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Design of Aptamer-Gold Nanoparticles Based Colorimetric Assay for the Early Diagnosis of Breast Tumor

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Abstract: ***Combinatorial chemistry techniques based on the SELEX methodology were used for the identification of the specific aptamers. We have developed a highly specific colorimetric assay for breast tumor antigen (CA 15-3) that uses aptamer-gold nanoparticles (Apt-GNPs) conjugate, to combine the selectivity and affinity of aptamers and the spectroscopic advantages of gold nanoparticles to allow for the sensitive detection of CA 15-3. We have synthesized an Apt-GNPs conjugate that is specific to CA 15-3 and we used this conjugate system to detect CA 15-3 by monitoring the changes in the color and UV-vis extinction spectra of the Apt-GNPs that occur due to the aggregation of Goldnanoparticles. In this colorimetric assay we found, at very low concentration of CA 15-3 i.e. <30 units there is no change in the conjugate but when CA 15-3 was >30 units the red color of Apt-GNPs conjugate turned purple and at higher concentration of CA 15-3 i.e. >500 units there were very less extent of aggregation, which is not visible as a change in color. We have checked the change in flocculation intensity in the presence of different concentration of CA 15-3 by UV-vis extinction spectra. The results obtained in this assay would be much helpful in the practical applications of Apt-GNPs conjugate and breast tumor diagnosis.***

Keywords: Aptamer; Goldnanoparticle; Breast Tumor; colorimetric assay; CA 15-3.

1. Introduction

Recent advances in Nanoscience and Nanotechnology have provided new opportunities for the application of nanomaterials in biological analysis and disease diagnosis (Matsui *et al.*, 2004; Zhu *et al.*, 2004). Among nanomaterials, gold nanoparticles (GNPs) are especially attractive because of their unique physical and chemical properties (Storhoff *et al.*, 1998; Nath and Chilkoti, 2002; Thanh and Rosenzweig, 2002; Liu and Lu, 2004; Huang *et al.*, 2005; Lin *et al.*, 2005; He *et al.*, 2008). Due to the Plasmon resonance, Gold nanoparticles (GNPs) possessing strong distance dependent optical properties and high extinction coefficients have emerged as important colorimetric materials. Since the aggregation of DNA-modified GNPs and its application in DNA detection were reported (Mirkin *et al.*, 1996; Elghanian *et al.*, 1997), therefore aptamer –GNPs conjugate have attracted considerable attention for the development of colorimetric sensing devices (Huang *et al.*, 2005; Liu and Lu, 2006; Liu and Lu, 2007; Lee *et al.*, 2008; Zhao *et al.*, 2008).

Aptamers are oligonucleotide strands that bind to their targets with high affinity and selectivity, which rival antibodies in their diagnostic potential (Brody and Gold, 2000). Aptamers are able to fold into unique three dimensional conformations that allow them to bind to selected molecules ranging in size from small organic molecules to proteins (Ellington and Szostak, 1990; Famulok and Mayer, 1999; Fang *et al.*, 2001; Li *et al.*, 2002). Once selected the aptamers; it can be chemically synthesized for different functional groups or labeling molecules to be easily integrated. This property combined with their selectivity and affinity makes them ideal for use in diagnostic assays.

It is found ssDNA can bind to citrate capped GNPs through DNA base-gold interactions and stabilize GNPs electrostatically (Li and Rothberg *et al.*, 2004; Li and Rothberg *et al.*, 2005). In contrast, dsDNA formed by the hybridization of ssDNA and its complementary DNA target, shows little binding affinity to citrate-capped GNPs and, therefore it provides little stabilization because once hybridized the DNA bases are not free to bind to GNP surface. In other words, at an appropriate salt concentration (e.g. 200 mM NaCl), citrate-capped GNPs are stabilized in the presence of ssDNA, but aggregate in the presence of dsDNA (Zhao *et al.*, 2008).

Aptamer-GNP conjugate based colorimetric assays possess several substantial advantages e.g. desirable sensitivity and selectively; the detection can be accomplished at low cost; the colorimetric assay displays a rapid response that results from instant color change; it is very convenient to operate the assay and colorimetric systems make on-site real-time detection easier compared with fluorescent measurements (Liu and Lu, 2004; Huang *et al.*, 2005; Liu and Lu, 2007). In this assay we conjugated breast tumor antigen specific aptamer with Goldnanoparticles to combine the selectivity and affinity of aptamers and the spectroscopic advantages of gold nanoparticles to allow for the sensitive detection of breast tumor markers. In this work, the aptamers were selected using the modified SELEX methodology (Stoltenburg *et al.*, 2005) in which breast tumor antigen CA 15-3 served as the target. CA 15-3 is the most widely used serum marker in breast cancer (Duffy, 2001). The CA 15-3 assay measures the protein product of the *MUC1* gene. We have designed a colorimetric assay for the simple and specific detection of Breast tumor antigen that uses the Aptamer-GNP conjugate as a probe.

2. Material and Methods

2.1 Materials

All chemicals until specified were purchased from Sigma - Aldrich, Himedia, CDH, QIAgen and Invitrogen. Glassware's were purchased from Borosil (India). Plasticwares were procured from Tarsons products Pvt. Ltd. (India) and Nunc (Denmark). Breast Tumor Antigen (CA 15-3) from Human Cell Culture was purchased by Sigma –Aldrich. 3' thiol labeled aptamer was purchased by Sigma –Aldrich.
Aptamer sequence- GAAGTGAATATGACAGATCACAACT [ThiC3]

2.2 Synthesis of GNPs

20nm citrate capped Gold nanoparticles were synthesized. 50 mL of 0.01% $HAuCl_4$ solution (w/v) was heated to boiling with stirring in a sterile flask. 1000µL of 1% (w/v) of trisodium citrate solution was quickly added to the $HAuCl_4$ solution. After several minutes heating and stirring, the color of the solution was changed from yellow to black and then to red color. Final color of the solution depends on the different sizes of the nanoparticles. The color change is slower for larger nanoparticles compared with small nanoparticles. The amount of citrate solution determines the size of the nanoparticles. Smaller nanoparticles require more citrate solution and whereas larger nanoparticles require less citrate solution. Gold Nanoparticles were characterized by TEM, DLS and UV spectrophotometer.

2.3 Preparation of Apt-GNPs

The Aptamer Goldnanoparticles (Apt-GNPs) conjugate were designed using a modified method (Medley *et al.*, 2008). 5mL of the 20nm gold colloid nanoparticles (GNPs) was centrifuged for 15 min at 13000 rpm. The GNPs were washed three times with 1 mL aliquots of 5mM sodium phosphate buffer (pH 7.6). To wash the GNPs 1mL of 5mM sodium phosphate buffer (pH 7.6) were added in the pellet followed by dispersion through sonication, and centrifuged for 15 min at 13000 rpm in each wash round. After the final wash step, the GNPs were dispersed in 1 mL of the 5mM sodium phosphate buffer (pH 7.6). 50 µM stock of thiol-labeled DNA sequence (mercaptoethanol treated) was added to wash GNP sample to achieve final concentration 1.5µM and incubated for 24 hrs at 4°C. After completion of incubation, the sample was centrifuged at 13000 rpm for 5 min. the sample was washed three times as described previously with the 5mM sodium phosphate buffer (pH 7.6). After, the final wash step, sample was dispersed in 500µL of 5mM sodium phosphate buffer (pH 7.6), and stored at 4°C until used. The Apt-GNP conjugate synthesis was characterized through electrophoresis at 2.5% Agarose gel.

2.4 Assay development

The colorimetric assay for the diagnosis of breast tumor antigen was designed according to the following method. Different concentrations of breast tumor antigen (CA 15-3) i.e. 10, 30, 100, 250, and 500 units were added to 1mL of 1.5µM Apt-GNP conjugate in separate tubes and incubated for 30 minutes at 4°C. After completion of incubation 10µL of 500mM NaCl were added. Flocculation was detected visually and absorbance was measured using UV-vis spectrophotometer.

3. Results and Discussion

Early and accurate detection of cancer often requires time-consuming techniques and expensive instrumentation. To overcome these limitations, we developed a colorimetric assay for the direct detection of breast cancer. In the present research work we have synthesized 20nm size of Gold nanoparticles. Size of the gold nanoparticles was confirmed by UV-vis extinction spectra and TEM images (figure 1).

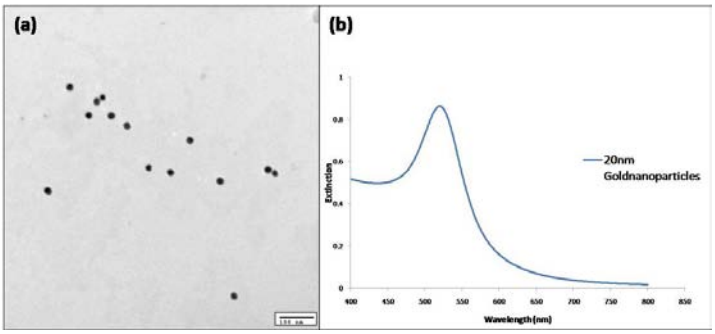

Figure 1: TEM image and UV-vis extinction spectra of 20nm citrate capped Gold nanoparticles.

CA 15-3 specific aptamers were screened from a purchased DNA library through a modified SELEX methodology. Thiolated form of the more stable screened aptamer was synthesized. In the present study we have prepared Apt-GNPs conjugate of 20nm GNPs and different concentrations of screened CA 15-3 specific aptamers. To verify assembling of the aptamer molecules on the surface of the GNPs surface, the washed conjugate sample were checked on 2.5% agarose gel electrophoresis, we found clearly visible bands of DNA in each conjugate. A conjugate having low aptamer concentration shows blurt or less visible band, while a conjugate with high aptamer concentration shows a sharp band on 2.5% gel (figure 2).

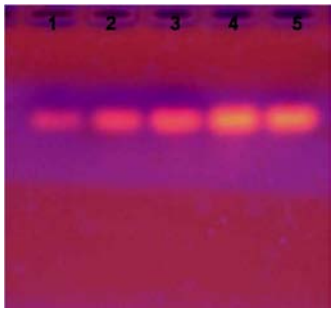

Figure 2: Image showing successful binding of different concentrations (1) 0.15µM, (2) 0.75µM (3) 1.5µM (4) 3.75µM and (5) 7.5µM of Thiolated aptamer with Gold nanoparticles on 2.5% Agarose Gel Electrophoresis.

To prepare a stable conjugate we have optimized concentration of aptamer and size of Gold nanoparticles. The conjugate of 1.5µM aptamer and 20 nm Gold nanoparticle was found more stable. There were no flocculation in the prepared conjugate even we added 10% NaCl. While this Apt-GNPs conjugate solutions was flocculated by adding 500mM NaCl and shows color change, cherry red to purple, in the presence of CA 15-3 (Breast Tumor Antigen). This is might be because of interparticle crosslinking. At low concentration of CA 15-3 no aggregation was observed while at high concentration of CA 15-3 clear aggregation and color change was observed (figure 3). This aggregation was confirmed by TEM images (figure 4).

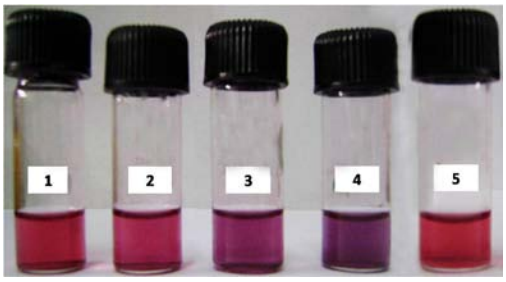

Figure 3: Color change in Apt-GNPs conjugate in presence of (1) 10 units, (2) 30 units, (3) 100 units, (4) 250 units and (5) 500 units of CA 15-3 & 500mM NaCl

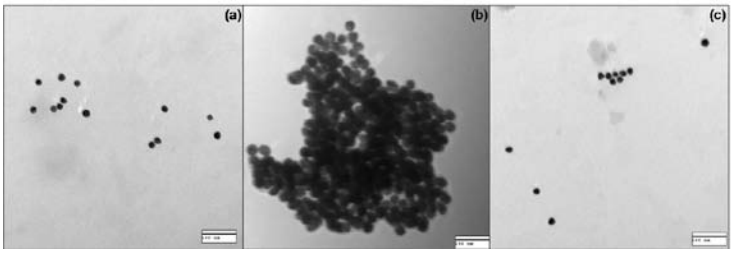

Figure 4: TEM images showing aggregation in the Apt-GNPs conjugate in the presence of (a) 30 units, (b) 250 units and (c) 500 units of CA 15-3.

We believe for the results obtained that, at low concentration of CA 15-3 there were very low interparticle crosslinking, which was not visible, but in case of high concentration of CA 15-3 it was high enough to induce a greater degree of interparticle crosslinking (figure 5) and as a result of aggregation of Apt-GNPs conjugate we observed perfect color change.

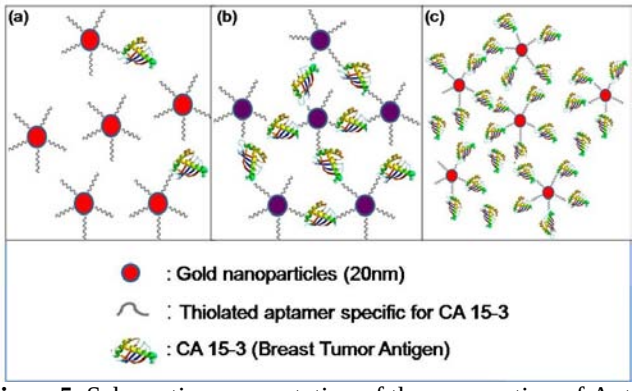

Figure5. Schematic representation of the aggregation of Apt-GNPs conjugates in presence of (a) Low, (b) high and (c) very high concentration of CA 15-3

The reason behind the results obtained was believe that the Apt-GNPs conjugate acts like a bridge in between two Apt-GNPs molecules, which causes the aggregation of conjugate sample. But again at very high concentration of marker we obtained no color change or slight color change i.e. No or very less extent of aggregation, which could be seen only in the data obtained by UV-vis spectrophotometer (figure 6) .

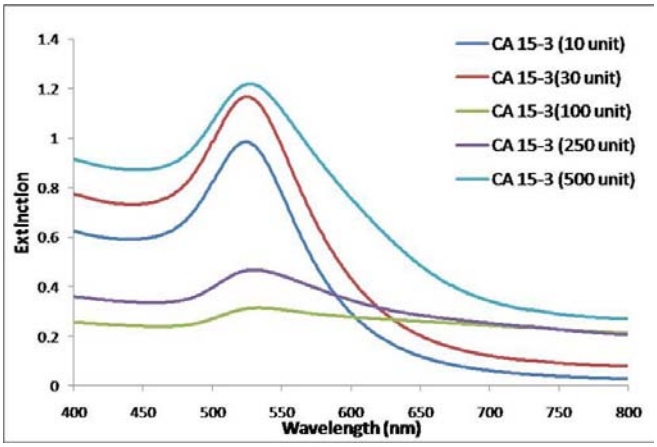

Figure6. UV-vis extinction spectra of Apt-GNPs conjugate in presence of different concentrations of CA 15-3 and 500mM NaCl.

We believe that this is because the aptamer molecules were saturated by the CA 15-3 molecules which causes repulsion in between two Apt-GNPs conjugates. Even it is mentioned in the literature that, the salt induced aggregation of oligonucleotide probe-modified GNPs can readily occur due to the sticky-end pairing effect while addition of target molecules favors the formation of the hairpin structure of probe sequences and substantially inhibits the nanoparticle assembly (Wu *et al.*, 2010). The non specific binding of the Apt-GNPs conjugates were also checked with BSA and no flocculation were found in the presence of BSA (1mg/mL) rather than there were clearly visible flocculation in presence of CA 15-3. Obtained results were also confirmed through UV-Vis spectrophotometer (figure 7).

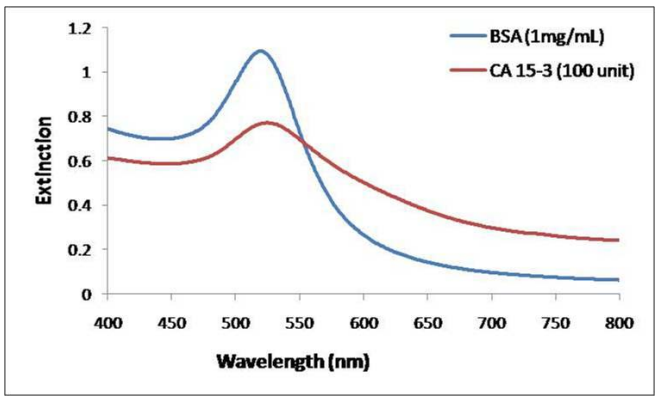

Figure 7: UV-vis extinction spectra of Apt-GNPs conjugate in presence of CA 15-3 & BSA containing 500mM NaCl.

4. Conclusion

The Apt-GNP conjugate have been demonstrated for the very sensitive and specific detection of CA 15-3 (Breast tumor antigen) through utilizing the unique optical properties of Gold nanoparticles and the excellent selective nature of aptamers. We have synthesized 20nm citrate capped Gold nanoparticles and thiol modified aptamer. We have used these 20nm GNPs and thiolated aptamer to synthesize Apt-GNPs conjugates to analyze CA 15-3. The increased concentration of CA 15-3 is easily available in serum if someone is suffering from Breast Cancer. Non specific binding of Apt-GNP conjugate were checked with BSA. In the 5mM sodium phosphate buffer (pH 7.6) and by using 500mM NaCl the Apt-GNPs conjugate act as a probe and specifically and sensitively detected CA 15-3 in patient's serum. The advantages of the present research work was to develop a simple and specific colorimetric assay to diagnose Breast Cancer in early stage, also it was well suited for protein analysis. The future aspects of this work are to develop a diagnostic kit for Breast Cancer by using very simple and specific colorimetric assay and to develop electrochemical biosensor based on Apt-GNPs conjugates.

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