Research Article

Gold Nanoparticle Sensor for the Visual Detection of Pork Adulteration in Meatball Formulation


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1. Introduction

Detection of selective DNA sequences is the key step in non-aggregated, genetic screening [1–3], food analysis [4–7], environmental monitoring, and forensic investigations [8]. Most of the sequence detecting assays, available at hand, rely on polymerase chain reaction (PCR) followed by electrophoretic visualization of PCR products [4–6, 8]. Although the use of PCR effectively amplifies DNA from single copy to easily detectable quantities, it is an expensive technique in the platforms of reagent and instrumental costs [3, 9]. Moreover, authentication of PCR products may further need identification of specific sequences within it by RFLP analysis [4, 6], southern blotting or sequencing [8, 9]. Therefore use of PCR is unwarranted where sample scarcity is not a concern.

The distinct surface plasmon resonance (SPR) characters of aggregated and biodiagnostics GNPs are interesting as they can be monitored by absorption spectroscopy and also visually [3]. Researchers have long exploited these distinctive optical properties of colloidal GNPs for sensing specific oligonucleotide sequences to address a wide range of biological issues such as biodiagnostics, genetics, and food analysis [2, 3, 10–19]. However, those studies are limited to a cross-linking mechanism with synthetic probes and targets. A cross-link-based DNA detection scheme requires surface modification of GNPs to immobilize two DNA probes. The immobilized probes are further needed to be interlinked by a complementary target to realize aggregation [10, 16, 19].

Detection of nucleotide sequences by a noncross-linking method is particularly interesting [2, 3, 16]. It does not involve any modification chemistry and target hybridization is considerably fast. Li and Rothberg [2] pioneered this work by detecting selective sequences and single nucleotide mismatch in PCR amplified DNA with 13 nm-GNPs. Mismatch detection is a challenging but necessary task for the early
diagnosis of cancers and other hereditary problems [2, 3]. However, spectroscopic supports in their findings were not adequate. In our last report, we have shown 40 nm GNP s can be used for visual identification of specific sequences and mismatches in PCR-products and also in nonamplified genomic DNA. We validated our visual findings by absorption spectroscopy [3]. In the current report, we use 20 nm GNP s for visual identification of pork adulteration in meatball formulations. We demonstrate that 20 nm colloidal particles produce more pronounced changes in color and absorption spectra than those of 40 nm counterparts. The absorption peak at 525 nm changes its position and appears in a new location between 555–580 nm depending on the degree of aggregation in 3 mM PBS (60 mM NaCl, pH 7.4). Stronger absorbance also remarkably appeared between 550–800 nm, making the identification more obvious. The detection limit (DL) of genomic DNA in heterogeneous mixture is also significantly reduced than that of 40-nm counterpart [3].

Meatball is a special type of restructured comminuted meat products [20, 21]. It is a favorite food in certain Asian countries such as Malaysia and Indonesia and also some European countries [20, 21]. Pork is a potential adulterant in beef and chicken meatballs due to its availability at cheaper prices. The mixing of pork or its derivatives in the Halal and Kosher foods is a serious matter as it is not permissible by the respective religious laws [20–22]. Unconscious consumption of pork may also ignite allergic reactions in certain individuals [4, 5]. Additionally, its high content of cholesterol and saturated fats are a concern for people with diabetes and cardiovascular diseases.

2. Materials and Methods

2.1. Swine Specific Probe Design. A 25 nt swine probe (567–(5’TAC CGC CCT CGC AGC GT ACA TCT C–(3’TAC CGC CCT CGC AGC GT ACA TCT C–(3’))–591) is designed by comparing Sus scrofa cytochrome b (cytb) gene (GenBank: GU135837.1) with Bos taurus (cow; GenBank: EU807948.1) and Gallus gallus (chicken; GenBank: EU839454.1) cytb genes by ClustalW multiple sequence alignment program (http://www.genome.jp/tools/clustalw/). NCBI BLAST (http://www.ncbi.nlm.nih.gov/nucleotide/blast) analysis against nonredundant nucleotide collection confirms the probe is unique for the pig as no other species shows similarities with it. The probe is purchased from the first BASE, Selangor, Malaysia.

2.2. Synthesis of Colloidal Gold Nanoparticles. Colloidal GNP s are synthesized by the citrate method described in bibliography [23]. The resultant particles are characterized by Hitachi 7100 transmission electron microscope (TEM) (Figure 1) and PerkinElmer Lambda 25 UV-vis spectrophotometer (Figure 2). The concentration and particles number are determined according to Haiss et al. [24]. All chemicals are procured from Sigma-Aldrich, USA, in the highest analytical grades and are used without further purification.

All solutions are prepared in 18.2 MΩ water (Sartorius) immediately before use. All glass wares are cleaned with piranha solution and are oven dried prior to use.

2.3. Preparation of Meatballs and DNA Extraction. Meatballs are prepared according to Rahman et al. [21] either with pure or mixed emulsified meats of pork, beef, and chicken, along with the addition of starch, seasonings, and salts in certain ratios. All the meatballs are cooked in boiling water for 20 min prior to DNA extraction. DNA extraction is performed from 100 mg of cooked meatball of each formulations using MasterPure DNA Purification Kit (Epicenter Biotechnologies, USA) as per the manufacturer instructions. The DNA concentration is determined with a biophotometer (Eppendorf, Germany) based on triplicate readings. The purity (A260/A280) of all DNA samples used in all experiments is 1.95–2.0.

2.4. Detection of Single-Stranded and Double-Stranded DNA. In four separate vials, labeled as (a)–(d); Figure 2), 100 µL of 1.8 mM colloidal GNP s is taken. Thirty microliters (30 µL) of 25-mer single-stranded (ss-) and double stranded (ds-) oligoprobes of 100 nM (1st BASE, Malaysia) are added into vials (c) and (d). Volume in vial (b) is adjusted with water (18.2 MΩ). All vials, except dsDNA containing one (d), are incubated in a water bath at 50°C for 3 min to facilitate ssDNA adsorption onto GNP s [2]. Vial (d), which contains dsDNA, is incubated at 25°C to avoid temperature-induced dehybridization of the complementary strands [3]. Then 300 µL of 10 mM PBS (0.2 M NaCl, pH 7.4) is added into each tube except vial (a) where the volume is homogenized with water. All tubes are vortexed immediately. Colloidal suspension in PBS (b) and dsDNA (d) turns into grey-purple within 3 min or immediately. However, GNP s in DI water (a) and ssDNA exposed vial (c) remain undisturbed. They retain their characteristic pinkish-red color. After 10 min, sufficient water is added into each vial to adjust the final volume to 1 mL and is characterized by transmission electron microscopy (Figure 1) and absorption spectroscopy (Figure 2). Thus the final concentration of probe, GNP s, and PBS buffer is made to 3 nM, 180 pM, and 3 mM, respectively. Stability of ssDNA-incubated colloidal particles in 3 mM PBS is studied for seven days keeping them at 4°C and is found unchanged.

2.5. Pork Identification in Beef and Chicken Meatballs. In order to detect pork contamination in processed meat products, meatballs are prepared with emulsified mixed meats of pork-beef, pork-chicken, and chicken-beef binary mixtures in 1:1 (w/w) ratios. Pure meatballs are formulated with pure meats of each species under identical conditions. After 20 min of cooking in boiling water, DNA extractions are performed. One hundred microliters (100 µL) of mixed genomic DNA (300 µg/mL–1) is taken in vials ((b)–(d); Figure 3). Equal portion of pure genomic DNA of pork, beef, and chicken is taken in vials (a), (e), and (f). All tubes are exposed to 30 µL of 100 nM swine probe (25 nt; inset of Figure 3) at 95°C for 3 min to allow denaturation. All mixtures are cooled down to 50°C for 2 min to favor
Figure 1: TEM images of colloidal particles before and after salt-induced aggregation. Shown are 180 pM gold colloids in DI water (a), in 3 mM PBS (b), in 3 mM PBS after 3-minute incubation in 3 nM ssDNA probe at 50°C (c), and in 3 mM PBS after the same-time incubation in equimolar 25-bp dsDNA at 25°C (d). All images are shown at a magnification of 100,000 times.

Figure 2: Absorption spectra of aggregated and non-aggregated GNPs. Shown are absorption spectra of 180 pM gold colloids in DI water (blue curve (a)), in 3 mM PBS (pink curve (b)), and in equimolar PBS after incubation with 3 nM ssDNA probes (red curve (c)), and with the equimolar dsDNA probes (green curve (d)). The vials in the inset shows the color photographs of the solutions in DI water (a), PBS buffer (b), PBS buffer plus ssDNA (c) and PBS buffer plus dsDNA (d).

perfectly matched annealing and mismatched nonannealing. Subsequently, 100 µL of 1.8 nM gold colloids is added to each vial and mixed for 2 min by mild shaking to allow adsorption of unhybridized probe onto GNP-surfaces. Finally, 300 µL of 10 mM PBS is added to induce aggregation of colloidal particles. All the swine DNA containing vials ((a)–(c)), either in pure (a) or mixed forms (b) and (d), immediately turn into purple-grey. However, the rest of the vials ((d)–(f)) that contain other species (chicken or beef) retain the characteristic color of colloidal particles. The final volume is adjusted to 1 mL with water and is characterized by absorption spectroscopy. Thus the final concentration of GNP, probe, genomic DNA and PBS is made to 180 pM, 3 nM, 30 µg/mL, and 3 mM.

2.6. Determination of LOD. To determine LOD, raw pork and beef are mixed in a ratio of 1:99, 3:97, 5:95, 10:90, and 15:85 (w/w). All mixtures are emulsified and meatballs are prepared. DNA is extracted from cooked meatballs of each formulation. One hundred microliters (100 µL) of mixed DNA (400 µg/mL) is taken into five separate vials ((a)–(e); Figure 4)). All vials are exposed to 15 µL of 100 nM
Figure 3: Identification of swine DNA in mixed meatballs. Vials ((a)–(f)) represent color of GNPs in genomic DNA extracted from meatballs prepared with pure pork (a), 1:1 (w/w) mixtures of pork-beef (b), pork-chicken (c), chicken-beef (d), pure beef (e), and pure chicken (f). The corresponding absorption spectra are labeled by respective alphabets. All vials are incubated at 95°C for 3 min and annealed at 50°C for 2 min before adding the colloidal particles and PBS. The top inset is the comparison of probe sequences with shown species. Mismatch bases are demonstrated by red.

swine probe (25 nt; Inset of Figure 3) at 95°C for 3 min and then annealed at 50°C for 2 min. After that 50 µL of 1.8 mM gold colloids is added to each vial and incubated for 2 min with mild shaking. Finally, 100 µL of 10 mM PBS is added to each vial. Vials ((a)–(c)) retain the pinkish-red color of monomeric GNPs with an increasing trend of fading. The fading of color proportionates the portion of pork in each vial. Vials (d) and (e) clearly turn into purple-grey, indicating clumping of colloidal particles. The final volume is made to 1 mL with water and is characterized by absorption spectroscopy. Thus the final concentration of probe, GNPs, mixed genomic DNA, and PBS is made to 1.5 nM, 90 pM, 40 µg/mL, and 1 mM. The concentration of swine DNA in vials ((a)–(e)) is calculated to be 0.4, 1.2, 2.0, 4.0, and 6.0 µg/mL.

3. Results and Discussion

3.1. Characterization of Gold Nanoparticles and Detection of DNA. The formation of gold nanoparticles is confirmed by TEM images (Figure 1) and UV-vis spectra (Figure 2). The size of the particles (diameter: 20 ± 5 nm) is assigned according to previously established methods [2, 3]. TEM images revealed that most of the particles are spherical in shape and homogeneously distributed throughout the bulk solution in water (a) and in ssDNA incubated 3 mM PBS (c), clearly showing particle isolation. A minor fraction of the particles are appeared in small groups sitting side by side or one on another in water (a), showing a very low level of aggregation in DI water. This is consistent with the findings of Li and Rothberg [2]. Negative coatings of citrate ions on GNP-surfaces electrostatically repel one other, keeping them separated. ssDNA adsorbed onto GNPs surfaces by van Waals interactions and adds negative charges on GNP surfaces with the exposed phosphate groups [2, 3]. Thus the GNPs are stabilized against salt-induced aggregation when they are previously exposed to ssDNA [3].

However, the huge aggregates of GNPs become obvious after the addition of salts (3 mM PBS; b) that induces clogging of particles by screening the repulsive negative charges on particle surfaces [2]. Particles aggregation is also found in dsDNA, containing 3 mM PBS (d). However, the size of the aggregates appeared to be smaller. This is probably due to the partial protection provided by a small fraction of ssDNA which is frequently present in dsDNA solution [3].

Unlike ssDNA, dsDNA cannot protect the particles from salt-induced aggregative stresses [2, 3]. This is contrary to the conventional wisdom as both of them are highly negatively charged due to the constituent phosphate back-bone. However, when the nitrogenous bases of uncoiled ssDNA face the
3.2. Detection of Pork Adulteration in Mixed Meatballs. In order to detect pork adulteration in beef and chicken meatballs, we design a 25 nt swine probe that bears full matching with swine cytb and 13 nt and 14 nt mismatching with the bovine and chicken cytb genes (Figure 3: inset). Thus the mismatching with bovine and chicken genes is 52% and 56%, respectively. The presence of mismatch bases has a remarkable effect on hybridization [2, 3, 9, 12, 13, 16]. Mismatches reduce melting temperature (Tm) significantly, making hybridization difficult [2, 3]. Therefore, it is highly unlikely for the probe to hybridize with bovine and chicken DNAs that contain more than 50% mismatch nucleotides at or near temperature where perfectly match hybridization is possible [2, 3]. Consequently, the probe should be available to interact with GNPs if it is annealed with bovine and chicken genes at or near its melting temperature (64°C).

We mix the probe with an excess of pure and mixed genomic DNAs of pork, beef and chicken extracted from ready to consume meatballs of respective species as shown in Figure 3. We denature the mixtures at 95°C to induce strand separation of all genomic DNAs [4–8]. Afterwards, we cool down the mixtures to 50–60°C to allow complementary base-pairing between the strands and the probe. Previous studies demonstrated shorter DNA hybridizes before the longer counterparts due to steric reasons [2]. Thus the limited probe should be completely engulfed by the excess genomic DNA if it bears complementary targets within it. However, the probe does not hybridize with mismatch bearing targets if they are not forced to do so by the excessive reduction of annealing temperature.

The inset of Figure 3 clearly shows that the probe is consumed completely by the pork DNA in pure (vial (a)) or
mixed forms (vials (b) and (c)). Thus the colloidal particles in all pork containing vials (a)–(c) experience clogging upon the addition of salts as they are not protected by the ssDNA probe. This is clearly depicted by the dramatic change of color from pinkish-red to grey purple. The UV-vis spectra demonstrate a huge red-shift of ∼100 nm and appearance of a collective plasmon peak at 575 nm, confirming the visually determined result. However, probe is not engulfed at all by the huge mismatch (>50%) containing beef or chicken DNA either in pure (vials e & f) or mixed formulation (vial d). Thus the probe is available in vials ((d)–(f)) to adsorb onto GNPs surfaces to provide them withstanding strength in salt solution. Consequently GNPs of these vials do not undergo aggregation upon the addition of equimolar PBS. This is clearly revealed by their retention of characteristic pinkish-red color and plasmon peak of isolated colloidal particles at 525 nm.

3.3. Determination of LOD. The absorption spectra and visually detected color of GNPs in various percentages of pork containing beef meatballs are shown in Figure 4.

It is very clear from visually observed results as well as spectroscopic data that 1% pork containing vial (a) retains almost 100% original color of colloidal particles (pink curve: spectrum (a)). However, original pinkish-red color of GNPs in 3–5% pork containing vials (b) and (c) considerably disappeared, reflecting partial aggregation. This is confirmed by the appearance of collective plasmon peak near 535 nm and considerably stronger absorption between 550 and 650 nm ((red curve: spectrum (b) and green curve: spectrum (c))). On the other hand, 10% and 15% pork containing vials ((d) and (e)) change color from pinkish-red to purple-grey simulating aggregation. Absorption spectra of vials (d) and (e) display the collective surface plasmon features of 20 nm aggregated particles between 550 and 700 nm with a collective plasmon peak near 555 nm. Concentration of swine DNA in 10% pork containing vial is 4 µg/mL. Thus the determined LOD is 4 µg/mL swine DNA in processed beef meatballs. It is observed that some of the particles (∼3–5%) retain their colors in vials (d) and (e) that contain 10% and 15% swine DNA. These are most likely the unconsumed probe-bound particles that withstand the salinity stresses.

3.4. Efficacy and Limitation of the Current Assay. The current assay directly determines swine-specific sequences in a population of nonamplified mixed genomic DNA. The mixed population of genomic DNA is obtained from meatballs, prepared with the emulsified meats of chicken, beef, and pork. The method is capable of detecting target sequences just by visually observed color change of GNPs. The visually determined results are sufficient to make a concrete decision. However, it can be further authenticated by a relatively inexpensive and easily available absorption spectroscopy. This eliminates any sort of color blindness errors that may arise from visual findings. Sensitivity of the assay is also improved as revealed by a low LOD (4 µg/mL).

Both the color and absorption spectra of 20 nm GNPs are more remarkable than the earlier report [3], making them a more suitable candidate for the analysis of targets in processed meat products. In earlier report, we have shown absorption peak of 40 nm colloidal particles at 530 nm fall down commensurating the degree of aggregation [3]. However, the current study has shown the absorption peak of 20 nm particles change its position and appears in a new position proportioning particle clumping. Absorption between 550–800 nm regimes is also significantly increased following aggregation. Thus a well-defined change in the peak position of aggregated and non-aggregated particles can be easily detected avoiding any ambiguity. These features probably make 20 nm counterparts more sensitive than 40 nm particles.

The LOD of the assay is higher than that of the real-time and conventional PCR [5–7]. However, PCR-based methods need comparatively longer targets which are reported to break down during the chemical and physical stresses of food processing, causing template crisis in PCR assay [9]. On the other hand, the present assay uses DNA target (25 nt) that is comparable with the size of a typical PCR-primer [5–7]. As shorter targets are more stable than the longer one [7], the method can be applied to analyze highly degraded samples where PCR may lose its candidacy. The probe deign is also much simpler than that of a PCR assay. Moreover, PCR-electrophoresis is a clumsy technique and sometimes needs self-authentication by RFLP-analysis [4], sequencing, or blotting [8]. The LOD of the assay can be decreased by using increased amount of DNA mixtures to ensure sufficient targets for the probe. Using increased amount of targets is not problematic in food analysis because here sample scarcity is not a concern.

The presence of single stranded nucleic acids (DNA or RNA) interferes with target detection by sticking to GNPs and interfering particle aggregation. However, by using appropriate purification technique [26], the single-stranded nucleic acid can be easily removed from the degraded samples.

The method cannot provide quantitative information of the target DNA. TaqMan fluorogenic probe can detect, quantify, and amplify specific sequences by real-time PCR without the need of electrophoresis and blot analysis [7]. However, the TaqMan probe, real-time PCR, and the master-mix used in real-time PCR are highly expensive and ordinary laboratories cannot afford them. On the other hand, UV-vis spectroscopy is available in most laboratories and can authenticate the visually identified results of colloidal gold.

4. Conclusion

A rapid (less than 10 min), reliable, and cheap method for the selective detection of target DNA sequences in processed meat products is developed. It does not need any instrument or surface modification chemistry and directly detects target DNA in nonamplified mixed genomic DNA. The procedure is very simple and relies on the color change of 20-nm GNPs following salt addition. The visual finding is solid and can be further confirmed by an inexpensive, available, and reliable technique, absorption spectroscopy which incurs
only the instrumental cost and reusable cuvette. The use of absorption spectroscopy increases sensitivity and eliminates any sort of color-blindness error or ambiguity in visual detection by producing well-defined bands of aggregated and non-aggregated colloidal particles. The assay needs a shorter probe whose design is simpler than PCR primers. The method is applicable to analyze extensively degraded sample which may not be possible by PCR which require longer targets.

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