Can Gold Nanoparticles Aid Electrophoretic Detection of Sulphur in Biomolecules? Development of Gold-PAGE

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The prevalence, distinctive reactivity, and biological significance of sulphur-based groups in proteins and nucleic acids means that analysis of sulphur is of prime importance in biochemistry, biotechnology, and medicine. We report steps in the development of a method to detect these moieties using gold nanoparticles as adjuncts in polyacrylamide gel electrophoresis (Gold-PAGE).

The chemistry of sulphur is key in biological systems. In proteins, the correct formation of disulphide bonds is frequently required for folding, and hence function, and the distinctive nucleophilicity of thiols is routinely exploited within chemical biology to enable labelling of proteins via cysteine.[1] In nucleic acids, natural modifications of RNAs include sulphurised nucleobases,[2] and the phosphorothioate linkage is an essential tool for increasing the biostability of nucleic acid therapeutics[3] – a field boosted very recently by the first Phase III success of an RNAi therapy. Sulphur groups in DNA are also widely used in structural nanotechnology.[4,5] There is therefore an important need for tools to analyse the presence and chemical state of sulphur in proteins and nucleic acids. Mass spectrometry, usually the first port-of-call, can often be challenging under native conditions, while other methods for assessing sulphur chemistry are either destructive (e.g. ICP-MS) or indirect (e.g. circular dichroism). Polyacrylamide gel electrophoresis (PAGE), on the other hand, can be run under native conditions. The inclusion of a mercury layer within PAGE has been shown to identify sulphurised RNAs by diminished mobility mediated by soft-soft Hg-S interactions.[6] In principle, this could give structural information, since the metal-sulphur affinity depends upon accessibility and oxidation state. However, the technique utilises highly toxic organomercury building blocks, and which are embedded within a crosslinked slab of gel, greatly amplifying waste disposal costs. Gold nanoparticles (AuNPs) are far less toxic[7] and are well known for their affinity for sulphur; the replacement of weakly bound ligands by thiols, disulphides,[8] and thioethers[9] is widely employed in their functionalisation. The integration of AuNPs into PAGE (Fig. 1a) could therefore be used to provide information on the number and chemistry of sulphur atoms in proteins and nucleic acids (Fig. 1b). This would mirror the use of boronic acids in PAGE to detect glycation.[10] AuNPs have been incorporated into hydrogels previously, but not for electrophoresis.[11]

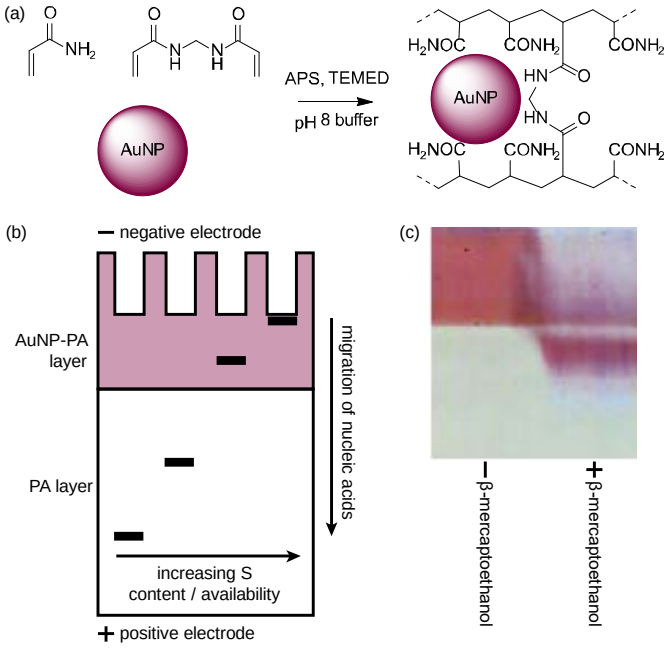

Figure 1. (a) Polymerisation of acrylamide and bisacrylamide to give crosslinked gels containing AuNPs. APS = ammonium persulphate; TEMED = tetramethylethylenediamine. (b) Resultant architecture of gel slabs for electrophoresis and expected alterations in migration according to sulphurous functionality. (c) Image of gel contain citrate AuNPs run in the absence and presence of excess thiol.

To achieve this integration, we initially looked into creation of thiolated acrylamide monomers to ensure that any overall charge on AuNPs (dependent upon surface capping) did not result in their own migration under a voltage. These monomers proved difficult to isolate due to thiol-ene self-reactivity. However, during preliminary screening of conditions it became apparent that concern about nanoparticle migration was unwarranted (Fig 1c): citrate-coated AuNPs were retained in within their original gel layer under normal electrophoresis conditions. The addition of β-

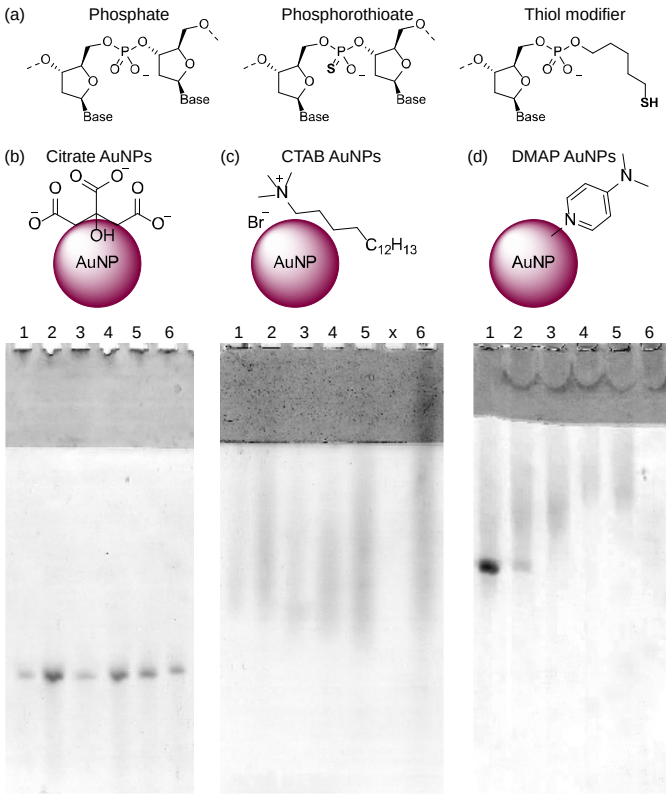

Figure 2. (a) Sulphur-modified DNA used herein. (b-d) Gold-PAGE using three types of AuNP. Lanes: 1 = **0S**; 2 = **1S-term**; 3 = **1S-cent**; 4 = **2S**; 5 = **3S**; 6 = **SH**; x = unused. All gels run under denaturing conditions (urea) in TBE buffer at room temperature. (b) Citrate AuNPs (1 mg Au mL^{-1}) in 8% PAGE. (c) CTAB AuNPs (1 mg Au mL^{-1}) in 8% PAGE. (d) DMAP AuNPs (10 mg Au mL^{-1}) in 12% PAGE.

mercaptoethanol (a common additive used to reduce protein disulphides) was required to transport the AuNPs through the gel. This finding also verified the displacement of weakly coordinating ligands by thiols within the gel matrix.

Having established both AuNP entrapment within the polymer gel and ligand exchange, we examined the scope of nanoparticle capping systems which could be used (Fig. 2). Citrate-capped AuNPs have an overall negative charge, and are routinely used as precursors to functionalised AuNPs in aqueous conditions. They were synthesised using the Frens method,[12,13] and their size verified by dynamic light scattering (DLS, r = 9.9 ± 2.6 nm) and a citrate-specific UV-visible spectroscopy method[14] (r = 10 nm). Cetyltrimethylammonium bromide (CTAB) capped nanoparticles are also water soluble, but have a surfactant-type coating, and ligand exchange is known.[15] They were synthesised by a modified version of the Brust-Schriffen synthesis[16] and sized by DLS (r = 12.5 ± 4.2 nm). Dimethylaminopyridine (DMAP)-capped AuNPs are stabilised by weak coordinative bonds and have been used as precursors to thiolated AuNPs.[17] They were produced by Brust-Schriffen reduction followed by phase-transfer ligand exchange, and sized by DLS (r = 10.2 ± 2.9 nm) and TEM (r = 4.4 ± 2.1 nm); the TEM measurement is considered to be more accurate. All AuNP solutions displayed the typical red-purple colouration due to the plasmon resonance band and were stable in solution at 4 °C on the order of months. Gold-PAGE gels were cast for electrophoresis consisting of a standard polyacrylamide layer topped with a AuNP-containing layer (Fig. 2b-c). A series of DNA samples (Fig. 2a) was then used to assess the ability of the gels to selectively retain sulphurous functionalities. These 54mer strands (taken from an aptamer[18]) had identical nucleobase sequences, but differing degrees and types of sulphur: none (**0S**), a single phosphorothioate linkage before the terminal nucleoside (**1S-term**), a single phosphorothioate in the centre of the strand (**1S-cent**), two and three of the same linkages (**2S** and **3S** respectively), and a terminal thiol-modifier (**SH**).

The citrate AuNPs were found to remain well dispersed throughout the polymerisation process in pH 8 tris-borate-EDTA (TBE) buffer. However, there was no visible change in the retention of the strands with respect to their degree or type of sulphur chemistry (Fig. 2b). This remained the case despite variation of AuNP size (d = 8 – 40 nm) and concentration (1 – 60 mg Au mL^{-1}). Noting that both DNA and citrate AuNPs are polyanionic and therefore could experience too much electrostatic repulsion to interact, we switched to a pH 8 tris-acetate-

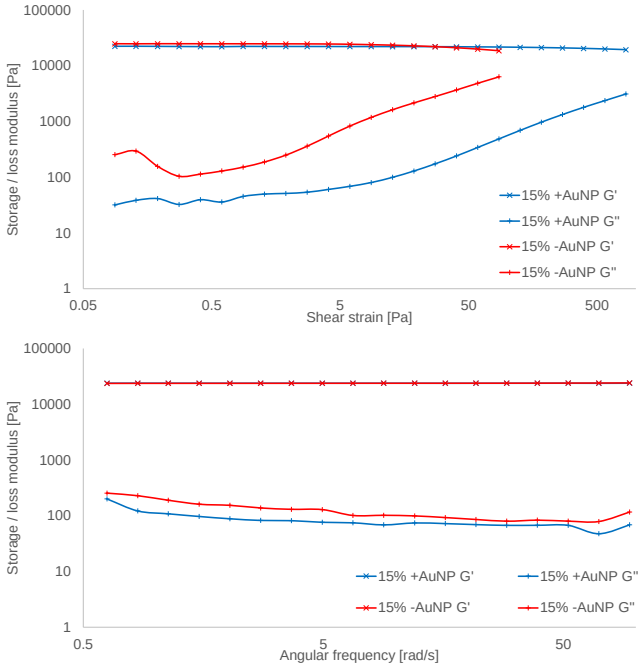

Figure 3. Comparison of rheology of gels with (+AuNP) and without (-AuNP) gold nanoparticles. Strain sweep (top) and frequency sweep (bottom) of 15 % PA gel. In the lower graph the two G' curves follow nearly exactly the same path.

magnesium (TAMg) buffer in which the dications could act to screen the charge (akin to use of Mg^{2+} in native PAGE to observe DNA hybridisation). However, this resulted only in aggregation of the AuNPs (presumably via neutralisation of the negatively charged citrate shell) to form large clumps within the gel (ESI Fig. S7), which did not affect DNA migration. CTAB-AuNPs were also found to aggregate during the polymerisation process, even in TBE buffer – this may be due to the weakly capped AuNPs having non-innocent interactions with the free radicals.[19] However, in this case the oligonucleotide migration was altered with respect to a non-Au gel – they now appeared as diffuse smears - but there was no discrimination according to sulphur content (Fig. 2c). This confirms that the oligonucleotides can interact attractively with the AuNPs entrapped within the gel matrix, although at this stage the importance of sulphur is not implicated. We then assessed the DMAP AuNPs in Gold-PAGE. The colloids remained well dispersed through to the end of the gelation period, although seemingly batch-sensitive changes in rate of gelation occurred. This was addressed by increasing the amount of the APS initiator used (see ESI). It was found that at a concentration of 10 mg mL^{-1} of Au within the upper gel layer, a progressive retention of the DNA strands according to their sulphur content could be observed, albeit in very diffuse bands (Fig. 2d). Importantly, the thiol-modified strand was retained significantly more than the phosphorothioates, indicating sensitivity to both number and type of sulphur functionality. With DMAP AuNPs, a direct interaction of the gold surface with sulphur functionality can be confirmed. However, clarity of the result varied upon repetition, and we therefore endeavoured to examine the gel itself in more detail.

Firstly, the rheological properties of the gel were examined (Fig. 3) to assess whether the presence of DMAP AuNPs resulted in alteration of the gel matrix which might affect the way in which the DNA strands pass through. An amplitude sweep was performed on TBE gels at acrylamide concentrations between 5 and 20 %, and with and without 2.5 mg Au mL^{-1} AuNPs. These studies revealed negligible variation in the storage modulus (G') between +Au and -Au at the same acrylamide concentration, however the loss modulus (G'') was significantly lower in the presence of gold. Frequency sweeps were then performed within the linear viscoelastic region as determined by the amplitude sweeps. Again, the difference made by the

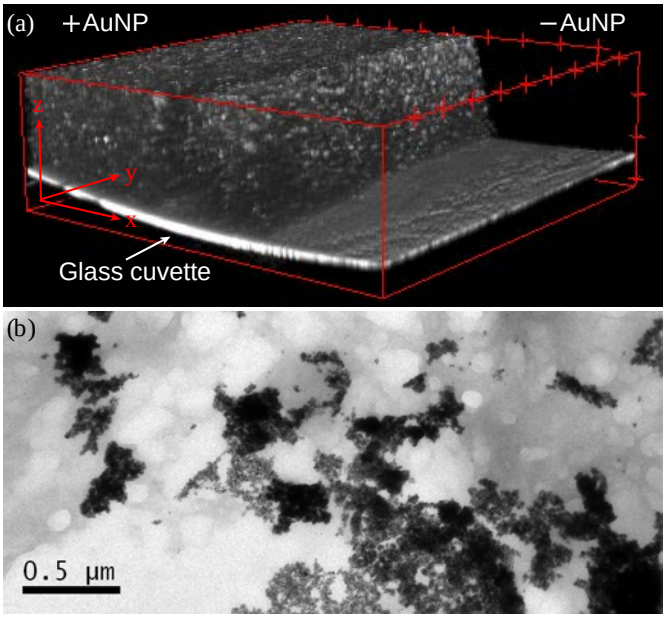

Figure 4. (a) OCT volume illustrating inhomogeneity of +AuNP layer compared with that of the pure acrylamide (-AuNP) layer (scan of 1.5 mm x 1.5 mm lateral x 1.5 mm, measured in air). (b) TEM image of DMAP AuNPs forming large aggregates within the PA gel matrix.

presence of AuNPs was only seen in the loss modulus, which was both lower and more consistent across the range of frequencies in the presence of AuNPs. These results show that the gel retains its elasticity but loses just a little of its viscosity when AuNPs are present. For the purposes of Gold-PAGE, we can therefore conclude that the smearing is due to the chemistry of the AuNPs rather than a bulk effect on the physical properties of the gel.

To further examine the structure of the gel matrix, we used optical coherence tomography (OCT). OCT can be used to non-invasively create micrometre resolution 3D models of a transparent sample based upon variations in its refractive index along the depth, without physical contact.[20] Gels consisting of an AuNP and Au-free layer were cast within a glass cuvette and volumes were produced using spectral domain OCT.[21] The interface between the layers could be clearly resolved (Fig. 4a) – the non-gold gel provided no contrast, whereas the gold layer had a strong, speckled appearance. This is indicative of variations in gold concentration across the gel, resulting in modulation of refractive index. To understand this observation, we undertook transmission electron microscopy of the gels (Fig. 4b), which revealed large aggregates of AuNPs within the gel matrix, and in some places, partial agglomeration. This inhomogeneity may well be responsible for the smeared nature of the bands in Gold-PAGE in its current form: the amount of gold which each DNA strand 'sees' as it passes

through the gel varies greatly, resulting in the elongated bands seen in Fig. 2d.

Conclusions

We have demonstrated the entrapment of AuNPs within polyacrylamide gel matrices, and shown that they are capable of binding sulphurous moieties within that matrix. DMAP-AuNPs were able to distinguish different levels of sulphur modification in oligonucleotides in electrophoresis, albeit with poor band quality. We anticipate that the quality of AuNP dispersal within the gel matrix is critical to reproducible and accurate detection of sulphur by Gold-PAGE. To make this method more fully applicable will therefore require embarkation on fine tuning of buffer, polymerisation conditions, and AuNP ligand structure.

Conflicts of Interest

There are no conflicts to declare.

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