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Colloidal aggregate detection by rapid fluorescence measurement of liquid surface curvature changes in multi-well plates

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Abstract

A simple fluorescence method is reported for the detection of colloidal aggregate formation in solution, with specific applications to determination of the Critical Micelle Concentration (CMC) of surfactants and detection of small molecule promiscuous inhibitors. The method exploits the meniscus curvature changes in high density multi-well plates associated with colloidal changes in solution. The shape of the meniscus has a significant effect on fluorescence intensity when detected using a top read fluorescence plate reader, because of the effect of total internal reflection on fluorescence emission through a curved liquid surface. A dynamic range of 60% is calculated and observed, and is measured with a relative sensitivity of 2%. Facile determination of the CMC of a variety of surfactants is demonstrated, as well as a screening assay for aggregate forming properties of small drug-like compounds, a common cause of promiscuous inhibition in HTS enzyme inhibitor assays. Our preliminary results show a potential HTS assay with Z' factor of 0.76, with good separation between aggregating and non-aggregating small molecules. The method combines the high sensitivity and universality of classic surface tension methods with simplicity and high throughput determination, enabling facile detection of molecular interactions involving a change in liquid or solid surface character.

We describe a simple fluorescence assay for sensitive detection of colloidal aggregation based on the pronounced capillarity observed in high density (384, 1536) multi-well plates used in high throughput screening. Aqueous colloidal systems form when amphipathic (surfactant) molecules are dispersed in water. The molecules form a monolayer at the air-water interface, with their non-polar tails extended away from the polar liquid medium. The result is a lowering of the surface tension of the solution, and an increase in downward curvature (wetting) in a tube made of hydrophobic material (Evans and Wennerstrom 1999). This is capillarity, a function of solid surface tension, liquid surface tension, solid-liquid interface tension and gravity. Above the critical micelle concentration (CMC), the surface monolayer is saturated, surfactant molecules form micelles and no further changes in surface curvature occur. Of particular interest in drug screening is the identification of small molecules in synthetic drug libraries that cause promiscuous inhibition of enzymes by virtue of colloidal aggregate formation (McGovern et al. 2002; McGovern et al. 2003; McGovern and Shoichet 2003). We surmise that such molecules should be readily identified in a screen which detects changes in capillarity.

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The fluorescence assay is based on the effect of a curved meniscus on spectrophotometric measurements using a plate reader with a vertical light path, unlike a regular spectrometer, where the light path is horizontal and does not pass through an air-water interface. The effect is normally avoided in high throughput screening by adding a constant concentration of non-denaturing surfactant to the wells of the plate. Cottingham et al (Cottingham et al. 2004) were the first to recognize the optical effects of surface curvature in a vertical beam system, utilizing it to develop a spectrophotometric method for assessing surface tension in 96 well plates. Here we provide a quantitative analysis of the effect of surface curvature changes on fluorescence emission intensity in 96, 384 and 384 micro-well plates using the top-read mode of a fluorescence plate reader, determining the dynamic range and sensitivity of the measurement. We also demonstrate use of the method for accurate determination of the critical micelle concentration (CMC) of a measured surfactant, and for sensitive detection of colloidal aggregate-forming promiscuous enzyme inhibitors. We further suggest an extension of Cottingham's spectrophotometric method for determination of absolute surface curvature.

Experimental Methods

Materials

Standard polystyrene black untreated 384 square well plates and 384 tapered clear bottom microwell plates were obtained from Greiner Bio-one. Black 96 cylindrical well plates were obtained from Whatman. Lucifer Yellow Iodoacetamide (LY) was purchased from Invitrogen-Molecular Probes. Other chemicals were purchased from Sigma-Aldrich.

Fluorescence measurements

Fluorescence measurements were made using a Spectramax Gemini XPS Plate Reader (Molecular Devices), a Spectramax M5 (Molecular Devices) or a Shimadzu RF-5301PC spectrofluorimeter. Sample volumes were 100 μ l, 30 μ l and 15 μ l respectively in 96, 384 and 384-micro well plates, and 2ml in a standard cuvette measured using the spectrofluorimeter. Excitation and emission wavelengths for LY were 425 and 540 nm respectively.

Calculation of total internal reflection

Total internal reflection was calculated using the diagrams in Figure 2. The angle α in Figure 2 was obtained from geometric analysis of the triangle ABC, with angles A, B and C and opposite sides of length a, b and c. $A=\alpha$, $B=\theta_c-\alpha$, $C=\pi-\theta_c$, $a=R$, $b=L$, $c=R+h$. $\theta_c=48.6$ is the critical angle of reflection from water to air. h is the distance from point A in the well to the liquid surface. The following geometric equations were used to solve for α and the cone length L for a given surface curvature radius R, and depth h:

$$0.5 * (b+c-a)=r * \cot (0.5 * A) \quad (1)$$

$$0.5 * (c+a-b)=r * \cot (0.5 * B) \quad (2)$$

$$0.5 * (a+b-c)=r * \cot (0.5 * C) \quad (3)$$

$$A+B+C=\pi \quad (4)$$

r is the radius of the internal circle of triangle ABC. An additional restriction on α arises from limitation of the cone size to the well width, ie. $h/\cot(\alpha) \leq r_w$, the radius of the well. The relative detectable fluorescence from point A is the ratio of the spherical cap surface in Figure 2C to the total surface of the sphere, which is geometrically equivalent to the ratio of the height of the spherical cap, h_1 , to the diameter of the sphere:

$$h_1 = L - L \cos(\alpha) \quad (5)$$

$$\eta = 0.5(1 - \cos(\alpha)) \quad (6)$$

η is the fraction of fluorescence that is detectable. At a given h and R , η can be calculated from equations (1) - (6) (Mathcad, Mathsoft). Integrating from 0 to the liquid depth gives the relative fluorescence intensity for a particular value of curvature R .

Screening assay for promiscuous inhibitors

Standard 384-well plates were used. The upper bound of the assay was measured using assay buffer: 50 mM phosphate, 50mM NaCl, pH 7.1, 0.5 μ M LY. The lower bound of the assay was measured using control buffer: 50 mM phosphate, 50mM NaCl, pH 7.1, 0.5 μ M LY and 1 mM Triton X-100. 2.5% DMSO was added to the buffers prior to measurement of upper and lower bounds. 144 measurements of upper and lower bounds were made, and the Z' factor determined according to the literature definition (Zhang et al. 1999). Compounds were evaluated at 50 μ M concentration by dilution from 2mM stock solution in DMSO into assay buffer and into control buffer. Relative fluorescence was measured as the ratio of top-read fluorescence intensity in assay buffer to control buffer, further scaled to give a unit value for assay buffer signal. For the compound data, an LY concentration of 1 μ M was used.

Results and Discussion

A series of solutions of gradually decreasing surface tension was prepared using increasing concentrations of detergent in consecutive wells, with sample volume and fluorescence dye (Lucifer Yellow, LY) concentration kept constant. The liquid surface was nearly flat for buffer solution without surfactant and formed a concave meniscus as surfactant concentration was increased, an effect that was easily seen by eye when the well plates were examined. Figure 1a shows the result on fluorescence intensity for four surfactants in a standard 384-well plate. The capillary effect was significant, as expected for the 3.683mm diameter square wells of the plate. The fluorescence intensity decreased with increasing concentration of surfactant, reaching 50% of the original at saturation. A discontinuity occurred at the CMC. The fluorescence intensity change was diminished in the larger wells of a 96-well plate (30% reduction), and enhanced in 384-well microplates (60% reduction), where wells have a smaller diameter than in regular 384 rectangular well plates (Figure 1b). Curvature variation with well size is simply a result of gravitational effects.

A series of control experiments confirmed that this effect was caused by changes in surface curvature, and not by liquid depth changes or interaction of the dye with the surfactant. LY is not environmentally sensitive and does not interact with the surfactant. The fluorescence emission maximum and intensity of LY was unchanged as a function of surfactant concentration when measured in a cuvette with a horizontal light path (figure 1a). Experiments using different liquid volumes showed that liquid depth change was not a factor in the observed fluorescence changes, since the ratio of fluorescence of buffer and high surfactant solutions remained unchanged over a wide range of volumes. This was confirmed

by using the bottom-read mode of the fluorescence plate reader (figure 1b). Very little variation in fluorescence intensity occurred across the range of surfactant concentrations when the fluorescence emission did not pass the curved liquid surface to reach the detector.

An explanation for the effect of surface curvature on fluorescence emission, and, by corollary, the sensitivity of fluorescence in determination of surface curvature in multi-well plates, was obtained by investigating the light path of fluorescence emitted through a curved liquid surface. Figure 2 shows that light emitted from point A in the well passes through the solution, refracts at the liquid-air boundary and travels to the detector. Detectable emission is limited to fluorescent light that hits the liquid - air boundary within the critical angle $\theta_c = \arcsin(n_1/n_2)$, above which the light is reflected back into the solution (total internal reflection). n_1 and n_2 are the refractive indices in air and liquid respectively. Figure 2 shows that detectable emitted light is restricted to a cone of angle 2α . For a flat liquid surface, the normal at the point of refraction is vertical, so $\alpha = \theta_c$ as in figure 2a. When the liquid surface is curved, the normal of the liquid surface tilts towards the centre of the curved surface. In a concave meniscus, $\alpha < \theta_c$, resulting in less detectable emitted light from point A (figure 2b).

We have applied this model to estimate the detectable emission from point A at depth h and curvature radius R as shown in figure 2, and integrated the emission over all h to obtain relative fluorescence intensity. For curvature variation from a flat surface ($R=\infty$) to the maximum possible curvature ($R = \text{radius of the well}$), the calculation predicted a relative fluorescence intensity change of 60% between these two extremes, which was observed in the smallest wells (384-well microplates, Figure 1b). The agreement between observed and calculated data supports the hypothesis that the change was due to total internal reflection. The calculation was based on a cylindrical well and assumed that detectable emission at all depths has an equal chance to reach the detector. Variation in angular distribution of emitted light from different depths was neglected, on the basis of experimental observations of a constant ratio of fluorescence between a curved and flat surface at different volumes.

The fluorescence intensity change was highly reproducible, and could be measured with a relative standard deviation of 0.02 in standard 384-well plates on the Spectramax Gemini XPS plate reader. Total internal reflection is independent of fluorescent dye since n_2 is essentially invariant over a wide range of wavelengths (Djurisic and Stanic 1998). A similar result was obtained using fluorescein as a reporter. A fluorescent dye with strong environmental sensitivity should be avoided because of competing effects of surfactant concentration on fluorescence intensity.

Determination of Critical Micelle Concentration

Current CMC determination methods include measurement of surface tension (del Mar Graciani et al. 2005), fluorescence intensity (Nakahara et al. 2005) or anisotropy (Thorsteinsson et al. 2005) of micelle-adsorbed dyes and Hyper-Rayleigh scattering (Ghosh et al. 2003). Direct measurement of the curvature induced fluorescence change in 384-well plates has led to a sensitive HT-ready method for surfactant CMC determination, as shown in Figure 1a. The phase transition was readily detected from the intersection of two straight lines which fit the data above and below the CMC. CMC values of 1.86 mM, 0.16 mM, 0.054 mM and 0.029 mM were obtained for SDS, Triton X-100, Tween-20 and N-hexadecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate (SB3-14) respectively, consistent with literature values (del Mar Graciani et al. 2005; Nakahara et al. 2005; Thorsteinsson et al. 2005). In the general case, samples may be prepared by 2X serial dilution in a row of 24 wells in a 384-well plate, which will cover a concentration range of 10^7 and allow CMC determination with an error less than a factor of 2. The precision will be even greater using 1.5X dilution in 48 wells of a 1536 well plate.

The linearity of the fluorescence intensity change with the logarithm of surfactant concentration at sub-CMC levels is not understood. Surface tension is linear with the log of surfactant concentration (del Mar Graciani et al. 2005), but there is no established theoretical basis for the linearity between surface tension and fluorescence. Similar results were reported using other techniques for CMC determination. The precision of the technique compares favorably with existing methods, including measurement of surface tension (del Mar Graciani et al. 2005), fluorescence intensity (Nakahara et al. 2005) or anisotropy (Thorsteinsson et al. 2005) of micelle-adsorbed dyes, and Hyper-Rayleigh scattering (Ghosh et al. 2003). Direct surface tension measurements are precise but low throughput, requiring complex preparation and expertise in sample handling (Evans and Wennerstrom 1999). Fluorescence anisotropy measurements have the potential for high throughput determination, but are not universally applicable for all surfactants because of a dependence on dye and micelle compatibility (Nakahara et al. 2005). CMC determination using the fluorescence method described here is precise, rapid and universal, since modifying the hydrophilic character of water solution is a common property of all surfactants.

Determination of Promiscuous Inhibitors in Drug Libraries

The fluorescence assay has been used to detect compounds in drug libraries that cause promiscuous inhibition in enzyme assays (Feng et al. 2005; McGovern et al. 2002; McGovern et al. 2003; McGovern and Shoichet 2003). Amphiphilic or hydrophobic properties of the molecules cause the formation of colloidal aggregates which sequester the enzymes used in the assays, causing false positives. Figure 3 shows that aggregators are easily detected in the fluorescence assay, due to the change in surface tension that accompanies aggregate formation. The upper and lower bounds of the assay for aggregators were determined using buffer and Triton-X-100 saturated buffer, respectively, each containing 0.5 μ M LY and 2.5% DMSO. Triton X-100 causes maximum liquid surface curvature change at its CMC, providing an ideal positive control for the assay. A Z' factor of 0.76 was obtained. The robustness of the assay was verified by testing 14 known aggregators and 8 known non-aggregators at 50 μ M concentration (McGovern and Shoichet 2003; Seidler et al. 2003). Relative fluorescence was measured as a ratio of the observed fluorescence of compound in buffer to that of the compound in the 1mM Triton solution, in order to alleviate any compound-specific absorption or fluorescence. A clear distinction between the two types of small drug-like molecules was obtained. The increase in curvature and associated decrease in fluorescence observed for aggregators is caused by a decrease in surface tension of the solution containing the colloidal aggregates. The compound sulconazole is an outlier, because of a specific association between LY and sulconazole aggregates. The result is LY fluorescence enhancement that is not cancelled out by the Triton-X-100 control, since Triton breaks up the sulconazole aggregates (Feng et al. 2005). Using a second fluorophore (fluorescein) eliminated this spurious effect, as did changing the control from Triton-containing sample in a second well to the bottom-read fluorescence measurement of the original well, a measurement that is invariant with curvature (Figure 1b).

The result suggests an extremely simple HTS assay for molecules that form aggregates in any biological or chemical system. Synthetic compound libraries can be screened to detect colloidal aggregators at very low cost, tagging them as potential non-specific inhibitors. A built-in control scan corrects for the effects of any colored compounds on the measurement (Figure 3). DMSO concentrations up to 8% did not affect the curvature changes. Furthermore, the concentration dependence of aggregation, thought to be associated with a critical point phenomenon (Feng et al. 2005), could easily be followed using a dose response curve in the fluorescence assay.

There is no limitation on the optical characteristics of the fluorescent probe, but its key feature must be environmental insensitivity. An occasional outlier may present due to aggregate - probe binding, because of the effect of detergent on aggregates, as seen with sulconazole in figure 3. False negative or positive results can be alleviated by using an additional fluorophore or by using the bottom-read fluorescence measurement as the control. The bottom-read control may provide superior results because of simplified sample handling (no second set of wells required) and concomitant higher Z' factor. It does, however, require instrumentation capable of performing the dual measurement. The Triton-X-100 control and bottom-read control measurements will be the subject of an expanded study to evaluate the advantages of each in compound ranking, avoidance of false positives or negatives and sensitivity to aggregate detection.

An optical density method for surface curvature measurement

The curvature-induced fluorescence intensity change in multi-well plates was first noted by Cottingham et al (Cottingham et al. 2004). They alleviated the effect by adding a high concentration of detergent, and instead exploited the lens effect of a curved liquid surface using a UV detector, experimentally connecting their observation with surface tension measurements. They assumed the effect arose from defocusing of the light beam through a concave liquid surface. Our calculations show the main cause of their observation should be deflection of the light beam. For the high curvature limit, light is expected to totally escape detection in their system. Instead they observed an eight-fold reduction; residual observed light may have arisen from light scattering in the liquid. Our calculation on their model predicts a potentially sensitive and straightforward method to measure liquid surface curvature from the horizontal displacement of the light beam. A geometric relationship exists between the light beam displacement and the surface curvature radius, which would allow a simple read off of meniscus curvature in an instrument modified to read the position of the light beam below the wells.

Conclusion

In conclusion, we have demonstrated the theoretical and experimental basis for a simple HT fluorescence method to study the liquid surface curvature change associated with changes in surface forces at the solid-liquid interface. The fluorescence measurement has a dynamic range of 60% and a relative sensitivity of 2%. The method has been applied to universal rapid surfactant CMC determination, an important characterizing feature of colloidal solutions and an area of ongoing technique development (del Mar Graciani et al. 2005; Ghosh et al. 2003; Nakahara et al. 2005; Thorsteinsson et al. 2005). Additionally, the method was used to detect promiscuous enzyme inhibitors by virtue of the change in surface tension accompanying their colloidal aggregate formation (McGovern et al. 2002). An assay that can identify compounds that consistently cause false positives in enzyme assays is of great value for pre-screening large synthetic compound libraries. The fluorescence assay is simple and cost effective compared to currently existing assays involving light scattering or enzyme assays (Feng et al. 2005).

A broad range of applications is possible for this sensitive liquid surface curvature measurement. It is directly related to the change of contact angle of liquid on a solid surface, which contains important information on solid surface tension, liquid surface tension and liquid-solid interface tension. Any colloidal or other process that changes these forces can be potentially monitored in this way. We have predicted an additional optical method, based on the studies of Cottingham et al (Cottingham et al. 2004), which would enable direct determination of absolute surface curvature.

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REFERENCES

- Cottingham MG, Bain CD, Vaux DJ. Rapid method for measurement of surface tension in multiwell plates. Laboratory investigation; a journal of technical methods and pathology. 2004; 84:523–529. [PubMed: 14767490]
- del Mar Graciani M, Rodriguez A, Munoz M, Moya ML. Micellar solutions of sulfobetaine surfactants in water-ethylene glycol mixtures: surface tension, fluorescence, spectroscopic, conductometric, and kinetic studies. Langmuir. 2005; 21:7161–7169. [PubMed: 16042437]
- Djurisic AB, Stanic BV. Modeling the wavelength dependence of the index of refraction of water in the range 200nm to 200 μ M. Appl Opt. 1998; 37:2696–2698. [PubMed: 18273213]
- Evans, DF.; Wennerstrom, H. The colloidal domain: where physics, chemistry, biology, and technology meet. Wiley-VCH; New York: 1999.
- Feng BY, Shelat A, Doman TN, Guy RK, Shoichet BK. High-throughput assays for promiscuous inhibitors. Nature chemical biology. 2005; 1:146–148.
- Ghosh S, Krishnan A, Das PK, Ramakrishnan S. Determination of critical micelle concentration by hyper-rayleigh scattering. Journal of the American Chemical Society. 2003; 125:1602–1606. [PubMed: 12568621]
- McGovern SL, Caselli E, Grigorieff N, Shoichet BK. A common mechanism underlying promiscuous inhibitors from virtual and high-throughput screening. Journal of medicinal chemistry. 2002; 45:1712–1722. [PubMed: 11931626]
- McGovern SL, Helfand BT, Feng B, Shoichet BK. A specific mechanism of nonspecific inhibition. Journal of medicinal chemistry. 2003; 46:4265–4272. [PubMed: 13678405]
- McGovern SL, Shoichet BK. Kinase inhibitors: not just for kinases anymore. Journal of medicinal chemistry. 2003; 46:1478–1483. [PubMed: 12672248]
- Nakahara Y, Kida T, Nakatsuji Y, Akashi M. New fluorescence method for the determination of the critical micelle concentration by photosensitive monoazacryptand derivatives. Langmuir. 2005; 21:6688–6695. [PubMed: 16008375]
- Seidler J, McGovern SL, Doman TN, Shoichet BK. Identification and prediction of promiscuous aggregating inhibitors among known drugs. Journal of medicinal chemistry. 2003; 46:4477–4486. [PubMed: 14521410]
- Thorsteinsson MV, Richter J, Lee AL, DePhillips P. 5-Dodecanoylamino fluorescein as a probe for the determination of critical micelle concentration of detergents using fluorescence anisotropy. Analytical biochemistry. 2005; 340:220–225. [PubMed: 15840494]
- Zhang JH, Chung TD, Oldenburg KR. A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. J Biomol Screen. 1999; 4:67–73. [PubMed: 10838414]

Figure 1.

(a) Relative fluorescence intensity of 1μMLY as a function of the concentration of non-ionic detergents Triton-X-100 (●) and Tween-20 (□), anionic detergent sodium dodecyl sulfate (▲), and zwitterionic surfactant SB3-14 (◆) in buffer (25mM Tris, 25mM acetate, pH 7.0). Experiments were repeated in triplicate in standard 384-well plates with the detector on top of the plate. Also shown is the fluorescence intensity of 1μMLY as a function of Triton-X-100 concentration as measured in a 3ml cuvette on a regular spectrofluorimeter (○). In all cases, the fluorescence is relative to that obtained in a buffer solution without detergent. (b) Effect of well-specification and read mode on the fluorescence intensity as a function of Triton-X-100 concentration. Shown are top-read measurements in a 96 cylindrical well plate

with well radius 3.6mm (Δ), a 384 square well plate with well radius 1.84 ($\bullet$), a 384 tapered microwell plate with upper and lower radius of 1.65 and 0.92 mm respectively ($\blacktriangle$), and bottom read measurements on the small volume plate ($\circ$).

Figure 2.

Detectable fluorescence emission in a well. The dot at point A and depth h indicates the fluorescent molecules. θ_c is the critical angle of reflection from liquid to air (48.6°), 2α is the angle of a cone of side length L, indicated in white, within which the fluorescence emission can pass through the liquid-air interface and become detectable. (a) Detectable emission through a flat liquid surface, where $\alpha = \theta_c$. (b) Detectable emission through a concave meniscus, where $\alpha < \theta_c$. (c) The surface area of the spherical cap of the cone defined by α and L determines the fraction of spherically emitted fluorescence that is detectable. The ratio of spherical cap to sphere surface area = $h_1 / 2L$.

Figure 3.

Potential high throughput screening assay for promiscuous inhibitors due to aggregate formation. Upper (○) and lower (▲) bounds were measured using assay and control buffer respectively (Materials and Methods). Compounds were assayed at a concentration of 50μM. Signals from literature-defined non-aggregators (○) and aggregators (▲) are shown the figure. Error bars indicate the standard deviation of measurements made in triplicate.

Compounds from left to right: 1. Palatine Chrome Black[§], 2. Quercetin, 3. Sulfadiazine, 4. Sulconazole, 5. Estradiol, 6. Congo Red[§], 7. Diflunisal, 8. Tetraiodophenolphthalein, 9. Cibacron Blue 3GA[§], 10. Primidone, 11. Clotrimazole, 12. Benzyl benzoate[†], 13. Fluconazole, 14. Protriptyline, 15. Carbamazepine, 16. Prednisone, 17. Carisoprodol, 18. Nicardipine, 19. Trypan Blue[§], 20. DY2O[§], 21. 4BPAP[§], 22. Rottlerin[§], 23. 2.5% DMSO control, 24. non-DMSO control. Underlined compounds were aggregators in an enzyme assay (McGovern and Shoichet 2003; Seidler et al. 2003).

[§]Colored compound; [†]Not an aggregator at 50μM concentration (Shoichet, personal communication)