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Effectual Gold Nanoprobe Sensor for Screening Horse Adulteration in Meat Products

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Abstract

A gold nanoparticle (AuNP) probe strategy for testing meat authenticity was developed, which relies on the colorimetric differentiation of a particular DNA sequence, due to the differential aggregation profiles exhibited by the AuNPs in the presence or absence of specific target hybridization. Gold nanoparticles were conjugated with thiolated oligonucleotides for specifically identifying a 69 bp fragment of the horse *cytochrome b* gene. In the presence of a complementary target preventing aggregation of the AuNPs when acid was added, the reaction mixtures retained the original pink colouration of the colloidal particles, whereas they turned purple in the opposite event. Fresh meatballs, prepared using pure bovine meat, were used as blanks, producing a purplish coloured solution with a peak at ≥570nm. Horse meat was used as positive control and the pink colour obtained after hybridization exhibited maximum absorption at 524 nm. Both the specificity and sensitivity of the tests performed were 100%. Visual observations and spectroscopic data indicated that the coloration produced by the AuNPs (positive-pink, negative-purple) was very stable, showing no change under normal laboratory conditions. The use of AuNPs for the colorimetric detection of DNA targets from undeclared species in meat products provides an inexpensive and easy-to-perform alternative to common molecular assays. The technology described here can be further developed to accommodate detection of many cases of adulteration and fraudulent practices.

Keywords: Gold nanoparticles, food authentication, horse meat adulteration

1. Introduction

Fraudulent practices involving substitution of high-priced meats by lower commercial value species or undeclared non-meat ingredients are becoming a serious problem in the meat processing sector. Although generally sporadic, these practices seem to have escalated in recent years, challenging consumers' confidence in the integrity and safety of the meat supply chain, as well as impacting purchasing and eating behaviour (Barnett et al., 2016; Stanciul, Stanciul, Dimistrascu, Ion, & Nistor, 2013). Ground meat products and precooked, ready-to-eat meat items seem to be more prone to adulteration, due to the lack of external diagnostic traits for species identification. Several factors appear to contribute to this growing phenomenon, including economical gain, increasing diversity of products, complexity of international food trading networks, deficiencies in traceability systems for ethnic foodstuffs, as well as lack of effective methods to identify meat sources, particularly in thermally processed products (Rahmati, Julkapli, Yehye, & Basirun, 2016; Armani et al., 2015; Ercsey-Ravasz, Toroczkai, Lakner, & Baranyi, 2012). Regardless of the motive or the level at which adulteration takes place, the repercussions of this act are manifold, far exceeding economic fraud. A multitude of practical, ethical, religious, and safety issues may be introduced as a result of fraudulent meat substitution in the processing and merchandising chain (Iammarino, Marino, & Albenzio, 2017; Rahmati et al., 2016; Ali, Razzak, & Hamid, 2014). Combating mislabelling requires that sensitive and inexpensive diagnostic tests for rapidly identifying meat species are developed and integrated within sector-specific and generic traceability systems. Most of the methods currently in use for monitoring food authenticity are based on the detection of species-specific proteins and DNA analyses. Molecular methodologies are especially useful in food authentication testing, thanks to the ubiquitous presence of DNA molecules in all biological tissues, and their stability under the production and processing operations applied along the food-chain (Asensio, Gonz ález, Garc á, & Martin, 2008; Taylor et al., 2012). DNA-hybridization techniques used for identifying sequences in the

genomic DNA provide a far more effective approach in authentication tests (Rahmati et al., 2016). Thus, Chen, Wei, Chen, Zhao, & Yang (2015) developed a duplex PCR assay for the identification of horse, donkey and mule species in raw and heat-processed meat products, based on the amplification of a fragment of the mitochondrial DNA. According to the authors, the target meat species could be detected at a level of 1%. Similarly, Ali et al. (2015) and Ilhak and Arslan (2007) were able to determine the origin of several meat species (beef, sheep, pork, goat, horse, cat, dog, monkey, and rat) by multiplex PCR assays, using amplified species-specific fragments of the mitochondrial and *cytochrome b* genes. Currently, a new challenge in the identification of meat origin in foods has emerged, which involves the development of specific nanoparticle-based probes, capable of detecting several-fold shorter DNA target sequences found in highly processed products (Ali et al., 2014). Designing DNA hybridization detection assays that do not depend on target-amplification by the PCR reaction offers higher flexibility and greater multiplexing capabilities, as well as ignoring the requirement for time-consuming electrophoresis that sometimes needs self-authentication by RFLP-analysis, sequencing, or blotting (Hill & Mirkin, 2006). Therefore, the development of a simplified, cost-effective and accurate procedure for detection of trace amounts of fraud, not requiring complex instrumentation, seem to be imperious in order to overcome the time delay and allow rapid and sensitive detection. Nanotechnology and, more specifically, gold nanoparticle chemistry provides revolutionary opportunities for the rapid and simple diagnosis of authenticity, being able to detect trace amounts of fraud, due to their unique optical properties. Gold nanoparticles have been used successfully as colorimetric sensors for visually identifying pork adulteration in beef and chicken meatball preparations (Ali, Hashim, Mustafa, Che Man, & Islam, 2012). The aim of the present study was to design and construct functionalized gold nanoparticles (AUNPs) that could be incorporated into an easily applicable DNA detection methodology for the identification of horse meat adulteration in meat products.

2. Method

2.1 Meat Products and Controls

A total of 40 meat items were collected from local super markets and fast food restaurants in the area of Athens. The sampled products included fresh meat cuts (5 items), slices of roasted beef and pork (14 items), ready to eat meatballs (8 items), and country style sausages (13 items). Fresh meatballs prepared in the laboratory using pure bovine meat were used as blanks, whereas horse meat was used as positive control. Bovine meatballs containing different levels (0.1-50%, w/w) of horse meat were also analyzed, in order to determine the assay's detection limit of horse meat adulteration.

2.2 Preparation of Gold Nanoparticle Probes

Twenty nm gold colloid nanoparticles (AuNPs) were purchased from BBI Solutions (Cardiff, UK). The oligonucleotides specified sense GAA GCA TAA TAT TCC GG and Antisense primer TTA GTG TCA GTA AGT CTG CC were used (specific for the identification of the *cytochrome b* gene). All oligonucleotides were thiolated (modified with 10 dATP in the 5' end of the primer). The AuNP merging with the oligonucleotides was performed by adding 1 ml of an aqueous solution of AuNPs to 4 nmol of the thiolated oligonucleotides, specific for the *cytochrome b* gene, using a previously described protocol (Li & Rothberg, 2004). Briefly, the thiol modified oligonucleotides were initially incubated with the AuNPs overnight, using an orbital shaker at room temperature. The solution was then brought to 9 mM phosphate buffer (pH 7) and SDS solution (0.1%, w/v) was added to prevent aggregation. The total volume of salting buffer (2 M NaCl in 10 mM PBS) required to bring the AuNPs solution to a final concentration of 0.3 M NaCl was divided into six doses that were added over the next 48 hours. After centrifugation, the precipitate was washed with 500 μl of 10 mM PBS (pH 7.4), 150 mM NaCl, and 0.1% SDS, followed by centrifugation and re-suspension in 500 ml of the same buffer. The gold nanoprobes were placed in glass vials and stored in the dark, at room temperature (Hill & Mirkin, 2006).

2.3 DNA Isolation

DNA extraction from horse meat (designated positive control) and commercial meat products was performed using the NucleoSpin Food kit (Macherey-Nagel, GmbH & Co. KG, Germany), according to the manufacturer's instructions. The extracted DNA was quantified spectrophotometrically at 260 nm, and serial tenfold dilutions were prepared (C_1=1,23 ng/μL, C_2=123 pg/μL, C_3=12,3 pg/μL, C_4=1,23 pg/μL, KC_5=123 fg/μL, KC_6=12,3 fg/μL) for evaluating the analytical sensitivity of the DNA extraction method. DNA samples from bovine and pork meat were used for specificity confirmation (designated specificity negative controls).

2.4 PCR Amplification

PCR was performed according to a previously published protocol (Chisholm, Conyers, Booth, Lawley, & Hird, 2005) in 50 μl final volume solutions, using the GoTaq Hot Start Master Mix (Promega Gmbh, Mannheim,

68199, Germany), 1 mM each of the primers Forward: GAC CTC CCA GCT CCA TCA AAC ATC TCA TCT TGA TGA AA and Reverse: CTC AGA TTC ACT CGA CGA GGG TAG TA amplifying a 439 bp gene, and 10 µl of eluted DNA. PCR products were separated in 2% agarose gel, stained with ethidium bromide (0.5 µg/ml) and documented under UV illumination.

2.5 Direct Hybridization and Colour Detection in Food Samples

In order to obtain an indication of the method's performance on food samples, the optimized assay was applied for detecting horse meat adulteration in the food samples referred to above. For the detection of horse meat, 15 µl of the DNA extracted from the food were added to 10 µl of the AuNP-oligonucleotide solution, followed by two incubation steps, one denaturation step at 95℃ for 5 min, and one annealing step at 55℃ for 5 min. The presence of complementary DNA prevented aggregation of the nanoprobes when 2 µl 0.1 N HCl were added in the reaction mixture, followed by incubation for 5 min at room temperature, so that the solution remained pink; in the opposite event (no presence of complementary DNA), the reaction mixture turned purple. The colour change could be detected visually and it was further confirmed by absorption spectra. The solutions were photographed after standing for 5–15 min at room temperature for full colour development. All experiments were performed in triplicate and the results were compared with those obtained by PCR assays. To assess the repeatability of the method for the specific types of samples, testing with the proposed assay was repeated three times for each DNA extract.

3. Results and Discussion

Spherical gold nanoparticles in the size range of 13–20 nm, showing absorbance peak at around 520 nm, have been employed in biosensors due to ease of synthesis. The AuNP-oligonucleotide solution exhibits a pink colour because of surface plasmon resonance at an absorbance peak of ~525 nm. In the absence of the specific DNA target, the addition of HCl enhances aggregation of the AuNP-oligonucleotide probes, leading to a change in colour from pink to purple. The visually detected changes are strongly supported by the remarkable features in UV-Vis spectrum (Li & Rothberg, 2004). Using horse meat (positive control), the proposed assay produced positive results, with the sensitivity being 100%. Serial 10-fold dilutions of the positive control DNA, starting from 1.23 ng/µL (C_1), indicated that the lower detection limit (LOD) of the assay was 12.3 fg/µL of DNA, as compared to the 123 fg/µL LOD of the PCR method, thus indicating that the AuNP-oligonucleotide assay performed better results than the PCR in the range of one 10-fold dilution. PCR-based methods need comparatively longer targets, which are known to break down during chemical and physical stresses induced by food processing, causing template crisis in the PCR assay (Ali et al., 2011). The UV-Vis spectroscopic data obtained supported the hypothesis of aggregation-induced visual discrimination of the samples. As shown in Figure 1, samples containing horse genomic DNA exhibited the characteristic absorbance peak of the AuNPs at 520 nm, caused by the collective excitation of the free conduction band electrons of the dispersed particles, known as the surface plasmon resonance. On the other hand, the wide absorbance spectrum exhibited by negative (bovine and/or pork meat) samples is indicative of the peak shift towards longer wavelengths (≥ 550nm), due to the coupling in the surface plasmons of the particles in the aggregates (Figure 2). Thus, the collective plasmon peak was intensified and appeared in a new position, between 560 and 800 nm, depending on the degree of aggregation and concentration of the AuNP-oligonucleotides. The specificity of the assay was 100% (true negative rate).

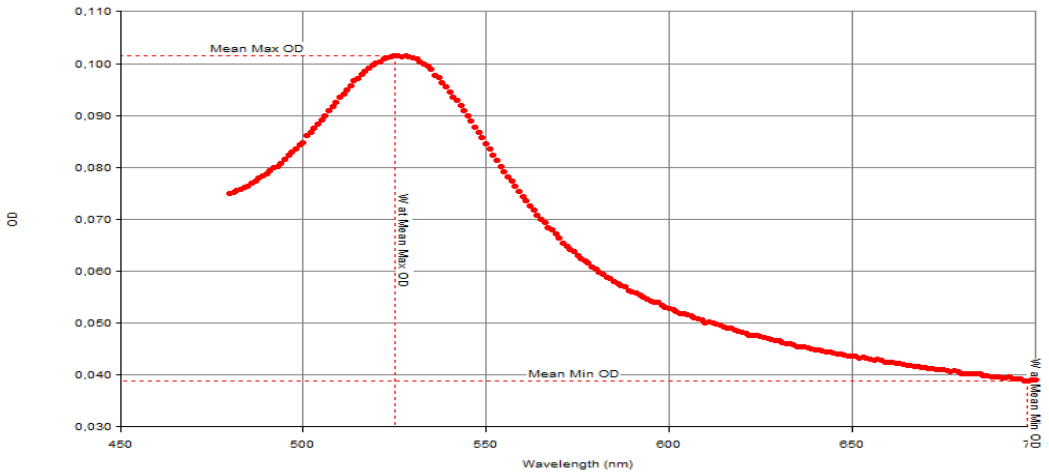

Figure 1. UV-Vis spectrum from sample containing horse genomic DNA, showing characteristic absorbance peak of AuNP at 520 nm

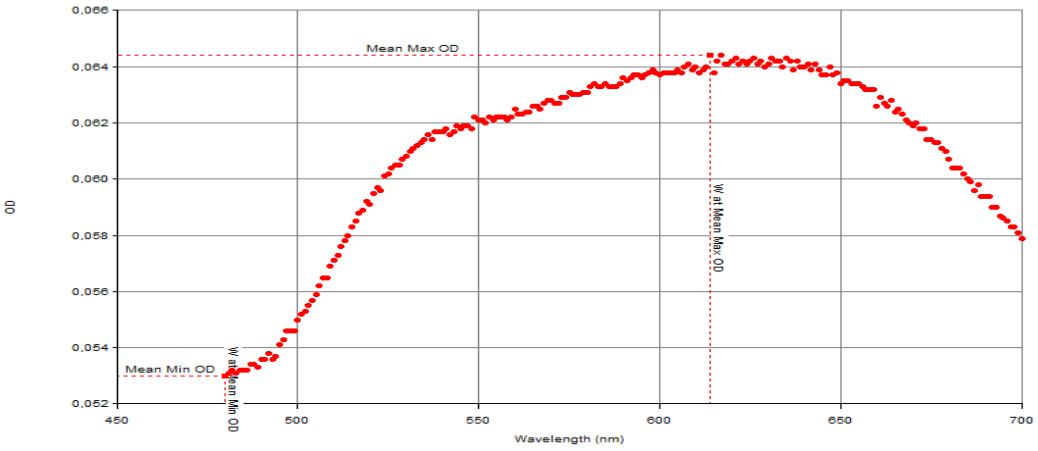

Figure 2. Negative sample exhibiting wide absorbance spectrum longer wavelength (≥600nm)

The method was repeatable, producing identical results all three times it was performed. Interestingly enough, when left overnight at room temperature, positive samples retained their colour, indicating long time stability of the AuNP-oligonucleotide probe hybridization with the target sequence. This feature can be particularly useful when prolonged read-out capability is required for high-throughput applications. The colouration exhibited by AuNP-oligonucleotides obtained from bovine meatballs containing different levels (0.1-50%) of horse meat, is shown in Figure 3. It was clear from visual observations and spectroscopic data that the colloidal particles retained their original coloration practically unaffected, regardless of the horse meat level. By contrast, the pinkish-red colour of the AuNP-oligonucleotides turned into purple, when only beef was present, indicating aggregation of the AuNP-oligonucleotide probes. This was confirmed by the appearance of a collective plasmon peak near 535 nm and a considerably stronger absorption between 550 and 650 nm. Of the 40 items tested in this study, 16 (40%) were positive by the AuNP-oligonucleotide assay; the horse meat gene was detected in 6 out of the 8 (75%) meatball items, as well as in 10 out of the 13 (77%) country style sausages. The sensitivity of the assay was 100% (true positive samples). When using the PCR assay, only 10 out of the 40 items (25%) were positive. The sensitivity of the PCR assay was 62.5%, probably due to the inefficiency of the method when applied to highly degraded samples.

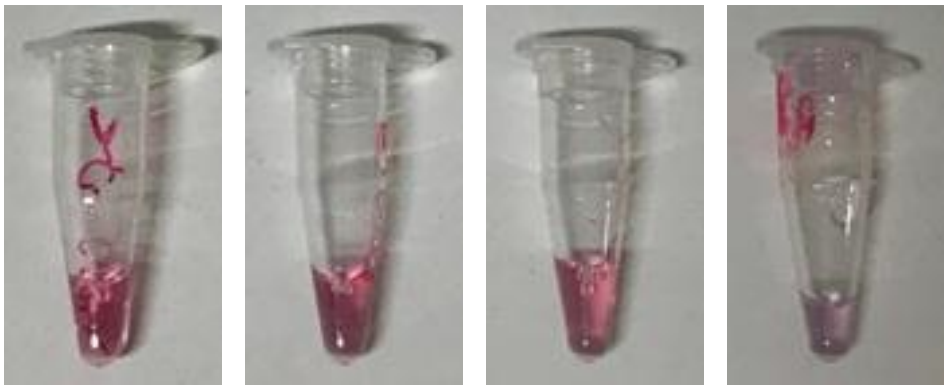

Figure 3. Horse-beef mixtures in 0.1: 99.9, 10: 90 and 50: 50 ratios (w/w), along with a negative control (100% beef)

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