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Affordable and simple method for separating and detecting ovarian cancer circulating tumor cells using BSA coated magnetic nanoprobes modified with folic acid

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ABSTRACT

The detection of circulating tumor cells (CTCs) offers a noninvasive method for early-stage cancer diagnosis. Although this is a challenging process, the detection and enumeration of CTCs are essential for assessing cancer progression. In this study, we demonstrated an inexpensive and simple method that used folic acid, FA, as targeting probe to replace antibodies for the enrichment and isolation of ovarian cancer CTCs. Bovine serum albumin (BSA) was decorated on magnetic nanoparticles (MNP) to form BSA-MNP, while FA was conjugated on the BSA-MNP surface through a PEG_{2K}-linker. The FA-BSA-MNP complex showed efficient capture of ovarian cancer CTCs as low as 20 cells/mL. Moreover, this FA-BSA-MNP complex showed minimal nonspecific adsorption between the modified magnetic nanoprobes and ovarian cancer cells, resulting in high specific enrichment. More importantly, the MNP-BSA-FA complex was nontoxic to ovarian cancer cells. The isolated cancer cells remained highly viable, were readily grown *in vitro* without significant death rate with a viability of 92.7%, and without microscopically visible contamination. These results suggested that the FA-BSA-MNP complex could successfully be used for the capture of ovarian cancer CTCs from whole blood. Moreover, when combined with immunocytochemical staining method, the FA-BSA-MNP captured CTCs could be differentiated from the blood cells.

1. Introduction

Epithelial ovarian cancer is the leading cause of death among gynecologic malignancies in women [1–3]. Early-stage diagnosis of ovarian cancer can significantly improve the patient's five-year survival rate [4,5]. However, early diagnosis remains technologically challenging because the ovary is located deep in the pelvis, thereby, making detection difficult that leads to delay in diagnosis, which is one of the major causes for poor survival rate despite advances in surgical and chemotherapeutic options [6,7]. Circulating tumor cells (CTCs) draw significant attention because they are considered indicators of impending malignancies and could be used for early cancer detection [8]. However, CTCs occur at extremely low levels in the blood (as low as $1–10^2$ cells per 10^6 nucleolate blood cells). Therefore, enrichment of the CTCs is a key step before detection.

Over the past decades, immunomagnetic separation methods have been widely applied to CTCs enrichment process due to their easy manipulation and specific reorganization of CTCs with magnetic particles that are modified with antibodies, as well as convenient coupling with identification methods, such as immunocytochemical staining[9], fluorescence activated cell sorting (FACS) analysis[10], and gene mutational investigation[11]. In immunomagnetic separation, epithelial cell adhesion molecules (EpCAM) are widely used as capture target because EpCAM are overexpressed in malignant tumor cells and absent from blood cells. The CellSearch™ system, which is FDA-approved and commercially available, is based on the immunomagnetic separation methods using anti-EpCAM antibody-modified ferrofluid (120–200 nm) for separating CTCs from the whole blood of breast, colorectal, and prostate cancer patients[12–14]. However, the EpCAM-based immunomagnetic separation methods have drawbacks. Antibodies are large molecules that limit the number of antibody molecules that anchor on nanoparticles and a non-directional orientation of antibodies linked on the magnetic nanoparticles may be unsuitable for antibodies to bind to antigens on the CTCs, thereby, lowering the immunoreaction efficiency. Moreover, antibodies are expensive making immunomagnetic separation costly, particularly for large-volume blood sample screening in clinics. For example, the CellSearch assay requires the screening of 7.5 mL whole blood sample for CTCs capture, enumeration, and detection. More importantly, during metastasis, the tumor cells undergo

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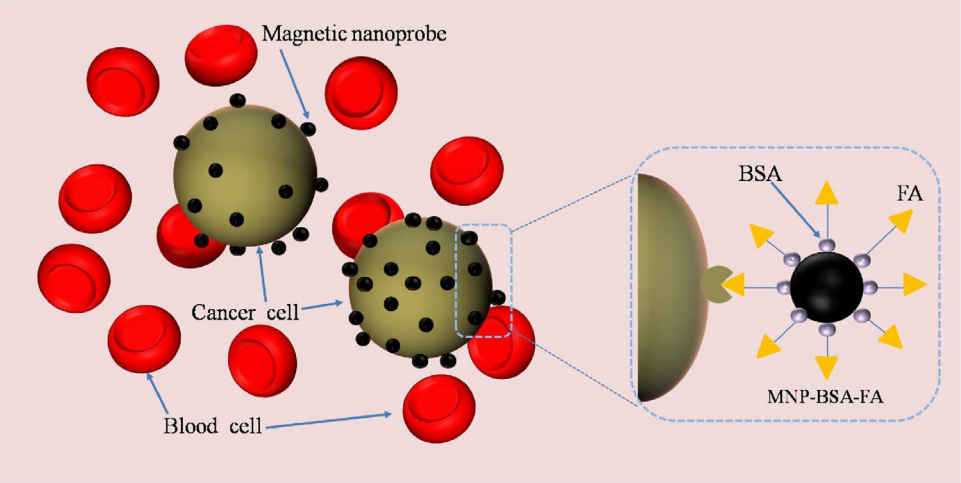

Scheme 1. Schematic diagram for CTCs labeling and separating with the FA decorated nanoprobes in blood.

epithelial–mesenchymal transition that causes down regulation of the EpCAM expression in CTCs [15]. Therefore, EpCAM-based targeting technologies may not be highly efficient for CTCs enrichment. As such, finding other alternative markers to replace EpCAM for a highly efficient capture of CTCs is necessary for the early detection of various types of cancer including ovarian cancer.

Recently, small molecule probes have drawn significant interest due to their capability of mediating strong interactions between nanoprobes and target cells. Studies have reported that using small molecules as probes to decorate nanoparticles could effectively label pathogenic bacteria and cancer cells [16–22]. Folic acid (FA) is a smaller molecule compared with antibodies and possesses only a few functional groups for bioconjugation. Therefore, controlling the architecture of the FA anchored on the nanoparticle surface in order to expose its functional groups can be accomplished more easily than that of the big antibodies. FA is an essential vitamin in cell nuclear glucoside synthesis, inexpensive, and has less immunogenicity compared with antibodies. Moreover, FA can bind to folate receptors (FAR) with high affinity (K_d = 0.1 nM) via its γ-carboxyl group. Thus, it has been widely used as specific targeting probe for cancer cell labeling, targeting, treatment, and detection [23–26]. Furthermore, FAR, a 38 kDa glyco-polypeptide, is a folate-binding protein which is overexpressed in many types of cancer cells [24,27,28]. In particular, 95% of ovarian cancer cells overexpress FAR [29]. Thus, FAR may be an ideal alternative target that could replace EpCAM for isolating ovarian cancer CTCs.

Given the possible steric hindrance between FA attached to the surface of small nanoparticles, the FA modification on the nanoparticles surface may result in poor contact with FAR on the cell surface and affect the recognition efficiency with target cells which may result in reduced capture efficiency. Moreover, the large surface-to-volume ratio of the nanoparticles easily leads to high nonspecific adsorption for cell targeting and low purity [9], making subsequent identification of the CTCs time consuming. Thus, in CTCs enrichment processes, methods to avoid the nonspecific adsorption of the cells to improve the specific capture efficiency are important for downstream CTCs identification. Jo et al. [11] suggested that utilizing a PEG-linker could improve nanoparticles stability and reduce nonspecific binding. Therefore, using a linker as a space arm to couple FA on the nanoparticles surface may make the FAs more available for attachment to FAR on CTCs surfaces which can improve FAR recognition by the nanoprobes.

Bovine serum albumin (BSA) is a widely used blocking agent in immunoreactions to minimize nonspecific adsorption. To improve biological applications of the nanoparticles and to decrease nonspecific separation efficiency for CTCs capture, BSA could be used as coating when conjugated on the magnetic nanoparticles surface. Furthermore, BSA is a highly soluble protein and possesses 59 lysine 3-amine groups and numerous carboxyl groups for conjugation. Therefore, it could be used as a carrier to conjugate more target molecules as well as increase nanoprobes bioapplicability [30–32]. In our previous study, we used BSA coated magnetic nanoparticles as nanocarrier for vancomycin conjugation and achieved a very specific capture for *Staphylococcus aureus* cells [33]. Moreover, the use of a big biomolecule such as BSA as a coating on the surface of the magnetic nanoparticles may remarkably decrease the cytotoxicity of the nanoprobes to the cell [32,34,35].

In this study, an affordable and simple method for the enrichment of CTCs and their subsequent detection was developed. BSA was first conjugated onto the surface of the magnetic nanoparticles and then FA was linked on the BSA through a PEG_{2K} spacer (Scheme 1). The FAR-overexpressing SKOV3 cell line was selected as a model ovarian cancer CTCs. Combined with immunocytochemical staining, ovarian cancer CTCs were isolated and identified from whole blood cells.

2. Materials and methods

2.1. Materials

Magnetic nanoparticles (50 nm) were bought from Ocean NanoTech (San Diego, CA, USA). Bovine serum albumin (BSA) was purchased from Biosharp (Beijing, China). RPMI-1640 cell culture medium and 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) were obtained from Solarbio (Beijing, China). Fetal bovine serum (FBS) and trypsin were purchased from ExCellBio (Shanghai). Bisbenzimide H.33342 dye, Sulfo-N-hydroxysuccinimide (Sulfo-NHS) and 1-ethyl-3, 3-dimethylaminopropyl carbodiimide hydrochloride (EDC) were provided by Sigma-Aldrich (St, MN, USA). FA-PEG_{2K}-NHS was purchased from Ponsure (Shanghai, China). CMFDA and CMRA cell tracker dyes were obtained from Invitrogen (Carlsbad, CA, USA). Goat polyclonal antibody anti-HE4 was purchased from Santa Cruz Biotechnology and FITC labeled rabbit anti-goat IgG was bought from EarthOx LLC (Millbrae, CA, USA).

2.2. Cell culture and blood preparation

The human ovary carcinoma cell line SKOV3, human chronic myeloid leukemia cell line K562 and HL-60 cells were kindly gifted from the Medical Research Center of the Second Affiliated Hospital to Nanchang University. Cell lines K562 and HL-60 were grown