of VE-cadherin antibody was performed by amide bonding (**a-4**) and deactivated using ethanolamine solution (**a-5**). Eventually, unbounded areas on the bare gold surface were blocked with BSA solution. Pre-concentration assays were done by immobilization buffer at four different pH values (**b**). SPR curves shift in bare gold surface, after MUA modification and after immobilization step (**c**).

solution containing 2 mM MUA in ethanol and DDW at the ratio of 7:3 at room temperature in a closed container. All thiol and disulfide adsorptions were performed in ethanol solution. After 24 h, the SAM was formed on the gold chip surface and then MUA-chip was soaked 3 times with ethanol and PBS solution.

Further, the gold slides were dried by using N_2 gas and then submitted for VE-cadherin antibody immobilization through either pre-prepared or on-line immobilization approaches as described in Section 2.4.4. Thereafter, the glass surface of each sensor slide was exactly cleaned with ethanol solution and optical tissue to prevent any contamination of prism in direct contact of glass side chip and prism during SPR test. Finally, freshly MUA-modified gold chips were placed in SPR slide holder and the related SPR curve of MUA surface and the initial scan were recorded.

2.4.3. Pre-concentration assay

Pre-concentration assay was done after MUA monolayer preparation on the gold surface and before the activation of carboxyl surface. VE-cadherin antibody was diluted to a final concentration of 25 µg/ml in the immobilization buffer at different pHs (3, 4, 5 and 6) and injected to the chip surface modified with MUA. The regeneration of the sensor chip surface was carried out with glycine/HCl solution and 50 mM NaOH to remove the ligand.

2.4.4. Covalent immobilization of antibody with amine coupling

The covalent immobilization of VE-catherin antibody on the sensor surface was carried out by amine coupling method. In this strategy, the immobilization process was performed on the sensor surface before inserting the chip into the sensor-slide-holder in the SPR instrument. Briefly, the modified MUA-Au chips were immersed in 0.05 M N-hydroxysuccinimide (NHS)+0.2 M 1-ethyl-3-(3- dimethylaminopropyl) carbodiimide (EDC) for one hour. Then, they were washed 3 times with PBS and coated with 25 µg/ml VE-cadherin antibody in acetate buffer (pH=5) for one hour. After washing with PBS, the deactivation was performed using 1.0 M ethanolamine-HCl pH 7.5 for 15 min. To block free sites on the chip surface, 0.5% BSA was used for 30 min.

2.4.5. BSA chips for non-specific binding (NSB)

After activation of the MUA chips with EDC/NHS solution, they were coated with BSA which was used as the blocking agent for 30 min. Different concentrations of BSA (0.01%, 0.1%, 0.5% and 1% w/w in PBS) were used to find the optimized concentration of BSA. Then, the chips were rinsed three times with PBS and distilled water and dried in air.

2.4.6. Cell capturing on biochips

Prior to cell injection, cells from each phenotype was counted using Neubauer slide (Precicolor HBG, Germany). A number of 10^5 cells per ml was rinsed in PBS and resuspended in HEPES buffer. All cell injections were carried out in HEPES as the running buffer in pH 7.4 at 30 °C with the flow rate of 50 µl per min. After cell injection, the chips were rinsed with buffer to remove the unbound cells. Data were analyzed using data viewer and TraceDrawer™ 210A SPR Bionavis software.

2.4.7. Evaluation of cell viability during the SPR study

In order to show the cell survival rate during SPR experiment, we collected the content of waste tubes. It is believed that these cells could not attach antibody-coated surfaces on the chip at the time of injection and thereby passed through the fluidic system and pooled in waste tubes. Cell viability was measured by Trypan Blue staining exclusion method. According to our preliminary results, the most of the retained cells had a high viability at the end of the experiment (data not presented).

2.5. Flow cytometry assay

Prior to flow cytometry analysis, cells were collected by 0.25% Trypsin-EDTA solution. Following centrifugation at 1500 rpm for 10 min and washing with PBS, the cells were blocked with 1% BSA for 20 min. Then, 1 µl of Alexa Fluor488-conjugated anti-human VE-cadherin antibody was added to cell solution and incubated for 1 h at 4 °C. After twice washing, flow cytometry analysis was performed using a FACSCalibur (BD Bioscience) system and data were processed by FlowJo software ver.7.6.1. HUVECs and HepG2 cells were respectively

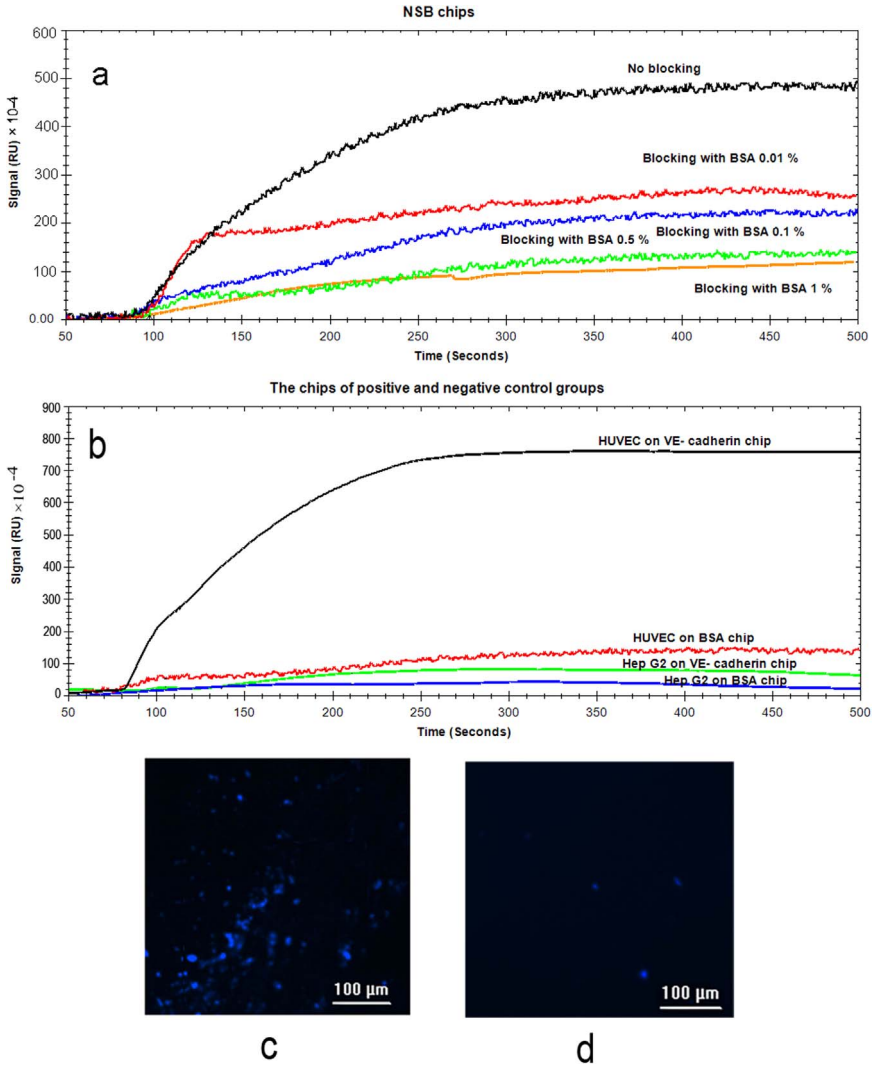

Fig. 2. Sensograms obtained from the injection of 10^5 cells onto pre-incubated chips with 0.01%, 0.1% and 0.5% BSA to find the optimum concentration of BSA as the blocking agent (flow rate 50 µl per min; injection time 5 min) **(a)**. In the panel b, cells were injected onto the VE-cadherin-coated and 1% BSA-blocked surface at a flow rate of 50 µl per min during 5 min (HUVECs were used as the positive control with 750 RU intensity and HepG2 cells were used as the negative group with 90 RU value) **(b)**. Biochip-bounded cell imaging with Cytation™ 5 cell imaging, BioTek: HUVECs **(c)** and HepG2 **(d)**.

used as positive and negative controls.

2.6. Immunofluorescence imaging

Immunofluorescence analysis was performed on HUVEC, HepG2 and hAMSC at various stages in endothelial differentiation on day 0, 3, 5, 7 and 14. Briefly, 10^4 cells were plated in each well of chamber slides (SPL). Thereafter, the cells were fixed by 4% paraformaldehyde for 20 min and incubated with 1% BSA for 30 min. Cells were kept in 1% BSA solution containing 20 µg/ml of anti-human CD144 overnight at 4 °C and then incubated with PE anti-mouse (dilution: 1:200) for 1 h. For background staining, 1 µg/ml DAPI was used. Finally, cells were visualized by fluorescence microscopy (Olympus, BX51).

3. Result and discussion

3.1. Random immobilization of VE-cadherin antibody on surface

The sensitivity of immune-biosensors is dependent on the extent of the immobilized antibodies, and thereby it is necessary to apply an optimal method for the antibody immobilization on the sensor surface. The entire procedure on the modified gold surface chip was performed according to our previous work (Fig. 1a) (Fathi et al., 2016).

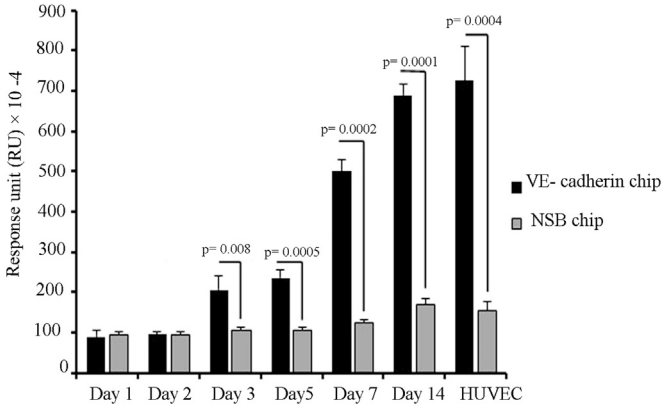

Fig. 3. RU value of VE-cadherin- and a BSA-coated sensor for the exclusion of non-specific binding (NSB). The HUVECs were used as the positive control to study hAMSCs differentiation on days of 1, 2, 3, 5, 7 and 14. Statistical analysis revealed a significant increase ($p < 0.01$) in the VE-cadherin-based RU values on days 3, 5, 7 and 14 after endothelial differentiation of hAMSCs. All values are expressed as mean ± SD (n=3).

Alkanethiols with long side chain like 11-MUA has been extensively applied for preparing self-assembled monolayers (SAMs) and antibody immobilization (Kraziński et al., 2011; Wang et al., 2005). Due to

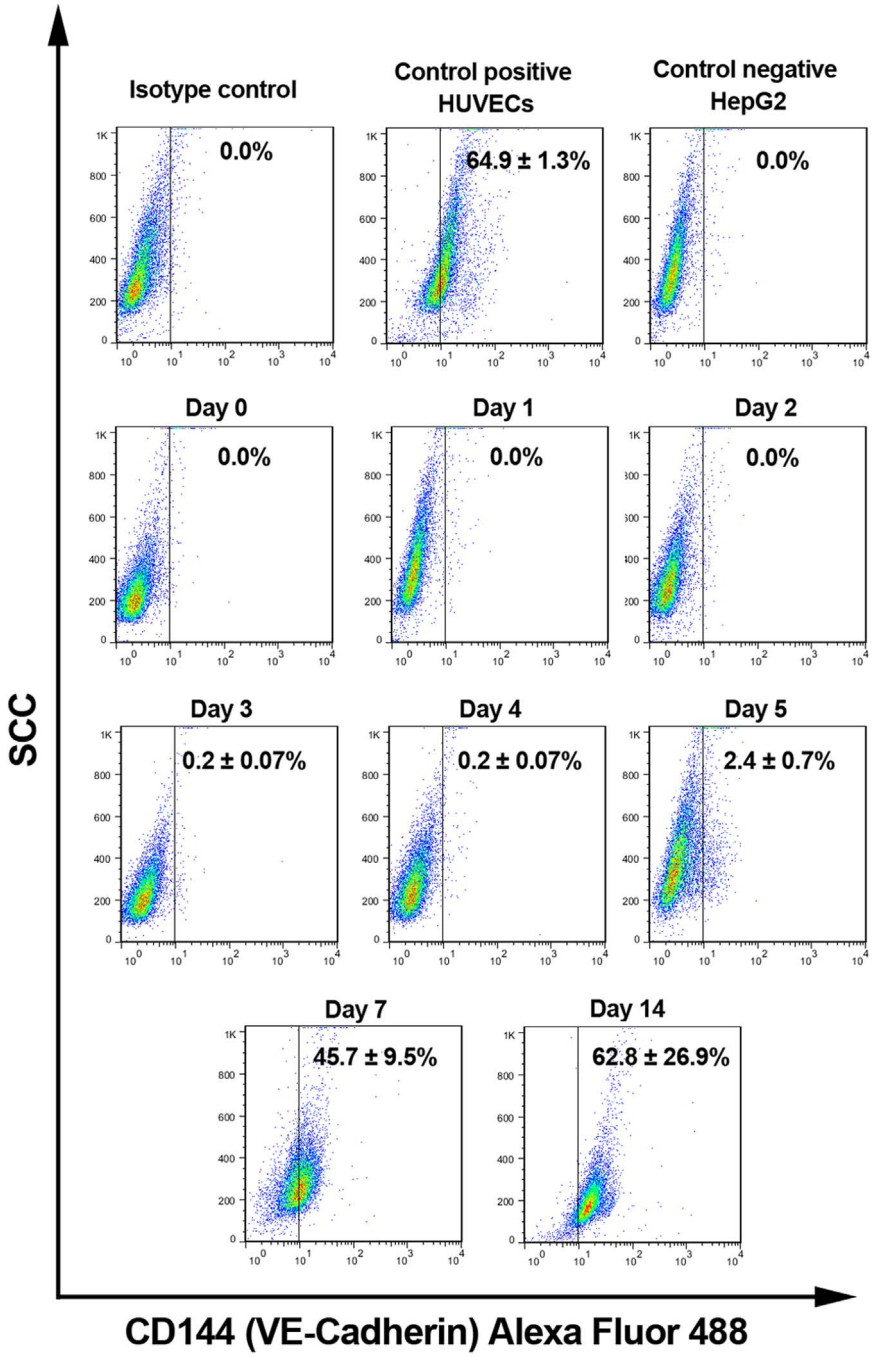

Fig. 4. Flow cytometric analysis of VE-cadherin expression during endothelial differentiation of hAMSCs over a period of 14 days (HUVECs: positive control; HepG2 cells: negative control).

having a long chain in the molecular structure of 11-MUA, the ability of formation broader three-dimensional space on top of the surface is more than short chain alkanethiols like 3-mercaptopropionic acid (MPA). This feature increases the probability of the protein molecules attachment. Accordingly, MUA was used for SAMs on the chip. Following the formation of negatively charged surface with carboxylic acid (COOH) SAMs, pre-concentration assay was carried out before the activation of the surface with NHS/EDC solution. Selection of the efficient immobilization processes not only depends on the charge of functional terminal groups on the desired surface, but also the charge and pI value of antibodies used. Human VE-cadherin peptide possesses 784 amino acid (aa) residues with a 546 aa extracellular domain located between amino acids 48 and 599 (Suzuki et al., 1991). The extracellular sequence of VE-cadherin is available online in http://www.uniprot.org and the VE-cadherin antibody used in the current experiment was directed against Asp48-Gln593 that it is thought to be sensitive in identifying extracellular compartment. In a pre-concentration experiment, the 25 μg/ml of VE-cadherin antibody was diluted in several immobilization buffers that differed by one pH unit. The optimum concentration of VE-cadherin antibody was determined by testing 12.5, 25 and 50 μg/ml of the antibody in the immobilization step (S. Fig. 1). As, both 25 and 50 μg/ml concentrations produced almost similar results, we used 25 μg/ml as the optimized concentration of VE-cadherin antibody. Also we concluded that buffers in acid regions (pH=5) below the pI value of the VE-cadherin antibody yields a fast and high pre-concentration performance (Fig. 1b). In other pH values above pI point, negatively charged amino acid residues were elicited in the immunoreactive antibody. As a result,

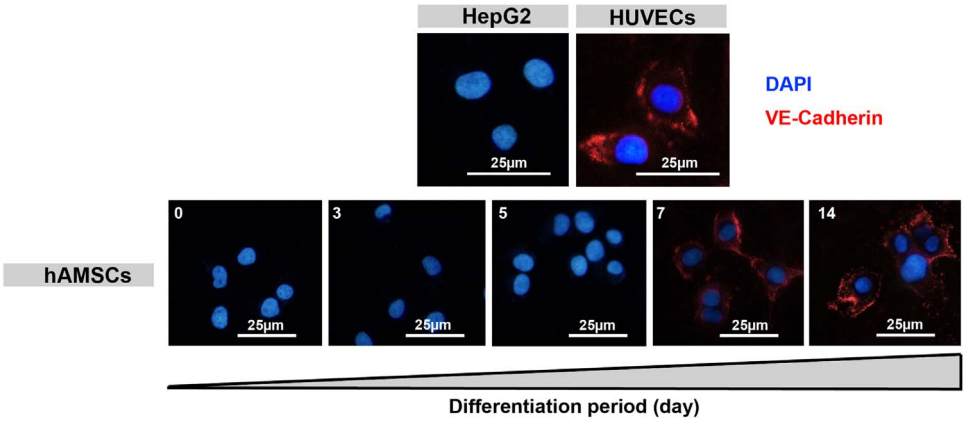

Fig. 5. Immunofluorescent imaging of differentiation markers: VE-cadherin in hAMSCs over a period of 14 days (HUVEC: positive control; HepG2 cells: negative control). For background staining, cells were incubated with DAPI.

these features have the ability to repulse antibody for closing the surface. Therefore, the pH of immobilization buffer was adjusted at 5 to increase the density of VE-cadherin antibody binding at the negatively surface of the sensor chip. This strategy resulted in a remarkable increase in the ligand density through electrostatic forces required to form covalent coupling and a reduction in the level of protein for injection, which is a desired requirement for a low-cost SPR experiment design (Drescher et al., 2009; Quinn et al., 1999). Based on our data, almost 400 m° (milidegree) shift was observed in the SPR curve after antibody immobilization (Fig. 1c). Adsorption of 1 ng/mm2 of the ligand on the sensor surface correlated with the generation of 100 m° SPR signal (de Mol, 2010). Accordingly, the amount of VE-cadherin antibody attached to the chip surface was calculated to be 4≈ng/mm2.

3.2. Blocking solution for minimizing NSB

The blocking reagents concentration affects the binding of biomolecules. In order to minimize NSB, it is necessary to block unoccupied antibody binding sites on MUA chip prior to injection of cell samples. For this purpose, BSA, Tween 20 or milk have been generally used for reducing electrostatic and non-electrostatic NSB on the surface of chip (Kyo et al., 2005; Lausted et al., 2008; Shen et al., 2012). In the present study, BSA was used as the blocking agent. This molecule not only has a good stability and low price, but also is able to block the non-target sites by saturating the chip surface (Cecchetto et al., 2015). The RUs values obtained for 0.01%, 0.1%, 0.5% and 1% BSA were 240, 220, 120 and 110 RU×10^{-4}, respectively during HUVECs injection for 5 min (Fig. 2a). No significant difference was observed in the signal intensity recorded for both 0.5% and 1% BSA solution when the same number of cells (10^5 cells) was injected on the chip surface coated with BSA. The injection of the same cell population on the bare gold sensor represented signal intensity around 480 RU×10^{-4}. This indicates that 0.5% BSA yields 4-fold reduction in the rate of NSB compared to the bare gold surface. Therefore, we used 0.5% BSA for NSB. Also, to find the optimum flow rate, the cell injection was carried out at three different flow rates of 10, 50 and 90 μl/min. After setting the cell number at 10^5 cell individually for each profile injection, cell samples were injected on top of the surface over a period of 5 min. As shown in Supplementary Fig. 2, the most appropriate result was obtained with flow rate of 50 μl/min. Therefore, the cell injections were performed at this flow rate.

3.3. Determining cell binding capacity to the antibody-coated chip by SPR

To measure the selectivity of a VE-cadherin-coated gold biosensor for binding of pure cell populations, HUVEC, as a positive cell line, and HepG2, as a negative cell line, were injected on the surface. We recorded 750 and 90 RU×10^{-4} values for HUVECs and HepG2 cells, respectively, following the injection on the VE-cadherin-coated chip surface (Fig. 2b). After subtracting NSB response, a marked decline in the RU values of HUVECs from 610 to 50 RU×10^{-4} in HepG2 cell was observed. Unlike endothelial cells (ECs) that express VE-cadherin on their surface, HepG2 are negative for VE-cadherin expression. The lack of these molecules on the surface of HepG2 accounts for the lower signal intensity observed in the injection of HepG2 on the VE-cadherin-coated chip surface in comparison to the HUVECs injection. At the end of each cell injection, the SPR sensor was examined under cell imaging instrument for qualitative evaluation of the presence of cells on the chip surface following the Ab-Ag interaction. The cells were also fixed on the biosensor surface and stained with DAPI solution for cell visualization. Interestingly, the number of bound cells was proportional to the signal intensity and the RU recorded (Fig. 2c and d). No marked differences ($p < 0.05$) were observed in the amount of RU following injection of HUVEC, HepG2, hAMSCs on the BSA chips due to the lack of any specific cell binding sites on the chip surface (Fig. 3). Considering high-level expression of the specific marker in EC lineage, HUVECs expressed a high amount of VE-cadherin, while HepG2 cells and hAMSCs, during the first and second days of differentiation, and did not show detectable levels of VE-cadherin ($p < 0.05$) (Fig. 3). All changes in the rate of NSB on BSA-coated sensor were in the range of 90–150 RU×10^{-4}, but it increased at the end of endothelial differentiation of hAMSCs (days 1–14). Due to a direct relationship between the mass size on the sensor surface and the RU values, the RU intensities were amplified as the cell morphology and size of the cells changed through the differentiation procedure. It is critical to note that in sensors coated with VE-cadherin antibody, the RU values in HUVECs were distinguishable from those recorded for HepG2 and differentiating hAMSCs on day 3, 5, 7 and 14 ($p < 0.05$) (Fig. 3 and Supplementary Fig. 3). Consistent with endothelial differentiation of SCs, a high level of VE-cadherin was encoded on the cell surface resulting in an increased attachment of the cells to the immobilized antibody on the chip surface. This attachment, in turn, caused profound changes in the refractive index values in the detection region which were reflected as increased RU intensities during differentiation stages. After subtracting the NSB responses, we recorded 0, 80, 120, 360, 510 and 610 RUs×10^{-4} for differentiating hMSCs following 1, 2, 3, 5, 7 and 14 days, respectively (Supplementary Fig. 4).

The sensitivity of the VE-cadherin-coated biosensor and the number of bounded cells on the biochip was also evaluated by DAPI staining and visualization with Cytation™ system after cell injection. The number of bounded cells was found to be ≈27 cells per mm^2 in VE-

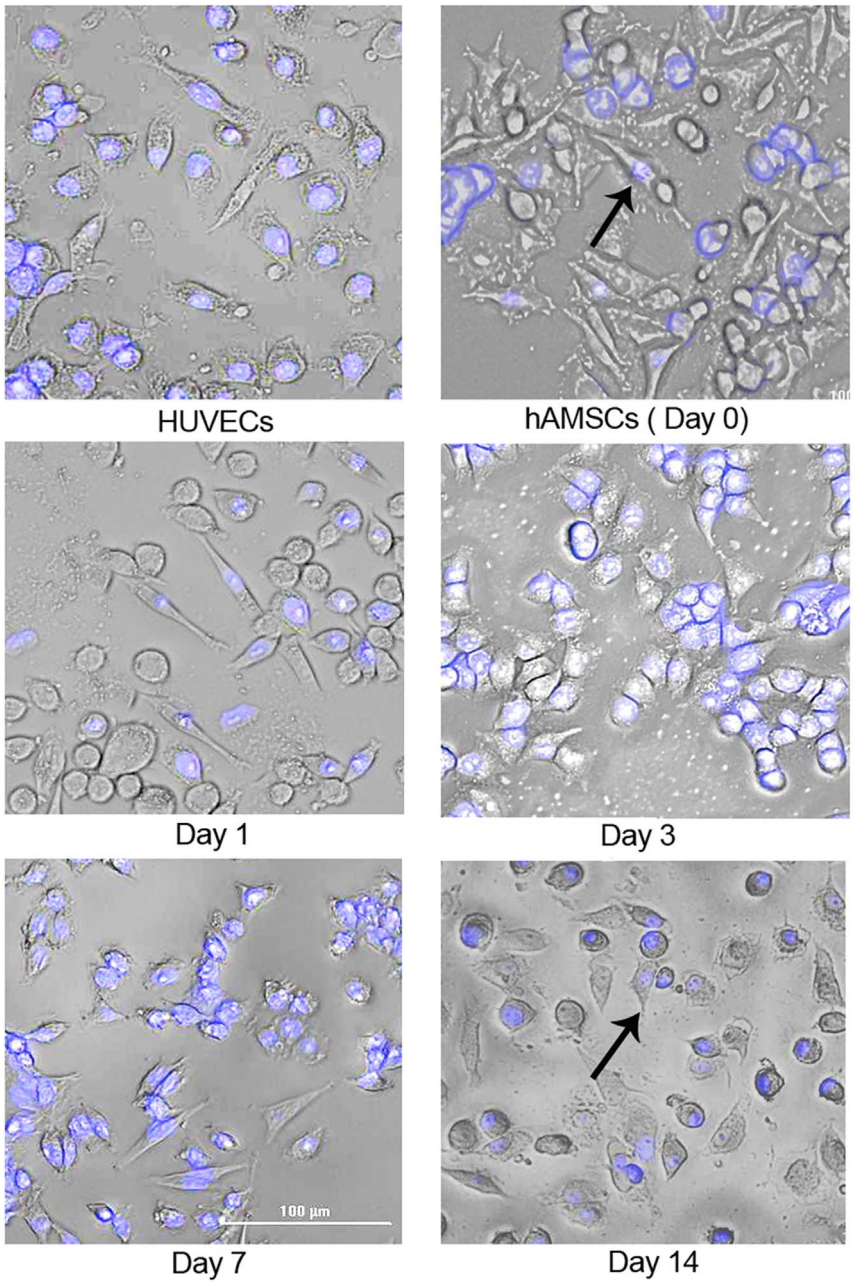

Fig. 6. The morphological change of hAMSCs during differentiation into the endothelial lineage. Cells were transformed from spindle-shape into an epithelial-like appearance by day 14 as indicated by the arrows.

cadherin-coated surface on the 3rd day of differentiation (Supplementary Fig. 5). Also more than 75% of the cells were found to be alive on the biochip surface during SPR analysis.

The ability of the proposed method to detect ≈27 cells per mm^2 during 5 min is a noticeable achievement. Usually, the number of cells quantified by SPR techniques is higher than ≈27 cells/mm^2 detected in the present study. Cortes and colleagues (Cortès et al., 2011) have been able to detect 700 murine macrophage cells/mm^2 during 40 min by a technology associating antibody microarrays with surface plasmon resonance imaging system. VE-cadherin can be also identified through ELISA (Gulino et al., 1998) and Western blot (Doulgere et al., 2015) analysis in cell lysate. However, these methods require more than 1,000,000 cells and fluorescent labeling techniques but this biosensor in label free form able to detect of ve-cadherin expression in live cells through 100,000 cell injection on top of chip.

3.4. VE-cadherin expression analyzed by flow cytometry

Flow cytometry is able to determine a small numbers of desired fluorescent cells population against a high background of non-fluorescent cells. To compare the sensitivity of SPR technique with flow cytometry in detecting differentiating cells, we also used a fluorescent-tagged antibody against VE-cadherin. The expression of VE-cadherin increased during differentiation toward endothelial lineage and reached a maximum level at the end of the experiment as compared to the initial time (62.8 ± 26.9% vs. 0%) (Fig. 4). The flow cytometric method was unable to discriminate the hAMSCs expressing VE-cadherin during the first 4 days of the differentiation, especially on day 0, 1 and 2, toward endothelial lineage. It seems that the developed SPR method is more sensitive than the flow cytometry in detection of the differentiating cells at the early stage.

3.5. Immunofluorescence imaging

Similar to the results obtained by flow cytometry, immunofluorescence visualization confirmed that the detectable levels of VE-cadherin were distinguishable in the later stage of the differentiation period (Fig. 5). Noticeably, no obvious levels of endothelial specific markers were visualized during first 5 days. During 7 and 14 days of differentiation, we imaged a large number of immunoreactive cells. As above-mentioned, it is noteworthy to acclaim that both the flow cytometric analysis and immunofluorescent staining are incapable of differentiating cells into an endothelial lineage at the early stage indicating the higher sensitivity of the SPR method developed in the present study compared to both flow cytometric and immunofluorescent staining techniques.

3.6. Morphological analysis of during endothelial differentiation of hAMSCs

At the initial time, the adherent hAMSCs showed a fibroblast-like morphology with whirlpool patterns (Tamagawa et al., 2007) (Fig. 6). We observed that endothelial differentiation of hAMSCs coincided with morphological changes into epithelial-like appearance (Fig. 6). The morphological changes along with the expression pattern for VE-cadherin are identical to the endothelial differentiation.

4. Conclusion

In the present study, the developed SPR technique could sense the early stage differentiation of hAMSCs on day 3 in label-free form without affecting cell viability, but flow cytometry and fluorescent microscopy methods were not able to detect the cell differentiation at the same time. This sensitive method presents hopeful views for monitoring and identification of rare and specific cell populations like tumor cells, cancer stem cells and etc. Furthermore, the application of SPR based biosensor for differentiation of stem cells can be further improved with enhancement technique of SPR by nanoparticles in order to achieve more effective evaluation of cell surface markers. In the current study, we did not monitor real-time changes in the level of VE-cadherin over a period of experimental procedure. Considering different endothelial lineage markers during cell differentiation could help us for better understanding of phenotypic shift.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bios.2017.05.018.

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