Artefacts at the liquid interface and their impact in miniaturized biochemical assay

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Droplet microfluidic technology has the potential to significantly reduce reagent use, and therefore, lower costs of assays employed in drug discovery campaigns. In addition to the reduction in costs, this technology can also reduce evaporation and contamination which are often problems seen in miniaturized microtitre plate formats. Despite these advantages, we currently advise caution in the use of these microfluidic approaches as there remains a lack of understanding of the artefacts of the systems such as reagent partitioning from droplet to carrier oil and interaction of the biological reagents with the water-oil interface. Both types of artefact can lead to inaccurate and misleading data. In this paper, we present a study of the partitioning of a number of drug-like molecules in a range of oils and evidence of protein binding at the water-oil interface which results in reduced activity of a cytochrome P450 enzyme. Data presented show that the drug-like molecules partitioned the least into fluorocarbon oils and the interaction of the 1A2 cytochrome at the water-oil interface resulted in a lower or complete absence of enzyme activity. This loss of activity of cytochrome 1A2 could be restored by the use of secondary blocking proteins although changes in the pharmacology of known 1A2 inhibitors were observed. The artefacts described here due to reagents partitioning into the carrier oil or protein binding at the water-oil interface significantly impact the potential use of these microfluidic systems as a means to carry out miniaturized biological assays, and further work is needed to understand the impact and reduction of these phenomena. © 2015 AIP Publishing LLC.

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INTRODUCTION

Drug discovery and droplet microfluidics (DMF)

Miniaturisation of existing microtitre plate assays to sub-microlitre volumes can be achieved using DMF. In addition to lower reagent costs, other desirable benefits may include increased sterility and reduced evaporation as droplets are in an isolated biphasic environment. In addition, the “concentration effect” of working with small droplet volumes may lead to gains in assay sensitivity and faster enzyme reaction rates.¹ As part of drug discovery cascades, drug-drug interactions are often studied using *in vitro* assays based on recombinant human P450 cytochromes using fluorogenic substrates to aid easier assay and analysis. Assays of this type have been conducted in 96, 384, and 1536 microtitre plate formats.² Miniaturisation of these

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assays to a droplet format is attractive to increase throughput and deliver significant reductions in reagent requirement, lowering the unit cost of large scale drug library screening.

Artefacts in typical microtitre plate assays can affect the observed data, and it is assumed that artefacts can also affect DMF assays. However, such artefacts are not fully described and consequently, DMF systems are not yet widely applied within screening laboratories.

Partitioning

In DMF systems, where droplets are formed by shear-force interaction between two immiscible liquids, it has been shown previously that substances may partition between phases by three mechanisms: direct partitioning;³ bilayer transport via interface-bound surfactant when droplets are close-packed;⁴ and via transport in reverse micelles formed in the carrier phase.⁵ Partitioning between phases in DMF may be significant for pharmaceutical products where lipophilicity is high. This could be reduced through the use of fluorinated oils, but it is often necessary to use surfactants in order to stabilise droplets, which in turn may exacerbate partitioning via reverse micellar transport. Whilst the leakage of fluorescent dye molecules in DMF has been reported,^{6,7} few reports study the specific wider impact the droplet leakage has on the use of DMF in bioassays.

Protein surface adsorption

Binding of proteins and enzymes to fluid interfaces and the potential for subsequent conformational change are well documented;⁸ however, there is limited understanding of how protein adsorption may affect bioassays run in DMF systems.

In this paper, we investigate: (i) the potential for leakage of drug-like molecules from aqueous droplets for a range of carrier oils and drugs and (ii) the design of a DMF P450 1A2 cytochrome enzyme inhibition assay with qualitative assessment of the effect of protein binding at the droplet interface in this assay.

REAGENTS AND MATERIALS

Imipramine, chlorpromazine, 3,5-dichlorophenol (DCP), indomethacin, trazodone, lidocaine, tolbutamide salicylic acid, perfluorodecalin, perfluoroperhydrophenanthrene (PHP), perfluorooctanol, dodecane (DOD), octanol (OCT), hexadecafluorodimethylcyclohexane (HDFDMCH), polydimethylsiloxane (PDMS), potassium hydrogen phosphate, disodium phosphate, fluvoxamine, nicotinamide adenine dinucleotide phosphate (NADPH), 3-cyano-7-ethoxycoumarin (CHC), 3-cyano-7-hydroxycoumarin gelatin, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO). Olive oil was purchased from Tesco (Cheshunt, UK). FC-40 was provided by 3M (Bracknell, UK), and FC-70 was purchased from Apollo Scientific Ltd. (Stockport, UK). Recombinant 1A2 P450 cytochrome was purchased from Life Technologies (Carlsbad, CA, USA).

A custom made fluorosurfactant (AZF) was prepared at AstraZeneca Pharmaceuticals plc. (Macclesfield, UK). For this surfactant, PEG600, thioyl chloride, dimethylformamide, sodium azide, dichloromethane, tetrahydrofuran, 1,1,2-trichloro-1,2,2-trifluoroethane, toluene, triphenylphosphine, and oxalyl dichloride were purchased from Sigma-Aldrich. Krytox 143FSH was purchased from DuPont (Wilmington, DE). A fluorescein isothiocyanate (FITC) labelling kit (#53029, Life Technologies, Carlsbad, CA, USA) was used to label a protein (courtesy of AstraZeneca plc., London, UK) of ~67 kDa mass. Self-adhesive microtitre plate seals (#6575) were purchased from Corning Life Sciences (NY, USA). Bootlace ferrules (0.75 mm²) were purchased from RS Components (Corby, UK). A M35 CNC milling machine was used for chip production (Datron Technologies Ltd., Milton Keynes, UK). PTFE tubing (0.5 mm i.d.) was purchased from Cole-Palmer (London, UK). Gastight[®] syringes were purchased from Hamilton (Bonaduz, Switzerland). Harvard syringe pumps (Edenbridge, UK), high voltage power supply (Consort, bvba, Belgium), a UV-2401 spectrophotometer (Shimadzu, Kyoto, Japan), and

Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA) were supplied by AstraZeneca plc.

Solutions

A stock solution of phosphate buffer (PB) was made by dissolving disodium hydrogen phosphate (38.6 g) in water (1 l) and adding to this stirring continuously, a solution of potassium hydrogen phosphate (6.8 g in 0.5 L water), until reaching pH 7.4.

Stock solutions of all tested chemicals were made by dissolving in PB to 200 μ M, except fluvoxamine which was dissolved in DMSO to 2 mM. PFO surfactant was used at 10% by volume and AZF at 8.5 mM.

METHOD

Microchip fabrication and use

Microfluidic devices (chips) were designed using Autodesk Inventor[®] and fabricated by direct CNC machining in polymethylmethacrylate (PMMA) and sealed with cut sections of microtitre plate seals. Chips designed for 0 min on-chip droplet incubation (t_0) and a spiral design providing 13.5 min incubation ($t_{13.5}$) were fabricated (Figure 1). Both chips used T-junction geometry to generate droplets.

Fluids were delivered to the chips using PTFE tubing connected to the chips via bootlace ferrules and pumped using syringes on Harvard syringe pumps.

Droplets were excited using a solid-state 405 nm laser module via a bespoke dichroic mirror/filter block assembly and a connected photomultiplier Tube (PMT) (Hamamatsu, Japan) used to detect droplet fluorescence. An ADC logger (Picoscope, Cambridge, UK) was used to record peak data for quantification. ImageJ software (National Institutes of Health, Bethesda, MD) was used to analyse fluorescence in droplets. Absorbance data were acquired with a Nanodrop 2000 UV-VIS spectrophotometer (Thermo Scientific, Waltham, MA) or UV-2401 spectrophotometer (Shimadzu, Kyoto, Japan).

Aqueous phase leakage (glass bottle)

Test oil (4 ml) and aqueous test drug solution (1 ml) were added to a glass bottle such that a planar interface existed between the phases. Disposable syringes fitted with blunt needles were used to extract aqueous samples. Initial drug solution absorbance was measured before adding to the oil. Samples (350 μ l) were taken at 15, 30, 45, and 60 min intervals post addition to the oil and the absorbance (over 200 to 450 nm) recorded. Nine oils covering five classes of chemistry and eight compounds of acidic and basic chemistry were tested (Table I). The oil exhibiting the lowest degree of partitioning was re-tested using a custom made fluorosurfactant (see supplementary material).⁹ The percentage loss from the aqueous phase was calculated under all conditions tested. Due to the relatively large surface area of aqueous phase in contact with the glass bottle, the theoretical maximal number of adsorbed molecules per unit area for each compound tested was used to check for a change in aqueous phase concentration due to non specific binding to the glass.

FIG. 1. Chips used for *in situ* droplet partitioning: (a) T-junction to provide 0 min droplet residence time; (b) 13.5 min droplet residence time.

TABLE I. Compounds tested in the static and chip-based leakage tests (left); Oils investigated in the glass bottle aqueous phase leakage test (right).

Compounds	Molecule ion class	Oil	Oil chemistry
Indomethacin	Acid	Dodecane	Alkane
Dichlorophenol	Neutral	Olive oil	Triglyceride
Salicylic acid	Acid	Polydimethylsiloxane	Silicone oil
Tolbutamide	Acid	Octanol	Alcohol
Lidocaine	Base	Hexadecafluoro-1,3-dimethylcyclohexane	Fluorinated cycloalkane
Trazodone	Base	Perfluorodecalin	Fluorinated cycloalkane
Imipramine	Base	Perfluoroperhydrophenanthrene	Fluorinated cycloalkane
Chlorpromazine	Base	FC-40	Fluorinated tertiary alkylamine
		FC-70	Fluorinated tertiary alkylamine

Droplet leakage

Absorbance measurements of droplets *in situ* are challenging due to the small quantity of material and short optical path length available; however, Mohr *et al.* reported that measurements of this type are possible using a combination of on-chip droplet fusion and fibre-optic guided spectrophotometers.¹⁰ In the absence of this equipment, a method employing off-chip pooling of droplets and subsequent low volume ($\sim 2 \mu\text{l}$) UV-Vis analysis using a Nanodrop protein spectrophotometer was used. The pool was assumed to represent the average partitioning across a large population of individual experiments (droplets), and assuming a high precision of droplet size (expected due to constant flow rate and geometry), this was also considered to accurately represent any individual droplet within the population.

The experiment was conducted two ways: (i) Aqueous droplets were produced at a T-junction ($100 \times 100 \mu\text{m}$ for the aqueous inlet; $200 \times 300 \mu\text{m}$ for the corresponding carrier oil channel section (Figure 2(a)), and flowed directly for a defined collection time (1.5 min) into a glass vial, and coalesced by brief application of an electric field (2–3 kV); and (ii) droplets were produced at a T-junction as part of the long channel chip providing a 13.5 min residence time, prior to collection and high voltage coalescence (Figure 2(b)). The electric field disrupts the surfactant-stabilised droplet interface and induces electro-fusion.^{11,12} In both cases, samples

FIG. 2. Chips used for *in situ* droplet partitioning: (a) T-junction to provide 0 min droplet residence time; (b) 13.5 min droplet residence time. Droplets were collected as an emulsion in 0.5 ml Eppendorf vials and electrofused by brief application of a high voltage. The resultant fused solution was then sampled and analysed by absorbance on a Nanodrop 2000 analyser.

of coalesced aqueous phase ($\sim 3 \mu\text{l}$) were extracted and measured using a Nanodrop spectrophotometer. Compounds tested are detailed in Table I.

Cytochrome P450 enzyme inhibition

An existing microtitre plate assay¹³ for 1A2 cytochrome P450 enzyme inhibition studies was reduced in volume for DMF miniaturisation. Droplets of $\sim 200 \mu\text{m}$ diameter (volume $\sim 4.2 \text{ nl}$) comprising cytochrome 1A2 (10 nM), CEC substrate ($3 \mu\text{M}$), and NADPH ($250 \mu\text{M}$) without fluvoxamine as inhibitor of 1A2 were incubated at 34°C for 13.5 min in the spiral chip to provide maximal control activity data. This was repeated over a range of fluvoxamine concentrations from 0.01 to $0.3 \mu\text{M}$ to generate data for IC_{50} calculation. The experiment was repeated using a chip designed for 6 min incubation under the same flow conditions. All data were referenced against droplet series of standard CHC concentrations over the range 0.01 to $3 \mu\text{M}$ in the t0 chip to quantify fluorescent metabolite produced. The tests were repeated using the microtitre plate protocol for comparison. A pseudo-Hill analysis was applied to determine IC_{50} of fluvoxamine.

Enzyme adsorption at the droplet interface

Possible binding of the 1A2 enzyme to the droplet-oil interface was qualitatively studied by comparing the behaviour of droplets containing fluorescein solution ($0.8 \mu\text{M}$, phosphate buffered to pH 7.4) to those containing a FITC labelled protein ($0.8 \mu\text{M}$ FITC tag, phosphate buffer pH 7.4). A microfluidic chip was constructed enabling populations of droplets to be studied in stopped-flow conditions on a confocal microscope in both epifluorescent and laser-scanning modes (435 nm emission, $8 \mu\text{m}$ confocal slices), Figures 3 and 4, respectively.

FIG. 3. (a) Epifluorescence analysis of pH 7.4 phosphate buffered fluorescein-only containing droplets ($8 \mu\text{M}$) and corresponding ImageJ analysis of pixel intensity across droplet region indicated by yellow dotted line. Droplets exhibit a uniform distribution of fluorescein with no apparent localisation to the droplet perimeter; (b) Epifluorescence analysis of pH 7.4 phosphate buffered droplets containing FITC-labelled protein (FITC at $8 \mu\text{M}$) and corresponding ImageJ analysis of pixel intensity. Significant localisation of fluorescence is observed at the droplet perimeter consistent with adsorption of protein to the droplet-oil interface. The brighter central region is due to the higher fluorescence seen through the droplet diameter.

Enzyme activity recovery using blocking proteins

Titration of BSA and gelatin at 50, 500, and 1000 μM were added to the control activity experiments to observe any change in assay signal from the effect of adding the blocking proteins. A fluvoxamine titration was separately included with each blocking protein concentration to assess for any shift in calculated IC_{50} due to the presence of blocking protein. Control activity and IC_{50} data were compared to that obtained from corresponding microtitre plate assays.

RESULTS AND DISCUSSION

Aqueous phase leakage (glass bottle)

Partitioning in most non-fluorous oils was observed to be high with compounds of higher logD leaking the most. Leakage was lowest in fluorocarbon oils, which was expected due to the pronounced hydro- and oleo-phobic nature of these oils. At an aqueous phase concentration of 200 μM , this equates to $\sim 1.2 \times 10^{17}$ molecules in solution. Calculation of the available surface area of glass exposed to the aqueous phase ($2 \times 10^{-3} \text{ m}^2$) and approximate minimum area of each drug molecule (approximating heavy atom radii to 3 Å and using standard bond lengths) suggests an insignificant number of molecules bond to the bottle surface, thus not contributing to the observed decrease from the aqueous phase (Table II). For this reason, a reduction in aqueous phase absorbance was deemed to be wholly due to leakage into the oil phase. Surfactant addition to the fluorous phase (PFO at 10% v/v and independently AZF at 2% w/w) significantly increased leakage for most compounds studied, but most notably for more basic chemistry. Acidic compounds were not affected as much, most likely as a consequence of the differing extent of molecular ionisation at pH 7.4 for the compounds tested (Table II).

Aqueous phase leakage (DMF)

Fluorosurfactant was required to produce and stabilise droplets in the microfluidic chip (data not shown), and as such, it was only possible to compare partitioning directly between the bottle and chip methods in the presence of AZF or PFO. In DMF, droplets do not directly contact the chip; thus, non-specific binding was deemed not significant, and, due to a higher surface area-to-volume ratio, leakage was expected to be higher compared to the bottle tests. The results show greater leakage seemingly occurring during the 1.5 minute droplet collection phase of the experiment, rather than during the 13.5 minute on-chip containment, for both the t0 T-junction and t13.5 minute spiral chips (Table III). This is possibly due to turbulent mixing during droplet collection. As in the bottle tests, basic compounds having a higher logD were subject to greater leakage, suggesting that compound chemistry influences the degree of leakage. Further detailed physicochemical analysis is required to characterise this observation for a wider range of chemistries and to determine the significance of compound leakage in relation to high throughput drug discovery screening.

FIG. 4. (a) Selection of confocal slices ($8 \mu\text{m}$) taken in the Z-plane through pH 7.4 phosphate buffered fluorescein-only containing droplets ($8 \mu\text{M}$). The blue box indicates disturbance where the droplet was observed to contact the chip; (b) Selection of confocal slices ($8 \mu\text{m}$) taken in the Z-plane through pH 7.4 phosphate buffered FITC-labelled protein containing droplets (FITC at $8 \mu\text{M}$). The red bounded area indicates evidence of an emerging ring suggesting localisation of labelled protein at the droplet-oil interface. The emergence of a chip “contact patch” is seen as before in the epifluorescent data, indicated in blue bounded frames.

TABLE II. % loss from the aqueous phase determined by absorbance for a range of compounds and oils. Second time point for tests using fluorosurfactant were at 45 min.

			% Loss to oil (no surfactant)										10% PFO		2% w/w AZF
			Time				Olive	PFP-		HDFD-	FC-	FC-	FC-		
	Ion	Log D	(mins)	DOD	OCT	PDMS	oil	HP	PFD	MCH	40	70	70	70	
Chlor-promazine	B	3.36	15	51	70	32	70	0	14	15	13	3	55	57	
			30	60	80	39	77	13	17	17	14	9	65	68	
			60	72	89	53	79	25	23	25	17	13			
Trazodone	B	2.64	15	21	71	7	25	17	6	6	5	11	9	13	
			30	38	73	10	29	16	6	6	6	15	16	30	
			60	53	79	24	47	24	7	6	6	4			
Imipramine	B	2.49	15	30	53	10	20	17	6	2	3	0	10	15	
			30	39	67	13	35	13	6	2	3	0	19	27	
			60	44	85	41	64	0	7	2	4	8			
Lidocaine	B	1.62	15	0	0	0	0	2	1	0	0	0	10	7	
			30	0	0	0	0	0	0	0	0	0	32	20	
			60	1	1	0	0	1	0	0	0	0			
3,5-DCP	N	3.58	15	11	79	12	63	32	2	0	0	0	81	81	
			30	19	88	28	76	34	3	0	2	0	82	83	
			60	33	91	55	78	32	5	0	4	3			
Indomethacin	A	0.95	15	3	49	3	4	1	5	5	4	4	4	4	
			30	3	61	3	7	5	4	6	4	9	5	6	
			60	2	84	3	11	10	5	5	5	13			
Tolbutamide	A	0.43	15	0	1	0	0	6	0	1	0	0	0	0	
			30	1	1	0	0	0	1	0	1	0	0	0	
			60	1	2	0	0	0	...	0	0	...			
Salicyclic acid	A	−1.43	15	0	0	0	0	0	2	0	0	0	0	0	
			30	0	0	0	0	2	1	0	0	0	0	0	
			60	0	0	0	0	...	1	0	0	0			

Cytochrome P450 enzyme inhibition

Microtitre plate data yielded a good assay window for the control tests, showing excellent linearity over a 20 min reaction. Signal to noise (s:n) was high at ~18:1. Metabolic turnover of CEC to ~8% CHC at 13.5 min was determined by fitting data to a CHC calibration curve. IC₅₀ plots generated using a four point FLU titration revealed an IC₅₀ value for fluvoxamine of ~0.02 μ M which was comparable to literature data (data not shown). In comparison, the DMF

TABLE III. Loss of compound (%) from the aqueous droplet in the t0 and t13.5 min microfluidic chips. Base chemistry compounds with higher logD tend to leak to a greater extent, depicted by darker shading.

	% loss (t0 chip)	% loss (t13.5 chip)
Chlorpromazine	96	96
Imipramine	95	95
3,5-DCP	67	79
Trazodone	64	75
Indomethacin	27	25
Salicylic acid	7	0
Lidocaine	0	23
Tolbutamide	0	0

control activity reaction data proved to be difficult to repeat yielding a poor s:n of $\sim 2.3:1$ for a 13.5 min incubation. Calibration titration droplets of CHC run in the 13.5 min chip were quantifiable to $0.03 \mu\text{M}$ having good linearity, and thus, leakage of CHC metabolite during reaction was not considered to be a cause of the small signal observed (data not shown). Data obtained using the microtitre plate method from reagents held at room temperature for 3 h prior to reaction were similar to that obtained when using fresh reagents, which suggested that enzyme degradation was not a likely cause of low/no signal. With the 6 min chip, a reproducible signal was obtained which, although had a lower s:n than the microtitre plate test, was sufficient to generate IC_{50} data.

Interfacial protein adsorption

Droplets containing fluorescein solution were observed by epifluorescent microscopy and confirmed by laser scanning microscopy to have a relatively even distribution from the droplet edge to centre as would be expected (Figure 3(a)). In contrast, droplets containing labelled protein showed a distinct “ring” of fluorescence in epifluorescence mode around the droplet perimeters (Figure 3(b)). ImageJ analysis plots indicate the image pixel intensity difference across the droplet diameters (Figures 3(a) and 3(b)). In confocal scanning mode, contiguous scans across droplets in the Z-plane revealed a concentration of fluorescence at the droplet interface (Figures 4(a) and 4(b)). This observation is suggestive of protein binding to the droplet interface and a likely cause for the decreased enzyme activity observed in the P450 DMF enzyme inhibition experiment. The greater surface-area-to-volume ratio of $200 \mu\text{m}$ diameter droplets (30:1 *cf.* 2:1 in the microtitre plate) may result in a greater proportion of enzyme binding at the droplet-oil interface. Such binding may result in decreased activity via conformational change or interaction with the surfactant in such a way as to block the active site of the enzyme. Further studies would be required to confirm the precise nature of the mechanism(s) involved in the reduction of enzyme activity.

Enzyme activity recovery using blocking proteins

Titration of BSA or gelatin as blocking proteins in the 6 min DMF control activity experiment increased the signal compared to when not present. In the microtitre plate, the same conditions of blocking protein had no discernible positive or negative effect on the quantified signal for the same incubation time. In the case of gelatin, the signal recovery was slightly better than for BSA. Partial binding of the substrate to BSA may explain the weaker signal obtained compared to when using gelatin. The fluvoxamine pIC_{50} value obtained when using either gelatin or BSA was observed to be more variable at different concentrations of blocking protein and was also, on average, lower compared to when not using blocking protein. This may be due to competitive protein interaction with either the substrate (CEC) or fluvoxamine inhibitor, resulting in an apparent reduction of inhibitor potency (Figures 5(a) and 5(b)).

SUMMARY AND CONCLUSIONS

We have shown that drug-like compounds readily partition between the dispersed aqueous droplet and oil carrier phases, especially in high surface-area-to-volume ratio droplets. Surfactants, often necessary to produce robust droplets, were shown to be problematic in respect of biphasic systems, and close-packing of droplets may exacerbate partitioning by increasing the solubility of molecules in the fluoruous phase via the various mechanisms previously referred to. There exists a difficulty in quantifying compound concentrations within the droplet at any point in time. Finally, we have demonstrated non-specific localisation of protein/enzyme at the water-oil boundary and the impact this has on a 1A2 P450 cytochrome inhibition assay in the droplet format, and have demonstrated how blocking proteins can be used to reduce undesirable surface interactions, albeit with some change in pharmacology. We recognise that work is required in this field to further characterise the extent of partitioning within biphasic droplet environments and how different chemistries may be affected.

FIG. 5. (a) Control activity of 1A2-CEC NADPH mediated reaction in the microtitre plate in the presence of gelatin and BSA titrations independently co-administered to the enzyme-substrate solution. No significant impact on activity is seen for the same incubation time at any concentration of blocking protein; (b) Control activity seen in the equivalent DMF experiment. Addition of BSA or gelatin increases the reaction signal and exceeds that obtained for the 6 min DMF experiment. The signal magnitude appears to correlate with increasing gelatin concentration. For BSA, higher concentrations appear to cause a reduction signal after an initial increase, possibly due to non-specific binding of substrate or 1A2 to the BSA; (c) Addition of BSA or gelatin to the microtitre plate results in a reduction in fluvoxamine potency; (d) Fluvoxamine potency is reduced in the DMF. The pIC₅₀ value seen for BSA (1000 µg/ml) where potency is observed to be higher may either be erroneous, or at the higher BSA concentration, there are other binding events leading to enhanced inhibition of the enzyme.

We conclude that for the successful use of microfluidic systems in quantitative assays, in particular, those where longer incubations are required, careful consideration of the chemistry and biology used in the assay and the role of water-oil surface artefacts is essential.

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