2011 International Conference on Biomedical Engineering and Technology
IPCBEE vol.11 (2011) © (2011) IACSIT Press, Singapore

Optimization of influencing parameters for fabrication of gold nanoparticle-based nucleic acid lateral flow strip test

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Abstract. Gold nanoparticle (AuNP)-based nucleic acid lateral flow (NALF) strip test is currently a promising tool for the point-of-care testing of nucleic acid detection. In order to improve sensitivity and specificity of the test, it is important that the conjugation between DNA probes and AuNPs is stable. Here we aimed at evaluating some factors influencing the conjugation efficiency. The results showed that the type of sulfhydryl reductants, sonication procedure and the amount of thiolated probes affected the AuNP-probe conjugation proficiency. By optimizing the fabrication parameters of NALF strip tests, our platforms could detect DNA targets as low as 1.25 fmole by naked eye. In conclusion, this study suggests a modified protocol to enhance the conjugation efficiency of DNA probes and AuNPs, providing potential steps to further develop lateral flow strip tests with higher sensitivity.

Keywords: nanomedicine, biosensors, lateral flow, gold nanoparticles, reductants, sonication

1. Introduction

Gold nanoparticles (AuNPs) have been widely used in several biomedical applications including sensing of biomolecules, delivering of drugs/genes and molecular imaging.[1] As a tool for biosensing applications, the unique physical and chemical properties as well as the high surface area of AuNPs allow for surface modifications with a variety of molecules. Recently, AuNP-based nucleic acid lateral flow (NALF) strip test has been important in the point-of-care of DNA detection for clinical diagnosis and monitoring of diseases,[2,3,4] since it serve as a highly sensitive, yet simple, diagnostic method, which requires no tedious operations or expensive equipment for interpretating the results.

The basic principle of AuNP-based NALF detection system involves the design of thiol-modified specific oligonucleotide probes on AuNPs. If there are complementary targets that can form sandwich hybridization with both the thiolated probes on AuNPs and another target-specific probes on a test line of a NALF test strip, the functionalized-AuNPs will accumulate at the test line, revealing a distinct red color. A previous study reported the utilization of a high sensitivity AuNP-based NALF assay to detect human genomic DNA samples by naked eye, without the need for polymerase chain reaction (PCR) amplification.[5]

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Nonetheless, sensitivity and specificity of the test strips depend on the target-probe hybridization process. The stringency of a buffer system is one of the key determinants of the test result. Changes in ionic concentration in the buffer system can cause AuNP aggregation and non-specific binding of the nucleic acids. Here in this study, we aimed at improving the processes of AuNP-thiolated DNA probe conjugation to optimize the conditions at which the probes are best stabilized on AuNPs. Our findings suggest an alternate cost-effective protocol for the establishment of a high sensitivity NALF test strip.

2. Materials and methods

2.1. Materials

The experimental probe sequences were 5' GGT AAA GAT TTA TTG CTC GGT TTT TTT TTT-$(CH_2)_3$-(Thiol) 3' [probe#1 : detection probes], 5' GCA CTT GGT GTA GCA ATT AAT GCT G-$(CH_2)_3$-(Thiol) 3' [probe#2 : detection probes], 5' (biotin)-GGC CAA GTT AAA CTC TAT GCT GAC 3'[probe#3 : capture probes on the test line] and 5' CAG CAT TAA TTG CTA CAC CAA GTG C-(biotin) 3' [probe#4 : complementary sequence with probe#2 on the control line]. Synthetic target sequence was 5' AGC AGC ATT AAT TGC TAC ACC AAG TGC TCC TTA AGT CAG CAT AGA GTT TAA CTT GGC CGA 3'. All reagents were prepared with Milli-Q water (> 18 MΩ).

2.2. Gold nanoparticles synthesis

Gold nanoparticles (AuNPs) were synthesized by adding 0.94 mL of 38.8 mM trisodium citrate dihydrate ($Na_3C_6H_5O_7 \cdot 2H_2O$) in 25.0 mL of boiling 0.02%w/v hydrogen tetracholoaurate (III) trihydrate ($HAuCl_4 \cdot 3H_2O$) in Milli Q water under vigorous stirring. The solution turned deep-red within 2 min after which it was boiled for additional 15 min and then stored at 4°C away from light. The AuNPs were characterized by UV/Vis spectroscopy (Beckman coulter DU ® 800; maximum absorption at 520 nm) and transmission electron microscope (TEM; HITACHI Model H-7650 : 100 kV). Their absorption spectrum and TEM micrograph are shown in Fig.1(A) and Fig.1(B) respectively.

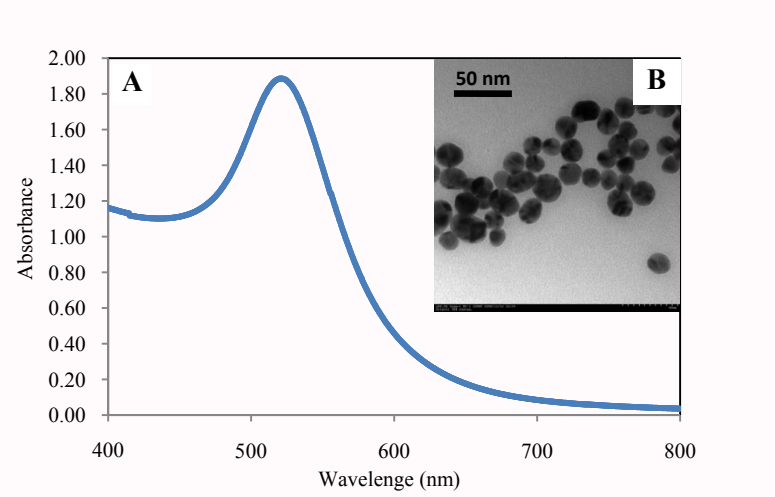

Fig.1: Characterization of AuNPs with (A) UV/Vis spectroscopy and (B) TEM (20-25 nm).

2.3. Conjugation of thiolated probes on AuNPs

DL-dithiothreitol (DTT) and tris (2-carboxyethyl) phosphine hydrochloride (TCEP) were used separately as a sulfhydryl reductant to reduce the disulfide linkages of the probes to the thiol forms prior to being conjugated with AuNPs. The conjugation was done in a conventional way as described in references[6,7,8] with a slight modification. Briefly, 100 µM of probe#1 (in its as-recieved form) were reduced with DTT. The probes had been purified with a NAP-5 column, prior to being added to AuNPs. They were finally incubated overnight under a gentle shaking. After the incubation, the shaking was continued while a salting buffer was periodically added once in every 8 h to obtain a final NaCl concentration of 0.7 M. After the first and the last 3 times of the salting additions, the conjugates were sonicated for 10 s. The final solution was centrifuged at 12,000 rpm at 4°C for 15 min and the sediment (AuNPs-probes) was washed with a washing buffer twice.

The supernatants (excess thiolated probes) from all three centrifugations were analyzed by UV/Vis spectroscopy and the results were used to calculate for the percentage of probe loss (i.e., % probe loss = [OD_{260nm} x 33 ng/µL x Volumn (µL)]/ initial thiolated probes (ng) x 100). The functionalized AuNPs were stored at 4°C. For the TCEP treatment, the procedure was similar to the mentioned DTT protocol. TCEP was added at a final concentration of 0.1 mM and no column purification was needed.

3. Results and Discussion

Table 1 : Experimental protocols studying the effects of reductants and sonication on the conjugation of thiolated probes on AuNPs.

Experimental conditions				Results	
Amount of thiolated probes* (nmole)	100 ppm AuNPs (µl)	Type of reductants	Sonication	% Probe loss	Average half-life** (min) (n =2)
1.6	400	DTT	Yes	86.72	16
1.6	400	DTT	No	84.80	8.75
1.6	400	TCEP	Yes	97.45	11.25
1.6	400	TCEP	No	98.08	7.5
0.8	200	-	Yes	68.80	N/A
0.8	200	-	No	70.59	7.5

(* The thiolated probes used in this step is probe#1 and, ** Half-life : The time duration taken for half of complete AuNP aggregration)

3.1. Effect of reductants on conjugation efficiency

Results from Table 1 indicate that all of the DTT-treated groups had the % probe loss values lower than those of the TCEP-treated groups. Although the use of TCEP has many advantages,[9] our study revealed that the DTT-treated groups have higher conjugation efficiency. The purification of the DTT-treated probes with the NAP-5 column removes the thiolated protecting caps, which may, otherwise, compete with the activated thiolated probes for the covalent adsorption on the AuNP surface as shown below.

3'oligo-(CH2)3-**S-S**-(CH2)3-OH + DTT/TCEP → 3'oligo-(CH2)3-**SH** + **HS**-(CH2)3-OH

[Unactivate disulfide probes] [Reductant] [Activated thiolated probes] [Thiolated protecting caps]

"Competitive adsorption on AuNPs"

3.2. Effect of sonication on conjugation efficiency

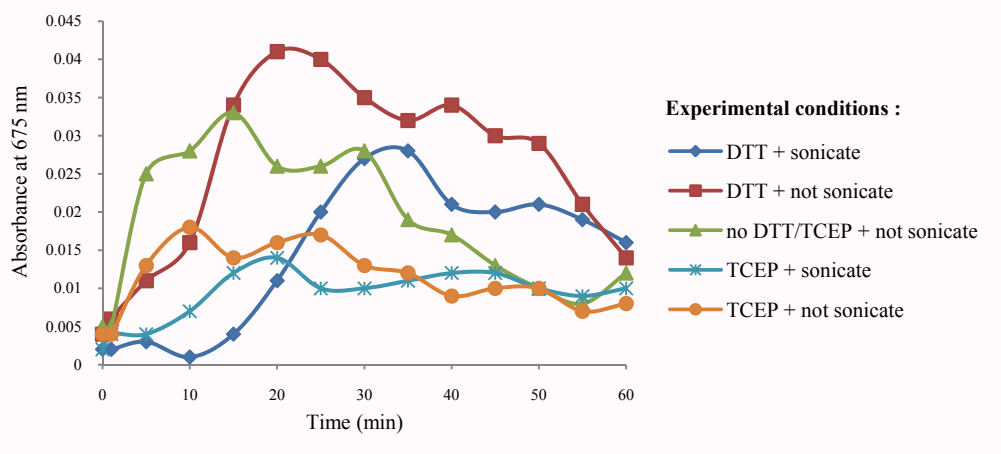

Fig. 2 : Deconjugation profiles after DTT treatment of the conjugates.

The stability of the conjugated probes was studied by using DTT as a deconjugating agent. Different conjugated probe samples were treated with DTT (at the final concentration of 10 mM) and incubated at 40°C throughout the experiment. A small portion of each sample was taken out every 5 min and analyzed for an increase in the absorbance at 675 nm. Since an increase in the agglomeration of AuNPs will shift the maximum absorbance wavelength from 520 nm to the range between 600-700 nm, the absorbance at 675 nm was chosen as a reference in this study. The measured absorbance at 675 nm was then plotted against time,

as shown in the deconjugation profiles of Fig. 2. From the deconjugation profiles, the initial rise in absorbance is due to the increased aggregation of AuNPs, after which the absorbance decreases when the aggregated AuNP precipitate, leaving a relatively more transparent solution.[10] The extent of stability could be obtained by examining the half-life values of each group. Based on the results shown in Table 1 and Fig. 2, half-lives of the sonication groups (16 and 11.25 min for DTT- and TCEP-treated groups, respectively) were greater than those of the non-sonication ones (8.75 and 7.5 min for DTT- and TCEP-treated groups, respectively). This indicates that sonication increases the stability of the conjugation process. As described in a previous work,[8] the sonication reduces undesirable electrostatic force and physical adsorption from bases of thiolated probes on AuNP surface, which would, otherwise, decrease the sensitivity of the system. When there is less adsorption of the base groups of the probes, more area of AuNP surface is available for a stronger covalent adsorption with the thiolated groups of the probes.[8] Such covalent adsorption is more stable than electrostatic force and physical adsorption, so it is more desirable.

3.3. Validation of thiolated probe quantity

Table 2 : Effect of amount of thiolated probes on AuNP conjugation.

Experimental conditions			Results		
Amount of thiolated probes* (nmole)	Type of reductants	Sonication	% Probe loss	Amount of conjugated probes on AuNPs (ng/µl)	Color after DTT added
1.0	DTT	Yes	83.05	6.18±1.17	Red
0.8	DTT	Yes	89.48	4.35±0.30	Red
0.5	DTT	Yes	76.52	4.68±0.40	Red
0.3	DTT	Yes	71.43	4.64±0.31	Purple
0.1	DTT	Yes	45.26	2.76±0.19	Purple

(* The thiolated probes used in this step is probe#1.)

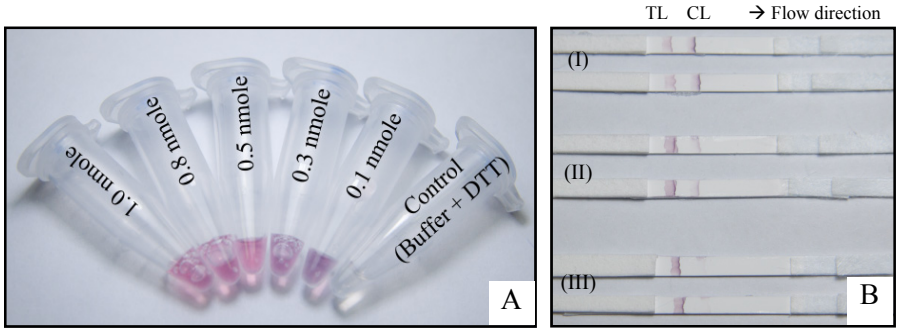

Fig. 3 : (A) Change in the color of conjugates after adding DTT. (B) Positive results of lateral flow test strip with DNA targets at (I) 125 fmole, (II) 12.5 fmole and (III) 1.25 fmole (TL = Test line, CL = Control line).

The conjugated gold colloid was dispersed in 10 mM phosphate buffer and 150 nM NaCl as the final buffer. After adding DTT (at the final concentration of 10 mM) to the conjugates at 40°C, the 0.1 and 0.3 nmole samples suddenly turned from red to purple (Table 2 and Fig. 3(A)). This suggests that these 2 conditions had too few thiolated probes to reach a proper AuNP stability. After complete aggregation of the conjugates, centrifugation was later performed in order to detect free thiolated probes in the supernatant. The % probe loss values were calculated and summarized in Table 2. Results showed that the % probe loss values were in accordance with the amounts of thiolated probes used in this study. Moreover, the amounts of conjugated probes on AuNP surface are very similar among the 0.3, 0.5, 0.8 nmole samples, whereas the change in the color of AuNPs, as seen by naked eye, was observed at 0.3 nmole. Therefore, this experiment indicates that the optimal quantity for thiolated probe conjugation on AuNPs (20-25-nm size) is approximately 0.5-0.8 nmole.

3.4. Detection of synthetic DNA targets with lateral flow strip test

We used another set of probes (probe#2, 3 and 4) to fabricate the lateral flow test strips, according to the method previously described by Mao *et al*. (2009).[5] Synthetic DNA targets at different concentrations were tested on a lateral flow platform in order to evaluate the sensitivity of the detection limit. The outcome

revealed that these test strips could detect the DNA target as low as 1.25 fmole (Fig. 3(B)). To our knowledge, our modified protocol results in the highest sensitivity of DNA detection by the lateral flow test.

4. Conclusions

In summary, although TCEP has many advantages over DTT, DTT treatment combined with gel filtration column to remove the thiolated protecting caps is more effective than TCEP treatment in the thiolated probe-AuNP functionalization. This work showed that the sonication promotes conjugation stability by enhancing the covalent bond formation of thiol moiety and AuNP surface. The initial amount of thiolated probes used is also critical to obtain the conjugation stability. Finally, we applied this conjugation protocol to construct lateral flow strip tests, results revealed that as low as 1.25 fmole of DNA target is detectable by naked eye detection.

5. Acknowledgements

The authors gratefully acknowledge the financial supports from the Chulalongkorn University Centenary Academic Development Project (Chula-MITC) and The National Research University Project of CHE and the Ratchadaphiseksomphot Endowment Fund (project code HR1166I) (to A.S.). We also thank Mr. Jeerawat Nakkuntod for his valuable suggestion on this work.

6. References

[1] P.C. Chen, S.C. Mwakwari , and A.K. Oyelere. Gold nanoparticles: From nanomedicine to nanosensing. *Nanotechnol. Sci. Appl*. 2008, **1**: 45-66.

[2] D.P. Kalogianni, *et al.* Dry reagent dipstick test combined with *23*S rRNA PCR for molecular diagnosis of bacterial infection in arthroplasty. *Anal. Biochem.* 2007, **361** (2): 169-175.

[3] D.P. Kalogianni, *et al.* Dry-reagent disposable dipstick test for visual screening of seven leukemia-related chromosomal translocations. *Nucleic Acids Res.* 2007, **35** (4): e23.

[4] I.K. Litos, *et al.* Multianalyte, dipstick-type, nanoparticle-based DNA biosensor for visual genotyping of single-nucleotide polymorphisms. *Biosens Bioelectron.* 2009, **24** (10): 3135-3139.

[5] X. Mao, *et al.* Disposable nucleic acid biosensors based on gold nanoparticle probes and lateral flow strip. *Anal. Chem.* 2009, **81**(4): 1660–1668.

[6] J. Zhang, *et al.* A gold nanoparticle-based chronocoulometric DNA sensor for amplified detection of DNA. *Nature Protocols*. 2007, **2** (11): 2888-2895.

[7] H.D. Hill, and C.A. Mirkin. The bio-barcode assay for the detection of protein and nucleic acid targets using DTT-induced ligand exchange. *Nature Protocols*. 2006, **1** (1): 324-336.

[8] S.J. Hurst, A.K.R. Lytton-Jean, and C.A. Mirkin. Maximizing DNA loading on a range of gold nanoparticle sizes. *Anal. Chem.* 2006, **78** (24): 8313–8318.

[9] E.B. Getz, *et al.* A comparison between the sulfhydryl reductants tris(2-carboxyethyl)phosphine and dithiothreitol for use in protein biochemistry. *Anal. Biochem*. 1999, **273**: 73-80.

[10] J.A. Dougan, *et al.* Enhanced oligonucleotide–nanoparticle conjugate stability using thioctic acid modified oligonucleotides. *Nucleic Acids Res.* 2007, **35** (11): 3668-3675.