Microwell methods for fast process development

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By

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Abstract

The combination of automated liquid handling robots (LHR) and microwell plates present a possible methodology for processing the large number of potential drug targets produced by drug discovery and combinatorial chemistry programs. Many biological processing operations do not require harsh physical environments and can therefore easily be completed in standard, off-the-shelf microwell supports. The key questions for automated microscale bioprocessing relate to the reproducibility and robustness of each unit operation carried out in a standard microwell support.

The utility of an automated liquid handling robot integrated with a microwell plate reader to enable the rapid acquisition of bioprocess kinetic data has been investigated. The relationship between the key parameters for liquid handling accuracy and precision and the sample detection period has been characterised for typical low viscosity (<2.0mPas) aqueous and organic phases and for a high viscosity aqueous phase (60mPas), all exhibiting Newtonian rheology. The use of a simple graphical method enables the suitability of a given automation platform to be assessed once these key parameters have been determined. Leading to a priori prediction of well usage within each microwell plate to maximise productivity.

Macro-mixing is commonly ignored by many laboratories when using microwell supports for analytical assays and bioprocess development. A microwell combined with a LHR is modelled using standard jet mixing theory. An inert food dye was used in conjunction with high-speed video technology to capture the stages of macro-mixing. Correlations have been produced for both 96-standard and 96-deep microwells. Thus allowing the prediction of the minimum addition volume needed to complete macro-mixing in a static microwell.
Abstract

Micro-mixing conditions with a reaction vessel have been shown to effect the product distribution in situations where multiple reaction pathways are available. An assessment of micro-mixing conditions has been made using a competitive chemical reaction system and response surface methodology techniques. The key parameters influencing the micromixing efficiency have been identified for each reactor configuration. Engineering design equations have also been produced allowing the prediction of the micromixing environment from initial operating conditions. Common micromixing efficiencies have been highlighted as a possible scale-up methodology.

A number of case studies demonstrating the applicability and implications of each of the three systems on the generation of bioprocess development data have been completed. These have highlighted the effects of reaction rates on the quality of data generated while measuring reaction kinetics, the need to complete macro-mixing within the jet lifespan to ensure quantitative data is produced and strategies for counteracting the effects of poor macro-mixing when high reagent costs prevent the completion of macro-mixing within the jet lifespan. A study of the effects of micromixing efficiency on plasmid DNA yield and quality during the chemical lysis of bacterial cells has also been detailed. This has demonstrated that chemical denaturation of plasmid DNA has an influence on the yield and quality of the plasmid and the micromixing efficiency plays a role in the determination of the denaturation rate. Caveats have also been discussed to suitability of using micromixing efficiency as a scale-up tool from the microscale to bench and pilot scales.
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Nomenclature

- **a**: Volume ratio of reactants
- **A**: Addition reagent
- **A_1**: Dimensionless constant
- **Abs**: Light adsorption
- **b**: Virtual origin of the jet (m)
- **B**: Bulk fluid environment
- **c_{Ao}**: Concentration of reagent A in reactor if well mixed and unreacted (M)
- **C_{Ao}**: Initial feed concentration of reagent A (M)
- **d**: Impeller diameter (m)
- **d_i**: Nozzle internal diameter (m)
- **D**: Reactor diameter (m)
- **D_o**: Reference vessel diameter (m)
- **D_{Da}**: Engulfment model Damköhler number
- **D_{Da_i}**: Incorporation model Damköhler number
- **DMSO**: Dimethyl sulphoxide
- **e**: Molar extinction coefficient (m^2 mol^{-1})
- **E**: Engulfment constant (s^{-1})
- **h**: Liquid height (mm)
- **I**: Ionic strength (M^{-1})
- **k**: Rate constant of iodate redox reaction (M^4 s^{-1})
- **k_2**: Rate constant of reagent A consuming reaction (m^3 mol^{-1} s^{-1})
- **K_B**: Equilibrium constant of tri-iodide / iodine equilibrium (M^{-1})
- **l**: Optical path length (m)
- **n**: Population size
- **N**: Impeller speed (rps)
- **OD_{353}**: Optical density at 353 nm
- **OD_{360}**: Optical density at 360 nm
- **OD_{595}**: Optical density at 595 nm
- **P_o**: Power number
- **r**: Rate of redox reaction (Ms^{-1})
- **Re_j**: Jet Reynolds Number (Re_j = u_o d_i \rho / \mu)
- **RO water**: Reverse osmosis purified water
- **t_c**: Critical addition time (s)
- **t_{inc}**: Incorporation time (s)
- **t_j**: Jet lifespan (s)
- **t_m**: Jet macro-mixing time (s)
- **t_{95}**: Time constant for reaching 95% homogeneity (s)
- **T**: Temperature (K)
- **u_c**: Axial jet fluid velocity (ms^{-1})
- **u_o**: Initial jet fluid velocity (ms^{-1})
- **V**: Vessel volume (m^3)
- **V_A**: Addition volume (µl)
- **V_{crit}**: Minimum addition volume to ensure homogeneity (µl)
- **V_i**: Initial fill volume (µl)
Nomenclature

\( V_{\text{injection}} \) Acid addition volume (\( \mu \text{l} \))
\( V_{\text{PM}} \) Volume of perfectly micro-mixed solution (\( \text{m}^{-3} \))
\( V_{\text{ST}} \) Volume of perfectly segregated solution (\( \text{m}^{-3} \))
\( x \) Axial distance from nozzle (m)
\( x_1 \) Disk thickness (m)
\( X \) Jet length (m)
\( X_s \) Segregation index
\( Y \) Ratio of number of moles of acid consumed by redox reaction
\( Y_{\text{ST}} \) Ratio of number of moles of acid consumed by redox reaction when total segregation conditions dominate
\( Z \) Cumulative standard Normal distribution

96-DRW 96 deep round well plate
96-SRW 96 standard round well plate
%CV Coefficient of variance

\( [\text{H}_2\text{BO}_3^-] \) Initial borate concentration (M)
\( [\text{H}^+]_0 \) Proton concentration of feed stream (M)
\( [\Gamma] \) Iodide concentration (M)
\( [\text{I}_2] \) Iodine concentration (M)
\( [\text{I}_3^-] \) Tri-iodide concentration (M)
\( [\text{IO}_3^-] \) Initial iodate concentration (M)

Greek letters

\( \alpha \) Micro-mixedness ratio
\( \varepsilon \) Local energy dissipation rate (\( \text{Wkg}^{-1} \))
\( \varepsilon' \) Mean energy dissipation rate (\( \text{Wkg}^{-1} \))
\( \phi \) Proportionality constant
\( \lambda_x \) Kolmogorov scale (m)
\( \mu \) Viscosity (Pas)
\( \nu \) Kinematic viscosity (\( \text{m}^2\text{s}^{-1} \))
\( \theta \) Confidence interval
\( \rho \) Density (kgm\(^{-3}\))
\( \sigma \) Standard deviation
\( \tau_m \) Micro-mixing time (s)
\( \omega \) Vorticity (s\(^{-1}\))
1 Introduction and the theory of turbulent mixing

1.1 Introduction

The financial pressures to deliver new medicines quickly and economically to market are increasing while the regulatory hurdles necessary to ensure product safety lengthen the development time and inflate costs (Littlehales, 1999). To help speed drug discovery, automation and miniaturisation technologies have been widely implemented by companies over the last decade. The impact of these, however, has been an explosion in the number of potential new drug candidates (Balkenhohl et al., 1996) and a shift in the product development bottleneck from discovery activities to process development (Pollard, 2001; Weinmann et al., 1999).

This thesis aims to take the automation and miniaturisation technologies developed within discovery programmes and apply them to process development. Engineering principles will be applied to the automation and miniaturisation technologies to develop reproducible systems which can be utilised for route scouting and as large-scale mimics, two of the main components of process development.

1.1.1 Process development

The drug development cycle for a typical biopharmaceutical product from initial discovery to the final market release is generally 7-8 years (Struck, 1994). This can vary dramatically and is product specific; insulin was released within 5 years while INF-B1b took nearly fourteen years to reach market (Gosse and Mannocchia, 1996). Within this wide range of development cycle times there is a standard pathway that all drugs must complete before commercial release (Figure 1-1). The ‘holy grail’ of drug development is to produce a unique, efficacious drug and to bring it to market.
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Figure 1-1: Timeline for a typical drug development cycle. The window for bioprocess development is shown by the arrows.

in the shortest possible time.

The explosion in potential drug candidates and intermediates has focused efforts within the drugs industry on finding new ways of reducing the amount of time and money invested in process development without reducing the quantity and quality of the data generated. The greatest pressure is currently on the first stage of process development, route scouting (Pollard, 2001), where a series of alternative process options must be identified and evaluated prior to final process selection. Route scouting involves the use of a large number of small-scale experiments to assess the effect of different operations, reagents and conditions. Commonly this involves simple “yes” / “no” or “better than” / “worse than” answers. The process identified does not necessarily have to be optimal but must show the capability for future optimisation during later scale-up. The small scale trials (1-10 mL) currently utilised for route scouting are also time-consuming and many experimental approaches do not lend themselves to easy automation and thus limit the rate of experimentation.

Though key parameter identification through route scouting would be the first stage of process development the ultimate goal would be to construct a model of each
unit operation, allowing the \textit{a priori} prediction of process performance with a given set of conditions. A number of groups have started to develop ultra-scale down models of large-scale unit operations such as fermentation (Kostov \textit{et al}, 2001; Lamping \textit{et al}, 2003), centrifugation (Mannweiler and Hoare, 1992; Boychyn \textit{et al}, 2000) and precipitation (Boychyn \textit{et al}, 2000). These represent an advance in reducing costs and speeding up the rate of bioprocess development when compared to the current techniques used within the pharmaceutical industrial but there are significant difficulties in the automation of these approaches.

1.1.2 Microscale bioprocessing techniques

Automated microscale processing techniques, defined as the combination of automated experimentation and bioprocess studies carried out in microplate formats (Lye \textit{et al}, 2003), have the potential to overcome the route scouting bottleneck and to provide valid large-scale mimics. Already a range of bioprocess unit operations have been demonstrated in microwell formats, most notably microbial fermentation (Doig \textit{et al}, 2002; Elmahdi \textit{et al}, 2003; Girard \textit{et al}, 2001; Maharbiz \textit{et al}, 2004; Minas \textit{et al}, 2000; Welch \textit{et al}, 2002). The potential for parallel experimentation (Weuster-Botz, 2005) and the operation of automated whole process sequences has also been shown (Lye \textit{et al}, 2003; Ferreira-Torres \textit{et al}, 2005).

The standardised geometry within microwell plates allows easy automation since the technology is commonly used already in high throughput screening (HTS) (Berg \textit{et al}, 2001; Delvin, 1997) and laboratory assays (Kitchen \textit{et al}, 2003; Zheng and Wozniak, 1997). Though microwell plates are routinely used in screening, their potential for process experimentation has hardly been investigated. Key to the success of bioprocess development utilising microscale technologies are the throughput of
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operations, reagents and conditions, the system flexibility, the system reproducibility and its cost.

Common first steps in a microscale approach would be the use of automated liquid handling robots and fast analytical devices, such as microwell plate readers. These are commonly utilised within drug discovery and combinatorial chemistry programs but have so far only had limited uses within bioprocess development (Lye et al, 2003). Automated liquid handling robots have been developed to allow the fast and accurate movement of liquid in the X, Y and Z planes. Commercial microplate formats (96-standard round well, 96-deep round well and 24-deep well) and reagent vessels, ranging from 100 mL to 10 mL, mounted onto a platform provide the sources and destinations for these liquid movements. To increase the productivity of liquid handling systems multiple pipette tips are utilised where the number of pipette tips is dependent on the model and manufacturer. A review of liquid handling robots currently on the market is given by Lye and co-workers (Lye et al, 2003). Additional equipment pieces are available that allow liquid temperature control and automated microwell filtration. The combination of this speed and range of platforms provide the liquid handling robot with great flexibility which can be applied to a range of uses.

Ensuring the reproducibility of data collected within microwell formats is crucial. To date, microwell experimentation has mainly been applied to high throughput drug discovery (Delvin, 1997; Lamsa et al, 2004; Wegener et al, 2003) and the routine performance of laboratory assays (Kolb, 1994; Wang et al, 2003). Here the focus has been on understanding the reliability and operation of the liquid handling robots used to perform experiments (Astle and Akowitz, 1996; Berg et al, 2001; Stevens et al, 1998). For bioprocess development studies it is equally important to understand how the physical environment within a microwell impacts on the
reproducibility and scalability of the data obtained (Berg et al, 2001; Lye et al, 2003). Recent engineering studies have therefore begun to address gas-liquid mass transfer and mixing phenomena in microwells (Doig et al, 2005; Duetz and Witholt, 2001; Hermann et al, 2001; John and Heinzle, 2001; Weiss et al, 2002;) and how to control the environment within microwells (Elmahdi et al, 2003). A number of guidelines have been produced as aids to speed up process development (Ager, 2001; Mukesh, 1999) and these have been utilised as a guide to developing the protocols discussed within this study.

The use of microwell technologies allow significant cost reductions to be made, as the quantities of reagents are minute compared to traditional pilot scale. Some key intermediates and reagents are prohibitively expensive and prevent a bioprocess from being fully developed at the pilot scale. The reduction in reagent costs must however be balanced against the initial capital cost of the liquid handling robot.

1.1.3 Integrated robotic systems

A liquid handling robot integrated with an analytical device represents a powerful tool for completing assay and bioprocess development. The integration of the two devices would increase the throughput of the system beyond using them as stand alone devices. Each machine would normally come with its own operating software and terminal but these can easily be integrated, through the use of macro software, so that both devices can be operated from one terminal, therefore increasing the user friendliness of the system. With their standardised geometry and ability to be easily automated, microwell formats lend themselves to the use of statistical design of experiment tools (DOE). This is a practical methodology by which multivariable experiments may be designed and results analysed to establish the effects of a set of
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independent factors on one or more response parameters (Anderson and Whitcomb, 2000; Hicks and Turner, 1999).

A number of analytical devices can be integrated with a liquid handling robot (e.g., microwell plate reader, particle sizer and mass spectrophotometer). In such a situation a microwell plate reader might be integrated for the purposes of measuring bioprocess kinetics and this will be developed as a potential application in this thesis. Microwell plate readers were initially developed for analytical applications in blood chemistry (Bednar et al., 1995), food science (Cameron et al., 1992) and microbiology (Metzger et al., 2001). A number of groups have looked at the measurement of process kinetics within microwell plates (Grant and Pattabhi, 2001; John and Heinzle, 2001; Metzger et al., 2001) but there are currently very few kinetic studies using liquid handling robots and microwell plates (Cameron et al., 1992; Doig et al., 2002). Therefore, we have taken the gathering of kinetic data, over the course of a bioprocess operation, as the first test of the utility of using integrated automation platforms in bioprocess development.

For such a microscale processing approach to be useful it must display the following features: (1) be able to sample wells reproducibly with a set frequency, (2) exhibit the flexibility to vary the sample detection period independently of the trial system and (3) maximise productivity within each microwell plate by a priori prediction of the degree of well utilisation. When using the combination of a liquid handling robot and a microwell plate reader, the minimum sample detection period is inversely proportional to the speed of liquid handling, the speed of detection by the microwell plate reader and the number of wells utilised on the plate. The relative importance of these three factors on the sample detection period will be investigated in this study. To be able to represent these interactions the data will be displayed
using operating windows (Woodley and Titchener-Hooker, 1996), which allow the simple visualisation of multivariate and complex design data. They also provide a convenient way to gain insight into a unit operation and can be applied to determine operational conditions or provide data for process design when scaling an operation.

1.1.4 Turbulent mixing

The maintenance of good mixing within a reaction vessel is fundamental to reproducibly complete a process operation. Extensive research in the biopharmaceutical and chemical industries has been completed at the large-scale into the maintenance of key component concentrations during processing (Hamby, 1992; van't Riet and Smith, 1973). The maintenance of a reproducible physical environment in microwell plates has hardly been investigated (Berg et al., 2001) and is often ignored when microwell plates are utilised during analytical techniques. Berg and co-workers observed that low volume additions completed by an automated liquid handling robot were not sufficient to ensure a well-mixed environment within the microwell. Macro-mixing was completed through the use of a secondary mixing step. A comparison between the poorly mixed and well-mixed environments within the microwells resulted in a significant difference in the final fluorescence reading of the assay. The well-mixed microwell produced fluorescence readings double that of poorly mixed microwells. Indicating that macro-mixing within the microwell can significantly alter the end result of a simple assay. To remove this inherent variability and for automated microwell technologies to be utilised in process development the physical environment within the microwell must be defined and controllable.
1.1.5 Gene therapy and DNA manufacture

Interest in gene therapy has blossomed since rapid advances in molecular biology and recombinant DNA techniques have allowed researchers to probe the methodology of cell control by genes and whether it is possible to manipulate these control pathways. Gene therapy involves the delivery of a functional gene for expression in somatic tissues for the selective correction or control of diseased states. Theoretically, the single administration of a modified gene would result in a cure or suppression of the disease (Freidman, 1997). This potential to utilise DNA vaccines as a treatment for genetic and acquired disorders has lead to a research explosion since the beginning of clinical trials (Marquet et al, 1995; Mountain, 2000).

For gene therapy to be effective the target gene must be identified and cloned into an expression vector which can then deliver the gene to the desired location and be expressed within the patient. Two types of expression vector have so far been utilised: viral vectors and DNA based vectors (Mountain, 2000). Viral vectors involve the transduction of the desired gene to the patients' cells from non-pathogenic viruses, which are rendered incapable of replication. Retroviruses and adenoviruses have been the most commonly used for phases I/II clinical trials (Mhasilkar et al, 2001). Plasmid DNA is used as the DNA vector either in its native form or with a protective layer, either phospholipids or conjugated polycations. This protective layer not only reduces the damage to the plasmid DNA during application but also increases the up-take and expression of the plasmid by the target cells (Nabel, 1993).

Plasmid DNA is found naturally as extrachromosomal entities within microorganisms. It consists of double stranded DNA conjoined at the ends to form a circle. Though non-essential for the survival of the micro-organism host they can often confer desirable traits to the host, such as antibody resistance. A number of
conformations of plasmid DNA exist and these have a direct influence on the activity of the plasmid genes. The supercoiled conformation is thought to be the most efficient at gene transfer and is therefore the desired final product. This form is negatively supercoiled through the DNA having fewer helical turns and is thus partially unwound, creating torsional tension within the plasmid. The open circle conformation is similar to the supercoiled in that it is a circle of DNA but one of the DNA chains has been broken and the supercoiling has unwound. Within micro-organisms this would be easily repaired through the use of a DNA gyrase. The final plasmid conformation is that of the linear form. Here the plasmid is no longer a circle as both DNA chains have been broken. In micro-organisms this could again be easily repaired to the supercoiled conformation but ligation with other linear DNA fragments would represent a danger. In a manufacturing environment the repair of the open circle and linear conformations are not possible and are so considered as impurities.

Due to the increase in the interest in gene therapy the manufacture of pure plasmid DNA to be used as vectors has become a priority (Ferreria et al, 2000; Levy et al, 2000; Meacle et al, 2004; Prazeres et al, 1999; Varley et al, 1999). Plasmid DNA is typically produced through the fermentation of an engineered E. coli strain, the cell ruptured and the plasmid DNA then purified. Key to this purification procedure is the chemical lysis of the bacterial cells. This must be achieved with minimal damage to the plasmid DNA while keeping impurities (Cell debris, protein, RNA, endotoxin and chromosomal DNA) to a minimum. The plasmid DNA is then isolated through selective flocculation and a number of chromatographic steps. The large size of the plasmid and chromosomal molecules effectively excludes the molecules from binding within pores in the chromatography resin (Ferreira et al, 1998). This significantly reduces the binding capacity of the resin and so increases the
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cost of chromatographic purification. The reduction in the impurities passed from the cell disruption phase to the chromatographic purification will greatly enhance the productivity of the protocol.

Cell disruption is commonly achieved by chemical lysis utilising the protocol based on the procedure originally described by Birnboim (Birnboim and Daly, 1979). This involves the use of an alkaline pH in conjunction with a detergent, sodium dodecyl sulphate (SDS). Initially, cells harvested from a fermentation are suspended in a Tris-EDTA buffer (pH 8.0). This provides a stable pH environment for released DNA (Middaugh et al, 1998) while the EDTA helps to disrupt the E. coli cell walls and reduces DNAase activity through the chelation of divalent cations. Upon addition of the alkaline detergent lysis solution the anionic SDS disrupts the non-covalent interactions in the cell wall (Scopes, 1994). The SDS binds to cationic sites on the wall and membrane proteins, causing denaturation. This protein denaturation leads to the solubilisation of the wall and membrane, releasing the intra-cellular contents. The alkaline pH of the lysis reagent leads to further protein denaturation and to the irreversible denaturation of the chromosomal DNA. Birnboim (Birnboim and Daly, 1979) reported that the irreversible denaturation of chromosomal DNA, without the denaturation of plasmid DNA requires a pH environment between 12.0-12.6. Later, the denaturation range of plasmids useful for gene therapy has been identified as being between 12.1 and 12.9 with only a range of ±0.2 pH units required to convert from soluble, supercoiled plasmid to a completely denatured form (Thatcher et al, 1997). The second stage of the chemical lysis involves neutralisation with ice-cold potassium acetate. The increase in the ionic strength causes the salting out of the SDS – protein complexes and the decrease in pH leads to the flocculation of SDS, denatured protein, cell wall debris and chromosomal DNA. The supercoiled plasmid
remains in solution and can be removed from the flocs through centrifugation or filtration.

The operation of a chemical lysis methodology utilising an alkaline SDS reagent is notoriously difficult. The optimal pH level of the lysis reagent is often determined by trial and error for each particular plasmid. The final protocol must ensure the complete denaturation of the chromosomal DNA while leaving the plasmid DNA unaffected. Chemical lysis has been demonstrated within stirred tanks at up to scales of 15 L (Chamsart et al, 2000; Meacle et al, 2004; Theodossiou and Dunnill, 1999; Varley et al, 1999), in static in-line mixers (Wan et al, 1998) and in opposed jet mixers (Ciccolini et al, 2002; Meacle, 2003). The effect of macro-mixing times on the yield of plasmid DNA has been demonstrated but the influence of micro-mixing efficiency, key to product segregation within chemical reactions, has not been fully investigated (Ng et al, 1996).

The physical environment within the reaction vessel also plays a key role in the impurity profile of the plasmid rich stream leaving the unit operation. Levy and co-workers (Levy et al, 1999a) stated that increased fluid stress lead to an increase in the chromosomal DNA contamination of up to 25% at a fluid strain rate of 760 s\(^{-1}\). Subsequent studies have demonstrated that though increased shear rates lead to the fragmentation of chromosomal DNA to smaller sizes this does not lead to significant chromosomal DNA contamination (up to 760 s\(^{-1}\) (Chamsart et al, 2001)) until high shear rates are reached (10\(^4\)-10\(^5\) s\(^{-1}\) (Meacle et al, 2004)).

The ideal mixer type for the completion of the chemical lysis of bacterial cells would involve a low shear device, preventing significant contamination by chromosomal DNA, with a high micro-mixing efficiency, minimising plasmid DNA denaturation by areas of high pH.
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1.2 The theory of turbulent mixing

Formal definitions of mixing efficiency and scale were produced early in the development of the field (Danckwerts, 1953a; Danckwerts, 1953b; Fossett and Prosser, 1949; van’t Riet, and Smith, 1973; Uhl and Gray, 1966) and the effects of bulk mixing (macro-mixing) and molecular scale mixing (micro-mixing) were quickly identified (van Krevelen, 1958; Danckwerts, 1958). These have been further refined to produce three distinct realms of mixing with their specific characteristics, macro-, meso- and micro-mixing (Baldyga and Bourne, 1992; Baldyga and Pohorescki, 1995; Baldyga et al., 1997). Macro-mixing can be defined as large scale flow processes up to the scale of the reaction vessel; meso-mixing has been identified as exchange between freshly entering material and its surroundings within the reaction zone. This exchange is coarse-scaled compared with micro-mixing but localised in comparison with bulk blending. Micro-mixing in liquids has been determined as the viscous-convective deformation of fluid elements, followed by molecular diffusion.

These three mixing regimes are interlinked and can be related to Kolmogorov’s theory of isotropic turbulence (Kolmogorov, 1941). The macro-mixing environment is characterised by anisotropic turbulence, low frequency primary eddies on the scale of the vessel with large velocity fluctuations, and determines the environmental concentrations for meso- and micro-mixing. Isotropic turbulence, high frequency small eddies which have lost the directional element of the main flow, is characteristic of meso-mixing and within liquid systems the inertial-convective disintegration of isotropic eddies proceeds with no effect on the molecular mixing. The structure of eddies within the inertial-convective subrange determines the micro-mixing environment. Micro-mixing is the final stage of turbulent mixing and is
1.2.1 Large scale homogeneity within reaction vessels

The rapid attainment and maintenance of liquid phase homogeneity is fundamental to the generation of reproducible data within microwell plates. At present, observations on mixing within microwells have mainly been reported for the types of shaken system used in fermentation studies (Duetz and Witholt, 2001; Hermann et al, 2001; John and Heinzle, 2001; Weiss et al, 2002). Many applications though, particularly those involving the study of enzyme kinetics and stability or biological assays are best carried out using static microwell plates (Berg et al, 2001; Lamsa et al, 2004; Lye et al, 2002; Aucamp et al, 2005). Here, rapid and complete macro-mixing must be achieved by the liquid additions made to each well by the pipette tips of the liquid handling robot.

1.2.1.1 Jet mixers

Jet mixing is well established in the chemical industries as a cost-effective method of mixing on the large scale (Baldyga et al, 1995a; Fossett and Prosser, 1949; Fox and Gex, 1956; Lane and Rice, 1981). Within jet mixing part of the bulk fluid is normally continuously recirculated by drawing it through a pump and returning it to the vessel through a nozzle. The resulting jet entrains the surrounding liquid and creates a circulating fluid flow pattern within the vessel. It is this fluid motion and entrainment of fluid in the jet itself that leads to macro-mixing of the vessel contents.

\[ \lambda_k = \left( \frac{v^3}{\varepsilon} \right)^{\frac{1}{4}} \]  \hspace{1cm} (1-1)
The early experiments by Fossett and Prosser (1949) proposed a simple correlation for the mixing time \( t_m \) which was independent of the jet Reynolds number as shown in Equation 1-2:

\[
  t_m = \frac{9.0D^2}{u_0d_i} \tag{1-2}
\]

Fox and Gex (1956) then extended this study to cover both the laminar and the turbulent flow regimes. They showed that the most important parameter in determining the mixing time was the momentum flux added to the vessel by the jet. They also determined that the mixing time is closely correlated to the jet Reynolds number in the laminar regime but is only weakly correlated in the turbulent regime. A number of contactor designs have been investigated and include the use of a vertical jet mixer (Hiby and Modigell, 1978) and a vertical mixer in a vessel with a hemispherical base (Lane and Rice, 1982). Work with hemispherically based tanks has been continued (Baldyga et al, 1995a) and has lead to a widely used correlation for estimating the mixing time in turbulent flow regimes:

\[
  t_{95} = \frac{4.48D^{1.5}h^{0.5}}{u_0d_i} \tag{1-3}
\]

A number of studies have investigated the flow patterns produced within a jet-mixed vessel and have proposed that the mixing time is a function of the jet Reynolds number and the longest jet length (Maruyama et al, 1982; Maruyama, 1986; Revill, 1992). An alternative approach also has been recommended, suggesting that the local turbulent energy dissipation rate at the end of the jet path controls the mixing rate for the whole vessel (Grenville and Tilton, 1996). A number of CFD models have been proposed that have emphasised the need to minimise or eliminate dead zones within a large vessel (Jayanti, 2001). These predict overall mixing times (Patwardhan, 2002;
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Zughbi and Rakib, 2002) and have investigated the effects of the jet position and the number of jets (Zughbi and Rakib, 2004) on macro-mixing within a vessel.

The typical structure of a free jet is shown in Figure 1-2. The three defined regions of fluid flow are also shown. Region 1 corresponds to the region containing the potential core; region 3 is defined by the production of a fully turbulent flow regime throughout the cross-section of the jet; region 2 has been identified as the transitional flow regime between regions 1 and 3.

Jet mixing theory can be applied to microwell experimentation by considering the system as a being a downward facing, vertical jet. However, instead of having a continuous re-circulation of liquid, true jet mixing will only occur during the lifespan of the jet ($t_j$). This jet lifespan will be determined by the liquid addition volume ($V_A$) and the volumetric flow rate of the addition. Ideally, complete macro-mixing should be achieved within the lifespan of the jet. There are two approaches that can be followed concerning novel microwell designs to achieve effective jet macro-mixing.

Figure 1-2: Typical structure of a free jet showing the potential core and the three defined fluid flow regimes. $D_i =$ nozzle diameter (m); $u_o =$ initial fluid velocity (ms$^{-1}$); $u_c =$ fluid velocity along jet axis (ms$^{-1}$); $A_1 =$ Dimensionless constant (Liepmann, 1991); $X =$ jet length (m).
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The first seeks to maximise fluid entrainment by ensuring a long jet path (X). This results in a microwell with an aspect ratio of 3:1 (Revill, 1992) and the jet remains free, unhindered by the vessel, throughout its length. The second seeks to maximise the local turbulent energy dissipation rate at the end of the jet resulting in a recommended well aspect ratio of $1/\sqrt{2}$ (Grenville and Tilton, 1996) with the jet impacting on the base of microwell.

The positioning of the jet nozzle is key to the performance of the jet mixer. The nozzle should be positioned below the liquid surface at a distance of $5d_i$ or less (Revill, 1992) or at a depth of 1mm below the surface when utilising microwell formats (J. Wrigley, Perkin Elmer Inc., personal communication).

A micro-mixing time ($t_{inc}$) and a characteristic mixing time ($t_m$) can be calculated. The micro-mixing time (Equation 1-4) is dependent on the kinematic viscosity and the energy dissipation rate (Fournier et al, 1996b) while the mixing time (Equation 1-5) is dependent on the axial distance from the nozzle ($x$) and the virtual origin of the jet ($b$). This has been shown to be in the range $d_i$ to $2d_i$ (Antonia et al, 1980), a value of $d_i$ will be utilised within this study (Baldyga et al, 1995b).

$$t_{inc} = 17.89 \left( \frac{\varepsilon}{\nu} \right)^{1/2} \quad (1-4)$$

$$t_m = \frac{0.1(x + b)^2}{d_i u_o} \quad (1-5)$$

The energy dissipation rate (Equation 1-6) within jet mixers can also be calculated from theory (Gholap et al, 1994) and is dependent on the axial distance from the nozzle ($x$).

$$\varepsilon = \frac{50d_i^3 u_o^3}{(x - 2d_i)^4} \quad (1-6)$$
The maximum energy dissipation rate has been shown to occur at $7d_i$ from the jet origin (Baldyga et al, 1994).

### 1.2.1.2 Stirred tank reaction vessels

Stirred tank reaction vessels have been well characterized (Hamby et al, 1992) with the macro-mixing being determined by the local energy dissipation rate within the reaction zone of the vessel. Standard mixing theory can be utilised to calculate this value for the range of conditions within this study. Within stirred tank vessels the local energy dissipation rate is ultimately determined by the stirrer size and speed. Though a number of intermediate steps are required for its calculation. The local energy dissipation rate is initially related to the mean energy dissipation rate ($\varepsilon'$) and the proportionality constant ($\phi$). The proportionality constant is dependent on the position of the feed inlet relative to the impeller. Within this study this is equal to 4 (Fournier et al, 1996a).

$$\varepsilon = \varepsilon' \phi \tag{1-7}$$

The mean energy dissipation rate is in turn determined by the power number ($P_o$), the vessel volume ($V$), the impeller diameter ($d$) and the impeller speed ($N$).

$$\varepsilon' = \frac{P_o N^3 d^5}{V} \tag{1-8}$$

The power number can be calculated for Rushton turbines from the disk thickness ($x_1$)

$$P_o = 2.5\left(\frac{x_1}{d}\right)^{-0.2} \left(\frac{D}{D_o}\right)^{0.065} \tag{1-9}$$

(Bujalski et al, 1987). Power numbers for propellers were interpolated from known data (Edwards et al, 1992).
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1.2.2 Micro-mixing efficiency within reaction vessels

Vessel homogeneity has been demonstrated to be important in determining the macro-mixing efficiency of a reaction vessel but this is unlikely to influence complex chemical reactions completed within the reaction vessel. Vessel homogeneity at the molecular scale will be the key influence on the product distribution of a chemical reaction. The effects of mixing efficiency on the product distribution and yield of a complex chemical reaction have economical and environmental significance within the chemical and pharmaceutical industries. The mixing efficiency will directly affect the raw material consumption, product purity and the cost and complexity of production purification. A number of groups have demonstrated an inter-relationship between product purity and selectivity when utilising a range of micro-mixing conditions (Akiti et al, 2005; Plasari et al, 1978; Suga et al, 2003; Yoshida et al, 2005).

1.2.2.1 Micro-mixing models

A number of micro-mixing models have been developed to predict the performance of reaction vessels and a review of micro-mixing models is given by Baldyga and Bourne (Baldyga and Bourne, (1999)). Two will be used in this thesis to compare the systems utilised within this study. Both models have been initially developed for application to stirred tank vessels but will be applied to both the stirred tank and jet mixed vessels within the study. The first model is based on the mechanism of mutual fine scale engulfment of the two fluids near the Kolmogorov scale (Figure 1-3). According to Kolmogorov all fine scale properties of turbulence are universal functions of the kinematic viscosity (ν) and the rate of dissipation of turbulent kinetic energy to heat (ε). The rate of engulfment was defined as
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Figure 1-3: Engulfment model of micro-mixing. A schematic representation of the formation of laminated structure through the action of vorticity ($\omega$). [Reproduced from Baldyga and Bourne, 1999]
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\[ E = 0.058\left(\frac{\varepsilon}{\nu}\right)^{0.5} \]  \hspace{1cm} (1-10)

and a characteristic micro-mixing time was defined as:

\[ \tau_m = E^{-1} = 17.24\left(\frac{\varepsilon}{\nu}\right)^{\frac{1}{2}} \]  \hspace{1cm} (1-11)

To apply the engulfment model to parallel reactions where the stoichiometric and volumetric ratios of the reagents are fixed the product distribution, \( X_s \), depends on a Damköhler number (Equation 1-12). The Damköhler number represents a ratio of the characteristic micro-mixing time by engulfment and the rate of reaction consuming reagent A. Here \( c_{A_0} \) represents the concentration of the reagent A if the reagent addition and mixing was complete before any reaction took place. \( k_2 \) is the rate of reaction of the A consuming pathway.

\[ Da = \frac{k_2 c_{A_0}}{E} \]  \hspace{1cm} (1-12)

When the volumetric ratios of the reactants are not fixed a volume ratio term \( a \) needs to be included (Balduyga and Bourne, 1990). This leads to the final equation for a first order A consuming reaction.

\[ Da = \frac{k_2 c_{A_0}}{E(1 + a)} \]  \hspace{1cm} (1-13)

The second, incorporation model, was developed by Fournier and co-workers (Figure 1-4; Fournier et al, 1996b). This model is based on the addition reagent (A) being divided into aggregates, which are progressively invaded by fluid from the surrounding environment. The characteristic incorporation time (\( t_{inc} \)) is assumed to be equal to the micro-mixing time. Damköhler numbers (Equation 1-14) for this model

\[ Da_i = t_{inc} k_2 c_{A_0} \]  \hspace{1cm} (1-14)

were calculated differently to the previous model and are dependent on the micro-mixing time, the rate of reaction of the consuming pathway and the initial
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Figure 1-4: The incorporation model of micro-mixing. Aggregates of the addition fluid (A) are progressively invaded by fluid from the bulk fluid environment (B) leading to increasing areas of well mixed fluid (chequered).

concentration of the feed reactant (A) before mixing. Both models are dependent on the prediction of the local energy dissipation rate in the reaction zone.

1.2.2.2 Determination of micro-mixing efficiency through a competitive reaction system

A number of chemical systems have been utilised to measure the micro-mixing efficiency within reaction vessels and are reviewed by Fournier (Fournier et al, 1996a). An iodate -iodide redox reaction with neutralisation is utilised within this study (Guichardon and Falk, 2000a). This system depends on competitive parallel reactions. The neutralisation reaction (Equation 1-15) is instantaneous while the redox reaction (Equation 1-16) is slower but in the range of the micro-mixing process.

\[
\begin{align*}
H_2B_03^- + H^+ &\leftrightarrow H_3BO_3 \\
5I^- + IO_3^- + 6H^+ &\leftrightarrow 3I_2 + 3H_2O
\end{align*}
\]  

This competitive reaction system leads to a segregation of the final destination of protons added during an acid addition. The segregation is measured through the additional reaction of the iodine produced (Equation 1-17).

\[
I_2 + I^- \leftrightarrow I_3^-
\]  

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The tri-iodide generated can be detected through its absorbance at 353 nm. The kinetics of the redox reaction have been determined and found to be second order in relation to the proton concentration (Guichardon and Falk, 2000b) (Equation 1-18).

\[ r = k[H^+]^2[I]_2[IO_3^-] \]  

(1-18)

The rate constant \((k)\) is a function of the ionic strength \((I)\) and within the conditions used within the study is determined by Equation 1-19.

\[ \log_{10}(K_B) = 9.28105 - 3.664\sqrt{I} \]  

(1-19)

In perfectly mixed conditions the acid addition is instantaneously dispersed within the reaction vessel and is consumed by the neutralisation reaction (Equation 1-15). When mixing conditions are poor the characteristic dissipation time of the acid aggregates, micro-mixing time, will be in the same range or larger than the reaction time of the redox reaction (Equation 1-16). These acid aggregates are then able to complete borate consumption and react via the redox pathway yielding iodine before fresh borate ions can be mixed into the aggregate. The selectivity of iodine is then a measure of the segregation state of the fluid. The borate ions have a secondary role in the reaction system as a buffer. Within the acid aggregates iodine formation becomes thermodynamically possible due to the low pH. If the mean pH within the reaction vessel falls below the iodine dismutation pH (Custer and Natelson, 1949) iodine is naturally formed in the absence of acid aggregates. The initial pH of the reaction mixture before the acid addition is also critical to the success of the system. Iodine is thermodynamically unstable at high pH values and will quickly dissociate in a high pH environment.

For an assessment of micro-mixing conditions within a reaction vessel to be made micro-mixing must be uncoupled from the effects of macro-mixing. This can be
achieved through the use of long addition times (Figure 1-5). During short addition times the segregation index is independent of the macro-mixing environment but after a critical addition time \( t_c \) the segregation index becomes constant and macro-mixing no longer plays a role in the determination of the segregation index (Baldyga and Bourne, 1992).

The use of this iodate – iodine reaction system to determine the micro-mixing efficiency and the use of the results to compare the models of micro-mixing requires an alteration to be made to the Damkohler number calculations. The equations to calculate the Damkohler numbers for the engulfment (Equation 1-13) and incorporation (Equation 1-14) models rely on the use of a first order reaction system. The key redox reaction has been shown to be second order relative to the proton concentration (Equation 1-18) and so the Damkohler number calculations for the iodate – iodide reaction system would now read as follows:

\[
D_{\text{f}} = \frac{k_2C_{A_0}^2}{E(1 + a)} \quad (1-20)
\]

\[
Da_{\text{i}} = t_c k_2 C_{A_0}^2 \quad (1-21)
\]

![Figure 1-5: Elimination of macro-mixing in the determination of the segregation index.](image-url)
1 Introduction and the theory of turbulent mixing

1.3 Organisation and aims of the thesis

The principle aim of the thesis is to develop automated microscale techniques that can be utilised for route scouting or as mimics of large-scale unit operations. A number of process systems will be investigated and their utility demonstrated through their application to real life process situations. The thesis has the following major objectives.

Objective 1: Investigation of the utility of integrated robotic systems (Chapter 3)

Automated liquid handling robots and analytical devices capable of utilising microplates are currently operated as separate entities. The integration of a liquid handling robot and a microwell plate reader to be utilised in the measurement of bioprocess kinetics is investigated. The reproducibility of liquid dispersion by the liquid handling robot is investigated along with the limitations imposed through the maintenance of precise and accurate liquid dispersion and the physical parameters of the equipment on the integrated format productivity.

Objective 2: Determination of macro-mixing times in a static microwell plate (Chapter 4)

Techniques utilising microwell plates commonly exhibit poor levels of reproducibility. This may be a result of large-scale in-homogeneities within the microwell. Microwells are often mixed by plate shaking to remove these in-homogeneity. This is not always an available option when using microwell plates for bioprocess development. Therefore, a protocol will be developed for the removal of in-homogeneities through modelling volume additions to microwell plates as a downward facing jet mixer. A high speed video technology will be used to measure
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macro-mixing times and to determine the minimum addition volume required to complete macro-mixing during the life span of the jet. The degree of in-homogeneity resulting from addition volumes below this minimum will also be investigated.

Objective 3: Calculation of micro-mixing efficiency in a range of mixer types and scales (Chapter 5)

A competitive chemical system will be utilised to measure the micro-mixing efficiency in microwell plates (96-standard round well plate and 96-deep round well plate) and in stirred tank reactors (500 mL and 10 L). Comparisons will be made to two theoretical micro-mixing models and their utility in predicting micro-mixing efficiencies from initial parameters will be discussed. It will be determined whether common micro-mixing efficiencies can be produced across the reactor types and scales and whether micro-mixing efficiencies in large-scale vessels can be mimicked in microwell plates.

Objective 4: Applications of microscale techniques for bioprocess development (Chapter 6)

To test the applicability of the microscale techniques developed within this thesis a number of test cases are investigated. The gathering of kinetic data from a number of biological systems with varying sample times is used to test the applicability of a liquid handling robot integrated with a microwell plate reader. The suitability of the integrated system and the effect of sample frequency on the productivity and data quality are investigated for each biological system.

The simple visualisation of the influence of addition volume on the homogeneity within a microwell will be completed for a number of microwell formats. Such a study will allow the prediction of minimum addition volumes
necessary for efficient macro-mixing and to identify when secondary mixing steps are required to remove in-homogeneities.

The effect of micro-mixing efficiency on the chemical disruption of *E. coli* cells for the isolation of plasmid DNA will be investigated. The utility of microscale technologies will be determined in ascertaining the relative importance of micro-mixing efficiency during plasmid DNA recovery. The ability to use microwell techniques to mimic large-scale unit operations will also be investigated.

**Objective 5: The bioprocess management, regulation and economics of microwell technology (Chapter 7)**

Microwell technology represents a potential solution to the bottleneck of process development within the drug discovery cycle. Route scouting and the use of large-scale mimics have been identified as the main two procedures of bioprocess development. These two procedures are implemented at different stages within the cycle and companies will place different emphasis on each procedure depending on their ultimate aim during process development. This will be discussed for a range of types of drug companies from big-pharma to niche biotech. The challenges presented by these varying goals and the ability of microwell technologies to meet these will be investigated.

Implicit to the success of microwell technologies must be the speeding up of process development and the reduction of costs, or at least an increase in the generation of bioprocess data for a fixed cost. A cost assessment for the determination of the influence of micro-mixing efficiency on the yield of plasmid DNA during the chemical disruption of bacterial cells is made for both microwell and bench scale formats.
1 Introduction and the theory of turbulent mixing

As the regulatory burden that must be met during drug development increases year-on-year and is increasingly brought to bear earlier within the discovery cycle so microwell technologies must comply with these directives to become a useful bioprocess development tool. The main areas requiring regulatory documentation are highlighted if an automated liquid handling robot where to be utilised as a process development tool.
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2.1 Evaluation of integrated robotic systems for the measurement of bioprocess kinetics

2.1.1 Materials

Generally accepted models of a low viscosity aqueous phase, RO water, and a low viscosity organic phase, DMSO (Sigma, Poole, Dorset, UK.), were used to determine liquid handling accuracy and precision (Astle and Akowitz, 1996; Stevens et al, 1998). To model a Newtonian, high viscosity liquid 80% w/w glycerol (Sigma, Poole, Dorset, UK.) was used (Olsen, 2000). The physical properties of the solvents are given in Table 2-1. These can have a large influence on the liquid handling ability and therefore must be taken into consideration during the operation of any liquid handling robot.

Table 2-1: Physical properties of the liquid phases used in this thesis. [Data compiled from CRC Handbook 2002-2003]

<table>
<thead>
<tr>
<th>Phase</th>
<th>Liquid</th>
<th>Temperature (°C)</th>
<th>Viscosity (mPas)</th>
<th>Density (kgm⁻³)</th>
<th>Surface Tension (mNm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low viscosity aqueous</td>
<td>RO Water</td>
<td>20</td>
<td>1.0019</td>
<td>998.0</td>
<td>72.75</td>
</tr>
<tr>
<td>Low viscosity organic</td>
<td>DMSO</td>
<td>25</td>
<td>1.987</td>
<td>1101.4</td>
<td>42.92</td>
</tr>
<tr>
<td>High viscosity aqueous</td>
<td>80% w/w Glycerol</td>
<td>20</td>
<td>59.78</td>
<td>1208.5</td>
<td>66.00</td>
</tr>
</tbody>
</table>

2.1.2 Automation platform and operation

Automated liquid handling was performed using a four tip Multiprobe II™ liquid handling robot ([LHR], Perkin Elmer Inc., Beaconsfield, Bucks. UK), while a
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Spectracount™ (Perkin Elmer Inc.) was used as the plate reader (wavelength range: 340-670nm) (Lye et al, 2003). Liquid was dispensed directly into the wells of a 96-standard round well plate (96-SRW, Sarstedt Inc., Newton, NC, USA) situated on the automated stage of the plate reader. The LHR software, WinPrep (Perkin Elmer Inc.), was integrated with the Spectracount™ Plate Reader Version 3.0 (Perkin Elmer Inc.) software through the use of Macro Express (Insight Software, Kaysville, UT, USA), allowing the automatic take in and reading of the plate after reagent addition.

Two types of pipette tips (Figure 2-1) are available for use on the robot, fixed (d_i = 0.54 mm) and disposable (d_i = 0.6 mm). When a single dispense per aspirate is performed, use of both of these tip types is time consuming. An alternative approach is to use a multiple dispense function with fixed tips. This involves the aspiration of the total desired volume of a reagent, for all wells to be utilised, in a single step. This is followed by a series of dispenses of the required volume into each well without the need to refill or change tips. This greatly reduces the number of robotic operations required and hence, the time taken to complete liquid handling.

To allow accurate and precise handling of liquids with different physical properties the LHR was set up using two different performance files. A range of variables such as dispense and aspiration speed (1-1866 μLs⁻¹), air gaps (0-10 μL) and waste volumes (30-100%) make up each performance file. The performance files used here were as per the manufacturers recommendations (J. Wrigley, Perkin Elmer Inc., personal communication) and are summarised in Table 2-2 (low viscosity) and Table 2-3 (high viscosity phases).

For multiple dispenses per aspirate, the total volume that can be dispensed into each well will be dependent on the volume capacity of the robot, the number of wells to be utilised and the influence of the performance file configuration. Components of
Figure 2-1: The Multiprobe II™ LHR and WinPrep™ software. (a) The Multiprobe II liquid handling robot with various reagent troughs and microwell plates loaded onto the deck, (b) The two types of pipette tips available for use with the Multiprobe II, disposable (clear tips) and fixed tips, (c) The WinPrep software, with drop down menus allowing easy visualisation of the sequence of liquid handling operations with source troughs (Aspiration) highlighted in red, the destination troughs / microwells (Dispension) highlighted in blue and the cleaning and washing station highlighted in green. A box of disposable pipette tips (solid blue) and a pipette tip shute are also shown.
Table 2-2: Details of performance files used for the handling of multiple and single dispenses per aspirate with low viscosity aqueous (RO water) and organic (DMSO) phases.

<table>
<thead>
<tr>
<th>Dispense Volume (µl)</th>
<th>Aspirate Speed (µls⁻¹)</th>
<th>Aspirate Delay (sec x10⁻³)</th>
<th>Dispense Speed (µls⁻¹)</th>
<th>Dispense Delay (sec x10⁻³)</th>
<th>Waste Volume (µl)</th>
<th>Waste Volume (% of aspirate)</th>
<th>Transport Air Gap (µl)</th>
<th>System Air Gap (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>10</td>
<td>200</td>
<td>400</td>
<td>200</td>
<td>5</td>
<td>100</td>
<td>3.0</td>
<td>5.0</td>
</tr>
<tr>
<td>30</td>
<td>25</td>
<td>200</td>
<td>400</td>
<td>200</td>
<td>10</td>
<td>30</td>
<td>3.0</td>
<td>5.0</td>
</tr>
<tr>
<td>50</td>
<td>50</td>
<td>200</td>
<td>400</td>
<td>200</td>
<td>15</td>
<td>30</td>
<td>3.0</td>
<td>5.0</td>
</tr>
<tr>
<td>100</td>
<td>75</td>
<td>200</td>
<td>400</td>
<td>200</td>
<td>30</td>
<td>30</td>
<td>3.0</td>
<td>5.0</td>
</tr>
<tr>
<td>250</td>
<td>125</td>
<td>200</td>
<td>400</td>
<td>200</td>
<td>75</td>
<td>30</td>
<td>3.0</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Table 2-3: Details of the performance file used for the handling of multiple dispenses per aspirate using a high viscosity phase (80% w/w Glycerol).

<table>
<thead>
<tr>
<th>Dispense Volume (µl)</th>
<th>Aspirate Speed (µls⁻¹)</th>
<th>Aspirate Delay (sec x10⁻³)</th>
<th>Dispense Speed (µls⁻¹)</th>
<th>Dispense Delay (sec x10⁻³)</th>
<th>Waste Volume (µl)</th>
<th>Waste Volume (% of aspirate)</th>
<th>Transport Air Gap (µl)</th>
<th>System Air Gap (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>500</td>
<td>150</td>
<td>500</td>
<td>500</td>
<td>5</td>
<td>100</td>
<td>0.0</td>
<td>5.0</td>
</tr>
<tr>
<td>30</td>
<td>500</td>
<td>150</td>
<td>500</td>
<td>500</td>
<td>10</td>
<td>30</td>
<td>0.0</td>
<td>5.0</td>
</tr>
<tr>
<td>50</td>
<td>500</td>
<td>150</td>
<td>500</td>
<td>500</td>
<td>15</td>
<td>30</td>
<td>0.0</td>
<td>5.0</td>
</tr>
<tr>
<td>100</td>
<td>500</td>
<td>150</td>
<td>500</td>
<td>500</td>
<td>30</td>
<td>30</td>
<td>0.0</td>
<td>5.0</td>
</tr>
<tr>
<td>250</td>
<td>500</td>
<td>150</td>
<td>500</td>
<td>500</td>
<td>75</td>
<td>30</td>
<td>0.0</td>
<td>5.0</td>
</tr>
</tbody>
</table>
the performance file, such as the inclusion of air gaps and waste volumes, ensure accurate and precision liquid dispensation but will also reduce the maximum dispense volume and increase the dispense time. The performance files used here (Table 2-2 and Table 2-3) resulted in a maximum dispense volume of 2089.5μL.

2.1.3 Dispense accuracy and precision

To determine the accuracy and precision of liquid handling over a range of operating conditions, the mean and coefficient of variance (%CV) of each dispense step were calculated. The difference between the measured mean volume and the target volume was used to calculate the precision (Equation 2-1), while the standard deviation (σ) around the measured mean volume was used to calculate the accuracy (Equation 2-2) (Astle and Akowitz, 1996; Olsen, 2000; Stevens et al, 1998):

\[
\text{Precision, } \%CV = \left(\frac{\text{Mean} - \text{Target}}{\text{Mean}}\right) \times 100 \tag{2-1}
\]

\[
\text{Accuracy, } \%CV = \left(\frac{\sigma}{\text{Mean}}\right) \times 100 \tag{2-2}
\]

The acceptable lower limit for an accurate and precise dispense was defined as one where both %CV values were below 5% (Olsen, 2000).

Initially a comparison of the mean and %CV for each of the four pipette tips on the Perkin Elmer robot was completed using the three types of solvent described in Table 2-1. 100μL of solvent was dispensed into dry, pre-weighed HPLC vials using the multiple dispense function and six dispenses per aspirate, as recommended by the manufacturer for the greatest dispense precision and accuracy (J. Wrigley, Perkin Elmer Inc., personal communication). After the dispenses were completed the vials were immediately capped and re-weighed. The process was then repeated using the upper limit of 24 dispenses per aspirate. A wider range of volumes (5-200 μL) and number of dispenses per aspiration (3-24) were then studied for each of the model
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liquids. For these later experiments the liquid handling accuracy and precision measurements were carried out using a single pipette tip.

2.1.4 Acquisition of bioprocess data

To gather bioprocess kinetic data from a given system it is necessary to monitor each well utilised in a plate regularly. This can be achieved using a single well but is highly time consuming and inefficient. To increase the productivity of an automation platform multiple wells run in parallel can be used but for this to be achieved the wells need to be co-ordinated such that they are initiated and monitored as a block rather than individually. To maximise the degree of well utilisation the majority of the reagents are added to each well and then a final initiating reagent is added to all wells in one dispense step (e.g. the addition of a substrate to an enzymatic bioconversion). The sample detection period, defined here as the time between each monitoring of a specific well on a plate, would then be determined by the number of data points required and the limitations imposed by the integrated robot and plate reader. The minimum sample detection period is dependent on which of the plate set up and plate monitoring time is rate determining, where:

Plate set up time = Dispense time + Reader initialisation time (2-3)

Plate monitoring time = Plate reading time + Reader reset time (2-4)

The dispense time is defined here as the time taken by the robot to dispense a known solvent volume into a specified numbers of wells. The degree of well utilisation on a plate (12.5%-100%), the dispense volume and the performance file (Table 2-2 and Table 2-3) each contribute to the dispense time. The utilisation of fewer than 12 wells (12.5%) was not investigated since there would be little or no gain in the operation time over manual liquid handling. For each level of plate
utilisation, the robot was programmed to perform one aspiration and consequently the number of dispenses per aspirate necessarily increased with well utilisation. The LHR used here (Figure 2-1) is fitted with 1mL syringes. To be able to dispense total volumes greater than 1mL it is necessary to empty the syringe contents into the robot's system tubing (volume capacity = 3 mL) displacing, but not mixing with, the system fluid (water). Once this is complete, additional reagent can be aspirated into the syringes; this process can then be repeated a number of times. Upon dispensing the reagent the procedure is put into reverse; the initial dispense is from the syringe, the syringes are reloaded from the system tubing and dispensing continues. The dispense time incorporating all these operations was measured for a range of dispense volumes (5-200µL).

The reader initialisation period was defined as the time elapsed from the final dispense step to the initial reading of the first well. The plate monitoring time was defined as the time between reading the first well on the first scan of the plate and the reading of the first well on the subsequent scan.

2.1.5 Bioprocess operating windows

To display the effects of dispense volume and the minimum sample detection period on the degree of well utilisation, the timings (Section 2.1.4) and liquid handling data (Section 2.1.3) were combined into bioprocess operating windows (shown later in Chapter 3). The degree of well utilisation per plate is represented by the number of bioprocess conditions that can be studied. Each bioprocess condition was performed in quadruplet to improve the statistical accuracy of the results obtained (a 100% well utilisation for a 96-SRW plate thus corresponds to 24 quadruplet measurements of different bioprocess conditions).
2.2 Quantification of jet macro-mixing times in static microwell plates

2.2.1 Design of individual microwell mimics

The three designs of microwell utilised during the work described in Chapter 4 are shown in Figure 2-2. The first was an identical copy of a single microwell from a 96-standard round well (96-SRW) microtitre plate. The geometry of the 96-SRW is at the lower end of the aspect ratio range recommended by Revill (Revill, 1992) and is very close to that recommended by Grenville (Grenville and Tilton, 1996). The two custom designed wells utilised the recommendations made by Revill regarding the optimal aspect ratio (0.75 < h/D < 3.0) and jet length (X/di > 50). The small designed microwell maintained the same fill volume (200 µL) commonly used in 96-SRW plates while maximising the jet length.

The large designed well used the same aspect ratio but the volume of the well was based on maximising the jet length (X) to jet nozzle diameter (di) ratio. This is similar in size to the deep, round well plate (96-DRW) format commonly used in microscale bioprocessing applications (Lye et al, 2003). Each microwell mimic was manufactured in polished Perspex™ with a square external face to prevent any image distortion during high speed video photography (as described in Section 2.2.3).

2.2.2 Jet macro-mixing time experiments

All automated liquid handling was performed using the Multiprobe II™ LHR described previously in Section 2.1.2. Two types of pipette tip were used, fixed tips (di = 0.54mm) and disposable tips (di = 0.6mm). The performance files (Table 2-2) governing the operation of each tip type were set to ensure that (i) both the accuracy and precision of liquid handling was within a 5% CV limit for all dispense volumes.
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Figure 2-2: Dimensions and maximum volumes of Perspex™ microwell mimics used for the study of macro-mixing times (Chapter 4)

(Section 3.2) and that (ii) the dispense speed was sufficient that turbulent jet fluid flow \((Re_j \geq 1000)\) was maintained throughout each addition. All additions to the wells were performed at a fixed distance of 1\,mm below the liquid surface, in accordance with the recommended jet nozzle position (Revill, 1992). The tip position remained fixed relative to the liquid surface throughout liquid addition by the use of the LHR tracking function; this smoothly moves the tip of the pipette vertically upwards in proportion to the speed of liquid addition.

RO water was used throughout as a model low viscosity, aqueous phase (Table 2-1). Visualisation of macro-mixing was achieved by adding approximately 50% v/v of an inert food dye (Super Cook, Leeds, UK). Control experiments showed that addition of the dye at this level did not significantly alter the physical properties of the liquid. The majority of the macro-mixing experiments were performed under conditions that ensured homogeneity was reached within the lifespan of the jet \((t_j)\).
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For experiments with fixed tips a number of addition volumes (45% and 50% of fill volume) and jet Reynolds number (1000, 2480, 3000 and 3960) were used with each microwell design. For experiments with disposable tips, the limit on the addition volume was 120μL i.e. the volume of the disposable tip itself. This restricted the use of these tips to the standard round well and small designed well geometries. With the disposable tips, an addition of 50% of the initial fill volume and jet Reynolds numbers of 1,000 and 3,960 were used. The upper jet Reynolds number was determined by the maximum achievable fluid velocity (7.33ms⁻¹) of the LHR. A further set of fixed tip experiments were performed to determine the macro-mixing time in cases where homogeneity was not reached during the lifespan of the jet. This involved addition volumes of 5, 10 and 20% of the fill volume at a jet Reynolds number of 1000 for all three microwell designs.

2.2.3 High speed video photography and image capture

Macro-mixing times have previously been determined using a number of methodologies. Grenville and Tilton (1996) utilised conductivity measurements while in microwells John and Heinzle (2001) have determined macro-mixing times using fluorescent indicator dyes. High-speed video photography has also been widely used with shaken microwell systems to measure oxygen transfer rates (Hermann et al, 2001) and to visualise liquid hydrodynamics (Lye et al, 2002).

In this study, the progress of macro-mixing within each microwell was recorded using a NAC HSV-500 high-speed, analogue camera (NAC Image Technology, Simi Valley, CA, USA) at a recording speed of 125 frames per second (fps) and a resolution of 496 x 104 pixels. Under these conditions recording could occur for up to one minute. The continuous video stream was manipulated through the
use of Virtual Dub software (www.virtualdub.org) to produce still images of the macro-mixing process.

To quantify the macro-mixing times within each experiment, the still images produced by the video stream where digitised using Leica QWin Image Pro image analysis software (Leica Microsystems, Cambridge, UK). For each image, a vertical profile was analysed, originating immediately below the liquid surface (termed pixel 0) and terminating at the base of each well as shown in Figure 2-3. The meniscus and any air bubbles introduced during liquid addition were avoided so as not to introduce any artificial heterogeneity. For each of the microwell designs the axial mixing profile was obtained as close to the centre point of the microwell while avoiding the jet itself. Control studies showed that macro-mixing times determined close to the edges of a microwell could vary by up to ±20%, however, all mixing time values reported here were obtained using the slightly off-centre profile as this represents the bulk of the liquid volume in a well. The QWin image analysis software subsequently generated grey scale intensity profiles along the axis of each well. These were then converted to a false colour intensity profiles to allow easier visualisation with the naked eye. This accounts for the decrease in the colour intensity profile as the colour increases within the microwell (see for example Figure 4-4)

2.2.4 Macro-mixing time analysis

The colour intensity profiles were analysed statistically assuming that the readings for each profile will be arranged in a Normal distribution around a mean value (Hines et al, 2003). The values of the confidence limits of the distribution can easily be calculated from the standard deviation (σ), the confidence interval (θ), the population (n) and the cumulative standard Normal distribution (Z) as in Equation 2-5:
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Confidence limit = \( Z_{a/2} \frac{\sigma}{\sqrt{n}} \) \( (2-5) \)

To calculate the percentage homogeneity for individual frames throughout the time course of a liquid addition a reference distribution and confidence limits are required. In all cases, the reference 95% confidence limits were calculated from the colour intensity profile immediately before liquid addition in each experiment. This assumes that the liquid initially in the well was homogeneous. The position of the 95% confidence limit from the reference distribution was then super-imposed onto the measured distribution for each time point as indicated in Figure 2-3. The degree of homogeneity at each time point is then calculated from the proportion of points found to be within the super-imposed confidence limits.

2.3 Quantification of micro-mixing efficiency in jet mixed static microwell plates

To quantify the micro-mixing environment within static microwell plates the competitive iodate / iodide reaction system described in Section 1.2.2.2 was utilised.

2.3.1 Preparation of borate / iodate / iodide bulk solution

A standard borate / iodate / iodide bulk stock solution (0.182M \( \text{H}_3\text{BO}_3 \) : 11.7mM KI : 2.3mM \( \text{KIO}_3 \) : pH 8.75-8.95) was prepared in accordance with the methodology outlined by Guichardon (Guichardon and Falk, 2000a). Care was taken with the order of solution preparation to ensure that the iodate and iodide ions remained in a basic solution at all times to prevent the thermodynamic formation of iodine. All chemicals were supplied by Sigma Aldrich (Poole, Dorset, UK) and were of analytical grade unless stated otherwise. Separate solutions of boric acid (0.4531M), sodium hydroxide (0.91M), potassium iodide (1.17M) and potassium
Figure 2-3: Illustration of the methodology for calculation of percentage homogeneity using a Normal distribution
iodate (0.023M) were also prepared. These were then combined in the following order and volume ratios; H$_3$BO$_3$ : NaOH : KI : KIO$_3$ (40:8:1:10). Finally, 41 volumes of RO water were added to complete the standard borate / iodate / iodide stock solution. The stock solution pH was then checked to ensure it was within the required range (pH 8.9-9.2). A pH too basic would result in an unstable tri-iodide signal after acid addition, while a pH too acidic would result in the thermodynamic iodine formation after acid addition (as described in Section 1.2.2.2). This pH adjusted solution will be referred to as the borate stock solution hereafter.

2.3.2 Microwell experiments and acid liquid additions

2.3.2.1 Implementation of tri-iodide micro-mixing technique at the microwell scale

The Multiprobe II™ LHR (Figure 2-1) was used for all liquid handling operations. As described in Section 2.1.3 accurate and precise liquid handling are essential for the production of quantitative data when utilising a liquid handling robot. Therefore, it is important to determine the accuracy and precision of liquid handling over the range of volumes (42 µL - 530 µL) used to when determining the micro-mixing efficiency. The methodology described in Section 2.1.3 was used to calculated the liquid handling reproducibility of single dispenses per aspirate with fixed pipette tips, at Re$_j$ = 1000 and for both the blowout and waste modes of operation. The performance files detailed in Table 2-2 (waste mode) Table 2-4 (blowout mode) were used throughout the analysis.

Once the liquid handling reproducibility had been determined the effect of the mode of operation and the pipette tip type on the micro-mixing efficiency was investigated. A sulphuric acid (pH 0.67, 0.214M) addition was made to 200 µL of the
Table 2-4: Details of the performance file used for handling low viscosity aqueous phases utilising a single dispense per aspirate and the blowout mode of operation. The dispense speeds were determined by the pipette tip used and the need to maintain a jet Reynolds Number ($Re_j$) of 1000.

<table>
<thead>
<tr>
<th>Dispense Volume (µL)</th>
<th>Aspirate Speed (µL s$^{-1}$)</th>
<th>Aspirate Delay (sec x 10$^{-3}$)</th>
<th>Dispense Delay (sec x 10$^{-3}$)</th>
<th>Blowout Volume (µL)</th>
<th>Blowout Delay (sec x 10$^{-3}$)</th>
<th>Transport Air Gap (µL)</th>
<th>System Air Gap (µL)</th>
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</thead>
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<td>200</td>
<td>200</td>
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<td>200</td>
<td>20</td>
<td>100</td>
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<td>200</td>
<td>20</td>
<td>100</td>
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<td>5.0</td>
</tr>
<tr>
<td>130</td>
<td>75</td>
<td>200</td>
<td>200</td>
<td>20</td>
<td>100</td>
<td>3.0</td>
<td>5.0</td>
</tr>
</tbody>
</table>
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borate stock solution in a static 96-SRW plate, at 1mm below the liquid surface using either the blowout or waste mode of operation. Acid addition volumes were determined by the minimum volume to ensure complete macro-mixing ($V_{\text{crit}}$) calculated in Section 4.2.1, for fixed tips ($V_{\text{crit}} = 42 \mu\text{L}$) and for disposable tips ($V_{\text{crit}} = 46 \mu\text{L}$). The performance file previously described in Table 2-2 was used for the waste model of operation while that described in Table 2-4 was used for the blowout mode. In each case the dispense speeds were altered to maintain a jet Reynolds number of 1000 (424 $\mu\text{Ls}^{-1}$ for fixed tips and 471 $\mu\text{Ls}^{-1}$ for disposable tips). The tri-iodide signal generated by the acid addition was measured through its absorbance at 360 nm ($\text{OD}_{360}$) using a Spectracount™ plate reader (Section 2.3.4.1).

In control experiments to determine the stability of the tri-iodide signal a number of time delays (0-30s) were introduced between the final acid addition and the activation of the plate reader. With a time delay of 0 s the first well would be read after 19 s (as explained in Section 3.2) and with a 30 s delay would read the plate after 49 s. Sulphuric acid (pH 0.67, 0.214M, 42 $\mu\text{L}$) was added to 200 $\mu\text{L}$ of the borate stock solution using the waste mode of operation, fixed pipette tips and $\text{Re}_j = 1000$. The $\text{OD}_{360}$ was then measured using a Spectracount™ plate reader (Section 2.3.4.1).

When utilising the 96-DRW plates (equivalent to the large designed microwell in Figure 2-2) it was not possible to measure the $\text{OD}_{360}$ with the microwell due to the incompatibility of the size of the well with the plate reader. Therefore samples had to be taken from each well and transferred to a 96-SRW plate. Further control experiments assessed the effect of the sampling position within the microwell needed to be completed. Sulphuric acid (pH 0.71 – 1.05, 0.195 – 0.089M, 290 $\mu\text{L}$) was added to 1.72 mL of borate stock solution using the waste mode of operation. Fixed tips
were used with a $Re_j = 1000$ and washed after the addition step was completed. Samples (200 µL) were taken from either at the surface (1 mm below the surface) or at the mid point within the well and transferred to a 96-SRW plate. The $OD_{360}$ was then measured using the Spectracount™ (Section 2.3.4.1).

The trials determining the effect of the mode of operation, the stability of the tri-iodide signal and the sample position were completed in quadruplicate using all four of the Multiprobe II™ pipette tips.

2.3.2.2 Experiments designed for 96 - standard round well plate (96-SRW)

Based on the initial control experiments (Section 2.3.2.1) sulphuric acid (pH 0.67 – 1.05, 0.214 – 0.089M) was added 1 mm below the liquid surface to 200 µL of the borate stock solution in a single well of a 96-SRW plate (Figure 2-2) using either fixed or disposable pipette tips. The acid concentrations were varied to ensure a constant number of moles of $H^+$ (8.97 µmoles) were added as the addition volumes were varied. The number of moles of protons added was fixed by the need to produce a significant and stable tri-iodide signal upon acid addition. The $OD_{360}$ was subsequently measured using a Spectracount™ plate reader (Section 2.3.4.1). Three factors influencing micro-mixing efficiency were investigated within the 96-SRW plate; pipette diameter, addition volume and jet Reynolds number. For the pipette diameter there were two options, $d_i = 0.6$ mm (disposable tips) and $d_i = 0.54$ mm (fixed tips). The lower addition volume (42 µL) was determined by the minimum volume to ensure homogeneity ($V_{crit}$) calculated in Section 4.2.1 while the upper limit (100 µL) was determined by the maximum volume of the microwell. The jet Reynolds number limits (1000 - 3960) where set by the upper dispense speed of the Multiprobe
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II™ (1877 μLs⁻¹, giving Rej = 3960 with disposable tips) and the need to maintain a turbulent jet throughout the addition (Rej ≥ 1000; Revill, 1992).

The limits for the factors were then utilised using factorial design and RSM to investigate their effects on the micro-mixing efficiency. A Box-Behnken design (see Appendix) was constructed using Design Expert 5.0 software (Stat-ease Corporation, Minneapolis, MN, USA) with five mid-points and analysed using the methodology described in Section 2.3.4.3. Runs generated by the Box-Behnken design which required a pipette tip diameter mid-point were not feasible and so were altered to an even number of either the upper or lower limit of the pipette tip diameter. When disposable pipette tips were used the minimum Vcrit volume was 46 μL and hence the value for the addition volume was altered from 42 to 46 μL when disposable pipette tips were used. All sets of conditions for the Box-Behnken design were completed in quadruplicate.

2.3.2.3 Experiments for 96 - deep round well plate (96-DRW)

To determine the micro mixing efficiency in 96-DRW plates sulphuric acid (pH 0.71 – 0.97, 0.195 – 0.107M) additions were made to 1.72 mL of the borate stock solution within a single well of a 96-DRW plate (Large designed well in Figure 2-2). The acid concentrations were varied to ensure a constant number of H⁺ (56.8 μmoles) were added to each well as the addition volumes were varied. A 200 μL sample was then taken from the mid-point of each well and transferred to a standard 96-SRW plate so the OD₃₆₀ could be measured using the Spectracount™ (Section 2.3.4.1). Disposable tips are not suitable for the volumes of the initial acid addition (290 μL – 530 μL), so only fixed tips and the waste mode of operation were used throughout.
Only two factors could therefore be investigated with this plate geometry, the jet Reynolds number and the acid addition volume.

A full, 2-level factorial design (see Appendix) with three mid-points was used to determine the effect of the two factors on the micro-mixing efficiency and analysed using the methodology described in Section 2.3.4.3. The lower addition volume (290 µL) was determined by the minimum volume to ensure homogeneity ($V_{crit}$) calculated in Section 4.2.1 while the upper limit (530 µL) was determined by the maximum volume of the microwell. The jet Reynolds number limits (1000 – 3960) were set by the by the upper dispense speed of the Multiprobe II™ LHR (1824 µLs$^{-1}$, giving $Re_{j} = 3960$) and the need to maintain a turbulent jet throughout the addition ($Re_{j} \geq 1000$; Revill, 1992).

2.3.3 Stirred tank experiments and liquid additions

2.3.3.1 500 mL bench scale stirred vessel

The 500 mL bench scale vessel is a simple, baffled stirred tank with a standard Rushton impeller (Figure 2-4). Once the borate stock solution (500 mL) was loaded into the vessel a suitable time gap (1 min) was allowed for the stabilisation of the fluid flow patterns produced by the impeller. Then, sulphuric acid (pH 1 - 3, 0.1 – 0.001M) was added through the use of a P-6000 Pharmacia pump (GE Healthcare, Milton Keynes, UK) as indicated in Figure 2-4. The significance of the three factors investigated, impeller speed, addition volume and addition time on the micromixedness ratio was determined through the use of factorial design and response surface methodology (Section 2.3.4.3). A Box-Behnken design (see Appendix) with five mid-points was utilised. This gave a total of 17 runs which were grouped into one block. The factor ranges were determined prior to being incorporated into the design.
Figure 2-4: Stirred tank reaction vessels and impeller geometries. a) 500 mL bench scale vessel, b) 10 L pilot scale vessel. X indicates acid addition point.
The impeller speed range (250 - 1750 rpm) was determined by the limits of the stirrer motor (Stuart Scientific SS10, Bibby Sterilin Ltd, Stone, Staffordshire, UK) and the need to maintain a turbulent flow regime within the vessel. The addition volume range (0.25 - 25 mL) was determined by the maximum and minimum volume of acid that could be added to the borate stock solution such that a significant and stable tri-iodide absorbance could be detected. This was measured at 353nm using a Jenway 6400 (EssLab, Hadleigh, Essex, UK).

During the experiments the total number of moles of protons added (25 µmoles) was kept constant throughout all the trials and so a range of acid concentrations (pH 1 - 3, 0.1 - 0.001M) were utilised as the addition volume varied. The addition time range (15 - 273 s) was determined by the range of the fluid flow rates of the Pharmacia pump and the need to allow a long addition time to ensure that the mixing regime within the vessel is independent of macro-mixing (Guichardon and Falk, 2000a). After each trial three samples were taken, with a time interval of a minute between each sample, and the absorbance at 353nm (OD₃₅₃) measured (Section 2.3.4.1).

2.3.3.2 10 L pilot scale vessel

The 10 L pilot scale vessel used was a baffled stirred tank with a six blade propeller (Figure 2-4). Once the borate stock solution (10 L) was loaded into the vessel a suitable time gap (1 min) was allowed for the stabilisation of the fluid flow patterns produced by the impeller. Sulphuric acid (pH 0.32 - 1.18, 0.479 - 0.066M) was added to the borate stock via the use of either a Watson-Marlow 101F/R pump (Watson-Marlow, Falmouth, Cornwall, UK) or a Masterflex L/S easy load II pump (Cole Parmer, Hanwell, London, UK) depending on the flow rate. A constant tube diameter
was used for the two pumps to prevent the nozzle geometry from influencing the degree of tri-iodide production.

Factorial design and RSM were used to assess the significance of three factors (Impeller speed: Addition volume: Addition time) using the same methodology as the 500 mL bench scale vessel. A Box-Behnken design (see Appendix) with 5 mid-points was utilised. The range of the factors were predetermined before being included within the design. The impeller speed range (40 – 280 RPM) was determined by the lower speed of the stirrer (Heidolph Instruments GmbH & Co, Schwabach, Germany) and the upper limit was determined by the point when excessive surface aeration caused foam formation. The addition volume range (25 – 180 mL) was determined by the maximum and minimum volume of acid that could be added to the borate stock, such that a significant, stable absorbance at 353nm could be detected. During the pilot scale experiments the total number of moles of protons added (12 mmoles) was kept constant throughout all the trails and so a range of acid concentrations (pH 0.32 – 1.18, 0.479 – 0.066M) were utilised as the addition volume varied. The addition time range (60 – 180 s) was determined by the fluid flow rates of the two pumps and the need to allow a long addition time to ensure that the mixing regime within the vessel is independent of macro-mixing (Guichardon and Falk, 2000a). Three samples were taken after the completion of the acid addition, with a time interval of a minute between each sample, and the OD$_{353}$ measured (Section 2.3.4.1). All experiments were completed on site at GlaxoSmithKline, Beckenham.
2.3.4 Data analysis for micro-mixing experiments

2.3.4.1 Iodide concentration measurement by spectrophotometry

The iodide concentration can be directly determined assuming the Beer-Lambert Law holds. This states that the light adsorption (Abs) is proportional to the tri-iodide concentration through the molar extinction coefficient (e) at 353 nm or 360 nm (Equation 2-6)

\[ [\Gamma_3] = \frac{\text{Abs}}{el} \quad (2-6) \]

where \( l \) is the optical path length. A range of values of the molar extinction coefficient have been calculated, the variation being attributed to the different apparatus used in each case (Guichardon and Falk, 2000a). In this study a value of \( e = 2575 \text{ m}^2\text{mol}^{-1} \) (Palmer et al, 1984) was used as the value of the extinction coefficient.

2.3.4.2 Determination of the segregation index (\( X_s \)) and the micro-mixedness ratio (\( \alpha \))

The segregation index and the micro-mixedness ratio can both be calculated from the tri-iodide concentration (Guichardon and Falk, 2000a) determined from the OD\(_{360} \) measurements (Section 2.3.3.1). The segregation index is a way of describing the micro-mixing efficiency. Values of \( X_s \) vary from 0 (perfect micro-mixing) to 1 (total segregation). Partial segregation, between these two extremes, can be calculated from the ratio of the number of moles of acid consumed by iodine formation divided by the total number of moles of acid added (\( Y \)) and the value of segregation when the micro-mixing process is infinitely slow (\( Y_{st} \)) where:

\[ X_s = \frac{Y}{Y_{st}} \quad (2-7) \]
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\[ Y = \frac{2V([I_2]^0+[I_3^-])}{V_{\text{Injection}}[H^+]_0} \]  \hspace{1cm} (2-8)

\[ Y_{ST} = \frac{6([IO_3^-]_0/[H_2BO_3^-]_0)}{6([IO_3^-]_0/[H_2BO_3^-]_0)+1} \]  \hspace{1cm} (2-9)

To calculate \( Y \) it is essential to know both the concentrations of \( I_3^- \) and \( I_2 \). \( I_3^- \) can be calculated from Equation 2-6 while the \( I_2 \) concentration must be calculated from a mass balance of the iodide (I) concentration (Equation 2-10) and the equilibrium constant (\( K_B \)) for the tri-iodide forming reaction (Equation 2-11).

\[ \frac{5}{3}[I_2]^2 + \left[I^-\right]_0 - \frac{8}{3}[I_3^-][I_2] - \frac{[I_3^-]}{K_B} = 0 \]  \hspace{1cm} (2-10)

\[ \log_{10} K_B = \frac{555}{T} + 7.355 - 2.575 \log_{10} T \]  \hspace{1cm} (2-11)

If the protocol of Villermaux (Villermaux, 1986) is followed the real fluid is composed of two typical fluids, one perfectly micro-mixed with volume \( V_{PM} \) and one that is perfectly segregated with volume \( V_{ST} \). These can be combined to form Equation 2-12.

\[ (V_{PM} + V_{ST})X_s = V_{PM}(X_s)_{\text{perfect micro-mixing}} + V_{ST}(X_s)_{\text{total segregation}} \]  \hspace{1cm} (2-12)

and with \((X_s)_{\text{perfect micro-mixing}} = 0\) and \((X_s)_{\text{total segregation}} = 1\) the micromixedness ratio was defined as:

\[ \alpha = \frac{V_{PM}}{V_{ST}} = \frac{1-X_s}{X_s} \]  \hspace{1cm} (2-13)

2.3.4.3 Response surface methodology and analysis

Response surface methodology is a statistical technique involving the identification of each factor and the range over which it is allowed to vary. The
methodology was designed to be able to assess the impact of a large number of factors and in this situation an initial screening experiment is completed before a more detailed assessment of relevant factors (Kalil et al., 2000; Lendrem et al., 2001; Mount et al., 2003).

In the experimental protocol each factor was allowed to vary in accordance with a predetermined pattern generated by Design Expert 5 (Stat-Ease Inc., Minneapolis, MN, USA) to fit either a Box-Behnken or a full, 2-level factorial design. Each factor had an upper and lower limit and a number of mid-points were added to test the reproducibility of the data. Where mid-points were not feasible (e.g. pipette tip diameter) the value was changed to either the upper or lower limit with an even spread better the two values. All the runs generated within the design were grouped into one randomised block and completed in one experimental session. The micro-mixedness ratio \( \alpha \) was then calculated from the tri-iodide concentration (as described in Section 2.3.2 for the microwell and Section 2.3.3 for the stirred tank experiments respectively) and incorporated into the Design Expert 5 data analysis software. The significant factors were identified and used to generate design and optimisation equations.

The significance of each model was assessed through the use of the ANOVA statistics function within Design Expert. T-values indicate the significance of each factor on the micro-mixing efficiency, while the coefficient estimate gives an indication of the relative effect of each factor. Factors with t-values greater than 0.1 are deemed as not having a significant effect while those with t-values less than 0.05 are designated as significant. The F-value is a measure of the significance of the model, the Probability > F is the probability of the null hypothesis being true, the Adj R\(^2\) is a measure of the amount of variation around the mean explained by the model.
and the Root MSE is the standard deviation of the experimental error. High F-values indicate that variance in the data can be described by the model while low F-values indicate that the variance may be due to experimental noise. Probability > F values < 0.05 indicate a significant model while values > 0.1 indicate the model is not significant. The closer the Adj $R^2$ value for the model is to 1 the better the model describes the experimental data. The lower the Root MSE value the more accurately the model describes the experimental data.

Typical perturbation plots for each factor are used to demonstrate the impact of each factor over its experimental range (highest value = +1 and lowest value = -1), while holding all the other factors constant at the mid-point (coded 0). Using the perturbation plots engineering design equations can be constructed for each of the tank configurations. Response surface plots were then generated to allow easy visualisation of the effects of each significant factor on the micro-mixing efficiency.

2.4 Influence of micro-mixing efficiency and scale on plasmid yield and purity during alkaline lysis of *E. coli* DH5-α

2.4.1 The influence of micro-mixing efficiency

To determine the effect of micro-mixing efficiency on plasmid yield and purity during the alkaline lysis of *E. coli* DH5-α a number of micromixedness ratios (Section 5.2.2.2) were used during the initial phase of alkaline lysis in 96-DRW plates (Fischer Scientific Ltd, Loughborough, UK). Starting from a standard alkaline lysis procedure (1 volume of 30mg/mL *E. coli* in TE buffer: 1 volume 0.2M NaOH, 1% w/v SDS, neutralized by 1 volume of 3M KAc. Birnboim and Doly, 1979) the micro-mixing efficiency was varied by varying the addition volume of the NaOH / SDS
solution. Addition volumes of 290 μL, 410 μL and 530 μL were used which correlated to α values of 3.07, 27.07 and 51.07 respectively (Section 5.2.2.2). To ensure that cell lysis was complete in all cases the number of moles of NaOH and SDS were kept constant with those added in the standard 1:1 lysis protocol as indicated in Table 2-5.

The bacterial system used to test the effect of micro-mixing efficiency was *Escherichia coli* DH5-α containing the 5.7kb gWiz plasmid (GlaxoSmithKline PLC, Beckenham, UK; Rock *et al*, 2003). All liquid handling was completed using the Multiprobe II™ LHR as described in Section 2.1.2 unless otherwise stated. Fixed pipette tips, Rej = 1000, the waste mode of operation and the performance file detailed in Table 2-2 were utilised. All dispenses were made at a depth of 1 mm below the liquid surface with 100% liquid level tracking. 1.72mL of a 30 mg/mL cell suspension in TE buffer (10 mM Tris HCL, 1 mM EDTA, pH 8.0) was dispensed into each well of a 96-DRW plate. The desired volume of lysis solution was then added to selected wells and left to equilibrate (5 min). The lysed cell suspension was then transferred (manual pipetting) to a 24- deep well plate (Whatman International Ltd, Maidstone, Kent, UK) and 1.72 mL of chilled 3M potassium acetate (KAc), pH 5.5 was added to neutralise the NaOH. The plate was left on ice for a further 10 min. The contents of each well were then transferred (manual pipetting) to 2mL Eppendorf.

Table 2-5: Concentrations of NaOH and SDS added in the lysis solution during alkaline cell lysis in 96-DRW plates. The standard lysis solution conditions (1:1 v/v) are shown for reference (Bimboim and Doly, 1979).

<table>
<thead>
<tr>
<th>Addition Volume (μL)</th>
<th>Cell Suspension : Lysis Solution Ratio (v/v)</th>
<th>[NaOH] (M)</th>
<th>[SDS] (%w/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>290</td>
<td>1 : 5.93</td>
<td>1.20</td>
<td>5.9</td>
</tr>
<tr>
<td>410</td>
<td>1 : 4.20</td>
<td>0.84</td>
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<td>530</td>
<td>1 : 3.25</td>
<td>0.65</td>
<td>3.2</td>
</tr>
<tr>
<td>1720</td>
<td>1 : 1</td>
<td>0.20</td>
<td>1.0</td>
</tr>
</tbody>
</table>
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tubes and centrifuged at 10,000 rpm for 30 min at 4°C (Eppendorf 5415R centrifuge, Eppendorf AG, Hamburg, Germany.). After centrifugation the samples were filtered through a Whatman No.1 filter paper (Whatman International Ltd, Maidstone, Kent, UK) and stored at 4°C. 1 mL samples were taken to assay the total protein concentration (Section 2.4.3.2). The clarified lysate was further purified by the standard protocol detailed in Section 2.4.3 prior to the analysis of plasmid DNA concentration and conformation (Sections 2.4.3.3 and 2.4.3.4). All conditions were repeated in quadruplicate using the four pipette tips available on the Multiprobe II™ LHR.

2.4.2 Alkaline lysis at common α values utilising differing reactor scales

An assessment of the effect of common α values at different scales was made by studying three scales: 200 μL in a 96-SRW plate, 1.72 mL in a 96-DRW plate and 500 ml in a baffled stirred vessel. An α value of 12.2 was used as it could be reproduced at all three scales. A common 30 mg/ml suspension of E. coli DH5-α containing the 5.7kb gWiz plasmid in TE buffer (10 mM Tris HCL, 1 mM EDTA, pH 8.0) was prepared for all three scales.

For microwell experiments all liquid handling was completed using the Multiprobe II™ LHR as described in Section 2.1.2 unless stated. Fixed pipette tips, Rej = 1000, the waste mode of operation and the performance file detailed in Table 2-2 were utilised. At the 96-SRW scale, 72 μL of the lysis solution (0.56M NaOH, 2.78% w/v SDS) and at the 96-DRW scale, 336 μL of the lysis solution (1.02M NaOH, 5.12% w/v SDS) were added. The liquid dispense was made at a height of 1mm below the liquid surface with 100% liquid level tracking. The microwells were
then left for 5 min and then the contents were transferred to a 24- deep well plate (manual pipetting). The NaOH was neutralised with 3M KAc, pH 5.5 and the left plate on ice for a further 10 min. The contents of each well were then transferred to 2 mL Eppendorfs (manual pipetting) and centrifuged at 10,000 rpm for 30 min at 4°C. The supernatants were removed and filtered through a Whatman No. 1 filter paper. Samples (100 μL) were taken to be assayed for total protein content while the remainder of the samples were further purified and analysed using the techniques detailed in Section 2.4.3. All conditions were repeated in quadruplicate using the four pipette tips of the Multiprobe II™ LHR.

For the 500 ml scale stirred tank experiment 450 ml of the same *E. coli* cell suspension was used but the lysis solution was added in stages. This is due to SDS being insoluble at the NaOH concentrations required. The cell suspension was added to the vessel (Figure 2-4 (a)) and the stirrer was set to 1000 rpm and left for 1 min for the fluid flow patterns to become stable. 50 mL of 20% w/v SDS was then added at the liquid surface. To prevent the SDS lysing the bacterial cells on its own, only a short time (20 s) was allowed for the SDS to become homogeneous throughout the stirred vessel before 50 ml of 4M NaOH was added at the addition point (Figure 2-4) over a 100 s time period. Once the addition had been completed the stirrer was switched off, to prevent possible damage to the plasmid and chromosomal DNA by the high shear environment in the impeller region (Ciccolini *et al*, 1998), and was then left for 5 min. The lysed cells were then added to 500 ml 3M KAc, pH 5.5 and left on ice for 10 min. To separate the cell debris from the soluble fraction the solution was centrifuged at 10,000 rpm (Beckman J2-MI, Beckman Inc., Palo Alto, CA, USA) for 30 min at 4°C. A 10 ml sample was taken and filtered through a Whatman No.1 filter paper. Three 1 ml samples were taken to be assayed for the total protein content.
(Section 2.4.3.2). Four further 1 mL samples were then taken from the filtrate and subjugated to the workup and DNA analysis procedures detailed in Section 2.4.3 prior to the analysis of plasmid DNA yield and conformation (Sections 2.4.3.3 and 2.4.3.4).

2.4.3 Plasmid DNA work-up and analysis

2.4.3.1 Isopropanol precipitation of DNA

The precipitation of DNA using isopropanol (Sigma, Poole, Dorset, UK), to remove any co-purified chromosomal DNA, was used to further purify plasmid samples prior to analysis. 0.7 mL of room temperature isopropanol was added to 1 mL of the clarified lysate in a 2 mL Eppendorf tube and mixed well. The samples were then centrifuged at 15,000 rpm for 10 min at room temperature. The supernatant was carefully decanted from the tube without disturbing the DNA pellet. The pellet was then washed with 1 mL of room temperature ethanol (70% v/v, Sigma, Poole, Dorset, UK) and centrifuged at 15,000 rpm for 15 min. The supernatant was again decanted off to isolate the pellet. The pellet was then air dried for 15 min before the dry DNA was redissolved in 100 µl TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8, 100 µg/mL RNAse A (Qiagen, Hilden, Germany)). This purified plasmid DNA was used for the DNA analytical techniques described below.

2.4.3.2 Bradford protein assay

The Bradford protein assay was used to determine the total soluble protein concentration of plasmid DNA samples after alkaline lysis had been completed (Sections 2.4.1 and 2.4.2). Protein standards were made up with bovine serum albumin (BSA) at concentrations from 0.1 to 1.0 mg/mL in 100 mM potassium
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phosphate buffer at pH 6.4. 100 μL of the protein standards were added to 2.9 ml of 1:5 Biorad solution (Pierce Biotechnology Inc., Rockford, IL, USA), mixed and left for 5 min. The OD was then measured at 595 nm (OD_{595}) against a blank of 3 ml 1:5 Biorad solution. Protein concentrations between 0.1 - 1.0 mg/ml gave a linear correlation between OD_{595} values of 0 - 1.0 (Figure 2-5). Values above 1.0 were diluted with 1:5 Biorad solution and remeasured.

The protein samples from the alkaline lysis experiments followed the same protocol for preparation as the BSA standards. Protein concentrations which resulted in OD_{595} values greater than 1.0 were further diluted with 1:5 Biorad solution as appropriate. Protein samples were analysed in triplicate with a maximum acceptable co-efficient of variance of 10%.

2.4.3.3 HPLC quantification of total DNA concentration and plasmid conformation

Analytical anion exchange chromatography was used with the methodology developed by Meacle and co-workers (Meacle et al., 2004). A Q-Sepharose HPLC column was loaded at 0.6M NaCl, 10mM Tris, 1mM EDTA, pH 8.0 and eluted over 25 min with a linear NaCl gradient (0.6-0.8M). The DNA was detected by its absorbance at OD_{260}. A peak area to [DNA] is shown in Figure 2-6 and a sample HPLC trace in Figure 2-7.

2.4.3.4 Determination of plasmid conformation by gel electrophoresis

Agarose gel electrophoresis was used here to determine the relative populations of the different conformational forms of a plasmid (supercoiled, open circular and linear) in alkaline lysis samples.
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A 0.6% w/v agarose solution in TAE (40mM Tris acetate, 1mM EDTA, pH 8.3) was made up by warming the suspension to dissolve the agarose. This was left to cool and ethidium bromide (1 μL ≥ 95.0% (HPLC grade), Sigma-Aldrich, Poole, Dorset, UK) was added before the gel set and thoroughly mixed. The mixture was then poured into the gel mold and left to set. Once the gel had set the dyed DNA samples (Novagen 6X DNA gel loading buffer, Merck Biosciences, Nottingham, UK) were added to the gel and run against a set of markers (Perfect DNA markers, 0.5-12kb, Novagen, Merck Biosciences, Nottingham, UK) using 60 V cm\(^{-1}\) for 40 min. The gel was then photographed using a digital camera (Gel Doc-It image system, Ultra-Violet Products Ltd, Cambridge, UK) and the relative fluorescence intensities of each of the bands calculated using Biol-imaging systems software (Ultra-Violet Products Ltd, Cambridge, UK). As open circular plasmid binds more ethidium bromide than the equivalent supercoiled plasmid form a correction factor of 2.5 (Ciccolini et al, 2002) was used to determine the relative concentrations.
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Figure 2-5: Calibration curve for the OD$_{595}$ readings over a range of BSA standards as measured by the Bradford protein assay.
Figure 2-6: Peak area vs [DNA] calibration curve utilising the Q-Sepharose HPLC column.
Figure 2-7: Typical HPLC chromatograph for plasmid gWiz in the absence of RNA. (RNaseA treated). Experiments performed as described in Section 2.4.3.3.
3 Evaluation of integrated robotic systems for the measurement of bioprocess kinetics

3.1 Introduction and aims

The use of integrated robotic systems for bioprocess development represents a possible solution to coping with the ever increasing number of potential drug candidates and active ingredients produced by high throughput screening (HTS) and combinatorial chemistry as described in Section 1.1 (Balkenhohl et al, 1996). The use of standardised microwell geometries and associated automation offer significant cost and speed advantages over the traditional methods of standard small and pilot scale bioprocess development. The technology is well documented and used regularly in HTS (Berg et al, 2001) and laboratory assays (Kitchen et al, 2003; Zheng and Wozniak, 1997) but has yet to be widely applied to bioprocess development applications.

The collection of kinetic data is required for most bioprocess operations of interest and therefore the gathering of kinetic data over the course of a bioprocess operation has been used as the first test of the utility of using integrated automation platforms in bioprocess development. Microscale processing techniques allow for the production of large quantities of data through the running of experiments in parallel (Weuster-Botz, 2005) but the usefulness of the data is highly dependent on its quality. The frequency of the measurements will determine the quality of the data generated and using the sample detection period, the time between measurements, the

influence of the operation of the LHR and plate reader on the data quality can be assessed.

For such a microscale processing approach to be useful it must display the following features: (1) accurate and precise liquid handling over a range of dispense volumes and liquid phases, (2) reproducibly well sampling with a set frequency, (3) the flexibility to vary the sample detection period independently of the trial system and (4) the maximisation of productivity within each microwell plate by a priori prediction of the degree of well utilisation.

When using the combination of a liquid handling robot and a microwell plate reader (as described in Section 2.1.2), the minimum sample detection period (as defined in Section 2.1.4) is inversely proportional to the speed of liquid handling, the speed of detection by the microwell plate reader and the number of wells utilised on the plate. The relative importance of these three factors on the sample detection period while maintaining accurate and precise liquid handling will be investigated in this chapter. To represent these interactions the data will be displayed graphically using operating windows (Woodley and Titchener-Hooker, 1996), which allow the simple visualisation of multivariate and complex design data. In the context of this work they also provide an assessment of the suitability of a given automation platform for the study of a particular bioprocess unit operation.

3.2 Accuracy and precision of liquid handling

Underlying the creation of microscale processing technologies (Lye et al, 2003) is the requirement for accurate and precise dispensing of a range of fluids into various microwell plate formats. An initial study of tip dispensing behaviour using a fixed target dispense volume of 100 µL showed that for model low viscosity aqueous (RO water), low viscosity organic (DMSO) and high viscosity aqueous (80% w/w
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glycerol) phases (Table 2-1), the liquid handling capability of each of the four pipette
tips on LHR (Section 2.1.2) were accurate and precise (Figure 3-1). That is, the
accuracy and precision was below the required 5% CV limit specified in Section 2.1.3
(Astle and Akowitz, 1996; Olsen, 2000; Stevens et al, 1998). Each pipette tip,
therefore, performed in a consistent and reproducible manner. This meant that it was
possible to complete the remaining liquid handling experiments with any one of the
pipette tips, rather than all four, thus reducing the total number of measurements
needed.

Results on the accuracy and precision of the robot over a wide range of
operating conditions are shown in Figure 3-2 (low viscosity aqueous), Figure 3-3 (low
viscosity organic) and Figure 3-4 (high viscosity aqueous). For all three solvents the
liquid handling precision decreased as the number of dispenses per aspirate increased.
At the lowest dispense volume, 5 μL the liquid handling precision was poor (>5% CV)
for all numbers of dispenses per aspirate. For all numbers of dispenses per
aspirate the precision increased with dispense volume. Both of the low viscosity phase
systems showed high precision at all volumes for six dispenses per aspirate (the
manufacturers recommendation for optimum liquid handling accuracy and precision).
Above a dispense volume of 10 μL the high viscosity aqueous system had a consistent
precision around 4% CV.

In contrast, the results for the liquid handling accuracy are more complex. The
accuracy for the low viscosity aqueous system increases with the number of dispenses
per aspirate and with increasing dispense volume. In the low viscosity organic system
it is harder to find clear trends. The accuracy again increases with increasing
dispenses per aspirate but for dispensing volumes of 50 and 100 μL the accuracy is
lower than that obtained for dispensing at smaller volumes. For the high viscosity
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Figure 3-1: (a) Pipetting accuracy and precision based on six dispense per aspiration and (b) pipetting accuracy based on 24 dispenses per aspiration for: (■) RO water, (■) DMSO and (■) 80% w/w glycerol solutions. Precision (blocks) and accuracy (error bars) of each pipette tip in dispensing a programmed volume of 100 μL. The acceptable band width of 5% CV is also shown. Experiments performed as described in Section 2.1.3. Performance files used as described in Tables 2-2 and 2-3.
Figure 3-2: (a) Multiple dispense precision and (b) multiple dispense accuracy with a low viscosity aqueous phase (RO water). The measured mean dispense volumes are shown for a range of programmed dispense volumes and number of dispenses per aspiration. The cut off for precise and accurate liquid handling, 5% CV is indicated by the dashed line. 3 dispenses per aspirate (■) 6 dispenses per aspirate (●) 12 dispenses per aspirate (▲) 24 dispenses per aspirate (▼) Experiments performed as described in Section 2.1.3. Performance files used as described in Tables 2-2 and 2-3.
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Figure 3-3: (a) Multiple dispense precision and (b) multiple dispense accuracy with a low viscosity organic phase (DMSO). The measured mean dispense volumes are shown for a range of programmed dispense volumes and numbers of dispenses per aspiration. The cut off for precise and accurate liquid handling, 5% CV is indicated by the dashed line. 3 dispenses per aspiration (■) 6 dispenses per aspiration (●) 12 dispenses per aspiration (▲) 24 dispenses per aspiration (▼). Experiments performed as described in Section 2.1.3. Performance files as described in Tables 2-2 and 2-3.
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Figure 3-4: (a) Multiple dispense precision and (b) multiple dispense accuracy with a high viscosity phase (80% w/w glycerol). The measured mean dispense volumes are shown for a range of programmed dispense volumes and number of dispenses per aspiration. The cut off for precise and accurate liquid handling, 5% CV is indicated by the dashed line. 3 dispenses per aspiration (■) 6 dispenses per aspiration (●) 12 dispenses per aspiration (▲) 24 dispenses per aspiration (▼). Experiments performed as described in Section 2.1.3. Performance files as described in Tables 2-2 and 2-3.
aqueous system, the results follow the same trends as the low viscosity aqueous system. However, in all cases the accuracy is much closer to the specified acceptable limit of 5% CV and does not vary significantly with dispense volumes above 10 µL.

Interestingly, it can be seen that there is a trend from the high viscosity aqueous phase through the low viscosity organic phase to the low viscosity aqueous phase of an increasing degree of accuracy and precision of liquid handling. This is despite modification of the performance files (Tables 2-2 and 2-3) that should have enabled optimum performance allowing for the changes in the physical properties of the fluids.

Table 3-1 summaries the liquid handling performance data for each of the liquid phases. This defines the minimum volume that can be dispense in each case with better than 5% CV accuracy and precision. Data collection from bioprocess operations relying on the dispensing of liquid volumes lower than specified in Table 3-1 would be inaccurate and only qualitative in nature.

Table 3-1: Minimum precise and accurate dispense volumes (variation below 5% CV in both cases) for the various phases used in this study. Values derived from Figures 3-2, 3-3 and 3-4 for RO water, DMSO and 80% w/w glycerol respectively.

<table>
<thead>
<tr>
<th>Number of Dispenses</th>
<th>Minimum Dispense Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RO Water</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>12</td>
<td>20</td>
</tr>
<tr>
<td>24</td>
<td>20</td>
</tr>
</tbody>
</table>

3.3 Kinetics of data acquisition

As described in Section 2.1.4, the minimum sample detection period available to acquire data on the kinetics of a given bioprocess operation is determined by the time taken to dispense the initiating reagent into a specified number of wells and the
time taken to take a spectral measurement using the integrated plate reader. To evaluate the relative significance of the plate set up time (Equation 2-3) and the plate monitoring time (Equation 2-4) the two values need to be determined. The plate set up time consists of the dispense time and the reader initialisation time. The reader initialisation time is fixed and was measured at 19s.

The dispense time varies as a function of dispense volume and the number of dispenses per aspiration as shown in Figure 3-5. The results for both the low and high viscosity systems exhibit distinct vertical steps in the dispense time at regular intervals. This is due to the extra time required for the robot to load the reagent back into the syringes from the system tubing as described in Section 2.1.4. The three main factors affecting the dispense time are the degree of well utilisation, the characteristics of the performance file used for a particular solvent and the dispense volume. From Figure 3-5 it can be seen that the degree of well utilisation is the most important of these factors. To change from 12.5% to 100% well utilisation, for 10 μL additions using a low viscosity phase leads to an increase of 80s in the dispense time. While to change from 10 μL to 200 μL additions at 100% well utilisation for a low viscosity phase leads to a 30s increase in dispense time and a change from a low viscosity to a high viscosity phase, at 100% well utilisation and 10 μL addition gives an increase of only 20s. The performance file and dispense volume do have a small effect on the dispense time but this varies with the degree of well utilisation. The greater the degree of well utilisation the greater the effect of the performance file and the dispense volume. The performance file affects the dispense time because of changes made to the dispense delay and the dispense speed (Tables 2-2 and 2-3) to ensure accurate and precise liquid handling, especially for more viscous liquids.

Finally, to be able to calculate the plate monitoring time (Equation 2-4) the
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![Graph showing dispense times](image)

Figure 3-5: (a) Liquid dispense times for low viscosity aqueous (RO water) and organic phases (DMSO) and (b) liquid dispense times for a high viscosity phase (80% w/w glycerol). The figures show the time taken using a single pipette tip to dispense a range of dispense volumes into a number of wells using the multiple dispense function. (■) 3 dispense per aspiration [12.5% well utilisation] (●) 6 dispenses per aspiration [25% well utilisation] (▲) 12 dispenses per aspiration [50% well utilisation] (▼) 24 dispenses per aspiration [100% well utilisation]. Experiments performed as described in Section 2.1.4. Performance files as described in Tables 2-2 and 2-3.
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plate reading time and the reader reset time are needed. The plate reading time can be seen to be an almost linear function of the degree of well utilisation (Figure 3-6) while the reader reset time was found to be constant at 7s. A comparison between the plate set up time and the plate monitoring time showed that the plate set up time is by far the most important and is thus rate limiting. This leads to the conclusion that the degree of well utilisation is the most important factor affecting the minimum sample detection period.

![Graph showing plate reading time vs number of wells]

Figure 3-6: Plate readings times for varying degrees of well utilisation. Experiments performed as described in Section 2.1.4. Performance files as described in Tables 2-2 and 2-3.

3.4 Operating windows for platform assessment

To clearly represent the relationship between the minimum sample detection period, the dispense volume and the degree of well utilisation, Windows of Operation (Woodley and Titchener-Hooker, 1996) were constructed for the low viscosity
Figure 3-7: Bioprocess operating window for low viscosity aqueous (RO water) and organic (DMSO) phases using a standard 96 round well plate (96-SRW). The figure shows the dependence of the number of bioprocess conditions (each completed in quadruplicate) which can be studied within a single microwell plate, on the accuracy and precision of liquid handling and the time between sampling. Sample detection periods that cannot utilise three or more bioprocess conditions are designated as being too slow. The upper capacity is based on the volume of the system tubing. Note that the indicated values are specific for the four pipette tip Multiprobe II™ LHR and Spectacount™ plate reader combination used in this study as described in Section 2.1.2. The operating window is based on data presented in Table 3-1 and Figure 3-5.
Figure 3-8: Bioprocess operating window for a high viscosity aqueous (80% w/w glycerol) phase using a standard 96 round well plate (96-SRW). The figure shows the dependence of the number of bioprocess conditions (each completed in quadruplicate) which can be studied within a single microwell plate, on the accuracy and precision of liquid handling and the time between sampling. Sample detection periods that cannot utilise three or more bioprocess conditions are designated as being too slow. The upper capacity is based on the volume of the system tubing. Note that the indicated values are specific for the four pipette tip Multiprobe II™ LHR and Spectracount™ plate reader combination used in this study as described in Section 2.1.2. The operating window is based on data presented in Table 3-1 and Figure 3-5.
3 Evaluation of integrated robot systems

(Figure 3-7) and high viscosity (Figure 3-8) phases. These are based on the data for pipetting precision and accuracy (Table 3-1) and the liquid dispense times (Figure 3-5). The operating windows show the lower limits of liquid handling accuracy and precision over the range of dispenses per aspiration studied as well as displaying the effects of dispense volume on the minimum sample detection period for each combination of well utilisation and dispense volume. The maximum working volume of the system tubing of the robot is 2089.5 ml and this sets the upper limit on the degree of well utilisation that can be achieved for each dispense volume.

Both windows show that the minimum sample detection period increases with increasing well utilisation and dispense volume for all three phase systems studied. The boundaries between the utilisation of 12-24 wells (the investigation of 3-6 separate bioprocess conditions, each in quadruplicate) and the 48-96 (6-12 bioprocess conditions) and the 48-96 wells (12-24 bioprocess conditions) show step increases in the sample frequency. This is a direct result of the need to load reagent from the syringes into the system tubing as described in Section 2.1.4.

A comparison of the operating window for the two low viscosity phases (Figure 3-7) with that for the high viscosity phase (Figure 3-8) shows that the boundaries between the various degrees of well utilisation have shifted to the right for the high viscosity system. The changes in the performance files (Tables 2-2 and 2-3), dictated by the physical properties of the fluid, lead directly to an increase in the minimum sample detection period for an equivalent degree of well utilisation. It can also be seen that only the nature of the initialising reagent has an influence on the minimum sample detection period and the degree of well utilisation. The bulk fluid having no effect on the sample detection period or degree of well utilisation and so
the measurement of bioprocess kinetics is independent of the bulk fluid of the trial system.

Finally, it should be noted that the sample detection periods show in Figures 3-7 and 3-8 are specific to the experimental set up used here. The use of another liquid handling robot with a different syringe size, number of pipette tips or operating speed would result in a change in the sample detection period for each degree of plate utilisation. The same would be true if a different microplate reader were to be considered. However, the approach to the construction of the windows of operation is generic and would give valuable insight into the utility and productivity of any automation set up.

3.5 Other liquid handling issues: contamination and liquid mixing

The use of automated liquid handling platforms as described here has the potential to reduce the time taken to complete the gathering of a kinetic profile of a bioprocess operation and increase the throughput of new target compounds through the development pathway. However, inherent to their use is the need to understand the general limitations of liquid handling equipment and those of integrated detection devices, such as microplate readers.

The most common liquid handling difficulties are tip carryover contamination (Astle and Akowitz, 1996), speed of liquid handling, liquid handling accuracy and precision (Olsen, 2000; Stevens et al, 1998) and the speed of the detection device. Speed of liquid handling, liquid handling accuracy and precision and the speed of the detection device have been addressed in Sections 3.3, 3.2 and 3.3 respectively. Tip carryover contamination can be avoided by dispensing above the liquid level in a well
with fixed tips. The use of disposable tips also overcomes this problem but increases the minimum sample detection period, due to the need to change tips after each dispense, by a factor of five in the case of the Multiprobe II™ LHR (data not shown) thus greatly reducing the degree of well utilisation.

For additions made from each type of tip it has been assumed throughout this study that when the initiating reagent is added to the bulk fluid within the well it will be rapidly mixed. This is a reasonable assumption when a small volume of reagent is dispensed within the bulk of a low viscosity fluid and then shaken (Duetz and Witholt, 2001; Stevens et al, 1998). At present there is no published data as to whether mixing in high viscosity phases in microwells will influence the measurement of bioprocess kinetics. For plates that remain static following the sample addition there is no published data on macro-mixing times. This problem will be addressed later in Chapter 4.

The use of integrated detection devices greatly increases the throughput of bioprocess development but can be restrictive on the type of microwell plate that can be utilised. The currently available microplate readers are only compatible with standard round well plates (Figure 2-2). From an analytical and HTS view point this does not present a problem but in many bioprocess development cases greater volumes are required and deep well plates (Figure 2-2 [Large designed round well]) have been found to be more suitable (Doig et al, 2002; Duetz and Witholt, 2001; Elmahdi et al, 2003). Currently sampling from deep well formats and the transfer of samples into a 96-SRW plate must therefore take place before analysis can be completed adding greatly to the sample detection period.
3.6 Discussion

It has been shown in this chapter that a liquid handling robot and a microwell plate reader can be effectively integrated. For the construction of an effective bioprocess development tool to measure process kinetics four features were identified in Section 3.1 and each of these features have been addressed. The dispense volumes that can be accurately and precisely dispensed utilising the multiple dispense per aspiration function have been defined for a range of liquid phases (Figures 3-2, 3-3 and 3-4). The minimum sample detection period for varying degrees of well utilisation have been determined (Figure 3-5) and it has been shown that minimum sample detection period is dependent on the fluid properties of the initialising reagent but is independent of the bulk fluid of the trial system (Section 3.4). The construction of the windows of operation (Figure 3-7 and Figure 3-8) for each of the liquid phases allows the a priori prediction of the degree of well utilisation from the two parameters of dispense volume and sample detection period.

The sample detection period has been shown to be the major influence on the degree of well utilisation within a plate. As the frequency of measurement increases, the number of wells that can be utilised within a plate decreases until a point is reached where the integrated LHR and plate reader offer no advantage over manual liquid handling (Figure 3-7 and Figure 3-8). The necessity of reducing the time of liquid handling also requires that multiple dispenses per pipette tip are used. The fluid properties of the initialisation reagent have been shown to have an effect on the degree of well utilisation. The need to maintain accurate and precise liquid handling leads to a reduction in the degree of well utilisation for a given sample detection period as the viscosity of the initialisation reagent increases. To demonstrate the suitability of the integrated robotic system for the measurement of bioprocess kinetics
a number of bioprocess case studies will be considered later in Chapter 6. These assess the suitability of the system depending on the sample detection period associated with each bioprocess unit operation and demonstrates how the quality of the process data will vary with the degree of well utilisation. Data with a short sample detection period, having a large number of data points, will have a lower degree of well utilisation than a data set utilising the same trial system but with a longer sample detection period.

Before that, however, the potential for poor macro-mixing within static microwells has been highlighted (Section 3.5) since this could have a significant effect on any kinetic data generated using this integrated robotic system. The conditions necessary to achieve effective jet macro-mixing within static microwell plates are therefore investigated in Chapter 4.
4 Quantification of jet macro-mixing times in static microwell plates

4 Quantification of jet macro-mixing times in static microwell plates*

4.1 Introduction and aims

As described in the previous Chapter automated liquid handling robots combined with standardised microwell formats are commonly used for analytical assays and are increasingly being utilised for bioprocess development applications. Integral to their successful use in both applications is the ability to generate reproducible and robust data. This is reliant on there being a homogeneous physical environment within each microwell. A number of groups have looked at jet macro-mixing within large-scale reaction vessels and have produced correlations for the macro-mixing times (Section 1.2.1.1). The macro-mixing times have been shown to be dependent on the vessel geometry, the jet nozzle diameter and the jet fluid flow regime (Equations 1-2 and 1-3), but currently there is no published data on macro-mixing times in static microwell plates. Therefore, in this chapter, the macro-mixing efficiency in a number of microwell formats with variable jet conditions is examined.

Initially a methodology will be developed for the reliable quantification of macro-mixing times in jet-mixed static microwell plates. Once the methodology has been established, macro-mixing times will be measured in a number of microwell geometries. These have been designed as models of commonly available microwell formats (96-SRW and 96-DRW) and utilising two parameters which are known to determine the macro-mixing efficiency, the maximisation of fluid entrainment and the maximisation of the energy dissipation rate at the end of the jet (Section 1.2.1.1).

+ The results in this chapter have been submitted for publication as: Nealon, A.J., O’Kennedy, R.D., Titchener-Hooker, N.J. and Lye, G.J. Quantification and prediction of macro-mixing times in static microwell plates. Chem. Eng. Sci.
Finally generic correlations will be produced which will allow the prediction of macro-mixing times in various microwell formats and utilising a range of automation equipment.

4.2 Visualisation of jet macro-mixing and calculation of macro-mixing times

4.2.1 Visualisation of jet macro-mixing

For microwell formats to be utilised reproducibly for analytical assays, HTS and bioprocess development applications a homogenous physical environment within the well is required. Experiments to determine the homogeneity within each of the three microwell designs (Figure 2-1) and utilising both the pipette tip types, disposable (d= 0.6mm) and fixed (d= 0.54mm), were performed over a range of addition volumes and jet Reynolds numbers as described in Section 2.2.2. Representative examples of the still images generated from the video footage are shown for the large designed well, small designed well and 96-SRW in Figures 4-1, 4-2 and 4-3 respectively. For all three microwell geometries the aim is for macro-mixing to be completed during the lifespan of the jet.

The images for the large designed microwell (Figure 4-1) show the reference frame, the beginning of the introduction of the inert dye (t=0.076s), two points during flow pattern development (t=0.145 s and t=0.348 s), and a point after the completion of the addition of the inert dye (t=1.22s). The images show the extent to which the jet will penetrate the liquid in the microwell. In this case it can be seen that under the conditions used the jet looses momentum and is dissipated before it reaches the base of the well. Initially, the dye is concentrated in the centre of the microwell (t = 0.076s) with the top and bottom of the microwell undisturbed. Next, the area at the base
4 Quantification of jet macro-mixing times in static microwell plates

Figure 4-1: Representative examples of jet formation and macro-mixing in a large designed microwell (Fixed tips, $Re_j = 2480$, $V_A = 859\mu l$). Well geometry as shown in Figure 2-2. Experiments performed as described in Section 2.2.2 and still images taken from high-speed video camera footage as described in Section 2.2.3.
Figure 4-2: Representative examples of jet formation and macro-mixing in a small design well (Fixed tips, $Re_j = 3960$, $V_A = 100\mu$L). Well geometry as shown in Figure 2-2. Experiments performed as described in Section 2.2.2 and still images taken from high-speed video camera footage as described in Section 2.2.3.
Prior to addition

$ t = 0.013s$

Figure 4-3: Representative examples of jet formation and macro-mixing in a 96-SRW plate (Fixed tips, $Re_j = 3000$, $V_A = 90\mu l$). Well geometry as shown in Figure 2-2. Experiments performed as described in Section 2.2.2 and still images taken from high-speed video camera footage as described in Section 2.2.3.
of the microwell becomes mixed by the circulating flow patterns generated by the jet (t = 0.145s) and finally the top third of the microwell becomes well mixed (t = 0.348s). In the top region of the microwell the jet initially has sufficient momentum that it penetrates through the bulk fluid with relatively little disturbance or entrainment, equivalent to region 1 in Figure 1-2. For this upper region to become well mixed the circulating flow patterns produced by the jet must have enough momentum to reach the liquid surface.

The visualised behaviour of the jet is in good agreement with corresponding colour intensity plots shown in Figure 4-4. At t = 0.076s the inert dye is clearly located in the centre of the microwell as indicated by the marked decrease in the colour intensity between pixels 60 and 190. The liquid in the top third and at the base of the microwell remain largely undisturbed at this point with colour intensity values close to those prior to liquid addition. The liquid at the base of the microwell is the next region to be mixed (t = 0.145s) followed by that at the top of the microwell.

The same pattern of macro-mixing phenomena was observed for jetting conditions studied in the small designed microwell (Figure 4-2 and Figure 4-5), in that the circulatory flow patterns and the same sequence of mixing in different regions in the microwell were observed.

In contrast to the two microwell geometries specifically designed for efficient jet mixing, the data for the standard round well geometry shows somewhat different results as seen from the still images in Figure 4-3. In this case, the liquid height is considerably less and the jet has sufficient momentum to rapidly reach the base of the microwell and impact upon it (t = 0.002s). This leads to much faster mixing at the base of the standard well than observed with the other two designed of microwell. Again, the top layer of the microwell initially remains relatively undisturbed by the jet (t = 0.013s). Circulating flow patterns next start to develop as the injection continues resulting in the inert dye flowing
Figure 4-4: Colour intensity plots corresponding to the sequence of still frames shown in Figure 4-1 for a large designed microwell (Fixed tips, Rej = 2480, V_A = 859μl). Pixel 0 represents liquid immediately below the liquid meniscus in each case. Colour intensity determined as in Section 2.2.3.
Figure 4-5: Colour intensity plots corresponding to the sequence of still frames shown in Figure 4-2 for a small designed microwell (Fixed tips, $Re_t = 3960$, $V_A = 100\mu l$). Pixel 0 represents liquid immediately below the liquid meniscus in each case. Colour intensity determined as in Section 2.2.3.
Figure 4-6: Colour intensity plots corresponding to the sequence of still frames shown in Figure 4-3 for a 96-SRW plate (Fixed tips, Re_j = 3000, V_A = 90µL). Pixel 0 represents liquid immediately below the liquid meniscus in each case. Colour intensity determined as in Section 2.2.3.
up the side walls of the microwell and then across the liquid surface. This leads to a small section of unmixed fluid forming in the centre of the microwell, which is the last region to become well mixed \((t=0.034s)\). Again, the corresponding colour intensity profiles shown Figure 4-6 nicely confirm the visual analysis.

4.2.2 Quantification of macro-mixing times

In order to quantify the actual macro-mixing times for the well geometries and conditions shown in Figures 4-1, 4-2 and 4-3 the calculated percentage homogeneity is plotted as a function of time in Figures 4-7, 4-8 and 4-9 respectively. These values were calculated as described in Section 2.2.3. The liquid homogeneity is seen to decrease rapidly once jet addition of the dyed liquid commences but then increases as liquid circulation around the well is established. The macro-mixing time, \(t_{95}\), is designated as the first time point at which the liquid homogeneity returns to 95\%. The calculated \(t_{95}\) macro-mixing times for the conditions used with the large designed well (Figure 4-7), the small designed well (Figure 4-8) and the standard round well (Figure 4-9) are 0.335s, 0.061s and 0.043s respectively.

4.2.3 Mixing following jet addition

A number of experiments were also performed at low volume additions, 5-20\% of initial well fill volume, where \(t_{95}\) is not reached within the lifespan of the jet. Such additions are typical of the conditions reported for many microwell-based assays used in drug discovery applications (Berg et al, 2001; Delvin, 1997; Lamsa et al, 2004). In these cases (Figure 4-10), the video recordings indicate large and persistent heterogeneities within the liquid contents of the wells. In the worst cases, for the two designed microwells, there is distinct segregation with the fluid in the top third of the wells being completely unaffected during jet addition. Macro-mixing times under these conditions
4 Quantification of jet macro-mixing times in static microwell plates

Figure 4-7: Calculated percentage homogeneity during the course of an injection into a large designed microwell (Fixed tips, Re_j = 2480, V_A = 859 μL). The lifespan of the jet addition and the point taken to represent t_{95} in each case are also indicated. Percentage homogeneity calculated as in Section 2.2.4 from the colour intensity profiles shown in Figure 4-4.
Figure 4-8: Calculated percentage homogeneity during the course of an injection into a small designed microwell (Fixed tips, Re_j = 3960, V_A = 100 μL). The lifespan of the jet addition and the point taken to represent t_{95} in each case are also indicated. Percentage homogeneity calculated as in Section 2.2.4 from the colour intensity profiles shown in Figure 4-5.
Figure 4-9: Calculated percentage homogeneity during the course of an injection into a 96-SRW (Fixed tips, Re_j = 3000, V_A = 90 µL). The lifespan of the jet addition and the point taken to represent t_{95} in each case are also indicated. Percentage homogeneity calculated as in Section 2.2.4 from the colour intensity profiles shown in Figure 4-6.
Figure 4-10: (a) Video still and (b) the corresponding colour intensity plot for a low volume addition in a large designed well 0.018 s after the termination of the addition (Fixed tips, \( Re_j = 1000, V_A = 85.9 \, \mu L \)). The fluid flow patterns have not been given enough time to fully develop and distinct segregation can be observed. Experiments performed as described in Section 2.2.2.
4 Quantification of jet macro-mixing times in static microwell plates

could be of the order of minutes and generally increased with decreasing addition volume
and decreasing jet lifespan. Weiss and co-workers also found that such long mixing times
could occur particularly with very low addition volumes (Weiss et al., 2002). In some
cases, particularly for the standard round well, the momentum flux from the jet was
sufficient to complete macro-mixing in a reasonable time even after jet addition had
ceased. In these cases, however, the macro-mixing time was significantly longer than for
larger volume additions as shown in Figure 4-11. For a low volume addition of 10μl the
calculated t_{95} value was 0.260s, which is six times slower than for the conditions used in
Figure 4-9.

4.3 Variation of macro-mixing times with well geometry and
jetting conditions

All the t_{95} values determined in Section 4.2.2 are summarised in Table 4-1. The
majority of the macro-mixing times ensure that homogeneity is reached during the
lifespan of the jet addition. Only when utilising the small designed microwell, an addition
volume of 100 mL and Re_j = 3960 is homogeneity reached after the jet addition is
complete. In general the fastest macro-mixing is achieved in the standard round well,
followed by the small designed well and finally the large designed microwell. In the
majority of cases, however, macro-mixing is extremely rapid with t_{95} being reached
within half a second. These mixing times are of the same order of magnitude, though
generally shorter than those reported by Weiss and co-workers who studied macro-mixing
times using fluorescent indicators (Weiss et al., 2002). This difference is most likely due
to the momentum flux added to the system, the addition times and the fluid flow pattern
development. The momentum flux added by a single jet addition is greater than the
momentum flux added by the pulsing addition methodology used by Weiss, the addition
Figure 4.11: Calculated percentage homogeneity during the course of a low volume injection into a 96-SRW (Fixed tips, Re_j = 1000, V_j = 10µL). The lifespan of the jet addition and the point taken to represent t_{95} in each case are also indicated. Percentage homogeneity calculated from the colour intensity profiles, as described in Section 2.2.4.
Table 4-1: Calculated $t_{95}$ macro-mixing times for jet mixing in three different microwell geometries. Values are shown as a function of tip type and jet Reynolds number. All values were calculated as described in Section 2.2.4 from the colour intensity profiles shown in Figures 4-4, 4-5 and 4-6. Volumes in brackets represent the addition volumes ($V_A$) used in each experiment.

<table>
<thead>
<tr>
<th>Microwell Design and Tip Type</th>
<th>Jet Reynolds Number (Re$_J$)</th>
<th>1000</th>
<th>2480</th>
<th>3000</th>
<th>3960</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.705 s</td>
<td>0.355 s</td>
<td>0.267 s</td>
<td>0.228 s</td>
</tr>
<tr>
<td>Large Designed Well ($V_i = 1720 \mu L$)</td>
<td></td>
<td>(859 \mu L)</td>
<td>(859 \mu L)</td>
<td>(774 \mu L)</td>
<td>(859 \mu L)</td>
</tr>
<tr>
<td>Fixed</td>
<td></td>
<td>0.236 s</td>
<td>0.095 s</td>
<td>0.107 s</td>
<td>0.061 s</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(100 \mu L)</td>
<td>(100 \mu L)</td>
<td>(90 \mu L)</td>
<td>(100 \mu L)</td>
</tr>
<tr>
<td>Disposable</td>
<td></td>
<td>0.155 s</td>
<td></td>
<td>0.050 s</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(100 \mu L)</td>
<td></td>
<td>(100 \mu L)</td>
<td></td>
</tr>
<tr>
<td>Small Designed Well ($V_i = 200 \mu L$)</td>
<td></td>
<td>0.121 s</td>
<td>0.051 s</td>
<td>0.043 s</td>
<td>0.028 s</td>
</tr>
<tr>
<td>Fixed</td>
<td></td>
<td>(100 \mu L)</td>
<td>(100 \mu L)</td>
<td>(90 \mu L)</td>
<td>(100 \mu L)</td>
</tr>
<tr>
<td>Disposable</td>
<td></td>
<td>0.097 s</td>
<td></td>
<td>0.033 s</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(100 \mu L)</td>
<td></td>
<td>(100 \mu L)</td>
<td></td>
</tr>
</tbody>
</table>

times are considerably shorter when additions are made in a single jet and the large scale fluid flow patterns generated by the single addition are not found with the pulsing addition method. In accordance with the theories for conventional jet mixing (Fox and Gex, 1956; Revill 1992) $t_{95}$ values are seen to decrease with increasing jet Reynolds's number.

### 4.4 Correlation of jet macro-mixing times

In order to relate the jet conditions (Section 4.3) and the impact of microwell geometry (Figure 2-2) to the measured $t_{95}$ values (Table 4-1) it is useful to correlate the key parameters of initial jet velocity ($u_o$), jet nozzle diameter ($d_i$) and the microwell aspect ratio. A valid correlation would also allow the prediction of macro-mixing times within the jet lifespan, shown to be important in Section 4.2.3. To determine the effect of the key parameters on the macro-mixing time a comparison was made to the correlation
4 Quantification of jet macro-mixing times in static microwell plates

proposed by Baldyga and co-workers as shown in Equation 1-3 (Baldyga et al, 1995a).

This correlation was determined from data generated on the large scale (0.61m diameter, 
\( V_i = 250 \text{ L} \), hemispherically dished vessel). Given the way the pipette tips of the liquid 
handling robot track upwards during liquid addition, as described in Section 2.2.2, it is 
necessary to use an average value for the liquid height in the well (h) over the lifespan of 
the jet.

Figure 4-12 shows a parity plot of measured and predicted \( t_{95} \) values with the 
original Baldyga correlation (dotted line) representing the line of parity. Two new 
correlations are indicated, one is applicable for the two designed microwell geometries 
(Equation 4-1), and the other that only applies to the standard round well geometry 
(Equation 4-2). In both cases there is a good linear correlation between the measured 
and predicted values (the data having a coefficient of determination greater than 95% to 
the trendline) suggesting that the Baldyga correlation is of an appropriate form to 
describe jet macro-mixing in microwell plates. The two correlations differ only in the 
value of the proportionality constant which is known to be related to the vessel 
geometry (Baldyga et al, 1995).

\[
\text{Designed microwells: } t_{95} = \frac{4.95D^{1.5}h^{0.5}}{u_0d_i} \quad (4-1)
\]

\[
\text{Standard microwell: } t_{95} = \frac{2.60D^{1.5}h^{0.5}}{u_0d_i} \quad (4-2)
\]

Also shown in Figure 4-12 are \( t_{95} \) values predicted using the original Baldyga 
correlation (Equation 1-3). In the case of the two designed microwells the measured \( t_{95} \) 
values are within 10%, on average, of the values predicted by the original correlation. 
This similarity suggests that even at the very different scales of operation the liquid jets 
act in an unconstrained fashion, unhindered by the walls or the base of the vessel. The 
difference between the two correlations is most probably due to the hemispherically based
Figure 4-12: Parity plot of measured and predicted $t_{95}$ values: small designed microwell (▲) large designed microwell (■) 96-SRW (●). The reference correlation of Baldyga et al., (1995) is also shown (......). Experimental values taken from Table 4-1.
vessel used in the original work which is known to improve macro-mixing efficiency compared to flat bottomed vessels (Lane and Rice, 1981). It is interesting to note that the correlation appears to apply at a scale of over a one hundred thousand fold smaller than that for which it was originally determined. In contrast to the case of the two designed microwells, mixing times in the standard round microwell were on average 42% faster than those predicted by the original correlation. This is due to the end of the jet impinging on the base of the standard microwells (Figure 4-3) resulting in an increase in the local turbulent energy dissipation rate at the end of the jet (Grenville and Tilton, 1996).

4.4.1 Definition of minimum liquid addition volume (V_{C_{rit}})

The long \( t_{95} \) macro-mixing times determined for small volume additions and short jet lifespans (Section 4.2.3) will significantly affect the precision and accuracy of any bioprocess kinetic measurements made under such conditions. One important use of the correlations shown in Figure 4-12 is therefore the prediction of the minimum addition volume (\( V_{C_{rit}} \)), for given pipetting conditions, which will ensure that \( t_{95} \) is achieved within the lifespan of the jet. Based on the correlations established for the two designed microwells (using Equation 4-1) and the standard round well (using Equation 4-2) predictions of the minimum addition volume (\( V_{C_{rit}} \)) required to achieve homogeneity can be made for each tip type available for the LHR (Table 4-2). This based on ensuring that the addition time is equal to or greater than the minimum \( t_{95} \) value needed to complete macro-mixing. A comparison can be made between the two types of pipette tip in equivalent flow regimes. As expected, the fixed tips, having a smaller nozzle diameter, give a better macro-mixing efficiency at equivalent Reynolds numbers due to the greater momentum flux input (Fox and Gex, 1956).

Table 4-2 also shows \( V_{C_{rit}} \) as a function of the initial liquid volume in the well. The small designed microwell requires the largest relative addition volume while the
Table 4-2: Minimum liquid addition volume ($V_{\text{Crit}}$) required to achieve 95% homogeneity in various microwell formats ensuring $t_{95}$ is reached within the jet lifespan. Values determined as described in Section 4.2.1.

<table>
<thead>
<tr>
<th>Tip type</th>
<th>$V_{\text{Crit}}$ ($\mu$L)</th>
<th>Volume (% of fill)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) Large designed microwell ($V_i = 1720 \mu$L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fixed</td>
<td>289</td>
<td>17</td>
</tr>
<tr>
<td>b) Small designed microwell ($V_i = 200 \mu$L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fixed</td>
<td>72</td>
<td>36</td>
</tr>
<tr>
<td>Disposable</td>
<td>80</td>
<td>40</td>
</tr>
<tr>
<td>c) 96-SRW plate ($V_i = 200 \mu$L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fixed</td>
<td>42</td>
<td>21</td>
</tr>
<tr>
<td>Disposable</td>
<td>46</td>
<td>23</td>
</tr>
</tbody>
</table>

large designed microwell requires the smallest. Values for the standard round well fall between the two. These trends can be accounted for by consideration of bulk liquid entrainment in the jet and the local turbulent energy dissipation rate at the end of the jet (Greenville and Tilton, 1996; Revill, 1992). For the two microwell designs with the same initial fill volume of 200μl, the standard round well shows greater mixing efficiency than the small designed well due to the higher energy dissipation rate as the jet impinges on the base of the well (Figure 4-3). However, the jet length to nozzle diameter ratio ($X/d_i$) is also important since it affects mixing efficiency through the entrainment of bulk fluid in the jet and the promotion of liquid circulation. The large designed microwell has a much higher $X/d_i$ ratio than either of the two other designs (Figure 2-2) and hence has the lowest relative addition volumes that ensure $t_{95}$ is achieved within the life time of the jet.

Of the three microwell designs investigated here, it appears that the large designed microwell is the most efficient macro-mixing vessel although the shortest mixing times, as indicated in Table 4-1, are obtained with the standard round well geometry. It might also be interesting to examine microwell designs with hemispherical or V-shaped bases as these would be predicted to decrease mixing times and increase mixing efficiency. Not having a flat base however, such wells would not be suitable for use in the standard
4 Quantification of jet macro-mixing times in static microwell plates

Microtitre plate readers routinely used with absorbance or fluorescence-based assays (Cameron et al, 1992; Zheng and Wozniak, 1997).

4.4.2 Other considerations affecting choice of pipetting conditions

The previous sections have shown that rapid jet macro-mixing can be achieved in static microwell plates. For single, large volume additions (25-50% of initial well volume) by turbulent jets, complete macro-mixing can be achieved in many microwell geometries is less than half a second. While these represent optimum mixing conditions, in many cases reagent costs and the need to reduce cross-contamination of wells must also be considered. In very high throughput drug discovery applications as many as 250,000 microwell assays can be performed each day (Delvin, 1997; Lamsa et al, 2004). In such cases the utilisation of key reagents like enzyme substrates can be high, and therefore expensive, so low volume additions (5-10% of initial well volume) must be used. Under such conditions we have shown that macro-mixing times can be of the order of minutes (Section 4.2.3) which can lead to high variability and assay results that are more qualitative than quantitative. To improve assay performance it is possible to include a secondary mixing step where a large aliquot of the mixture is re-aspirated into the pipette tip and then dispensed back into the well. This has previously been shown to improved assay performance but at the expense of experimental throughput (Berg et al, 2001).

Similarly, it has been shown here that the most effective mixing is achieved using small nozzle diameters (Section 4.2.1). For most liquid handling robots this requires the use of fixed, rather than disposable, tips. With fixed tips however, there is a greater risk of cross-contaminating individual wells (Astle and Akowitz, 1996) when dispensing below the liquid surface (liquid carry over). In order to minimise cross-contamination in critical applications like microwell fermentations and cell-based assay, a wash step for fixed tips
can be included between additions or disposable tips can be used. Both approaches will reduce well-to-well contamination but at the expense of experimental throughput. It has been previously demonstrated that dispensing above the liquid surface can be used to increase the throughput and allows rapid and repeated sampling of the contents of individual wells for kinetic studies (Chapter 3). But this approach has potential for cross contamination, by aerosol formation, and the macro-mixing times calculated in this study are not applicable as they only apply to dispenses made below the liquid surface.

4.5 Conclusions

In this study, a high speed video technique for the accurate quantification of jet macro-mixing times in static microwell plates has been developed. Under optimum conditions complete macro-mixing could generally be achieved within half a second and, in some cases within one tenth of a second. $t_{95}$ mixing times were shown to decrease with increasing jet Reynolds number and increasing addition volumes. Macro-mixing was also studied in a range of standard and novel microwell geometries. In deep well formats it was shown that maximising the jet length to nozzle diameter ratio ($X/d_i$) promotes the most efficient macro-mixing due to entrainment and circulation of the bulk liquid in the well. Microwells of the standard 96-well plate geometry were shown to provide the most rapid macro-mixing due to increased energy dissipation rates as the jet impinges on the base of the well. A number of correlations have also been developed which enable the prediction of (i) $t_{95}$ mixing times for given pipetting conditions, and (ii) calculation of the minimum liquid addition volume necessary to ensure efficient macro-mixing within the jet lifespan ($V_{Crit}$).

It has been also shown that for some addition volumes below $V_{Crit}$, macro-mixing can be completed by residual momentum and molecular diffusion but in most cases homogeneity can not be reached without a secondary mixing step. As a consequence of
this data generated with addition volumes less than $V_{\text{Crit}}$ will be qualitative rather than quantitative. The implications of incomplete macro-mixing on bioprocess situations and the identification of optimum operating conditions will be further discussed in Chapter 6.

In summary this chapter has presented original data on jet macro-mixing times in static microwell plates and correlations to enable $t_{95}$ values to be reliably predicted based on well geometry and liquid handling conditions. While macro-mixing is important in all process applications, micro-mixing, i.e. mixing below the Kolmogoroff scale, is also important in cases where the balance between fast competitive reactions determines the yield of a process. This is investigated further in Chapter 5.
5 Assessment of micro-mixing efficiency in static microwell plates and stirred tank vessels

5 Assessment of micro-mixing efficiency in static microwell plates and stirred tank vessels

5.1 Introduction and aims

In the previous chapter an approach to quantifying macro-mixing in static microwell plates was described. Also important is the micro-mixing efficiency within a reaction vessel since this is known to influence the product profile within fast chemical reactions. Efficient micro-mixing effectively maintains product quality and limits the formation of reaction by-products (Plasari et al, 1978). In this chapter the micro-mixing efficiency will be quantified in static jet-mixed microwell plates and comparisons will be made to micro-mixing efficiencies determined in bench and pilot scale stirred tank reactors.

The micro-mixing efficiency in large-scale stirred vessels has been determined by a number of teams (Baldyga and Bourne, 1990; Guichardon and Falk, 2000a) through the use of competitive chemical reactions and a number of correlations have been generated for the determined of the micro-mixing efficiency from the physical environment within the vessel (Section 1.2.2.1). As yet there is no published data on the micro-mixing efficiency within microwell plates.

Initially the iodate-iodide reaction system (Section 1.2.2.2) will be tested to determine its suitability to measure the micro-mixing efficiency in static jet mixed microwell plates. The factors influencing the micro-mixing efficiency within a number of microwell plate formats (96-SRW and 96-DRW) will then be determined through the use of statistical design of experiments (DOE). The micro-mixing efficiencies will then be determined for a bench scale and pilot scale stirred tank reactors.
Assessment of micro-mixing efficiency in static microwell plates and stirred tank reactor. The engineering design equations generated by DOE will then be compared to theoretical micro-mixing models (Section 1.2.2.1) allowing an assessment of the reactor performance across geometries, mixing methodologies and scales to be made. The suitability of microwell formats to be utilised as a micro-mixing route scouting tool and as a mimic of large scale vessels will then be determined.

5.2 Results

The tri-iodide micro-mixing technique utilises the competition between two chemical reactions to make an assessment of the micro-mixing efficiency within a reaction vessel (Section 1.2.2.2). Acid additions made to a borate stock solution initially quench the borate buffer within reaction zone and then proceed along the tri-iodide production pathway. Fresh borate ions are introduced into the reaction zone by mixing with the surrounding fluid. The relative rates of this mixing and the tri-iodide production pathway allow a segregation index for the protons added to the system (\( \alpha \)) to be generated. This \( \alpha \) value is a direct representation of the micro-mixing efficiency within the reaction vessel. The acid addition volumes and concentrations directly influence the calculated segregation index. High addition volumes and concentrations lead to the precipitation of iodine and an invalid representation of the segregation index. Low volumes and additions can lead to a low concentration of tri-iodide being generated with a correspondingly high signal to noise ratio. Therefore the acid addition volume and concentration have been tailored for each vessel format to produce a significant signal without iodine precipitation.
5 Assessment of micro-mixing efficiency in static microwell plates and stirred tank vessels

5.2.1 Implementation of tri-iodide micro-mixing technique at the microwell scale

To measure the micro-mixing efficiency within microwell plates it is essential that any method be accurate and reproducible. Figure 5-1 shows the liquid handling accuracy and precision determined using the methodology described in Section 2.1.3. Both the blowout and waste modes of operation were investigated. It can be seen that for all the acid addition volumes used in this work (42–530 µL) that liquid handling is accurate and precise for both modes of operation. Figure 5-2 demonstrates that the two modes of operation have very little influence on the micro-mixing efficiency. The blowout mode of operation gives slightly higher micro-mixing efficiencies for both fixed and disposable pipette tips but the variation is within the experimental error for each condition. The disposable pipette tips give greater micro-mixing efficiencies than the fixed tips independent of which mode of operation is used. This is consistent with the macro-mixing efficiencies measured in Section 4.2.2. From Figure 5-2 it can be concluded that neither the mode of operation nor any air introduced into the system, either by the blowout mode of operation or from surface entrainment (see Figure 4-1), will have an effect on the calculated micro-mixing efficiency. Consequently the liquid handling conditions utilised for determining the micro-mixing efficiency in the 96-SRW and the 96-DRW plates (Section 2.3.2) the waste mode were used as default. The significance of the $\alpha$ values themselves will be discussed later.

The 96-DRW plates are not compatible with commercially available microwell plate readers so samples must be taken and transferred from the deep well plates to a 96-SRW plate for analysis. The effect of sample position within the well was investigated and the results shown in Figure 5-3. It can be seen that the
Figure 5-1: Accuracy and precision of liquid handling using single dispenses per aspirate and either the blowout or waste modes of operation. The cut off point for accurate and precise liquid handling, ≤ 5% CV (----) is also shown. ■ Blowout mode accuracy, ● Blowout mode precision, ▲ Waste mode accuracy, ▼ Waste mode precision. The methodology is described in Section 2.1.3 for the performance files detailed in Section 2.3.2.1.1.
Figure 5-2: The effect of the mode of operation and tip type on calculated micro-mixing efficiency in static 96-SRW plates. □ Blowout  ■ Waste. Sulphuric acid additions (42 μL for fixed tips and 46 μL for disposable tips) were made to 200 μL of the borate stock solution. Each addition was made 1mm below the liquid surface with $Re = 1000$. The $\alpha$ values were calculated using the equations detailed in Section 2.3.3.2. Error bars represent the standard deviation of $\alpha$ across the four repeats of each condition.
Figure 5-3: The effect of pH and sample position when determining the micro-mixing efficiency in 96-DRW plates. (■ Surface sampling • Well mid-point sampling.) Fixed pipette tips were used for the acid addition and for sampling. The acid addition volume was 290 μL and the Re_j = 1000 as detailed in Section 2.3.2. Error bars represent the standard deviation of α across the four repeats of each condition.
5 Assessment of micro-mixing efficiency in static microwell plates and stirred tank vessels

Micro-mixing efficiency is dependent on the sampling position. Mid-point sampling gives higher micro-mixing efficiencies than for surface sampling. At the higher acid concentrations the difference is small but becomes larger as the acid concentration falls. This is however most likely to be due to exaggeration of the size of errors by the method of \( \alpha \) value calculation when the tri-iodide signal is small rather than due to physical differences in the mixing efficiency. Mid-point sampling was utilised when determining the micro-mixing efficiency in 96-DRW plates.

Figure 5-4 shows the effect of the sampling time after acid addition on the stability of the tri-iodide signal. It can be seen that the tri-iodide signal is stable for the detection period investigated and therefore any variation in the calculated \( \alpha \) value is independent of the sampling time after the acid addition has been made.

The effects of the physical parameters of liquid handling accuracy and precision, mode of operation, sample position and the stability of the tri-iodide signal on the micro-mixing efficiency have been investigated here. It has been demonstrated that these parameters have no influence on the micro-mixing efficiency but it has not yet been determined whether the variations in the experimental \( \alpha \) values can be attributed purely to changes in the micro-mixing environment within the microwell.

The \( \alpha \) values have also been shown to vary greatly between reactor geometry, a range of 2-4 in the 96-SRW but a range of 20-250 in the 96-DRW, suggesting that the \( \alpha \) values are dependent on the reactor efficiency and hence on the microwell geometry. Macro-mixing has also been shown to influence the experimental \( \alpha \) values (Guichardon and Falk, 2000a) but using conditions that ensure macro-mixing is complete within the lifespan of the jet addition (Table 4-2) will remove macro-mixing as a factor that can influence the experimental \( \alpha \) values.
Figure 5-4: Stability of the tri-iodide signal used for determining the micromixing efficiency in microwell plates. (■ 96 deep well plate ▲ standard 96 well plate). Fixed pipette tips and $Re_j = 1000$ were used for both well types. Acid addition volumes = 42 μL (standard 96-well plates) and 290 μL (96 deep round well plates). Sampling for the deep well plate was at the mid-point within the well. Error bars represent the standard deviation of the $\alpha$ value of four repeats of each condition.
5 Assessment of micro-mixing efficiency in static microwell plates and stirred tank vessels

5.2.2 Quantification of micro-mixing efficiency using response surface methodology

The micro-mixing efficiency was measured over a range of combinations of the factors utilised for each reactor geometry (Section 2.3.2 (microwell plates) and Section 2.3.3 (stirred tank reactors) using Design Expert software (Stat-Ease Inc, Minneapolis, USA). The calculated $\alpha$ values (See Appendix) were used to obtain predictive engineering equations built by the deletion of non-significant terms from a general linear model. Table 5-1 lists all the factors from each of the tank configurations with their regression coefficients and t-values. The ANOVA statistics for each model, described in the following sections, are shown in Table 5-2.

Table 5-1: Regression coefficients and t values for the design factors used in each of the vessel configurations described in Section 2.3.2 and 2.3.3.

<table>
<thead>
<tr>
<th>Tank configuration</th>
<th>Factor</th>
<th>Coefficient estimate</th>
<th>Probability &gt; t</th>
</tr>
</thead>
<tbody>
<tr>
<td>96 standard round well</td>
<td>Jet Reynolds number (A)</td>
<td>-2.25</td>
<td>0.4285</td>
</tr>
<tr>
<td></td>
<td>Acid volume (B)</td>
<td>11.45</td>
<td>0.0022</td>
</tr>
<tr>
<td></td>
<td>Tip diameter (C)</td>
<td>5.41</td>
<td>0.0195</td>
</tr>
<tr>
<td>96 deep round well</td>
<td>Jet Reynolds number (A)</td>
<td>7.25</td>
<td>0.2002</td>
</tr>
<tr>
<td></td>
<td>Acid volume (B)</td>
<td>24.55</td>
<td>0.0116</td>
</tr>
<tr>
<td>500 mL stirred tank</td>
<td>Impeller speed (A)</td>
<td>4.47</td>
<td>0.0030</td>
</tr>
<tr>
<td></td>
<td>Acid Volume (B)</td>
<td>3.12</td>
<td>0.0344</td>
</tr>
<tr>
<td></td>
<td>Addition time (C)</td>
<td>0.40</td>
<td>0.7663</td>
</tr>
<tr>
<td>10 L stirred tank</td>
<td>Impeller speed (A)</td>
<td>2.02</td>
<td>0.0032</td>
</tr>
<tr>
<td></td>
<td>Acid volume (B)</td>
<td>3.19</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Addition time (C)</td>
<td>0.50</td>
<td>0.3873</td>
</tr>
</tbody>
</table>

Table 5-2: ANOVA for the response surface reduced linear model utilising the vessel configurations described in Section 2.3.2 and 2.3.3. (Adj $R^2$ = adjusted correlation coefficient, Root MSE = root mean square error).

<table>
<thead>
<tr>
<th>Tank Configuration</th>
<th>F - value</th>
<th>Probability &gt; F</th>
<th>Adj $R^2$</th>
<th>Root MSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>96 standard round well</td>
<td>17.09</td>
<td>0.0002</td>
<td>0.6679</td>
<td>7.69</td>
</tr>
<tr>
<td>96 deep round well</td>
<td>21.63</td>
<td>0.0097</td>
<td>0.8049</td>
<td>10.56</td>
</tr>
<tr>
<td>500 mL stirred tank</td>
<td>10.42</td>
<td>0.0020</td>
<td>0.5568</td>
<td>3.29</td>
</tr>
<tr>
<td>10 L stirred tank</td>
<td>15.38</td>
<td>0.0001</td>
<td>0.7295</td>
<td>1.58</td>
</tr>
</tbody>
</table>
5.2.2.1 96 standard round well plate

From Table 5-1 it can be seen that there are two significant factors that influence micro-mixing efficiency in the 96-SRW plate; the acid volume and the pipette tip diameter. The influence of each factor on the value of alpha can be seen in the perturbation plot Figure 5-5. Both the factors have a positive influence on the values of \( \alpha \), an increase in either factor leads to an increase in \( \alpha \), and hence the micro-mixing efficiency. The acid volume has a greater effect on micro-mixing efficiency than the pipette tip diameter. The combined effects of the two factors are shown in Figure 5-6 and the engineering design equation in Equation 5-1. The model’s significance is demonstrated in Table 5-2. The model does have a relatively high degree of variance that cannot be explained by the model (Adj R\(^2\)) and a high degree of experimental error (Root MSE).

\[ \text{Alpha} = -113.07 + (0.39 \times B) + (180.22 \times C) \quad (5-1) \]

The effect of the acid volume on the micro-mixing efficiency can easily be explained by reference to the chemical system (Guichardon and Falk, 2000) and to other systems used to determine the micro-mixing efficiency (Baldyga et al., 1998). If the same number of moles of acid is added but in a more dilute solution (as happened here) a greater proportion of the acid will be quenched by the borate neutralisation reaction (Equation 1-15) and less acid will react through the slower tri-iodide side reaction pathway (Equation 1-16). It has also been observed that whenever the volume ratio of the reactants was decreased the micro-mixing efficiency increased (Baldyga et al., 1998). This was explained by the shorter mixing time giving less chance for the slow side reaction to proceed and thus increasing the selectivity (\( \alpha \)).

The effect of the pipette tip diameter can be explained with reference to the energy dissipation rate at the base of the well. It has been shown that there are two

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Figure 5-5: Perturbation plot showing the effect of the Reynolds number (A), acid addition volume (B) and tip diameter (C) on the micro-mixing efficiency (Alpha) in a 96-SRW plate. Deviations from a midpoint (0.000) are shown with –1 and +1 being the upper and lower limits of the variable range. Experiments performed as described in Section 2.3.3.2.
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Figure 5-6: Response surface plot showing the effect of acid addition volume and pipette tip diameter on the micro-mixing efficiency in a 96-SRW plate. (The acid volume units are mL and the tip diameter is measured in mm). Response surface generated using Equation 5-1 from detail given in Table 5-1 and Table 5-2
factors which influence the micro-mixing efficiency within a reaction vessel, the
degree of fluid entrainment (Revill, 1992) and the energy dissipation rate at the end of
the jet (Grenville and Tilton, 1996). Within this reaction system the degree of fluid
entrainment is independent of the pipette tip type and is fixed by the liquid height
within the microwell. The energy dissipation rate within the reaction vessel decays
from end of the potential core (Figure 1-2) as a function of the initial jet velocity ($u_0$),
the jet nozzle diameter ($d_i$) and the length of the jet path length (X) (Equation 1-6).
Using this equation the energy dissipation rates at the base of the well were calculated
for the two pipette types and a greater energy dissipation rate was predicted with the
disposable tips. This would lead to a greater mixing efficiency and a higher $\alpha$ value,
as experimentally observed.

5.2.2.2 96 - deep round well plate

The reduced number of factors investigated with the 96-DRW plate well
format required a full, two level factorial design to be conducted. As shown in Table
5-1, the Reynolds number was found not to be significant and therefore, as there is
only one significant parameter, it is not possible to produce a response surface plot.
Instead, only a perturbation plot is shown (Figure 5-7). The results for this well format
follow the pattern already discussed for the 96-SRW plate. The more dilute acid
solution (increasing acid addition volume) added leads to an increase in the micro-
mixing efficiency. This is consistent with the results observed for the 96-SRW plate,
in the more dilute solution a greater proportion of the acid is quenched by the borate
neutralisation pathway (Equation 1-15) and less acid reacts via the slower tri-iodide
production pathway (Equation 1-16). An engineering design equation (Equation 5-2)
was produced and the validity of the model is demonstrated in Table 5-2. This shows
Figure 5-7: Perturbation plot showing the effect of the Reynolds number (A) and acid addition volume (B) on the micro-mixing efficiency (Alpha) in a 96-DRW plate. Deviations from a midpoint (0.000) are shown with −1 and +1 being the upper and lower limits of the variable range. Experiments performed as described in Section 2.3.3.2.
a greater tendency for the null hypothesis to be true compared to the 96-SRW model but the model is still significant. The model can explain more of the variance seen in the results but there is a greater spread of experimental errors than for the previous system.

\[ \text{Alpha} = -54.93 + (0.20 \times C) \] (5-2)

The numerical values of \( \alpha \), and hence the micro-mixing efficiencies, are significantly higher in the 96-DRW than in the 96-SRW. This is due to the large increase in the degree of fluid entrainment in the 96-DRW associated with the much longer jet length \( (X) \) in the 96-DRW compared to the 96-SRW. This result is consistent with the observations of macro-mixing efficiency made in Section 4.2.2 that the 3:1 aspect ratio reaction vessel is a more efficient mixer than the 1:1 aspect ratio vessel.

5.2.2.3 **Bench and pilot scale stirred tank reactors**

Data for the bench and pilot-scale stirred tank reactors can be analysed together as they both display the same trends. Assessment of the significance of each of the factors investigated (Table 5-1) lead to the identification of two significant factors in each case; the impeller speed and the acid volume. When using the stirred tank reactors it is necessary to determine whether the calculated micro-mixing efficiency is also influenced by the macro-mixing environment. The identification of the addition time as being a non-significant factor in both cases (Table 5-1) demonstrates that macro-mixing is not playing a role in the determination of the micro-mixing efficiency (Figure 1-4).

From the perturbation plots of the two systems (Figure 5-8 and Figure 5-9) it can be seen that the impeller speed is the more important factor in the 500 mL bench
Figure 5-8: Perturbation plot showing the effect of the impeller speed (A), acid addition volume (B) and addition time (C) on the micro-mixing efficiency (Alpha) in a 500 mL bench scale stirred tank. Deviations from a midpoint (0.000) are shown with -1 and +1 being the upper and lower limits of the variable range. Experiments performed as described in Section 2.3.3.3.
Figure 5-9: Perturbation plot showing the effect of the impeller speed (A), acid addition volume (B) and addition time (C) on the micro-mixing efficiency (Alpha) in a 10 L pilot scale stirred tank. Deviations from a midpoint (0.000) are shown with -1 and +1 being the upper and lower limits of the variable range. Experiments performed as described in Section 2.3.3.3.
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scale but the acid volume is more important in the 10 L pilot scale. This may be due to the relative ranges of the two factors and their influence on the micro-mixing efficiency. Due to the high degree of surface aeration achieved at high impeller speeds in the pilot scale vessel the range of impeller speeds investigated was low compared to the bench scale vessel. The plots also show that higher micro-mixing efficiencies can be achieved in the bench scale reactor against the pilot scale reactor.

The combined effect of the two factors are shown in the response surface plots for each configuration (Figure 5-10 and Figure 5-11). The validity of the models is given in Table 5-2 and the engineering design equations in Equation 5-3 (500 mL bench scale) and Equation 5-4 (10 L pilot scale). Both designs produce significant models though the 10 L configuration is the more significant, has less variance that cannot be described by the model and has less experimental error.

\[
\text{Alpha} = -0.026 + (5.967 \times 10^{-3} \times A) + (0.25 \times B) \quad (5-3)
\]

\[
\text{Alpha} = -3.86 + (0.017 \times A) + (0.041 \times B) \quad (5-4)
\]

The significance of the impeller speed has been demonstrated by a number of other groups (Guichardon and Falk, 2000a; Baldyga and Bourne, 1994) and is dependent on the energy dissipation rate within the vessel. This is in turn dependent on the vessel and impeller geometries, the feed position and the impeller speed (Section 2.3.4). The energy dissipation rate will increase as the impeller speed increases and therefore the observed increase in the micro-mixing efficiency as the impeller speed increases is expected. The increase in the micro-mixing efficiency with the increasing acid volume is explained by the reactant volume ratio. A decrease in the volume ratio leads to a shorter mixing time giving less chance for the slow side reaction to proceed and so increasing the selectivity (Baldyga et al, 1998).
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Figure 5-10: Response surface plot showing the effect of acid addition volume and impeller speed on micro-mixing efficiency in a 500 mL stirred tank reactor. (The acid volume units are mL and the tip diameter is measured in mm). Response surface generated using Equation 5-3 from detail given in Table 5-1 and Table 5-2.
Figure 5-11: Response surface plot showing the effect of acid addition volume and impeller speed on micro-mixing efficiency in a 10 L stirred tank reactor. (The acid volume units are mL and the tip diameter is measured in mm). Response surface generated using Equation 5-3 from detail given in Table 5-1 and Table 5-2.
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5.3 Discussion

In order to relate the models of micromixedness for each of the reactor configurations generated in Section 5.2.2 to their scale, mixing methodology and reactor geometry it is useful to make a comparison to a number of known micro-mixing models (Section 1.4.2). To this end the α values predicted from the engineering design equations (Equations 5-1, 5-2, 5-3 and 5-4) have been compared to calculated Damköhler numbers from two micro-mixing models. An engulfment model (Equation 1-20: Baldyga and Bourne, 1989) and an incorporation model (Equation 1-21: Fournier et al, 1996) were considered. Initially, due to the different mixing methods employed, the two reactor categories are considered separately before a comparison between the different reactor geometries and mixing methodologies is made.

5.3.1 Stirred tank reaction vessels

The relationship between Da and α for the two stirred tanks are shown in Figure 5-12 (engulfment) and Figure 5-13 (incorporation). Using the engulfment model it can be seen that there is a linear relationship between α and Da. The 10 L, pilot scale reactor produces data where Da can be used to predict α for all addition volumes. The 500 mL bench scale reactor, however highlights a limitation in this model. The data for the two higher acid volumes can be correlated together while the data from the lowest addition volume is distinct. It has been shown that the engulfment model is only valid for very small volume fractions of the feed (Baldyga and Bourne, 1999). Significant differences in the volume fraction lead to the over-estimation of the reaction rates as the model assumes that all engulfment will be between the feed and the bulk fluid. At higher volume fractions self-engulfment of the
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Figure 5-12: Comparison of Damköhler numbers calculated using the engulfment model with micro-mixing efficiencies generated using the engineering design equations produced in Section 5.2.2 in stirred tank reactors. Data is shown for both the 500 mL vessel (---) with a Rushton impeller and the 10 L vessel (-----) fitted with a propeller. Different acid addition volumes are shown for each reactor. In each case the total number of moles of acid added remains constant (25 µmoles for the 500 mL stirred tank and 12 mmol for the 10L vessel). Sulphuric acid addition volumes: (■) 0.25 mL, (●) 12.62 mL, (▲) 25 mL, (►) 102.5 mL, (▲▲) 180 mL.
Figure 5-13: Comparison of Damköhler numbers calculated using the incorporation model with micro-mixing efficiencies generated using the engineering design equations produced in Section 5.2.2 in stirred tank reactors. Data is shown for both the 500 mL vessel (——) with a Rushton impeller and the 10 L vessel (-----) stirred with a propeller. Differing acid addition volumes are shown for each reactor. In each case the total number of moles of acid added remains constant (25 mmol for the 500 mL stirred tank and 12 mmol for the 10L vessel). Sulphuric acid addition volumes: (■) 0.25 mL, (•) 12.62 mL, (▲) 25 mL, (►) 102.5 mL, (▼) 180 mL.
feed can occur. This is likely to explain the separation of the low acid addition volume
data. The Damköhler numbers for the two higher addition volumes both over estimate
the reaction rate.

Comparisons with the incorporation model (Figure 5-13) demonstrate the
difference between the two approaches in that there is a separate correlation for each
set of reactant concentrations and acid addition volumes. Each of the correlations, for
a given reactor, are parallel, however suggesting the micro-mixing mechanism in each
set of conditions is consistent. The correlation gradients display similar trends to the
engulfment model; the 500 mL reactor having a steeper gradient, indicating a more
efficient mixer, than the 10 L reactor.

Both models can be use to predict α values from known physical conditions
within the reactor vessel but a number of conditions limit their applicability. The
engulfment model can be used to predict α values when the volume fraction of the
feed remains small. Large changes in the dilution factor lead to a shift in the
correlation by an unknown degree due to the over estimation of the engulfment rate
and so cannot be used for α value prediction. With the incorporation model the
correlations for each of the reactant conditions are parallel and are likely to be shifted
by the dilution factor in a predictable manner. Therefore the α values could be
predicted from the Damköhler numbers within the range of the dilution factors used to
generate Figure 5-13. When appropriately applied, both models could be used to
predict operating parameters that would generate common alpha values in reaction
vessels of various scales.
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5.3.2 Microwell reaction vessels

The engulfment and incorporation micro-mixing models produce very similar correlations when a comparison between the two microwell formats is made (Engulfment: Figure 5-14 and Incorporation: Figure 5-15). For the 96-SRW plate the two pipette types produce parallel, linear correlations between the $\alpha$ values and $Da$, indicating that the model is able to predict $\alpha$ values but that it can not explain the influence of the pipette tip diameter. This is not surprising as both models were constructed for use in stirred tank reactors and not for jet mixers. In both types of vessel the micro-mixing efficiency is determined by the energy dissipation rate within the reaction zone. In the stirred tank this is close to the feed inlet and is governed by the impeller speed. In the jet mixed microwells the reaction zone is located at the end of the jet and the energy dissipation rate is fixed by the decay of the initial fluid velocity. This rate of decay is a function of the nozzle diameter and the length of the jet. The 96-DRW plate produced a simple linear relationship for each model. The steeper gradient of the data correlation indicates that the 96-DRW format facilitated more efficient micro-mixing than the 96-SRW format. The volume fraction, which was seen to have an important role in the stirred tank reactors (Figure 5-14), does not seem to play such a significant role in the jet mixed reactors. The only difference being a small change in the gradient of the correlations.

The relative efficiencies of each of the reactor geometries is consistent with standard jet mixing theory. Though the 96-SRW format has a high energy dissipation rate at the end of the jet, since the jet is short, the 96-DRW plate achieves a much greater micro-mixing efficiency due to the high level of fluid entrainment generated by the long jet length. The energy dissipation rate at the end of the jet has been shown to be important (Grenville and Tilton, 1996) but it is the fluid entrainment which has
Figure 5-14: Comparison of Damköhler numbers calculated using the engulfment model with micro-mixing efficiencies generated using the engineering design equations produced in Section 5.2.2 in jet mixed microwells. Data is shown for both the 96-SRW (----) and the 96-DRW (-----) plates. Different acid addition volumes are shown for each reactor. In each case the total number of moles of acid added remains constant (8.97 μmol for the 96-SRW plate and 56.8 μmoles 96-DRW plate). 96-SRW plate: (■) fixed tips, (•) disposable tips. 96-DRW plate: (▲) fixed tips.
Comparison of Damköhler numbers calculated using the incorporation model with micro-mixing efficiencies generated using the engineering design equations produced in Section 5.2.2 in jet mixed microwells. Data is shown for both the 96-SRW (——) and the 96-DRW (-----) plates. Different acid addition volumes are shown for each reactor. In each case the total number of moles of acid added remains constant (8.97 μmol for the 96-SRW plate and 56.8 μmol 96-DRW plate). 96-SRW plate: (■) fixed tips, (○) disposable tips. 96-DRW plate: (▲) fixed tips.
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the greater influence on the mixing efficiency (Revill, 1992; Section 4.2.2).

5.3.3 Comparison across mixing methods and scales

Having established the relationship between $\alpha$ values and $Da$ in Section 5.3.2 for each of the vessel types, a comparison between the jet mixed vessels and the stirred tank reactors could be made. The engulfment model (Figure 5-16) predicts a significant difference in micro-mixing efficiency between the 10 L pilot scale reactor and the 96-SRW plate. A similar comparison using the incorporation model (Figure 5-17) can be made with the 500 mL bench scale reactor and the 96-deep round well plate. Both comparisons demonstrate that much higher $\alpha$ values can be generated within the microwell format and that jet mixing at the small scale is more efficient than mixing by mechanical agitation in the larger tanks.

Further more, the plots identify a limited range of conditions where similar $\alpha$ values can be obtained between the microwell formats and the stirred tank vessels. This potentially represents an opportunity to utilise $\alpha$ values as a methodology for the study of large-scale micro-mixing conditions within microwell plate formats.

5.4 Conclusions

Experiments on four reactor vessels (96-SRW, 96-DRW, 500 mL stirred tank and 10 L stirred tank) have shown that the competitive chemical reaction system, described in Section 1.2.2.2, can be used to assess the micro-mixing efficiency in stirred tank and jet mixed reactor configurations. Response surface methodologies have then been utilised to determine the factors significant in influencing the level of mixing and engineering design equations have been constructed to predict the micro-mixing efficiency over a range of factor values. In the jet-mixed reactors the
Figure 5-16: Comparison of Damköhler numbers calculated using the engulfment model with micro-mixing efficiencies generated using the engineering design equations produced in Section 5.2.2. Data is shown for both the 10 L pilot scale stirred vessel (——) and the 96-SRW plate (-----). The total number of moles of acid added remains constant for each reactor (8.97 μmol for the 96-SRW and 12 mmol for the pilot scale reactor). Different tip types are shown. (▼) Fixed tips, (▲) Disposable tips. Acid addition volumes: (■) 25 mL, (●) 102.5 mL and (▲) 180 mL.
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Figure 5-17: Comparison of Damköhler numbers calculated using the incorporation model with micro-mixing efficiencies generated using the engineering design equations produced in Section 5.2.2. Data is shown for both the 500 mL bench scale stirred vessel (---) and the 96-DRW plate (-----). The total number of moles of acid added remains constant for each reactor (56.8 µmol for the 96-DRW and 25 µmol for the bench scale reactor). Different acid addition volumes are shown: (■) 0.25 mL, (●) 12.625 mL and (▲) 25 mL. (▼) 96-deep round well plate
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fluid entrainment and the energy dissipation rate at the end of the jet were both shown to influence the micro-mixing efficiency. For stirred tank vessels, the fluid environment within the reaction zone, close to the addition point, determined the micro-mixing efficiency of the whole vessel. The relative efficiencies of the reaction vessels have also been investigated with the jet-mixed reactors being able to produce significantly higher micro-mixing efficiencies than the stirred tank vessels.

A comparison with theoretical micro-mixing models allows an assessment of the impact of the vessel geometry and scale to be made. Variation of the aspect ratio within the microwell plate formats produced different micro-mixing efficiency profiles, with the 96-DRW plate being more efficient than the 96-SRW plate, as small changes in the Damköhler number lead to significantly larger changes in the $\alpha$ value within the 96-DRW plate. This indicates that fluid entrainment is more important than the energy dissipation rate at the end of the jet in determining the micro-mixing efficiency of the whole vessel. The influence of the scale of geometrically similar stirred tank reactors demonstrated that micro-mixing efficiency is independent of scale but dependent on the physical environment within the reaction zone surrounding the addition point. The volume fraction was shown to influence the $\alpha$ value through the over-estimation of the reaction rates and large changes in the volume fraction should be avoided.

Common micro-mixing efficiencies have been identified across the various scales and reactor types for a limited range of microwell operating conditions. This potentially enables the use of a liquid handling robot as a methodology for the scaling down of micro-mixing conditions from large reaction vessels for bioprocess development.
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The validity of using $\alpha$ values as a design tool when route scouting a bioprocess in microwells will be investigated in Chapter 6 through application to a biological system. The virtues of using microwell bioprocessing technology to mimic micro-mixing conditions within larger scale operations will also be studied.
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6.1 Introduction and aims

This final chapter assess the applicability, and hence the usefulness, of the conclusions drawn from the earlier studies on automation platform operation and the fundamental mixing characteristics of jet mixed microwells. It highlights how the insights gained can be utilised for the experimental evaluation of real bioprocess operations and tests their ability to provide fast bioprocess design data.

The influence of the bioprocess being investigated on well utilisation and the quality of the data generated when measuring bioprocess kinetics using an integrated robotic system (Chapter 3) is first demonstrated. Operations demonstrating fast kinetics, such as the chemical lysis of bacterial cells, are shown not to be suitable for investigation with the integrated robotic system when large quantities of data need to be collected in short time frames. Only when the frequency of data collection required falls does the integrated robotic system provide productivity benefits over manual pipetting.

The macro-mixing environment within a microwell (Chapter 4) is next assessed for situations when low volume additions, common in high throughput assays, are made to a static microwell plate. Operating windows have been generated to allow the easy identification of cases where secondary mixing steps will be required to complete the macro-mixing of added volumes of material. Strategies to prevent the introduction of variability into any data generated through poorly macro-
mixed wells, either by plate shaking or secondary pipetting steps, have been identified.

Finally, the influence of micro-mixing efficiency at a range of scales from 1.5 mL to 500 mL on the final yield and quality of plasmid DNA from the chemical lysis of bacterial cells is investigated. The micro-mixing efficiency is shown to influence both plasmid yield and quality, playing a key role in the design of larger scale lysis vessels and in determining the extent of further downstream processing required. Micro-mixing efficiencies, common to both the microscale and larger scale reaction vessels have been identified, allowing microwell formats to be utilised in the prediction of plasmid yield and quality in large scale vessels.

6.2 Integrated robotic systems for the measurement of bioprocess kinetics

In this section the importance of the operating conditions, displayed in Figures 3-7 and 3-8, in relation to some typical bioprocess operations that can be studied using microscale bioprocessing techniques is considered (Lye et al, 2003). These are considered as a series of case studies which exhibit a range of kinetics that can be used to determine the applicability of integrated automated platforms for the collection of bioprocess design data.

6.2.1 Case study 1: Alkaline lysis of bacterial cells

During the production of plasmid DNA gene therapy vectors and DNA vaccines *E. coli* cells are chemically lysed through the action of a detergent in an alkaline environment (Levy et al, 2000). This process is characterised by an initial rapid chemical rupture of the cell membrane in 0-30s which reaches complete product liberation after approximately 120s. Cell disruption is followed by a further chemical
degradation of released chromosomal DNA until a pseudo-steady state is reached after 200-400s (Ciccolini et al, 1998). The process is characterised by a marked increase in the viscosity of the broth from 3mPas to 35mPas during cell lysis, due to the release of the chromosomal DNA, and then a steady decrease to 20mPas as the chromosomal DNA is degraded by shear (Ciccolini et al, 1998). In this example the collection of data for the initial, rapid process of cell disruption is examined.

The lysis of bacterial cells by the addition of 0.2M NaOH containing 1% w/v SDS is characterised by rapid kinetics (Ciccolini et al, 1998). To be able to use an operating window to predict well utilisation the required sample detection period and final dispense volume need each to be specified. The final dispense volume (of the NaOH / SDS solution) for microscale alkaline lysis can realistically be fixed at 150 μL (when added to a 150 μL of an E. coli suspension). The quantity of kinetic data that needs to be generated next needs to be established since this will have a significant influence on well utilisation. Two examples can be taken to demonstrate this significance; the collection of 10 or 3 equally spaced data points over a period of 120s. If 10 data points are required, then the maximum sample detection period is 12s, if only 3 are required then the sample detection period is 40s. The well utilisations for these two sample detection periods vary greatly (Figure 3-7 for a low viscosity addition). In this case it can be seen that a sample detection period of 12s is too slow to reach the required minimum degree of well utilisation. The liquid handling function can be completed accurately and precisely but the integrated robot and plate reader are simply not fast enough to set up and take readings from a sufficient number of wells to make automation worthwhile. If this is compared to the situation when the sample detection period is 40s, however, then the operating window predicts a degree of well utilisation (up to 25%) allowing up to six bioprocess conditions to be varied
simultaneously (each in quadruplet). The use of the windows of operation concept not
only allows the researcher to predict the degree of well utilisation that can be attained
with the integrated robot but allows the productivity of the platform to be tailored to
the quality of the data required. This kind of trade off will always be necessary for
process exhibiting rapid kinetics.

6.2.2 Case study 2: Aerobic bacterial fermentation

The aerobic fermentation of micro-organisms in microwell plates is a good
example of a process characterised by slow kinetics (John et al, 2003; Lye et al, 2003;
Kostov et al, 2001; Maharbiz et al, 2004; Minas et al, 2000). Fermentation processes
are characterised by relatively slow kinetics with growth and product formation
occurring over a period of 6-8 hours or longer.

The monitoring of the aerobic fermentation of E. coli for the production of an
enzyme used in a Baeyer-Villiger bioconversion (Doig et al, 2001) is examined. Cell
density and the rate of product formation can both be typically monitored using a
microwell plate reader (Duetz et al, 2000) in order to quantify cell growth kinetics
(Minas et al, 2000) or the optimum time for the induction of enzyme expression (Doig
et al, 2001). The sample detection period for fermentations is typically long, in the
range of 30 minutes since the overall process will take many hours. This process has a
typical sample detection period of 30 min. The broth viscosity is unlikely to increase
above 10mPas.

In this case, it can be seen that for both low viscosity additions, e.g. NaOH for
pH control, (Figure 3-7) and high viscosity additions, e.g. concentrated glucose
solutions during fed-batch operation, (Figure 3-8), the sample detection period is too
large to influence the degree of well utilisation. The suitability of the automation
platform is now determined solely by the liquid handling functionality; with lower
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limits determined by accuracy and precision and upper limits set by the capacity of
the robot. The ability to perform automated parallel microwell fermentations could
potentially provide a rapid approach to the establishment of process operating
conditions such as medium composition, pH (Elmadhi et al, 2003), and temperature.
Current work on the quantification of oxygen transfer rates (Duetz and Witholt, 2001;
Lye et al, 2003; John et al, 2003) will further enable the performance of aerobic
microwell fermentations to be related to larger scales of operation.

6.2.3 Case study 3: Equilibrium stage separation processes

Equilibrium stage separations, such as liquid-liquid extraction and solid-liquid
extraction (Welch et al, 2002), are kinetically independent systems where the key
design parameters, distribution coefficients and adsorption constants, are based on
single measurements made once the system has come to equilibrium. The viscosities
of the liquid phases will depend upon the particular solvents selected but will
generally be below 2mPas.

No intermediate measurements are generally necessary unless it is required to
have in-process samples to ensure a true equilibrium is reached. This will free the
automation platform from the constraint of the sample detection period, allowing
100% plate usage in all instances. In such cases the only factors influencing the
operation of the system would be the liquid handling accuracy and precision.

6.2.4 Implications for microscale bioprocess development

The integration of a liquid handling robot and a plate reader (Figure 2-1)
represents a powerful platform for automated route scouting. When compared to
traditional methods of route scouting it allows small volumes of reagent and sample to
be used and at greater throughputs, whilst maintaining high levels of reproducibility
and sensitivity. The results in this thesis have shown that the operating window concept can be applied successfully to a number of bioprocess operations with varying process characteristics provided the two key variables of dispense volume and sample detection period are known. It has also been demonstrated (Section 6.2.1) that the quality of data needed for a system with rapid kinetics is linked with the productivity or throughput of the experiment. A reduction in the number of data points required leads to a greater degree of well utilisation and hence greater experimental throughput. The operating window approach (Figure 3-7) may be used to visualise the trade-offs implicit in determining the suitability of a given process step to being translated into a microwell format and can then be applied so as to reduce the length of any experiment by maximising well usage within each plate, prior to carrying out any scale-up studies.

6.3 Quantification and prediction of jet macro-mixing times in static microwell plates

Previously the minimum addition volume \( V_{\text{CrI}} \) required to ensure the completion of macro-mixing within the lifespan of jet addition has been identified (Section 4.4.1) and the extent of macro-mixing quantified when addition volumes less than this minimum are used (Section 4.2.3). To enable the easy visualisation of these interactions operating windows are constructed here for the 96-SRW plate (Figure 6-1) and the large designed microwell (Figure 6-2) described previously Chapter 4.

Both of these windows show three distinct regions each reflecting different macro-mixing conditions within the microwell. The first region (when \( V_A > V_{\text{CrI}} \)) is that in which macro-mixing is complete within the lifespan of the jet addition. In this region the addition time is greater than the macro-mixing time. Any assay or
Figure 6-1: Operating window showing the impact of addition volume ($V_A$) and time on jet macro-mixing in a 96-SRW plate. Window constructed based on a 200 μL initial volume using fixed pipette tips for dispensing 1 mm below the liquid surface at $Re_j = 1000$. Experimental data taken from Table 4-1 and Section 4-3.
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Figure 6-2: Operating window showing the impact of addition volume ($V_A$) and time on jet macro-mixing in a 96-DRW plate. Window constructed based on a 1720 μL initial volume using fixed pipette tips for dispensing 1 mm below the liquid surface at $Re_j = 1000$. Experimental data taken from Table 4-1 and Section 4-3.
bioprocess data generated using these conditions can be expected to be reproducible and quantitative in nature (except for processes with time constants << 1s). In the intermediate region, $V_A < V_{\text{Crit}}$, macro-mixing is incomplete within the jet lifespan but is completed shortly after through post-injection momentum. Data generated in this region is likely to be reproducible but more qualitative in nature. In many ways, however, the time needed to complete macro-mixing by post-injection momentum may be too short to have any effect on the bioprocess being investigated again allowing the collection of quantitative data. Finally there is a region where macro-mixing times can be many seconds or indeed $t_{95}$ may never actually be reached within the time frame of the experimental trial. High degrees of variability would be expected to be found in data generated under these conditions and results would be highly qualitative without further mixing steps being utilised.

6.4 Impact of micro-mixing efficiency on plasmid DNA recovery during alkaline cell lysis

In Chapter 5 an assessment of micro-mixing efficiencies was made in a range of reactor configurations. Here a biological system is used in order to demonstrate the applicability of generating bioprocess design data from an operation with rapid process kinetics when a range of micro-mixing efficiencies prevail i.e. the release of plasmid DNA from bacterial cells by alkaline cell lysis. In addition an assessment of using micro-mixing efficiency across a range of reactor configurations has been made.

6.4.1 Quantification of plasmid DNA yield and quality

It has been shown that high pH, > 13.0, (Thatcher et al, 1997) can cause the irreversible denaturation of supercoiled plasmid DNA. Standard alkaline lysis protocols (Birnboim and Doly, 1979) commonly utilise an initial sodium hydroxide
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centration of 0.2M and it has therefore been postulated that poor micro-mixing conditions within a reactor will expose the supercoiled plasmid from lysed cells to regions of high pH, leading to irreversible supercoiled plasmid degradation. Improvement of the micro-mixing efficiency during the cell lysis process should lead to a reduction in any chemical damage of the supercoiled plasmid.

Experimental results for DNA releases during alkaline lysis of E. coli cells in a 96-DRW plate over a range of micro-mixing efficiencies are shown in Table 6-1. Total plasmid DNA concentrations were calculated by HPLC (Section 2.4.3.3). A general trend of increasing plasmid DNA concentration with increasing micro-mixing efficiency can be seen. This confirms the original hypothesis that avoiding exposure to regions of high pH leads to an increase in the recoverable plasmid DNA.

An assessment of the quality of the plasmid DNA released with varying \( \alpha \) values in a 96-DRW plate was also performed (Table 6-1). This shows a decrease in the ratio of supercoiled to open circle plasmid but an increase in the total supercoiled plasmid recovered as the micro-mixing efficiency increases. This can be explained by the process of DNA denaturation in high pH environments. Initial contact with a high

Table 6-1: The supercoiled plasmid yield, the relative populations of supercoiled and open circle plasmid and protein impurity concentration after alkaline lysis was completed at a range of micro-mixing efficiencies and reactor configurations. Experiments performed as described in Section 2.4.3.

<table>
<thead>
<tr>
<th>Reactor</th>
<th>Micro-mixing efficiency ((\alpha))</th>
<th>pDNA ((\mu g/mL))</th>
<th>SupercOiled /open circle ratio</th>
<th>SupercOiled plasmid ((\mu g/mL))</th>
<th>[Protein] ((mg/mL))</th>
</tr>
</thead>
<tbody>
<tr>
<td>96-DRW</td>
<td>3</td>
<td>19.3</td>
<td>9.21</td>
<td>17.4</td>
<td>0.101 ± 0.004</td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>23.1</td>
<td>8.14</td>
<td>20.6</td>
<td>0.098 ± 0.005</td>
</tr>
<tr>
<td></td>
<td>51</td>
<td>28.5</td>
<td>7.98</td>
<td>25.3</td>
<td>0.102 ± 0.005</td>
</tr>
<tr>
<td>96-SRW</td>
<td>12.2</td>
<td>7.91</td>
<td>6.54</td>
<td>6.9</td>
<td>0.032 ± 0.001</td>
</tr>
<tr>
<td>96-DRW</td>
<td>12.2</td>
<td>7.91</td>
<td>6.54</td>
<td>6.9</td>
<td>0.032 ± 0.001</td>
</tr>
<tr>
<td>500 mL</td>
<td>STR</td>
<td>12.2</td>
<td>23.3</td>
<td>5.58</td>
<td>19.8</td>
</tr>
</tbody>
</table>
pH region leads to the 'nicking' of the phosphate – sugar backbone of a double stranded DNA chain. Once this has occurred the supercoiling within the native state of the plasmid is allowed to unwind leading to conversion to the open circle configuration. Further 'nicking' will lead to the formation of the linear plasmid configuration. This configuration is removed by the purification workup (Section 2.4.3.1) and so is not quantified in the final sample. Under more efficient micro-mixing conditions the rates of the two degradation reactions, supercoiled to open circle and open circle to linear, are reduced. This results in the increase in both the supercoiled and open circle plasmid populations seen in the data. Micro-mixing appears to have a greater influence on the open circle to linear reaction leading to a relative increase in the population of the open circle configuration. An assessment of the total protein impurity found in the lysate before further purification demonstrated that this is independent of the micro-mixing efficiency (Table 6-1).

These results are consistent with those from other groups that have investigated the chemical lysis of bacterial cells. Thatcher and co-workers (Thatcher et al., 1997; Varley et al., 1999) determined that the pH environment within the reaction vessel must be kept below an upper pH limit to prevent the denaturation of supercoiled plasmid DNA. To control the pH within the reaction vessel a pH probe was utilised but fouling by the cell debris released during lysis remained a particular problem. Meacle and co-workers (Meacle 2003; Meacle et al., 2004) confirmed that high pH conditions can lead to the permanent denaturation of supercoiled plasmid DNA and identified that mixing conditions within a reactor, utilising simple stirred tanks and impinging jet vessels, have an influence on the yield of supercoiled plasmid DNA. The control of the micro-mixing environment within the reaction vessel allows
the pH environment within the reaction vessel to be controlled without the use of a pH probe and the associated fouling problems.

6.4.2 Effect of micromixedness ratio at different scales of operation

Finally, experiments were performed at constant micromixedness ratios to examine whether experiments performed in microwells could provide insight into micro-mixing effects in larger scale, stirred tank vessels. Three reactor configurations were investigated and a common micro-mixing efficiency was generated in each reactor configuration (experimental conditions given in Section 2.4.2). The results on the yield of supercoiled plasmid DNA and protein impurities are shown in the bottom half of Table 6-1.

If the micro-mixing efficiency were the only factor influencing the plasmid DNA yield then common results would be expected when a common micro-mixing efficiency was used in various reactor geometries. This is obviously not the case and another mechanism must affect the plasmid recovery. This could be due to physical damage to the plasmid by high shear forces as this has been shown to damage large macromolecules in solution (Davison, 1959; Levy et al, 1999a; Levy et al, 1999b; Nguyen and Kausch, 1992). A comparison of the energy dissipation rates in the reaction zones (calculated from equations 1-6 and 1-7) of each of the reactor configurations with the total plasmid DNA release produces a strong correlation. The higher the energy dissipation rate the lower the total plasmid DNA released at common micro-mixing efficiencies. The yield of supercoiled plasmid to open circle plasmid show slightly different results. Again the 96-deep round well is the most efficient reactor configuration but the stirred tank produces a lower ratio of supercoiled to open circle plasmid than the 96-standard round well. The protein
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analysis shows an increased protein concentration in the crude lysate as the scale of the reactor increases but the differences are small compared to the protein concentration in the 96-DRW with varying micro-mixing efficiencies.

6.4.3 Implications for microscale bioprocess development

From this data it is possible to draw a number of conclusions. From Section 6.4.1 two main conclusions can be drawn. Firstly that the yield of plasmid DNA obtained by the chemical lysis of bacterial cells is dependent on the micro-mixing efficiency. Both the total populations of supercoiled and open circle plasmid are affected by the micro-mixing efficiency. The relative populations of each plasmid configuration are also affected by the micro-mixing efficiency and this will have implications for secondary purification further downstream. Secondly, it has been shown that microscale processing techniques can be utilised as a route scouting tool to identify parameters that will influence product yield and quality.

Section 6.4.2 demonstrates the usefulness of microscale bioprocess data but does highlight that caveats must be applied when utilising such information. A full understanding of the system being studied is essential before a reaction system can be scaled utilising a single parameter. In the case of alkaline lysis, the micro-mixing efficiency within the reactor vessel and a second parameter, probably the shear degradation of the supercoiled plasmid, are key parameters in determining the overall yield of the process operation. Though common micro-mixing efficiencies can be reproduced in various reactor configurations the extent of shear degradation is reactor-specific and conclusions as to the effect of micro-mixing efficiency on the supercoiled plasmid yield and quality must be made in light of this. Therefore it would be unwise to use only bioprocess data gathered in a microwell environment to
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determine a likely product yield and quality in pilot-scale or larger operations, where a different reactor configuration is likely to be used.

6.5 Conclusion

Using the windows of operation generated in earlier chapters it has been possible to demonstrate the applicability of the systems described to 'real life' process operations. The targeted data quality for kinetic systems has been shown to be dependent on the speed of the reaction being investigated and in turn determines the degree of well utilisation. The degree of well utilisation ultimately determines the suitability of the automated liquid handling robot for the measurement of process kinetics. With prior knowledge of the degree of well utilisation it is possible to plan to plan the construction of the experiments or tailor the quality of the data such that automation becomes a viable option.

The of macro-mixing behaviour has demonstrated the observation initially made by Berg and co-workers (2002), that macro-mixing is incomplete at low addition volumes. This has been taken further and the boundary between poorly mixed and well mixed environments has been defined. This information can then be utilised when planning and designing experiments based upon microwell formats. The ideal operating region would be that when macro-mixing is completed within the jet lifespan. When high reagent costs, such as when using enzyme substrates, enforce the need for small addition volumes (5-10% of initial well volume) additional secondary mixing steps, either through the shaking of the plate or through re-circulation through the pipette tip, must be utilised.

The micro-mixing environment within a reaction vessel has been shown to influence the yield and quality of plasmid DNA during the chemical disruption of *E. coli* cells by reducing the chemical damage to the plasmid DNA by regions of high
pH. Microwell formats have been utilised to demonstrate the route scouting of micro-mixing efficiency as a significant factor in the reaction pathway. Mimics of large scale reaction vessels have been generated and it has been demonstrated that a full understanding of all the significant parameters affecting a process step are necessary and that a single parameter can not be studied in isolation.
Chapter 6 has highlighted the use of microscale techniques within bioprocess development. Specific case studies were investigated and the applicability and limitations of the microscale models demonstrated. In this chapter the positioning of microscale technology within the overall scheme of drug development and the likely regulatory implications of utilising this technology are discussed. These issues must be addressed before the technology can be utilised in an industrial environment.

### 7.1 Management of microscale development technologies

#### 7.1.1 The traditional drug development cycle

The drug development cycle for a typical biopharmaceutical product from initial discovery to the final market release of the product is generally 7-8 years (Struck, 1994). This can vary dramatically and is product-specific; insulin was released within 5 years while INF-B1b took nearly fourteen years to reach market (Gosse and Mannocchia, 1996). Within this wide range of development cycle times is a standard pathway that all drugs must complete before commercial release (Figure 7-1). The holy grail of drug development is to produce a unique, efficacious drug and to bring it to market in the shortest possible time. The advances in high throughput screening and combinatorial chemistry techniques have lead to an explosion in the number of potential drugs candidates and active ingredients. This increase has in turn lead to the bottleneck in drug development being shifted toward process development (Pollard, 2001). With the constant commercial pressure to shorten the drug development cycle and the potential for an increase in the length of clinical trials, in
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7.1.2 Bioprocess research and development

Bioprocess R&D has been described as ‘... to move from a process that has been adapted for laboratory development work and that is complex, inefficient and potentially unsafe to one that is practical, efficient, robust and safe’ (Pisano, 1997). The scale of the task is highlighted for a typical small chemical entity below (Table 7-1). The significant differences between the initial process and that used for final commercial production represent a significant challenge to be solved during a highly constrained period of process development.

The process development of biopharmaceutical products present a number of issues that are commonly not encountered with small chemical entities. Namely low production volumes (est. 2001 demand: Epogen 2 kg and Rituxan 210-230 kg (Carroll et al, 1998)), higher risk products as critical care applications predominate, high facility capital costs and extensive regulation. Though these factors increase the risk associated with biopharmaceuticals this is offset to a degree by their high market
Table 7-1: Comparison of initial and commercial production processes (Pisano, 1997)

<table>
<thead>
<tr>
<th></th>
<th>Initial discovery process</th>
<th>Final commercial production process</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of purification steps</td>
<td>15</td>
<td>7</td>
</tr>
<tr>
<td>Equipment</td>
<td>Test tubes – 1 litre flasks</td>
<td>7000 – 15000 litre vessels</td>
</tr>
<tr>
<td>Batch size (output)</td>
<td>~ 1 g</td>
<td>~ 100-200 kg</td>
</tr>
<tr>
<td>Operators</td>
<td>PhD chemists</td>
<td>Technicians, semi-skilled plant workers</td>
</tr>
<tr>
<td>Purity</td>
<td>1-10 %</td>
<td>&gt;99.9 %</td>
</tr>
<tr>
<td>Cost ($/kg)</td>
<td>20,000 – 50,000</td>
<td>~ 3,500</td>
</tr>
<tr>
<td>Process design criteria</td>
<td>Biological activity; patent issues</td>
<td>Cost; quality; operability; regulatory compliance</td>
</tr>
</tbody>
</table>

value, though even this is constantly under pressure. Bioprocess development can be used to reduce this risk through obtaining real cost data at the commercial scale to determine a realistic cost profile and by establishing a cost model based on the proposed process. An assessment of the economical viability of a product can then be conducted. This must be completed relatively early in the drug development cycle as the manufacturing process must be fixed before the commencement of Phase III clinical trials. Any subsequent alterations in the manufacturing process can lead to delays in the regulatory approval of the drug and hence extend the development cycle.

Bioprocess development can be broken down into a number of tasks with different levels of activity depending on the stage of the development cycle; route scouting during pre-clinical trials, the initial determination of the purification strategy; early stage development, from the production of small quantities for toxicity studies to low cost cGMP manufacture for Phase I and II trails; late stage development, intensive development program to produce a cost effective manufacturing operation. Though each of these stages must be completed for the development cycle to be completed it is not necessary for each stage to be undertaken by one company and in
many instances this will not be the case. Companies have adapted to become niche players within different parts of the development cycle. This specialisation has lead to separate demands being placed on bioprocess development. The different company types, with examples, and their bioprocess development needs are summarised in Table 7-2. The company types investigated are as follows:

- Large pharmaceutical (GlaxoSmithKline)- Bioprocess development can be completed in-house as well as outsourced. In-house development teams are assigned to specific projects commonly with a fixed time frame. Bioprocess development technology must be applicable to a range of bioprocess situations as projects are quickly dropped when determined to be uneconomical or non-viable for clinical reasons.
  - Development aim: the fast definition and optimisation of a bioprocess at bench, pilot and manufacturing scale of a wide potential range of products.

- Small Biotech (Cambridge Antibody Technology)- Likely to complete all stages of development in-house but will only have a small range of products within one product family. Route scouting will be completed for the initial product but then a common production strategy will be tailored to future products. Bioprocess technology will be used with a small number of process operations.
  - Development aim: the fast optimisation of a bioprocess at bench, pilot and manufacturing scale of a group of products within one drug family.

- Contract manufacturer (Avecia)- Commonly only complete late stage development with the scale-up of a process to manufacturing scale.
Significant range of products so flexible bioprocess development technology is required. The profitability of a contract is often dependent on the success of bioprocess development.

- Development aim: the fast optimisation of a bioprocess from pilot to manufacturing scale.

- Drug discovery and early development (Exelixis Inc) - Identification and initial route scouting of a wide range of products. Potential drug candidates are then sold on, commonly to large pharmaceutical companies. The identification of potential drug candidates is key but the fast route scouting of a suitable production process is essential.

- Development aim: Fast identification of potential drug candidates with a basic small scale manufacturing process for the production of material for toxicity and early clinical trials.

Commonly the greatest research activity is focused on the late stage development of a product. At this stage there is a good chance the product will receive regulatory approval and the expenditure of resources can be justified. Inherent with this approach is the problem of the final determination of the manufacturing process.

To reduce the drug development cycle the final determination should be fixed as

Table 7-2: The different stages of bioprocess development completed by a range of company types. Early stage development is defined as being up to the point of administration into man. Late stage development is defined as being from the point of administration into man up to the final fixed manufacturing process.

<table>
<thead>
<tr>
<th></th>
<th>Route scouting</th>
<th>Early process development</th>
<th>Late process development</th>
<th>Flexibility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large Pharma</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>Small Biotech</td>
<td>Limited</td>
<td>√</td>
<td>√</td>
<td>X</td>
</tr>
<tr>
<td>Contract manufacturer</td>
<td>X</td>
<td>X</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>Discovery</td>
<td>√</td>
<td>√</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>
quickly as possible but the complete development of a bioprocess, which may give a product a competitive advantage when it reaches market, will lengthen the cycle. For a biopharmaceutical product there are three main methods of gaining a competitive advantage; being first to market, having an efficient manufacturing process (high yields, purities and reproducibility) and designing a low cost manufacturing process. The standard thinking is based on small chemical entities and being first to market predominates. This approach is suitable when the drug is relatively simple to manufacture and process development only involves a few major challenges. This is not however a suitable approach for biopharmaceuticals where complex production processes are common and bioprocess development cannot be quickly completed prior to the commencement of Phase III clinical trials.

7.1.3 The management problem

The need to reduce the drug development cycle, to minimise the risk of loosing capital investment through the regulatory failure of a drug and the need to have an early product launch invariably precludes biopharmaceutical companies from fully developing a manufacturing process. Changes can be made after the product launch but would involve the resubmission of data to the regulatory authorities and rarely occurs. This can result in the production process being inefficient, unwieldy and unnecessarily costly. Accompanying the problems associated with a poorly developed manufacturing process is the increasing attention regulatory authorities are placing on process definition early in the development cycle. To meet the three challenges of an increased number of potential drug candidates, removal of bioprocess development from the critical path and the increasing regulation of bioprocess development biopharmaceutical companies are seeking ways to enable bioprocess development earlier in the drug development cycle.
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7.1.4 Microscale technologies – the answer?

Microscale technologies, such as those described within Chapters 3, 4 and 5 of this thesis can be applied to a number of bioprocess situations as discussed in Chapter 6. The two main areas; of bioprocess development and the reproducibility of the technology have been addressed. Bioprocess development can be split into two categories; route scouting and the establishment of larger scale bioprocess mimics. Route scouting often only requires simple ‘yes / no’ or ‘more than / less than’ answers and the models for kinetics and micro-mixing developed in Chapters 3 and 5 respectively, can easily be utilised to determine the importance of these parameters within a process step. The kinetic model from Chapter 3 can be combined with data determining the minimum addition volumes required for a homogeneous environment (Chapter 4) in order to generate kinetic data which would be applicable to large scale process operations. Chapter 5 highlighted the difficulties involved in mimicking pilot scale unit operations within microwells but demonstrated that this approach could be used to suggest alternative reactor geometries at the pilot scale.

The increasing regulation during bioprocess development demands a greater definition of the process earlier within its development. Process variability which was previously tolerated must now be reduced, defined and explained. The macro-mixing times defined in Chapter 4 allow inhomogeneities within the microwell to be eliminated as a source of process variability when utilising microscale technologies for bioprocess development.

To conclude, microscale technologies represent a viable methodology for fast, flexible early stage bioprocess development and after further research could prove useful as mimics of pilot-scale operations.
7.2 Validation of microscale bioprocessing technology

In order for a process or product to be utilised or released onto the market it must received regulatory approval from the relevant authorities in the country of release. To be granted regulatory approval the product must be tested in order to ensure that it is efficacious and safe for administration to humans. Guidance for the use of pilot scale facilities have been produced by the regulatory authorities and robustness and reproducibility must be comprehensively tested (Schultz, 1995). Validation of the manufacturing process provides the documentary evidence that it is operated under defined conditions and that it performs to a consistent level.

Microscale development techniques do offer substantial benefits over standard larger scale bioprocess development but an additional workload would be placed on any company utilising these technologies. This results from any data generated from microscale mimics used to predict large scale operating conditions having to be validated. As this is a new technology the complete range of validation documentation would be required. Once the technology had been proven as validated approach, this burden would be reduced but would include:

- Process qualification (PQ): Documentation of errors inherent to the system being used. This would involve the yield and purity of the product being processed.
- Equipment validation: The standard error determination for all equipment pieces over their operating range.
- Operational qualification (OQ): Ensuring variability cannot be introduced by the operators.
- Installation qualification (IQ)
The bioprocess management, regulation and economics of microwell technology

- Cleaning: To ensure that material from a specific product from one process development trial is not retained within the system and contaminates a subsequent development run.

- Change management: To ensure that material from a specific product from one process development run is not retained within the system and contaminates development runs from an alternative product.

These areas each present different challenges to microscale bioprocessing technologies. Though data from validated drug discovery programmes, where liquid handling robots are commonly used, could be utilised for a number of the validation stages.

- Process qualification:
  1) Purity and yield data for each specific process investigated over the range of operating conditions investigated.
  2) Documentation to demonstrate that the microscale technology accurately mimics the larger scale process.
  3) GLP compliance.
  4) Reproducibility of physical environment during bioprocess development. Steps have been made to the completion of this through the definition of macro-mixing times (Chapter 4) and the micro-mixing efficiency (Chapter 5).

- Equipment validation:
  1) Liquid handling robot software validation (likely to have been completed for use within drug discovery programmes)
2) Liquid handling robot parameter reproducibility eg dispense volume and dispense speed reproducibility (likely to have been completed for use within drug discovery programmes)

3) Liquid handling robot maintenance schedule

4) Microwell plate (provided by manufacturers)

5) Analytical device validation (unless a new device is utilised validation documentation can be used from either drug discovery programmes or analytical departments)

- Operational qualification: Generation of standard operating procedures (SOPs) and operator training to ensure SOPs are followed.
- Installation qualification: Likely to already be filed as no new equipment pieces are being utilised.
- Cleaning validation: Production of cleaning protocols for the liquid handling robot and analytical devices along with the scheduling of regular cleaning to ensure no cross-contamination between development runs. Cleaning validation would not be needed for the microwell plates as they are disposable. Documentation would also be required to demonstrate the effectiveness of the cleaning protocol.
- Change management: Similar procedure to the cleaning validation but must be more rigorous as the tolerance levels for contamination between processes are much lower when a process is operated within its specified process conditions.

The pharmaceutical industry is inherently conservative due to the high degree of regulation and the validation procedures listed here clearly represent an obstacle to the use of microscale bioprocessing technologies. Though investment must be made
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to ensure any data generated using these technologies can be utilised within a product or process submission this would be offset by the substantial benefits gained through a well defined and developed process once the product reaches the market place.

7.3 Economic benefits of microwell technology

To demonstrate the economic benefits of microwell technology an assessment of the route scouting of micro-mixing as a significant parameter during the chemical lysis of bacterial cells will be made. The bounds of the assessment will be the production of a micro-mixing model for a 96-DRW plate and a 500 mL stirred tank reactor to generate a known range of micro-mixing efficiencies, which will then be utilised to assess the chemical lysis.

The DOE model utilised in Section 2.3.2.3 to determine the range of micro-mixing efficiencies in the 96-DRW plate system was used to assess the economics of route scouting. In conjunction with the DOE model the liquid handling and sample reproducibility studies from Section 2.3.2.1 were included to ensure that the micro-mixing conditions described by the DOE model were valid. Once the model development had been completed, three micro-mixing efficiencies were chosen over the range of the model to demonstrate the effect of micro-mixing during the chemical lysis. Each condition was repeated in quadruplicate with the lysate solution being purified using the protocols from Section 2.4.3.

To assess the 500 mL stirred tank the same procedure was repeated. The DOE model from Section 2.3.3.1 was utilised. No additional sample reproducibility studies were required as the exclusion of macro-mixing as a significant parameter is included within the DOE model. Once the model of micro-mixing efficiencies had been constructed three micro-mixing conditions were utilised, in triplicate, to assess the
influence on the chemical lysis. The lysate work-up detailed in Section 2.4.3 was utilised to isolate the supercoiled plasmid DNA.

To assess the economics of a technology a number of cost factors must be taken into consideration; the reagent costs to complete each trial condition and analyse the data, operating / utility costs, man hours required to complete the route scouting and the fixed capital costs.

The reagent costs for the three main areas of the route scouting, production of the micro-mixing model, the chemical lysis in defined micro-mixing conditions and the purification work-up and analysis are detailed in Table 7-1. RO water has been considered as free as likely to come from a filtered source on site. The only major cost not included in this analysis is the cost of the bacterial cell paste. This is extremely difficult to quantify as the cost of the production of a bacterial strain with genetic construct can vary greatly. Once a strain has been produced and assessed production of a Master cell bank can cost between £40,000-60,000 while production for a Phase I clinical trial can be over £1 million depending on regulatory documentation (R. O’Kennedy, personal communication). Therefore this cost is excluded but it can be noted that there is a scale factor between the use of the 500 mL stirred tank and the 96-DRW for route scouting. The 500 mL stirred tank required approximately 65 times more cell paste than the 96-DRW plate.

Table 7-3: Reagent cost for the route scouting of micro-mixing as a significant parameter during the chemical lysis of bacterial cells (Based on prices from Sigma-Aldrich, 2005)

<table>
<thead>
<tr>
<th>Reactor configuration</th>
<th>Micro-mixing model (£)</th>
<th>Chemical lysis (£)</th>
<th>Work-up and analytical techniques (£)</th>
<th>Total reagent cost (£)</th>
</tr>
</thead>
<tbody>
<tr>
<td>500 mL stirred tank</td>
<td>48.96</td>
<td>191.40</td>
<td>0.48</td>
<td>240.84</td>
</tr>
<tr>
<td>96-DRW</td>
<td>0.10</td>
<td>1.23</td>
<td>0.89</td>
<td>2.22</td>
</tr>
</tbody>
</table>
The utility / operating costs are likely to be negligible for both the reactor systems. No specific waste disposal is required for either system and electricity consumption is not expected to be significant.

Route scouting for each of the two systems can be completed by one researcher though the time taken to complete the experiments varies greatly. It has been assumed that the researcher has a full understanding of the liquid handling robot programming and does not require any training. The total time to complete the route scouting utilising the 96-DRW methodology will include the automated platform programming, sample reproducibility studies, DOE model trails, chemical lysis experiments, purification work-up, analysis of purified lysate and data interpretation. The total time to complete the route scouting utilising the 500mL stirred tank reactor includes the DOE model, chemical lysis experiments, purification work-up, analysis of purified lysate and data interpretation. Using estimates of the times taken to complete each of these phases it has been estimated that route scouting utilising the 96-DRW system could be complete within 7.5 hrs while utilising the 500 mL stirred tank reactor route scouting would need 14.75 hrs to complete.

The capital costs of the two methodologies vary greatly with the 500 mL stirred tank reactor having significantly lower capital costs. This methodology utilises relatively cheap off the shelf equipment that can be sourced easily from an equipment supplier. On the other hand, the automated platform is a highly specialised and complex piece of equipment and hence has a price tag to match. Depending on the model and manufacturer this can vary from £50,000-100,000. The capital costs of the analytical devices can be ignored as they are common to both methodologies.

In summary, the use of an automated liquid handling platform present significant reagent cost and researcher time benefits over the standard bench-scale
methodology but is handicapped by its high initial capital cost. If a long term approach is taken to the automated platform paying off its initial capital cost, the opportunity cost of route scouting in half the time and the likelihood that a delay to the market launch of a drug can easily cost upwards in £1 million / day in lost sales included in the analysis, it can be seen that the initial capital cost of the automated platform is relatively small.
8 Conclusions and future work

8.1 Conclusions

A number of conclusions can be drawn from each of the objectives outlined in Section 1.3 on the nature of microscale bioprocess development and the applicability of the systems developed on the specific case studies investigated in this study.

Objective 1: Investigation of the utility of integrated robotic systems

- The operating limits of liquid handling accuracy and precision and the sample detection period were investigated and it was determined that, under optimum conditions, the liquid handling accuracy and precision can be reduced to below 5% CV (Section 3.2). Operating windows for low and high viscosity solvents were constructed allowing the \textit{a priori} prediction the degree of well utilisation from the initial operating parameters (Section 3.4).

- The need to dispense the final addition volume above the liquid surface to ensure high degrees of well utilisation, introducing variability in the physical environment within the microwell, and the integrated system having no benefit over manual pipetting when the sample detection period was short were identified as limitations of the integrated system (Section 3.5).
Objective 2: Determination of macro-mixing times in a static microwell plate

- Macro-mixing times were successfully measured through the use of a high-speed video technique for a number of microwell geometries (Section 4.2). Correlations for each of the microwell geometries have been constructed allowing the a priori prediction of macro-mixing times from the original operating conditions.

- An assessment of the microwell geometry and its influence on the macro-mixing times has been completed with microwells having a 3:1 h:D ratio being more efficient at macro-mixing than the √2:1 h:D ratio found in typical operating volumes within standard 96 well plates (Section 4.4.1).

- Minimum addition volumes ($V_{crit}$) to ensure macro-mixing was completed within the lifespan of the jet addition have been identified for each of the microwell geometries. Strategies to complete macro-mixing for addition volumes below $V_{crit}$ have been highlighted (Section 4.4.1).

Objective 3: Calculation of micro-mixing efficiency in a range of mixer types and scales

- The micro-mixing efficiency within a range of reactor types and scales (96-SRW, 96-DRW, 500 mL stirred tank and 10L stirred tank) was defined over a range of operating conditions using a competitive chemical reaction system. Engineering design equations for each of the reaction vessels were produced allowing the a priori prediction of the micro-mixing environment from the original operating conditions (Section 5.2.2).
8 Conclusions

- Fluid entrainment and the energy dissipation rate at the end of the jet were identified as the key parameters in determining the micro-mixing efficiency within the reaction vessel, with the former being identified as the most significant (Section 5.2.2.1).

**Objective 4: Applications of microscale techniques for bioprocess development**

- Integrated robotic systems were successfully demonstrated to allow the measurement of bioprocess kinetics. A trade-off was made with the quality of the data to ensure the integrated robotic system had a material benefit over manual pipetting (Section 6.2).

- The construction of an operating window allowed the *a priori* prediction of homogeneity within a microwell. Experiments could then be designed to ensure macro-mixing was completed during the jet lifespan or secondary mixing steps could be incorporated when this was not possible, removing macro-mixing as a factor in the experimental outcome (Section 6.3).

- Microwell technology was successfully demonstrated to allow the identification of micro-mixing efficiency as significant parameter during the chemical lysis of bacterial cells for plasmid DNA manufacture (Section 6.4).

- Mimics were constructed within microwell plates that were able to successfully reproduce micro-mixing conditions in large-scale vessels. These mimics were not able to reproduce process conditions found during the chemical lysis of bacterial cells as the mimics did not control the energy dissipation rate (Section 6.4).
Objective 5: The bioprocess management, regulation and economics of microwell technology

- The utilisation of microwell technology for route scouting and as large-scale mimics to increase the throughout within process development has been demonstrated. The applicability of microwell technology as a process development tool for a range of types of pharmaceutical companies, from drug discovery to large multinational, has also been investigated (Section 7.1).

- The need for microwell technology to meet regulatory validation requirements before it can be effectively used as a process development tool have been investigated. A protocol highlighting the steps within a validation procedure has been constructed (Section 7.2).

- A simple cost analysis has been completed for the route scouting of micro-mixing as a factor in the yield of plasmid DNA during the chemical lysis of bacterial cells (Section 7.3). This has demonstrated that microwell technology has a basic reagent cost advantage of a factor of 108 and could be completed in half the time of route scouting utilising a bench scale reactor. Though the high capital costs of the liquid handling robot represented a draw back in the utilisation of the technology.

8.2 Future work

- Integrated robotic systems

A liquid handling robot was successfully integrated with a microplate reader to measure reaction kinetics. The integration of the liquid handling robot with other analytical devices, such as a particle sizer and a mass spectrometer, could be
investigated to expand the range of process operations and improve the quality of data analysis available when utilising microscale bioprocessing.

- **Quantification and prediction of macro-mixing times in static microwell plate**

  Low volume additions into microwell plates have been identified as resulting in a non-homogeneous environment. The need for secondary mixing steps has been highlighted with plate shaking and fluid recirculation through the pipette tips being identified as a possible mixing method. The assessment of the effectiveness of each mixing methodology could be completed.

  Macro-mixing times were studied in three microwell formats but there are a large range of other formats and geometries commercially available. These include U and V shaped microwells at the 200 µL scale and 12 well plates, with a 20 mL working volume. U and V shaped microwells were developed for improved mixing within the plate but there is currently no data on the effectiveness of these geometries to decrease macro-mixing times. 24 well plates offer an intermediate scale of operation between 96-deep round well plates and the bench scale.

- **Assessment of micro-mixing efficiency in static microwell plates and stirred tank vessels**

  A range of micro-mixing efficiencies can be predicted and reproduced within the microscale environment. This allows the determination of the relative importance of micro-mixing within a bioprocess operation but it does not allow the microscale format to be utilised to predict large scale operating conditions. Further investigation of the scaleability of micro-mixing efficiency through the use of alternative large scale reactor types could be completed.
• Applications in microscale bioprocess development

Microscale bioprocess development has been demonstrated as a useful tool to speed up the identification of key parameters within a bioprocess operation. To increase the usefulness of the tool protocols for a wider range of unit operations could be built up. This would allow a standard protocol for fast key parameter determination to be applied to any process situation. This data would allow the researcher to quickly focus on the important parameters with the process operation.

The production of reproducible large scale mimics in microwell formats would significantly increase the knowledge of a process operation and decrease costs when completing bioprocess development.
9 References


9 References


References


9 References


9 References


9 References

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the iodide-iodate reaction system. Part I: experimental procedure. Chem. Eng. Sci., 55,
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determination of the oxygen transfer capacity of small bioreactors based on sulfite

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9 References


References


9 References


References


Appendix
### DOE model for assessment of micro-mixing efficiency in a 96-SRW plate

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