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RESEARCH PAPER

High Performance Liquid Chromatographic Analysis of Reduction Products of a Thiolated DNA for Immobilization on Gold Nanoparticles

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ABSTRACT

DNA-based nano-biosensors are emerging scope in the field of biosensors. Many synthetic single stranded functional DNAs have been applied for development of such sensors, recently. Immobilization of DNA oligonucleotides on the surface of gold nanoparticles is a key step in the development of most colorimetric nano-biosensors. The bound DNA is usually thiolated and forms Au-S covalent bond to the surface of gold nanoparticles. To this endeavor, the DNA must get reduced prior to immobilization. There are a variety of approaches for reduction of thiolated DNAs that mostly employ Dithiothreitol (DTT). DTT-based DNA reduction is not always complete and the sulfhydryl DNA (DSH) is not the sole product of the reaction. The results of the reduction of a thiolated DNA with DTT is analyzed in this paper with high performance liquid chromatography (HPLC) in order to find an optimal condition for DTT based reduction. Finally, the optimal condition is compared to TCEP-based reduction for the efficiency of DNA immobilization on the surface of gold nanoparticles.

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INTRODUCTION

Nucleic acids are well known as structural elements for genetic data transfer among generations and species. This insight was valid until the first ribozymes have been discovered in 1982 (1). Since then, numerous functional nucleic acids have been either discovered in nature or have been selected in vitro. Guide RNAs in clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 systems (2), riboswitches (3) , aptamers (4) , ribozymes (5) and deoxyribozymes (6) are major classes of functional nucleic acids. Functional nucleic acids bear a specific prevalence for application which is the possibility of rational design based on their predictable secondary structures e.g. Watson-Crick, Hoogsteen and wobble base pairings, triple helix formation among poly-purines and poly-pyrimidines (7) , i-motifs (8) and etc. Hence, many reports have been focused to utilize nucleic acids in various fields including biosensors.

By definition, biosensors consist of a recognition element, a transducer element and a readout system. The recognition elements of biosensors have been traditionally enzymes, antibodies or even whole cells. However, in recent years, single stranded DNAs (9) , molecular beacons (10) , aptamers (11) and deoxyribozyme (12) have become a major research topic in the field of biosensors. The transducer element depends on the readout system which accompanies the biosensors. For instance, an electrochemical readout requires a gold electrode (13) or a colorimetric readout may depend on the ability of

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gold nanoparticles to assemble or aggregate in a controlled fashion (14).

In the two given examples above, the bio-macromolecules must get immobilized on the surface of a gold electrode or gold nanoparticles. Such immobilization may be a trivial step in the case of proteins, since they normally bear cysteines. The free thiol functional groups in the cysteines may form covalent bonds with the surface of gold electrode or gold nanoparticles. However, in the case of nucleic acids the procedure is not that trivial since they do not bear a sulfur atom *A priori*.

It is noteworthy, that thiol containing macromolecules are able to be conjugated to fluorophores that are accompanied by α,β-unsaturated ketones, maleimides or other Michael acceptors. As in the case of macromolecule immobilization on gold surfaces, this conjugation is trivial for proteins, since they bear the sulfur atom in their amino acid sequence, but is a challenge in nucleic acid oligonucleotides.

DNA molecules that are utilized in biosensors are usually synthetic and single stranded. The standard procedure of nucleic acid synthesis is known as solid phase synthesis and occurs on the surface of insoluble polymer such as controlled pore glass (CPG). The synthesis is performed in the 3' to 5' direction by several round of sequential addition of building blocks that is followed by a series of reactions i.e. coupling, capping, oxidation, detritylation (removal of a 4,4'-dimethoxytrityl group or namely DMT group) (Fig. 1a). The building blocks are protected phosphoramidites. As an example a cytosine phosphoramidite for DNA synthesis is marked blue in Fig. 1a. Modifications such as insertion of sulfur containing functional

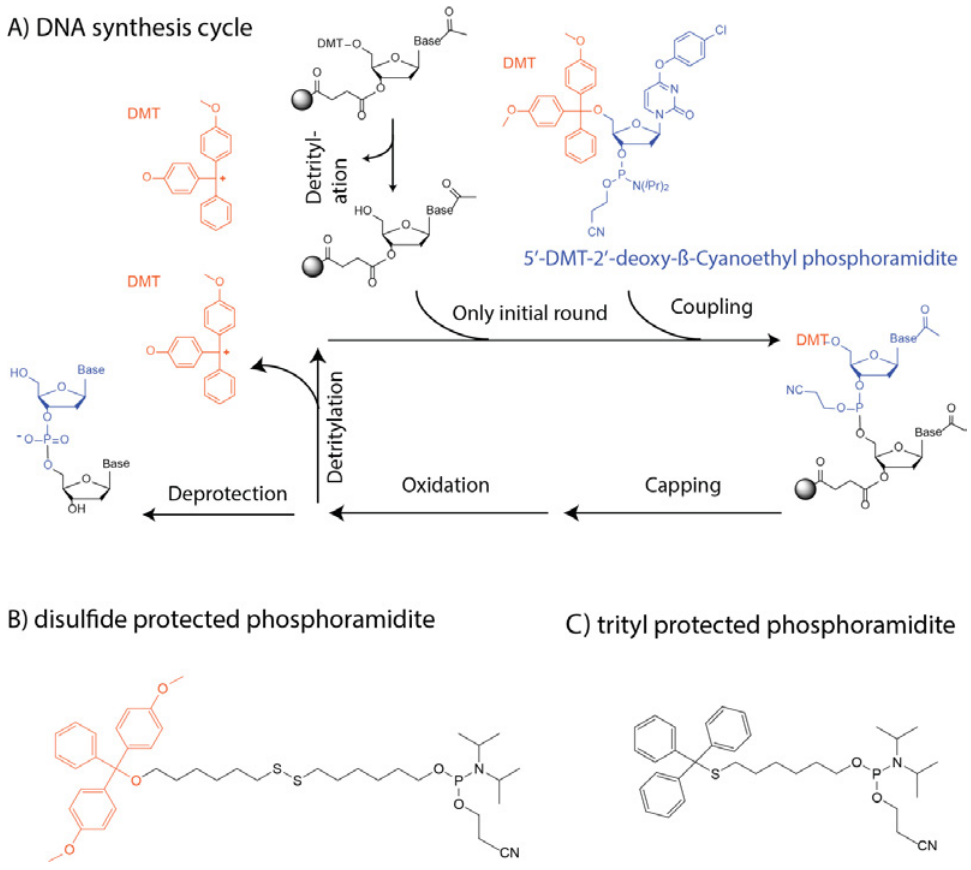

Fig. 1. Schematic representation of DNA oligo synthesis procedure and its building blocks. A) The synthesis cycles start with removal of DMT group (deteritylation) from a surface attached phosphoramidite and continues with cycles of coupling, capping, oxidation and detritylation of a newly arriving phosphoramidites. Final round finishes with deprotection of the synthesized oligo i.e. separation of the synthesized oligo from the solid resin and base protection groups. As an example a standard cytosine phosphoramidite is depicted in blue color in this figure. All DMT groups are marked in orange color. B and C) phosphoramidites that are incorporated in synthetic oligonucleotides for 3' and 5' end labeling of DNA with thiol group. The disulfide protected form is utilized often and the trityl protected form is used rarely.

groups are amenable by applying a solution of modified phosphoramidite in a specific round of synthesis. Fig. 1b and c, depict two examples of thiol modifier phosphoramidites which are commercially available. Finally, the synthesized products undergo through a final deprotection step which removes the acetyl and benzyl groups of incorporated phosphoramidites and in addition, separates the synthesized DNA from the solid phase.

In the case of application for biosensors, the thiol modifier is usually placed on the 3'or 5' end of the synthesized DNA. In rare cases, the protection group is not a disulfide bond but a trityl group (Fig. 1c). In these cases, the trityl group is removed by silver nitrate ($AgNO_3$). Excess DTT is then used to remove the silver nitrate. In the final step, the DTT itself must be removed either by ethyl acetate extraction or a gel filtration (desalting) column (e.g. a NAP-10 column).

Since the thiol groups are sensitive to oxidation, in most commercial cases, the thiol modified DNAs are shipped as semi-protected disulfide containing DNA, DSSR, as indicated in the Fig. 2A. Thus, the final deprotection step, that is reduction of the disulfide bond to the active sulfhydryl group must be performed prior to the desired application of such modified DNAs. The reduction of the DNA is performed either by Dithiothreitol (DTT) or Tris(2-carboxyethyl)phosphine (TCEP), Fig. 2B. The reduced DNA is then capable for covalent attachment to the surface of gold nanoparticles or gold electrodes as schematically depicted in Fig. 2C.

The choice between DTT or TCEP is affecting the experimental protocols. While most commercial providers suggest DTT for DNA reduction and provide a variety of different methods for its utility, application of DTT has several drawbacks. DTT bears two thiol groups and thus is capable of formation of Au-S covalent bonds with the desired gold nanoparticles or gold electrodes and thereby competes with the thiolated DNA for binding. Therefore, DTT must get removed prior to the immobilization step of the thiolated DNA on the gold nanoparticle. Otherwise, most of the immobilized species would be DTT molecules instead of thiolated DNA. In the other hand, TCEP is not capable of bond formation with gold and is not a competitor for DNA in the immobilization step. In the procedure of deprotection of a thiolated DNA, reaction productss are formed with differences in molecular weight, i.e. removal of the RSH group, changes the total molecular weight of the DNA. This phenomenon was utilized as a key factor for dissociation of reduced products in this study. HPLC and gel electrophoresis are the most common approach for such analysis. This report analyses different conditions for DTT reduction using high performance liquid chromatography (HPLC) and finally compares the efficiency of immobilization on gold nanoparticles using the two approaches.

MATERIALS AND METHODS

Chemicals

All chemicals for synthesis of gold nanoparticles including $AuHCl_4$, sodium citrate, HCl and HNO_3, as well as ingredients of HPLC buffers including Tris-HCl, $NaClO_4$ and urea were purchased from Sigma

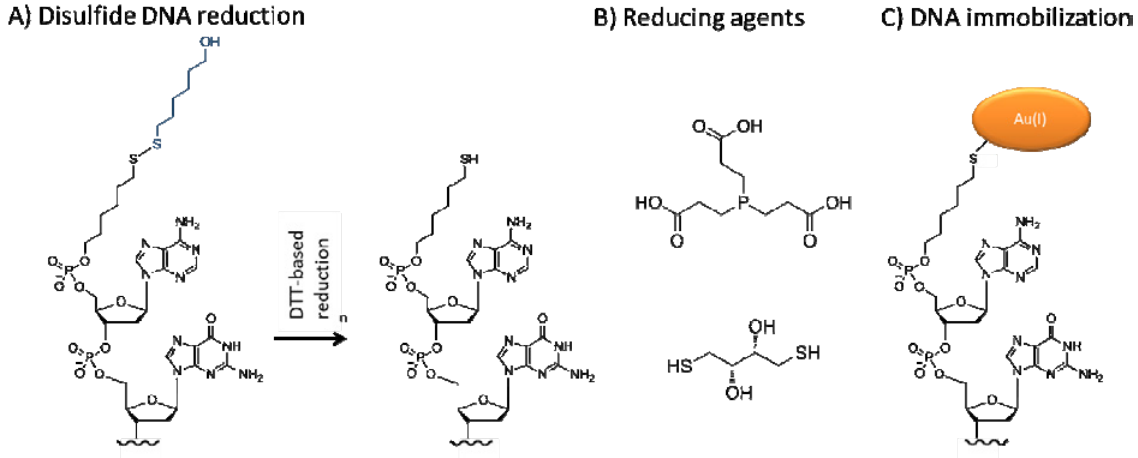

Fig. 2. Reduction of thioated DNA and its immobilization on the gold surfaces. A) Thiolated DNAs are shipped in disulfide oxidized form and are subject to reduction prior to application. B) The reducing agents are usually TCEP (above) or DTT (bottom). C) The reduced thiolated DNAs may form a covalent Au-S bond to the surface of gold nanoparticles or gold electrodes.

Aldrich. The two reducing agents, DTT and TCEP and ethyl acetate were product of Merck. The ultrapure water (18 MΩ·cm^{-1}) was purchased from Hava Kesht Aria, Isfahan, Iran.

DNA reduction procedure

The 17 mer 5'-thiolated DNA with the sequence of (GTAAAACGACGGCCAGT) were initially run on a poly acrylamide gel electrophoresis and subsequently were visualized by UV-shadowing system. The bands were cut and purified by crush and soak method. For the reduction procedures 50 pmol of the purified DNAs were incubated in the volume of 25 µl in presence of 50 mM DTT or 10 mM TCEP and for the indicated time om each images. The DTT and TCEP were both in excess to the concentration of DNA (by 3 orders of magnitude). DTT was finally removed by ethyl acetate extraction prior to HPLC.

HPLC analysis

Äkta purifier (GE healthcare) was used for HPLC analyses of this report. Anion-exchange chromatography on a Dionex DNAPac PA200 Analytical column was performed by increasing gradient of eluent B (25 mM Tris-HCl pH 8, 0.5 M $NaClO_4$, 6 M urea,) in eluent A (25 mM Tris-HCl pH 8.0, 6 M urea). All the HPLC analyses have been performed at 80°C.

Gold nanoparticle synthesis and DNA immobilization

All glassware were washed with aqua regia (3:1 concentrated HCl:HNO_3) for 30 min and subsequently rinsed with generous amount of deionized water and then ultrapure water (18.2 MΩ·cm^{-1}). Synthesis of gold nanoparticles was according to reported literature (15). To start synthesis, 100 ml of 1 mM $HAuCl_4$ was brought to reflux on a heater with a magnetic stirrer, consequently 10 ml 38.8 mM sodium citrate was added. The color change from pale yellow to deep red was observed within 1 min. The system refluxed for further 20 min and then allowed to be cooled down to room temperature while still stirring. For DNA immobilization, salt aging method was applied (15).

RESULTS AND DISCUSSION

The effect of pH

The reduction of the thiolated DNAs are mandatory prior to application on the field of nano-biosensors. The reduction is usually performed by DTT or TCEP. The provider companies suggest a variety of methods for reduction step which vary a lot in the sense of time, pH and temperature. The main goal of this study was to report the optimal conditions for reduction of thiolated DNAs. In the first step, DTT was incubated for 60 min with the thiolated DNA in presence of 50 mM HEPES buffer pH 7.5 or 8.0. Consequently, excess DTT was removed and the samples were injected on HPLC.

Comparison of the DTT-treated samples with the starting material (untreated) revealed formation of two peaks with 280 nm absorbance. The starting material had a disulfide bond in the middle of two C6 spacers and is thereby abbreviated RSSD in this report. The RSSD had a retention time of 12.9 min. Upon reduction, two peaks emerged with the retention times of 12.5 and 15.3 min. The peak at 12.5 min corresponded to the reduced thiolated DNA, abbreviated as DSH in this report. DSH was the desired species for the immobilization on the gold nanoparticles. The peak at 15.3 min was the dimer of thiolated DNAs in the head-to-head orientation (DSSD) that were oxidized after removal of DTT, prior to HPLC injection (Fig. 3). Formation

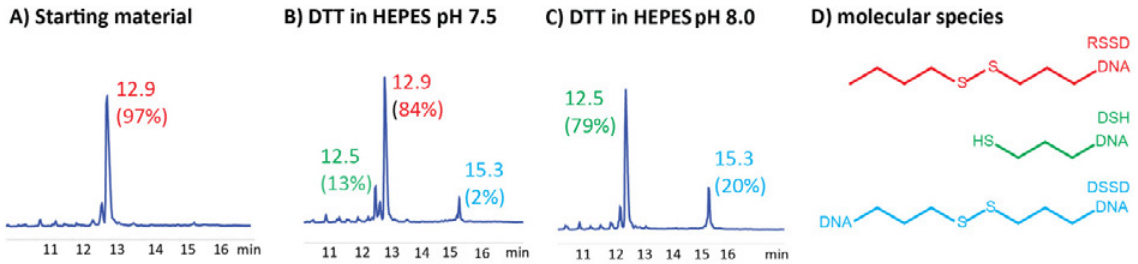

Fig. 3. Formation of thiolated reduced DNA and oxidized dimer DNA. A) Starting material, RSSD, protected thiolated DNA with a disulfide bond. B) The results of incubation of the sample with 50 mM DTT in presence of 50 mM HEPES buffer pH 7.5 for 60 min. C) The results of incubation of the sample with 50 mM DTT in presence of 50 mM HEPES buffer pH 8.0 for 60 min. in B and C DTT was removed prior to HPLC. D) Schematic representation of the starting material, RSSD (red), the desired thiolated reduced DNA, DSH (green) and the side product dimer of DNAs, DSSD (blue). The same color codes are valid through all chromatograms of this report. In this figure, retention times are depicted with the same color code as in D, above each peak. The numbers in parenthesis indicate the percentage of each peak over total peaks.

of sulfur bridge dimeric form of thiolated DNA after DTT removal is consistently reported earlier (16).

The preliminary data showed that the desired species, DSH, and the side product, DSSD, got formed in different ratios when the pH was increased. The results depicted that increasing pH, leads to complete conversion of RSSD, i.e., almost no peak at 12.9 min was visible in pH 8.0. However, the oxidized dimer (DSSD) had higher percentage at this pH (20%). The ratio of formation of the side product, DSSD, to the desired products, DSH, was increased from 0.15 to 0.25 when the pH increased from 7.5 to 8.0. Thus further experiments were performed to dissect the effect of pH to seek an optimal condition. The optimal condition must guarantee highest DSH percentage, and least RSSD/DSH and DSSD/DSH ratios.

The effect of DTT incubation time

In the next step, samples incubated with the same concentration of DTT (50 mM), in two buffer systems i.e. HEPES pH 7.5 or HEPES pH 8.0, for either 30 min or 2 h. Fig. 4 depicts the outcome of this experiment. It is clearly visible that at pH 7.5, formation of the desired product, DSH was time dependent. The ratio of DSH over total peaks increased from 12% to 53% when incubation time increased from 30 min to 2 h (Fig. 4 A and B). However, formation of DSH in pH 8.0, was clearly so fast that no RSSD (starting material with retention time 12.9 min) was observed only after 30 min incubation time (Fig. 4, C and D).

The ratio of the peak area of RSSD to the peak area of DSH (RSSD/DSH) indicates incomplete reduction procedure while the ratio of peak area of DSSD over the peak area of DSH (DSSD/DSH) depicts oxidation to dimer. In the other words, the lowest outcome of these two ratios indicates that higher DSH is available for formation of Au-S covalent bond with the surface of gold nanoparticles. In pH 7.5, RSSD/DSH decreased from 6.4 to 0.5 by an increase in incubation time. This decrease was an indication of reduction of RSSD. However, not all reduced species remained as DSH since the peak area of DSSD was increased from 8% to 15% and DSSD/DSH were ca. 0.3-0.7 in pH 7.5.

The situation differed in pH 8.0. Since almost no RSSD was remained even after 30 min incubation, the RSSD/DSH was zero in both incubation times. The peak area of DSSD was 20% in 30 min incubation time and 14% after 2 h. The DSSD/DSH were ca. 0.15 to 0.25 in pH 8.0. Thus, in summary the DTT reduction at pH 8.0 resulted in faster removal of all RSSD, and least DSSD/DSH.

Time dependent formation of DSSD

DTT based reduction of thiolated DNA requires presence of excess DTT. DTT is applied in millimolar range while the concentration of the thiolated DNA is in the range of micromolar. Subsequent to reduction step, the majority of DTT

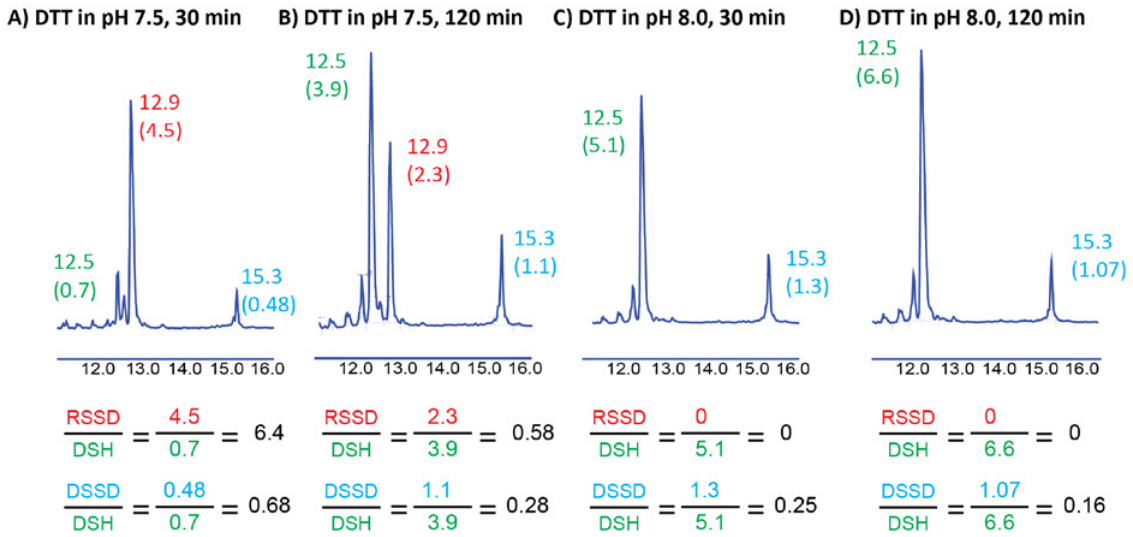

Fig. 4. The effect of incubation time with DTT in formation of the desired product or the side product. The numbers in parenthesis are peak area of each peak analyzed by One-manager unite of HPLC. The ratios RSSD/DSH and DSSD/DSH indicate incomplete reduction procedure and oxidation to dimer, respectively. The color codes of this figure are as indicated in Fig 3, D.

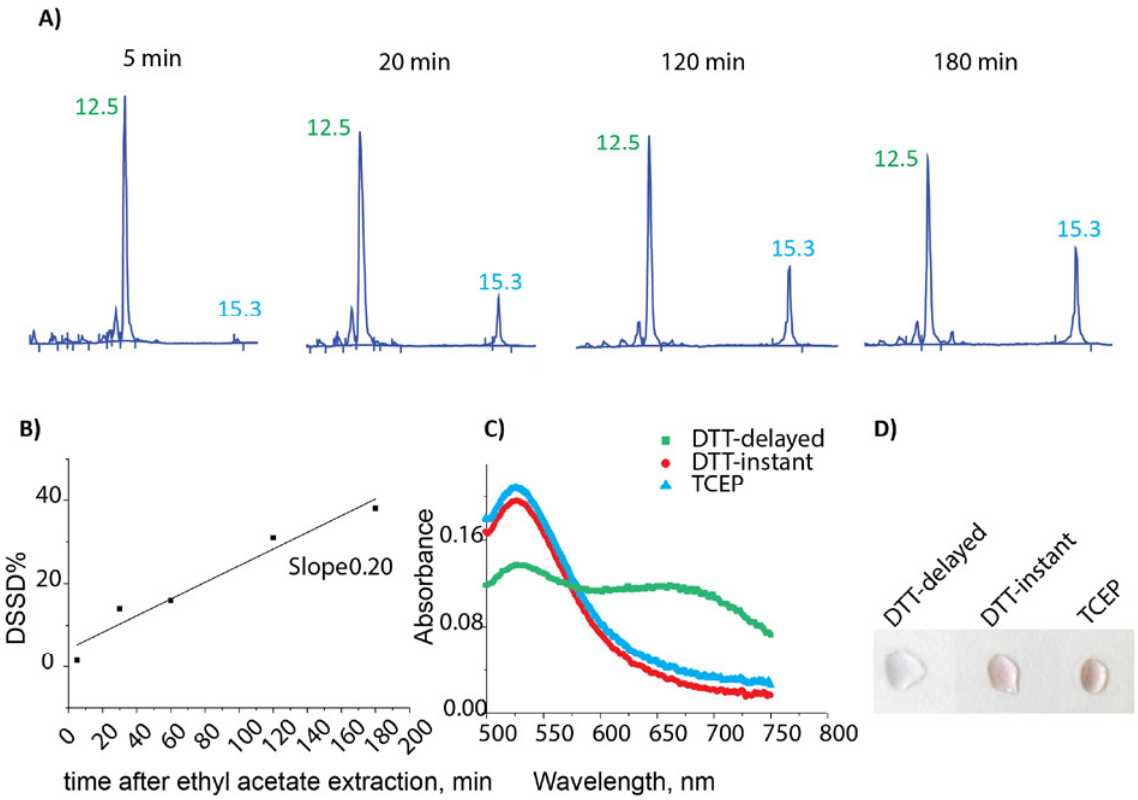

Fig. 5. Time dependent formation of DSSD and its effect on DNA immobilization A) HPLC chromatograms of DTT treated DNAs (50 mM DTT in 50 mM HEPES pH 8.0) after 5, 20, 120 and 180 min of DTT removal by ethyl acetate. The reduced form of DSH with 12.5 min retention time gradually converted to DSSD with 15.3 min retention time. B) The rate of formation of DSSD after DTT removal. C) The spectra of gold nanoparticles that were incubated with DTT or TCEP after addition of 100 mM NaCl. D) The appearance of gold nanoparticle droplets on a surface of glass after addition of 100 mM NaCl. The nanoparticles were incubated with DTT and TCEP treated DNA prior to addition of NaCl.

molecules are still in the reduced state and only a small fraction have been consumed for DNA reduction. Thus, a milimolar concentration of DTT molecules with two thiol groups are available for immobilization to the surface of gold nanoparticles which competes strongly with reduced thiolated DNAs which are available only in the range of micromolar concentration. Thus, DTT must be removed prior to incubation of reduced DNA with gold nanoparticles. Removal of DTT, however, has a strong drawback, which is formation of disulfide oxidized DNAs. Two reduced DNAs get oxidized and form a disulfide bond in the head-to-head orientation to form a side product, DSSD.

Here, we analyzed the rate of disulfide DSSD formation, after removal of DTT (Fig. 5A). The data indicated that even only after 20 min, the population of DSSD side product was conspicuous. The population of oxidized form increased as the time passed after DTT removal by the percentage rate of 0.20 min^{-1} (Fig. 5B). Subsequently, the reduced DNAs were subjected for AuNP immobilization by salt aging method. The reduced DNAs with 5 and 180 min after removal of ethyl acetate were utilized for immobilization on the surface of gold nanoparticles. The immobilized gold nanoparticles that used thiolated DNA instantly after DTT removal (only with 5 min delay time, for preparation) were resistant to NaCl induced aggregation, while the nanoparticles that were immobilized with 180 min delay were sensitive to aggregation. In the case of delayed immobilization (180 min), the spectrophotometric ratio of 670 nm/520 nm, which is an indication of aggregation (17), (18), (19), increased clearly (Fig. 5C) and the color of gold colloid changed to blue upon this aggregation (Fig. 5D).

An alternative choice for reduction of thiolated DNAs is utilization of TCEP. In contrast to DTT, TCEP does not bear thiol groups and is

not a competitor of reduced DNA for formation of Au-S covalent bonds. Thus, there is no need for removal of TCEP after reduction. Here, we compared the immobilization efficiencies of DTT-treated thiolated DNAs with TCEP treated ones. The spectrophotometric results were almost the same as DTT-treated thiolated DNA that were instantly used after DTT removal (Fig. 5C). In the same line, no aggregation of gold nanoparticles was observed and the color of gold colloid stayed red (Fig. 5D).

CONCLUSION

Synthetic thiolated DNA oligonucleotides have a great potential for application in the field of nano-biosensors. Sulfhydryl groups have the ability to form a covalent bond with gold atoms and thus immobilize the DNA oligonucleotide on the surface of gold nanoparticles or gold electrodes. Thiolated DNAs must get activated prior to immobilization on gold surfaces. The activation process includes application of a reducing agent to form a sulfhydryl from a disulfide protected shipped DNAs. Provider companies suggest application of DTT as a reducing agent with a variety of protocols and methods. This paper reports an optimal condition for DTT-based reduction which is incubation of the thiolated DNA with 50 mM DTT in 50 mM HEPES pH 8.0 for 30 min. The DTT must be removed by ethyl acetate to prevent DTT competition with DNA for gold immobilization.

This study showed that the reduced thiolated DNA converts to an inactive disulfide form in a time dependent manner. Thus it is suggested that the reduced DNA get applied to gold nanoparticles, directly after removal of DTT. In addition, this paper showed that TCEP-based reduction of thiolated DNA has equal efficiency as DTT-based reduction and does not encounter the challenge of removal of the reducing agent, since TCEP is not a competitor for thiolated DNA. In summary, this report suggests application of DTT in 50 mM concentration in 50 mM HEPES pH 8.0 for 30 min, followed by ethyl acetate extraction and direct application to the gold nanoparticles for immobilization. Alternatively, utilization of TCEP in 1 mM concentration for 1 h is recommended for best immobilization efficiencies.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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