

## Technical Report

# Rapid method for measurement of surface tension in multiwell plates

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**We describe an optical method for quantifying surface tension in 96-well microtitre plates. Absorbance and fluorescence measurements in vertical beam systems as in 96-well plate photometers are complicated by the interaction of the light with the curved surface of the liquid. If the samples do not all have the same meniscus, errors in the data proportional to the degree of curvature are produced, which may confound interpretation of the data. We have harnessed this effect by modifying a 96-well plate spectrophotometer, and show that the apparent optical density measurements correlate very closely with surface tension. The method is much quicker than conventional methods for measuring multiple samples and requires considerably less technical skill. We demonstrate the applicability of the method to the study of biomolecules by investigating the surfactant properties of two peptides (A $\beta$  and AChE<sub>586–599</sub>) that form amyloid fibrils *in vitro*, in one case replicating previously published results, and in the other case highlighting the similarity, and possibly generic nature, of the biophysical properties of these amyloidogenic peptides.**

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Disposable microplates with 96 or more sample wells are widely used in the modern laboratory for biochemical and molecular biological experiments. Fluorescent or coloured reaction products can be analysed in the plate wells with a dedicated multiwell plate spectrofluorimeter or spectrophotometer. The light-path of these devices runs through the surface of the sample liquid, rather than, as in the case of conventional instruments, through the parallel walls of an optical cuvette containing the sample. The optical characteristics of the system are therefore contingent upon the configuration of the sample surface, which may be flat or curved, depending on the size of the meniscus present at the air–liquid interface. In this paper, we report that differences in the menisci of samples in a 96-well plate introduce errors into fluorescence and absorbance measurements. While this effect can be a serious confounding factor in the interpretation of results, we demonstrate that it can also be harnessed for use as an exceptionally quick and straightforward way of measuring surface tension, by

simple modification of a 96-well plate spectrophotometer.

There is a wide variety of different ways of measuring surface tension of aqueous solutions,<sup>1,2</sup> but only a few of these are of sufficient generality and precision as to form the basis of commercial instrumentation. Traditional methods such as the du Noüy ring and Wilhelmy plate rely on the direct measurement of the force exerted by the surface on a solid object. Bubble pressure measurements have their basis in the Laplace equation, which relates the excess pressure inside the bubble to the surface tension and the radius of the bubble. Drop volume techniques balance the gravitational force on a drop and the force exerted by the surface tension of the drop on the needle. More recently, drop shape analysis techniques have been introduced, in which a digital image of the drop is fitted to the Laplace equation. All these methods are capable of a precision of 0.1 mN/m in expert hands. Some, such as drop shape analysis, yield surface tensions in a fraction of a second and require only microlitres of solution. All these methods are, however, essentially serial techniques, in which the needle, plate, ring, cuvette or other measuring device needs to be rigorously cleaned or replaced between measurements on different samples. They do not lend themselves readily to the automated measurement of a large number of biological samples. Furthermore,

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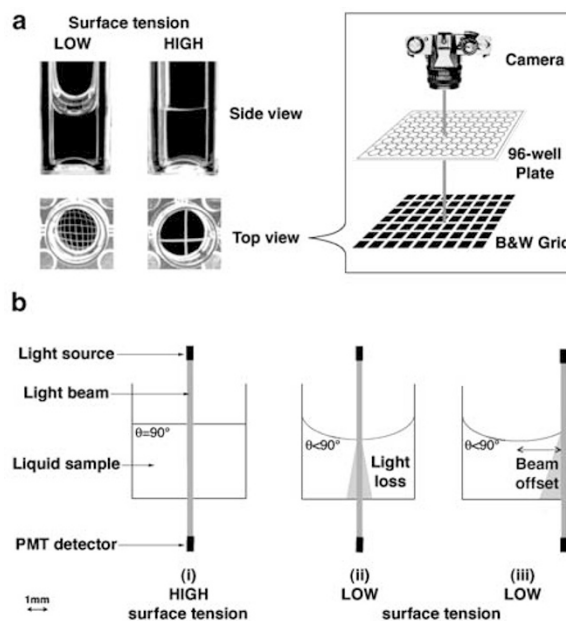
biological samples are frequently highly dilute, resulting in lengthy equilibration times (many minutes) before equilibrium properties are reached. In conventional techniques, this equilibration time is required before each measurement. In the novel approach we describe below, all the samples are formed at the beginning of the experiment and equilibrate simultaneously. Only one equilibration time is required, after which all the samples can be rapidly read out.

Our method is based on the Young equation (eq. (1)), which relates the contact angle,  $\theta$ , at the solid–liquid–vapour contact line to the surface tension of the liquid,  $\gamma_{lv}$ , and the solid–liquid and solid–vapour interfacial tensions,  $\gamma_{sl}$  and  $\gamma_{sv}$ , respectively.

$$\gamma_{lv} \cos \theta = \gamma_{sv} - \gamma_{sl} \quad (1)$$

The value of  $\theta$  determines the curvature of the meniscus. When  $\theta = 90^\circ$ , the meniscus is flat; when  $\theta < 90^\circ$ , the meniscus is curved upwards at the edges of the cuvette; when  $\theta > 90^\circ$ , the meniscus is curved downwards. Adsorption of a surfactant at the surface of the solution reduces  $\gamma_{lv}$  and, other things being equal, increases  $\cos \theta$  in order that the Young equation is still satisfied. Measurements of  $\theta$  therefore provide a means of measuring the surface tension. We note two comments in this regard. First, the minimum value of  $\theta$  is zero ( $\cos \theta = 1$ ). Once this value is reached, there is no sensitivity to further decreases in the surface tension. It is therefore not desirable to use microplates fabricated of a material that is too easily wetted by pure water. Second, adsorption of a surface-active molecule to the walls of the well reduces  $\gamma_{sl}$  and hence reduces  $\theta$  also. The relationship between surface tension and contact angle will therefore depend not only on the nature of the microplate (which affects both  $\gamma_{sv}$  and  $\gamma_{sl}$ , but which can be allowed for by calibration with a standard surfactant solution of known surface tension) but also the extent of adsorption to the surface of the plate-well, which will vary for different biological surfactants. The effect of variations in  $\gamma_{sl}$  on  $\theta$  can be minimised by choosing a plate material that is resistant to adsorption of the species of interest (eg by having the same charge).

Rather than measuring the contact angle directly, our method relies upon the ability of a meniscus of a translucent aqueous solution to act as a lens (see Figure 1a). When fluorescence intensity or optical density measurements are made in 96-well plates containing samples with different surface tensions, the lensing effect of the meniscus reduces the amount of light able to reach the detector: the lower the value of  $\theta$  ( $< 90^\circ$ ), the greater the curvature of the meniscus and the lower the intensity of the light detected. This effect is depicted in parts (i) and (ii) of Figure 1b. We initially observed the effect when attempting to measure in a 96-well plate the fluorescence intensity of differing concentrations of a fluorescein-labelled peptide that, as described later, happened to be highly surface active. In this



**Figure 1** Principle of method. Panel a: The ability of a meniscus to act as a lens. The surface of water is nearly flat in polystyrene vessels such as disposable cuvettes and microtitre plates, but a meniscus is produced when the surface tension is lowered (from 72 to  $\sim 35$  mN/m) by the addition of detergent. The resulting curved surface evident in the side view allows the liquid to act as a demagnifying lens. This phenomenon is demonstrated in the photograph of a grid of 20-mm squares taken through the samples in a 96-well plate positioned 100 mm above the grid. Panel b: The extent of the lens-like effect of a solution meniscus can be quantified by measuring the relative intensity of a light beam passed through the liquid. When the surface is flat (i), refractive and reflective light losses are minimal. When the surface is a curved meniscus (ii), some light is lost due to these processes and does not reach the detector. This effect can be enhanced by passing the light beam through the sample near the edge of the well (iii), where the surface curvature is greater. The light intensity varies as a function of the contact angle ( $\theta$ ) of the liquid and the side of the vessel, which is in turn related to surface tension.

case, the degree of fluorescence appeared lower when more peptide was present; only when an excess of detergent was added to all wells to produce equal menisci was the expected dependence of fluorescence intensity upon peptide concentration unmasked. In the absorbance mode, the meniscus-induced decrease in intensity is reflected in an increased value for the optical density. Absorbance measurements are usually made with the beam positioned at the centre of the well; in this configuration, the maximal effect of a meniscus upon the absorbance read-out is 0.1–0.2 absorbance units—certainly enough to affect interpretation of results. For measurement of surface tension, the light beam can be offset, as shown in part (iii) of Figure 1b, from the centre of the well to the edge, where the effect of the meniscus curvature is greater. This results in an increase in the effect of up to 0.8 absorbance units, and extends the dynamic range of the system.

In this paper, we investigate and assess the ability of our method to measure surface tension accurately under circumstances of varying charge both of the plate construction material and of the detergent used to control surface tension. While there is clearly a correlation of the apparent optical density with the surface tension, it is not the case that a given value for the optical density corresponds to a particular surface tension for any surfactant in any plate. We have not therefore attempted to derive an equation relating the apparent optical density to  $\theta$ , since the relationship between  $\theta$  and  $\gamma_{iv}$  is not unique. Nevertheless, our method, while limited for this reason to relative rather than absolute surface tension measurements, has a number of appealing features. First, as discussed above, the parallel nature of the sample preparation and equilibration leads to greatly reduced measurement times on multiple samples. Second, the volume of solution required for each measurement is small ( $\sim 0.1$  ml). Third, the instrumentation required is familiar to all biochemical researchers and no special training is required, whereas considerable technical skill is required for accurate measurements with most commercial surface tensiometers. Fourth, the standard of cleanliness and uniformity of disposable microwell-plates and micropipettes is very high, leading to excellent reproducibility. There is no risk of errors introduced by incomplete cleaning of specialised surface tensiometry equipment.

Even for researchers not interested in studying surface tension itself, an understanding of the influence of the meniscus upon the optical properties of a 96-well plate-based assay is crucial, if the samples in question are not identical with respect to surface tension. We therefore provide a simple means of establishing whether observed readings are an effect of analyte concentration or surface curvature.

## Materials and methods

### Detergents and Synthetic Peptides

AOT was purchased from BDH and  $C_{10}E_8$  and DTAB from Sigma. The synthetic peptides AChE<sub>586–599</sub> (AEFHRWSSYMVHWK),  $A\beta_{1–16}$  (DAEFRHDSGYEVHHQK) and  $A\beta_{1–40}$  (DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGV) were purchased from Anaspec. Peptides were dissolved in ultrapure water, and diluted into buffer (0.1 M sodium acetate pH 3.0 or 0.1 M sodium phosphate pH 7.0 for AChE<sub>586–599</sub> and 0.1 M Tris pH 7.0 for  $A\beta$  peptides). Detergents were diluted in ultrapure water.

### Conventional Surface Tension Measurements

Surface tension was measured either using an electronic du Noüy ring tensiometer (Krüss; AOT and  $C_{10}E_8$ ) or a digital drop shape analysis apparatus

(ITConcept; DTAB and AChE<sub>586–599</sub>). The average of three measurements was taken; error bars in graphs show the standard error of the mean.

### Optical Surface Tension Method

A BMG Polarstar 96-well plate combined spectrophotometer/fluorimeter was adapted to measure surface tension (see below). Samples of detergents or peptides of 200  $\mu$ l amount were pipetted into the wells of a microplate, which was briefly shaken in an orbital shaker prior to being placed in the machine. The mean of multiple samples was taken and error bars in graphs represent standard error of the mean.

### How to Modify a Plate Reader to Measure Surface Tension

The light path of the BMG Polarstar plate reader consists of a xenon flash bulb, a filter wheel, a 0.25-mm fibre optic terminating 10 mm above a robotic plate carrier, a multiple fibre optic bundle positioned 10 mm below the plate carrier, and a photomultiplier tube. We used a 450 nm filter, but any wavelength may be employed, provided the sample is at least partially transparent at that wavelength. The BMG Fluostar 64 software supplied with the machine was modified to position each well such that the beam passed 2.4 mm from the centre of the well rather than through the centre. The plate is robotically positioned according to a text file specifying the number of 0.1 mm motor steps required (in *x* and *y* directions) to move different types of plate from the standby position to (i) a position such that well A1 is centred in the beam and (ii) a position such that well H12 is centred in the beam. These positions were altered by defining new 'imaginary' microplates with increased or decreased values for the two parameters, so that they appear as options under the plate-type menu in the method window of the software. The optimum figure of a 2.4 mm offset in the *x* direction (corresponding to the longer, horizontal plate dimension) was obtained empirically.

We have not investigated how to modify other types of plate reader to perform similar measurements, although in theory this should be possible, if reproducible measurement at a fixed offset can be achieved. The optimum offset may differ depending upon the beam dimensions (0.25 mm for the BMG). Where there is a software look-up table of different plate dimensions, a procedure similar to that outlined above might be efficacious. Simpler machines (eg Labsystems Multiskan) utilise a slotted metal bar attached to the plate carrier to contact a switch that identifies the central measurement position of each column of wells. This device is usually designed to be adjustable for different types of plate, so such machines should be simple to modify. We have

demonstrated the effect in an Anthos htII machine, which has a 'scan mode' enabling 3360 measurements to be taken across a plate, such that even the areas between the wells are included in the measurements. One could subsequently select a subset of these for quantifying meniscus curvature, either manually or via a more complex software modification. The simplest possibility, which is simply to malposition deliberately the plate within the carrier, is usually not permitted by the design of most machines, in order to prevent unintentional errors.

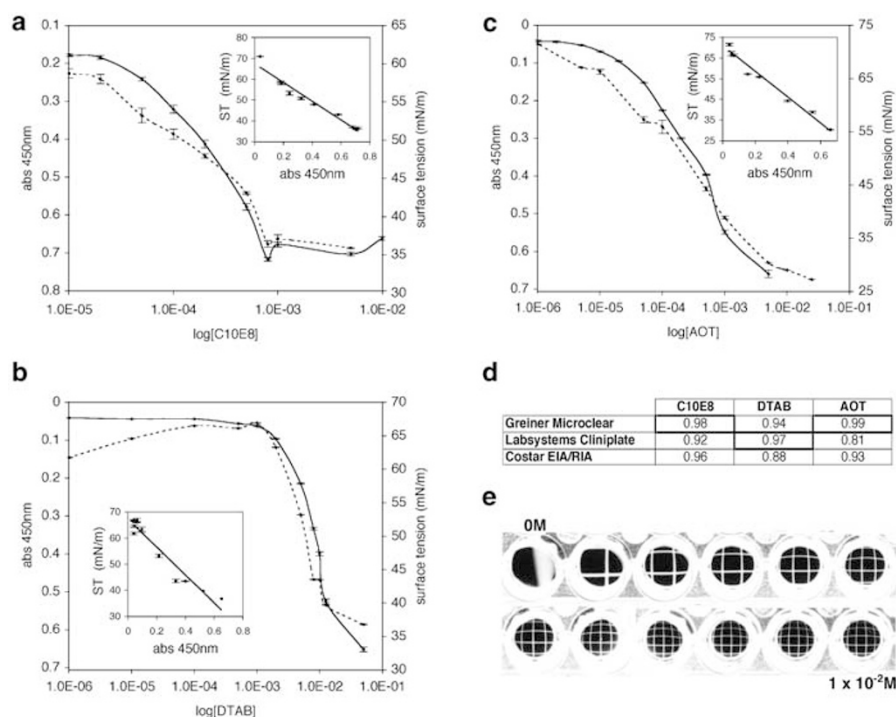
## Results

### Validation of Method using Detergents

In order to evaluate the utility of our method, we compared actual surface tension measured by du Noüy tensiometry or drop shape analysis with the values obtained using the novel optical method (expressed in absorbance units as apparent optical density, aOD). To test the nature of the relationship under different conditions, three detergents were used to control surface tension: one nonionic,  $C_{10}E_8$  (octaethylene glycol monodecyl ether), one cationic, DTAB (dodecyltrimethylammonium bromide) and one anionic, AOT (Aerosol OT, sodium bis(2-ethylhexyl) sulphosuccinate). In addition, three

types of polystyrene 96-well plate were evaluated, the Labsystems Cliniplate, which has little or no charge; the Costar EIA/RIA plate, which is negatively charged and has high protein-binding capacity; and the Greiner Microclear plate, which has intermediate to low negative charge, but is the only plate used that possesses opaque black well walls. The results of these experiments are shown in Figure 2. The relationship between surface tension and aOD was analysed by linear regression, and the calculated multiple- $R$  values are displayed in Figure 2d. Note that a higher aOD corresponds to a lower surface tension (more curved meniscus), and that the axes for the aOD are inverted.

For all three detergents, it was possible to select a plate able to produce an excellent correlation ( $R > 0.97$ ) between surface tension and apparent optical density, although this plate was not the same for each detergent. The Cliniplate performed very badly with AOT and the Costar plate very badly with DTAB, most probably due to electrostatic interactions between the surfactants and the surface of the plates. All three plates performed well with the nonionic detergent  $C_{10}E_8$ . The charge of the surfactant is a confounding factor, but not an insuperable one, since the Greiner plate performs almost equally well with all three detergents, although this could be attributable to its black walls rather than to its intermediate negative charge. A



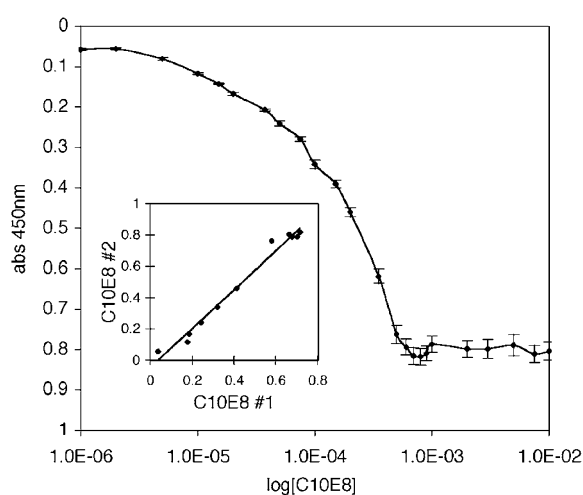
**Figure 2** Validation of the method. Panels **a–c**: Comparison of data obtained by the novel optical method (—), expressed in absorbance units, with surface tension as determined directly (----), expressed in mN/m, for three detergents,  $C_{10}E_8$  (**a**), DTAB (**b**) and AOT (**c**). Inset graphs show line fit plots of the two data sets. Panel **d**: Effect of different types of microwell plate upon the method, expressed as multiple  $R$ -values calculated by linear regression analysis. Darker outlining indicates the  $R$ -values for the selected data shown in panels **a–c**. Panel **e**: Photograph of the samples of  $C_{10}E_8$  taken using the method shown in Figure 1a, showing the dependence of the lensing effect of the meniscus upon surface tension.

feature not apparent from the  $R$ -values in Figure 2d is the limited dynamic range of the highly charged Costar plate, in which even pure water (surface tension = 72 mN/m) has a pronounced meniscus, and therefore an elevated aOD ( $\sim 0.2$ ) compared to the other plates (aOD  $\leq 0.05$ ).

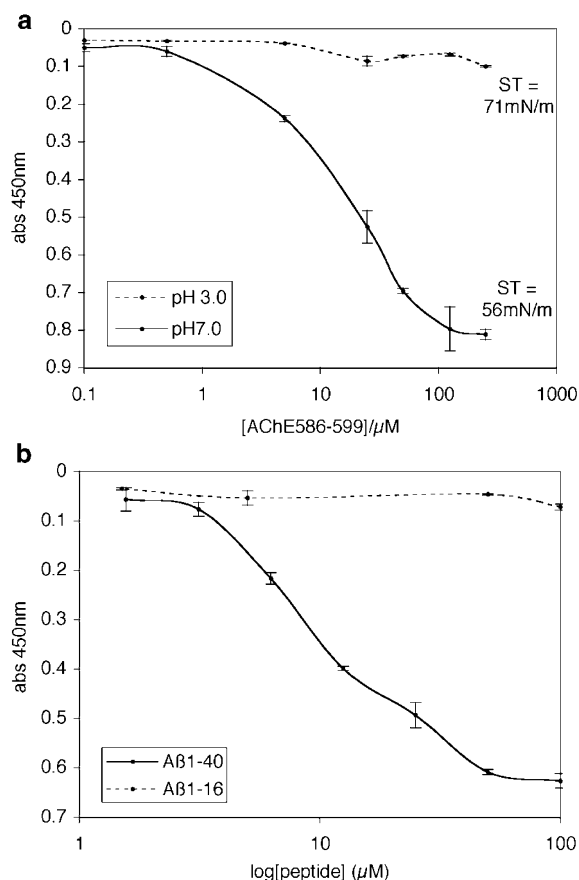
We note that the absorbance measurements seem smoother, and conform more closely to the classical curve shape, than the conventional surface tension measurements. This probably arises from inexperience in avoiding systematic errors in the latter measurements: much of the scatter in the insets in Figure 2a–c arises from the tensiometry. To verify this, as well as the reproducibility of the technique, we repeated the  $C_{10}E_8$  measurements using a fresh stock solution and a wider range of dilutions, as well as a separate lot of Greiner Microclear plates (Figure 3). The two data sets (Figures 3 and 2a) are almost identical ( $R=0.99$ ), and both exhibit the inflection near the critical micelle concentration (at  $10^{-3}$  M). The slight discrepancy is probably due to inaccuracy in preparation of the stock solution. It is also notable that the maximum aOD is slightly higher in the second experiment (Figure 3), suggesting that there may be small differences in the charge of 96-well plates in different batches.

### Application of Method to Biological Samples

The surfactant properties of the 40-amino acid peptide  $A\beta$  have been previously described.<sup>3</sup> This peptide is formed as an aberrant proteolytic product in Alzheimer's disease and rapidly self-assembles *in vitro* into structures known as amyloid fibrils. The kinetics of amyloid fibril formation are highly dependent upon concentration and a critical con-



**Figure 3** Reproducibility of method. Repeat determination with additional data points of the  $C_{10}E_8$  surface tension vs concentration curve using a fresh stock solution, new dilutions and new Greiner Microclear 96-well plates. The inset graph shows a line fit comparing these data and the data shown in Figure 2a ( $R=0.99$ ).



**Figure 4** Surfactant properties of amyloid polypeptides. Panel a: Effect of the peptide  $AChE_{586-599}$  at pH 3.0 and pH 7.0 upon surface tension, measured using the optical method and expressed in absorbance units. The surface tension as measured by drop shape analysis is shown for the highest concentration at each pH. Panel b: Effect of peptides  $A\beta_{1-16}$  and  $A\beta_{1-40}$  upon surface tension at pH 7.0, also expressed in absorbance units.

centration of  $\sim 25 \mu\text{M}$  is required for fibril formation *in vitro*.<sup>4</sup> Soreghan *et al* used a Wilhelmy plate apparatus to show that this critical concentration is analogous to the critical micelle concentration of detergents: the surface tension decreases with increasing concentration up to  $25 \mu\text{M}$ , where fibrils begin to form, but remains constant at higher concentrations. Furthermore, they showed that the shorter peptide,  $A\beta_{1-16}$ , which consists of the N-terminal region of the  $A\beta_{1-40}$  peptide and which does not form amyloid fibrils, is not surface active. We have repeated their experiments using the optical method (Figure 4b) with Labsystems Cliniplates, which were found to give the highest dynamic range and best sensitivity with proteinaceous surfactants. The results are identical to the published data, and show the surface tension plateau at  $\sim 25 \mu\text{M}$  for  $A\beta_{1-40}$  and the absence of any surfactant activity of  $A\beta_{1-16}$ . The plateau lies at an aOD of  $\sim 0.6$ , which corresponds therefore to the previously measured surface tension of 60 mN/m.

Figure 4a shows the effect upon surface tension of a similar fibrillogenic peptide,  $AChE_{586-599}$ .<sup>5,6</sup> Fibril

formation by this peptide is highly dependent upon pH, and it only self-assembles in a narrow window around neutral pH, while under acidic conditions it remains monomeric. Its effect upon surface tension is similarly dependent upon pH: at neutral pH, it reduces the surface tension in a similar manner to  $A\beta$ , with a plateau at about  $50 \mu\text{M}$ . This concentration is also the critical concentration required for self-assembly into amyloid fibrils.<sup>5,6</sup> Conversely, at pH 3, conditions under which it does not form fibrils, the peptide is not surface active; this situation mirrors that of the  $A\beta_{1-16}$  peptide. The surface tension of the highest concentration of AChE<sub>586-599</sub> was measured by drop shape analysis (Figure 4a). The aOD of  $\sim 0.8$  for  $200 \mu\text{M}$  AChE<sub>586-599</sub> at pH 7 corresponds to a surface tension of  $56 \text{ mN/m}$ . We note that the lower limiting surface tension of AChE<sub>586-599</sub> compared to  $A\beta_{1-40}$  ( $56$  vs  $60 \text{ mN/m}$ ) (3) is also correlated with a higher absorbance ( $0.8$  vs  $0.6$ ), suggesting that differences in  $\gamma_{\text{sl}}$  are not of major importance in this case.

## Discussion

We tested three detergents in three types of 96-well plate in order to investigate whether the optical method of quantifying meniscus curvature is a good measure of surface tension. The method proved to be considerably easier and more reproducible than either of the alternative accepted methods that were used, as well as requiring considerably less skill, time and volume of sample. However, we conclude that the method is limited to determination of relative surface tension, since there is no absolute correlation between the apparent optical density and the surface tension. Instead, it is necessary to calibrate the system for each surface-active analyte by measurement of the absolute surface tension for at least one point on the curve. Furthermore, it is necessary to determine empirically the best type of plate to use for a given surfactant, although we have not found it necessary to go any further than the Greiner Microclear black-wall plate and Labsystems Cliniplate for any analyte. Once the system has been set up, it is accurate, precise, reproducible and easily adapted to high-throughput applications. We have also demonstrated its suitability for studying problematic biological specimens, such as amyloid peptides. Owing to the long equilibration times (they are large molecules with low diffusion coefficients and are present only at low bulk concentrations), analogous measurements with conventional serial techniques would have been very time-consuming.

The technique relies upon the lens-like effect of the meniscus at the surface of aqueous solutions to diffuse a beam of light. To maximise this effect, a microplate spectrophotometer was modified to pass the beam near the edge of the sample well, making the effect useful for quantification of surface

curvature, and hence, surface tension (see Materials and methods). The measurement of the surface tension of solutions that themselves absorb light can therefore present a problem. However, even if a wavelength at which the sample does not absorb cannot be found, it is possible to overcome this problem by measuring both at the centre of the well and at the offset position. Whereas the meniscus-induced increase in absorbance varies over this distance, the absorbance due to the true optical density of the solution differs only very slightly (it is not constant because of the tiny difference in the path length). When the true optical density is small ( $< 0.2$ ) and the meniscus effect large ( $> 0.8$ ), one can subtract the centre value from the offset value to obtain the contribution of the meniscus to the apparent OD.

Conversely, when measuring absorbance conventionally, through the centre of the well, solution menisci can produce errors in the optical density data, although the effect is smaller than that obtained at the offset position. We have not investigated whether this error occurs in other plate readers, although most common instruments have a similar optical arrangement. This consideration is not important for the vast majority of biological assays, since it is unusual that the surface tensions of the samples will differ substantially; however, it was just such a difference that led to the developments described herein. Where the concentration of components in the final step of an assay is not identical across all wells, as may well be the case, for example, in high-throughput screening of drug candidates, it is very important to check the effect of solution menisci upon optical measurements in microplates.

Verifying that menisci are not a confounding factor in absorbance measurements can readily be accomplished by comparing signals taken when the incident beam is centred and offset, since, as described above, meniscus curvature, and hence lensing effects, will vary over this distance. We have not attempted this comparison using instrumentation other than the BMG Polarstar plate reader, but some suggestions for simple user modification of other instruments are made in the Materials and methods section. Alternatively, the lensing effect of the menisci could be checked using the photographic method employed in Figures 1c and 2e, provided that the solutions are sufficiently translucent. It has not escaped our notice that this photographic technique could also be harnessed for measurement of surface tension in microplates via automated digital image analysis, although we have found (not shown) that the spectrophotometer-based method is superior in terms of sensitivity and variability to measurements of the dimensions of a demagnified grid image. Nevertheless, since the new generation of tensiometers are based on automated digital drop shape analysis, it is possible that deconvolution of

such images may represent a useful alternative to the method described here.

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