Metal-Enhanced Immunofluorescence Assays for Detection of Carcinoembryonic Antigen

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***Abstract*—Carcinoembryonic antigen (CEA) is a glycosylphosphatidylinositol cell surface anchored glycoprotein that is a well-known, broad spectrum biomarker related to various cancers and it is also an indicator of disease recurrence. In this work, metal-enhanced fluorescence (MEF) is utilized to lower the detection limit of CEA in immunofluorescence assays. Silver nanocubes (AgNCs) of 50 nm edge-length were incubated to plasmonically enhance fluorescence intensity. This increased sensor sensitivity by a factor of 6 and lowered the limit of detection to below 1 ng/mL in fluorescence detection of the antigen.**

Keywords—metal-enhanced fluorescence; Silver nanocubes; carcinoembryonic antigen

I. INTRODUCTION

Tumor markers have been developed to help diagnose cancers and monitor disease progression by their concentration levels in body fluids [1]. Gold and Freedman first introduced carcinoembryonic antigen (CEA) as the tumor associated antigen in 1965 [2]. Although the clinical value of CEA has not been clarified completely, it has been accepted widely as the marker of gastrointestinal cancers for over the past 40 years. According to many reports, 2.5 ng/mL of CEA is accepted to be the critical value to estimate the possibility of having gastrointestinal cancers or malignancy [3-5].

Immunofluorescence (IF) assays are widely used because both location and quantitative information on proteins can be gathered by visualizing specific antigen/antibody interactions with fluorophores [6]. However, there are some limitations in this technique, primary ones being the high limit of detection (LOD) compared to other detection techniques, and low working efficiency.

To detect CEA at the clinically significant level, metal-enhanced fluorescence (MEF) was utilized in this work to lower the LOD of the IF assays to nanograms/mL levels using silver nanocubes (AgNCs). MEF as a phenomenon is now known for a few years, and has been drawing attention in fluoroscopy. The ability of several metal particles to enhance the intensity in IF, especially gold and silver nanoparticles, has been reported [7-11]. MEF is a powerful tool in biotechnology in detecting proteins or DNA in cells in vitro. Many factors can significantly affect the enhancement including the distance between metal particles and fluorophores, the size and shape of metal nonoparticle, the type of fluorophores labeled, and the compatibility of proteins and DNA [7, 11, 12]. Generally, the interactions between excited states of the fluorophores and the induced surface plasmons of metal nanoparticles result in increased quantum yield and decreased lifetime of fluorophores. While the former causes enhancement of intensity, the latter improves photostability of the fluorophores. In this work, AgNCs were found to effectively increase the signal by around 4.6 times and improve photobleaching. With the enhancement provided by MEF, the detection limit is lowered down to nanogram/mL levels, the clinically significant range.

II. EXPERIMENTAL

A. Reagents and Apparatus

The materials and reagents utilized in our experiments and their sources are: CEA capture antibody (Fitzgerald, 10-C10D), CEA detection antibody labeled with Alexa-488 (Fitzgerald, 10-C10E) (tagged with Alexa Fluor® 488 Protein Labeling Kit, Thermo Fisher), CEA antigen (Abcam, ab742), PBS (Life Technologies, pH 7.4), and bovine serum albumin (BSA, from Fisher Scientific). The materials used to synthesize the AgNCs included silver trifluoroacetate (CF3COOAg, from Sigma-Aldrich), HCl (37% in weight, from Sigma-Aldrich), ethylene glycol (Sigma-Aldrich), NaSH (Sigma-Aldrich) and poly (vinyl pyrrolidone) (PVP, from Alfa Aesar). Reagent grade deionized water (DI water) with 18.2 MΩ resistance was produced in the laboratory using a Millipore system.

The instruments utilized in the experiments were: Hitachi S-800 Scanning electron microscope (SEM); Leica DMI4000 B fluorescence microscope.

B. Preparation of Silver Nanocubes

Silver nanocubes were synthesized using the protocol developed by Zhang *et al*. [13] This method utilizes ethylene glycol as the solvent and CF_3COOAg as the precursor in a nucleation and growth solution process. The synthesized AgNCs were stored in DI water.

C. Antigen Immobilization and Detection with MEF

Two groups of glass slides were set up and cleaned using a piranha solution (4 parts of concentrated sulfuric acid and 1 part of 30% hydrogen peroxide solution). The solution of 3-aminopropyltriethoxysilane (3-APTES, from Sigma-Aldrich) in pure ethanol (1% in volume) was prepared to pretreat the

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surface of the slides and immobilize the AgNCs and the capture antibody. After drying with nitrogen, both groups of slides were treated with the 3-APTES solution on the surface and 30 µL of AgNC solution was immobilized on the surface of one group of slides overnight. 30 µL of 10 µg/mL CEA capture antibody was added to the surface of the glass sides in both groups, and incubated for 1 hour at room temperature followed by thorough rinsing with PBS three times. PBS rinsing followed every incubation procedure in all experiments. 1% of BSA in PBS solution was used to block the extra binding sites of 3-APTES on the surface by adding 30 µL of BSA and incubating for 1 hour. CEA was diluted to the desired concentrations of 1 ng/mL, 10 ng/mL, 100 ng/mL, 1 µg/mL and 10 µg/mL with PBS solution. After rinsing, 20 µL of different concentrations of CEA antigen from 1 ng/mL to 10 µg/mL were added and incubated for 45 minutes. Following this, 10 µL of CEA detection antibody labeled with Alexa-488 of approximate concentration 10 µg/mL were added and incubated for another 45 minutes. A Leica DMI4000 B microscope was utilized for fluorescence measurements. The concentration of CEA capture antibody and CEA detection antibody labeled with dye were kept constantly in all experiments. The experiment was repeated three times for each concentration of CEA antigen. Fig. 1 illustrates the MEF experiments schematically.

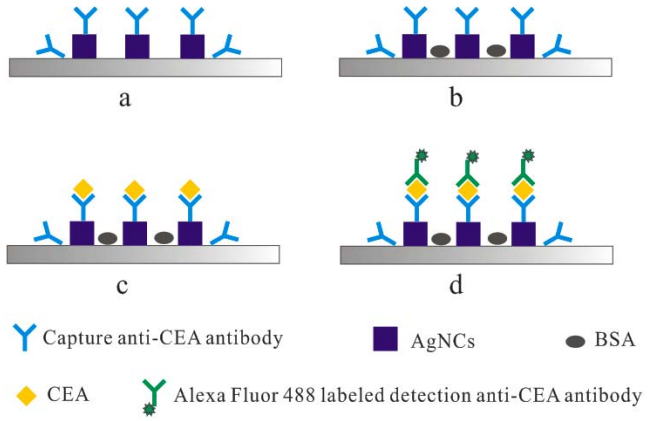


Fig. 1. Schematic diagram of the MEF experiment. a) Ag nanocubes were immobilized on the surface of the glass slides via APTES and CEA capture antibody was incubated above them; b) BSA was added to block the excess binding sites; c) CEA antigen was then added and bound to the capture antibody; d) CEA detection antibody labeled with Alexa-488 was added and bound to the CEA antigen.

III. Results and Discussion

A. Characterization of Siver Nanocubes

AgNCs with edge length of about 50 nm were targeted in the synthesis. Scanning electron microscope was used to image the AgNCs. An SEM image of these AgNCs is shown in Fig. 2, which gives the edge length of the AgNCs to be approximately 50 nm with a relatively narrow distribution. Most particles are cubes with some spheres and irregular shapes present.

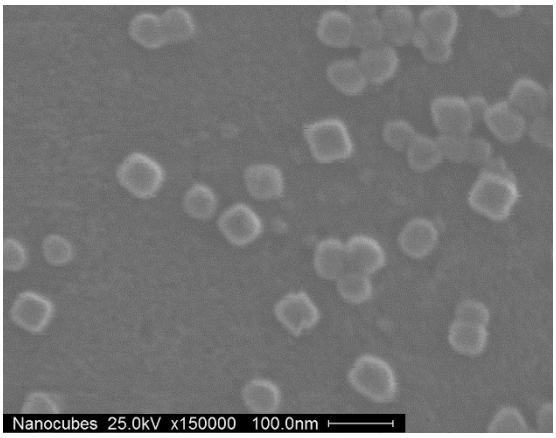


Fig. 2. SEM image of the synthesized silver nanocubes under 150000 magnification.

B. MEF Results

Fluorescence images of each slide were taken by the Leica microscope and the pixel intensity was determined and color coded with the software ImageJ. Fig. 3 summarizes the MEF results. Five concentrations of CEA antigen were detected with and without AgNCs to quantify MEF. Triplicate measurements were made, which exhibited small standard deviations.

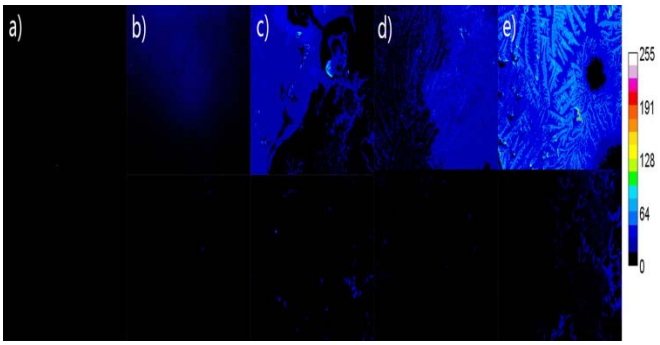
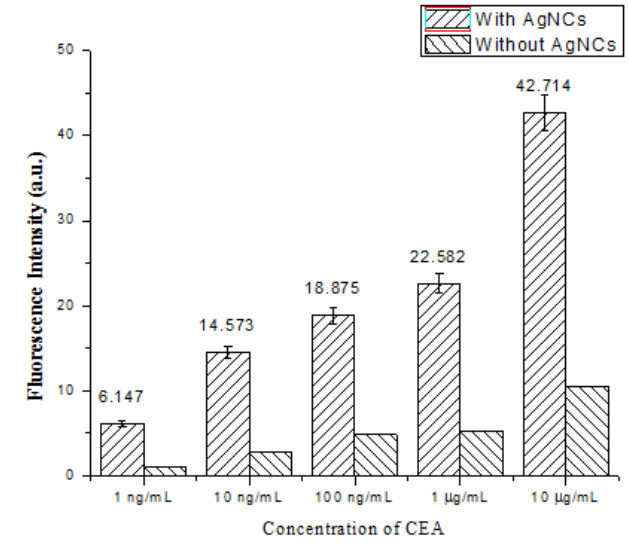
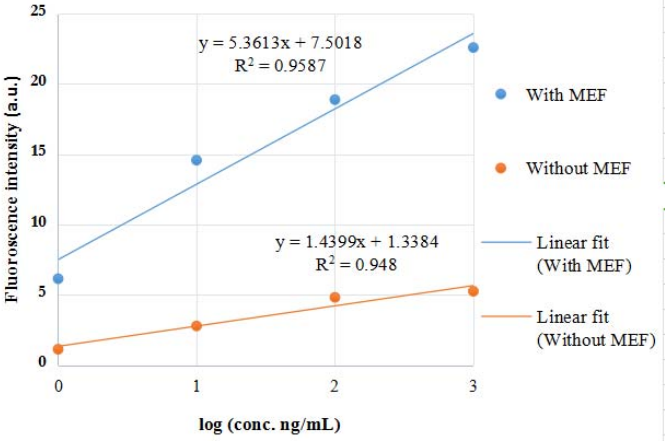


Fig. 3. Results of the MEF experiment. The top row of the fluorescence images are for the group with AgNCs of approximately 50 nm edge length. The bottom row is for the corresponding controls, i.e., with no AgNcs. The concentration of CEA antigen used was a) 1 ng/mL, b) 10 ng/mL, c) 100 ng/mL, d) 1 µg/mL, and e) 10 µg/mL.

The intensity enhancement factors for different concentrations varied in the range of 3.9 to 5.4 (Fig. 4a). It is apparent from these results that AgNCs enhance signal intensity sufficiently to lower the detection limit to below 1 ng/ml. Fluorescence intensity is linear in log (conc ng/ml) over the range of 1-1000 ng/mL as shown in Fig. 4b. Sensitivity of the sensor with AgNCs is calculated to be about 6 times larger than that without, due to MEF. The signal at 100 pg/mL is measurable and larger than the measurement error. Hence, this device exhibits a limit of detection lower than 1 ng/mL. Without MEF, the intensity exhibited for 1 ng/mL would require over 100 ng/ml, indicating 2-3 orders of magnitude lowering of the LOD with MEF. These results are encouraging and clearly indicate that an optimized device with MEF could lower the LOD to less than 100 pg/mL CEA in the solution.



(a)



(b)

Fig. 4. (a) Histogram of fluorenscence intenstiy; (b) Calibration curve for logarithmic concentration of target CEA in the conditions with and without AgNCs

IV. Conclusion

To summarize, this work focused on the detection of CEA antigen at clinically significant levels with metal-enhanced immunofluorescence assays. The limit of detection of CEA antigen was successfully brought from 1 µg/mL down to sub-nanogram levels. This study enables construction of sensor substrates and IF assays for sensitive detection and quantification of cancer biomarkers in clinically relevant sub-nanogram/ml levels in body fluids such as blood and urine.

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