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Label-free colorimetric estimation of proteins using nanoparticles of silver

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Metallic nanoparticles have received considerable attention in bioassays and diagnostics due to their unique surface plasmon resonance (SPR) properties. Gold nanoparticles have been employed for the development of SPR-based colorimetric bioassays. In the present report we have described a sensitive colorimetric approach for estimation of proteins, within a detection limit of 10~80 µg/mL, using unmodified silver nanoparticles. Besides the common advantages of colorimetric assay such as simplicity, high sensitivity, and low cost, our method has a label-free design and provides an important and attractive alternative to classical sensing probes and systems. The present work will contribute to the development of nanotechnology-based diagnostic tools.

Keywords: Silver nanoparticles; Agglutination; Surface plasmon resonance; Colorimetric estimation; Bovine serum albumin; Immunoglobulin

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Colorimetric bioassay based on nanomaterials has received considerable attention in last few years. Gold nanoparticles have been explored mostly among metallic nanomaterials, and variety of colorimetric sensors have been developed against analytes, such as DNA, metal ions, carbohydrate and proteins [1-10] employing unique optical properties of nanogold. These assays have employed either modified (type I) or unmodified nanoparticles (type II) [8]. Use of DNA-modified gold nanoparticle conjugate as indicator pioneered development of type I sensors [1], while Rothberg et al. [2,3] developed type II sensors employing unmodified nanogold. Gold-based sensors have provided comparable or even better sensitivity and selectivity than their conventional fluorescent counterparts [8].

Owing to inherent photostability, ease of synthesis, biocompatibility, ability to conjugate to biological molecules and innate anti-bacterial as well as anti-platelet properties, nanosilver has established its biomedical potential [11-13]. However, though silver nanoparticles possess unique optical properties similar to nanogold, little attention has been paid on nanosilver-based colorimetric assays. Only a few reports are available in literature describing the use of functionalized nanosilver coupled with appropriate ligands in colorimetric detection of DNA, metal ions and proteins [11,14-17]. Functionalization of nanosilver can cause its chemical degradation rendering it to be easily oxidized [8]. On the contrary silver nanoparticles have the advantage of higher extinction coefficient as compared to gold particles of comparable sizes.

In our earlier reports we have described synthesis of highly stable, biocompatible nanoparticles of silver and have analyzed their anti-bacterial, anti-platelet and protein stabilizing properties in absence of any modification. Unmodified silver nanoparticles have been shown to be ideal sensors for enzymatic reactions involving dephosphorylation of adenosine triphosphate (ATP) by calf intestine alkaline phosphatase and peptide phosphorylation by protein kinase A [8]. In the present study we describe a sensitive colorimetric assay for quantification of proteins employing unmodified nanoparticles of silver. Our method is based on unique surface plasmon resonance (SPR) of these particles in dispersed and aggregated states and provides a

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sensitive approach of estimation of proteins in range of about 10~80 μg/ml, thus potentially broadening the applicability of nanosilver-based sensors.

Material and Experiments

Chemicals and Reagents

Silver nitrate, Sodium hydroxide, sodium chloride, hydrazine, liquid ammonia (30%), and D-glucose were procured from Merck India. Bovine serum albumin (BSA) (fraction V) and immunoglobulin G (IgG) were purchased from Sigma Aldrich. Filters (pore size 0.2 μm) were purchased from Sartorius. All other chemicals were of analytical grade. Milli-Q grade deionized water (Millipore) was used for preparation of the solutions.

Synthesis of silver nanoparticles

Preparation and characterization of highly stable biocompatible nanoparticles of silver have been described in our earlier reports [11-13]. Briefly, silver nitrate (17 mg) was dissolved in 100 ml deionized water, to which ammonia was added at final concentration of 0.01 M. The pH of the solution was adjusted to 7.4 using citric acid. A blend of reducing agents like D-glucose (0.01 M) and hydrazine (0.01 M) was added drop wise to the solution of silver salt under constant stirring to ensure complete reduction of silver ions to nanoparticles. The final solution of nanosilver at concentration of 540 μg/ml (pH 7.4) was stored in air tight bottles till further experiments. Size, morphology and distribution of silver nanoparticles were characterized using transmission electron microscope (Technai-12) and UV-Vis spectrophotometer (Pharmacia Biotech). Nanoparticles were found to be spherical in shape with average size of 10~15 nm. The solution of nanoparticles was sonicated (Labsonic 2000 U, B. Braun) for 2 min and passed through filters of 0.2 μm pore size (Sartorius) before each experiment.

Colorimetric estimation of proteins using unmodified silver nanoparticles

Incubation mixtures (final volume 500 μl), containing globular proteins (BSA or IgG) over concentration range 0~250 μg/ml, silver nanoparticles (50 μg) and NaCl (10 g/100 ml), were incubated in siliconized glass tubes for 10 min at RT to produce color change. Aliquots were diluted 1:4 and absorbance was recorded at 407 nm, the characteristic absorption maximum for disperse nanosilver of size 10~15 nm. A concentration-dependent curve was obtained using these values.

For detection of unknown protein, 400 μl of test sample was treated identically (final volume becomes 500 μl) and absorption at 407 nm was recorded. Absorption values for test sample were extrapolated on standard concentration curves for BSA or IgG in order to obtain concentration of the unknown proteins.

Transmission electron microscopy

Size and morphology of silver nanoparticles and nanosilver-BSA complex were analyzed under Technai-12 transmission electron microscope equipped with SIS Mega View III CCD camera. Samples were prepared by placing a drop of specimen solution onto carbon-coated copper grids followed by air drying. Grids were monitored under the microscope operated at an accelerated voltage of 100 kV. Measurements were carried out using AnalySIS software (SIS, Germany).

Absorption spectrophotometry

Absorption spectra of silver nanoparticles, BSA, IgG and conjugates were recorded at wavelengths ranging from 220 to 500 nm in a Beckman spectrophotometer (model DU-640B) equipped with constant temperature cell holder.

Results and Discussion

Nanosilver has propensity to interact with proteins [8,11]. To test that unmodified nanoparticles of silver could act as sensing probes for protein estimation, agglutination of nanosilver was investigated in presence of increasing concentrations of protein (see Fig. 1). BSA was elected as the representative protein for the study due to its well characterized structure and properties as well as its immense physiological significance [18-20]. Colloidal solution of 50 μg nanosilver was incubated with increasing concentrations of BSA (0~250 μg / ml) and nanoparticle agglutination was induced by NaCl (10

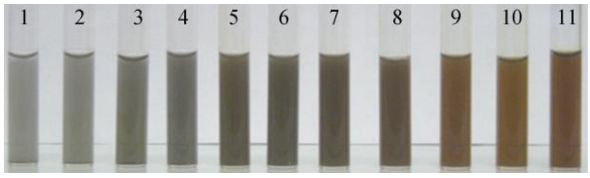

FIG. 1. Agglutination of silver nanoparticles. Tubes 1~10, nanosilver incubated with 0, 1, 10, 30, 50, 70, 90,110, 150 and 250 μg/mL BSA, respectively, and treated with 10 gm % of NaCl. Tube 11, control solution of nanosilver in absence of NaCl.

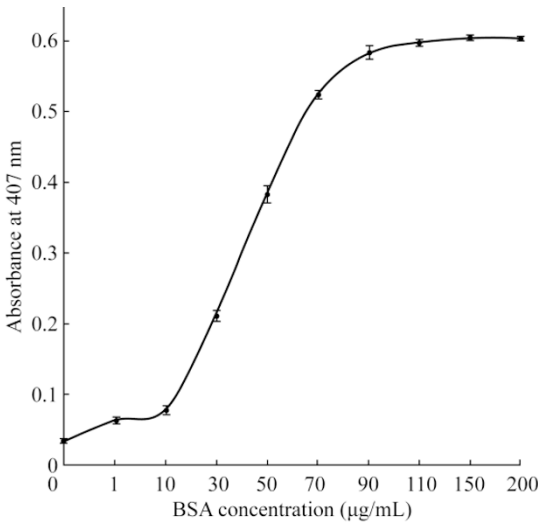

FIG. 2. Concentration of BSA versus absorbance at 407 nm.

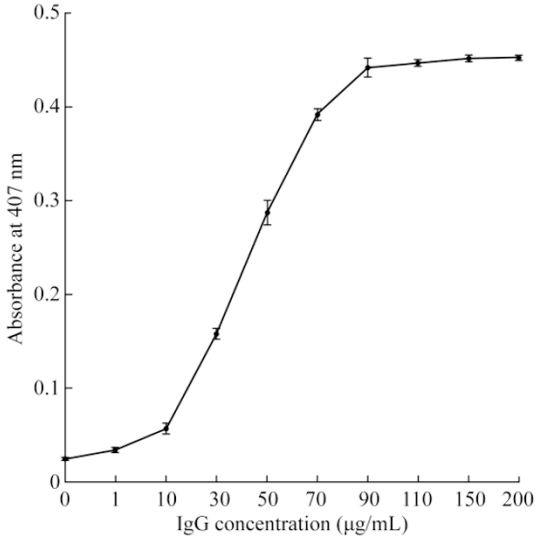

FIG. 3. Concentration of IgG versus absorbance at 407 nm.

gm % final concentration). Pure solution of nanosilver changed color from dark orange to grey upon addition of NaCl, indicative of altered SPR following aggregation of nanoparticles. Incubation with ascending concentrations of BSA demonstrated progressive resistance to color change ensued upon NaCl treatment. Nanosilver solution supplemented with 150 or 250 μg/ml of BSA retained the pristine dark orange color after addition of NaCl attesting the dispersed distribution of particles in solution. Above observations suggested that BSA interacts with silver nanoparticles and prevents agglutination of particles in a concentration-dependent manner. The extent of nanosilver aggregation determines the SPR, which is reflected from specific color of nanoparticle solution. Protein content of a solution can be estimated conveniently based on such color change. Similar colorimetric approach using unmodified gold nanoparticles has been attempted, where nanoparticles were shown to exhibit resistance to salt-induced aggregation in presence of ATP [21]. However, there has been no report describing protein estimation employing unmodified nanoparticles.

In order to estimate protein concentration a standard curve of absorbance (at 407 nm) of BSA-nanosilver complex in presence of NaCl versus BSA concentration was generated. Absorbance was found to increase linearly with BSA concentration in the range from 10~80 μg/ml, beyond which it turned into a plateau (see Fig. 2). We were able to determine accurate concentrations of unknown solutions of BSA using this curve. We also obtained a similar curve using another globular protein, IgG, where absorbance (at 407 nm), too, increased linearly over protein concentration 10~80 μg/ml (see Fig.3).

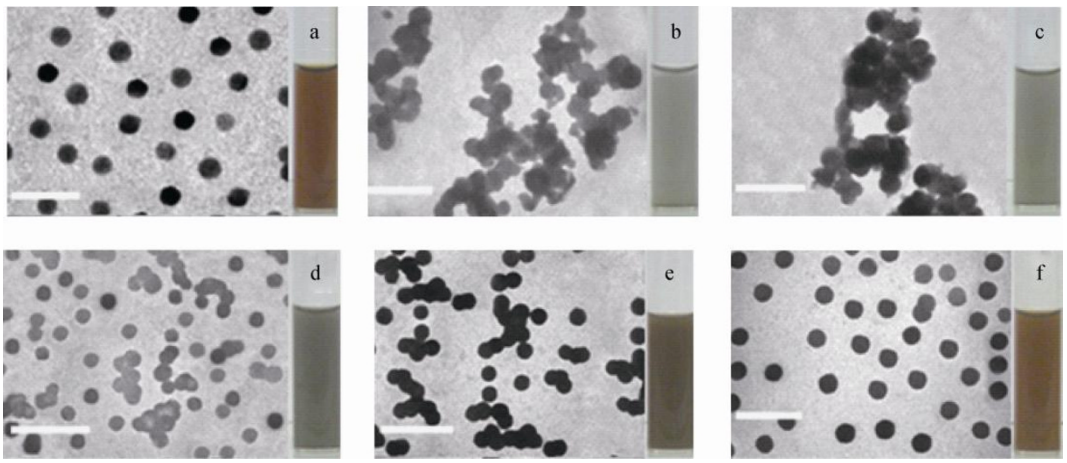

FIG. 4. Electron microscopic analysis of nanosilver agglutination. a) Spherical, uniformly sized and dispersed nanoparticles of silver in absence of salt. b-f) nanoparticles of silver in presence of NaCl and 0, 1, 10, 50 110 μg/ml BSA, respectively. Scale bar; 50 nm. Inset in each figure shows corresponding color of nanosilver solution.

Subsequently, electron microscopy was performed to examine details of interaction between silver nanoparticles and BSA (see Fig. 4). Spherical, uniformly sized nanoparticles of silver remained well dispersed in solution in the absence of NaCl (see Fig. 4a). Upon exposure to salt aggregates of nanoparticles were evident and free nanoparticles were not visible (see Fig. 4b). Addition of 1 μg/ml BSA did not bring about any change in agglutination of nanoparticles (see Fig. 4c). However, nanosilver solution supplemented further with increments of BSA (10, 50 and 110 μg/ml) exhibited progressive resistance to nanoparticle aggregation (see Fig. 4d-f). Thus, BSA at 10 μg/ml completely prevented aggregation of nanoparticles in response to NaCl treatment and retained uniform distribution of particles identical to nanosilver distribution in absence of salt having enhanced absorbance at 407 nm (see Fig. 4f). Electron microscopic analysis suggests that interaction between proteins and nanosilver prohibits agglutination of latter in response to salt treatment. As inhibition of agglutination is dependent on amount of protein, the characteristic SPR at 407 nm can be used as indicator of protein concentration in the solution.

Conclusions

In the presented report, we have described a sensitive colorimetric approach for estimation of proteins, within a detection limit of 10~80 μg/ml, using unmodified nanoparticles of silver. Besides the common advantages of colorimetric assay such as simplicity, high sensitivity, and low cost, our method has a label-free design and provides an important and attractive alternative to classical sensing probes and systems. The present work may contribute to future application of nanoparticle-based technologies to the development of diagnostic tools.

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