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Nanomechanical Detection of Antibiotic-Mucopeptide Binding in a Model for Superbug Drug Resistance

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ARTICLE

The alarming growth of the antibiotic-resistant superbugs methicillin-resistant Staphylococcus aureus (MRSA) and vancomycin-resistant Enterococcus (VRE) is driving the development of new technologies to investigate antibiotics and their modes of action. We report the label-free detection of vancomycin-mucopeptide interactions on cantilever arrays, with 10 nM sensitivity and at clinically relevant concentrations in blood serum. Differential measurements quantified binding constants for vancomycin-sensitive and vancomycin-resistant mucopeptide analogues. Moreover, by systematically modifying the mucopeptide density we gain new insights into the origin of surface stress. We propose that stress is a product of a local chemical binding factor and a geometrical factor describing the mechanical connectivity –percolation- of regions affected by local binding. Our findings place BioMEMS devices in a new class of percolative systems. The percolation concept will underpin the design of devices and coatings to significantly lower the drug detection limit and may also impact on our understanding of antibiotic drug action in bacteria.

KEY WORDS: **surface stress, vancomycin, mucopeptides, serum, percolation, BioMEMS**

When biochemically specific interactions occur between a ligand immobilized on one side of a cantilever and a receptor in solution, the cantilever bends due to a change in surface stress[1-9]. The general applicability of this novel nanomechanical biosensing transduction mechanism has been shown for sequence-specific DNA hybridization[1-5,8], single base mismatches[1], DNA quadruplex[5], protein recognition[1,3,7,9] and recently the detection of interferon alpha induced I-8U gene expression in total human RNA, a potential marker for melanoma progression and viral infections[8]. However, to date, multiple cantilever arrays have not been applied to quantify drug-target binding interactions, despite offering considerable advantages. First, cantilevers require no reporter 'tags' or external probes and so biomolecules can be detected rapidly in a single step reaction. Second,

cantilever arrays can screen multiple drug-target interactions and reference coatings in parallel and under identical experimental conditions. Third, we have previously shown that quantitative ligand-receptor binding constants can be measured on cantilever arrays[2]. Moreover, the nanomechanical signal is not limited by mass, in contrast to evanescent techniques such as surface plasmon resonance (SPR) that detects mass-related changes in the dielectric constant[10-13]. Cantilevers are therefore unique as probes of small molecule drug binding interactions and, by virtue of their miniaturized dimensions they are amenable for parallelization[14,15] for high-throughput screening of thousands of drugs per hour.

Here we report the first quantitative differential nanomechanical investigation of drug-target binding interactions on multiple cantilever arrays focusing on the antibiotic vancomycin (Figure 1). Today vancomycin is one of the last powerful antibiotics in the battle against resistant bacteria and the 'hospital superbug' MRSA[16-27]. It 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. Vancomycin binds to the C-terminus of peptidoglycan mucopeptide precursors terminating in the sequence Lysine-D-Alanine-D-Alanine[16-18,20,21] as shown in Figure 1. This interaction blocks the action of bacterial transpeptidases and transglycosylases, which catalyse the cross-linking of the growing bacterial cell wall, resulting in cell lysis[16-27]. Unfortunately, due to the over-use of antibiotics, resistance to vancomycin is rapidly increasing and now poses a major international public health problem[22, 24,26]. Bacterial resistance in Enterococci can arise due to the subtle change of an amide linkage to an ester linkage in the growing bacterial cell wall, resulting in the deletion of a single hydrogen bond from the binding pocket, rendering the antibiotic therapeutically ineffective[16-27] (Figure 1). The development of novel methods to detect and quantify the binding of antibiotic–mucopeptide interactions is thus of significant clinical importance. In addition the structure and binding properties of vancomycin-mucopeptide complexes

have been extensively studied both at surfaces and in free solution[16-27] and thus serve as a model system to evaluate the capabilities of cantilevers in small molecule drug-target detection.

To probe the in-plane nanomechanics of antibiotic drug-target interactions, multiple arrays of eight rectangular silicon cantilevers were coated on one side with a thin film of gold and functionalized with alkanethiol self-assembled monolayers (SAMs) of (i) the drug-sensitive mucopeptide analogue $HS(CH_2)_{11}(OCH_2CH_2)_3O(CH_2)(CO)NH(CH_2)_5(CO)$-L-Lys-(ε-Ac)-D-Ala-D-Ala, herein termed ***D*Ala**; (ii) the mutated sequence which confers vancomycin resistance in VanA and VanB resistant Enterococcal phenotypes, $HS(CH_2)_{11}(OCH_2CH_2)_3O(CH_2)(CO)NH(CH_2)_5(CO)$-L-Lys-(ε-Ac)-D-Ala-D-Lac, termed ***D*Lac**. Our previous studies[2,5,6,8] have emphasized the importance of acquiring differential measurements using a reference cantilever and here we use a cantilever coated with an 'inert' SAM terminating in triethylene glycol $HS(CH_2)_{11}(OCH_2CH_2)_3OH$ termed ***PEG,*** which is known to resist biomolecule adsorption on surfaces[28-30]. The Supplementary Material describes the synthesis of ***D*Ala, *D*Lac** and ***PEG***. The absolute deflection at the free-end of each cantilever Δz_{abs} was measured using a time-multiplexed optical detection system in different buffer and blood serum environments under constant flow. The bending signal was subsequently converted into a differential surface stress between the upper and lower sides of the cantilever $\Delta\sigma_{abs}$, using Stoney's equation[31]

$$\Delta\sigma_{abs} = \frac{1}{3}\left(\frac{t}{L}\right)^2 \frac{E}{1-\nu}\Delta z_{abs} \qquad \textbf{Equation 1}$$

where L is the effective length of the cantilever up to 500 μm, t is the thickness ~ 0.9 μm, and $E/(1-\nu) = 180$ GPa is the ratio between the Young's modulus E and Poisson ratio ν of Si (100)[32]. We used a home-built gravity flow system to control the exchange of up to six different vancomycin solutions (10 nM – 1 mM), 100 mM sodium phosphate buffer and 10 mM HCl regeneration solutions, via an automated valve (Supplementary Material).

The aim of our investigations was to ascertain whether cantilever arrays have the sensitivity to quantify vancomycin–***D-Ala*** binding interactions and detect the deletion of a single hydrogen bond associated with antibiotic resistance to the mutated peptide analogue, ***D-Lac***. Moreover, we examined the sensitivity of antibiotic detection in blood serum at clinically relevant concentrations[33] of 5-40 μg/ml, which corresponds to 3-27 μM. In addition, we sought to alter the surface peptide density in order to optimize drug detection sensitivity and to investigate the underlying mechanotransduction mechanism. Nanomechanical biosensors can best be exploited only if we develop a fundamental understanding of what causes the cantilever to bend and this knowledge will aid the development of new devices with significantly improved drug detection sensitivity.

Nanomechanical Detection of Vancomycin-Mucopeptide Interactions

The nanomechanical force exerted by vancomycin-peptide interactions was investigated on microfabricated cantilevers. The deflection of an array of cantilevers coated with ***D-Ala, D-Lac*** or in-situ reference ***PEG*** SAMs, was monitored in parallel upon injection of different concentrations of vancomycin in sodium phosphate buffer (pH 7.4, 0.1 M). Typical absolute bending signals for one array comprising two ***D-Ala***, three ***D-Lac*** and two ***PEG*** coated cantilevers are shown in Figure 2a. In buffer, we observed that all cantilevers showed a stable baseline. Upon injection of 250 μM vancomycin, both ***D-Ala$_1$*** and ***D-Ala$_2$*** coated cantilever rapidly bent downwards (illustrated in Figure 1a), reaching a stable equilibrium absolute compressive bending signal of -180 nm and -172 nm respectively after 30 mins under constant flow conditions. Conversely the ***D-Lac*** cantilevers showed a small absolute downwards bending signal of -38, -31 and -31 nm for ***D-Lac$_1$***, ***D-Lac$_2$*** and ***D-Lac$_3$***, respectively. The two reference ***PEG*** coated cantilevers, ***PEG$_1$*** and ***PEG$_2$*** showed a small downwards bending signal of -14 nm and -13 nm. Upon injection of the buffer, the signals were observed to converge back towards the stable 'zero stress' baseline. This step was then followed by

10 mM HCl, which is known to dissociate vancomycin-peptide complexes and regenerate the free peptides for further antibiotic studies.[13]

It is known that the absolute bending signals are a convolution of biologically *specific* binding events and *non-specific* influences, including reactions occurring on the underside of the cantilever, liquid injection spikes, changes in refractive index and temperature[1,2,5-8]. These non-specific effects will affect ***DAla, DLac*** and ***PEG*** signals to the same extent, and are therefore removed by taking a differential measurement using a reference cantilever with an inert coating.[2,5-8] The differential measurements, shown in Figure 2b revealed the surface forces induced by biochemically *specific* vancomycin-peptide interactions. Upon injection of 250 μM vancomycin, the differential surface stress signal for $\textbf{\textit{DAla}}_1$ ($\textbf{\textit{DAla}}_1$ - $\textbf{\textit{PEG}}_1$) and $\textbf{\textit{DLac}}_1$ ($\textbf{\textit{DLac}}_1$ - $\textbf{\textit{PEG}}_1$) were found to be -35.3 mN/m and -5.1 mN/m respectively.

To examine the reproducibility of the nanomechanical signals, we performed more than 100 measurements on four different cantilever arrays, each composed of eight cantilevers. The raw bending signals were analyzed using automated data software developed in our group to rapidly examine large data sets and remove bias[6]. The average nanomechanical surface stress signal for 250 μM vancomycin on one array was -34.6 ± 0.9 mN/m for ***DAla*** and -4.2 ± 0.5 mN/m for ***DLac***. The mean signals across four arrays was -34.2 ± 5.9 mN/m for ***DAla*** and -3.8 ± 1.5 mN/m for ***DLac***. The high reproducibility of *within-array* measurements, and an increased variance associated with *between-array* measurements, agrees with our previous findings[6].

The dynamic range and sensitivity was investigated by varying the vancomycin concentration in solution [Van]. The differential ***DAla*** bending signal scaled with increasing [Van] ~ 10, 100 and 1000 nM, giving equilibrium differential signals of -8, -29 and -114 nm respectively (Figure 2c).

The lowest [Van] to be detected was 10 nM giving rise to a ***D-Ala*** differential bending signal of -9 ± 2 nm on three cantilevers (Figure 2d).

The capacity of cantilever to detect antibiotics in serum was investigated in the clinically relevant concentration range[33] of 3-27 μM. Figure 3 shows the differential signal for ***D-Ala*** and ***D-Lac*** coated cantilevers upon injection of 7 μM vancomycin in serum (90 % fetal calf serum plus 10 % sodium phosphate buffer pH 7.4). The differential signal for ***D-Ala*** in serum was 105 ± 4 nm and no significant bending was detected for ***D-Lac***.

Drug-Target Nanomechanical Percolation

We monitored the nanomechanical response of cantilevers with systematically varied peptide densities p (where p is defined as the ***D-Ala*** surface coverage fraction on the cantilever determined by X-ray photoelectron spectroscopy (XPS) as described in the Supplemental Materials) to a series of [Van]. Figure 4 shows all of the stress data as a function of p and [Van] for both ***D-Ala*** and ***D-Lac***. The nanomechanical signal was much larger for ***D-Ala*** than ***D-Lac***, and steeply increased as a function of [Van] followed by saturation, whereas it increased more gradually as a function of p. For fixed $p = 1$, where the cantilever is coated with only ***D-Ala,*** the nanomechanical response saturates for [Van] > 50 μM when most accessible vancomycin binding sites are occupied, consistent with binding equilibrium. This effect is a measure of the specific chemical interactions between the vancomycin and the peptide. On the other hand, for fixed [Van], upon increasing ***D-Ala*** from $p = 0$ to $p = 0.1$ no detectable nanomechanical signal was measured, whereas from $p = 0.1$ to $p = 1.0$ there is an approximately linear increase. What this means is that the stress transduction is actually a *collective phenomenon*, requiring a relatively large fraction of the surface to be covered so as to establish connectivity between chemically transformed regions of the surface. Assuming that the local

chemistry and geometric effects responsible for the collective build-up of strain are separable, we can write a general product form for the cantilever response,

$$\Delta\sigma_{\mathrm{eq}} = \frac{a \cdot [\mathrm{Van}]}{K_{\mathrm{d}} + [\mathrm{Van}]} \left(\frac{p\text{-}p_{\mathrm{c}}}{1\text{-}p_{\mathrm{c}}} \right)^{\alpha} \quad , \text{for } p > p_c. \qquad \textbf{Equation 2}$$

and zero otherwise. The first term is the Langmuir Adsorption Isotherm, accounting for drug-target binding events and the second term is the power law form describing the large-scale mechanical consequences of stressed network formation. The constant a corresponds to maximum surface stress when all the accessible binding sites are occupied and K_d is the surface equilibrium dissociation constant on the cantilever. The build-up of surface stress follows from the connectivity of the chemically transformed network as well as the interactions between nodes of the network[34]. The power law α is associated with the elastic interactions between chemically reacted regions on the cantilever. For short range interactions, such as steric neighbour-neighbour repulsive interactions there will be a finite percolation threshold p_c beyond which there will be a connected network which can produce an apparent bending of the cantilever. On the other hand, for long range interactions such as ideally elastic interactions, p_c=0.

We have carried out a series of least-squares fits of Equation 2 to our data to find the key parameters as well as to ascertain the validity of the product form. The parameters p_c and α, which characterize the collective behavior are coupled in a statistical sense. Therefore, to determine what their best values might be, we have chosen not to rely on the multi-parameter fitting routine but instead have examined directly how the fit changes as a function of p_c, looking at both the resulting values of α and squared deviation between the data and the fit. Figure 4b shows the outcome, which reveals a percolation threshold p_c = 0.075, and a concomitant preference for a power α close to 1.3. We were able to ascertain the validity of the product form, Equation 2, by comparing the global fit

values for K_d and a, or α and p_c, with the values obtained from subsets of the data at constant p or constant [Van]. The analysis shown in the Supplementary Materials shows that the values do not vary outside the experimental error limits, and that therefore, within the errors of our present experiments, local drug-target chemical interaction effects decouple from the collective elastic phenomenon ultimately responsible for the bending of the entire cantilever. Having established this, we can find K_d, a, α, and p_c using all available data. The outcome of the global fit is superimposed onto the measured differential stress signals in Figure 4a. Table 1 shows a summary of a and K_d for both ***D-Ala*** and ***D-Lac*** peptides and makes the comparison with reported values from solution phase UV spectroscopy[20,21] and SPR measurements[11-13].

DISCUSSION

Our experiments show that cantilever arrays have the sensitivity to detect and quantify the binding affinity of the antibiotic vancomycin to drug-target mucopeptide analogues: Lysine-D-Alanine-D-Alanine and Lysine-D-Alanine-D-Lac. The former occurs in the peptidoglycan precursors found in vancomycin sensitive bacteria and the latter in those precursors in VanA and VanB vancomycin resistant bacteria[16-27]. Differential measurements could successfully discriminate between the two peptide sequences, detecting the deletion of a single hydrogen bond from the drug binding pocket, which is associated with drug resistance. This gave rise to an 800-fold increase in K_d of ***D-Lac*** compared to ***D-Ala***, in agreement with measurements made by SPR[11-13]. We find that the minimum detectable vancomycin concentration was 10 nM and comparable to SPR studies, which have reported the detection of 30 nM vancomycin[11-13]. Furthermore, we show that cantilevers can detect and quantify vancomycin in blood serum at clinically relevant concentrations, which is important for pharmacokinetic/dynamic drug profiling, personalized medicine and forensic applications.

The molecular binding events occurring between vancomycin in solution and ***D-Ala*** were found to generate a repulsive compressive surface stress. The origin of the biochemically induced surface stress is the subject of much scientific debate and interest[1-9]. Our experiments reveal a finite percolation threshold p_c = 0.075 below which the macroscopic bending is effectively zero. This means that a critical number of ***D-Ala*** and vancomycin binding events are required to yield observable stress and demonstrates a local short range transduction mechanism. For $p > p_c$, the nanomechanical signal increases with a power law α of 1.3 and so is approximately linearly proportional to the number of ***D-Ala*** molecules on the cantilever. Figure 5 shows the operation of this mechanism, which begins with the steric forces generated by insertion of the vancomycin into the ***D-Ala*** SAM. 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 vancomycin. As the number of such regions grows they will interact to produce bending of the entire cantilever. The antibiotic-mucopeptide triggered changes in surface stress differ significantly from previous studies of the Young's modulus of macroscopic two-dimensional model random elastic media[35,36]. Our findings establish nanomechanical cantilever biosensors as the hosts for new universality classes of percolating systems, where the elasticity resides in the coupled multilayered, multiscale system[37] - buffer/Van/***D-Ala/PEG***/gold/chromium/silicon. Our findings suggest that the structure and mechanics of the underlying ***D-Ala/PEG*** SAMs play a major role in mechanotransduction. Future work[38] will investigate the extent of mixed monolayer phase separation[39,40] on cantilevers and the decoupling of chemical and geometric factors. These findings will aid rational design of novel devices and surface chemistries for improving the sensitivity of cantilevers to chemical binding events such as those in our current drug testing application. Interestingly we show that the maximum stress signal is obtained at high ***D-Ala*** packing densities, conditions that are traditionally considered to be unfavourable for other surface sensing techniques such as SPR[12, 13] (For p=1.0 we estimate 10^{11} ***D-Ala*** per cantilever, with a single footprint of 44 $Å^2$, as described in the XPS

measurements detailed in the Supplementary Material). Thus, beyond our key result that cantilevers are a very useful tool in antibiotic research, our systematic experiments on model SAMs provide a new framework for understanding and eventually engineering the response of cantilevers to biochemical signals.

We close by speculating that nanomechanical percolation may play an important role not only in sensor response but also in the glycopeptide antibiotic mode of action in real bacteria. Williams and co-workers proposed that glycopeptide antibiotic dimerisation impacts on antimicrobial efficacy and that antibiotic dimers show unusually strong binding constants[16-18]. We propose further that drug-target binding events act *collectively* to disrupt the mechanical properties of the bacterial peptidoglycan cell wall and plasma membrane (Figure 5b). Dimeric glycopeptides with membrane-insertive hydrophobic tails such as biphenylchloroeremomycin and oritavancin[18], may possess greater potency than vancomycin because of their propensity to self-associate and diffuse in the membrane to create extended networks. Our results should therefore clearly motivate future work to investigate the percolation of drug action in lipid membranes, including eventually those of living bacteria, and test the more general validity of the hypothesis (underlying Equation 2) of decoupling between chemical binding and geometric factors.

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TABLE

Mucopeptide	***a*** (mN/m)	K_d **Cantilever** (μM)	K_d **SPR** (μM)		K_d **solution** (μM)	
D-Ala	29.7 ± 1.0	1.0 ± 0.3	1.1 ± 0.1	Ref. 13	0.7 ± 0.1	Ref. 21
D-Lac	14.1 ± 3.0	800 ± 310	526 ± 139	Ref.13	94. 2	Ref.25

Table 1: Equilibrium dissociation constant K_d and saturation stress signals, *a*, of vancomycin-mucopeptide interactions on cantilever arrays in comparison to literature solution phase and SPR measurements.

Figure 1

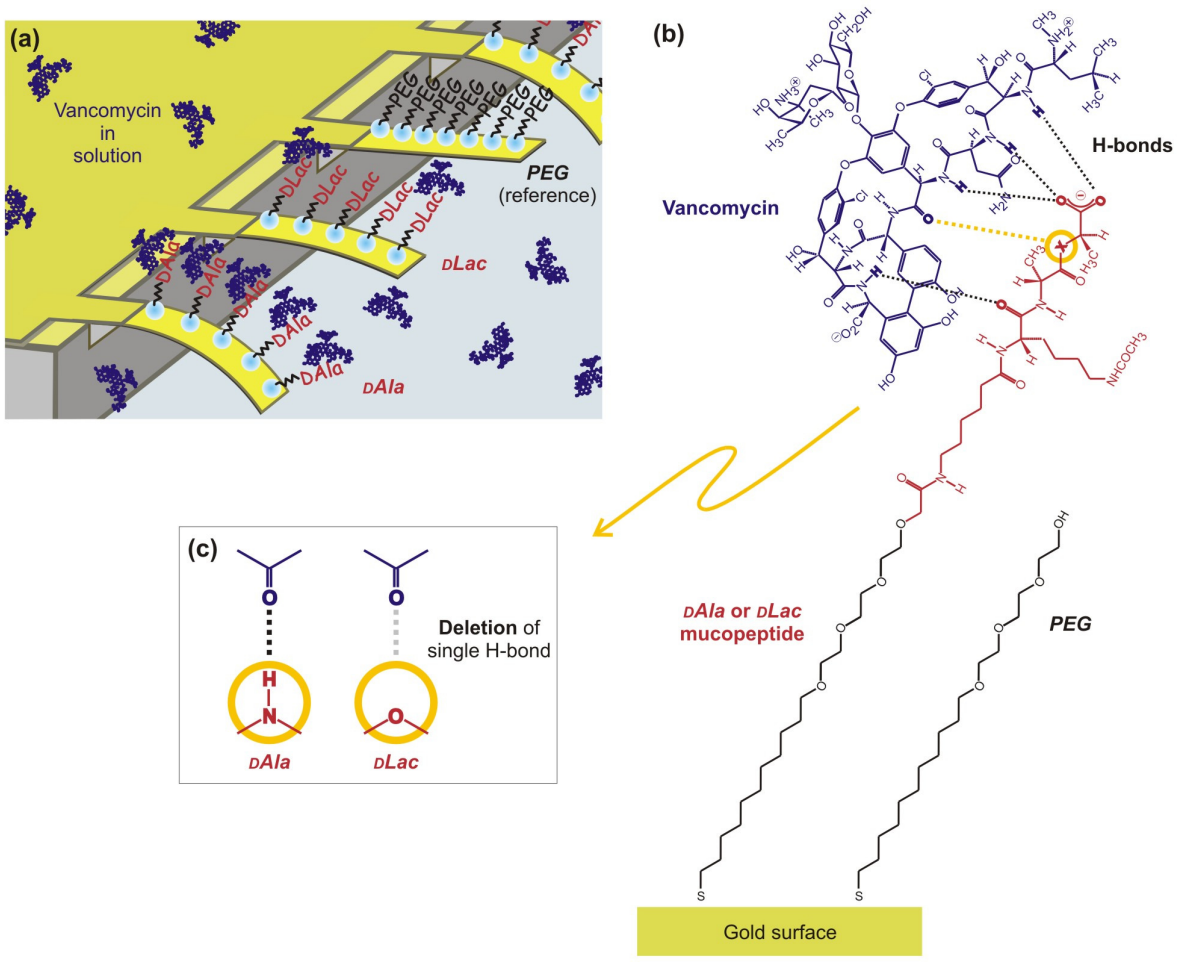

Figure 1*: The nanomechanical detection of vancomycin-mucopeptide analogue interactions on multiple cantilever arrays.* ***(a)*** *Schematic diagram to show cantilevers coated with* ***DAla*** *(vancomycin sensitive),* ***DLac*** *(vancomycin resistant) or* ***PEG*** *(reference) alkanethiol SAMs. Vancomycin is injected in solution and binds specifically to the mucopeptide analogues causing the cantilever to bend downwards due to a compressive surface stress.* ***(b)*** *The chemical binding interaction between vancomycin and the bacterial mucopeptide analogue,* ***DAla.*** *It is known from solution phase studies that the specificity of this complex arises due to (i) the interaction of the C-terminal free carboxylate of the peptide with three amide bonds in the vancomycin backbone (ii) the formation of two C=O----H-N hydrogen bonds and (iii) hydrophobic interactions of the alanine methyl groups with aromatic residues of vancomycin. The dashed lines represent the 5 intermolecular hydrogen bonds. The yellow dashed line represents the hydrogen bond associated with bacterial resistance;* ***(c)*** *The deletion of a single H bond in mutated* ***DLac*** *mucopeptides gives rise to drug resistance. The binding pocket of vancomycin is represented schematically and the grey dotted line represents the deleted hydrogen bond and electrostatic repulsion between the oxygen lone pairs of electrons*

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Figure 2

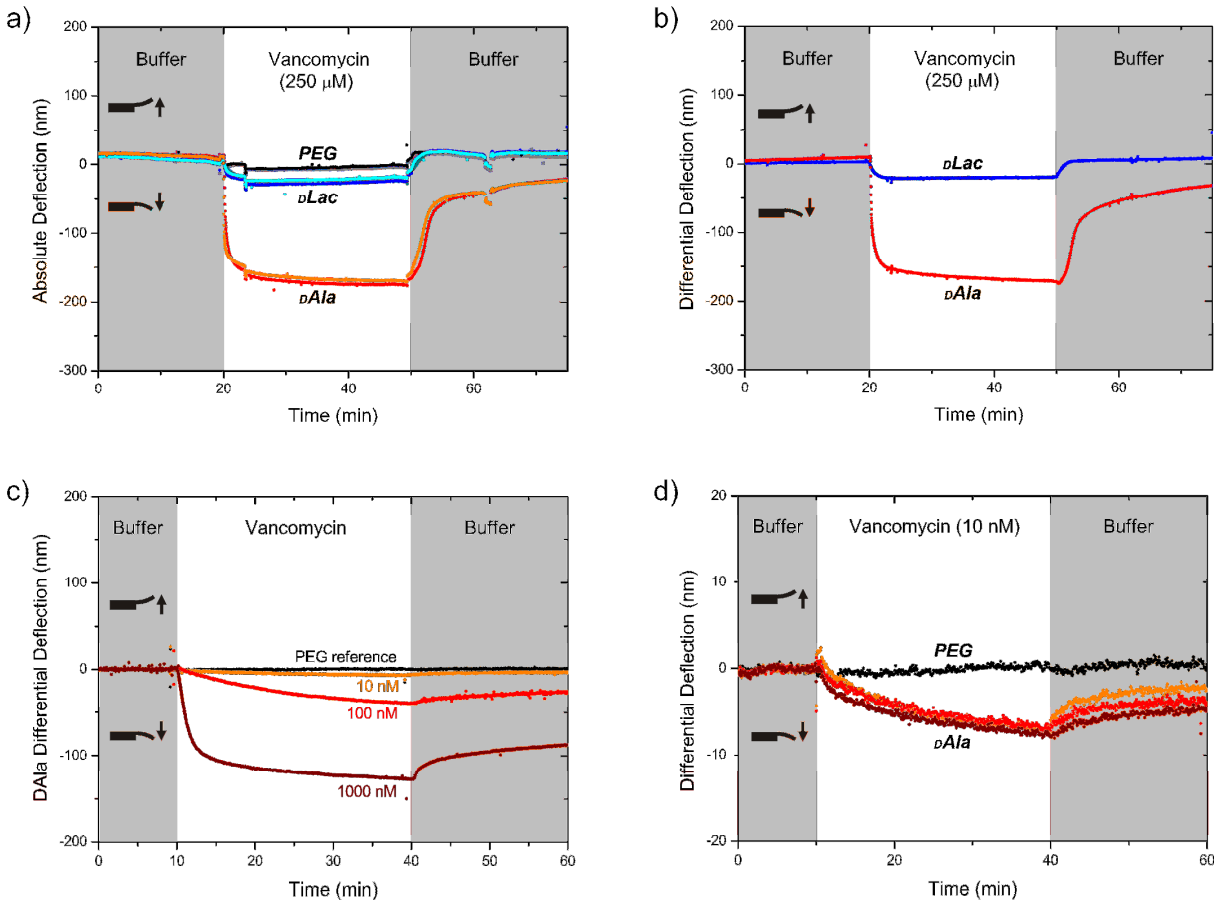

***Figure 2**: **(a)** Absolute bending signal of **DAla$_1$** (red), **DAla$_2$** (orange), **DLac$_1$** (light blue), **DLac$_2$** (dark blue), **DLac$_3$** (dark green) and in-situ reference cantilevers **PEG$_1$** (black) and **PEG$_2$** (grey) coated cantilevers to phosphate buffer, 250 μM vancomycin, and return to phosphate buffer. A negative signal corresponds to a compressive surface stress and the downwards bending of the cantilever, as illustrated in Figure 1a; **(b)** The corresponding differential bending signals of **DAla$_1$** (**DAla$_1$** minus **PEG$_1$**, shown in red) and **DLac$_1$** (**DLac$_1$** minus **PEG$_1$**, shown in blue); **(c)** Differential **DAla** signals for 10, 100, 1000 nM vancomycin. The differential **PEG** reference signal is shown (**PEG$_2$** – **PEG$_1$** black); **(d)** Differential signals of three **DAla** cantilevers for 10 nM vancomycin. The differential **PEG** reference signal is shown (black).*

Figure 3

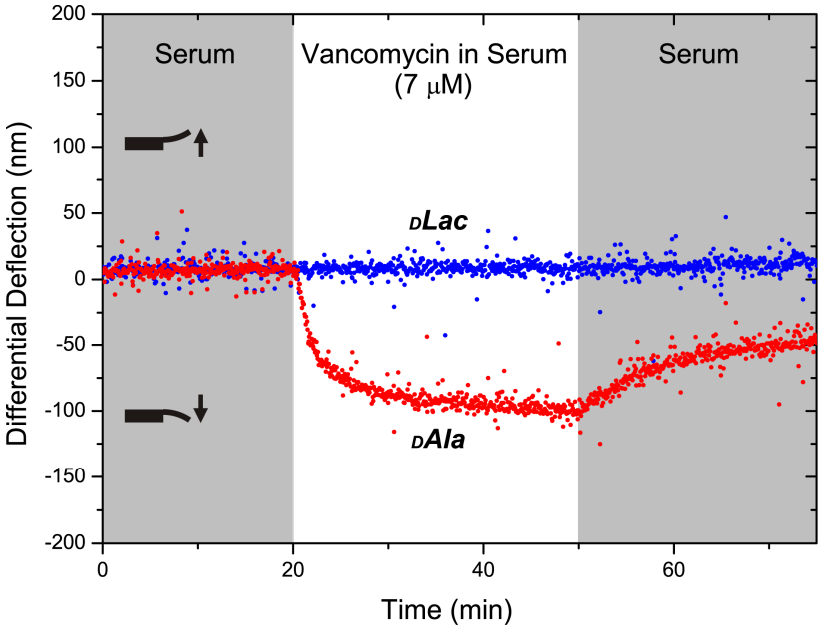

***Figure 3**: Nanomechanical detection of antibiotics in blood serum at clinically relevant concentrations. Differential bending signal of* $_{D}Ala_{1}$ *(red) and* $_{D}Lac_{1}$ *(blue) upon injection of 7 μM vancomycin in 90 % fetal calf serum.*

Figure 4

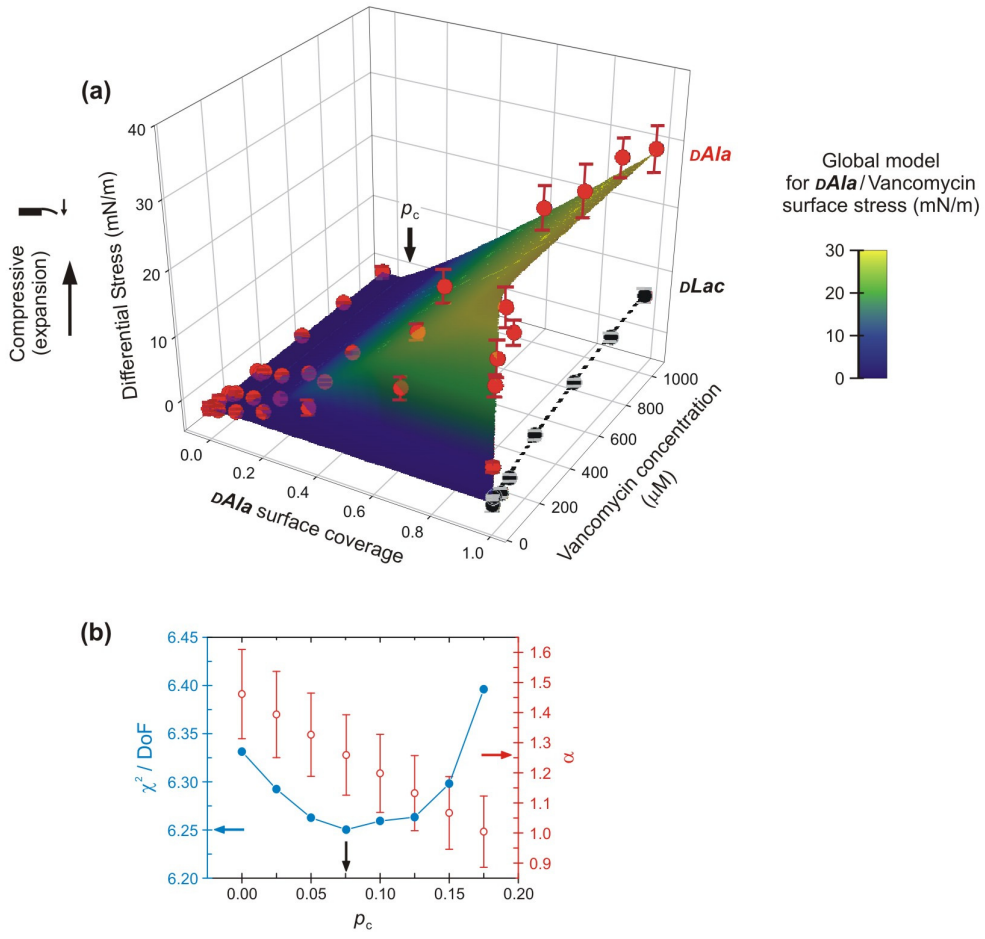

***Figure 4**: **(a)** A three dimensional graph showing the measured differential surface stress response for **DAla** and **DLac** coated cantilevers as a function of vancomycin concentration in solution [Van] and **DAla** surface coverage p, superimposed with the results of the global fit according to Equation 2; (b) Least-squares analysis to determine the best values for p_c and α;*

Figure 5

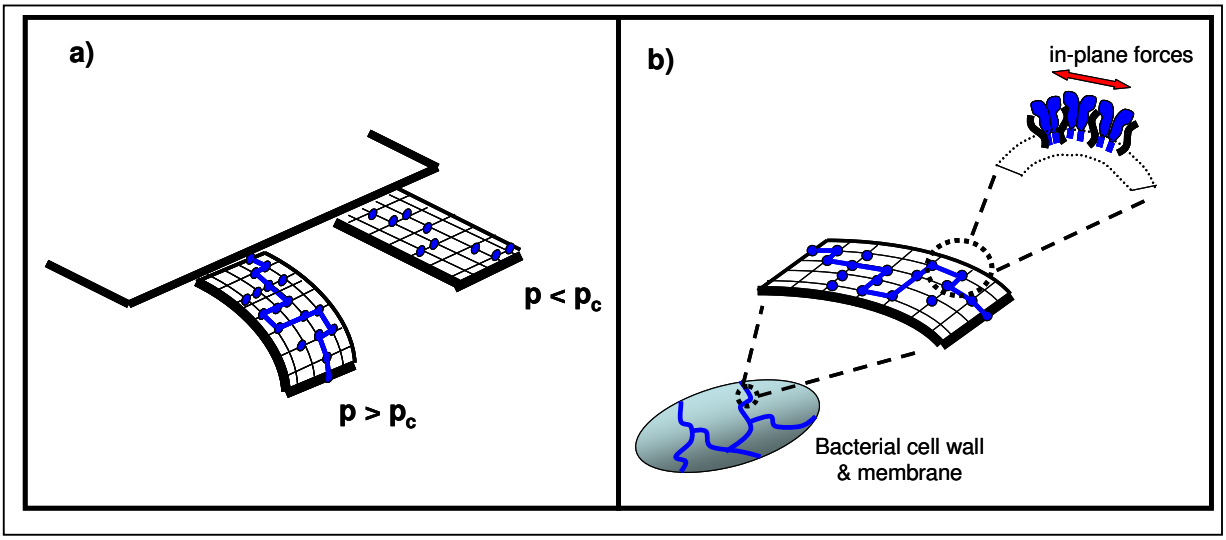

Figure 5 *Concepts underpinning nanomechanical antibiotic transduction.* ***(a)*** *A schematic to show the concept of percolation on a cantilever array;* ***(b)*** *A schematic to illustrate nanomechanical drug-target percolation on a bacterial membrane and cell wall.*