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Nano-particle labelling of nucleic acids for enhanced detection by inductively-coupled plasma mass spectrometry (ICP-MS)

Samantha Louise Kerr and Barry Sharp*

Liquid Chromatography–Inductively Coupled Plasma–Mass Spectrometry (HPLC-ICP-MS).

Inductively coupled plasma - mass spectrometry (ICP-MS) is the most sensitive and versatile analytical technique for providing elemental and isotopic information. Most elements in the periodic table can be ionised in the ICP source including biologically important elements such as P and S and as a result, ICP-MS has been increasingly employed in the analysis of nucleic acids and proteins. ICP-MS provides complementary information to conventional organic MS and has many additional advantages. Firstly, ICP-MS offers very low limits of detection, which generally range from pg/l - µg/l depending on the analyte. Calibration and quantification are much easier and require only inorganic elemental standard solutions. In addition, because ICP-MS measures the total concentration of an element, regardless of chemical form, it facilitates mass balance calculations, which are vital for establishing analyte recovery and method validation. Although the ICP is a hard ionisation source, molecular information can be obtained, for example by measuring P/S ratios in proteins, or by employing it as a selective detector coupled to separation techniques.

Despite the advantages of ICP-MS, P and S are problematic elements because they have high first ionisation potentials (10.5 eV and 10.4 eV respectively), which results in incomplete ionisation (~35% and 15% for P and S respectively), and they suffer from polyatomic interferences e.g. 16O2 and 18O2. Thus whilst metallic elements can readily be detected at pg/l levels, the detection limits for these elements are much higher. The collision reaction cell method converts the analytes P and S to their oxides at m/z 47 and 48 yielding detection limits of 1 and 1.5 µg/l for 31P and 32S respectively. For a sector instrument, running at 4000 40C, the limits of detection for these elements are much lower. Avidin proteins bind strongly to biotin with association constants in the region of 10^{15} M^{-1} in what is known as the strongest non-covalent interaction. This property makes these two molecules ideal linkers for biomolecule labelling. The limits of detection for Au are much lower than those of P and S due to an absence of interferences and a lower first ionisation potential. In addition, the biomolecule signal is further enhanced since each nanoparticle contains approximately 80 gold atoms. The ICP-MS analysis of Au labelled DNA has been reported elsewhere, however, the method employed DNA hybridised with a peptide sequence. Gold labelled secondary antibodies were used to label a monoclonal antibody which was located at the peptide site on the hybrid biomolecule. The method detailed in this communication is site specific but not base sequence specific or dependent upon peptide sequences. It is therefore generic and can be applied to any nucleic acid that is biotinylated. In addition, this method can be applied to small nucleic acids, such as dimers which are produced in the post labelling assay, which are not suitable for PCR amplification.

For quantitative analysis of the labelled oligonucleotides, it is essential to know how many gold atoms have been bound and where. To satisfy this criteria, site specific labelling is required, which was achieved by employing DNA biotinylated at the 5’ end. Oligonucleotides containing 24 bases and biotinylated at the 5’ end were obtained from Biotez (Berlin, Germany). The sequence of the oligonucleotides was as follows; 5’ Biotin-TAT CTG TTC ACC

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GCA AAT CTG TGG 3’. Alexa Fluor-488 FluoroNanogold Streptavidin (SFNG) was obtained from Nanoprobes (New York, USA). The attached gold nanoparticles are 1.4 nm in diameter and contain approximately 80 Au atoms which are covalently attached to a streptavidin protein and Alexa Fluor-488 fluorophore. The manufacturer states that there is an average of one nanogold particle to one streptavidin protein. Although streptavidin contains four biotin binding sites, only one or two of these sites may be accessible to the biotin in this case due to the steric restraints created by the presence of the nanogold and fluorophore. The labelling of the biotinylated DNA was achieved by adding an excess of SFNG to the biotinylated DNA in a polypropylene vial. The reaction mixture was prepared by adding SFNG (1.33 µM, 83 µl) to the 5’ biotinylated DNA solution (2.24 µM, 26 µl). Thus the reaction mixture contained; 58.7 pmol biotinylated DNA and 110 pmol streptavidin which equated to 442 pmol of biotin binding sites (assuming a total of 4 biotin binding sites per streptavidin molecule) and 17.12 mg/l gold. This reaction mixture therefore contained a potential 7.5 fold molar excess of biotin binding sites. The mixture was stored for approximately 48 hours at 4°C.

Following the 48 hour reaction period, the reaction mixture was diluted so that the final solution contained 5.55 µg/l gold. This
diluted sample was then injected onto a HPLC column in order to separate the bound and unbound SFNG (see Table 1).

A C18 column was chosen to provide a rapid separation based on size exclusion coupled with a small degree of hydrophobic interaction. A HP 1090 HPLC system (Agilent Technologies, Waldbronn, Germany) was employed; the column outlet was connected directly to the ICP nebuliser via a short length of 150 µM internal diameter Teflon tubing. The eluent flow was split post-column with a T-piece so that ~300 µl/min of the mobile phase entered the nebuliser. The remainder of the mobile phase was sent to waste. An Element 2XR high resolution ICP-MS instrument (Thermo Finnigan, Bremen, Germany) was employed for the determination of ¹⁹⁷Au. The operating parameters are summarised in Table 1.

Table 1 Instrument Parameters

<table>
<thead>
<tr>
<th>HPLC Parameters</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>Waters C18 µBondpak 300 mm x 3.9 mm</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>5% methanol in deionised water</td>
</tr>
<tr>
<td>Stop time</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Injection volume</td>
<td>10 µl</td>
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<tr>
<td>Flow rate</td>
<td>1 ml/min</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ICP-MS Parameters</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Analyte</td>
<td>¹⁹⁷Au</td>
</tr>
<tr>
<td>Scan type</td>
<td>E-Scan 196,900-197,032</td>
</tr>
<tr>
<td>Magnet rest mass</td>
<td>196,906</td>
</tr>
<tr>
<td>Cones</td>
<td>Pt sampler and skimmer</td>
</tr>
<tr>
<td>Nebuliser</td>
<td>PFA-CLC (Elemental Scientific, Omnila, USA)</td>
</tr>
<tr>
<td>Spray Chamber</td>
<td>Cyclonic (Glass Expansions, Victoria, Australia)</td>
</tr>
</tbody>
</table>

A SFNG standard containing 3.66 µg/l Au was prepared from the stock SFNG solution. This standard solution was used for quantification and to establish the retention time of the unbound SFNG. Figure 1 shows the ¹⁹⁷Au chromatogram of the SFNG standard, which had a retention time of 1.57 minutes. The void volume of the column was determined with uracil, which had a retention time of 3.87 minutes. This indicates that the SFNG was totally excluded from the pore space in the column. A very small peak was observed in the SFNG standard chromatogram at 1.06 minutes, which was attributed to carry over between samples. Gold has the disadvantage of being 'sticky' in nature and thus it can be difficult to completely remove all traces of the element between injections.

Figure 1 also shows the ¹⁹⁷Au chromatogram of the DNA-SFNG reaction mixture which contains two peaks. The first peak at 1.06 minutes corresponds to the DNA-SFNG conjugate and the unbound SFNG. The second peak is observed in the chromatogram since each additional oligonucleotide added to the DNA molecule was occupied. This is the optimum labelling of one oligonucleotide. If more than one oligonucleotide bound to DNA, then more than one nanogold particle per protein would increase the average number of Au atoms per particle and there may also be a small fraction of nanogold not bound to streptavidin. If either of these species were present they should be resolved from the DNA-SFNG conjugate during HPLC separation. No evidence of additional peaks was observed in any of the chromatograms. The figure of 86 Au atoms per nanogold particle was used for further calculations, although this assay was only applied to one batch of SFNG, it is unknown whether the figure varies between batches.

From the chromatogram, it can be concluded that 53.5% of the nanogold and hence streptavidin was bound to DNA. Therefore, the number of moles of bound streptavidin in the reaction mixture was 58.9 pmol. If one biotinylated DNA molecule bound to one streptavidin protein, then 58.9 pmol of DNA also bound. Considering 58.7 pmol of DNA in the reaction mixture, 100.3% of the DNA was labelled with SFNG. This data indicates that with a 7.5 molar excess of potential binding sites, or a 7.5/4 ≈ 2 fold molar excess of nanogold probe, only one binding site per protein molecule was occupied. This is the optimum labelling stoichiometry since it equates to one Au nano-particle (86 Au atoms) per oligonucleotide. If more than one oligonucleotide bound to SFNG, more than one conjugate peak would have been expected in the chromatogram since each additional oligonucleotide added to the SFNG would have resulted in an increase of 8KDa to the...
The background was consistent at approximately 12000 counts per second (cps), equivalent to ~70 ng/l of Au. This continuous flux of Au was thought to be hydrophobically bound SFNG from previous injections slowly eluting from the column. Repeated injections of nanogold produced an accumulation of Au at the head of the column that could be reduced, but not totally removed by back flushing. The observed background Au signal may also have been enhanced due the presence of 5% methanol in the mobile phase which was shown to increase Au signals by ~16%. It has been suggested by Larsen et al.,16 that the presence of carbon facilitates the ionisation of certain analytes in the plasma.16 This effect is well known for some elements with high ionisation potential such as Se and As.16 Rodushkin et al.,17 have also reported that the addition of methane to the spray chamber increased the signal intensity of some elements including Au.17 In addition, the presence of the organic solvent reduces the surface tension of the Au solution, which may improve nebulisation efficiency.16

The chromatograms shown in Figure 1 exhibit a dip in the baseline at approximately 1.4 minutes. This reduction in signal was consistent and observed in all chromatograms, suggesting it was an injection related event. The mobile phase consisted of 5% methanol but the samples were diluted in deionised water. From the order of elution in Figure 1, it can be established that the DNA-SFNG conjugate eluted first followed by the sample solvent, which contained a higher proportion of water compared to the mobile phase, and finally the unbound SFNG eluted. If the background Au was hydrophobically bound then the elution of the water solvent (a weaker eluent than methanol) would transiently reduce the Au flux from the column. Further, reduced carbon load and higher surface tension of the water would reduce the Au signal further causing the temporary reduction in baseline signal.

The final aspect considered was the enhancement in signal due to SFNG labelling compared to that obtained by direct monitoring of 31P. To calculate the enhancement factor, two calibration curves were prepared; one for 197Au and one for 31P. The slopes of the calibration equations were 265765 and 1082 for 197Au and 31P respectively with the concentration axes expressed in µg/l. By comparing the gradients from the two sets of data, it was established that Au gave a 246 times greater response compared to P. The sensitivity of ICP-MS depends upon the number of similar isotopes in the sample, so the number of P and Au atoms also has to be considered. Each DNA-SFNG conjugate contained 24 P atoms from the DNA phosphate backbone and 86 Au atoms. Hence, each DNA-SFNG conjugate will have had 3.6 times more Au atoms compared to P. Therefore, an 882 times increase in signal was observed for the Au labelled oligonucleotides. As indicated above, the detection limit for 31P is typically 50 ng/l, or given that P accounts for ~10% by mass of DNA, 500 ng/l for DNA. The amplification achieved here lowers that to ~500 ng/l or an estimated 5 fg for a 10 µl injection. The amplification factor improves for smaller nucleic acids, but deteriorates for larger nucleic acids in proportion to the number of P atoms.

The enhancement in signal requires that the nanogold particles are completely ionised in the plasma and thus behave as Au atoms. Once the average number of Au atoms per nanogold particle was established, Au calibration curves for SFNG and elemental Au standards were compared. The gradients for the two sets of calibration data were 266226 and 265217 (x axis expressed as µg/l in both cases) for SFNG and elemental Au respectively. The calibration data confirmed that SFNG gave a similar response to the atomic Au solutions and is therefore ionised efficiently in the plasma. Recent work has suggested that for the plasma to process particles in a truly composition independent fashion requires them to be less than 90 nm in size (based upon the analysis of glass)18 which is much greater than the 1.4 nm in diameter of the nanoparticles.

It has been demonstrated that nucleic acids can be labelled with Au nanoparticles and subsequently analysed by ICP-MS. Nanoparticle labelling coupled with elemental MS has several advantages. The problems of polyatomic interferences that are encountered with 31P detection are avoided. The bio-molecule signal is greatly enhanced by the higher sensitivity for 197Au compared with 31P and the presence of ~86 gold atoms per oligonucleotide. The data shown in this communication also demonstrates that, unlike other techniques, ICP-MS can be utilised to establish mass balance for the nano-particle label thereby ensuring that all the labelled species from the reaction are both detected and quantified. A further benefit of the elemental analysis approach is that, post-separation, the stability of the molecular species is not important since only the label is detected and at this stage quantification can be undertaken using simple acidic Au standards.2 The data presented here show that ICP-MS is an ideal complement to organic MS for the study of nucleic acids and offers enhanced detection and quantification.

References
