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Research Article

Effectual Gold Nanoprobe Sensor for Screening Cow Milk Adulteration in Goat Milk

Jose Manuel Llopis Ortiz*, Dimitra Panagiotis Houhoula, Efstathia Tsakali and Sophie Pytel

Department of Food Technology, Technological Educational Institution of Athens, Greece

***Corresponding Author:** Jose Manuel Llopis Ortiz, Department of Food Technology, Technological Educational Institution of Athens, Greece.

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Abstract

Different methods of detection have been used to detect milk adulteration, but in recent years the use of nanomaterials has demonstrated to be an interesting tool in this field, because of their interesting optical properties. A gold nanoparticle (AuNP) probe strategy for testing milk 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 which specifically amplify a 271 bp fragment of cow mitochondrial DNA. In the presence of a complementary target preventing aggregation of the AuNPs when acid was added, the reaction mixtures retained the original pink coloration of the colloidal particles, whereas they turned purple in the opposite event. Negative and goat reaction mixtures showed a purplish coloured solution with a peak at ≥570nm, while samples containing bovine DNA have an absorbance closer to the characteristic peak of the AuNPs at 520-525 nm. Presence of bovine milk even at traces levels was detected, achieving a level of detection comparable with PCR + Electrophoresis. The use of AuNPs for the colorimetric detection of DNA targets from undeclared species in milk products provides an inexpensive and easy-toperform alternative to common molecular assays. However, the tested oligonucleotides only proved to be effective under very concrete conditions, due to their low specificity. The technology described here can be further developed and more specific oligonucleotides are advised to be tested. Even though, this method offers the possibility to accommodate for detection of many cases of adulteration and fraudulent practices in different food matrices.

Keywords: Gold Nanoparticles; Food Authentication; Goat Milk Adulteration; Detection Limit

Introduction

Food adulteration has always been a major problem in relation with the food industry. Substitution of high-prices materials by other ones with lower commercial value or addition of ingredients non related with the nature of the product are practices that have been reported since years ago and which cause the lack of confidence of the public in the Food Industry [1]. Natural milk is considered as an important nutritive food, being a proper source of carbohydrates, fats, quality proteins, minerals and vitamins [2]. Its beneficial effects for mothers, infants, children and elderly people are due to its easy digestibility and absorption, and its proteins are a significant source of amino acids needed for a correct growth [3]. Nevertheless, when adulterated, there are not only a decrease in the quality and an economic fraud, but also can be dangerous for consumers. Bovine milk is commonly used for these practices because of its predominant production in the world and its cheaper cost in comparison with other kinds [4]. This circumstance supposes a risk for consumers affected with allergies to cow milk components in the case they would drink impure goat milk [5-7], because milk proteins, even when found at low concentrations, are potential allergens [8,9]. This fraud has an important impact considering than in occidental countries goat milk consumption is gaining importance. Previous studies have shown that adulteration of dairy products with cow milk is a common practice, especially in developing countries [10-12].

Due to the aforementioned social and health impact of milk adulteration, there is a vast number of publications about different methods for its detection. Over time, new methods appeared and have been perfected, but still the majority of methodologies to

identify dairy products' authenticity are based in the study of major milk proteins [13]. Other analytical strategies are immunological [14-16], electrophoretical [17], chromatographic [18]. Among all the studied techniques, PCR is the most widely employed method to identify milk adulteration [19,20] due to its level of sensitivity and the repeatability of the results. In the literature there are examples of researchers who have been able to detect up to 0,5% [10] and 0,1% [21] of cow milk in other species' milk.

At present, they are appearing new strategies for the identification of the origin of foods, related to the development of precise nanoparticle-based probes, competent for detecting DNA target sequences [22-25]. Nanotechnology and, specially, gold nanoparticles (AuNPs) chemistry offers innovative opportunities for quick and easy analysis of authenticity, being capable to evidence small amounts of adulteration, thanks to their particular optical properties [26]. AuNPs technique, used effectively as colorimetric sensors for visual identification, supposes a cheaper and simple alternative to other molecular methods, and it has be proven to work with other food matrices [22-25]. The intention of the present research paper is to demonstrate that functionalized AuNPs can be used to develop a simple DNA detection procedure for the identification of cow milk adulteration in goat milk, and to compare its sensitivity with the PCR, the most used method.

Method

Selection and preparation of samples

Authentic milk samples from cow (*Bos taurus*) and goat (*Capra hircus*) were acquired directly from the animals of a local dairy farm and collected into collection tubes. These samples were transported to the laboratory in refrigeration conditions and were stored frozen at -18ºC until used.

Different dilutions of cow milk in goat milk were prepared (traces, 1%, 2%, 5%, 10%, 20% and 50%). Also pure goat milk was used as blank and cow milk as positive control.

Preparation of gold nanoparticle probes

Twenty nm gold colloid nanoparticles (AuNPs) were purchased from BBI Solutions (Cardiff, UK). The AuNPs were conjugated with specific oligonucleotides for cow: sense primer GCC ATA TAC TCT CCT TGG TGA CA and antisense primer GTA GGC TTG GGA ATA GTA CGA (Invitrogen™, USA), which amplify a 271 bp fragment of mitochondrial DNA) [5,27,28]. To test the possibility to work with only one primer, part of the nanoprobes were prepared conjugating only with the sense primer. One of the objectives of the present project, is to find a cheaper alternative to current employed methods, so if it is possible to work with only one primer instead of two, the price of the technique will be lower.

The oligonucleotides were thiolated, which is an addition of 10 dATP (deoxyadenosine triphosphate) at the 5' end of the primer. This is because thiols show an important affinity for gold surfaces (due to the formation of sulfide bonds) (Figure 1) [29]. The thiolated oligonucleotides can be conjugated by direct mixing with AuNPs, followed by a "salt-aging" process which makes the binding of the primers to the gold surface easier. 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 using a previously described protocol (Li and 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 and Mirkin, 2006).

The obtained functionalized AuNPs solution was measure with a Spectrophotometer Epoch of BioTek®. The result was consequent with these previous observations, the maximum absorbance was around 520-530 nm. This indicates a proper conjugation of Gold NP with the oligonucleotides.

DNA isolation

1,5 ml of the different milk dilutions were centrifugated (10' at 12000 x g) to obtain a pellet.

DNA extraction from these pellets was performed using the NucleoSpin Food® kit (Macherey-Nagel, GmbH and Co. KG, Germany), according to the manufacturer's instructions. The extracted DNA was quantified spectrophotometrically at 260 nm.

PCR amplification and electrophoresis

PCR was performed according to a previously published protocol described by Mašková and Paulíčková (2006) [30] in 50 µl final volume solutions using MeltDoctor™ HRM Master Mix (Thermo Fisher Scientific™, USA) and carried out in a Veriti® 96 Well Therman Cycler (Applied Biosystems®): Initial denaturation: 94°C, 1 min; 40 cycles with the following stepcycle profile: denaturation 94ºC, 30s; annealing 60ºC, 30s; extension 72ºC, 30s; Final extension 72ºC, 5 min. The chosen primers amplify cow DNA fragments of the size of 274 bp: forward primer: 5' GAC CTC CCA GCT CCA TCA AAC ATC TCA TCT TGA TGA AA 3'; reverse primer: 5' CTA GAA AAG TGT AAG ACC CGT AAT ATA AG 3'. PCR products were separated in 2% agarose gel, stained with ethidium bromide (0.5 µg/ml) and documented under UV illumination using MiniBIS Pro device (DNR Bio-Imaging Systems Ltd., Israel).

Direct hybridization and colour detection in food samples

This method is based on the specific target hybridization of the AuNPs with an specific DNA sequence. Later on, it is possible to induce the aggregation on the NPs with the addition of acid, which leads to a colorimetric discrimination. In general, colloidal solutions of AuNPs with diameters ranging between 5 - 20 nm show a pink color (red when very concentrated), due to its optical absorption peak at around 520 - 525 nm, caused by the collective excitation of the free conduction band electrons of the dispersed particles, known as the surface plasmon resonance [25,31,32]. The aggregation of the AuNPs displaces the absorption peak to a longer wavelength (≥ 570 nm) and the color of the colloidal solution turns purple, as a result of the coupling in the surface plasmons of the particles in the aggregates [33]. Therefore, the degree of aggregation of AuNPs in suspension determines the color of the gold colloid [34], and this aggregation can be simply induced with the addition of hydrochloric acid (HCl) [25]. AuNPs were conjugated with thiolated oligonucleotides (primers) to specifically identify a cow specific DNA sequence (a 271 bp fragment of bovine mitochondrial DNA). In the presence of the complementary target, the functionalized AuNPs hybridize with the cow DNA and do not aggregate. Therefore, after the addition of acid, if in the sample there is cow DNA, the reaction mixture remain with its original pink coloration, whereas if there is not, the mixture changes to purple, due to the aggregation of the AuNPs. 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.

The definitive protocol was designed as follows:

- **Denaturation step:** 20 µl of DNA extract in PCR tube (20 µl of water for the Negative tube). In the thermocycler (same device used for the PCR experiments), 95ºC during 5 minutes;
- **Annealing step:** Addition of 20 µl Au-NPs-oligonucleotides solution and 10 µl of phosphate buffer (prepared as described by Hill and Mirkin, 2006). Incubation in the thermocycler: 5 minutes at 55ºC.
- **Aggregation step:** Addition of 8 µl HCl 1 M to cause the aggregation of the NPs. Incubation at room temperature. Wait 5 minutes to see color change. If not perceptible color change, addition 1 µl of HCl each 5 minutes till observe color change in the negative tube.

If the color remains pink, the sample is positive (presence of cow DNA), because the NPs have found the target DNA sequence and they do not aggregate. If the color turns to purple, the sample is negative (no cow DNA), because the NPs have not found any DNA sequence to hybridize with and they aggregate, causing the color change. It is important always to prepare a Negative tube to compare. Later on, results can be confirmed by UV-Vis spectroscopic analysis.

Results

Concentration of every DNA extracts was measured with the spectrophotometer at 260 nm. The test proved to work when the concentrations were between 10 - 12 ng/µl and all the samples must have a similar concentration. It turned out to be of vital importance to avoid the formation of drops and bubbles inside the PCR tubes (normal situation when handling such small amounts). When this happens, it avoids the proper interaction between reagents, therefore it is crucial to perform all the steps of the protocol gently. In this regard, it is imperative to highlight that during the Aggregation step, after the initial 8 µl of HCl, the following microliters must be added 1 at a time, because a minimal addition of HCl can change the result of the test. For the interpretation of the results, it must be clarified what was considered as a positive and what was considered as a negative result. Theoretically, according to previous studies [25], the reaction mixtures could turn purple (no bovine DNA) or remain pink (presence of bovine DNA). During this project it was evidenced that an intermediate situation could occur: the reaction mixture changes to pink-purple (Figure 1a and 1b).

Figure 1a: Samples with the characteristic light pink color previous to the addition of HCl.

This happens because even in the presence of cow DNA, some AuNPs do not attach to the target sequences, so they aggregate giving to the solution a darker color than the original pink. So, they will be considered as positive all those samples that even being a bit darker than the original reaction mixture, are still perfectly distinguishable with a naked eye from the purple color of the Negative and goat samples. A last experiment was performed with all

Figure 1b: Positive samples exhibit a darker color than the original pink, but still perfectly distinguishable from the purple color of negative samples.

the studied dilutions to evidence how all of them (having different degrees of pink color) can be clearly differentiable from the sample with no cow DNA (purple) (Figure 2).

Figure 2: Last experiment showing the results of the protocol applied to all the studied dilutions.

This difference was further confirmed by absorption spectra. As it can be seen in figure 3, it is appreciable that the absorbance peak of the negative and goat samples are higher than the ones with cow DNA. These negative samples produce a purplish colored solution with a maximum absorbance at ≥ 570 nm [25], while samples containing bovine DNA have an absorbance closer to the characteristic peak of the AuNPs at 520 - 525 nm [32]. Therefore, it was confirmed that following this protocol it is possible to find even traces of cow milk in goat milk. However, it is necessary to indicate that when left overnight at 4ºC, positive samples changed their color to purple, indicating that the reaction mixtures are not stable in time.

These results are consequent with the ones obtained with the PCR, with which it was also possible to detect traces amount of cow milk in goat milk (Figure 4). Therefore, the gold nanoprobe exhibited the same detection potential than the most employed molecular method. It is important to emphasize that the results of this biosensor were the same when functionalized with both primers and when working with only the forward primer.

Figure 3: UV Analysis of samples from the last experiment. The wavelengths at maximum absorbance are: A1 (Negative): 594 nm; A2 (Cow): 530 nm; A3 (Traces): 547 nm; A4 (1%): 532 nm; A5 (2%): 546 nm; A6 (5%): 536 nm; A7 (10%): 531 nm; A8 (20%): 554 nm; A9 (50%): 539 nm; A10 (Goat): 570 nm.

Figure 4: Electrophoretic analysis of the PCR products obtained from DNA extracts of different dilutions of cow milk in goat milk, obtained with NucleoSpin® Food. (A) Using 200 µl of milk; (B) After centrifuging 1,5 ml of milk. Negative; G: Goat; T: Traces; C: Cow. Percentages show the proportion of cow milk contamination.

Discussion and Conclusion

Consumers may be cheated or suffer diseases because of the consumption of adulterated milk. Quality control inspections are a substantial step to guarantee unadulterated milk for consumption. Detecting the adulteration of milk is a difficult task as indicators of adulteration can change according to several factors (biological, climatic, agronomic...), specially after processing, which can considerably alter milk composition. It is a challenge to develop easy and cost-effective techniques for detection of milk adulteration, and specially, these techniques must have a high degree of repeatability [19,20].

The studied technique is an useful screening test to detect the presence of cow milk in goat milk, even at traces levels. But aside from sensitivity, it is considerable the advantage of time saving and from an economic point of view, AuNPs assay is more cost-effective. This is explained because if the concentration is too low, it will be a small amount of DNA to hybridize with the NPs. Therefore, there will be a lot of free NPs that will precipitate giving to the sample a purple color, masking the pink color of the AuNPs hybridized with the available target DNA. In the other hand, if the concentration is too high, the NPs will find where to attach even in the goat DNA. This shows than the chosen primers were not very specific, because when using a very concentrated goat DNA, they could find sequences to hybridize with, giving a false positive. For that reason, it is better to work with lower concentrations, because in that way the primers will hybridize only with cow DNA.

This procedure is perfectly open to later modifications by other researchers and it will be possible to find other valid working conditions. What it is recommended is to use the minimum possible amounts of reagents. Not only for an economic purpose, but also for practical reasons. The more employed DNA, AuNPs and Assay Buffer, the more HCl will be necessary to cause the aggregation. That means more consumption of time and also more dilution, so the color will be less intense and more difficult to distinguish with the naked eye. For future experiments it will be advisable to use another pair of primers.

Despite these considerations, gold nanoparticles functionalized with 1 or 2 primers have been used to obtain a valid method for fast, cheap and easy detection of nucleic acids. Their cost effective synthesis, simple functionalization and durable stability are all characteristics that will allow to convert the laboratory assays into commercial detection kits for real-world food samples, especially interesting in underdeveloped territories. In spite of the vast amount of different scientific works, the research in gold nanoparticles field is not finished yet, and it will benefit from the full exploitation of their optical and binding properties.

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