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Application of surface enhanced Raman spectroscopy as a diagnostic system for hypersialylated metastatic cancers

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ABSTRACT

Early diagnosis of metastatic cancers could greatly limit the number of cancer-associated deaths. Aberrant surface expression of sialic acid (hypersialylation) on tumors correlating with metastatic incidence and its involvement in tumorigenesis and progression is widely reported; hence detection of hypersialylated tumors may be an effective strategy to identify metastatic cancers. We herein report on the application of phenylboronic acid-installed PEGylated gold nanoparticles coupled with Toluidine blue O (T/BA-GNPs) as SERS probes to target surface sialic acid (*N*-acetylneuraminic acid, Neu5Ac). Strong SERS signals from metastatic cancer cell lines (breast cancer; MDA-MB231 and colon cancer; Colon-26) were observed, contrary to non-metastatic MCF-7 cells (breast cancer). The detected SERS signals from various cancer cell lines correlated with their reported metastatic potential, implying that our T/BA-GNP based SERS system was capable of distinguishing the metastaticity of cells based on the surface Neu5Ac density. T/BA-GNP based SERS system could also significantly differentiate between hypersialylated tumor tissues and healthy tissues with high SERS signal to noise ratio, due to plasmon coupling between the specifically aggregated functionalized GNPs. Furthermore, we also confirmed reduction in SERS signals from MDA-MB231 surface upon treatment with our original reactive oxygen species (ROS)-scavenging polymeric micelle, nitroxide-radical containing nanoparticles (RNPs). The ROS-mediated abrogation of sialylation by impairing the activation of NF-κB-sialyltransferase signaling cascade upon RNP treatment was confirmed by expression studies and the T/BA-GNPs based SERS system. The aforementioned findings thus, establish T/BA-GNPs based SERS as a potential cytodiagnostic system to detect hypersialylated metastatic tumors and RNPs as anti-metastatic cancer drug candidates.

1. Introduction

Cancer is one of the most serious diseases worldwide and its early diagnosis, which can prevent cancer-associated deaths, is therefore, highly required. Enzyme-linked immunosorbent assay (ELISA), latex immunoassay (LIA), and radioimmunoassay (RIA) are promising techniques to diagnose cancer by detecting cancer-specific biomarker proteins in the blood [1–4]. Since the blood levels of the biomarker correlate with cancer progression, the aforementioned techniques fail to diagnose cancer until the biomarkers are released into the bloodstream. On the contrary, cytodiagnosis and histological diagnosis involve detection of cancers in the early-stage by staining tissue biopsies with hematoxylin and eosin (H&E) stain and Papanicolaou (Pap) stain [5,6]. Immunostaining of receptors that are overexpressed in different kinds of

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breast cancer, such as human EGFR-related 2 (HER2), estrogen receptor (ER), progesterone receptor (PR) and Ki-67, is also a popular method to detect and categorize breast cancer [7–11]. ER^-, PR^-, and $HER2^-$ triple-negative breast cancers (TNBC) are reported to be more aggressive and associated with poorer prognosis compared to other breast cancer types [12]. However, TNBC cell lines/tissues are heterogeneous; for example, MDA-MB231 shows high metastasis, whereas MDA-MB468 is comparatively less metastatic [13–15]. Thus, classification of so far uncategorized TNBC cell lines/tissues can facilitate defining their degree of metastatic risk.

Recent advances in metastatic cancer research have clearly demonstrated that high cell surface density of sialic acid in malignant transformed cells correlates with their metastasis incidence [16–18]. Sialic acids consist of 5-amido-3,5-dideoxy-D-glycero-D-galacto-nonulosonic acid (neuraminic acid) covalently bound (the reaction is catalyzed by sialyltransferase) to terminal oligosaccharides attached on lipid or protein moieties. *N*-acetylneuraminic acid (Neu5Ac) is the most ubiquitous biosynthetic precursor of sialic acids [19]. Many findings suggest that aberrant sialylation on tumors contributes to tumorigenesis and tumor progression by evading apoptosis, promoting invasion and metastasis, and conferring resistance to chemo- and radio- therapies [16]. Yogeeswaran and Salk reported that the sialylation on murine cell lines in culture positively correlated with their metastasis potential [20]. It has also been reported that sialyltransferase inhibitor soyasaponin I significantly impaired the metastatic potential of MDA-MB231 and enhanced the adhesion ability of MCF-7 [21]. Since Neu5Ac on the surface of normal cells is involved in many versatile functions in our body, such as cell proliferation, cell adhesion, and angiogenesis [22], a new diagnostic system is required that can detect the baseline Neu5Ac expression level on cancer cells associated with their metastatic potential.

Furthermore, suppression of sialic acid-mediated metastasis via inhibition of sialyltransferase could be a potential strategy to inhibit metastasis-associated cell death. Hatano and coworkers reported that sialic acid levels in human castration-resistant prostate cancer cells are regulated by NF-κB via the action of sialyltransferase [23]. It has also been reported that reactive oxygen species (ROS) activate NF-κB via the mitogen-activated protein kinase (MAPK) signaling cascade [24–26]. Scavenging of aberrant levels of vital ROS in tumors may decrease metastasis by impairing the activation of the MAPK-NF-κB signaling cascade, which consequently may lead to the inhibition of sialylation.

Recently, applications of functionalized gold nanostars based surface-enhanced Raman scattering (SERS) technique in cancer theranostics and biomedical sensing is gaining momentum. Gold-based SERS has been reported to provide an excellent multimodality platform *in vivo*, owing to non-invasiveness and high sensitive chemical analysis due to plasmon coupling between the functionalized gold particles, which results in amplification of the SERS enhancement factor, e.g. application of gold nanostars to monitor real-time drug delivery in *in vitro* and *in vivo* models based on SERS as proposed by Tial et al., and gold nanostar-based nanocomposite for application in photothermal tumor therapy in conjunction with magnetic resonance-SERS bimodal imaging proposed by Gao et al. [27,28].

Thiol-ended PEG is commonly used for surface modification of gold to form gold-sulfur bonds; however, such linkages can be dissociated by oxidation of thiol group [29,30], which may cause unwanted aggregation of GNPs before application and may disturb suitable SERS detection. We previously reported the synthesis of ligand-installed PEG-b-polyamine block copolymers [31] and confirmed that PEG-b-polyamine block copolymer gave high dispersion stability to gold nanoparticle developed from auric acid [32]. This information prompted us to develop phenylboronic acid-installed PEGylated GNP as SERS probes characterized by high dispersion stability to prevent non-specific aggregation of GNPs for its effective application in detection of surface Neu5Ac associated cancer metastaticity. In this study, we developed surface modified GNPs by the autoreduction of $HAuCl_4$ using the base polymer poly(ethylene glycol)-block-[poly(2-(*N*,*N*-dimethylamino)ethyl methacrylate)] (PEG-*b*-PAMA) as previously reported [32,33]. 3-Aminophenylboronic acid terminated PEG-*b*-PAMA (APBA-PEG-*b*-PAMA) was prepared to attain Neu5Ac targeting characteristics, as boronic acid selectively recognizes Neu5Ac [34]. To demonstrate the performance of this SERS-based detection system, Toluidine blue O (TBO, a Raman reporter) and APBA-PEG-*b*-PAMA co-immobilized on GNPs (T/BA-GNPs) were tested in *in vitro* and *ex vivo* experiments (Fig. 1). As mentioned previously, surface Neu5Ac exists in normal cells also; therefore, during application of this system to monitor the Neu5Ac levels on the surface of cancer cells, the signal originating from the low levels of Neu5Ac on the surface of normal cells should be eliminated to avoid false detection. T/BA-GNPs selectively attach to Neu5Ac, and since cancer cells are hypersialylated, the aggregated GNPs should produce a higher SERS signal to noise ratio (S/N) due to plasmon coupling [35], than monodispersed GNPs on normal cells [36].

We have also investigated if ROS-scavenging polymeric micelles, pH-insensitive radical containing nanoparticles (RNP^O), and pH-sensitive radical containing nanoparticles (RNP^N) modulated the expression of sialyltransferase and if the T/BA-GNPs SERS system could detect RNP-mediated reduction in sialylation. RNP^O and RNP^N are nitroxide radical-containing nanoparticles, consisting of the self-assembling amphiphilic block copolymers, methoxy-poly(ethylene glycol)-b-poly(4-[2,2,6,6-tetramethylpiperidine-1-oxyl]oxymethylstyrene)] (MeO-PEG-b-PMOT) and poly(ethylene glycol)-b-poly[4-(2,2,6,6-tetramethylpiperidine-1-oxyl)amino-methylstyrene] (MeO-PEG-b-PMNT), respectively [37,38]. Various therapeutic applications of RNPs mediated by their ROS scavenging characteristics, have been previously reported in various oxidative stress-induced disease models, such as intracerebral hemorrhage, ulcerative colitis, ischemia reperfusion injuries and various cancer models, with negligible adverse effects, longer blood circulation tendency, and higher biocompatibility and bioavailability than their low molecular weight ROS scavenging counter parts [39–48]. Here, abrogation of the crucial ROS-mediated suppression of sialic acid by RNPs via NF-κB pathway inhibition was studied in metastatic breast cancer cells, which was confirmed by expression studies and T/BA-GNPs-SERS system, thus establishing RNPs as potential anti-metastatic cancer drug candidates.

2. Materials and methods

2.1. Materials, reagents, and instruments

Tetrahydrofran (THF), 2-(*N*,*N*-dimethylamino)ethyl methacrylate (AMA), 2-propanol (IPA), tetrachloroauric acid tetrahydrate ($HAuCl_4 \cdot 4H_2O$), chloroform and benzene were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. 3,3-Diethoxypropanol and 3-aminophenylboronic acid were purchased from Sigma-Aldrich Co., St. Louis, MO, USA. Ethylene oxide (EO) was purchased from Air Water, Inc., Osaka, Japan. Prior to their use, THF, AMA, and EO were distilled after dehydration and 3,3-diethoxypropanol was distilled after reflux following conventional methods. All other chemicals were used as received.

Gel permeation chromatography (GPC) measurements to evaluate molecular weights were carried out using a Shodex GPC-101 system equipped with gel columns (Shodex KF-801, KF-803, KF-805). Proton nuclear magnetic resonance (^1H NMR) spectra were obtained with a JEOL JNM-ECS 400 (JEOL Ltd., Japan) instrument at