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Imaging of epidermal growth factor receptor on single breast cancer cells using surface-enhanced Raman spectroscopy

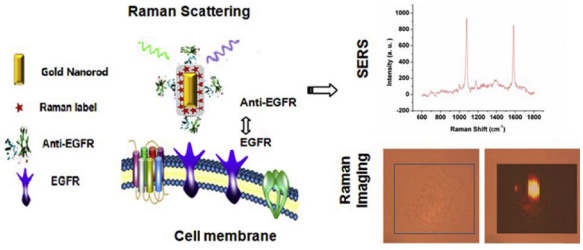
Lifu Xiao [a], Sitaram Harihar [b], Danny R. Welch [b], Anhong Zhou [a,*]

[a] Department of Biological Engineering, Utah State University, Logan, UT 84322-4105, USA
[b] Department of Cancer Biology, The University of Kansas Medical Center and The University of Kansas Cancer Center, Kansas City, KS 66160, USA

HIGHLIGHTS

- Gold nanorods based SERS probe was successfully developed for biomarker detection.
- Cancer biomarker EGFR was detected on single cancer cells.
- SERS probe was applied to detect EGFR expression in four cancer cell lines.
- SERS offers noninvasive and dynamic imaging modality for biosystems.

GRAPHICAL ABSTRACT



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ABSTRACT

Epidermal growth factor receptor (EGFR) is widely used as a biomarker for pathological grading and therapeutic targeting of human cancers. This study investigates expression, spatial distribution as well as the endocytosis of EGFR in single breast cancer cells using surface-enhanced Raman spectroscopy (SERS). By incubating anti-EGFR antibody conjugated SERS nanoprobes with an EGFR-over-expressing cancer cell line, A431, EGFR localization was measured over time and found to be located primarily at the cell surface. To further validate the constructed SERS probes, we applied this SERS probes to detect the EGFR expression on breast cancer cells (MDA-MB-435, MDA-MB-231) and their counterpart cell lines in which EGFR expression was down-regulated by breast cancer metastasis suppressor 1 (BRMS1). The results showed that SERS method not only confirms immunoblot data measuring EGFR levels, but also adds new insights regarding EGFR localization and internalization in living cells which is impossible in immunoblot method. Thus, SERS provides a powerful new tool to measure biomarkers in living cancer cells.

1. Introduction

Epidermal growth factor receptor (EGFR), a receptor tyrosine kinase, is overexpressed in a variety of human cancers, including all breast cancer subtypes [1]. Overexpression of EGFR in breast cancer is generally associated with poor prognosis and high recurrence rates [2]. Since EGFR status is related to cancer progression, there has been extensive research to develop agents targeting EGFR and its corresponding signaling pathways [3–5]. Therefore, improved methods to quantify and measure function of EGFR in breast cancer cells could improve diagnosis and treatment of breast cancer.

Currently, the most commonly used methods to assess EGFR status in clinical cancer specimens are immunohistochemistry and immunofluorescence staining [6–8]. Quantification can be done using immunoblotting. However, these methods either need cell

Abbreviations: SERS, surface-enhanced Raman spectroscopy; EGFR, epidermal growth factor receptor; BRMS1, breast cancer metastasis repressor 1.

* Corresponding author. Tel.: + 1 435 797 2863; fax: 1 435 797 1248.
E-mail address: Anhong.Zhou@usu.edu (A. Zhou).

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fixation (immunohistochemistry and immunoblotting), or face the problem of photobleaching (immunofluorescence), rendering them non-suitable for measuring dynamic alterations of cell receptors and ligands.

Surface-enhanced Raman spectroscopy (SERS) is a powerful analytical tool in biological applications which has attracted considerable attention recently. SERS offers extremely high enhancement and turns the weak inelastic scattering effect of photons into a structurally sensitive nanoscale probe [9]. In turn, one can realize ultrasensitive levels of detection and non-invasive tagging of specific bioanalytes in living cells and animals [10]. A key to the SERS technique is the metal nanoparticle (NP, e.g., AuNP or AgNP) encoded with sensitive Raman reporter molecules followed by the coating of mono- or multi-layer protective polymers (e.g., silica, polyelectrolyte and PEG) which improve stability and biocompatibility [11–14]. Several studies have reported using SERS probes to target cancer cells in vitro or in vivo [11,14–19], including measurement of EGFR [11,15,19]. However, very little SERS studies were focused on EGFR cellular distribution, EGFR-mediated bioprocess, and how EGFR is regulated by metastasis suppressors.

Metastasis suppressors are a relatively recently described family of molecules that suppress the development of cancer metastasis without blocking primary tumor growth (reviewed in [20]). Of the approximately 30 metastasis suppressors (genes) identified to date, BRMS1 has been well characterized for its ability to regulate molecules that alter cellular response to microenvironmental signals which can be different between orthotopic sites (i.e., the mammary gland for breast cancer) and ectopic sites (i.e., sites of metastasis) [21], thought to explain why metastasis suppressors allow primary tumor growth, but not metastatic colonization. BRMS1 regulates EGFR [21] and osteopontin [22] expression, phosphoinositide [23], NFκB [24] and PKA [25] signaling, connexin expression and gap junctional intercellular communication [20,26], all of which play significant roles in cancer progression. The mechanism by which BRMS1 does these myriad things is thought to be as part of SIN3 histone deacetylase regulation of chromatin structure [27].

Understanding how BRMS1 directly impacts cellular responses to signals from the microenvironment is thought to be key to defining the critical mechanisms of action. Unfortunately, the tools to measure ligand-receptor or antibody–antigen interactions are suboptimal for this purpose. Therefore, we designed a SERS probe based on polyelectrolyte-coated gold nanorods (GNRs) to specifically recognize and detect EGFR molecules (via antibody–antigen interaction) on the cell surface of breast cancer cells. Using an EGFR over-expressing cell line (e.g., A341), we validated the ability of the antibody-conjugated SERS probe to measure EGFR distribution and internalization on single cancer cells. Then, using BRMS1-expressing cells and comparing them to their parental breast cancer counterparts, we demonstrated that our constructed SERS probe is able to distinguish EGFR levels in different cancer cells and to provide spatial information of EGFR expressed on single cancer cell surface.

2. Materials and methods

2.1. Materials

Ultrapure water ($18\,M\Omega\,cm^{-1}$) was used in this work. All chemicals were purchased from commercial source and were used as received: gold nanorods (5.1×10^{11} particles mL^{-1}, Nanopartz Inc., USA), monoclonal anti-EGFR antibody (Invitrogen). A431 cell line was obtained from American Type Culture Collection (ATCC). MDA-MB-435 (435), MDA-MB-231 (231), MDA-MB-435 expressing BRMS1 (435^{BRMS1}) and MDA-MB-231 expressing BRMS1 (231^{BRMS1}) were described previously [21]. Cell culture media and supplies were purchased from Thermo Fisher Scientific Inc. (Waltham, MA). Other chemicals were purchased from Sigma–Aldrich (St. Louis, MO) at the highest available purity.

2.2. Instrumentation

The morphology of the gold nanorods (GNRs) SERS probe was determined by a FEI Titan 80–300 transmission electron microscope (TEM) in a bright-field mode. Extinction spectra of the GNRs were taken by an Agilent Cary 60 UV–vis spectrophotometer controlled by Cary WinUV software. Dark field images of cell samples were obtained by using an Olympus IX71 Inverted Microscope equipped with an oil-immersed dark field condenser (NA = 1.5) and a 100× objective lens. Images were acquired using DPController software (Olympus).

2.3. Preparation of the SERS probe

As shown in Fig. 1a, the synthesis of the SERS probe includes three steps: (1) bare GNRs and Raman reporter molecules 4-mercaptobenzoic acid (MBA) were mixed together with a molar ratio of 1:10,000, conjugating the reporter molecules onto GNRs through Au-S interaction; (2) polyallylamine hydrochloride (PAH) solution ($28\,mg\,mL^{-1}$, 200 μL) and NaCl solution (1 mM, 100 μL) were added to 1 mL of GNRs solution containing 1 nM MBA-GNRs and reacted for 3 h; and (3) after removing the excess PAH by centrifugation, monoclonal antibody anti-EGFR ($0.21\,mg\,mL^{-1}$, 10 μL) was added to the solution and incubated for 1 h. Excess antibody was removed by centrifugation. The SERS probe was stable for several days at 4 °C in solution.

2.4. Cell culture

Cell lines were grown in a mixture of Dulbecco's-modified Eagle's medium (DMEM) and Ham's F-12 medium (1:1) supplemented with 5% fetal bovine serum (Atlanta Biologicals, Atlanta, GA) in a humidified atmosphere at 37 °C with 5% CO_2. Cells were at 80–90% confluence when used for experiments.

2.5. Immunoblotting

Cells were rinsed twice with ice-cold PBS and lysed in a buffer containing 25 mM Tris–HCl (pH 7.4), β-glycerol phosphate (50 mM), EDTA (0.5 mM), glycerol (5%), triton X-100 (0.1%), sodium orthovanadate (1 mM), benzamidine (1 mM), and a protease inhibitor cocktail containing aprotinin, leupeptin, and phenylmethylsulfonyl fluoride (Roche, Indianapolis, IN). Protein concentration was determined using a BCA assay (Pierce, Rockford, IL). Protein was denatured with Laemmli's buffer at 95 °C for 5 min and lysate (50 μg) was loaded to each well. Proteins were separated using 10% SDS-PAGE gel electrophoresis and resolved proteins were transferred to PVDF before incubating in Tris-buffered saline containing Tween-20 (0.05%) and fat-free dry milk (5%) for 1 h at room temperature. Membranes were incubated with primary antibodies to EGFR (Cell Signaling, Danvers, MA) β-Actin (Sigma, St. Louis, MO) and BRMS1 overnight at 4 °C and subsequently with HRP-conjugated secondary antibody at room temperature for 1 h. Signals were visualized using ECL (Pierce, Rockford, IL) following manufacturer's instructions.

2.6. Immunofluorescence imaging

To evaluate EGFR localization, MDA-MB-435/231 and 435^{BRMS1}/231^{BRMS1} cells grown on coverslips for 24 h were fixed using 4% para-formaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 20 min, and permeabilized using 0.1% Triton X-100 (Union Carbide Corporation, Texas City, TX) for 10 min.