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Novel surface-enhanced Raman scattering-based assays for ultra-sensitive detection of human pluripotent stem cells

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ABSTRACT

Human pluripotent stem cells (hPSCs) are a promising cell source for regenerative medicine, but their derivatives need to be rigorously evaluated for residual stem cells to prevent teratoma formation. Here, we report the development of novel surface-enhanced Raman scattering (SERS)-based assays that can detect trace numbers of undifferentiated hPSCs in mixed cell populations in a highly specific, ultra-sensitive, and time-efficient manner. By targeting stem cell surface markers SSEA-5 and TRA-1-60 individually or simultaneously, these SERS assays were able to identify as few as 1 stem cell in 10^6 cells, a sensitivity (0.0001%) which was ~2000 to 15,000-fold higher than that of flow cytometry assays. Using the SERS assay, we demonstrate that the aggregation of hPSC-based cardiomyocyte differentiation cultures into 3D spheres significantly reduced SSEA-5^+ and TRA-1-60^+ cells compared with parallel 2D cultures. Thus, SERS may provide a powerful new technology for quality control of hPSC-derived products for preclinical and clinical applications.

1. Introduction

Owing to their distinct self-renewal and differentiation properties, human pluripotent stem cells (hPSCs), either human embryonic stem cells (hESCs) or human induced pluripotent stem cells (iPSCs), hold great potential as a cell source for regenerative medicine. However, these properties also make them potentially tumorigenic—even a small number of undifferentiated hPSCs are sufficient to generate teratomas when transplanted *in vivo* [1,2]. Hence, the risk of tumorigenesis is a major concern for the clinical translation of all hPSC-derived products [3]. Animal studies documenting the risk of teratoma formation following transplantation of hPSC-derivatives have spurred efforts to evaluate and enhance the safety of hPSC-based therapies [4–10]. For clinical safety, a highly sensitive and specific quality control assay is required to determine the number of undifferentiated cells in hPSC-derived products.

In current practice, cell-based assays such as flow cytometry can detect undifferentiated cells when present at ~0.1% or higher in a mixed cell population [11], which is insufficient sensitivity to ascertain that a cell preparation for transplantation contains a number of hPSCs below the threshold for teratoma formation. A study in mice reported that 10^4 undifferentiated cells were sufficient to produce tumors *in vivo* [2]. Accordingly, if an estimated 10^9 cells are required for a single transplantation for heart failure [12], the sensitivity of assays used to detect residual undifferentiated cells needs to be 1 stem cell in a background of 10^5 cells (0.001%), which is unachievable via flow cytometry.

A prevailing method to evaluate the risk of teratoma formation is to inject cell products into SCID mice and evaluate tumor formation after at least 3 months [3,13–15]. While this method may provide a direct assessment of tumorigenicity, it is highly impractical as a quality-control assay due to its non-quantitative, non-

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scalable, costly, and time-consuming nature. Therefore, an assay that is fast, highly sensitive, and efficient in detecting a trace number of undifferentiated cells is imperative for assessing the safety of hPSC-derived products.

Nanoparticle-based surface-enhanced Raman scattering (SERS) technology is gaining momentum in biomedical applications such as molecular multiplex detection, pathogen and cell detection, and *in situ* imaging [16–21]. When conjugated with biomolecular targeting ligands, Raman reporter-labelled gold (Au) nanoparticles can be used to detect specific molecules with high specificity and sensitivity [19,21–23]. SERS detection produces a sharp, fingerprint-like spectral pattern that is distinct from other interference patterns in a complex biological environment. This is uniquely advantageous when detecting a low number of cells, since conventional fluorescence signals may be masked by the scattering signals of background cells [20,21]. In this study, we developed SERS-based assays targeting the hPSC surface markers stage-specific embryonic antigen-5 (SSEA-5) and TRA-1-60 to detect residual undifferentiated hPSCs with high specificity and sensitivity. Using our newly developed assays, we efficiently detected SSEA-5^+ and TRA-1-60^+ cells at sensitivities several orders of magnitude higher than flow cytometry assays. As such, these assays represent a rapid, efficient, and economic method for assessing the safety of hPSC-based products for pre-clinical and clinical applications.

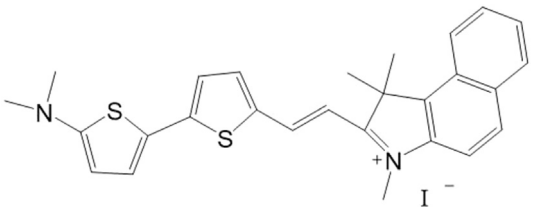
2. Materials and methods

2.1. Materials

Ultrapure water (18 MΩ cm^{-1}) was used to prepare all aqueous solutions. The following chemicals were used without further purification: 60 nm citrate-stabilized gold nanoparticles (2.6×10^{10} particles/mL) (Ted Pella Inc.), black hole quencher (BHQ) (Biosearch Technologies), PEG-SH (MW = 5,000 and 20,000 Da) (Rapp Polymere, Germany), SSEA-5 IgG1 antibody (Stemcell Technologies), and TRA-1-60 IgM antibody (Millipore). All other reagents were obtained from Sigma-Aldrich at the highest purity available.

2.2. BIDI reporter molecule

The molecular structure of (E)-2-(2-(5'-(dimethylamino)-[2,2'-bithiophen]-5-yl)vinyl)-1,1,3-trimethyl-1H-benzo[e]indol-3-ium iodide (BIDI) is shown here. The synthesis of BIDI will be reported later in another work.



BIDI ^{1}HNMR (DMSO, 500 MHz): δ = 8.61–8.64 (d, 1H, $C_{10}H_6$), 8.36–8.38 (d, 1H, $C_{10}H_6$), 8.21–8.23 (d, 1H, C_2H_2), 8.15–8.17 (d, 1H, $C_{10}H_6$), 8.04–8.05 (d, 1H, C_4H_2S), 7.97–7.99(1, H, C_2H_2), 7.74–7.77 (m, 1H, $C_{10}H_6$), 7.63–7.66 (m, 1H, $C_{10}H_6$), 7.56–7.57 (d, 1H, C_4H_2S), 7.37–7.38 (d, 1H, C_4H_2S), 6.84–6.87 (d, 1H, $C_{10}H_6$), 6.16–6.17 (d, 1H, C_4H_2S), 4.05 (s, 3H, CH_3), 3.08 (s, 6H, CH_3), 1.98 (s, 6H, CH_3).MALDI-TOF-MS: *m*/*z*433.0 ($M\text{-}I^-$).

2.3. Preparation of SSEA-5-conjugated and TRA-1-60-conjugated nanoparticles

Au nanoparticles were labelled with Raman reporters as described previously [24], conjugated with SSEA-5 (IgG1) or TRA-1-60 (IgM) antibodies, and then coated with polyethylene-glycol (PEG). Amine function group of TRA-1-60 IgM antibody was modified to couple with a streptavidin linker for 3 h at room temperature. Excess glycine was used to quench the un-reacted linker. The bioconjugation of SSEA-5 or TRA-1-60 antibodies with nanoparticles was carried out using previously reported procedures [24]. Briefly, the 60 nm citrate-stabilized Au nanoparticles were labelled with BHQ reporter molecules via adsorption to the negatively charged Au nanoparticle surface through electrostatic interaction.

To prepare Au nanoparticles conjugated with SSEA-5 or TRA-1-60 antibodies, Au-BHQ nanoparticles first were reacted with varying quantities of antibodies (10, 25, 50, 100, 200 antibodies/ligands per particle). The reaction was performed at room temperature with shaking for 2 h and the mixture was incubated at 4 °C overnight. Complete PEGylation of the unreacted gold surface required the introduction of excess 10 μM PEG-SH into the reaction mixture and was performed at room temperature with shaking for 1 h. The reaction mixture was centrifuged at 4000 × g for 20 min, followed by removal of the supernatant fraction that contained unreacted antibodies and PEG-SH. Antibody-conjugated nanoparticles formed a pellet at the bottom of the reaction vial. The pellet was washed three times with 1 mL of 1 × PBS and repetitive centrifugation. The final concentration of the bio-conjugated Au nanoparticles was determined by the measurement of UV–Vis absorption spectrum using a Raman detection system. For the multiplexing assay, SSEA-5 antibodies were conjugated with Au nanoparticles labelled with the BIDI reporter and TRA-1-60 antibodies with Au nanoparticles labelled with the BHQ reporter.

Antibody-conjugated nanoparticles were characterized as described previously [24] with minor modification. UV–Vis absorption spectra were recorded on a Shimadzu (UV-2401) spectrometer using disposable polyacryl cuvettes. Transmission electron micrographs (TEM) were taken with a Hitachi H7500 high-magnification electron microscope. For the preparation of samples for TEM, nanoparticle samples (5 μL each) were dropped onto copper 200 mesh grids that were pre-treated with UV light to reduce static electricity. After 30 min, the solvent was drained with a filter paper.

SERS spectra were recorded on a compact Raman system (Sierra, Snowy Range Instruments) using 785 nm (70 mW) excitation. The scanning laser beam has a footprint of 1.7 mm diameter with 25 μm focal size. SERS intensities were normalized to the Raman spectra of cyclohexane and polystyrene to correct for variations in optical alignment and instrument response. The spectral resolution was approximately 4 cm^{-1}. Typical spectrum acquisition time is 1 s.

2.4. Cell culture

Human induced pluripotent stem cells (IMR-90 iPSCs, WiCell-Research Institute) and human embryonic stem cells (H7 hESCs [25], NIH registration no. 0061) were maintained in Essential 8™ (E8) medium (Life Technologies) on growth factor-reduced Matrigel™ (1:60, v/v, Fisher Scientific Inc.). NIH3T3 fibroblasts were maintained in DMEM medium supplemented with 10% FBS. Primary rat cardiomyocytes were isolated as described previously [26]. All cultures were under standard culture conditions (5% CO_2, 37 °C).