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Steric Hindrance Assay for Secreted Factors in Stem Cell Culture

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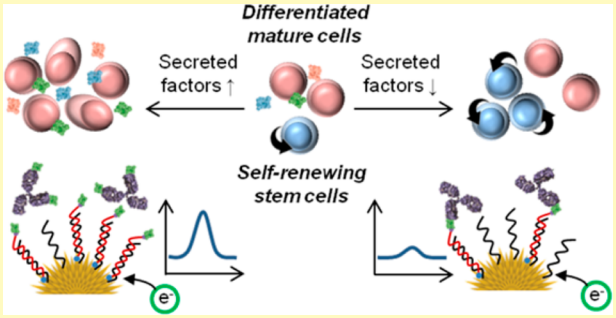
Supporting Information

ABSTRACT: The ex vivo expansion of hematopoietic stem cells is significantly inhibited by secreted proteins that induce negative feedback loops. The ability to effectively monitor these factors is critical for their real-time regulation and control and, by extension, enhancing stem cell expansion. Here, we describe a novel monitoring strategy for the detection of soluble signaling factors in stem cell cultures using a DNA-based sensing mechanism on a chip-based nanostructured microelectrode platform. We combine DNA hybridization engineering with antibody-capturing chemistry in an amplified steric hindrance hybridization assay. This method enables the quantification of important secreted proteins, showcased by the detection of 10 pg·mL^{-1} level concentrations of three proteins in stem cell culture samples. This approach is the first universal nonsandwich technique that permits pg·mL^{-1} level quantification of small proteins in stem cell culture media without signal loss.



KEYWORDS: *biosensors, DNA, electrochemistry, nanostructures, proteins, stem cells, steric hindrance*

Hematopoietic stem cell (HSC) transplantation is used as a clinical therapy for hematological pathologies including blood cancers and immune system disorders.[1,2] Umbilical cord blood is an appealing source of HSCs,[1,3] but its clinical use is limited by the low cell numbers available,[4] prompting the need for ex vivo expansion. Expansion is made especially difficult by the accumulation of endogenously produced signaling protein factors secreted from off-target cell populations, which promote unwanted differentiation.[5−7] Sensitive and specific detection of the various secreted proteins that regulate HSC expansion would enable control over their concentrations, improving ex vivo HSC growth.

Strategies to promote HSC expansion include attempts to minimize the influence of mature cells[7−10] through the supplementation of additional factors[11−14] and regular media exchange to slow the accumulation of secreted proteins.[7] Even at low concentrations, these factors have a strong impact on cell fate decisions,[15−17] with signals from mature blood cells leading to a net negative effect on HSC expansion (Figure 1).[6,7] The quantification of signaling factors would allow for the development of process control strategies to monitor and regulate the concentrations of these proteins. An integrated sensor to provide sensitive, real-time feedback on secreted proteins would thus be highly attractive as it would reduce the impact of secreted factors on HSC differentiation and enhancing HSC expansion.

The enzyme-linked immunosorbent assay (ELISA) is the current gold standard method for protein quantification, but the long process times involved, along with the labels and equipment required, make this method less convenient for in-line monitoring applications. Several other techniques exist for protein analysis, such as selected reaction monitoring,[18] Raman spectroscopy,[19] and surface plasmon resonance,[20] but these methods either do not lend themselves easily to integration due to the complex equipment required or do not have sufficiently low limits of detection. Other sensors are based on improving reaction kinetics to reduce process times and make use of assays employing aptamers[21] or microbeads,[22] but are limited in their widespread use due to the considerable development involved, high costs, and variability. Specific pg·mL^{-1} protein quantification in complex media using techniques amenable to automation is difficult to achieve and poses a significant challenge.

Electrochemical sensors are an attractive option for protein monitoring due to their versatility, integration capability, and excellent sensitivities/low limits of detection.[23−38] In particular, chip-based platforms that make use of modified surfaces such as

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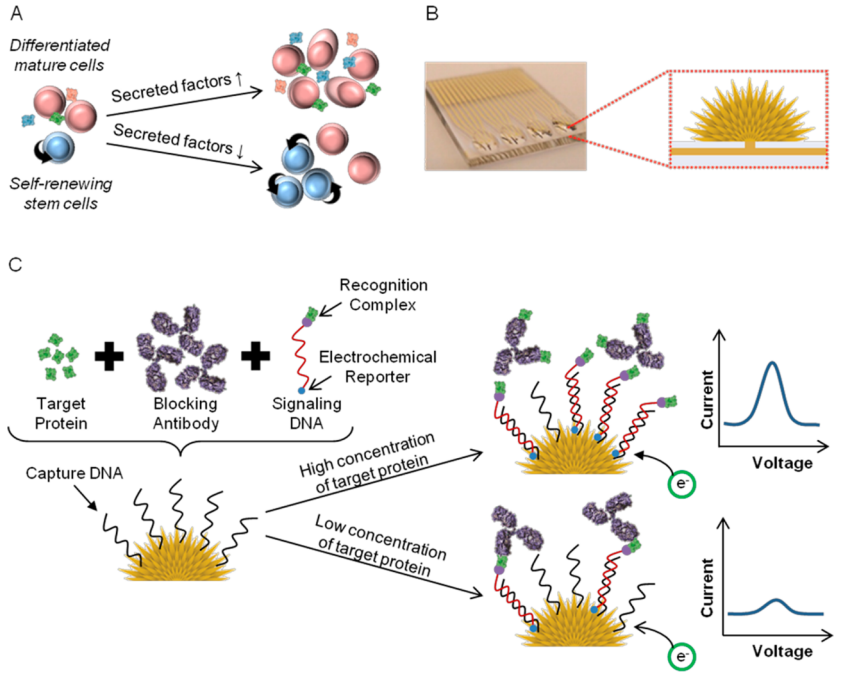

Figure 1. Schematic showing the influence of secreted factors on stem cell culture and an overview of the chip-based electrochemical detection scheme. (A) Simplified schematic of the interactions between soluble factors and cell subpopulations. As the concentrations of signaling proteins increase, mature cells (pink) accumulate as HSCs (blue) tend toward differentiation. As the concentration of secreted factors is decreased through media dilution, their impact is reduced, promoting the proliferation and self-renewal of HSCs. (B) Chip layout. Contacts are formed from circular apertures in a layer of SU-8 covering a gold pattern on chip surface. (C) Schematic representation of ASHHA on NMEs. Samples containing the target protein are preincubated with the blocking antibody. The samples are then mixed with signaling DNA strands labeled with both the electrochemical reporter and the recognition element before on-chip incubation.

nanostructured microelectrodes (NMEs) can achieve multiplexed immunosensing of several factors and have improved sensitivity compared to that of planar surfaces. While several blocking assays or sandwich assays have been developed for the analysis of proteins using antibody-modified sensors,[39−42] the detection of low molecular weight proteins at low concentrations has remained difficult. Given that most secreted factors involved in stem cell differentiation in culture are small proteins with 100 amino acids or fewer, new assay configurations are needed to target the important application of stem cell culture engineering.

Here, we describe a novel method for the quantification of signaling proteins in primary stem cell cultures using a sensitive on-chip detection strategy. Drawing inspiration from the design of a recently developed assay that uses steric hindrance effects to detect large proteins, namely, antibodies and streptavidin,[27] we report on a powerful approach to the analysis of small secreted proteins. Only by combining the use of size-controlled DNA hybridization engineering on three-dimensional gold NMEs with an alternative competitive antibody attachment scheme, we were able to improve on the original assay to enhance steric hindrance effects in a new amplified steric hindrance hybridization assay (ASHHA) for the detection of small proteins. We present a highly specific protein capturing system and engineer a wide dynamic range from 10 pg·mL^{-1} to 10 ng·mL^{-1} for a number of targets that are important for stem cell expansion.

The glass microchips used as the sensor platform and the protein detection strategy are illustrated in Figure 1. Gold contacts covered in SU-8 with 5 μm apertures formed the templates for the 100 μm electroplated NMEs. The surface of the NMEs is functionalized with immobilized thiolated capture DNA strands to form a high density DNA monolayer.

As shown in Figure 1, the detection of small secreted proteins is accomplished by monitoring the competitive binding of a blocking antibody. The target protein is attached to a strand of DNA, which is also labeled with the redox-active reporter methylene blue; this is referred to as the signaling DNA. If the target protein is present in solution at a high concentration, the blocking antibody will not bind to the conjugated target on the signaling DNA, and this molecular species is therefore free to bind to the electrode surface, producing a significant level of electrochemical current. If the target protein is not present, the blocking antibody binds to the signaling DNA, suppresses the hybridization of the DNA at the sensor surface because of steric hindrance, and decreases the level of electrochemical current observed. For intermediate levels of protein, the amount of current would then be proportional to the concentration of target protein. This approach links increases in current with increased concentrations of protein in solution irrespective of the size of the target. We take advantage of the competition chemistry in the ASHHA approach and, through the incorporation of the NME platform and the inclusion of a large antibody, enable the sensitive detection of small secreted proteins at low concentrations.

We selected three analytes to demonstrate the effectiveness of this approach: (1) regulated on activation, normal T cell expressed, and secreted (RANTES); (2) macrophage-derived chemokine (MDC); and (3) transforming growth factor-β1 (TGF-β1). All three are secreted factors deleterious to HSC expansion.[43,44] These factors are significant modulators in stem cell culture and therefore monitoring and controlling the

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concentrations of these proteins is important. As TGF-β1 is only present in culture in latent form and cannot be measured through antibody detection without an activation step, we used the latency-associated peptide (LAP) as a surrogate. LAP binds to TGF-β1 to form the Small Latent Complex[45,46] and its concentration correlates very well to that of TGF-β1.[22] We generated target/DNA complexes corresponding to all three proteins and monitored the steric effects resulting from the binding of the blocking antibody to the DNA signaling reporter by measuring the differences among electrochemical signals obtained upon hybridization (Figure 2A). The DNA–protein

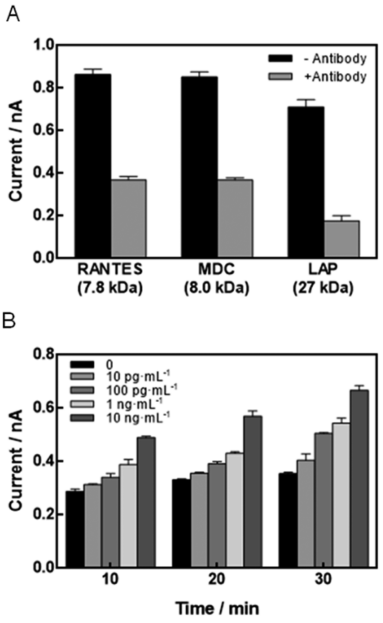

Figure 2. (A) Signal changes upon conjugation of the RANTES, MDC, and LAP recognition complexes and their respective antibodies to the DNA signaling strands. (B) Electrochemical signals obtained as a function of time and concentration for the RANTES assay.

conjugates were incubated on-chip at a concentration of 30 nM and the signal was measured after 40 min. Square wave voltammetry was used to scan the sensors for the detection of methylene blue, with the reduction potential peak located at −0.25 V versus Ag/AgCl.

As shown in Figure 2, the presence of antibodies against RANTES, MDC, or LAP all caused large changes in the electrochemical current in assays featuring corresponding DNA–protein signaling conjugates. Addition of the blocking antibodies caused signal changes of 58%, 59%, and 75% in terms of gain reduction for RANTES, MDC, and LAP, respectively, confirming the ability of the sensor to detect each of the three target proteins (Figure 2A). The largest protein, LAP, produced the largest change in signal, but the smaller proteins also produced measurable signal changes. We also investigated the time and concentration dependence of the assay for RANTES (Figure 2B), and observed that discernible signal changes could be detected as early as 10 min.

To determine the dynamic range of this sensor for the detection of signaling proteins, a concentration series was performed using RANTES and a human RANTES antibody (Figure 3). A sample of each concentration of RANTES was mixed with the blocking RANTES antibody and then incubated with the RANTES-bound signaling strands. The solution was

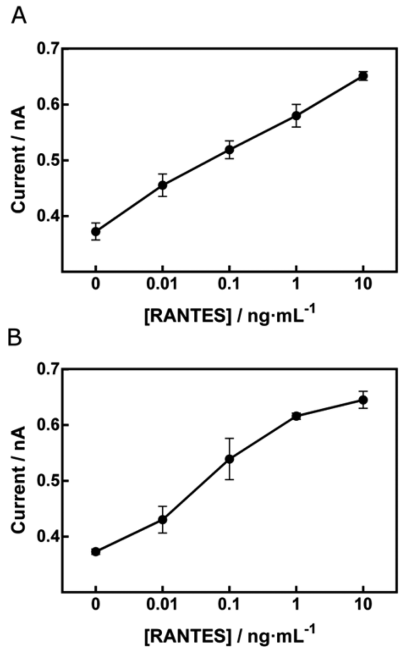

Figure 3. Square wave voltammetry-derived currents detected at electrodes with varying concentrations of RANTES in (A) buffer and (B) cell culture media.

pipetted onto chips containing NMEs functionalized with capture strands and each electrode was scanned after 30 min. In buffer solution, the sensors were able to detect concentrations ranging from 10 pg·mL^{-1} to 10 ng·mL^{-1} (Figure 3A). This range of detection sensitivity has practical significance as these proteins are typically present in culture at concentrations between 10 pg·mL^{-1} and 1 ng·mL^{-1}.[47] The change in current was measured for each concentration of protein, and the greatest reduction in current was observed for the lowest protein concentrations.

In order to demonstrate the utility of ASHHA for in-line monitoring of signaling proteins, detection was also performed in cell culture media, with RANTES spiked into media (Figure 3B). The assay is shown to be sensitive even in media, with no signal drift or change in current. Furthermore, it is specific enough to function selectively in a heterogeneous solution.

Electrochemical sensors have the advantage of being versatile, and can be easily integrated into a culture system with the potential for multiplexing. We highlighted our approach by using these sensors to analyze samples drawn from a HSC fed-batch bioreactor culture system that was made to dilute soluble factors (Figure 4A).[47] Fresh media was added to the culture each day to dilute the solution, lowering the concentration of the secreted factors and thus minimizing their impact on HSC expansion. Samples were taken from the culture every 4 days over the course of 12 days, with the sample from day 0 containing no secreted factors and treated as the baseline signal control sample. The electrochemical current levels of each of the signaling proteins of interest (RANTES, MDC, and TGF-β1) were measured from these samples, compared to measured calibration curves, and converted to concentrations, the results of which were validated through comparison with measurements from an ELISA (Figure 4). The results obtained using the electrochemical strategy compared very favorably with the ELISA method, indicating that this new

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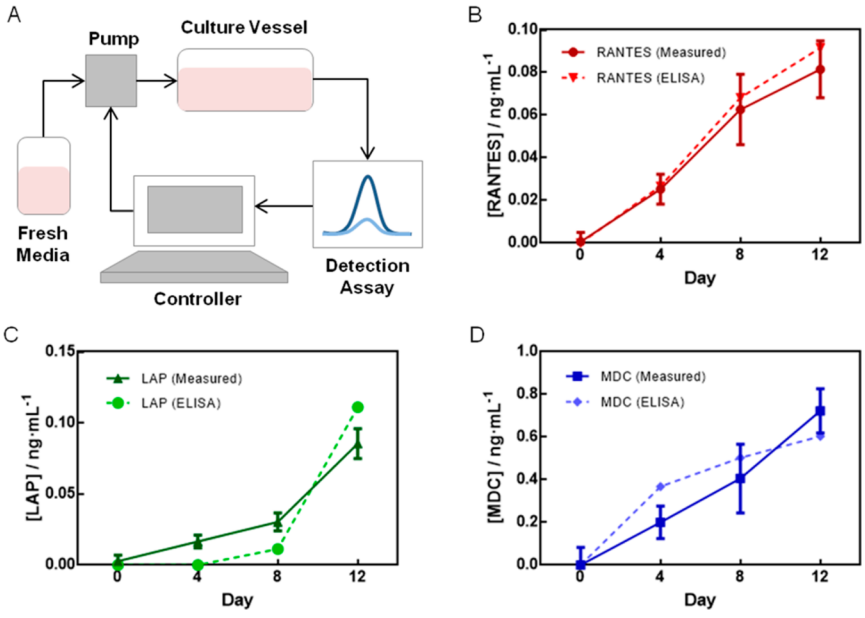

Figure 4. Electrochemical detection directly in cell culture media samples. (A) At-line protein monitoring schematic. Measured concentrations obtained for specific detection of (B) RANTES, (C) LAP, and (D) MDC in culture samples compared against ELISA measurements from the same samples. Currents obtained from electrochemical measurements for each factor were normalized according to calibration curves and converted to concentrations.

approach displays comparable accuracy relative to the gold standard.

The protein sensing strategy described here employs a novel approach for the sensitive detection of small proteins and specifically for monitoring soluble signaling factors in stem cell cultures. The integration of ASHHA on a nanostructured microelectrode platform and the enhancement of steric effects by size-controlled surface hybridization engineering are imperative to the detection strategy. The approach reported is the first universal nonsandwich technique that permits the specific and sensitive pg·mL^{-1} level quantification of small proteins in complex stem cell culture media without loss or drift in signal. We have demonstrated the analysis of a number of proteins of different sizes with sensitive detection limits in complex media, and the ability to produce data comparable to the established ELISA approach. Along with the potential for integration with a bioreactor to improve HSC expansion, this approach presents an attractive option for at-line sensitive protein quantification.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssensors.7b00136.

Materials, chip and sensor fabrication, conjugation of protein to DNA, electrochemical and ELISA measurements, and MDC, LAP, and extended RANTES binding curves (PDF)

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Notes

The authors declare no competing financial interest.

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