COMMUNICATION www.rsc.org/chemcomm | ChemComm

Quantitation of biomolecules conjugated to nanoparticles by enzyme hydrolysis†

Fiona McKenzie,‡ Victoria Steven,‡ Andrew Ingram and Duncan Graham*

Received (in Cambridge, UK) 23rd December 2008, Accepted 16th March 2009
First published as an Advance Article on the web 31st March 2009
DOI: 10.1039/b823057a

A novel method for quantifying biomolecules immobilised onto gold and silver nanoparticles is reported; fluorescent-labelled antibodies and DNA are hydrolysed on the surface of the nanoparticles by the addition of trypsin and DNase I, respectively, resulting in the release of the quantifiable fluorescent label into the bulk solution.

Noble metal nanoparticles are finding much use in bioanalytical applications due to their near-field and far-field optical properties. Their intense colours, arising from their capacity to strongly absorb and scatter light at the plasmon resonance wavelength, renders them highly suitable as staining and visualisation agents[1,2] and for sensing in plasmon resonance[3,4] and SERS detection techniques;[5,6] whilst their inert nature and ability to be naturally internalised by cells means they are finding use in the delivery of molecules.[7,8]

Nanoparticles can be functionalised with a variety of biomolecules including proteins,[9] antibodies[10,11] and DNA[12] using several different conjugation techniques such as electrostatic adsorption,[13,14] thiol–gold interactions[12] and *via* nanoparticle surface ligands.[15,16] However, it is important that the resultant conjugate is adequately characterised for each method and specific biomolecule. Protein structures are susceptible to denaturation and nanoparticle conjugation has been shown to affect the protein structure,[17,18] whilst excessive biomolecule coverage can reduce functionality due to steric crowding.[19] In addition to controlling biomolecule coverage to maintain functionality, it is important to quantify numbers of adsorbed biomolecules to determine the response per immobilised biomolecule.

Characterisation has been achieved for protein nanoparticle conjugates using fluorescence resonance energy transfer (FRET),[20] stepwise photobleaching,[21] and adsorption isotherms.[17] Absorption spectroscopy has also been used for quantitation of immobilised protein molecules by subtracting the nanoparticle contribution from the extinction spectrum of the conjugated nanoparticles[18] and also by analysis of protein concentration remaining in the bulk solution following conjugation to nanorods.[10] This method is less than ideal as it requires post-analysis data correction or observation of a decrease in the extinction intensity. Demers *et al.* developed a method for quantifying numbers of single stranded DNA molecules on gold nanoparticles by incorporating a fluorescent label at the terminus of the DNA sequence.[19] DTT was used to displace the surface-bound strands allowing quantitation by fluorescence spectroscopy. This technique is simple and sensitive however it relies on the competition of ligands for the nanoparticle surface and in cases where both types of ligands (the DNA and the displacing molecule) have a similar affinity for the surface, an equilibrium may be established where displacement of the fluorescent-labelled biomolecule is not entirely efficient.

This communication presents a method for quantifying biomolecules attached to gold and silver nanoparticles which utilises enzyme hydrolysis of the biomolecular structure. Only one bond needs to be hydrolysed to release the fluorescent label into the bulk solution and therefore 100% efficiency of the enzyme is not required for this method to be effective. Since this is not a displacement technique, it is not reliant on which species has a greater affinity for the nanoparticle surface which provides greater flexibility in terms of the range of surface-bound species that can be quantified. This method may be applied to any fluorophore-labelled biomolecule. To demonstrate this, we have used a fluoroscein isothiocyanate labelled anti leukocyte function associated antigen-1 (LFA-1) and FAM labelled thiol-DNA.

The anti LFA-1 was conjugated to gold and silver nanoparticles using a cyclic disulfide linker incorporating a surface-enhanced resonance Raman scattering (SERRS) active near-infrared chromophore (Fig. 1).[22] Addition of DTT to anti LFA-1 FITC conjugated to gold, for use in the Demers quantitation method,[19] did not induce aggregation of the nanoparticles which is the usual indication that the DTT has displaced the biomolecule and has rendered them unstable in the conjugation buffer. Fig. 2 illustrates the extinction spectrum for gold nanoparticles modified with the linker that had been incubated with DTT. The λ_{max} and peak width for the plasmon resonance extinction remains relatively

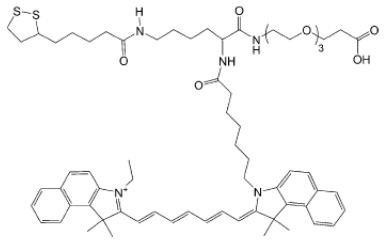


Fig. 1 Cyclic disulfide linker used to conjugate biomolecules to nanoparticle surfaces.

Centre for Molecular Nanometrology, WestCHEM, Pure and Applied Chemistry, University of Strathclyde, 295 Cathedral Street, Glasgow, UK G1 1XL, E-mail: duncan.graham@strath.ac.uk; Tel: +44 (0)141 548 4701

† Electronic supplementary information (ESI) available: Experimental details. See DOI: 10.1039/b823057a

‡ These authors contributed equally to this work.

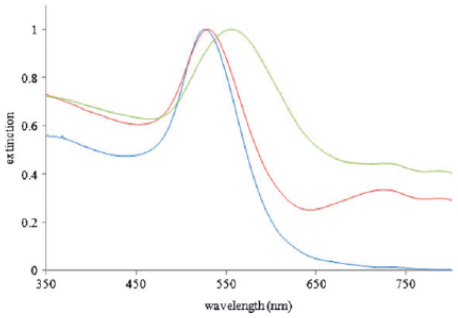


Fig. 2 UV-visible spectra of unmodified gold nanoparticles (blue), linker-modified nanoparticles incubated with DTT in 0.1 M PBS (red), and linker-modified nanoparticles incubated with trypsin in 0.1 M PBS (green). The broad absorbance ~700 nm corresponds to the near-infrared linker.

unchanged, confirming that the nanoparticles still exist in a monodisperse state. It was therefore assumed that due to the stability of the resultant conjugate afforded by the cyclic disulfide linker, the DTT was unable to displace the biomolecule. There has been significant effort to develop surface complexing linkers to improve the stability of biomolecule conjugates in bioapplications, therefore it is important that a technique can be employed that is sufficient in characterising these superior particles. DTT was substituted with trypsin, an enzyme that hydrolyses peptide backbones following lysine and arginine residues. In this approach, the fluorescent label is released upon digestion of the biomolecule instead of the entire biomolecule, including the surface complexing group, being displaced from the surface (Fig. 3). The addition of trypsin to linker-modified nanoparticles results in a shift and broadening of the plasmon resonance peak (Fig. 2), presumably due to neutralisation of the repulsive electrostatic charges on the nanoparticle surface.

Comparison of the concentration of nanoparticles and fluorescent label allows quantitation of the number of biomolecules per nanoparticle. This is shown for anti LFA-1 FITC on gold nanoparticles in Fig. 4. The number of anti LFA-1 FITC molecules per 13 nm Au nanoparticle was found to be 0.21 ± 0.02 (Table 1). This suggests that some nanoparticles are not functionalised with antibody which is consistent with data that indicates polyvalent biomolecule functionalisation follows a Poisson distribution.[20] To corroborate this technique, the DTT displacement method was also performed for anti LFA-1 FITC conjugated to silver nanoparticles. Silver nanoparticles functionalised with protein *via* the cyclic disulfide linker are less stable than the analogous gold nanoparticles and therefore the linker may be displaced from the surface.[22] The number of anti LFA-1 FITC molecules per 35 nm silver nanoparticle, using the DTT displacement method, was found to be 1.82 ± 0.10. This was in close agreement with the number calculated using the trypsin hydrolysis method: 1.85 ± 0.50 (Table 1).

The technique was also applied to DNA functionalised nanoparticles. Quantitation of FAM labelled DNA strands on gold nanoparticles was achieved using the endonuclease, DNase I. DNase I catalyses the hydrolysis of phosphodiester linkages in the DNA backbone, preferentially adjacent to pyrimidine nucleotides. Hydrolysis of any bond along the DNA molecule will allow the release of the fluorescein label into the bulk solution. Again, the calculated number of DNA

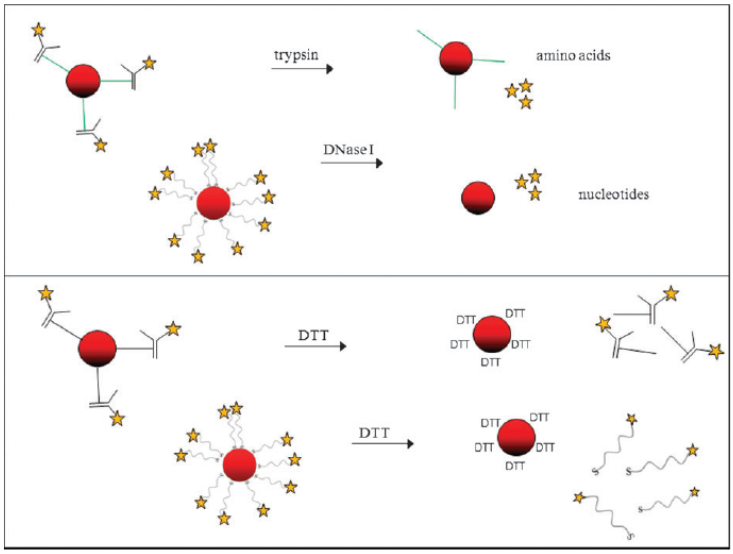
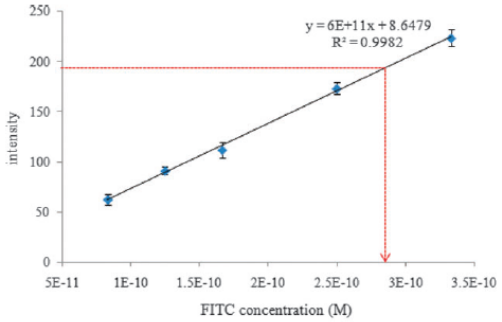


Fig. 3 Enzyme hydrolysis method (top): the tryspin and DNase I will hydrolyse the antibodies and DNA, respectively, leaving the surface linker in-tact with the nanoparticle surface. DTT displacement method (bottom): the DTT has a higher affinity for the nanoparticle surface than the linkers and biomolecules so it will displace them.



sample	fluorescence intensity	FITC concentration using calibration equation	concentration prior to sample dilution	nanoparticle concentration	antibodies per nanoparticle
A	205.84	3.29×10^{-10}	9.13×10^{-10}	3.87×10^{-9}	0.23
B	155.83	2.45×10^{-10}	6.81×10^{-10}	3.26×10^{-9}	0.21
C	184.21	2.93×10^{-10}	8.13×10^{-10}	4.21×10^{-9}	0.19
D	168.02	2.66×10^{-10}	7.38×10^{-10}	3.61×10^{-9}	0.20
E	195.19	3.11×10^{-10}	8.64×10^{-10}	4.05×10^{-9}	0.21

Fig. 4 Calibration graph for FITC-labelled anti LFA-1 illustrating the intensity for unknown sample C. The anti LFA-1 FITC concentration was determined for each sample using the calibration equation. Evaluation of the number of antibodies per nanoparticle was achieved by dividing the value for the antibody concentration by the nanoparticle concentration.

Table 1 Calculated number of FITC labelled anti LFA-1 molecules and FAM labelled oligonculeotides immobilised onto Au and Ag nanoparticles

Method	Number of antibodies per Au NP	Number of antibodies per Ag NP	Number of oligonucleotides per Au NP
Enzyme hydrolysis	0.21 ± 0.02	1.85 ± 0.50	24.52 ± 1.26
DTT displacement	Not possible	1.82 ± 0.10	24.47 ± 1.49

strands adsorbed onto the nanoparticles using the enzyme digestion method was consistent with the values obtained using the DTT displacement method (Table 1).

In summary, we have presented a novel enzyme-based technique for quantifying biomolecules on nanoparticles. It allows quantitation when complexing groups of high surface affinity are used for conjugation of the biomolecule and displacement methods are unsuitable. It is possible that the accessibility of the incoming enzyme may be hindered for nanoparticles with high biomolecule surface coverage and as such may affect the efficiency of hydrolysis. However, the similarity between the calculated quantitation values for our nanoparticle conjugates indicates that the enzyme hydrolysis method technique is equally as effective for quantifying biomolecules on nanoparticles as the DTT displacement method and supersedes it in circumstances where biomolecules are unable to be displaced.

Notes and references

1 I. O. Ellis, J. Bell and J. D. Bancroft, *J. Histochem. Cytochem.*, 1988, **36**, 121–124.
2 M. De Waele, W. Renmans, E. Segers, V. De Valck, K. Jochmans and B. Van Camp, *J. Histochem. Cytochem.*, 1989, **37**, 1855–1862.
3 N. Nath and A. Chilkoti, *J. Fluoresc.*, 2004, **14**, 377–389.
4 A. J. Haes, D. A. Stuart, S. M. Nie and R. P. Van Duyne, *J. Fluoresc.*, 2004, **14**, 355–367.
5 S. Nie and S. R. Emory, *Science*, 1997, **275**, 1102.
6 K. Kneipp, Y. Wang, H. Kneipp, L. T. Perelman, I. Itzkan, R. R. Dasari and M. S. Feld, *Phys. Rev. Lett.*, 1997, **78**, 1667–1670.
7 P. Ghosha, G. Hana, M. Dea, C. K. Kima and V. M. Rotello, *Adv. Drug Delivery Rev.*, 2008, **60**, 1307–1315.
8 D.-R. Chen, C. H. Wendt and D. Y. H. Pui, *J. Nanopart. Res.*, 2000, **2**, 133–139.
9 M.-E. Aubin-Tam and K. Hamad-Schifferli, *Biomed. Mater.*, 2008, **3**, 1–17.
10 C. X. Yu and J. Irudayaraj, *Anal. Chem.*, 2007, **79**, 572–579.
11 J. Y. Chang, H. M. Wu, H. Chen, Y. C. Ling and W. H. Tan, *Chem. Commun.*, 2005, 1092–1094.
12 R. Elghanian, J. J. Storhoff, R. C. Mucic, R. L. Letsinger and C. A. Mirkin, *Science*, 1997, **277**, 1078–1081.
13 L. A. Dykman, V. A. Bogatyrev, B. N. Khlebtsov and N. G. Khlebtsov, *Anal. Biochem.*, 2005, **341**, 16–21.
14 W. D. Geoghegan and G. A. Ackerman, *J. Histochem. Cytochem.*, 1977, **25**, 1187–1200.
15 R. L. Letsinger, R. Elghanian, G. Viswanadham and C. A. Mirkin, *Bioconjugate Chem.*, 2000, **11**, 289–291.
16 J. A. Dougan, C. Karlsson, W. E. Smith and D. Graham, *Nucleic Acids Res.*, 2007, **35**, 3668–3675.
17 A. A. Vertegel, R. W. Siegel and J. S. Dordick, *Langmuir*, 2004, **20**, 6800–6807.
18 M.-E. Aubin-Tam, H. Zhou and K. Hamad-Schifferli, *Soft Matter*, 2008, **4**, 554–559.
19 L. M. Demers, C. A. Mirkin, R. C. Mucic, R. A. Reynolds, R. L. Letsinger, R. Elghanian and G. Viswanadham, *Anal. Chem.*, 2000, **72**, 5535–5541.
20 T. Pons, I. L. Medintz, X. Wang, D. S. English and H. Mattoussi, *J. Am. Chem. Soc.*, 2006, **128**, 15324–15331.
21 D. Casanova, D. Giaume, M. Moreau, J.-L. Martin, T. Gacoin, J.-P. Boilot and A. Alexandrou, *J. Am. Chem. Soc.*, 2007, **129**, 12592–12593.
22 F. McKenzie, A. Ingram, R. J. Stokes and D. Graham, *Analyst*, 2009, **134**, 549–556.