

Biological nanostructures: platforms for analytical chemistry at the sub-zeptomolar level

Graham J. Leggett

DOI: 10.1039/b403882g

The analysis and manipulation of molecules at the sub-zeptomolar level (*i.e.* from 1 to 600 molecules) remains the unconquered frontier of analytical chemistry. While some techniques offer *sensitivity* to single molecules, there are no established tools for the *manipulation* of such small quantities of material. Scanning probe lithography has begun to provide practicable means to manipulate biological organisation on length scales of 100 nm and less, and three promising approaches (dip-pen nanolithography, nanoshaving and scanning near-field photolithography) are reviewed. Each offers extraordinary spatial resolution combined with the capability for use under ambient and, in some cases, fluid conditions. These techniques offer a multitude of strategies that may at last make the manipulation of handfuls of molecules—and perhaps single molecules—a practical possibility for the analytical chemist.

While the miniaturisation revolution of the past two or three decades was initially driven by the electronic device industry, its impact upon analytical chemistry has already been far-reaching. One of the most important developments in our field has been the emergence of micro-total analysis systems,¹ and a variety of microtechnologies have emerged that are based upon the adaptation of electronic device manufacturing methods. New methodologies such as soft lithography² have been developed to drive down cost and demystify micro-fabrication, taking it from the hands of specialist engineers and placing it at the disposal of chemists. Microfluidic channels pump small volumes of liquid from place to place on miniaturised chips, where analysis stations deliver data from tiny specimens, which may, for example, be a specified region of a single cell.³

The explosion of interest in genomics, and the concomitant and apparently unquenchable thirst for sequence data, has driven the development of high throughput sequencing systems, for which miniaturisation in some form is a *sine qua non* given the sheer size of even a restricted portion of a genome. With the emergence of proteomics, the degree of interest in high-throughput methods, in combination with data-mining and other

informatics developments, seems unlikely to abate. In the analysis of proteins, many of the technical challenges are brought into sharp focus. Sensitivity is a key issue. In the case of DNA analysis, polymerase chain reaction (PCR) technology is an essential ingredient in high-throughput analysis, amplifying samples that would otherwise be too small to yield meaningful data. For proteins, no equivalent methodology exists. Miniaturisation is thus even more at a premium in protein analysis: a suitable high-throughput analysis system should be able to deal with sample sizes significantly smaller than any addressed by current systems designed for DNA analysis. How much smaller? The Holy Grail of biomolecular analysis is the manipulation and detection of single molecules. While methods exist for the interrogation of single molecules under highly controlled conditions in fundamental experiments (for example, the optical tweezers and the atomic force microscope may be used to measure forces in single molecules), this is a world away from a high-throughput analytical system. Currently, mass spectrometry is the technique of choice in ultra-high sensitivity proteomic investigations, but while it is capable of single molecule detection, the interface with the biological specimen is woefully

crude. Typically, sample preparation is *via* a 2D gel, and the problems associated with these are widely documented.

There is thus a huge technology gap. The field of high-throughput analysis is not unique in experiencing difficulties. Fluorescence techniques offer the possibility of obtaining spectroscopic data from single biological molecules, but the preparation of suitable samples for such exquisite analysis remains a substantial challenge. While techniques exist that can detect signals from single molecules, our ability to manipulate molecules is floundering a large number of orders of magnitude behind. To get a feel for the extent of the problem, consider a popular if flawed paradigm for high-throughput analysis: the microarray. To confuse matters further there is a great deal of hyperbole surrounding any miniaturised technology, and these devices are often termed nanoarrays because they may have nanogram sensitivities. However, there is nothing “nano” about the technology used to fabricate them; indeed in a typical “nanoarray” the features may well be visible to the naked eye. Let us suppose that the system has a sensitivity of 1 ng and we are analysing a protein of molecular weight 10^5 g mol^{-1} . This represents a sensitivity of 10 fmol. Impressive though this

sounds, multiplication by Avogadro's constant yields a detection limit of 6×10^9 molecules. This is significantly poorer than single molecule detection! What is required to service the requirements of modern biomolecular analysis is a quantum leap in sensitivity. Analysis at the attomolar level (10^{-18} mol) currently presents major challenges, but to meet the needs of proteomic analysis by high-throughput methods, we require a system with sub-zeptomolar (10^{-21} mol) sensitivity. Miniaturisation may provide part of the solution to these problems. For example, if we shrink the spots in an array from $50 \mu\text{m}$ to 50 nm in diameter, then *ceteris paribus*, a 10^6 gain in sensitivity is possible provided we have a technique to read the array with single molecule sensitivity (for example, confocal microscopy could detect fluorescence from single molecules placed 300 nm apart; near-field scanning optical microscopy could resolve material at much smaller separations and, following the recent work of Humphris *et al.*, at very high scan rates⁴). Similar advantages may be realised in other areas. For example, the ability to precisely locate single molecules on a solid surface would greatly facilitate their interrogation by techniques such as AFM and optical tweezers; miniaturised sensors may be developed to function downstream of microscopic culture wells used for single cell culture and may be incorporated into microfluidic networks to take micro-total analysis to higher levels of sensitivity. The most significant challenge in all of these areas is the development of adequately powerful, versatile methods for the positioning and attachment of biological molecules on small length scales.

Electron beam lithography offers very high spatial resolution and is a widely used nanofabrication tool in semiconductor device research. However, the principal drawbacks are that modification occurs under ultra-high vacuum conditions, terminal for the retention of biological functionality. The only suitable strategy for using electron beam lithography to create biological surface structures is first to pattern a molecular monolayer,⁵ and then subsequently attach the biological species of interest. One demonstration of this has been provided by Harnett *et al.*, who used electron beam lithography to selectively

remove molecular adsorbates from gold and silicon substrates and then back-fill the bare regions with a reactive SAM to which biotin could be attached as an anchor for biological species.⁶ However, when one contemplates the feasibility of fabricating an extended, multicomponent array (even if the problem with vacuum-compatibility could be overcome) the practical obstacles become very clear. Alternative approaches are required that facilitate either the parallel deposition of multiple different species or the rapid, sequential deposition of biomolecules. To realise the latter approach, operation in a fluid medium is a necessity. All of these considerations suggest that a method based upon scanning probe microscopy (SPM) is likely to provide the solution, and several methods have begun to emerge as feasible solutions.

Dip-pen nanolithography (DPN) utilises the simplest concept of all (Fig. 1). An atomic force microscope (AFM) tip is inked in a solution of the molecule of interest and traced across the sample surface, leaving behind it a trail of molecules wherever it travels.⁷ The technique offers a simple analogy with the age-old technology of writing with a pen and ink: the pen is dipped in the ink and used to transfer the ink to a suitable surface to create a pattern. Alkanethiols make ideal inks if gold is employed as the paper. The thiol–gold interaction is strong and provides a driving force for molecular attachment and organisation. Transfer of the thiol to the surface occurs *via* the liquid capillary bridge that forms between an AFM tip and a solid substrate under ambient conditions. The

resolution of DPN depends upon a variety of experimental parameters, including the writing speed, the tip radius, the ambient humidity, the sample roughness and the nature of the ink. For example, by working under a controlled environment, it is possible to influence the relative humidity and hence the feature size. Humid atmospheres lead to larger features; reducing the ambient humidity reduces the diameter of the capillary, improving resolution until at very low humidities fluid transfer becomes difficult. The morphology of the substrate has a critical influence. Best results on gold are achieved for atomically flat substrates, and the ultimate linewidth achievable using DPN is *ca.* 15 nm .⁸ Linewidths are poorer for polycrystalline surfaces such as those commonly used in surface chemistry applications. However, the demonstration of the ability under optimal conditions to match electron beam lithography using an ambient technique based around direct deposition of liquid-phase reagents is a remarkable achievement. Once the pattern has been formed, the rest of the surface can be functionalised by overwriting with a contrasting thiol, facilitating control over the whole substrate's properties.⁹ Alternatively, thiol nanopatterns may be used as resists to etch gold nanoelectrodes by subsequent exposure of the sample to an etchant for the underlying substrate; underivatised regions of gold are removed while those functionalised with thiols by DPN are protected from the etchant.¹⁰

DPN is not restricted to gold surfaces, or to thiol deposition. Liu and co-workers

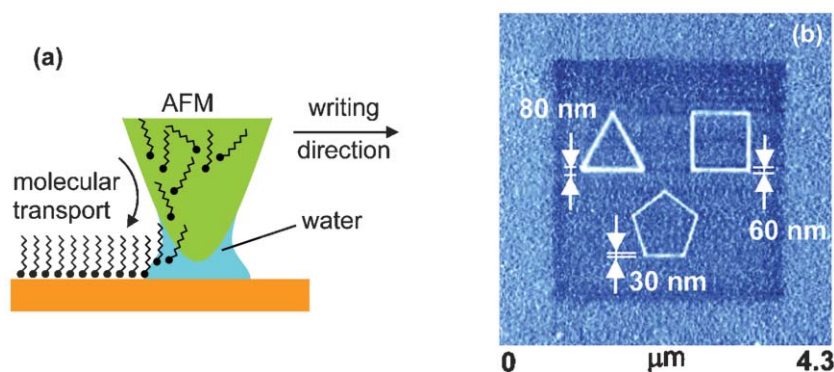


Fig. 1 (a) Schematic diagram illustrating the basic principles behind dip-pen nanolithography. (b) Polygons drawn by depositing mercaptohexadecanoic acid onto gold using DPN. The area around the polygons has been overwritten with a monolayer of octadecanethiol. Reproduced with permission from ref. 7.

have realised sharp features with small dimensions used aqueous metal salts as inks, and the native oxide layer of silicon as the substrate.¹¹ By applying a potential difference between the AFM tip and the substrate, it is possible to deposit material electrochemically, reducing metallic ions in the one-dimensional electrochemical cell that is established in the meniscus and leading to the deposition of metal nanostructures. Polymeric structures may also be fabricated. Maynor *et al.* used a conducting AFM tip to deposit 3,4-ethylenedioxythiophene (EDOT) under electrochemical conditions,¹² leading to the formation of electropolymerised semiconducting polymeric nanostructures. A line width of 50 nm was achieved.

DPN offers several approaches to the deposition of biomolecules to form miniaturised arrays. One approach is to pattern the surface to introduce chemistries with specific organising influences on biomolecules. For example, Lee *et al.* fabricated arrays of spots of mercaptohexadecanoic acid (MHA) by DPN, and then passivated the regions in between them using a highly protein-resistant oligo(ethylene glycol) (OEG) terminated thiol, before exposing the samples to a solution of protein.¹³ Protein adsorbed to the regions functionalised by MHA, but not to the OEG functionalised regions resulting in structures as small as 100 nm. A problem associated with the physical adsorption of proteins is their propensity to denature. In solid-phase immunoassay systems, as many as 95% of the antibodies coated on well bottoms may be inactive because of adsorption-induced conformational change. A more sophisticated approach promising much better control of conformation was reported by Hyun *et al.*,¹⁴ who also patterned MHA onto gold by DPN, but then used this as an anchor for the attachment of biotin and, subsequently, streptavidin. The widespread availability of biotinylated biological molecules makes this platform one of quite general utility.

The direct deposition of biomolecules is also possible. Demers *et al.* demonstrated two alternative approaches capable of yielding linewidths smaller than 100 nm.¹⁵ First, hexanethiol-modified oligonucleotides were deposited onto

gold surfaces. Second, oligonucleotides with 5'-terminal acrylamide groups were deposited onto silicon dioxide layers functionalised with 3'-mercaptopropyltrimethoxysilane. The direct deposition of proteins is also possible. Lee *et al.* transferred lysozyme and IgG to a surface by DPN.¹⁶ The conformational activity of the transferred proteins was demonstrated by the bonding of antibody-functionalised gold nanoparticles, which could be readily observed as relief features in AFM images, providing an illustration of the types of approach that might be used to read a miniaturised biological array. In an alternative approach, Lim *et al.* created passivated tips by the direct attachment of 2-[methoxypoly(ethyleneoxy)propyl]trimethoxysilane to the silicon oxide layer present on the tip surface.¹⁷ The tip was then inked with a protein layer. The presence of the passivating coating meant that protein adsorbed weakly to the tip and was thus readily transferred to the sample surface. Attachment to the surface was enhanced by either pretreatment of silicon dioxide with base (rendering it negatively charged) or by attachment of an aldehyde to an aminosilane monolayer. Feature sizes as small as 55 nm were reported.

While DPN offers the advantage of ambient operation using an experimental platform of significantly lower cost and ease of implementation than electron beam lithography, a significant major challenge is the requirement that it be possible to deposit multiple different biological molecules in an array. Approaches to parallel writing based upon simultaneously actuated arrays of multiple cantilevers bound to a single silicon nitride chip have been described.¹⁸ Recently, a report has been published of an array of *separately* thermally actuated probes.¹⁹ This represents a useful step forward. However, a further problem concerns the inking of probes with different materials. For DPN to deliver multiple different molecules, it will be necessary either to overwrite complementary patterns one-by-one on the same region of the substrate (which would place stringent demands on piezo stability during fabrication of an extensive array) or to separately ink each member of a very large array of cantilevers. While the latter approach seems the most likely

to succeed, it remains to be seen how practicable a route this will be to a highly miniaturised array.

Mechanical removal of adsorbates offers an alternative approach that also avoids all of the complications and expense associated with electron beam lithography, and which seems to hold real promise for the fabrication of miniaturised biological structures. In nanoshaving, an AFM tip is used to scratch a line into a SAM by scanning the surface at elevated load. If the operation is carried out under an ethanolic solution of a second thiol, then the scratch will be in-filled in the wake of the tip creating a pattern of heterofunctional regions that may be as small as a few nm in size.²⁰ An alternative approach, termed "nanopen reader and writer", is an amalgam of nanoshaving and DPN.²¹ The tip is precoated in a solution of the second molecule, and traced across the surface at elevated load. Mechanical removal of adsorbates is followed immediately by deposition of molecules from the tip. Using this approach, a patch of octadecanethiol only $2 \times 4 \text{ nm}^2$ in size was grafted into a decanethiol monolayer.

The application of such methodologies to the fabrication of biological nanostructures has been effectively demonstrated. Liu and co-workers have shown that several approaches may be used for the immobilisation of protein molecules. For example, carboxylic acid terminated thiols may be patterned onto a surface and exposed to a solution of lysozyme. At pH 7, the protein has a net positive charge, and a small fraction of the carboxylic acid groups exposed at the surface are ionised, resulting in an attractive electrostatic interaction.²² For protein binding, a high density of attractive interactions is not required—a few percent of groups at the surface capable of interacting strongly with the protein will be sufficient to immobilise it at the surface. An alternative method is to pattern aldehyde functionalised thiols at the surface; these bind protein through the formation of imine linkages with lysine residues (which possess a free amine group).^{22,23} The electrostatic binding of proteins to samples patterned using nanoshaving was also studied by Zhou *et al.*²³ When they introduced regions terminated with neutral (methyl)

or positively charged *N*-(6-mercaptohexyl) pyridinium bromide) into an OEG-terminated SAM, lysozyme did not adsorb at the surface, while regions patterned with negatively charged mercaptopropionic acid exhibited a uniform coverage of adsorbed protein. Carbonic anhydrase and protein G were patterned by similar methods, with the immobilised biomolecules providing a template for the construction of three dimensional architectures in the latter case, *via* the subsequent attachment of IgG and then anti-IgG. The immobilisation of single stranded DNA sequences, functionalised with a thiol group at the 5' end was demonstrated by Liu *et al.*²⁴ and Zhou *et al.*²⁵ The binding of complementary oligonucleotides to DNA patterns formed this way may be observed directly using AFM.²⁶

In terms of ultimate spatial resolution, these techniques offer superior performance to DPN. The principal drawbacks are the rather severe nature of the process (loads of 100 nN are typically required) and its inevitably rather poorly defined nature, and the restriction that moderately labile surface functionalities are required. Effectively this appears to be a restriction to the alkanethiol-on-gold system, for which the balance between the rates of energy dissipation at "elevated" loads accessible in a commercial AFM system and the activation barrier to displacement of species from the surface appear to be well-matched. Thus an analytical system based upon miniaturised arrays of biological molecules would suffer a limitation to gold if mechanical fabrication methods were used. Against this, it would appear that nanoshaving might be readily conducted in a flow cell provided this obstacle was not insuperable. The rapid serial fabrication of biological nanostructures may thus be a possibility.

A final, and more recent, approach is based upon photolithography. The photo-patterning of SAMs was first demonstrated just over ten years ago, by Tarlov and Hemminger and co-workers.^{27,28} Exposure of a SAM to UV light through a mask in the presence of oxygen leads to the conversion of alkanethiolate adsorbate species to weakly bound disulfides in exposed regions. These may be displaced by solution-phase thiols to yield clean, well ordered molecular patterns

that have excellent edge-definition. Although this approach was generally overlooked in the explosion of interest in soft lithography that occurred around the same time, a number of groups have demonstrated the efficacy of photopatterning for the control of surface molecular function. But how might photochemical methods be used to pattern surfaces at the nanometre scale? It is not possible to arbitrarily reduce the dimensions of features fabricated photolithographically, because when the openings in the mask reach dimensions equal to half the wavelength of the light being used, diffraction starts to occur, leading to ill-defined patterns (the diffraction limit). This constitutes a major problem for semiconductor device manufacturers, who currently rely on photolithography as the main tool for chip manufacturing and who are currently fabricating structures close to the diffraction limit in commercial microprocessors. One approach to the resolution of this apparently fundamental obstacle that has been explored in the author's laboratory is the use of near-field optical methods. In particular, exposure of a SAM of alkanethiols on gold to light from a frequency doubled argon ion laser (244 nm) delivered through an optical fibre-based near-field scanning optical microscope (NSOM) (termed scanning near-field photolithography (SNP)) resulted in nanoscale photochemical oxidation and facilitated the routine fabrication of molecular patterns with widths of only 30–40 nm²⁹ (Fig. 2). Rather surprisingly, this represents a linewidth smaller than the aperture in the fibre used to excite photo-oxidation (*ca.* 50 nm at best). As a first approximation, one might have expected that feature sizes would be limited by the diameter of the aperture used to expose the monolayer. Moreover, the best feature sizes achieved to date, 13 nm (nearly $\lambda/20$), represent a level of performance significantly beyond this. Clearly the physical phenomena involved are complex. One possible explanation is that the polycrystalline nature of the gold films used plays a critical role. Recently there has been growing interest in the use of apertureless NSOM techniques, in which a metal tip is brought very close to a surface and irradiated with light, leading to a pronounced enhancement of the electric field in the near field region

under the tip apex. The result is a highly confined region of as much as several orders of magnitude greater field strength than is measured in the far-field region. Recently we have hypothesised that the grains in the gold film function as an array of asperities;³⁰ these are able to interact non-radiatively with the electric field associated with the NSOM aperture leading to a focussing of the field and the exceptional resolution we observe. While this hypothesis remains in need of further testing, the main conclusion is clear: SNP provides a method for rivalling the performance of electron beam lithography but under ambient conditions and using photochemical processes. This represents a potentially very powerful capability.

Using this approach, a variety of strategies have been developed for the creation of functional molecular architectures. SNP-patterned SAMs have been used as resists to etch three-dimensional structures into gold films,³¹ and to attach proteins and DNA in nanometre scale patterns. For example, the oxidation of lines in an OEG-terminated SAM, followed by the adsorption of carboxylic acid functionalised thiols, resulted in the formation of nanopatterned regions to which IgG could be attached covalently using well-established methods involving the use of carbodiimide reagents to generate active ester intermediates. Importantly, the process is extendable to silicon surfaces, and may also be implemented under a fluid medium. Recent studies by other workers have pointed to future advances through the application of apertureless NSOM instruments³² and through the exploitation of sophisticated photochemical mechanisms (for example, two photon absorption³³) to excite highly specific reactions. Given the extensive range of strategies based upon photochemistry available for the attachment of biological molecules to surfaces, it seems that SNP may provide a versatile route to surface nanostructures that is capable of combination with a flow cell to facilitate the sequential deposition of multiple different biomolecules.

Conclusions

From the foregoing discussion it will be clear that there remain some outstanding

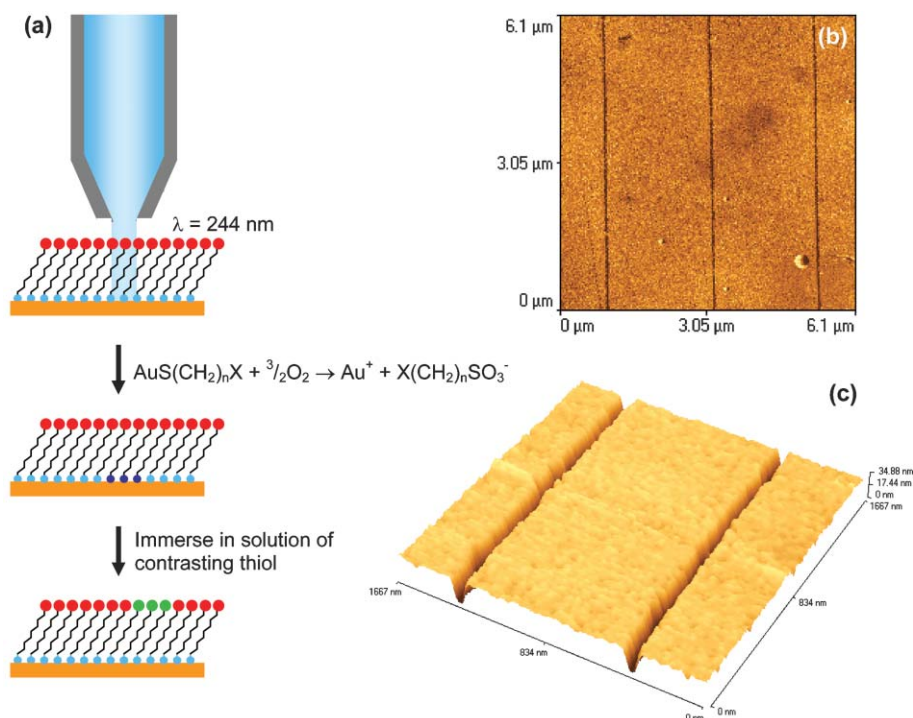


Fig. 2 (a) Schematic diagram illustrating the basic principles behind scanning near-field photolithography. (b) 40 nm wide lines of $\text{HS}(\text{CH}_2)_{11}\text{CH}_3$ written into a monolayer of $\text{HS}(\text{CH}_2)_{10}\text{COOH}$ by SNP and imaged using friction force microscopy. (c) Nanotrenches etched into gold using an SNP-patterned SAM as the resist.

problems associated with the techniques reviewed here. In the case of DPN, there are questions concerning the practicality of fabricating extensive multicomponent arrays. In the case of nanoshaving, the restricted range of chemistries accessible is problematic, while SNP remains comparatively less well explored. However, it is also clear that all of these methods provide significant promise. All have proved capable of fabricating biological structures with dimensions on the order of 100 nm or less, all are capable of implementation under ambient conditions and, in the case of nanoshaving and SNP, there is also the possibility of combination with a flow cell to yield novel reactive nanolithographies.

Acknowledgements

The author wishes to thank the EPSRC and the RSC Analytical Chemistry Trust Fund for their financial support.

Graham J. Leggett

Department of Chemistry, University of Sheffield, Brook Hill, Sheffield, UK S3 7HF.
E-mail: Graham.Leggett@shef.ac.uk

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