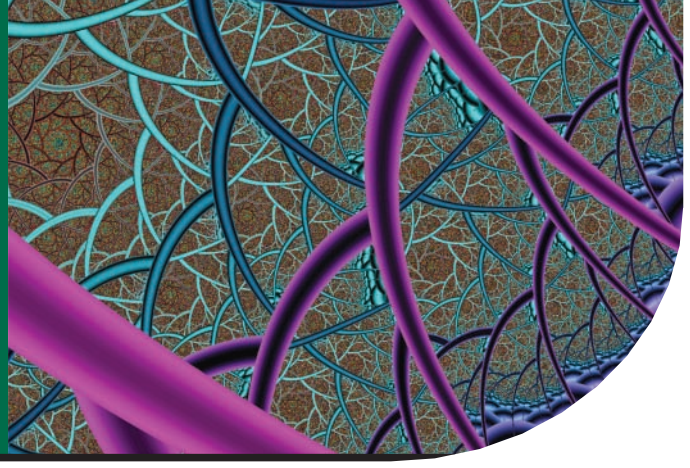

Antibiotic Resistance - Nanotech to the Rescue: Nanomechanical detection of drug-target interactions

Drug resistance has evolved from being an infrequent and manageable occurrence in the treatment of microbial infections, to a healthcare problem on a global scale.
The last five years has seen a meteoric rise in the cases of life-threatening infections by mutated forms of common bacteria, such as methicillin-resistant Staphylococcus aureus (MRSA) and vancomycin-resistant Enterococcus (VRE). Eradication of such multi-drug resistant (MDR) strains is proving difficult since there are not enough effective antimicrobials on the market, and very few in the development pipelines. There is though, a concerted effort to develop new compounds capable of combating these infections effectively.
A novel drug-target binding detection system is proving itself to be a big hit in this search. It is based on micro-cantilever technology and provides exquisite information on the nature of a drug-target interaction. Furthermore, it can be applied on a high throughput basis making the search for new products much quicker too.

> ***"The differential measurements generated enabled clear discrimination between the two peptide sequences. This showed that the system can detect the subtle binding changes induced by the deletion of a single hydrogen bond"***

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BASIC PRINCIPLES

By immobilising a ligand on one side of a cantilever and adding the relevant receptor in solution, the cantilever bends in response to the change in surface stress when the ligand and receptor interact. This nanomechanical biosensing transduction mechanism has been used for measuring a number of biochemically specific interactions such as sequence-specific DNA hybridisation, single base mismatches, DNA quadruplex, protein recognition and has also been used for the detection of interferon-alpha-induced I-8U gene expression in total human RNA - a potential marker for melanoma progression and viral infections. This technology has now been applied very successfully to quantify drug–target binding interactions.

Cantilever advantages

Cantilever-based sensing offers a number of advantages over existing technologies. For example it does not require the use of any reporter 'tags' or external probes, and can therefore be used to detect biomolecules rapidly in a single-step reaction. Arrays of cantilever sensors can be constructed to screen multiple drug–target interactions and reference coatings in parallel, under identical experimental conditions. Furthermore, quantitative ligand–receptor binding constants can be measured on cantilever arrays. Due to the nature of the detection, the nanomechanical signal generated from a cantilever is not limited by mass, a restriction commonly associated with evanescent techniques such as surface plasmon resonance.

In summary, cantilever-based sensing provides a unique and highly precise method of measuring small-molecule drug-binding interactions and is ideal for parallelisation, enabling high-throughput screening of thousands of drugs per hour.

EXAMPLE APPLICATION

Vancomycin (Van) is a vital therapeutic drug used worldwide for the treatment of infections with Gram-positive bacteria, particularly those Staphylococci and Enterococci responsible for post-surgical infections. It is also, essentially, the last chance antibiotic in the battle against MRSA super-bug infections. Van is a very potent bactericidal compound, binding to the C-terminus of peptidoglycan mucopeptide precursors terminating in the sequence 'Lysine—D-Alanine—D-Alanine', as shown in *Figure 1*. As a result it blocks bacterial transpeptidases and transglycosylases, which catalyse the crosslinking of the growing bacterial cell wall, resulting in cell lysis. With the rapid turnover and mutation capabilities of many bacterial species, coupled with the misuse of antibiotics, resistance to Van has rapidly increased and continues to do so at an alarming rate. As a result, infections from Van resistant species now pose a major international public health problem, with growing morbidity and mortality.
Developing resistance to Van in Enterococci only requires the change of an amide linkage to an ester linkage in the growing bacterial cell wall – a subtle change. This results in the deletion of a single hydrogen bond to the binding pocket, rendering the antibiotic therapeutically ineffective (*Figure 1*).

A recent series of investigations [1] by an international team of scientists led by Dr Rachel McKendry, London Centre for Nanotechnology, University College London, has demonstrated a novel and highly precise method to detect and quantify the binding of antibiotic–mucopeptide interactions.

INVESTIGATION SET-UP

Multiple arrays of eight rectangular silicon cantilevers were developed to probe the nanomechanics of antibiotic drug–target interactions. The cantilever arms were coated on one side with a thin film of gold and functionalised with alkanethiol self-assembled monolayers (SAMs) of either:

1. Lysine-D-Alanine-D-Alanine – found in Van-sensitive bacteria (DAla); or
2. Lysine-D-Alanine-D-Lac - found in Van-resistant bacteria - (DLac); or
3. Polyethylene glycol - PEG (control cantilever).

The absolute deflection at the free end of each cantilever

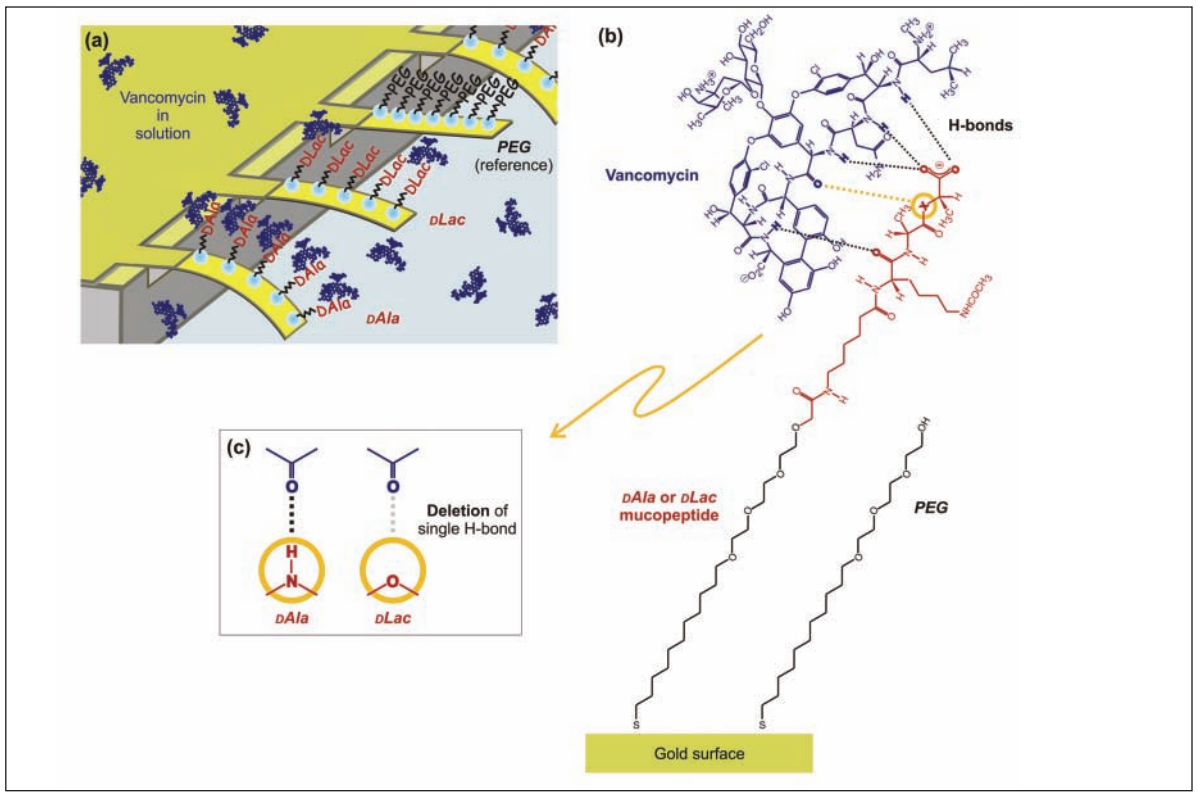

Figure 1. Binding of Vancomycin to mucopeptide receptors, showing the single atom change that causes Van resistance.

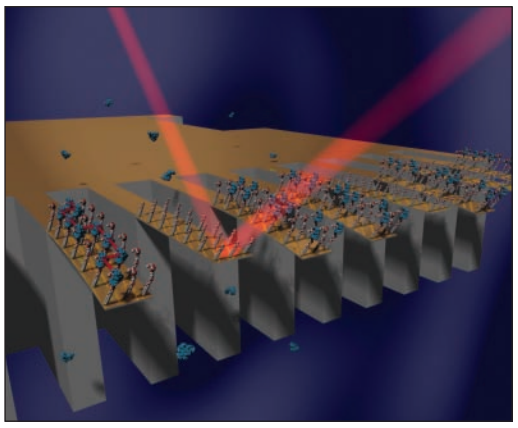

Figure 2. Schematic of microcantilever detection of molecular binding causing surface stresses, which are measured by the angle of laser reflection.

was measured using a time-multiplexed optical detection system in different buffer and blood serum environments under constant flow. From the bending signal the differential surface stress between the upper and lower sides of the cantilever was calculated.

The investigation was designed to provide clear evidence that cantilever arrays have the sensitivity to quantify Van–DAla binding interactions and detect the deletion of a single hydrogen bond associated with antibiotic resistance to the mutated peptide analogue, DLac. Moreover, this was tested in a clinically relevant situation; looking at antibiotic detection in blood serum at concentrations of 5–40 µg ml^{-1} (corresponds to 3–27 µM). In addition, the investigation also looked at the affect of altering the surface peptide density in order to optimise drug detection sensitivity and to examine the underlying mechano-transduction mechanism. In this way the investigation should also enable a better understanding of what causes the cantilevers to bend and therefore how nanomechanical biosensors can best be exploited for drug detection sensitivity.

RESULTS

The deflection of an array of cantilevers coated with DAla, DLac or PEG SAMs was monitored in parallel upon injection of different concentrations of Van in sodium phosphate buffer (pH 7.4, 0.1 M). In the buffer all cantilevers showed a stable baseline, but on injection of 250 µM Van, the DAla cantilevers rapidly bent downwards (illustrated in *Figure 1a*), reaching an average stable equilibrium absolute compressive bending signal of -176 nm, for the 30-minute injection under constant flow conditions. Conversely, the DLac cantilevers showed a much smaller average absolute downwards bending signal of -33 nm. The PEG cantilevers showed an even smaller average downward bending signal of -14 nm. When the buffer was flushed through, the signals were observed to converge back towards the stable 'zero stress' baseline. These absolute bending signals are generated within the experimental system by a combination of the biologically specific binding events and non-specific influences, such as reactions occurring on the underside of the cantilever, liquid injection spikes, changes in refractive index and temperature. Since these affect all the cantilever signals to the same extent, their effect can be removed by taking a differential measurement using a reference cantilever with an inert coating. The differential measurements revealed the surface forces induced by biochemically specific Van–peptide interactions. 250 µM Van induced differential surface stress signals for DAla and DLac of -35.3 and -5.1 mN m^{-1}, respectively.

Reproducibility

Reproducibility of the nanomechanical signals is very important to establish and therefore 100 measurements were made across four different cantilever arrays, each composed of eight cantilevers. Specialised software capable of handling the quantities of data concerned showed that both 'within' and 'between' array reproducibility was excellent, with slightly higher variance between arrays than within.

Dynamic range & Sensitivity

The system showed a very broad dynamic range, with 10 nM being the lower detection limit, giving rise to a differential bending signal of -9 ±2 nm. Furthermore, the capacity of cantilevers to detect antibiotics in serum was investigated in the clinically relevant concentration range of 3–27 µM. Injection of 7 µM Van in serum produced a differential signal for DAla in serum of 105 ±4 nm, with no significant bending detected for DLac.

Cantilever loading

In a further system quantification study, the amount of ligand loaded on to the cantilever arms (p, where p = fraction of the surface covered with ligand) was varied to assess the effect of peptide densities on nanomechanical response. The nanomechanical signal was much larger for DAla than DLac, steeply increasing as Van concentration increased followed by saturation, but increased more gradually as a function of p. With the cantilever surface completed coated with DAla (p = 1), the nanomechanical response saturates with Van concentrations greater than 50 µM. At this concentration most of the accessible Van binding sites are occupied. This effect is a measure of the specific chemical interactions between Van and the peptide.

With the concentration of Van fixed, no nanomechanical signal was detected from p = 0 to p = 0.1, whereas from p = 0.1 to p = 1.0 there was an approximately linear increase. These tests showed that stress transduction is a collective phenomenon, requiring a relatively large fraction of the surface to be covered to establish connectivity between chemically transformed regions of the surface. From this, it was possible to determine the critical percolation threshold (p_c = 0.075) for the system and then resolve the dissociation constant (K_d) for the interactions.

CONCLUSIONS

This series of investigations showed that cantilever arrays have the sensitivity to detect and quantify the binding affinity of the antibiotic Van to the drug–target mucopeptide analogues: Lysine-D-Alanine-D-Alanine – found in Van-sensitive bacteria, and Lysine-D-Alanine-D-Lac - found in Van-resistant bacteria.

The differential measurements generated enabled clear discrimination between the two peptide sequences. This showed that the system can detect the subtle binding changes induced by the deletion of a single hydrogen bond from the drug binding pocket, which is associated with drug resistance. The effect of this deletion was clearly indicated by the technology, since it gave rise to an 800-fold increase in K_d for DLac compared to DAla, which is in agreement with measurements made using other technologies.

The minimum detectable Van concentration was 10 nM and the technology can detect and quantify Van in blood serum at clinically relevant concentrations. This is important for pharmacokinetic/dynamic drug profiling, personalised medicine and forensic applications.

Cantilever bending

The molecular binding events were found to generate a repulsive-compressive surface stress, the origin of which is the subject of much scientific debate and interest[2–10]. These experiments showed a finite percolation threshold p_c = 0.075, below which the macroscopic bending is effectively zero. This means that a critical number of DAla and Van binding events are required to yield observable stress and demonstrates a local short range transduction mechanism. Above this threshold the nanomechanical effect increases almost linearly in proportion to the number of DAla molecules on the cantilever. The resulting complexes will induce a local strain in the silicon as well as carry an electrostatic charge, which in the neutral pH conditions of this study is +1 for Van. As the number of such regions grows they will interact to produce bending of the entire cantilever.

A NEW MEASUREMENT TECHNOLOGY

This research has established that nanomechanical cantilever biosensors can be the basis for new classes of percolating systems. The results of these investigations will also aid in the rational design of novel devices and surface chemistries for improving the sensitivity of cantilevers to chemical binding events. The system also showed excellent dynamic range, with the maximum stress signal obtained at high DAla packing densities (for p = 1.0 it is estimated that that there are 1 x 10^{11} DAla per cantilever), conditions that are traditionally considered to be unfavourable for other surface sensing techniques, such as surface plasmon resonance. Therefore, as well as showing that cantilevers are a very useful tool in antibiotic research, these investigations have also provided a new framework for understanding and eventually engineering the response of cantilevers to biochemical signals.

COMMERCIAL PROMISE

This cantilever technology has been used very successfully by Bio Nano Consulting during investigations into the mechanism of action of a novel antibiotic on behalf of Targanta Therapeutics of Cambridge MA (now part of The Medicines Company), showing that this nanoscale technology provides a new tool in the fight against 'superbugs'. Bio Nano Consulting (BNC) is a specialist research and development consultancy operating in the convergent field of bionanotechnology. A joint venture of Imperial College London and University College London, BNC is funded through the Technology Strategy Board (TSB) with additional support from the London Development Agency (LDA). Along with its partner organisation, the National Physical Laboratory, BNC offers a service to the biomedical and healthcare industries in microsystems and nanotechnology. This encompasses design, 3-D modelling and visualisation, rapid prototyping, and characterisation.

REFERENCES

[1] Ndieyira, J. W. et al. Nanomechanical detection of antibiotic–mucopeptide binding in a model for superbug drug resistance. Nature Nanotech. 3, 691-696 (2008).
[2] Fritz, J. et al. Translating biomolecular recognition into nanomechanics. Science 288, 316–318 (2000).
[3] McKendry, R. A. et al. Multiple label-free biodetection and quantitative DNA-binding assays on a nanomechanical cantilever array. Proc. Natl Acad. Sci. USA 99, 9783–9788 (2002).
[4] Savran, C. A., Knudsen, S. M., Ellington, A. D. & Manalis, S. R. Micromechanical detection of proteins using aptamer-based receptor molecules. Anal. Chem. 76, 3194–3198 (2004).
[5] Calleja, M. et al. Highly sensitive polymer-based cantilever-sensors for DNA detection. Ultramicrosc. 105, 215–222 (2005).
[6] Shu, W. et al. DNA molecular motor driven micromechanical cantilever arrays. J. Am. Chem. Soc. 127, 17054–17060 (2005).
[7] Watari, M. et al. Investigating the molecular mechanisms of in-plane mechanochemistry on cantilever arrays. J. Am. Chem. Soc. 129, 601–609 (2007).
[8] Backmann, N. et al. A label-free immunosensor array using single-chain antibody fragments. Proc. Natl Acad. Sci. USA 102, 14587–14592 (2005).
[9] Zhang, J. et al. Rapid and label-free nanomechanical detection of biomarker transcripts in human RNA. Nature Nanotech. 1, 214–220 (2006).
[10] Wu, G. et al. Bioassay of prostate-specific antigen (PSA) using microcantilevers. Nature Biotechnol. 19, 856–860 (2001).