Characterisation of the Fundamental Function(s) of Bovine Serum Albumin in the Culture of NS0 cells

Submitted in accordance with the academic requirements to the Department of Mechanical, Aeronautical and Biomedical Engineering, Faculty of Science and Engineering, University of Limerick in fulfilment of the requirements for the degree of

Doctor of Philosophy

By

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DECLARATION

I hereby declare that I am the sole author of this thesis and that it has not been submitted to any other University or higher institution, or for any other academic award in this University. References and acknowledgements have been made, where necessary, to the work of others.

Signature:       Date:

--------------------------------------------------------------------------                                           -------------------------------------

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Liam
Dedicated to Nanny, with love
ABSTRACT

The large-scale production of therapeutic monoclonal antibodies (mAbs) is commonly carried out using NS0 cells, which are a cholesterol deficient mouse myeloma cell line. The non-ionic surfactant Pluronic F-68 is routinely added to large-scale cell cultures to protect cells from damage experienced in the bioreactor, however there have been reports detailing limitations of Pluronic F-68. Bovine Serum Albumin (BSA) is also added to media used for the large-scale cell culture of some cell lines (including NS0 cells); however there has been limited research published to support the function of BSA in large-scale cell culture. BSA has been suggested to have a number of possible functions in large-scale cell culture media, including; i) offering protection against damage experienced by cells in the bioreactor, ii) acting as a carrier of nutrients (e.g. cholesterol) and iii) acting as an antioxidant. The addition of BSA to media increases the risk of contamination with adventitious agents and requires additional controls by regulatory agencies to ensure patient safety.

In this project two hydromechanical stress models (moderate and extreme hydromechanical stress) were developed in baffled shake flasks to investigate the effects of BSA on NS0 cells. High concentrations of an effective lot of Pluronic F-68 (1g/L) completely protected the NS0 cells in both hydromechanical stress models irrespective of the presence of BSA. The results showed that BSA protects NS0 cells from damage in both baffled shake flask models in media with low concentrations of Pluronic F-68 (≤0.1g/L). Although BSA was shown to offer protection to NS0 cells experiencing laminar shear stress, BSA offered greater protection to the cells in the hydromechanical stress models. This suggests that BSA may have a number of different protective mechanisms of action for NS0 cells experiencing damage.
The protective effect of BSA was shown to be rapidly afforded and lost in both baffled shake flask models, suggesting that BSA offers physical or fast-acting biological protection for NS0 cells experiencing hydromechanical stress. Investigations into the possible physical or fast-acting biological protective mechanism(s) of action of BSA (in media with reduced concentrations of Pluronic F-68) showed that the BSA reduces the surface tension of the NS0 cell culture medium. The protection afforded by BSA was also shown to be independent of cholesterol supplementation and protein tertiary structure (using denatured BSA). BSA was shown to coat the NS0 cells in culture and the NS0 cell membrane hydrophobicity was reduced in the presence of BSA.

Methyl cellulose, a non-animal sourced surfactant, was shown to protect NS0 cells from hydromechanical stress similar to BSA, therefore the protective effect of BSA may be substituted by a non-animal sourced surfactant. The findings in this project show that BSA protects NS0 cells from damage by a number of physical or fast-acting biological mechanisms when the protective effect of Pluronic F-68 is limited. The results suggest that the large-scale cell culture of NS0 cells would be possible in media not supplemented with BSA if the protective effect of Pluronic F-68 is not limited (or if the protection afforded by BSA is replaced with a non-animal derived surfactant).
NOMENCLATURE

Abbreviations and Acronyms

ADCC  Antibody-Dependent Cellular Cytotoxicity
Approx.  Approximately
BSA  Bovine Serum Albumin
CDC  Complement-Dependent Cytotoxicity
CDR  Complementary Determining Region
CFD  Computational Fluid Dynamics
CHMP  Committee for Medicinal Products for Human Use
CHO  Chinese Hamster Ovary
CLC  Cholesterol Lipid Complex (1000X)
conc  Concentration
CMV  Cytomegalovirus
CO₂  Carbon Dioxide
deg  Degree
DHFR  Dihydrofolate Reductase
DO  Dissolved Oxygen
EF  Elongation Factor
EMA  European Medicines Agency
FDA  United States Food and Drug Administration
GPCR  G Protein Coupled Receptor
GS  Glutamine Synthetase
HEK  Human Embryonic Kidney
IgG  Immunoglobulin G
<table>
<thead>
<tr>
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<th>Full Form</th>
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<tbody>
<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate Dehydrogenase</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid Chromatography</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal Antibody</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>MRE</td>
<td>Mean Residue Molar Ellipticity</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium Hydroxide</td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>Ammonium</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>PAGE</td>
<td>Poly-Acrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>PES</td>
<td>Polyethersulfone</td>
</tr>
<tr>
<td>ProG</td>
<td>Protein G</td>
</tr>
<tr>
<td>PTM</td>
<td>Post Translational Modifications</td>
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<tr>
<td>RPM</td>
<td>Revolutions Per Minute</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulphate</td>
</tr>
<tr>
<td>STB</td>
<td>Stirred Tank Bioreactor</td>
</tr>
<tr>
<td>STDev</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>STEP</td>
<td>Stress Induced Energy Production</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris Buffered Saline containing Tween-20</td>
</tr>
<tr>
<td>TCD</td>
<td>Total Cell Density</td>
</tr>
<tr>
<td>USD</td>
<td>Ultra-Scale Shear Device</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VCD</td>
<td>Viable Cell Density</td>
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<tr>
<td>Vol</td>
<td>Volume</td>
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### Units and Measures

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<tr>
<th>Symbol</th>
<th>Description</th>
<th>Unit</th>
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<tbody>
<tr>
<td>G</td>
<td>Acceleration due to gravity (9.81 m s$^{-2}$)</td>
<td>m s$^{-2}$</td>
</tr>
<tr>
<td>LU</td>
<td>LDH Activity unit</td>
<td>nmol/min/10$^6$cells</td>
</tr>
<tr>
<td>EDR</td>
<td>Energy Dissipation Rate</td>
<td>W/m$^3$</td>
</tr>
<tr>
<td>$K_{LA}$</td>
<td>Volumetric Oxygen Transfer Coefficient</td>
<td>h$^{-1}$</td>
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<tr>
<td>vol/vol</td>
<td>Volume/volume</td>
<td></td>
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<tr>
<td>w/vol</td>
<td>Weight/volume</td>
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</table>

| $\pi$  | Pi                                       |           |

- Dalton (atomic mass)  
  - Da
- Normal (concentration)  
  - N
- Pascal (pressure)  
  - Pa
- Watt (power)  
  - W
- Centimetre (length)  
  - cm
- Centimetre cubed (volume)  
  - cm$^3$
- Decimole  
  - dmol
- Gram (mass)  
  - g
- Metre (length)  
  - m
- Millilitre (volume)  
  - ml
- Millimetre (length)  
  - mm
<table>
<thead>
<tr>
<th>Unit Description</th>
<th>Symbol</th>
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<tbody>
<tr>
<td>Millimolar</td>
<td>mM</td>
</tr>
<tr>
<td>Dynamic Viscosity</td>
<td>mPa.s</td>
</tr>
<tr>
<td>Nanomole</td>
<td>nmol</td>
</tr>
<tr>
<td>Microlitre (volume)</td>
<td>µl</td>
</tr>
<tr>
<td>Micron (length)</td>
<td>µm</td>
</tr>
<tr>
<td>Degrees Celsius (temperature)</td>
<td>°C</td>
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Figure 3.6: Increasing orbital shaker speeds causes a reduction in NS0 cell growth in baffled shake flasks in media with low Pluronic F-68 concentrations. Parental NS0 cells were cultured with 0.025g/L Pluronic F-68 in 125ml baffled shake flasks with a working volume of 24ml. Figure 3.6A shows the growth profiles for cells cultured at 110 RPM in media with 0 and 1g/L BSA. Figure 3.6B shows growth profiles for cells cultured at 110, 135 and 150 RPM in media with 0g/L BSA. Figure 3.6C shows the doubling times for the cells cultured at 110, 135 and 150 RPM in media with 0g/L BSA after approximately 3 days in culture, p=0.0007 (**), student T-test, n=3. Error bars depict the standard deviation of three replicate flasks.

Figure 3.7: Baffled shake flasks cause reduced NS0 cell growth compared to non-baffled shake flasks under moderate hydromechanical stress conditions. Parental NS0 cells were exposed to moderate hydromechanical stress (135 RPM) in media with 0g/L BSA and 0.025g/L Pluronic F-68 in baffled and non-baffled shake flasks with working volumes of 24ml. Figure 3.7A shows the growth profiles for cells cultured at 135 RPM over 4 days. Figure 3.7B shows the doubling times for cells cultured in non-baffled and baffled shake flasks after approximately 3 days in culture, p=0.0006 (**), student T-test, n=3. Figure 3.7C shows the LDH Activity for cells cultured at 135 RPM after approximately 2 days in culture, p=0.0002 (**), student T-test, n=3. Error bars denote the standard deviation of three replicate flasks.

Figure 3.8: Conditions for the moderate hydromechanical stress model in baffled shake flasks. This figure shows the conditions for exposing NS0 cells to moderate hydromechanical stress in 125ml baffled shake flasks.

Figure 3.9: NS0 cells are susceptible to damage in baffled shake flasks at high orbital shaker speeds in media with low Pluronic F-68 concentrations. Parental NS0 cells were exposed to various orbital shaker speeds in media with 0.025g/L Pluronic F-68 and 0g/L BSA in 125ml baffled shake flasks with a working volume.
of 24ml. Figure 3.9A shows the results for cells that were exposed to 110, 150, 175 and 200 RPM for 60 minutes at an initial cell density of 1x10^6cells/ml, 175 RPM p=0.00004 (****), 200 RPM p=0.00003 (****), student T-test, n=3. Dotted line indicates LDH Activity after 30 minutes exposure. Error bars show the standard deviation of three replicate flasks. Figure 3.9B shows the reduction in VCD for cells exposed to 150, 155, 160, 165 and 175 RPM. Dotted line indicates LDH Activity after 30 minutes exposure. Error bars show the standard deviation of two replicate flasks…………………………………………………….…………

Figure 3.10: Exposure time and initial cell density affect the damage experienced by NS0 cells in baffled shake flasks at high orbital shaker speeds. Parental NS0 cells were cultured with 0.025g/L Pluronic F-68 and 0g/L BSA in 125ml baffled shake flasks with a working volume of 24ml. Figure 3.10A shows the reduction in VCD with increasing exposure time for cells exposed to 175 RPM at an initial seeding density of 1x10^6cells/ml. Figure 3.10B shows the reduction in VCD for cells exposed to 175 RPM for 60 minutes at various seeding densities: 1, 4 and 8x10^6cells/ml. Error bars show the standard deviation of three replicate flasks….91

Figure 3.11: Baffled shake flasks cause more damage to NS0 cells compared to non-baffled shake flasks under extreme hydromechanical stress conditions. Parental NS0 cells were exposed to extreme hydromechanical stress (175 RPM) in media with 0g/L BSA and 0.025g/L Pluronic F-68 in baffled and non-baffled shake flasks with working volumes of 24ml. Figure 3.11A shows the percentage reduction in VCD for cells exposed to 175 RPM for 60 minutes, p=0.01 (*), student T-test, n=3. Figure 3.11B shows the LDH Activity for cells exposed to 175 RPM for 30 minutes, p=0.001 (**), student T-test, n=3. Error bars show the standard deviation of three replicate flasks……………………………………………………………………….93

Figure 3.12: Conditions for the extreme hydromechanical stress model in baffled shake flasks. This figure shows the conditions for exposing NS0 cells to extreme hydromechanical stress in 125ml baffled shake flasks…………………………………….95

Figure 3.13: BSA protects NS0 cells experiencing moderate hydromechanical stress in baffled shake flasks in media with low Pluronic F-68 concentrations. Parental NS0 cells were cultured at 135 RPM in 125ml baffled shake flasks with a working volume of 24ml in media with 0.025g/L Pluronic F-68 and varying concentrations of BSA (0, 0.1, 0.5, 1 and 2g/L). Figure 3.13A shows the growth profiles for the NS0 cells over 4 days culture. Figure 3.13B shows the doubling times for the cells after approximately 3 days in culture, 0.5g/L BSA p=0.05 (*), 1g/L BSA p=0.05 (*), 2g/L BSA p=0.03 (*), student T-test, n=3. Figure 3.13C shows the LDH activity of the NS0 cells cultured after approximately 2 days of culture, 0.1g/L BSA p=0.002 (**), 0.5g/L BSA p=0.003 (**), 1g/L BSA p=0.001 (**), 2g/L BSA p=0.0005 (**), student T-test, n=3. Error bars denote the standard deviation of three replicate flasks………………………………………………………………………………….98

Figure 3.14: BSA affords dose dependent protection for NS0 cells exposed to extreme hydromechanical stress conditions in media with low Pluronic F-68 concentrations. Parental NS0 cells were exposed to 175 RPM for 60min in 125ml baffled shake flasks at a seeding density of 1x10^6cells/ml in media with 0.025g/L Pluronic F-68 with various concentrations of BSA (0, 0.1, 0.5, 1 and 2g/L). Figure
3.14A shows the reduction in VCD after 60 minutes exposure, 0.5g/L BSA p=0.00005 (****), 1g/L BSA p=0.002 (**), 2g/L BSA p=0.000007 (**), student T-test, n=3. Figure 3.14B shows the LDH activity for samples taken after 30 minutes exposure, 0.1g/L BSA p=0.0001 (**), 0.5g/L BSA p=0.000004 (****), 1g/L BSA p=0.000001 (****), 2g/L BSA p=0.00001 (**), student T-test, n=3. Error bars denote standard deviation of three replicate flasks ………………………………..100

Figure 3.15: BSA protects NS0 cells against extreme hydromechanical stress at all exposure times and cell densities tested. Parental NS0 cells were exposed to 175 RPM in 125ml baffled shake flasks with a 24ml working volume in media with 0.025g/L Pluronic F-68 with either 0 or 1g/L BSA. Figure 3.15A shows the percentage reduction in VCD for NS0 cells seeded at 1x10^6 cells/ml with varying exposure times (5, 15, 30, 60, 90, 120 and 180 minutes), 30 min p=0.007 (**), 60 min p=0.0006 (**), 90 min p=0.009 (**), 120 min p=0.002 (**), 180min p=0.008 (**), student T-test, n=3. Figure 3.15B shows the percentage reduction in VCD for cells seeded at 1, 4 and 8x10^6 cells/ml after 60 minutes exposure, 1x10^6 cells/ml p=0.002 (**), 4x10^6 cells/ml p=0.002 (**), 8x10^6 cells/ml p=0.0007 (**), student T-test, n=3. Error bars depict the standard deviation of three replicate flasks………101

Figure 3.16: BSA protection is only evident at reduced concentrations of Pluronic F-68 for NS0 cells exposed to extreme hydromechanical stress. Parental NS0 cells were exposed to 175 RPM shaking conditions in 125ml baffled shake flasks with a 24ml working volume in media with 0 or 1g/L BSA and various concentrations of Pluronic F-68 (0, 0.025, 0.05, 0.1 and 1g/L). Figure 3.16A shows the percentage reduction in VCD for samples after 60 minutes exposure, 0g/L Pluronic F-68 p=0.00002 (****), 0.025g/L Pluronic F-68 p=0.00001 (**), 0.05g/L Pluronic F-68 p=0.00007 (**), 0.1g/L Pluronic F-68 p=0.02 (*), student T-test, n=3. Figure 3.16B shows the LDH Activity results after 30 minutes exposure, 0g/L Pluronic F-68 p=0.001 (**), 0.025g/L Pluronic F-68 p=0.00002 (****), 0.05g/L Pluronic F-68 p=0.00007 (**), 0.1g/L Pluronic F-68 p=0.0005 (**), student T-test, n=3. Error bars denote standard deviation of three replicate flasks…………….104

Figure 3.17: BSA promotes the growth of mAb-producing NS0 cells experiencing moderate hydromechanical stress in media with low Pluronic F-68 concentrations. MAb-producing NS0 cells were exposed to moderate hydromechanical stress (135 RPM) in 125ml baffled shake flasks with a working volume of 24ml. Media with 0 and 1g/L BSA was tested with 0.025 and 1g/L Pluronic F-68. Error bars depict the standard deviation of three replicate flasks. Figure 3.17A shows the growth profiles for NS0 cells. Figure 3.17B shows the LDH Activities for NS0 cells after approximately 2 days in culture. Error bars depict the standard deviation of at least two replicate flasks…………………………….108

Figure 3.18: BSA protects mAb-producing NS0 cells exposed to extreme hydromechanical stress in media with low Pluronic F-68 concentrations. MAb-producing NS0 cells were exposed to extreme hydromechanical stress (175 RPM) in 125ml baffled shake flasks with a working volume of 24ml. Media with 0 and 1g/L BSA was tested with 0.025 and 1g/L Pluronic F-68. Figure 3.18A shows the percentage reduction in VCD for NS0 cells, p=0.0003 (**), student T-test, n=3. Figure 3.18B shows the LDH Activity after 30 minutes exposure for NS0 cells.
Chapter 4:

**Figure 4.1: BSA has no effect on the amino acid levels in media during NS0 cell culture.** MAb-producing NS0 cells were cultured with 0 or 1g/L BSA in 250ml shaking flasks with a working volume of 100ml under standard growth conditions; 1g/L Pluronic F-68, 10% CO₂, 37°C and 110 RPM and 0.3x10^6 cells/ml seeding density. Samples were taken on day 1, 6, 10 and 14 for NMR analysis to quantify the amino acid levels in the media.

**Figure 4.2: BSA binds to cholesterol in NS0 culture media supplemented with CLC.** This figure shows the cholesterol bound to BSA which was purified by IP from NS0 cell media supplemented with 1g/L BSA and 2ml/L 1000X CLC. Two control IPs were used in this experiment: i) Media with 0g/L BSA and 2ml/L CLC, and ii) Media with 1g/L BSA and 0ml/L CLC. The cholesterol content of the immunoprecipitated samples was quantified using an Amplex Red Cholesterol Quantification Kit.

**Figure 4.3: BSA protective effect is independent of CLC supplementation for NS0 cells exposed to extreme hydromechanical stress.** Parental NS0 cells exposed to extreme hydromechanical stress conditions (175 RPM) in baffled shake flasks in media with various concentrations of 1000X CLC (0, 2 and 4ml/L) in media with 0.025g/L Pluronic F-68 with 0 and 1g/L BSA. The p-value for the cells exposed in media with 0ml/L CLC is p=0.0003 (***), and the p-value for the cells exposed in media with 2ml/L CLC is p=0.003(**), student T-test, n=3. Error bars denote the standard deviation of three replicate flasks.

**Figure 4.4: High concentrations of Pluronic F-68 mask the protective effects of CLC on NS0 cells exposed to extreme hydromechanical stress.** Parental NS0 cells were exposed to extreme hydromechanical stress (175 RPM) in media without CLC supplementation. Media with 0g/L BSA and various concentrations of Pluronic F-68 (0.025, 0.1 and 1g/L) were tested. Figure 4.4A shows the percentage reduction in VCD after 60 minutes exposure. Figure 4.4B shows the LDH Activity results after 30 minutes exposure. Error bars denote standard deviation of three replicate flasks.

**Figure 4.5: BSA protection for NS0 cells exposed to moderate hydromechanical stress is afforded rapidly for cells originally cultured in media with 0g/L BSA.** Parental NS0 cells were exposed to hydromechanical stress by shaking at 135 RPM in 125ml baffled shake flasks with a working volume of 24ml. Media with 0.025g/L Pluronic F-68 and 0 or 1g/L BSA was used in this experiment. NS0 cells originally cultured with 0g/L BSA were exposed to hydromechanical stress in media with 0 and 1g/L BSA. Figure 4.5A shows the growth profiles for NS0 cells. Figure 4.5B shows the doubling times for the NS0 cells after approximately three days in culture, p=0.0009 (**), student T-test, n=3. Figure 4.5C shows the LDH Activity for cells after approximately 2 days in culture, p=0.000002 (****), student T-test, n=3. Error bars denote standard deviation of three replicate flasks.
Figure 4.6: BSA protection for NS0 cells exposed to moderate hydromechanical stress is rapidly lost for cells originally cultured in media with 1g/L BSA. Parental NS0 cells were exposed to hydromechanical stress by shaking at 135 RPM in 125ml baffled shake flasks with a working volume of 24ml. Media with 0.025g/L Pluronic F-68 and 0 or 1g/L BSA was used in this experiment. NS0 cells originally cultured with 1g/L BSA were exposed to hydromechanical stress in media with 0 and 1g/L BSA. Figure 4.6A shows the growth profiles for NS0 cells. Figure 4.6B shows the doubling times for the NS0 cells after approximately three days in culture, \(p=0.0009\) (**), student T-test, \(n=3\). Figure 4.6C shows the LDH Activity for cells after approximately 2 days in culture, \(p=0.000004\) (****), student T-test, \(n=3\). Error bars denote standard deviation of three replicate flasks.

Figure 4.7: BSA protection for NS0 cells exposed to extreme hydromechanical stress is afforded and lost rapidly. Parental NS0 cells were exposed to hydromechanical stress by shaking at 175 RPM for 60 minutes in 125ml baffled shake flasks with a working volume of 24ml. Media with 0.025g/L Pluronic F-68 and 0 or 1g/L BSA was used in this experiment. NS0 cells originally cultured with 1g/L BSA were exposed to hydromechanical stress in media with 0 and 1g/L BSA. NS0 cells originally cultured with 0g/L BSA were exposed to hydromechanical stress in media with 0 and 1g/L BSA. Figure 4.7A shows the percentage reduction in VCD for cells originally cultured with 0g/L BSA, \(p=0.003\) (**), student T-test, \(n=3\). Figure 4.7B shows the LDH Activity for cells originally cultured with 0g/L BSA, \(p=0.002\) (**), student T-test, \(n=3\). Figure 4.7C shows the percentage reduction in VCD for cells originally cultured with 1g/L BSA, \(p=0.0006\) (**), student T-test, \(n=3\). Figure 4.7D shows the LDH Activity for cells originally cultured with 1g/L BSA, \(p=0.004\) (**), student T-test, \(n=3\). Error bars denote standard deviation of three replicate flasks.

Figure 4.8: BSA affords limited protection for NS0 cells experiencing laminar shear stress. Parental NS0 cells were exposed to 2.5Pa of laminar shear for 3 hours with an initial seeding density of \(1\times10^6\) cells/ml. These results show the percentage reduction in TCD for NS0 cells exposed to the laminar shear in media with 0 and 1g/L BSA and 0.025g/L Pluronic F-68, \(p=0.013\) (*), student T-test, \(n=3\). Error bars denote standard deviation of three replicate tests.

Figure 4.9: Protection afforded by Pluronic F-68 is limited for NS0 cells exposed to laminar shear stress. Parental NS0 cells were exposed to 2.5Pa of laminar shear for 3 hours in media with 0g/L BSA and varying concentrations of Pluronic F-68 (0, 0.025, 0.1, 0.5, 1 and 2g/L). The results show the percentage reduction in TCD for the samples.

Figure 4.10: BSA reduces the surface tension of NS0 cell culture media at low concentrations of Pluronic F-68. Figure 4.10A shows the surface tension of NS0 cell culture media with 0g/L Pluronic F-68 and various concentrations of BSA (0, 0.1, 0.5, 1, 2, 3 and 4g/L), 0.5g/L BSA \(p=0.04\) (*), 1g/L BSA \(p=0.04\) (*), 2g/L BSA \(p=0.03\) (*), 3g/L BSA \(p=0.02\) (*) and 4g/L BSA \(p=0.006\) (**), student T-test, \(n\geq 3\). Figure 4.10B shows the surface tension of NS0 cell culture media with 0.025g/L Pluronic F-68 and 0 or 1g/L BSA, \(p=0.00002\) (****), student T-test, \(n\geq 3\). Figure 4.10C shows the surface tension of NS0 cell culture media with 1g/L Pluronic F-68.
and 0 or 1g/L BSA. Error bars denote standard deviation of at least three replicate tests.

**Figure 4.11:** Shaking NS0 cell culture media in baffled shake flasks at low orbital shaker speeds (110 and 135 RPM) does not result in bubble formation. This figure shows 24ml of NS0 cell culture media after 1 hour shaking at 110 and 135 RPM. Figure 4.11A shows media with 1g/L Pluronic F-68 and 0 and 1g/L BSA shaken at 110 RPM. Figure 4.11B shows media with 0.025g/L Pluronic F-68 and 0 and 1g/L BSA shaken at 110 RPM. Figure 4.11C shows media with 1g/L Pluronic F-68 and 0 and 1g/L BSA shaken at 135 RPM. Figure 4.11D shows media with 0.025g/L Pluronic F-68 and 0 and 1g/L BSA shaking at 135 RPM.

**Figure 4.12:** Pluronic F-68 and BSA stabilise bubbles formed in NS0 cell culture media shaken in baffled shake flasks at high orbital shaker speeds (150 and 175 RPM). This figure shows 24ml of NS0 cell culture media after 1 hour shaking at 150 and 175 RPM. Figure 4.12A shows media with 1g/L Pluronic F-68 and 0 and 1g/L BSA shaken at 150 RPM in baffled shake flasks. Figure 4.12B shows media with 0.025g/L Pluronic F-68 and 0 and 1g/L BSA shaken at 150 RPM in baffled shake flasks. Figure 4.12C shows media with 1g/L Pluronic F-68 and 0 and 1g/L BSA shaken at 175 RPM in baffled shake flasks. Figure 4.12D shows media with 0.025g/L Pluronic F-68 and 0 and 1g/L BSA shaking at 175 RPM in baffled shake flasks.

**Figure 4.13:** BSA associates with NS0 cells in a dose dependent manner during cell culture. Parental NS0 cells were cultured in media with 0g/L BSA and 1g/L Pluronic F-68. The cells were then washed and resuspended in media with 0, 0.1, 0.5, 1 and 2g/L BSA and 0.025g/L Pluronic F-68 and incubated for one hour. 1x10^6 cells were centrifuged and washed twice using 100µl of media (with 0g/L BSA and 0g/L Pluronic F-68). The NS0 cells were then lysed in 100µl of lysis buffer. Washes and lysates were run on 12% SDS-PAGE gels and transferred to nitrocellulose membranes and probed for BSA. Figure 4.13A shows the BSA in the cell washes. Figure 4.13B shows the BSA in the cell lysates. Beta-actin is used as a loading control in this experiment.

**Figure 4.14:** Pluronic F-68 does not affect the association of BSA with NS0 cells during cell culture. Parental NS0 cells were cultured in media with 0g/L BSA and 1g/L Pluronic F-68 from thaw. The cells were then resuspended in media with 1g/L BSA and either 0.025 or 1g/L Pluronic F-68 and incubated for one hour. 1x10^6 cells were centrifuged and washed twice using 100µl of media (with 0g/L BSA and 0g/L Pluronic F-68). The NS0 cells were then lysed in 100µl of lysis buffer. Washes and lysates were run on 12% SDS-PAGE gels and transferred to nitrocellulose membranes and probed for BSA. These results show BSA in the cell lysates. Beta-actin is used as a loading control in this experiment.

**Figure 4.15:** BSA association with NS0 cells is removed by culturing the cells in media with 0g/L BSA for one hour. Parental NS0 cells were cultured in media with 0 and 1g/L BSA. The cells cultured in media with 0g/L BSA were resuspended in media with 1g/L BSA and incubated for one hour. The cells cultured in media with 1g/L BSA were resuspended in media with 0g/L BSA and incubated for one hour. 1x10^6 cells were centrifuged and washed twice using 100µl of 0g/L BSA media.
The NS0 cells were then lysed in 100µl of lysis buffer. Washes and lysates were run on 12% SDS-PAGE gels and transferred to nitrocellulose membranes and probed for BSA. Figure 4.15A shows the BSA in the cell washes. Figure 4.15B shows the BSA in the cell lysates. Beta-actin is used as a loading control in this experiment.

**Figure 4.16: BSA causes a dose dependent reduction in NS0 cell membrane hydrophobicity in media with low Pluronic F-68 concentrations.** Figure 4.16A shows the effects of various concentrations of BSA (0, 0.1, 0.5, 1 and 2g/L) on the NS0 cell membrane hydrophobicity in media with 0g/L Pluronic F-68, 0.5g/L BSA p=0.004 (**), 1g/L BSA p=0.006 (**), 2g/L BSA p=0.0098 (**), student T-test, n=3. Figure 4.16B shows the effect of various concentrations of BSA (0, 0.1, 0.5, 1 and 2g/L) on NS0 cell hydrophobicity in media with 0.025g/L Pluronic F-68, 0.5g/L BSA p=0.01 (*), 1g/L BSA p=0.005 (**), 2g/L BSA p=0.007 (**), student T-test, n=3. Figure 4.16C shows the effect of 0 and 1g/L BSA on NS0 cell hydrophobicity in media with 1g/L Pluronic F-68. Error bars denote the standard deviation of three replicate tests.

**Figure 4.17: Denatured BSA offers improved protection to NS0 cells exposed to extreme hydromechanical stress in media with low Pluronic F-68 concentrations.** BSA was denatured at 100°C for 30 minutes. Parental NS0 cells were exposed to hydromechanical stress (175 RPM in 125ml baffled shake flasks) in media with 0g/L BSA and 1g/L of native or denatured BSA and 0.025g/L Pluronic F-68. These results show the percentage reduction in VCD after 60 minutes exposure to extreme hydromechanical stress, 1g/L denatured BSA vs 0g/L BSA p=0.0009 (**), 1g/L native BSA vs 1g/L denatured BSA p=0.005 (*) student T-test, n=3. Figure 4.17B shows the LDH Activity of the samples after 30 minutes exposure to extreme hydromechanical stress p=0.008 (%) student T-test, n=3. Error bars denote the standard deviation of three replicate flasks.

**Figure 4.18: Denatured BSA protects NS0 cells from laminar shear stress.** Parental NS0 cells were exposed to 2.5Pa of laminar shear for 3 hours in media with 0g/L Pluronic F-68 and either 0 or 1g/L BSA (native or denatured at 100°C for 30 minutes). These results show the percentage reduction in TCD for the samples.

**Figure 4.19: Denatured BSA associates with NS0 cells in culture and reduces the NS0 cell membrane hydrophobicity similar to native BSA.** Parental NS0 cells originally cultured in media with 0g/L BSA were cultured in media with 1g/L of native or denatured BSA and for one hour. 1x10^6 cells were centrifuged and washed twice. The NS0 cells were then lysed in 100µl of lysis buffer. Washes and lysates were run on 12% SDS-PAGE gels and transferred to nitrocellulose membranes and probed for BSA. Figure 4.19A shows the BSA in the cell lysates. Beta-actin is used as a loading control in this experiment. Figure 4.19B shows the effects of 1g/L native and denatured BSA (heat denatured at 100°C for 30 minutes) on NS0 cell membrane hydrophobicity in media with 0g/L Pluronic F-68, Native BSA p=0.005 (**), Denatured BSA p=0.003 (**), student T-test, n=3. Error bars denote the standard deviation of three replicate tests.

**Figure 4.20: Denatured BSA causes a greater reduction in the surface tension of NS0 culture media compared to native BSA.** This figure shows the surface tension.
of NS0 cell culture media with 0.025g/L Pluronic F-68 and 2ml/L CLC supplemented with native or denatured BSA (heat denatured at 100°C for 30 minutes), p=0.0004 (**), student T-test, n=5. Error bars denote standard deviation of five replicate tests.

Figure 4.21: Methyl cellulose protects NS0 cells from extreme and hydromechanical stress in baffled shake flasks in media with low Pluronic F-68 concentrations. Parental NS0 cells were exposed to extreme hydromechanical stress (175 RPM for 60 minutes in 125ml baffled shake flasks) at a seeding density of 1x10^6 cells/ml and moderate hydromechanical stress (135 RPM) in baffled shake flasks. The cells were exposed in media with 0g/L BSA and 0.025g/L Pluronic F-68 with 0 or 0.1g/L methyl cellulose. Figure 4.21A shows the growth profiles for the NS0 cells exposed to moderate hydromechanical stress. Figure 4.21B shows the doubling times for the NS0 cells exposed to the moderate hydromechanical stress p=0.03 (*), student T-test, n=3. Figure 4.21C shows the reduction in VCD for cells exposed to the extreme hydromechanical stress, p=0.0002 (**), student T-test, n=3. Figure 4.21D shows the LDH activity for samples taken after 30 minutes exposure to the extreme hydromechanical stress, p=0.000006 (****), student T-test, n=3. Error bars denote standard deviation of three replicate flasks.

Figure 4.22: Methyl cellulose has no effect on NS0 cell membrane hydrophobicity. This figure shows the relative membrane hydrophobicity for NS0 cells in media with 0.025g/L Pluronic F-68 and 0 and 1g/L methyl cellulose. Error bars denote the standard deviation of three replicate tests.

Figure 4.23: Methyl cellulose reduces the dynamic surface tension of NS0 cell culture media and stabilises bubbles in baffled shake flasks. Figure 4.23A shows the dynamic surface tension of NS0 cell culture media with 0.025g/L Pluronic F-68, 0g/L BSA and 0ml/L CLC supplemented with 0 and 0.1g/L methyl cellulose, p=0.000003 (****), student T-test, n =5. Error bars denote the standard deviation of at least three replicate tests. Figure 4.23B shows the bubbles formed in media with 0g/L BSA and 0.025g/L Pluronic F-68 supplemented with 0 or 0.1g/L methyl cellulose.

Chapter 5:

Figure 5.1: Schematic showing the protective effect of BSA is only evident in media with limited protection from Pluronic F-68. Flask (a) depicts media supplemented with BSA and an effective lot of Pluronic F-68. Flask (b) depicts media supplemented with an effective lot of Pluronic F-68. Flask (c) depicts media with limited protection from Pluronic F-68 supplemented with BSA. Flask (d) depicts media with limited protection from Pluronic F-68. Flask (e) depicts media supplemented with BSA. Flask (f) depicts media not supplemented with BSA or Pluronic F-68.

Figure 5.2: Schematic of the association of BSA and Pluronic F-68 with the air-liquid interface and with the NS0 cell membrane. Figure 5.2A depicts NS0 cells cultured in media with 0.025g/L Pluronic F-68 and 0g/L BSA. The red arrows indicate the affinity of the NS0 cells with the air-liquid interface, causing the cells to
be highly susceptible to damage. Figure 5.2B depicts NS0 cells in media supplemented with 0.025g/L Pluronic F-68 and 1g/L BSA. Media with 0.025g/L Pluronic F-68 and 1g/L BSA gives a ratio of Pluronic F-68 molecule to BSA molecule of approximately 1:5 as depicted in this figure. BSA and Pluronic F-68 have an affinity for the NS0 cell membrane and for the air-liquid interface. The red indicates a reduced affinity of the cells with the air-liquid interface, which causes a reduced susceptibility to damage. Figure 5.2C depicts NS0 cells in media supplemented with 1g/L Pluronic F-68 and 0 or 1g/L BSA. Media with 1g/L Pluronic F-68 and 1g/L BSA gives a ratio of Pluronic F-68 molecule to BSA molecule of approximately 8:1 as depicted in this figure. The red arrows indicate the reduced association of the NS0 cells with the air liquid interface, which results in low susceptibility to damage. The presence of BSA does not affect the damage experienced by the NS0 cells in media with 1g/L Pluronic F-68.

**Figure 5.3: Overview of the protective effect of BSA on NS0 cells.** This figure shows the physical and fast-acting biological protective mechanisms of action of BSA (native or denatured) on NS0 cells. It also shows that the protective effect of BSA may be replaced by a functioning surfactant (e.g. Pluronic F-68).

**Appendix 1:**

**Figure A.1: Immunoprecipitation of BSA from NS0 cell culture media.** BSA was purified from monoclonal antibody producing NS0 cell culture media supplemented with 1g/L BSA using a number of different immunoprecipitating methods: ProG beads, ProG beads with anti-BSA antibody and Agarose beads with anti-BSA antibody. Control IPs were carried out using media from NS0 cells cultured with 0g/L BSA. Samples were run on a 12% SDS-PAGE gel and stained with coomassie blue. M: protein marker, S: Sample pre-IP, ProG: ProG beads, ProG+mAb: ProG beads and Rabbit anti-BSA antibody, Agarose+mAb: Agarose beads and Rabbit anti-BSA antibody.

**Figure A.2: BSA offers dose dependent protection for NS0 cells exposed to laminar shear stress in media with low Pluronic F-68 concentrations.** Parental NS0 cells were exposed to various laminar shear conditions in media with 0.025g/L Pluronic F-68 and various concentrations of BSA. The results show the percentage reduction in TCD after exposure to the shear. Figure A.2A shows the results of NS0 cells exposed to 0.5, 1.5 and 2.5Pa of laminar shear for 3 hours in media with 0 or 1g/L BSA. Figure A.2B shows the results of NS0 cells exposed to 2.5Pa of laminar shear stress for 1, 2 and 3 hours in media with 0 or 1g/L BSA. Figure A.2C shows the results of NS0 cells were exposed to 2.5Pa of laminar shear stress for 3 hours in media 0, 1, 2 and 4g/L BSA. Error bars denote the standard deviation of two replicate tests.

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stress. These results show the percentage reduction in TCD for the samples

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**Figure A.7: Pluronic F-68 does not affect the amount of BSA in the first washes of NS0 cells.** Parental NS0 cells were cultured in media with 0g/L BSA and 1g/L Pluronic F-68. The cells were then resuspended in media with 1g/L BSA and either 0.025 or 1g/L Pluronic F-68 and cultured for one hour. 1x10^6 cells were centrifuged and washed twice using 100µl of media (with 0g/L BSA and 0g/L Pluronic F-68). The NS0 cells were then lysed in 100µl of lysis buffer. Washes and lysates were run on 12% SDS-PAGE gels and transferred to nitrocellulose membranes and probed for BSA. This figure shows the BSA detected in the first and second washes of the cells.

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Chapter 1: General Introduction
1.1 Therapeutic Monoclonal Antibodies

1.1.1 Monoclonal Antibodies and Disease Targets

The development of hybridoma technology in the 1970s by Köhler and Milstein enabled the development of highly specific therapeutic monoclonal antibodies (mAbs) (Köhler and Milstein, 1975). The general structure of mAbs or immunoglobulins (IgGs) is made up of a Fab domain and Fc domain. The Fab domain of the antibody functions as the antigen-binding site and contains the complementary determining regions (CDRs) which are complementary to the epitope on the antigen. Variations in the amino acid sequence in the CDRs results in a wide variety of highly specific mAbs (Breedveld, 2000). The Fc domain of the antibody determines the effector function of the antibody as described in Section 1.1.2 (Breedveld, 2000).

The first generation of mAbs were murine mAbs and had limited protective capabilities because they caused an immune response in humans due to their murine origins (Khazaeli et al., 1994), however these limitations were overcome by the use of recombinant engineering techniques to develop chimeric mAbs which contained human Fc domains and humanised mAbs which contained all human protein sequence except for the murine CDRs (Adams and Weiner, 2005). The current state of the art therapeutic mAbs are fully human mAbs which contain 100% human protein sequence (Lonberg, 2005). These fully human mAbs have a lower risk of immunogenic response than murine, chimeric and humanised mAbs (Hwang and Foote, 2005). The general structure of murine, chimeric, humanised and fully human mAbs are shown in Figure 1.1. The structure of mAbs consists of two light and two heavy chains, as shown in Figure 1.1.
The main targets for therapeutic mAbs include; cancer, autoimmune/inflammatory diseases, neovascular diseases, infectious diseases, haemostasis and rejection (Elvin et al., 2013). It is anticipated that over the coming years the number and types of diseases that will be treated with mAb products will continue to increase (Ecker et al., 2015).

![Figure 1.1: Structure of mAbs. The basic structure of murine, chimeric and humanised mAbs is shown in this figure. This figure was adapted from Brekke and Sandlie, 2003.](image)

### 1.1.2 Mechanisms of Action of Monoclonal Antibodies

There are a number of different mechanisms by which mAbs can elicit their therapeutic response once the mAb has bound to its specific antigen. Antibody-dependent cellular cytotoxicity (ADCC) is a mechanism of action of therapeutic mAbs which involves Fc receptors on the surface of immune effector cells associating with the mAb Fc domain once it has bound to its specific antigen on the target cell (Steplewski et al., 1983). There are a number of different immune effector cells which can have cytotoxic effects of the target cell, e.g. monocytes,
macrophages, natural killer cells, killer cells, and granulocytes (White et al., 2001). These cells can release compounds which cause cell death, or they can engulf the target cell by phagocytosis causing cell death. An overview of ADCC is shown in Figure 1.2A.

Complement-dependent cytotoxicity (CDC) is mechanism of action of mAbs which is mediated by the mAb binding to specific antigens on the cell surface and causing a signalling cascade controlled by complement proteins which eventually creates pores in the cell membrane causing cell death due to the formation of a Membrane Attack Complex which causes cell lysis (Adams and Weiner, 2005; Natsume et al., 2009). An overview of CDC is shown in Figure 1.2B.

Another mechanism of action of mAbs is through signal transduction alterations, causing apoptosis of the cells. The mAbs can also physically block the interaction of the receptor and its activating ligand (Sunada et al., 1986), or prevent dimerization of receptors which can inhibit signal transduction (Franklin et al., 2004). An overview of these mAb effector functions are shown in Figure 1.2A.
Figure 1.2: Overview of antibody effector functions. Figure 1.2A gives an overview of ADCC (1.), Signal transduction (2.) and Ligand blocking (3.) functions of antibodies. Figure 1.2B shows the steps involved in the CDC effector function of antibodies. These figures are adapted from Loureieo et al., 2015 and Natsume et al., 2009 respectively.
1.1.3 Monoclonal Antibody Market

The first approved therapeutic mAb used to treat humans, Muromonab, was approved in 1986 as a treatment against organ rejection (Todd and Brogden, 1989). Since the 1980s there has been approximately 50 mAbs (and antibody-related products, e.g. Fc-fusion proteins, antibody fragments and antibody-drug conjugates) approved for human treatment and there are approximately four mAb products approved every year, therefore it has been predicted that there will be 70 or more mAb products on the market by 2020 (Ecker et al., 2015).

According to the IMS Institute global spending on medicines will be almost $1.3 trillion by 2018, which is an increase of approximately 30% from the 2013 figure (IMS Institute for Healthcare Informatics, 2014). Other reports have shown that the global market sales of mAb products grew from approximately $39 billion in 2008 to approximately $75 billion in 2013 (Ecker et al., 2015), and the sales of mAb products have been predicted to increase to nearly $90 billion by 2017 (BCC Research, 2013).

The growth in mAb product sales has been attributed to the increasing and aging human population and the increasing standard of living worldwide (United Nations Development Programme, 2013). The increased demand for mAb products has resulted in greater manufacturing capacity devoted to the production of mAbs and improvements in the design and optimisation of mAb products (Ecker et al., 2015).
1.1.4 Development of Monoclonal Antibodies

1.1.4.1 Overview of Industrial Cell Culture Process Development

The development of an industrial cell culture process begins with the generation of cell lines and the selection of suitable clones, followed by optimisation of the culture process in small-scale, high-throughput systems including shake flasks and small-scale bioreactors (Li et al., 2010). Once the process is fully developed at the lab and pilot scales, then the mAb product can be commercialised which involves a number of steps, namely: process characterisation, scale-up, technology transfer and validation of the manufacturing process (Li et al., 2006).

1.1.4.2 Cell Line Development

The first step in the development of mAbs is the development of a stable cell line which produces the mAb of interest. The host cell line of choice is transfected with plasmids containing the antibody genes, as well as selectable markers before the cells are screened for high productivity (Li et al., 2010). Strong promoters/enhancers are commonly used to ensure high levels of antibody production, including the cytomegalovirus (CMV) promoter (Boshart et al., 1985) and elongation factor (EF) 1-alpha promoter (Deer and Allison, 2004). A Kozak sequence can also be placed before the first initiation codon to enhance translation of the mAb genes (Kozak, 1986). A signal peptide sequence is also placed in front of the antibody peptide to ensure that the antibody is secreted from the cell (Birch and Racher, 2006). A small number of biological selectable markers are commonly used in industrial cell line development to aid the selection of high expressing clones, including dihydrofolate reductase (DHFR) (Page and Sydenham, 1991) and glutamine synthetase (GS) (Cockett et al., 1990; Bebbington et al., 1992).
1.1.4.3 Cell Lines used for Monoclonal Antibody Production

Human insulin was the first recombinantly produced protein product approved by the United States Food and Drug Administration (FDA), which was produced in bacterial cells (E.coli) by Eli Lilly and Company (Williams et al., 1982). However, the first biotechnology product produced in animal cells, human plasminogen activator, was produced by Genentech using Chinese Hamster Ovary (CHO) cells (Kaufman et al., 1985). Today the production of mAb products is mainly carried out using mammalian cell lines including NS0 cells (mouse myeloma cell line) (Barnes et al., 2000; Spens and Häggström, 2007), CHO cells (Puck et al., 1958), Human Embryonic Kidney (HEK) cells (Graham et al., 1977) and PER.C6® cells (human embryonic retina cells) (Pau et al., 2001). The most commonly used cell lines are CHO cells, which are used to produce approximately 70% of all biopharmaceutical products (Birch and Onakunle, 2005). The use of mammalian cell lines is preferred for the production of mAbs (in the vast majority of cases) due to the mammalian cells providing human-like post translational modifications compared to bacterial cell lines (Page, 1988; Walsh and Jefferis, 2006). However, mammalian cells have been reported to be difficult to culture at a large-scale due to low product yield, medium complexity, serum requirement and greater sensitivity to damage (Li et al., 2010).

There are a number of factors taken into consideration when choosing the mammalian cell line used to produce a mAb product, including: i) productivity of the system, ii) capability of producing a mAb product with acceptable quality attributes (e.g. molecule integrity and aggregation, glycosylation profiles, charge heterogeneity) and iii) the historical experience of individual companies (Li et al.,
This thesis will focus on the NS0 cell line which is used in Eli Lilly to produce a number of therapeutic mAbs.

1.1.4.4 NS0 Cells

The NS0 cell line is one of the most commonly used cell lines for the production of mAb products (Barnes et al., 2000). There are a number of mAb products on the market which are produced in NS0 cells, including; Simulect™ produced by Novartis which was approved by the FDA in 2001 (FDA, 2009) and Synagis™ produced by MedImmune which was approved by the FDA in 1998 (FDA, 2010). There are a number of different factors which make NS0 cells very favourable for the large-scale production of biopharmaceuticals, including; the ability of NS0 cells to incorporate foreign DNA in a stable and productive manner, the lack of endogenous antibody production, the extensive regulatory pedigree associated with NS0 cells, the capability of single-cell dispersions in suspension culture and the fact that NS0 cells are capable of robust growth and high levels of production in a variety of media (Whitford, 2003). The fact that NS0 cells originate as a plasmacytoma has also been suggested to be responsible for the efficient production, processing, and secretion of antibody proteins by NS0 cells (Barnes et al., 2000). NS0 cells require the exogenous provision of glutamine due to the lack of endogenous glutamine synthetase (GS) expression as a result of gene silencing during cell line development (Bebbington et al., 1992). Therefore the GS selection system can be used very easily as a selectable marker to select for successfully transfected NS0 cells (Barnes et al., 2000). This innate lack of GS expression initially made NS0 cells an attractive cell line for the production of therapeutic mAbs; however the GS selection system has been adapted for use in some CHO cell lines by using high concentrations of a GS
inhibitor methionine sulphoximine to select for high mAb-producing cells (Birch and Racher, 2006). CHO cells have also been developed which have the GS gene knocked out in order to improve the selection of high producing cell lines (Fan et al., 2011). Therefore, the GS selection system is no longer unique to NS0 cells.

The development of the NS0 cell line began in 1962 when Potter and Boyce noticed that intraperitoneal injection of mineral oil into mice induced plasma-cell neoplasms (Potter and Boyce, 1962). NS0 cells are a myeloma cell line which was derived from a plasmacytoma cell line MOPC21, which was an immunoglobulin G-1 (IgG1) secretor (Potter et al., 1965). This MOPC21 cell line was used to establish a continuous tissue culture line (Horibata and Harris, 1970). These MOPC21 cells were transplanted in mice and this led to the development of P3K cells, which were also IgG1 secretors (Horibata and Harris, 1970). These P3K cells were cloned to give P3-X27 cells, which were also IgG1 secretors (Ramasamy et al., 1974). Two cell lines, 289-16 and P3-X63, were developed by re-cloning the P3-X27 cells (Ramasamy et al., 1974). The 289-16 cell line was not an IgG1 secretor; however it produced the antibody light chain and did not secrete it. The 289-16 cell line was renamed NS1 (non-secreting) (Cowan et al., 1974). These cells were cloned once more which generated the NS0 cell line which did not synthesise antibody heavy or light chains (Galfré and Milstein, 1981). The development of the NS0 cell line is summarised in Figure 1.3.
Although NS0 cells are commonly used to produce therapeutic mAbs, there are some limitations to the cell line, as outlined in the following paragraphs. Similar to their ancestral cell line, NS1 (Sato et al., 1984), NS0 cells are auxotrophic for cholesterol and therefore require the provision of cholesterol from the cell culture media for optimal growth (Keen and Steward, 1995). The cholesterol deficiency of NS0 cells is due to the lack of expression of the enzyme 17-Hydroxysteroid dehydrogenase type 7 which is involved in cholesterol synthesis (Seth, McIvor and Hu, 2006; Seth, Ozturk and Hu, 2006). NS0 cells are a myeloma cell line and it has been reported that cancer cells (e.g. myeloma cells) require more cholesterol than

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**Figure 1.3: Development of the NS0 cell line.** This figure was adapted from Barnes et al., 2000.
normal cells, with some reports showing that there is up to 7 times more cholesterol in the cell membrane of cancer cells compared to normal cells (Pederson, 1978; Coleman and Lavietes, 1981). Cholesterol is not soluble in water-based liquids and therefore requires a carrier in water-based cell culture media. It has been known since the early 1980s that a cyclic oligosaccharide, cyclodextrin, can solubilise cholesterol and provide cholesterol to cells in cell culture (Yamane et al., 1981; Christian et al., 1997; Härtel et al., 1998). Bovine serum albumin has also been added to cell culture media to aid cholesterol solubilisation and provision (as discussed later in Section 1.4.2.3). There have also been reports of the successful growth of some NS0 cell lines which are not cholesterol dependent (Birch et al., 1994; Keen and Steward, 1995; Seth, Ozturk, and Hu, 2006); however these cells were not cultured on an industrial scale.

As well as being cholesterol deficient, NS0 cells lack the expression of the protein Hsp70 (Aujame and Firko, 1988), which has been associated with an increased susceptibility to apoptosis (Lasunsgaia et al., 2003). Finally, cell lines derived from rodents lack the enzyme required for α(2-6) sialylation and murine cell lines also perform α(1-3) galactosylation (Baker et al., 2001), which may cause an immunogenic response in humans when therapeutic mAbs are produced in rodent cell lines. These challenges along with the development of GS-CHO cells have resulted in the declining use of NS0 cells for the production of therapeutic mAbs; however there are a number of therapeutic mAbs in clinical trials which are produced in NS0 cells and therefore NS0 cells remain an important cell line for the production of therapeutic mAbs.
1.2 Large-Scale Monoclonal Antibody Production

The treatment of diseases using therapeutic mAbs requires large doses of mAbs over extended periods of time; therefore biopharmaceutical companies need to carry out cell culture in large-scale bioreactors to produce sufficient quantities of therapeutic mAbs (Chadd and Chamow, 2001). The first reports of mammalian cell culture in suspension were in the 1950s, where it was shown that suspension cells could be grown in agitated systems, such as tumbling tubes and shake flasks (Owens et al., 1954; Earle et al., 1954). By the end of the 1960s mammalian cells were being cultured in bioreactors at the scales of hundreds of litres (Moore et al., 1968). Modern industrial cell culture can now be carried out in extremely large bioreactors with a volume of up to 25,000L (Bork et al., 2013). The two main reasons that such large bioreactors are required are the low product titre produced by mammalian cells and the high therapeutic dosage required for mAbs (Birch and Racher, 2006).

A recent report stated that there was almost 10 metric tons of mAbs produced in 2013 compared to approximately 8.6 metric tons of other recombinant protein products (Ecker et al., 2015). Another report estimated the development of each biopharmaceutical product costs $1.6-6.3 billion, therefore there is continued research being conducted to reduce the development time and cost of mAb production, while also increasing the productivity of the mAb production process (Drakeman, 2014).

1.2.1 The Monoclonal Antibody Production Process

The production of mAbs is generally split into upstream and downstream operations. The function of the upstream operation is to grow the mAb-producing cell line so that the mAb will be produced. An overview of the upstream process is depicted in
Cells are thawed from a cell bank and expanded in shake flasks until there are sufficient cell concentrations to inoculate a series of sequentially larger “Seed Reactors” and then these grow sufficient cell densities to inoculate a “Production Reactor” where the therapeutic mAb of interest is produced. There are a number of different bioreactor operating modes used; however the main methods used are fed-batch, batch and perfusion (or continuous) cell culture (Bibila and Robinson, 1995). In fed-batch cell culture the bioreactor is supplemented with nutrients (e.g. glucose and amino acid feeds, as shown in Figure 1.4) which aid the cell growth and antibody production, whereas there are no nutrients added to batch cell culture (Birch and Racher, 2006). In perfusion (or continuous) cell culture there is a continuous addition of fresh medium and removal of spent medium (containing the mAb product), therefore the cells remain in the bioreactor which leads to very high cell concentrations (Voisard et al., 2003). The chosen operating mode varies from product to product (Lim et al., 2006); however fed-batch is currently the most common operating mode in the production of therapeutic mAbs (Birch and Racher, 2006). The downstream operation involves the purification of the mAb product by centrifugation, filtration, viral clearance and chromatography steps. This work will focus on the upstream process of NS0 cells.

Improvements in the upstream cell culture process over the past few decades have led to an increase in product titres produced by mammalian cells. Mammalian cell culture titres have increased from 50mg/L in 1984 to >10g/L in 2009 (Wurm, 2004; Kelley, 2009). Although there have been reports of titres >10g/L and there other reports of continuous cell cultures with product titres of up to 25g/L (Chon and Zarbis-Papastoitis, 2011), these titres have not yet been achieved in large-scale mAb production. The current average reported large-scale titre is estimated at
2.56g/L, and is predicted to increase to ≤7g/L with further improvements in downstream yields which currently stand at approximately 75% (Rader and Langer, 2015).

Figure 1.4: Overview of the upstream industrial cell culture process. This figure shows the upstream cell culture process including: development of cell bank, vial thaw, flask and seed reactor expansion and production reactor with required feeds (for fed-batch bioreactors).

1.2.2 Cell Culture in Large-Scale Bioreactors

There are a number of different bioreactor systems which can be used for large-scale mammalian cell culture, including stirred, airlift and disposable bioreactors. Each bioreactor system relies on good mixing, as the concentration gradients experienced by cells depends on the level of mixing in the bioreactor (Nienow, 1996). Concentration gradients in bioreactors can cause reduced cell growth (Osman et al., 2002) and can also cause problems with parameter controls, e.g. pH (Nienow, 1999). Stirred-tank bioreactors (STBs) are currently the most commonly used system for the
production of therapeutic monoclonal antibodies (Chu and Robinson, 2001), which are agitated using a number of impellers and aerated using a sparger as depicted in Figure 1.5. The operation and design of STBs requires temperature, pH and dissolved oxygen (DO) probes and the design of STBs for mammalian cell culture was originally adapted from microbial stirred tanks (Marks, 2003). The pH of the bioreactors is commonly controlled by the addition of CO$_2$ (to lower the pH) and base (NaOH – to raise the pH).

**Figure 1.5: Diagram of standard stirred tank bioreactor.** This schematic shows the typical set-up for a stirred tank bioreactor with pH, DO and temperature probes as well as a sparger and agitator.
1.2.3 Sources of Cellular Damage in Bioreactors

The term “shear stress” is commonly used to describe the damaging conditions in large-scale bioreactors, however it is impossible to quantify the exact stress that cells are experiencing in a bioreactor at any one time as the cells are subjected to time varying shear and elongational forces (Garcia-Briones and Chalmers, 1994). The sensitivity of animal cells to damage has been shown to vary from cell line to cell line (Handa et al., 1986); however animal cells have been suggested to be more susceptible to shear damage in a bioreactor than microbial cells due to the lack of a protective cell wall (Marks, 2003). Mammalian cells cultured in suspension have been shown to be much less sensitive to damage than cells cultured on microcarriers (Ma et al., 2002). Nevertheless, shear associated damage has a significant economic impact in animal cell culture industry (Chisti, 1998); therefore there has been a great deal of research carried out into the protection of cells against damage in the bioreactor.

The first reports of cells being damaged in bioreactors were published when early attempts of the scale-up of mammalian cell culture were being carried out in the 1960s (Bryant et al., 1960; Telling et al., 1965). Since the late 1980s it has been reported that sparging mammalian cell cultures can have a detrimental effect on cell number and viability (Oh et al., 1989; Kunas and Papoutsakis, 1990a). These studies showed hybridoma cells could withstand high stirring speeds as long as there is no air introduction to the culture (from sparging, or bubble entrainment from the central vortex). Therefore agitation of the cell culture is not thought to be the main source of damage to cells in a bioreactor, however it has been demonstrated that high agitation speeds (≥700RPM) in a bioreactor can reduce the viability of hybridoma cells when there are no bubbles present (Kunas and Papoutsakis, 1990b), which suggests that
there is a critical point above which the cells experience damage due to the hydromechanical stress caused by the high agitation speeds.

There are three potential regions in the bioreactor where cell-bubble interactions could cause damage to mammalian cells, i) at the sparger during bubble formation and detachment (Tramper et al., 1987; Zhu et al., 2008), ii) bubble break-up at the impeller region (Oh et al., 1992) and iii) bubble burst at the air-liquid interface (Handa et al., 1986). However, it is generally accepted that the bubble burst event at the air-liquid interface is the region in the bioreactor where most cell damage occurs (Handa et al., 1986; Handa-Corrigan et al., 1989; Chisti, 2000).

There has been extensive research conducted into the damaging bubble rupture events in sparged bioreactors. It has been shown that as the bubble approaches the air-liquid interface the bubble raises the liquid surface and the liquid film which is formed by the bubble begins to drain, as shown in Figure 1.6A and Figure 1.6B (Chalmers and Bavarian, 1991; Cherry and Hulle, 1992). As the film gets thinner, a hole eventually forms and the liquid film forms a toroidal ring and rapidly enters the bulk of the liquid, as shown in Figure 1.6C (Michaels et al., 1995b; Chisti, 2000). This rapid flow has been postulated to cause damage to cells in the vicinity of the bubble (Chalmers and Bavarian, 1991). When the bubble ruptures and the fluid rapidly moves down both sides of the interior walls of the bubble cavity and an impact zone is formed immediately below the bubble where this rapidly moving fluid meets (Michaels et al., 1995b; Chisti, 2000). The high pressure produced by the two streams impacting causes the formation of a jet of fluid to form above the air-liquid interface and a jet of fluid to form in the bulk of the liquid (Michaels et al., 1995b; Chisti, 2000), as shown in Figure 1.6D. The extent of the damage caused by bubble bursting event has been associated with a number of
factors; the number of cells associated with the bubble (Wang et al., 1994), the rate of bubble rupture in the liquid and at the air-liquid interface (Wang et al., 1994), the size of the bubble; with smaller bubbles causing more damage due to a greater energy dissipation rate during bubble burst (Boulton-Stone and Blake, 1993; Garcia-Briones et al., 1994) and the thickness of the film between the bubble and the air-liquid interface at the time of rupture; with thicker films causing more damage (Chalmers and Bavarian, 1991).

In an experiment carried out by Garcia-Briones et al. 95% of the cells in the upward jet produced during bubble burst were non-viable even when the viable cell density in the bulk liquid was ≥90% (Garcia-Briones et al., 1994), suggesting that the bubble burst event is extremely damaging to cells. The results from another study showed that the majority of cells in the upward jet were dead, with an average of approximately 1050 cells are killed per 2 mm diameter bubble burst (Trinh et al., 1994). In this study it was also shown that the cell concentration in the upward jet of the bubble burst was twice the concentration in the culture, suggesting that the cells have an inherent attraction to the air-liquid interface (Trinh et al., 1994).
Figure 1.6: Diagram of bubble bursting events. This diagram shows the events which occur during a bubble burst in cell culture. Figure 1.6A depicts a bubble approaching the air-liquid interface in culture. Figure 1.6B shows the formation of a film as bubbles approach the air-liquid interface. As the film gets thinner, a hole eventually forms and the liquid film forms a toroidal ring and rapidly enters the bulk of the liquid, as shown in Figure 1.6C. Figure 1.6D shows the formation of a jet of fluid to form above the air-liquid interface and a jet of fluid to form in the bulk of the liquid due to the high pressure produced by the impact of the streams formed during bubble burst. Figure adapted from Michaels et al., 1995b and Chisti, 2000.

1.2.4 The Cellular Responses to Damage

Many studies have been carried out to investigate the responses of cells to damaging shear forces, and a number of different methods have been used to characterise the cellular response to the damaging forces, including; the effects of the damage on cell growth rate (Moo-Young et al., 1992; Moo-Young et al., 1993; Meijer et al., 1994)
and cell viability (Ramírez and Mutharasan, 1990), the release of intracellular material from the cells (e.g. Lactate Dehydrogenase enzyme) (LDH) (Märkl et al., 1991; Meijer et al., 1994; Zhang et al., 1995) and alterations in the morphology of the cells (Makagiansar et al., 1993; Dunlop et al., 1994; Zhang et al., 1995). The majority of these studies were carried out on adherent cell lines (e.g. endothelial cells and smooth muscle cells) exposed to well-defined shear stress conditions.

A wide range of responses have also been published for cells exposed to non-lethal shear stress, e.g. i) the re-arrangement of the cell’s cytoskeleton (Papadaki and Eskin, 1997), ii) activation of proteins (integrins, ion channels, receptor tyrosine kinases, GPCRs and G proteins, PECAM-1) causing signal transduction in the cells which can lead to increased gene expression, proliferation or apoptosis, increased cell membrane permeability etc. (Li et al., 2005) and iii) increased glucose consumption and mitochondrial activity (Al-Rubeai et al., 1995). Although few of these observations have been published for cells cultured in suspension, the results provide an insight into the potential responses of suspension cells exposed to shear stress in large-scale bioreactors.

1.3 Lab-Scale Cell Culture Models

1.3.1 Shake Flasks (Non-Baffled and Baffled)

Shaken systems are often used for initial screening and development experiments for industrial cell lines as they allow for numerous experiments to be run at the same time (Betts and Baganz, 2006; Bareither and Pollard, 2011). It has been reported that over 90% of all industrial cell culture experiments are conducted in shake flasks (Büchs, 2001); however the industry standard for experiments mimicking large-scale cell culture is still the lab-scale STB (Nienow, 2013). This may be due to reports
showing differences in bio-performance between cells cultured in an agitated and aerated bioreactor and cells cultured in shake flasks (Hewitt and Nienow, 2007).

The use of baffled shake flasks has been discouraged in the past due to the splashing motion wetting the sterile plug in the flasks leading to impaired oxygenation of the culture (McDaniel et al., 1965; Henzler and Schedel, 1991). Also, early studies showed that baffled shake flasks had greater variability than non-baffled shake flasks (McDaniel et al., 1965). Baffled shake flasks have also been shown to have a highly complicated and chaotic fluid flow regime which is difficult to characterise (Büchs, 2001). However, there have been numerous studies published using baffled shake flasks with improved mixing and oxygenation being the main reasons cited for using baffled flasks instead of non-baffled flasks (McDaniel and Bailly, 1969; Dekker and Barbosa, 2001; Bermek et al., 2004; Galindo et al., 2004). Baffled shake flasks are also used rather than non-baffled shake flasks if a higher level of hydromechanical stress is required (Büchs, 2001). A recent report showed that the use of baffled shake flasks identified ineffective lots of the shear protectant, Pluronic F-68 using an industrial mammalian cell culture process (Peng et al., 2014).

The power input into the liquid (kW/m³) is a method used to characterise the culture conditions in baffled shake flasks (Klöckner and Büchs, 2012), which will be discussed later in Section 1.4.3.

1.3.2 Small-Scale Bioreactors

During process development, the scale-up of a mammalian cell culture is a difficult and complex process (Nienow, 2006; Marks, 2003). The development of lab-scale models (or scale-down models) for process optimisation and investigation involves the same issues as scaling-up, however the difficulty with the development of lab-
scale models is with mimicking the conditions experienced in the large-scale bioreactors so that there is comparability in cell growth and product quality in the small-scale models (Berridge et al., 2009). The main issues with developing lab-scale models of large-scale bioreactors tend to involve developing models with similar oxygen transfer, mixing and CO₂ stripping conditions (Xing et al., 2009).

A number of different mechanisms are used to aid the scale-up and scale-down of bioreactors, including; power input, mixing time, impeller tip speed, impeller shear rate and specific impeller pumping rate (Varley and Birch, 1999; Yang et al., 2007).

There are a number of different types of small-scale bioreactors which have been used as lab-scale models of large-scale bioreactors. The two main types of small-scale bioreactors which have been used are; i) stirred tank bioreactors (STB) (Kunas and Papoutsakis, 1990a; Kunas and Papoutsakis, 1990b; Oh et al., 1992) and ii) Air-lift bioreactors (Birch et al., 1987; Hülscher et al., 1988; Hülscher et al., 1990; Martens et al., 1992). The typical set up for a lab-scale STB was shown previously in Figure 1.5.

Advances in technology have led to the development of mini-bioreactor systems where large numbers of mini-bioreactors can be cultured in a limited amount of space (Betts and Baganz, 2006; Marques et al., 2010; Bareither and Pollard, 2011). These mini-bioreactor systems have similar attributes to traditional small-scale bioreactors, e.g. agitation, sparging of gas and control of pH and dissolved gasses (Nienow et al., 2013). An example of these mini-bioreactor systems is the AMBR™ System (Sartorius), which has a maximum volume of 15ml and can be bought with the capability of culturing 24 or 48 reactors at once. The AMBR system is the only mini-bioreactor system which uses an impeller to agitate the cultures and
also has the additional benefit of automated control of pH and dissolved gasses (Bareither and Pollard, 2011; Nienow et al., 2013). A diagram of the AMBR15 bioreactor is shown in Figure 1.7.

Figure 1.7: AMBR15 mini-bioreactor. This picture shows an AMBR15 mini-bioreactor containing; an agitator and impeller, sparger and pH and DO probes. This figure was adapted from Nienow, 2013.

1.4 Methods of Exposing Cells to Damage

As outlined in Section 1.3 small-scale bioreactors (air-lift, STB and AMBR) and shake flasks (non-baffled and baffled) are routinely used to expose cells to culture conditions similar to those experienced in large-scale bioreactors. However, it is difficult to define the stress conditions in large-scale bioreactors; therefore several other devices have been designed to expose cells to defined conditions. Some of these devices and methods of quantifying the damage experienced by cells will be outlined in the following sections.
1.4.1 Laminar Shear Devices

There are a wide variety of devices which can be used to expose cells to laminar shear (e.g. cone and plate rheometers and concentric cylinder rheometers). These devices have been widely used to test the effects of shear stress on cells, as the devices can expose the cells to well defined levels of shear for extended periods of time (Joshi et al., 2006). The majority of investigations into the effects of shear on cells have been carried out using laminar shear devices (results discussed earlier in Section 1.2.4), and a novel concentric cylinder shear device was designed in the University of Limerick to be used during this project.

Although laminar shear devices have been used to investigate the effects of shear on cells, there are a number of limitations associated with the use of laminar shear devices, as outlined by Joshi et al.: i) the devices have limited oxygenation capabilities; therefore experiments must be restricted to short periods, ii) normal stresses experienced by cells in a bioreactor (e.g. extensional flow, damage at air-liquid interface) cannot be tested, iii) steady state laminar shear stress may not be as damaging as the hydromechanical stress fluctuations experienced by cells in a bioreactor and iv) there is difficulty correlating the results obtained in the laminar shear devices with the results seen in turbulent stress models (Joshi et al., 1996). However, these laminar shear devices have been used to further our understanding of the effects and cellular responses involved when cells are exposed to shearing conditions (Schürch et al., 1988; Goldblum et al., 1990; Mardikar and Niranjan, 2000).
1.4.2 Hydrodynamic Stress Devices

A “torture chamber” device was designed by Chalmers and co-workers to expose cells to hydrodynamic stress due to contraction through a passage with a diameter of as little as 225µm (Ma et al., 2002). It was possible to quantify the hydrodynamic force (energy dissipation rate (EDR)) to which the cells were exposed to while passing through the chamber, therefore these devices were used to determine the sensitivity of some cell lines used for biopharmaceutical production (e.g. CHO, Bel-2, hybridoma, and PER.C6 cells) (Ma et al., 2002; Mollet et al., 2007). The first studies carried out using this device cells were carried out by passing cells through the torture chamber once, therefore the effects of extended exposure to the hydrodynamic stress could not be tested. This was overcome by the development of a loop where the cells were constantly being circulated from a lab-scale bioreactor through the device and back into the bioreactor (Godoy-Silva et al., 2009).

Another lab-scale device, called an Ultra-Scale Shear Device (USD), was designed in University College London using a spinning disk inside a chamber to mimic (on a lab-scale) the forces experienced when cells enter a large-scale disk-stack centrifuge (Levy et al., 1999). The forces experienced in the USD were shown to be similar to the forces in a large-scale disk-stack centrifuge using computational fluid dynamics (CFD) (Boychyn et al., 2004). The USD has been used for a number of purposes, including: assessment of the effects of the damaging forces in a disk-stack centrifuge on mAb Post Translational Modifications (PTMs) (Reid et al., 2010), optimising conditions for the clarification of cellular debris from harvest material by disk-stack centrifuges (Boychyn et al., 2004; Zaman et al., 2009) and testing the effects of shear forces on the recovery of plasmid DNA in E. coli cells (Levy et al., 1999).
1.4.3 Quantification of Damage

There have been a number of different methods used to quantify the damage experienced by cells in bioreactors and lab-scale models of damage, including; Kolmogorov eddy size (Croughan et al., 1987; Cherry and Papoutsakis, 1988; Croughan et al., 1989; Oh et al., 1989) and EDR or power input into the liquid (W/m$^3$ or kW/m$^3$) (Sumino et al., 1972; Büchs et al., 2000; Ma et al., 2002; Mollet et al., 2009).

The Kolmogorov eddy size has been used to quantify the damage experienced by cells since the 1980s (Cherry and Papoutsakis, 1986; Croughan et al., 1987). The Kolmogorov eddy size, $\eta$, is evaluated using the local EDR, $\varepsilon$, and the media kinematic viscosity, $\nu$, using the following equation:

$$\eta = (\nu^3/\varepsilon)^{1/4}$$

This method of quantifying damage is based on the formation of damaging eddies in turbulent flow where kinetic energy is converted into heat dissipation. It has been suggested that Kolmogorov eddies become damaging when the eddy is the same size or smaller than the cell exposed to the damage (Kunas and Papoutsakis, 1990a). This method of quantifying damage is not as widely used as EDR, as it has a number of limitations, e.g. turbulent flow is required and the volume in which the energy is dissipated cannot be derived from first principles (Godoy-Silva et al., 2013).

The EDR is commonly used to quantify the damage experienced in bioreactors (Kresta, 1998) and laminar shear devices (Mollet et al., 2004; Mollet et al., 2007). The EDR, $\varepsilon$, can be calculated from first principles (for a Newtonian
fluid) using the following equation, where $\tau$ is the stress tensor and $\nabla U$ is the velocity gradient tensor (Brodkey, 1995; Bird et al., 2006):

$$\varepsilon \equiv \tau \cdot \nabla U$$

The EDR of bursting bubbles was quantified using computer simulations and the EDR was used to compare the damage caused by bubble bursting to the damage caused by mixing in a typical STB (Boulton-Stone and Blake, 1993; Garcia-Briones et al., 1994). These studies concluded that the EDR of bursting bubbles was much higher than the EDR caused by mixing alone, which supports the findings discussed previously showing that bubble rupture is more damaging than agitation alone (Handa-Corrigan et al., 1989; Chisti, 2000).

The power input per unit volume (kW/m$^3$) has been used during scale-up of cell cultures in bioreactors and has also been used to characterise the culture conditions in non-baffled and baffled shake flasks (Sumino, et al., 1972; Büchs et al., 2000; Peter, Suzuki, Rachinskiy et al., 2006; Peter, Suzuki and Büchs, 2006). The first report attempting to quantify the power input into liquid in shake flasks used temperature increase in heat-insulated flasks as the readout (Sumino et al., 1972). The interpretation of the results was difficult as the temperature increase was quite low and there were complications due to undefined thermal conduction to the parts of the flask not in contact with the liquid (Sumino et al., 1972). Another method used to determine the power input into the liquid in shake flasks was carried out by Kato et al. where the total electric power consumption of the shaking machine was determined (Kato et al., 1995), however this was conducted in flasks where the liquid height was equal to the vessel diameter and using this method to quantify the power input in commercially available shake flasks was reported to be highly
inaccurate (Büchs et al., 2000). Büchs et al. described a method of determining the power input into the liquid in shake flasks by determining the mechanical torque counteracting liquid friction on the wall of the shake flask (Büchs et al., 2000). The device used by Büchs et al. measures the torque acting on the drive shaft of the shaker machine (Büchs et al., 2001). This mechanism was used to show that the power input into the liquid in the is greater in baffled shake flasks compared to non-baffled shake flasks under the same operating conditions (Peter, Suzuki, Rachinskiy et al., 2006). Increased power input into the liquid is seen with deeper baffles, elevated viscosity, increased orbital shaker diameters, increasing orbital shaker speeds and decreasing fill volume (Büchs et al., 2000; Büchs et al., 2001; Peter, Suzuki, Rachinskiy et al., 2006).

1.5 Protective Additives for Industrial Cell Culture

Industrial cell culture media can contain over 70 ingredients, with the concentrations of these ingredients requiring optimisation using spent medium analysis or high-throughput screening experiments (Hodge, 2005; Liu and Chang, 2006). The biopharmaceutical industry is moving towards the use of protein-free and even chemically defined media for the production of mAbs; however the development of these media is generally considered to be a difficult task (Merten, 2002; Zhang and Robinson, 2005). It has also been suggested that although it would be favourable, it is probably impossible to develop a single chemically defined medium which would give optimal growth and antibody production for a number of cell lines (Merten, 2002; Li et al., 2010).

There have been various different approaches attempted to reduce bubble-associated damage to cells cultured in bioreactors; i) sparging in cell-free
compartments of bioreactors (Chisti and Moo-Young, 1993b; Chisti and Moo-Young, 1994), ii) the use of bubble free oxygenation (Schneider et al., 1995; Velez-Suberbie et al., 2013), iii) the use of liquid oxygen vectors, e.g. perfluorocarbons (Ju et al., 1991) and iv) use of surface aeration (Kunas and Papoutsakis, 1990a; Kunas and Papoutsakis, 1990b). However, direct sparging of the bioreactor is the most widely used method of aeration and this is not likely to change in the near future (Chalmers, 1998).

The most commonly used method of protecting cells from damage caused by sparging is by the addition of shear protectants to the cell culture media. The protective effect of many different media additives have been tested for cells experiencing damage in aerated and non-aerated conditions, including; serum, proteins (e.g. Bovine Serum Albumin (BSA)), pluronic polyols (e.g. Pluronic F-68), methyl cellulose, polyethylene glycols, dextrans and polyvinyl alcohols (Papoutsakis, 1991). Pluronic F-68 is the most commonly used protectant in large-scale cell culture, and it will be described in detail in the following section.

1.5.1 Pluronic F-68
Poloxamer 188, trade name Pluronic F-68®, is the most widely used protectant in industrial cell culture media and is commonly added in the range of 0.5 to 3g/L (Chisti, 2000). Pluronic F-68 is a member of a large group of pluronic polyols which are block co-polymer non-ionic surfactants consisting of a centre block of polyoxypropylene (hydrophobic) and two end blocks of polyoxyethylene (hydrophilic) as shown in Figure 1.8 (Murhammer and Goochee, 1990b). Pluronic F-68 is made up of approximately 20% polyoxypropylene and 80% polyoxyethylene
with an average molecular weight of 8400 Da and a critical micelle concentration of approximately 9.2 g/L (Murhammer and Goochee, 1990b; Chisti, 2000).

Pluronic polyols (including Pluronic F-68) are synthesised in a two-step process; i) A polyoxypropylene block is synthesised using propylene oxide and propylene glycol and ii) the polyoxyethylene blocks are then added to both ends of the polyoxypropylene using an alkaline catalyst, e.g. potassium hydroxide. A variety of Pluronic polyols are produced by varying the size of the polyoxypropylene and polyoxyethylene blocks. The various Pluronic polyols produced vary in average molecular weight and also in their effects on cells, with some Pluronic polyols causing cell lysis (e.g. Pluronic L-121), inhibition of cell growth (e.g. Pluronic P-65) and protection against bubble damage (e.g. Pluronic F-68, Pluronic L-35 and Pluronic F-38) (Murhammer, 2013). The letters L, P and F in the Pluronic polyol name represent liquid, paste and flake and describe the natural form of the Pluronic polyol (Schmolka, 1977).

![Figure 1.8: General structure of Pluronic polyols.](image)

This figure shows the general structure of Pluronic polyols with a hydrophobic centre block of polyoxypropylene and two hydrophilic end blocks of polyoxyethylene. This figure was adapted from Murhammer and Goochee, 1990b.

The first report of the use of Pluronic F-68 to protect cells from the detrimental effects of sparging was published in the late 1960s by Kilburn and Webb (Kilburn and Webb, 1968) and by the late 1980s Pluronic F-68 was commonly added to media to protect various different cell types experiencing damage from sparged
cell culture conditions (Murhammer and Goochee, 1988; Handa-Corrigan et al., 1989; Murhammer and Goochee, 1990a). The studies carried out by Handa-Corrigan et al. also showed that the protective effect of Pluronic F-68 was dose-dependent, and that 1g/L Pluronic F-68 was sufficient to protect cells under the most damaging conditions tested (Handa et al., 1986; Handa-Corrigan et al., 1989). This concentration of Pluronic F-68 (1g/L) is still commonly used today in to protect cells from sparging damage (Velez-Suberbie et al., 2013). These studies conducted by Handa-Corrigan et al. showed that the survival of cells in the presence of sparging depends on a number of factors; i) cell type, ii) bubble size and frequency (smaller bubbles and higher flow rates were shown to be more damaging) and iii) concentration of Pluronic F-68 (Handa et al., 1986).

1.5.1.1 Pluronic F-68 Protective Mechanisms of Action

The protective effect of Pluronic F-68 was shown to be afforded immediately for cells exposed to sparging damage; therefore it was concluded that the protective effect of Pluronic polyols in sparged conditions was due to a physical or fast-acting biological protection mechanism of action (Michaels et al., 1991). There have also been some biological protection mechanisms proposed for Pluronic F-68.

Pluronic F-68 has been shown to protect insect cells from laminar shear damage (Goldblum et al., 1990); however there are other reports which suggest that Pluronic F-68 does not afford protection to mammalian hybridoma cells experiencing laminar shear (Michaels et al., 1991). This result supports the fact that the protective effect of Pluronic F-68 is cell line dependent (Handa et al., 1986).

It has been known for many years that Pluronic F-68 causes increased foaming in bioreactors (Murhammer and Goochee, 1990a). Therefore, Pluronic F-68
acts as a bubble stabiliser, which has been a proposed physical protective mechanism of action of Pluronic F-68 (Handa-Corrigan et al., 1989), as stabilising foam can protect cells by allowing the cells to drain out of the bubbles before the damaging bubble rupture event occurs (Michael et al., 1995a; Michaels et al., 1995b; Chisti, 2000). Stabilising bubbles can also allow the film formed by the bubble to become thinner, thus resulting in a less damaging bubble-burst event (Handa-Corrigan et al., 1989).

Pluronic F-68 has also been shown to reduce cell-bubble interactions, therefore affording protection as the cells are not in the vicinity of the damaging conditions caused by the bursting bubbles (Handa et al., 1987; Chalmers and Bavarian, 1991; Bavarian et al., 1991). The interruption of cell-bubble interaction by Pluronic F-68 was confirmed by Chalmers and Bavarian by taking pictures of bubbles formed at the air-liquid interface in media with and without Pluronic F-68, as shown in Figure 1.9 (Chalmers and Bavarian, 1991). The bubble in Figure 1.9A was formed in media with 0g/L Pluronic F-68 and the picture shows cells attached to the film layer at the air-liquid interface. The bubble in Figure 1.9B was formed in media with 2g/L Pluronic F-68 and the picture shows that there are no cells attached to the film layer at the air-liquid interface.

Pluronic F-68 has been shown to reduce the cell membrane hydrophobicity of insect and mammalian cells (Wu et al., 1997; Ghebeh et al., 2002), which reduces the affinity of cells to the damaging bubbles and air-liquid interface (Meier et al., 1999). Pluronic F-68 is a non-ionic surfactant; therefore it causes a reduction in the surface tension of cell culture media (Murhammer and Goochee, 1988; Michaels et al., 1995b). Pluronic F-68 has been shown to reduce the surface tension of several media, which is a suggested mechanism of protection as it makes it less
thermodynamically favourable for cells to attach to the bubbles and therefore reduces the damage caused by the bubble burst event (Chattopadhyay et al., 1995a; Chattopadhyay et al., 1995b).

**Figure 1.9: Pluronic interrupts cell-bubble attachment.** Figure 1.9A shows a single bubble at the air-liquid interface in media with 0g/L Pluronic F-68. Figure 1.9 B shows a single bubble at the air-liquid interface in media with 2g/L Pluronic F-68. This figure was adapted from Chalmers and Bavarian, 1991.

Pluronic polyols have been shown to interact with the cell membrane, which may protect cells from damage (Ramírez and Mutharasan, 1990). Murhammer and Goochee showed that pluronic polyols with low hydrophilic-lipophilic balance values cause cell lysis, which supports the observations that Pluronic polyols interact directly with the cell membrane (Murhammer and Goochee, 1990a; Murhammer and Goochee, 1990b). It has also been shown that Pluronic F-68 can increase the membrane bursting tension of cells, suggesting that Pluronic F-68 reinforces the cell membrane (Zhang et al., 1992). Another study suggested that the addition of Pluronic F-68 to cell culture medium reduced the leakage of a positively charged dye, fluorescein diacetate, from the cells due to a biochemical association with the cell membrane (Al-Rubeai et al., 1993).

Plasma membrane fluidity is a measure of the freedom of movement of the plasma membrane and is a factor which has been shown to influence the
susceptibility of cells to damage (Ramírez and Mutharasan, 1990). Decreasing the plasma membrane fluidity has been shown to correlate with increased resistance to damage (Ramírez and Mutharasan, 1990). Pluronic F-68 has been shown to decrease the plasma membrane fluidity of hybridoma membranes, which in turn caused an increased resistance to shear (Ramírez and Mutharasan, 1990; Ramírez and Mutharasan, 1992).

1.5.1.2 Issues Associated with Pluronic F-68
Pluronic F-68 has been crucial for the development of large-scale monoclonal antibody production processes over the last couple of decades, however as industry moves towards higher cell concentrations and higher product titres it is the issues rather than the benefits associated with the use of Pluronic F-68 that are coming to light for biopharmaceutical companies (Ma et al., 2004; Peng et al., 2014; Apostolidis et al., 2015). Therefore, alternative protectants may be required in industrial cell culture media to maintain the current production processes for therapeutic mAbs (Hu et al., 2008; Chalmers, 2015). The main issues associated with the supplementing media with Pluronic F-68 are outlined in the following paragraphs.

There have been reports of batch-to-batch variability associated with Pluronic F-68 due to its stochastic mechanism of production (Passini and Goochee, 1989; Peng et al., 2014; Apostolidis et al., 2015). This batch-to-batch variability has been shown to cause a reduction in cell growth in the large-scale bioreactor and may require investigations into raw materials to determine the source of the problem. A recently published article by Peng et al. (Peng et al., 2014) described a lab-scale investigation into batches of Pluronic F-68 with reduced protective effects using
baffled shake flasks. Pluronic polyols have also been shown to contain low molecular weight impurities, e.g. aldehydes, formic acid and acetic acid (Bentley, Davis et al., 1989; Bentley, Gates et al., 1989). Trace contaminants have been suggested to be present in Pluronic F-68 for many years and have been associated with a reduction in cell growth (Passini and Goochee, 1989).

As mentioned earlier, Pluronic polyols (including Pluronic F-68) act as bubble stabilisers, which can lead to increased foaming in bioreactors (Murhammer and Goochee, 1990a; Murhammer and Goochee, 1990b). Excessive foaming in a bioreactor can be problematic as it can cause contamination of the culture (Varley et al., 2004) or there can also be issues with over-pressure if the foam blocks an air filter (Routledge, 2012). There are a number of different mechanisms employed to reduce foaming levels in bioreactors, including the use of mechanical foam breakers and the addition of chemical antifoaming agents (or “antifoams”) (Varley et al., 2004).

Pluronic F-68 has been shown to lose its protective effect at high cell densities, as seen by the association of cells with bubbles at high cell densities (Ma et al., 2004). This loss of protection may become an issue for large-scale mammalian cell culture, as there have been recent reports of cell densities of up to $10^7$ cells/ml (deZengotita et al., 2000; Wurm, 2004; Nienow, 2006). Therefore, there is research being carried out into alternative protectants which could be added into large-scale bioreactors to protect the increasing concentrations of mammalian cells from damaging conditions (Hu et al., 2008; Chalmers, 2015). A number of cell lines (including CHO cells) have also been shown to endocytose Pluronic F-68 during cell culture, causing the concentration of Pluronic F-68 to reduce over time, which could
further reduce the protective effect of Pluronic F-68 in the cell culture media (Gigout et al., 2008).

1.5.1.3 Alternatives to Pluronic F-68

Pluronic F-68 is the most commonly used protective additive in industrial mammalian cell culture; however other protective agents have been investigated in recent years due to the limitations of Pluronic F-68 outlined earlier. It has been demonstrated that many other Pluronic polyols can act as protectants, e.g. Pluronic F-38, Pluronic F-108 (Murhammer and Goochee, 1990b). It has also been shown that other additives which reduce the surface tension of media can protect cells, e.g. methyl cellulose and polyethylene glycol (Chattopadhyay et al., 1995a; Wu et al., 1995). Maltopyranosides are a group of non-ionic small molecule surfactants which have been suggested to be capable of replacing the protective effect of Pluronic F-68 (Hu et al., 2008).

1.5.2 Serum and BSA

Serum has been historically added to cell culture media in the range to 1-20% vol/vol. Serum provides a number of nutrients and proteins for cell culture (e.g. growth factors, hormones, fatty acids, cholesterol etc.) as well as acting as an antioxidant and cell protectant (Barnes and Sato, 1980; Michaels et al., 1991; Martens et al., 1992; Sandstrom et al., 1994; Liu and Chang, 2006; Francis, 2010). The main components of serum are outlined in Table 1.1 (Brunner et al., 2010). Various types of serum have also been shown to have a dose-dependent protective effect for numerous animal cell types in aerated and bubble-free cell cultures (Croughan et al., 1989; Handa-Corrigan et al., 1989; Ramirez and Mutharasan,
The development of serum-free media started in the 1970s (Keay, 1975; Barnes and Sato, 1980); however the first serum-free media contained animal-derived proteins, e.g. albumin, insulin and transferrin (Barnes and Sato, 1980; Murakami et al., 1982).

Table 1.1: Overview of the main components of serum. This table was adapted from Brunner et al., 2010.

<table>
<thead>
<tr>
<th>Main components of serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>growth factors, hormones and cytokines</td>
</tr>
<tr>
<td>binding and transport proteins (e.g. BSA)</td>
</tr>
<tr>
<td>attachment and spreading factors</td>
</tr>
<tr>
<td>additional amino acids, vitamins and trace elements</td>
</tr>
<tr>
<td>fatty acids, lipids and cholesterol</td>
</tr>
</tbody>
</table>

1.5.2.1 Serum Offers Physical and Biological Protection

The precise protective effect of serum against damage has not been clearly shown (Chisti, 2000). However, initial investigations into the protective effect of serum showed that the protection was physical or fast-acting biological (as termed by Michaels et al., 1991) in nature, due to cells being immediately protected upon the addition of serum to the medium (Michaels et al., 1991; van der Pol et al., 1992;
Martens et al., 1992). A biological protection has also been reported for serum in both aerated and non-aerated systems, this biological protection requires longer exposure to serum in order to take effect (Michaels et al., 1991; Martens et al., 1992). Therefore, serum may have both a physical and biological protective mechanism of action for cells exposed to damage (Michaels et al., 1991; Martens et al., 1992).

1.5.2.2 Potential Protective Mechanisms of Action of Serum

The main physical protective mechanisms associated with serum are involved with medium viscosity, bubble stabilisation and reduction of medium surface tension. Serum has been shown to cause a slight increase in the viscosity of cell culture media (Lavery and Nienow, 1987; Aunis and Henzler, 1993). Some reports have associated the protective mechanism of action of serum with this ability to increase the viscosity of the cell culture medium (Cherry and Papoutsakis, 1988; Croughan et al., 1989; McQueen and Bailey, 1989). However, there are also contrasting reports suggesting that the increased viscosity of the cell culture medium is not associated with a protective effect of serum (Kunas and Papoutsakis, 1990b; Goldblum et al., 1990). It has also been shown that serum stabilises bubbles, which allows cells to drain out of the bubbles before the bubble burst event occurs (Michaels et al., 1995a; Michaels et al., 1995b; Chisti, 2000). Serum proteins (including albumin) have also been shown to cause a reduction in liquid surface tension (Van Oss et al., 1981). As explained earlier, reducing media surface tension has been suggested as a mechanism of interrupting cell-bubble interaction, and therefore offering protection to cells from the bubble burst event (Chattopadhyay et al., 1995a; Chattopadhyay et al., 1995b).
The fast-acting biological protective mechanisms of action suggested for serum are mainly associated with serum altering the cell membrane resulting in a protective effect. It has been suggested that a protective mechanism of action of serum may be due to serum coating cells, therefore protecting the cells from damage (Croughan et al., 1989). Serum has also been shown to reduce the cell membrane hydrophobicity of insect cells (Wu et al., 1997). This reduction in cell membrane hydrophobicity causes a reduction in the association of cells with the air-liquid interface, which results in the cells avoiding damage caused in this region (Meier et al, 1999). In one set of studies serum was shown to decrease the plasma membrane fluidity of hybridoma membranes, which in turn caused an increased resistance to shear (Ramírez and Mutharasan, 1990; Ramírez and Mutharasan, 1992).

1.5.2.3 Serum Albumin

Albumin is the most abundant protein in serum where it is present at approximately 50mg/ml (Kaneko, 1997). BSA is made up of 583 amino acids with a molecular weight of 66430.3Da (Hirayama et al., 1990). The secondary structure of human serum albumin is comprised of 68% α-helices and does not contain β-folded sheets (Curry et al., 1998). Although the crystal structure of BSA has not been identified, detailed spectroscopic chemical cross-linking and other studies have been conducted to determine the 3-D structure of BSA modelled on the crystal structure of human serum albumin (HSA) (Huang et al., 2004). There is 76% sequence homology between BSA and HSA, therefore there may be some differences in specific physico-chemical properties, although the conservation of most of the residues which have been identified as important for binding various ligands (Francis, 2010). Serum albumin is comprised of three homologous domains (I, II and III), each domain
contains two α-helical subdomains (A and B) (Carter et al., 1994). Crystallographic studies have shown that many of the ligands are bound primarily within domains IIA and IIA (He and Carter, 1992; Carter and Ho, 1994). The use of BSA rather than human serum albumin in cell culture has been reported to be due to the greater supply and lower cost of BSA (Keenan et al., 1996).

There have been several functions proposed for BSA in the large-scale cell culture, including; transport of medium components (e.g. fatty acids, cholesterol etc.), detoxification functions, buffering functions and protection against shear stress (Merten, 2002). Although BSA is commonly cited as a shear protectant, there have only been a small number of reports of BSA having a protective effect on cells experiencing damage (Hülscher et al., 1988; Hülscher et al., 1990; Hesse et al., 2003). Hesse et al. showed that media supplemented with 1g/L BSA had a protective effect on cells in both bubble-free and aerated bioreactors compared to protein-free media; however there was also 10mg/L of transferrin and 10mg/L of insulin added to the media where BSA had a protective effect (Hesse et al., 2003). Therefore it cannot be concluded that the protective effect was completely due to the presence of BSA in the media.

Albumin is also known to associate with cholesterol in a low affinity and high capacity manner (Zhao and Marcel, 1996). Albumin has been shown to aid the transport of cholesterol to cells via lipoproteins similar to cyclodextrin (Zhao and Marcel, 1996; Sankaranarayanan et al., 2013) and this cholesterol provision has been a suggested function of BSA in the culture of NS0 cells (Francis, 2010).
1.5.3 Problems Associated with Serum and Serum Derived Proteins

There are several issues associated with the addition of serum or serum derived proteins to industrial cell culture processes, e.g. issues with purifying the product during downstream processing, variation of lots due to biological source, high cost of purified BSA and increased risk of contamination of the cell culture with bacteria, viruses, prions etc. (Merten, 2002).

The replacement of BSA with recombinant albumin is technically possible, however there have been reports that recombinantly expressed albumin does not have the same effect in cell culture as albumin sourced from serum (Keenan et al., 1997). Also, the manufacture of the recombinant BSA would be required to be carried out using a microbial fermentation free of animal-sourced raw materials to avoid possible contamination of the recombinant product with animal sourced viruses, bacteria, prions etc. (Merten, 2002).

The European Medicines Agency (EMA) and the Committee for Medicinal Products for Human Use (CHMP) have strict guidelines which must be adhered to relating to the use of bovine serum (and BSA) in the manufacture of human biological medicinal products (CHMP, 2013). These guidelines dictate the nature and quality of the serum (and BSA) which can be used for the production of human biological medicinal products (e.g. mAbs). The CHMP state that efforts should be made to replace bovine serum (and BSA) with a non-animal sourced material. However, the CHMP accept that some processes require the addition of serum (or BSA), and in this instance the CHMP have a list of tests which must be carried out to confirm that the serum (or BSA) is free from certain bacterial, mycoplasma, fungal and viral contaminants. The CHMP also state that serum (or BSA) must be irradiated before use in the manufacture of biological medicinal products for human use. All
these factors incur a great deal of cost for the manufacturer, however the guidelines must be strictly followed as patient safety is paramount for the pharmaceutical industry.

1.6 Study Aims and Objectives

As outlined previously, the addition of animal sourced raw materials to industrial cell culture processes is not favourable as it increases the risk of contamination of the cell culture and therefore has a potential impact on patient safety. There are numerous therapeutic mAbs approved for the market which are produced in media without BSA supplementation, however there is still a lack of understanding of the requirement for the supplementation of BSA in the large-scale cell culture media for NS0 cells. As described in the previous sections, a number of functions for serum and BSA have been proposed for cells cultured in large-scale mammalian cells culture, which mainly involve nutrient provision or protection against damage in the bioreactor.

The research carried out during this study was conducted as Eli Lilly want to further understand the function of BSA in industrial cell culture, as understanding its function is imperative if BSA is to be removed from the NS0 cell culture or replaced by a non-animal derived raw material. The specific objectives of this study are outlined as follows:

- To determine whether BSA is an essential component for the growth of NS0 cells under various culture conditions in industrial cell culture media. The NS0 cells used in this study were developed in media supplemented with BSA; therefore investigations will be carried out to assess whether BSA is
explicitly required for the successful cell culture of the cells. This work is described in Chapter 3.

- To develop lab-scale models to test whether BSA offers protection to NS0 cells experiencing damaging cell culture conditions. Small-scale models will be developed to expose NS0 cells to various magnitudes of damage to assess whether BSA offers protection to NS0 cells experiencing the damage. This work is described in Chapter 3.

- To assess whether BSA functions as a critical nutrient carrier for NS0 cells in culture. Investigations were carried out into whether BSA acts as a nutrient carrier for NS0 cells, as serum has been historically added to media as a source of nutrients. This work is described in Chapter 4.

- To identify the protective mechanisms of action of BSA on NS0 cells experiencing damage. Experiments will be conducted to assess whether the protective effect of BSA is afforded rapidly, or whether the cells require extended exposure to BSA for the protective effect to become evident. Further investigations will be carried out to determine the protective mechanism of action of BSA depending on whether its protective effect is afforded rapidly or after extended exposure. This work is described in Chapter 4.

- To test whether BSA could potentially be replaced by a non-animal derived compound. Following on from the results gathered on the protective effect and mechanisms of action of BSA, experiments will be conducted to assess whether BSA could potentially be replaced by a synthetic compound in industrial cell culture media. This work is described in Chapter 4.
Finally, the results obtained during this study will be discussed in detail in Chapter 5 and some overall conclusions will be drawn from the results. Options for future work will also be considered in this chapter.
Chapter 2:

Materials and Methods
2.1 Materials

2.1.1 Cell Lines

Parental NS0 cells (mouse myeloma cell line) were received from Eli Lilly, Branchburg, USA and were originally cultured in media supplemented with 10% Foetal Bovine Serum (FBS). This cell line is Glutamine Synthetase deficient, therefore requires the provision of Glutamine in media.

NS0 cells transfected with a monoclonal antibody gene and glutamine synthetase gene were also received from Eli Lilly, Branchburg, USA. This cell line was derived from the Parental NS0 cell line used in this project and these cells were developed in media supplemented with 1g/L BSA.

Research cell banks were laid down for the two cell lines in media supplemented with 1g/L BSA and 1g/L Pluronic F-68. These cell lines were stored in liquid nitrogen for the duration of the project.

2.1.2 Cell Culture Reagents

NS0 cells were cultured using an industrial media containing the following reagents: Proprietary Basal media powder (comprising of amino acids, glucose, minerals etc.) (Gibco), Bovine Serum Albumin (Gibco), Hy-Soy (Kerry), 500X Iron Chelate (Gibco), 1000X Cholesterol Lipid Complex (Gibco), Methyl cellulose (Sigma) and Pluronic F-68 (Sigma). All media was filter-sterilised by passing through a 0.1µm PES filter. Parental NS0 cells were supplemented with 10ml/L of 100X Glutamax (Gibco), due to the lack of a glutamine synthetase gene. The term “cell culture” in this thesis refers to the NS0 cells in suspension in media.
2.1.3 Media Formulations

A number of stock media were regularly made up in order to vary the concentrations of BSA, Pluronic F-68 and 1000X Cholesterol Lipid Complex (CLC) for studies. CLC is comprised of cholesterol bound to cyclodextrin which is commonly used to provide cholesterol to cholesterol auxotrophic NS0 cells. CLC was added to all media at a concentration of 2ml/L unless otherwise stated in the individual experiment.

The following stock media were formulated in order to aid the formulation of media with different concentrations of BSA and Pluronic F-68:

i. 4g/L BSA (0g/L Pluronic F-68 and 0ml/L CLC)
ii. 2g/L Pluronic F-68 (0g/L BSA and 0ml/L CLC)
iii. 0g/L BSA, 0g/L Pluronic F-68 and 0ml/L CLC

After the different media formulations were made up, CLC and Glutamax (100X) were added to the media in the required concentrations. The pH of the media was adjusted to 7.0 +/- 0.1 using 6N hydrochloric acid (J.T. Baker). The osmolality of the media was tested and maintained in the range of 350 to 400mM/kg using an Osmometer (Advanced Instruments, Inc. Osmometer Model 3250).

2.2 Methods

2.2.1 Cell Culture Conditions

The standard cell culture conditions used to expand cells were as follows: 37°C, 10% CO₂, passive humidity and shaking at 110 RPM in media with 1g/L Pluronic F-68. Cell culture was carried out in a Multitron Infors HT incubator. Media was warmed for at least one hour at 37°C in a water bath before being used.
Two vial thaws were carried out and the vials were mixed together and split into media with 0 and 1g/L BSA and 1g/L Pluronic F-68. The NS0 cells were passaged every two to three days. Cells between passage 5 and 20 were used for the experiments in this study.

The NS0 cells were cultured in T-flasks (up to a working volume of 60ml in a T175 flask) for the first 4 passages and then transferred to shaking E-flasks (up to a working volume of 400ml in a 1L flask). All cell counts were carried out using an automated Trypan Blue exclusion counter (Vi-cell XR, Beckman Coulter). Cell metabolites were measured when required using a Nova BioProfile 400 and ABL90 analyser (Radiometer).

2.2.2 AMBR™15 Mini-Bioreactor System

Monoclonal Antibody producing NS0 cells were cultured for 7 days in an AMBR15 mini-bioreactor system which is designed to have similar sparging and mechanical agitation to a 5L lab-scale bioreactor. The AMBR15 has been designed to mimic the 5L Bioreactors commonly used in pilot scale labs in industry in a 15ml mini-bioreactor.

The impeller tip speed of the AMBR15 mini-bioreactor was set to mimic the impeller tip speed of the 5L bioreactor process used to culture NS0 cells in Eli Lilly. The 5L bioreactor spins at 220 RPM which was calculated to be a tip speed of approximately 48m/min using the equation below, which calculates the tip speed from the radius of the impeller and the impeller rotational speed. The impeller used in the 5L bioreactor has a radius of 0.0345m, whereas the AMBR15 impeller has a radius of 0.0057m. Therefore, the AMBR15 bioreactor was set to 1350 RPM to
achieve a tip speed of approximately 48m/min (calculated using the equation below), as shown in Table 2.1.

\[
\text{Tip Speed (m/min)} = 2\pi(\text{impeller radius}) \times \text{Impeller RPM}
\]

In this experiment the NS0 cells were cultured in media with 0 and 1g/L BSA and various concentrations of Pluronic F-68 (0, 0.025, 0.1 and 1g/L). The cultures were sampled daily to measure the Viable Cell Density (VCD) and an LDH Activity sample was taken on day 2 to measure the cell membrane damage experienced by the cells in the mini-bioreactors. The dissolved oxygen (DO) levels were set to 50% and the pH was set to 7.2. The Nitrogen flow rate was set to 0.07cm\(^3\)/min and the oxygen and carbon dioxide flow rates were set to 0.01cm\(^3\)/min (within recommendations of manufacturer).

Table 2.1: RPMs of 5L bioreactor and AMBR15 bioreactors to achieve a tip speed of 48m/min.

<table>
<thead>
<tr>
<th>AMBR15</th>
<th>5L Bioreactor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Impeller radius (m)</td>
<td>0.0057</td>
</tr>
<tr>
<td>Impeller RPM</td>
<td>1350</td>
</tr>
<tr>
<td>Tip Speed (m/min)</td>
<td>48.35</td>
</tr>
</tbody>
</table>

2.2.3 NS0 Cell Culture in Baffled Shake Flasks

Baffled shake flasks (125ml and 1L Corning) were used to expose NS0 cells to various hydromechanical stress conditions. The baffled shake flasks were cultured on an Innova 2100 orbital shaker (New Brunswick Scientific), with an orbital
diameter of 19mm. The pictures in Figure 2.1 show the differences between the baffled and non-baffled shake flasks used in this project. The baffled shake flasks contain 4 baffles at the bottom of the flasks which increase the power input into the cell culture compared to non-baffled shake flasks (Peter, Suzuki, Rachinskiy et al., 2006; Peter, Suzuki and Büchs, 2006). The flasks have the same geometries except for the 4 baffles contained in the baffled shake flasks.

Figure 2.1: Baffled and non-baffled shake flasks. These figures show Corning 125ml baffled and non-baffled shake flasks. The baffled shake flasks have 4 baffles on the bottom of the shake flask. One baffle is indicated with a red arrow. Figure 2.1A shows the baffled and non-baffled shake flasks standing up-right. Figure 2.1B shows the bottom of the baffled and non-baffled shake flasks. Figure 2.1C shows the inside of the non-baffled shake flask. Figure 2.1D shows the inside of the baffled shake flasks.
In this thesis the term “hydromechanical stress” refers to cell culture conditions which cause damage to cells, e.g. increased power input due to greater agitation or damage at the air-liquid interface due to bubble burst or rapidly moving air-liquid interface. Two hydromechanical stress models were developed in baffled shake flasks to test the protective effect of BSA. Although these models were not developed to replicate the conditions in a bioreactor, the objective of the models was to determine the effects of BSA on NS0 cells experiencing different severities of hydromechanical stress. The two hydromechanical stress models are as follows:

- Model 1: Moderate hydromechanical stress

  The objective of this model was to expose the NS0 cells to hydromechanical stress where the cells were capable of proliferation, but the growth of the cells was reduced due to the hydromechanical stress. The term “moderate” was used to describe this model as the NS0 cells were capable of proliferation when exposed to the hydromechanical stress. This model could be used to test whether BSA promotes NS0 cell growth under moderate hydromechanical stress conditions. This model is relevant to industry as hydromechanical conditions which result in a reduction in cell growth would have a negative impact on product titre, and therefore the cells should be protected from these conditions if possible.

  The conditions for the moderate hydromechanical stress model were as follows; 125ml baffled shake flasks with a working volume of 24ml, 135 RPM orbital shaker speed, 0.3x10^6cells/ml seeding density and approx. 4 days exposure to the hydromechanical stress. These conditions did not cause the formation bubbles at the air-liquid interface. The readouts for this model
were cell growth (maximum VCD reached and doubling time) and a cell membrane damage assay (LDH Activity).

- Model 2: Extreme hydromechanical stress

The objective of this model was to expose the NS0 cells to extreme hydromechanical stress where the cells were not capable of proliferation and the VCD of the cells was reduced after short exposure to the hydromechanical stress. The term “extreme” was used to describe this model as the NS0 cells were not capable of proliferation under these conditions and the VCD of the cells was reduced after short exposure to the stress due to cell lysis. This model could be used to test whether BSA protects NS0 cells from damaging hydromechanical stress conditions where the NS0 cells are susceptible to lysis. This model is industrially relevant as protecting cells from hydromechanical stress conditions which cause cell lysis is important to maintain product titres.

The conditions for the extreme hydromechanical stress model were; 125ml baffled shake flasks with a working volume of 24ml, 175 RPM orbital shaker speed, 1x10^6cells/ml seeding density and 60 minutes exposure to the hydromechanical stress. The readouts for this model were the percentage reduction in VCD (calculated using the equation below) and a cell membrane damage assay (LDH Activity).

\[
\% \text{ Reduction in VCD} = \left(1 - \frac{\text{VCD pre-exposure}}{\text{VCD post-exposure}}\right) \times 100
\]
2.2.4 Lactate Dehydrogenase Activity Measurement

LDH Activity is an assay which can be used to measure the amount of damage experienced by cells, as LDH is an enzyme released when the cell membrane is damaged and the enzyme remains active after it has been released. In this study LDH Activity was measured using an LDH Activity Kit (Sigma). The LDH Assay is a kinetic colourimetric assay which detects the amount of NADH produced (by the LDH enzyme) in each sample over time.

Each sample was counted on a Vi-cell cell counter before the LDH Activity measurement was carried out. Cells were centrifuged from samples at 280G for 5 minutes. The supernatant was diluted 1:25 using the buffer supplied with the kit. A standard curve of different concentrations of NADH (0, 2.5, 5, 7.5, 10 and 12.5nM) was used to calculate the LDH Activity.

The samples were measured for absorbance at 450nm immediately after the substrate mix was added. Readings were taken every three minutes until the absorbance of the sample with the highest LDH Activity was higher than the 12mM NADH standard absorbance. The LDH Activity was then calculated using the following calculation:

\[
\text{LDH Activity} = \frac{\text{NADH conc} \times \text{Dilution factor}}{\text{Incubation time} \times \text{sample volume}}
\]

The LDH Activity was then normalised to the VCD of the samples, therefore the final LDH Activity unit is nmol of NADH produced per minute per 10^6 cells (nmol/min/10^6cells) and is referred to as LU (LDH Activity unit). The NS0 cells had a baseline LDH Activity which was typically ≤ 50 LU (there were slight
variations due to the fact that the samples were compared to a unique NADH standard curve for each assay conducted).

2.2.5 Nuclear Magnetic Resonance Analysis

A Bruker Nuclear Magnetic Resonance (NMR) instrument was used to quantify the levels of amino acids in the media of NS0 cells over time. This method of measuring amino acid levels in cell culture media was developed by Bradley et al. in Eli Lilly, Indianapolis (Bradley et al., 2010). NS0 cells were cultured for 14 days in media with 0 or 1g/L BSA (and 1g/L Pluronic F-68) and samples were taken for NMR analysis on day 1, 6, 9 and 14 (samples were frozen at -80°C until they were analysed). A broadband inverse probe was used to quantify the amino acids in the samples. The samples were prepared by adding 600µl of the sample to 200µl of NMR buffer before analysis. This work was kindly carried out in the NMR Fermentanomics lab in Eli Lilly, Kinsale by Kevin F. O’Sullivan.

2.2.6 NS0 Cell Lysate Formation

Lysates of NS0 cells were formed using 1% NP-40 lysis buffer. Cell pellets were formed by centrifuging at 280G for 5 minutes. The pellets were washed twice using media with 0g/L BSA and 0g/L Pluronic F-68 before they were lysed. A pellet of 1x10^6 cells was lysed in 100µl of lysis buffer with added protease and phosphatase inhibitors. Cell lysates were formed by incubating for 20 minutes on ice. Lysates were then centrifuged at 13300 RPM for 15 minutes at 4°C. The protein content of the lysates was quantified using a Bradford assay (Bio Rad) and using BSA solutions of known concentration as standards. The lysates were then diluted to the same protein content using purified water.
2.2.7 SDS-PAGE and Western Blotting

Protein samples for western blot analysis were separated using 4%/12% stacking Sodium Dodecyl Sulphate Poly-Acrylamide Gel Electrophoresis (SDS-PAGE) gels. (A 4%/8% stacking Native PAGE gel was also used to separate native and denatured BSA using the same technique; however no SDS was added to any of the reagents or gels.) Following separation of the gel, proteins were transferred onto a nitrocellulose membrane using electrophoresis and blocked for 1 hour at room temperature shaking in 5% milk (w/v) in TBS containing 0.5% Tween-20 (TBS-T). Membranes were incubated overnight at 4°C with primary antibody. Primary antibody dilutions were as follows:

Rabbit anti-BSA; 1:1000 in TBS-T/5% milk

Mouse anti-Actin; 1:1000 in TBS-T/5% milk

Appropriate secondary antibodies (IRDye® 680LT and 800CW – Infrared Dye coupled anti-rabbit or anti-mouse (LI-COR Biosciences)) were diluted 1:10000 in TBS-T/5% milk for 1 hour. Antibody reactive bands were detected with the Odyssey® infrared imaging system (LI-COR Biosciences).

2.2.8 BSA Immunoprecipitation (IP)

Three methods of immunoprecipitating BSA were carried out by adding 40µl of i) Protein G (ProG) beads, ii) ProG beads with 5µl of rabbit anti-BSA antibody added and iii) agarose beads pre-conjugated with rabbit anti-BSA antibodies (Sigma Aldrich) to 700µl of media. The mixture was incubated overnight at 4°C on a rotating wheel at 5 RPM. After incubation the beads were centrifuged at 3000 RPM for 3 minutes in a benchtop centrifuge set to 4°C. The beads were washed three times with ice cold lysis buffer.
2.2.9 **Amplex Red Cholesterol Quantification Assay**

An Amplex Red Cholesterol Quantification assay (Thermo Fisher) is a fluorescence based assay which was used to quantify the cholesterol bound to BSA in the NS0 cell culture media. The relevant reagents were made up as per the Amplex Red Assay protocols. A cholesterol standard curve was made up by serial dilution and was used to quantify the cholesterol in the samples. The samples were diluted using the reaction buffer provided with the kit and incubated for 60 minutes after adding the substrate mix. The readings were taken using a fluorescence plate reader using excitation in the range of 530–560nm and emission detection at ~590nm. The cholesterol was then quantified for each sample by comparing to the standard curve and correcting for the dilution factor.

The Amplex Red Cholesterol Quantification assay was used to quantify the cholesterol bound to BSA in media supplemented with Cholesterol Lipid Complex. BSA was immunoprecipitated using Agarose beads conjugated with anti-BSA antibody, as described in Section 2.2.8. After the IP was incubated overnight, the beads were washed and the cholesterol content was quantified using the beads.

2.2.10 **Laminar Shear Device**

A novel laminar shear device was designed and fabricated by Michael Collins in Stokes Institute, University of Limerick to test the protective effect of BSA on NS0 cells experiencing laminar shear stress. The device was fabricated using stainless steel 316L and utilises a combination of concentric cylinder and cone and plate geometries (at the top and the bottom of the device) to produce Couette flow. The device is comprised of one rotating inner cylinder with radius $R_1 = 0.08$ m and height $h = 0.24$m housed within a stationary outer cylinder with radius $R_2 = 0.08035$m,
resulting in a 0.35mm gap existing between the concentric cylinders. A schematic of the device is shown in Figure 2.2A. The laminar shear device can expose suspension cells to laminar shear within the range of 0 to 6.3Pa. The laminar shear magnitude was varied by increasing or decreasing the RPM of the internal cylinder using a motor, as shown in Figure 2.2B.

The device holds 50ml of cells and experiments were designed and carried out to test the protective effect of BSA under various magnitudes of laminar shear stress and exposure time. The Total Cell Density (TCD) refers to the total viable and non-viable cells in a sample. NS0 cells with a viability of >90% were used in these experiments. The percentage reduction in TCD was used to determine the damage experienced by the NS0 cells in the laminar shear device using the following formula:

$$\text{Reduction in TCD} = \left(1 - \frac{\text{TCD pre-exposure}}{\text{TCD post-exposure}}\right) \times 100$$

**Figure 2.2:** Concentric cylinder laminar shear device. These pictures show the device designed and fabricated in The University of Limerick which was used to expose NS0 cells to well-defined laminar shear. Figure 2.2A is a schematic of the laminar shear device showing shows the concentric cylinders with a 0.35mm gap where the cells are exposed to the laminar shear and showing the inlet for the cells. Figure 2.2B shows the device with a motor attached which controls the shear magnitude to which the cells are exposed.
2.2.11 Media Surface Tension Measurement

The effect of BSA and Pluronic F-68 on media surface tension was measured using a KSV CAM 200 Contact Angle and Surface Tensiometer (KSV Instruments, Finland). Different concentrations of BSA and Pluronic F-68 were added to media and tested on the CAM 200 device.

Droplets of the liquid being tested were formed through a syringe tip (1.27mm diameter) and several pictures of the droplet were taken using the KSV CAM 200 optical tensiometer as the droplets were forming and eventually falling from the syringe. The surface tension was calculated using the last picture of the droplet before it fell from the syringe. The surface tension was calculated using the software linked with the device using the Young-Laplace equation. At least three independent droplets were analysed for each sample. The surface tension was measured in mN/m.

2.2.12 Media Viscosity Measurement

The dynamic viscosity of the NS0 cell culture media was tested with varying concentrations of BSA and Pluronic F-68 using a Brookfield viscometer (Brookfield Viscometers Ltd, England). The viscometer was calibrated with a liquid of known viscosity before use. The viscosity of each media was tested at room temperature and the results are given in mPa.s.

2.2.13 Determination of Bubble Stabilisation

The formation of bubbles and the effects of BSA and Pluronic F-68 on the stabilisation of the bubbles were tested in 125ml baffled and non-baffled shake flasks containing 24ml of media. Two concentrations of Pluronic F-68 (0.025 and 1g/L)
were tested in media with 0 or 1g/L BSA. The flasks were shaken at 110, 135, 150 and 175 RPM for 1 hour and pictures were taken immediately after shaking (pictures were taken stationary).

2.2.14 Membrane Hydrophobicity Measurement

The NS0 cell membrane hydrophobicity was measured by determining the association of the NS0 cells with a hydrophobic liquid, hexadecane (Sigma). Parental NS0 cells were cultured in media with 0g/L BSA and 1g/L Pluronic F-68 and the cells were harvested by centrifugation (280G) and washed twice in media with 0g/L BSA and 0g/L Pluronic F-68. The cells were then resuspended in the test media at an initial Total Cell Density (TCD) of 1.5x10^6cells/ml. The cells were counted by trypan blue exclusion using an automated cell counter (Vi-cell XR, Beckman Coulter). The viability of the cell suspension was greater than 90% in all cases. 1ml of hexadecane was added to 1ml of the cells in a 5ml polyethylene tube (Sarstedt). Immediately after the addition of the hexadecane, the tube was vortexed at 50% of the maximum speed (using a Vortex Genie 2) for 15 seconds. The suspension was left for 40 minutes to allow for the two phases to separate. A 500µl sample of the cell suspension in the lower aqueous layer was removed using a sterile 1ml tip for counting. Care was taken into minimize the media and hexadecane mixing during this stage. The total cell concentration in this sample was determined using a Vi-cell XR. The relative hydrophobicity was determined by calculating the increase or decrease in total cell number of each sample and comparing it to two control samples: i) 0g/L BSA and 0g/L Pluronic F-68 (assumed to be 100% hydrophobic) and ii) 1g/L BSA and 1g/L Pluronic F-68 (assumed to be 0% hydrophobic)
The hydrophobicity was calculated using the following steps by deducting the final total cell count from the initial total cell count for the controls and samples. The relative hydrophobicity was then calculated using the following calculation:

A. The increase or decrease in total cell number for each sample being tested  
B. The decrease in total cell number for Control (i)  
C. The increase in total cell number for Control (ii)

\[
\text{Relative hydrophobicity} = \frac{(1-(A+B))(C-B)*100}{(C-B)}
\]

2.2.15 Heat Denaturation of BSA

A concentrated solution of BSA (40g/L) was made up and 5mls of the solution was denatured in a 15ml centrifuge tube (VWR). The BSA was denatured at 100°C for 30 minutes in a water bath. The denatured BSA solution was then added into media to a concentration of 1g/L BSA. As a control 5mls of the BSA solution was also heated to 37°C for 30 minutes.

2.2.16 Circular Dichroism

Circular Dichroism spectra were collected in both the near and far UV regions of native and denatured BSA using a Photophysics Chirascan circular dichroism spectrometer with dual polarising, dual dispersing monochromator and 150W air-cooled Xe arc lamp. The Pro-Data Chirascan, version 4.1.12 software was used. The instrument was calibrated using a 1mg/ml solution of camphor sulfonic acid (CSA) at A_{290.5}.

Protein samples at 2mg/ml BSA were analysed in a 0.5cm Hellma cuvette (p/n 110-QS) from 360 to 260nm for the near UV region. For the far UV region,
protein samples at 2mg/mL BSA were analysed using a circular 0.01cm path length Hellma cuvette (p/n 121.000-QS). For both regions a bandwidth of 0.5nm, 0.5nm step and time-per-point of 4 seconds were used. Three replicates in each region were collected.

The circular dichroism spectra were processed using Pro-Data viewer software, version 4.1.9. The three replicate spectra were averaged and the baseline was subtracted. The circular dichroism (mdeg) was converted to mean residue molar ellipticity (MRE) (deg cm$^2$ dmol$^{-1}$), using the BSA concentration (2mg/ml), respective near and far UV path lengths, a molecular weight of 66,000 Da and the number of amino acid residues in BSA (583) using the following equation (Miles and Wallace, 2006), where $C_p$ is the molar concentration of the protein, $n$ is the number of amino acid residues and $l$ is the path length in cm:

$$MRE = \frac{\text{Observed CD(mdeg)}}{C_pnl \times 10}$$

The $\alpha$-helix content of BSA can be estimated from the $MRE$ values at 208nm using the following equation, where $MRE_{\infty}$ is the observed $MRE$ value at 208nm, 4,000 is the gross $MRE$ of the $\beta$-sheet and random coil conformation at 208nm and 33,000 is the gross $MRE$ of a pure $\alpha$-helix at 208nm:

$$\alpha \text{ helical (\%)} = \frac{MRE_{208} - 4000 \times 100}{33000 - 4000}$$

This test was kindly carried out in the Quality Control lab in Eli Lilly, Kinsale by Edel Duignan and T.J. Nolan.
Chapter 3:

Development of lab-scale models to test the protective effect(s) of BSA on NS0 cells
3.1 Abstract

NS0 cells (mouse myeloma cell line) are routinely cultured in large-scale bioreactors for the production of human therapeutic mAbs. Mammalian cells have been shown to experience damage in large-scale bioreactors, mainly due to the agitation and sparging. Non-ionic surfactants, most commonly Pluronic F-68, are added to the media used for industrial mammalian cell culture to protect the cells from damage in the bioreactor. However, there are issues with some batches of Pluronic F-68 not affording protection to cells in large-scale bioreactors and there have also been reports that the protective effect of Pluronic F-68 can be exhausted in bioreactors with high cell densities. BSA is also supplemented in media for the large-scale production of some mAbs; however the function of BSA in the media is not fully understood. BSA has been suggested to protect mammalian cells from damage experienced in the bioreactor. Therefore, the objective of these experiments was to design lab-scale models to investigate whether BSA has a protective effect on NS0 cells experiencing damage in culture. The effect of BSA on NS0 cells was tested in shake flasks, an AMBR15 mini-bioreactor and in baffled shake flasks. BSA had no effect on NS0 cells in media supplemented with 1g/L Pluronic F-68 in these models, therefore the concentration of Pluronic F-68 was reduced to test whether BSA has an effect in media with limited protection from Pluronic F-68. There was an indication that the NS0 cells were susceptible to damage in AMBR15 mini-bioreactors in media with reduced concentrations of Pluronic F-68, however the effect was not absolutely clear. Two hydromechanical stress models were developed in baffled shake flasks to test the protective effect of BSA on NS0 cells. Model 1 was a moderate hydromechanical stress model, where the hydromechanical stress caused by the baffled shake flasks reduced the growth of the NS0 cells; however the cells
were still capable of proliferation. Model 2 was an extreme hydromechanical stress model where the NS0 cells were damaged by short exposure to the hydromechanical stress in the baffled shake flasks. These models were developed to test whether BSA protects NS0 cells from different hydromechanical stress conditions which could result in a lower product titre. BSA was shown to protect the NS0 cells from the moderate and extreme hydromechanical stress in baffled shake flasks in media with low concentrations of Pluronic F-68. This protective effect of BSA was shown for both Parental and mAb-producing NS0 cells. Therefore, it was concluded that BSA protects NS0 cells from damage in culture, and this protection may only be evident when the protective effect of Pluronic F-68 is exhausted.
3.2 Introduction

NS0 cells are a murine myeloma cell line commonly used for the production of therapeutic monoclonal antibodies (Galfrè and Milstein, 1981; Barnes et al., 2000). Although the NS0 cells in this study were developed in media supplemented with BSA, there have been several reports of NS0 cells cultured in serum-free and protein-free media (Keen and Hale, 1995; Zhang and Robinson, 2005; Burky et al., 2006), therefore it is possible to grow NS0 cells in media without BSA supplementation. The focus of this chapter is on developing models to test whether BSA has a protective effect on NS0 cells during culture.

There are a number of different lab-scale models used in the development and optimisation of industrial cell culture processes. Microscale bioreactors are a new technology used in industry for a number of developmental experiments, e.g. media optimisation and clone selection (Nienow et al., 2013). These microscale bioreactors have mechanical agitation, sparging and pH and dissolved oxygen probes similar to conventional lab-scale bioreactors (Bareither and Pollard, 2011). The AMBR®15 (Sartorius) microscale bioreactors are one of the most commonly used microscale bioreactors used in industry and there have been reports showing that the AMBR15 is capable of culturing CHO cells comparably with 5L bioreactor models (Nienow et al., 2013). These development and optimisation experiments can also be conducted in shake flasks (non-baffled and baffled) (Büchs, 2001; Peter, Suzuki, Rachinskiy et al., 2006; Peter, Suzuki and Büchs, 2006) and lab-scale bioreactors from 1 to 30L (Nienow, 2010). Baffled shake flasks have been shown to cause a greater power input (kW/m³) into the liquid in the flask compared than non-baffled shake flasks and therefore are potentially more damaging to cells (Peter, Suzuki, Rachinskiy et al., 2006; Peter, Suzuki and Büchs, 2006). The power input into the
liquid is a mechanism used to compare the conditions of shake flasks to those in bioreactors (Büchs et al., 2001).

Cell-protective additives are commonly added to industrial cell culture media to reduce the damage experienced by cells in bioreactors (Papoutsakis, 1991). Historically the most commonly used protectants in industrial cell culture are Pluronic F-68 and serum (Chisti, 2000; Hu et al., 2008). The protective effect of Pluronic F-68 has been shown to be concentration dependent; however the protective effect levels off at approximately 0.5g/L (Chisti, 2000). Pluronic F-68 has been shown to protect cells by suppressing the attachment of cells to bubbles and to the air-liquid interface (Chalmers and Bavarian, 1991; Bavarian et al., 1991; Chattopadhyay et al., 1995a). Although Pluronic F-68 is widely used in industry there have been reports of issues with batch-to-batch variability (Peng et al., 2014; Apostolidis et al., 2015) and loss of protective effect at high cell densities (Ma et al., 2004). Therefore, research has been conducted into potential alternative protectants to Pluronic F-68 (Hu et al., 2008). Serum has been historically added to media as a source of nutrients, hormones, growth factors and trace elements (Francis, 2010), however serum has also been reported to have a dose dependent protective effect on numerous mammalian suspension cell lines in both aerated and bubble-free cultures (Handa-Corrigan et al., 1989; Abu-Reesh and Kargi, 1991; Papoutsakis, 1991; Martens et al., 1992; Van der Pol et al., 1992). BSA is the most abundant protein in serum (Francis, 2010) and has also been shown to protect hybridoma cells in an airlift vessel allowing improved cell growth (Hülscher et al., 1990). Although the exact function of BSA in large-scale cell culture has not been identified, there have been a number of proposed functions, including transport of nutrients to the cells (fatty
acids, cholesterol, hormones, metal ions etc.), detoxification and buffering functions in the media, and protection against hydromechanical stress (Merten, 2002).

The addition of BSA to industrial cell culture media increases the risk of contamination of the cell culture with adventitious agents such as viruses and prions (e.g. bovine spongiform encephalopathy), which could potentially have an impact on patient safety (Merten, 2002). Biopharmaceutical companies must adhere to additional controls to assure patient safety when animal sourced materials (including serum and serum derivatives, e.g. BSA) are added to media used for the production of therapeutic biopharmaceuticals (Committee for Medicinal Products for Human Use (CHMP), 2013). Therefore, it is desirable for companies to remove BSA from cell culture media used for the production of human therapeutics, however this is a difficult task as the exact function of BSA in large-scale cell culture is not fully understood.

In this chapter investigations were carried out into the effect of BSA on NS0 cells in shake flasks, an AMBR15 mini-bioreactor and baffled shake flasks in a typical industrial cell culture media supplemented with various concentrations of Pluronic F-68. Two lab-scale hydromechanical stress models (moderate and extreme) were designed in baffled shake flasks. The objective of these hydromechanical stress models was to expose the NS0 cells to damaging conditions which would reduce the product titre of the cells. The hydromechanical stress models were then used to test the protective effects of BSA on NS0 cells in media with limited protection from Pluronic F-68.
3.3 Investigations into the effects of BSA on NS0 cells under various cell culture conditions in industrial cell culture media

3.3.1 BSA does not impact the growth of NS0 cells in non-baffled shake flasks under standard cell culture conditions

The NS0 cell banks used in this project (both Parental and mAb-producing cells) were originally cultured in an industrial media supplemented with 1g/L BSA and 1g/L Pluronic F-68; therefore the first objective was to determine whether the NS0 cells could grow in media without BSA supplementation. In this experiment mAb-producing NS0 cells were grown in non-baffled shake flasks under standard culturing conditions (110 RPM) in media with 0 and 1g/L BSA in an industrial media supplemented with 1g/L Pluronic F-68. This experiment was carried out over 14 days according to a fed-batch process (adding glucose and other nutrients according to a specific protocol).

The NS0 cells were not affected by the concentration of BSA in the media, as shown by the almost identical growth profiles (Figure 3.1A) and percentage viabilities (Figure 3.1B) for the cells in media with 0 and 1g/L BSA. Elevated levels of ammonium (NH$_4^+$) in cell culture media (due to protein metabolism) can have a negative effect on cell growth and metabolism (Lao and Toth, 1997). The results in Figure 3.1C show that there is no NH$_4^+$ produced by the NS0 cells for the first 4 days which is typical for these NS0 cells as the cells have low levels of protein metabolism at the early stages of the cell culture. The results also show that the presence of BSA in the media had no effect on the NH$_4^+$ levels for the NS0 cells cultured in these conditions.

It was concluded from these results that the NS0 cells are capable of normal growth in media with 0g/L BSA under standard shake flask conditions, and that BSA
has no effect on the growth of NS0 cells in these non-baffled shake flask conditions. It was hypothesised that the effect of BSA on NS0 cells may only be evident in stressful culture conditions (e.g. sparging, hydromechanical stress etc.); therefore the effects of BSA were next tested on NS0 cells in a sparged and agitated bioreactor system.

**Figure 3.1:** BSA has no effect on NS0 cell growth in standard non-baffled shake flask conditions. MAb-producing NS0 cells were cultured in media with 0 and 1g/L BSA. The cells were cultured in 250ml shaking flasks with a working volume of 100ml under standard growth conditions; 10% CO₂, 37°C and 110 RPM and 0.3x10^6 cells/ml seeding density. Figure 3.1A shows the growth profiles the NS0 cells. Figure 3.1B shows the percentage viability for the NS0 cells. Figure 3.1C shows concentration of NH4+ for the NS0 cells during culture. Error bars denote the standard deviation of the results from four replicate flasks.
3.3.2 BSA does not impact NS0 cell growth in a sparged and agitated mini-bioreactor system

After showing that BSA has no effect on NS0 cells in standard shake flask conditions, the effect of BSA on NS0 cells was next tested in a sparged and agitated AMBR15 mini-bioreactor system by culturing the cells in media 0 and 1g/L BSA in an industrial media supplemented with 1g/L Pluronic F-68. This experiment (and all experiments hereafter) was carried out as a batch process (i.e. no nutrient feeds were added to the media after inoculation), which results in a shorter culture period.

There was no significant difference in the growth profiles (Figure 3.2A) and the LDH Activities (Figure 3.2B) for NS0 cells cultured in the AMBR15 bioreactors in media with 0 and 1g/L BSA. Therefore, BSA had no effect on the growth or damage experienced by the NS0 cells cultured in the AMBR15 mini-bioreactor model. It was concluded from these results that the protective effect of BSA was not evident in this sparged and agitated bioreactor model in media supplemented with 1g/L Pluronic F-68. Therefore the next experiments focused on testing the effects of BSA on NS0 cells cultured in baffled shake flasks, which have been shown to have greater power input (kW/m$^3$) into the liquid in the flasks than non-baffled shake flasks (Peter, Suzuki, Rachinskiy et al., 2006; Peter, Suzuki and Büchs, 2006).
Figure 3.2: BSA does not affect NS0 cell growth in a sparged and agitated mini-bioreactor model in typical industrial cell culture media. MAb-producing NS0 cells were cultured in AMBR15 bioreactors for 7 days with 0 and 1g/L BSA in media with 1g/L Pluronic F-68. Figure 3.2A shows the growth profiles for the NS0 cells. Figure 3.2B shows the LDH Activity for NS0 cells after approximately 2 days in culture. Error bars depict the standard deviations of two replicate mini-bioreactors.
3.3.3 NS0 cell growth is reduced in baffled shake flasks at high orbital shaker speeds

Next, baffled shake flasks were used to test the effects of BSA on NS0 cells after a recent report showed that baffled shake flasks were capable of identifying lots of Pluronic F-68 which offer limited protection to cells in large-scale reactors (Peng et al., 2014). Increasing the orbital shaker speed has been shown to increase the power input into the liquid in baffled shake flasks (Peter, Suzuki, Rachinskiy et al., 2006; Peter, Suzuki and Büchs, 2006). Therefore, NS0 cells were cultured in media with 0 and 1g/L BSA (and 1g/L Pluronic F-68) under two conditions in baffled shake flasks; i) 135 RPM in 125ml baffled shake flasks and ii) 300 RPM in 1L baffled shake flasks to test the effects of BSA on NS0 cells in baffled shake flasks.

Although there was a slight differential in the growth profiles for NS0 cells cultured in media with 0 and 1g/L BSA in 125ml baffled shake flask at 135 RPM, as shown in Figure 3.3A, the difference in the doubling times (Figure 3.3B) was not statistically significant (p>0.05, student T-test, n=3). Furthermore, the LDH Activities (Figure 3.3B) of the cells in the 125ml flasks showed that the presence of BSA had no impact on the membrane damage experienced by the NS0 cells. The presence of BSA had no effect on the NS0 cell growth and LDH Activity in the 1L baffled shake flasks (at 300 RPM), as shown in Figure 3.3C and Figure 3.3D respectively. The results in Figure 3.3C show a reduction in the maximum VCD reached by the NS0 cells in the 1L baffled shake flasks at 300 RPM (maximum VCD of approx. 2.5x10^6cells/ml) compared to the cells in 125ml flasks at 135 RPM (maximum VCD of approx. 4x10^6cells/ml). It was concluded that the hydromechanical conditions in baffled shake flasks (likely due to increased orbital shaker speed) may damage the NS0 cells due to the greater power input into the cell
culture, the formation of bubbles (Büchs, 2001) or damage at the air-liquid interface due to the rapidly moving air-liquid surface (Chisti and Moo-Young, 1993a) causing a reduction in cell growth. Therefore, baffled shake flasks were chosen as a model to carry out further investigations into the effects of BSA on NS0 cells.

Figure 3.3: BSA has no effect on NS0 cells cultured in typical industrial cell culture media in baffled shake flasks. Parental NS0 were cultured in baffled shake flasks with 1g/L Pluronic F-68 and 0 or 1g/L BSA. Figure 3.3A shows growth profiles for cells shaken at 135 RPM in 125ml baffled shake flasks with a working volume of 24ml. Error bars show the standard deviation of three replicate flasks. Figure 3.3B shows the LDH Activity (bar chart) and doubling times (black dots) for the cells cultured at 135 RPM after approximately 48 hours culture. Figure 3.3C shows the growth profiles for cells cultured in 1L baffled shake flasks shaking at 300 RPM with a working volume of 200ml. Figure 3.3D shows the LDH Activity for the cells cultured at 300 RPM after approximately 48 hours culture.
3.4 Testing the effects of reduced Pluronic F-68 concentrations on NS0 cell growth under various cell culture conditions

The results gathered up to this point indicated that BSA had no clear effect on the damage experienced by NS0 cells. These results led us to hypothesise that the presence of 1g/L of the protective surfactant Pluronic F-68 in the industrial media may have been causing the protective effects of BSA to be redundant for NS0 cells exposed to damaging conditions. Therefore the next experiments were conducted to test whether BSA has an effect on NS0 cells in media with reduced protection from Pluronic F-68 (by using media with reduced concentrations of Pluronic F-68). This hypothesis was tested in the three systems used previously, i.e. non-baffled shake flasks, AMBR15 mini-bioreactor system and baffled shake flasks.

3.4.1 Reduced concentrations of Pluronic F-68 do not impact NS0 cell growth in standard non-baffled shake flask conditions

The first experiment carried out to test the hypothesis that high concentrations of Pluronic F-68 were causing the protective effect of BSA on NS0 cells to be redundant was conducted in non-baffled shake flasks under standard cell culture conditions (110 RPM) using media with 0g/L BSA and various concentrations of Pluronic F-68 (0, 0.01, 0.025, 0.05, 0.1 and 1g/L). The objective of this first experiment was to test whether reduced concentrations of Pluronic F-68 had an effect on the growth of the NS0 cells under standard non-baffled shake flasks conditions.

The reduced concentrations of Pluronic F-68 had no impact on the growth profiles (Figure 3.4A) or LDH Activities (Figure 3.4B) of the NS0 cells under these non-baffled shake flask conditions. Therefore, it was concluded that media with
reduced concentrations of Pluronic F-68 could be used to test the hypothesis that the presence of 1g/L Pluronic F-68 in the cell culture media causes the protective effect of BSA on NS0 cells to be redundant. Next, this hypothesis was tested using more damaging cell culture conditions, i.e. AMBR15 mini-bioreactor and baffled shake flasks.

**Figure 3.4:** Reduced concentrations of Pluronic F-68 do not affect NS0 cell growth under standard non-baffled shake flask conditions. Parental NS0 cells were cultured in 125ml non-baffled flasks under standard cell culture conditions in media with 0g/L BSA and varying concentrations of Pluronic F-68 (0, 0.01, 0.025, 0.05, 0.1 and 1g/L). Figure 3.4A shows the growth profiles for the cultures. Figure 3.4B shows the LDH activity for the cultures after approximately three days in culture. Error bars show the standard deviation of three replicate flasks.
3.4.2 BSA promotes NS0 cell growth in an AMBR15 mini-bioreactor system

Having shown that the growth of NS0 cells is not affected by decreased concentrations of Pluronic F-68 in non-baffled shake flasks, the next investigations focused on the effects of BSA on NS0 cells cultured in media with reduced concentrations of Pluronic F-68 in a sparged and agitated AMBR15 mini-bioreactor system. Earlier results showed that BSA had no effect on the NS0 cells cultured in the AMBR15 mini-bioreactors in industrial media supplemented with 1g/L Pluronic F-68 (Figure 3.2). MAb-producing NS0 cells were cultured for 7 days in the AMBR15 bioreactors using an industrial media supplemented with 0 and 1g/L BSA and various concentrations of Pluronic F-68 (0, 0.025, 0.1g/L). The cell growth was monitored by regular cell counts and the membrane damage was assessed after approximately 2 days in culture by performing an LDH Activity assay.

The NS0 cells cultured in media with 0g/L Pluronic F-68 had different maximum VCDs in media with 0 and 1g/L BSA, as shown in Figure 3.5A. The NS0 cells in media with 0g/L BSA had a maximum VCD of approx. 1.0x10^6 cells/ml, whereas the cells in media with 0g/L BSA had a maximum VCD of approx. 2.5x10^6 cells/ml. The NS0 cells cultured in media with 0g/L BSA and 0g/L Pluronic F-68 also entered the decline phase approx. 1.3 days earlier than the cells cultured with 1g/L BSA (decline phase beings at approx. 3.8 days for cells in media with 0g/L BSA and 5.1 days for cells in media with 1g/L BSA), as shown in Figure 3.5A. There was also an indication that BSA promoted NS0 cell growth in media with 0.025 and 0.1g/L Pluronic F-68 (Figure 3.5B and Figure 3.5C respectively), however the effect was not as clear as for the cells cultured in media with 0g/L Pluronic F-68. There was a decrease in LDH Activity for the NS0 cells with increasing Pluronic F-68 concentrations (Figure 3.5D); however there was no clear
indication that there was a protective effect caused by presence of BSA in the media. The results also indicated that there was reduced variability between replicates with increasing concentrations of Pluronic F-68 in the media. This suggests that the presence of increased concentrations of Pluronic F-68 in the media aids cell growth under these cell culture conditions.

These results indicated that BSA may potentially promote NS0 cell growth in media with reduced concentrations of Pluronic F-68 (≤0.1g/L). However, it is difficult to conclude this result due to the variability in the growth profiles of the replicate tests and due to the fact that the LDH Activities did not clearly support the hypothesis that BSA offers protection to the NS0 cells. The LDH Activity of the cells was tested after 2 days in culture; however it may have been more beneficial to conduct the LDH assay after approximately 4 days in culture as by that stage the growth profiles had diverged significantly for cells cultured in media with 0 and 1g/L BSA.

The AMBR bioreactors are an expensive and technically difficult method of exposing the NS0 cells to damaging conditions. Therefore the next investigations tested the protective effect of BSA on NS0 cells in baffled shake flasks, as baffled shake flasks are a more cost effective and user friendly method of exposing cells to damaging cell culture conditions and previous experiments carried out (Section 3.3.3) indicated that baffled shake flasks may offer a more stressful cell culture environment.
Figure 3.5: BSA promotes NS0 cell growth at low concentrations of Pluronic F-68 in AMBR mini-bioreactor system. MAb-producing NS0 cells were cultured in an AMBR mini-bioreactor system in media with various concentrations of Pluronic F-68 (0, 0.025, 0.1 and 1g/L) and 0 and 1g/L BSA. Figure 3.5A shows the growth profiles of the NS0 cells cultured in media with 0g/L Pluronic F-68. Figure 3.5B shows the growth profiles for the NS0 cells cultured in media with 0.025g/L Pluronic F-68. Figure 3.5C shows the growth profiles for the NS0 cells cultured in media with 0.1g/L Pluronic F-68. Figure 3.5D shows the LDH Activity after approximately 2 days in culture. Error bars depict the standard deviation of at least 2 replicate mini-bioreactors.
3.4.3 Increased orbital shaker speeds reduce NS0 cell growth in baffled shake flasks

Following on from the results gathered in the AMBR15 mini-bioreactors suggesting that BSA may have a growth promoting effect on NS0 cells in media with low concentrations of Pluronic F-68 and also based on the observations that baffled shake flasks may reduce the growth of NS0 cells when shaken at increased orbital shaker speeds, the next objective was to use baffled shake flasks to develop a model where the growth of the NS0 cells was reduced due to the hydromechanical stress in the baffled shake flasks. Media with a low concentration of Pluronic F-68 (0.025g/L) was chosen to test whether baffled shake flasks reduce the growth of NS0 cells, this concentration was chosen from the results gathered in Section 3.4.1 showing that reduced concentrations of Pluronic F-68 have no impact on NS0 cell growth in standard non-baffled shake flask conditions. Also, investigations in baffled shake flasks using media supplemented 0g/L Pluronic F-68 and 0g/L BSA resulted in cell clumping, however this clumping was not observed using media with 0.025g/L Pluronic and 0g/L BSA.

The first experiment was conducted to test the effect of BSA on NS0 cells cultured at the standard orbital shaker speed used to culture these NS0 cells (110 RPM) in 125ml baffled shake flasks in media with 0.025g/L Pluronic F-68 and 0 or 1g/L BSA. The NS0 cells were capable of growth in these baffled shake flask conditions and BSA had no effect on the cell growth, as shown in Figure 3.6A. It was hypothesised that the hydromechanical conditions in the baffled shake flasks at 110 RPM were not damaging the NS0 cells, thus BSA has no effect on the cells.

Next, the NS0 cells were exposed to greater hydromechanical stress in baffled shake flasks by culturing the cells using increasing orbital shaking speeds;
110, 135 and 150 RPM. As mentioned earlier, increasing the orbital shaker speeds in baffled shake flasks results in greater power input (kW/m$^3$) into the cell culture (Peter, Suzuki, Rachinskiy et al., 2006; Peter, Suzuki and Büchs, 2006), the formation of bubbles (Büchs, 2001) and potentially damaging conditions at the air-liquid interface due to the rapidly moving air-liquid surface (Chisti and Moo-Young, 1993a). Media with 0g/L BSA and 0.025g/L Pluronic F-68 was used to test whether the NS0 cells were damaged in the baffled shake flasks.

The NS0 cells were capable of growth in the baffled shake flasks shaking at 135 RPM; however the maximum VCD was reduced by approx. 25% (Figure 3.6B) and the doubling time increased by approx. 14% (Figure 3.6C) compared to cells cultured at 110 RPM. There was 100% cell death after 1 day in culture for cells shaken at 150 RPM (as shown in Figure 3.6B). This was seen by the VCD of 0x10^6 cells/ml after approx. 1 day in culture for the NS0 cells shaken at 150 RPM. Therefore it was concluded that the hydromechanical stress in these conditions was too extreme for the NS0 cells to proliferate. These results suggest that there is a critical point between 135 and 150 RPM where the power input into the baffled shake flasks is too extreme for these NS0 cells to be capable of proliferation and the cells are lysed by the hydromechanical stress after 24 hours exposure.

The orbital shaker speed of 135 RPM was chosen as the orbital shaker speed for the moderate hydromechanical stress model in baffled shake flasks where the NS0 cells were capable of growth, but the growth was reduced compared to cells shaken at 110 RPM. The purpose of this model was to test whether BSA promotes NS0 cell growth under moderate hydromechanical stress conditions. There were no bubbles formed 135 RPM, as shown later in Section 4.6.3.
Figure 3.6: Increasing orbital shaker speeds causes a reduction in NS0 cell growth in baffled shake flasks in media with low Pluronic F-68 concentrations. Parental NS0 cells were cultured with 0.025g/L Pluronic F-68 in 125ml baffled shake flasks with a working volume of 24ml. Figure 3.6A shows the growth profiles for cells cultured at 110 RPM in media with 0 and 1g/L BSA. Figure 3.6B shows growth profiles for cells cultured at 110, 135 and 150 RPM in media with 0g/L BSA. Figure 3.6C shows the doubling times for the cells cultured at 110, 135 and 150 RPM in media with 0g/L BSA after approximately 3 days in culture, p=0.0007 (***, student T-test, n=3. Error bars depict the standard deviation of three replicate flasks.
3.4.4 The presence of baffles in the shake flasks causes the reduction in NS0 cell growth

Having shown that the growth of NS0 cells is reduced in baffled shake flasks at increased orbital shaker speeds, the next objective was to test whether the reduction in cell growth experienced in the moderate hydromechanical stress model was due to the presence of baffles in the shake flask rather than the increase in orbital shaker speed. NS0 cells were exposed to the moderate hydromechanical stress conditions (135 RPM) in non-baffled and baffled shake flasks in media with 0g/L BSA and 0.025g/L Pluronic F-68.

The NS0 cells cultured in baffled shake flasks had reduced cell growth compared to the cells cultured in non-baffled shake flasks. The maximum VCD for the NS0 cells in baffled shake flasks was approx. 30% lower (Figure 3.7A) and the doubling time of the cells increased by approx. 16% (Figure 3.7B) compared to cells in non-baffled shake flasks. The LDH activity of the NS0 cells in baffled shake flasks was approx. 3.7 times higher than the non-baffled flasks (189.6 LU for baffled shake flasks compared to 51.9 LU for non-baffled flasks), as shown in Figure 3.7C, therefore cells were experiencing more membrane damage in baffled shake flasks compared to non-baffled shake flasks.

It was concluded from these results that the reduction in growth and increase in LDH Activity seen in the baffled shake flasks was due the hydromechanical stress caused by the presence of baffles in the flasks, which is likely due to the greater power input into the cell culture and potentially damaging conditions at the air-liquid interface due to the rapidly moving air-liquid surface (Chisti and Moo-Young, 1993a) in baffled shake flasks compared to non-baffled shake flasks.
**Figure 3.7:** Baffled shake flasks cause reduced NS0 cell growth compared to non-baffled shake flasks under moderate hydromechanical stress conditions.

Parental NS0 cells were exposed to moderate hydromechanical stress (135 RPM) in media with 0g/L BSA and 0.025g/L Pluronic F-68 in baffled and non-baffled shake flasks with working volumes of 24ml. Figure 3.7A shows the growth profiles for cells cultured at 135 RPM over 4 days. Figure 3.7B shows the doubling times for cells cultured in non-baffled and baffled shake flasks after approximately 3 days in culture, p=0.0006 (***) student T-test, n=3. Figure 3.7C shows the LDH Activity for cells cultured at 135 RPM after approximately 2 days in culture, p=0.0002 (***) student T-test, n=3. Error bars denote the standard deviation of three replicate flasks.
3.4.5 Model 1: Moderate hydromechanical stress

The objective of the moderate hydromechanical stress model was to expose the NS0 cells to hydromechanical stress where the cells were capable of proliferation; however there was a reduction in the cell growth compared to cells cultured at the standard cell culture orbital shaker speed (110RPM). This model was developed as a representation of hydromechanical stress conditions that cause a reduction in cell growth in large-scale bioreactors which would reduce the product titre of the process. The moderate hydromechanical stress model could then be used to test whether BSA promotes NS0 cell growth under conditions of hydromechanical stress which cause a reduction in NS0 cell growth. This model was named “moderate” hydromechanical stress as the NS0 cells were capable of proliferation under the conditions.

The conditions and readouts of damage for the moderate hydromechanical stress model are summarised in Figure 3.8. The conditions for the moderate hydromechanical stress model were as follows; 125ml baffled shake flasks with a working volume of 24ml, 135 RPM orbital shaker speed, 0.3x10^6 cells/ml seeding density and approx. 4 days exposure to the hydromechanical stress. These conditions did not cause the formation bubbles at the air-liquid interface. The readouts of damage for the moderate hydromechanical stress model were;

i) The reduction in cell growth as measured by;
   - Reduction in the maximum VCD reached during cell culture.
   
   This readout is relevant to industry as reduction in the maximum VCD reached in a bioreactor would likely result in a lower product titre. The maximum VCD that the Parental NS0 cells could reach during the 4 days of cell culture under standard shake flask cell
cultural conditions was approx. 3-3.5x10^6 cells/ml. The moderate hydromechanical stress model caused a reduction in maximum VCD of approx. 25-30% for Parental NS0 cells in media with 0g/L BSA and 0.025g/L Pluronic F-68.

- Increase in doubling time.

The doubling time of cells is commonly used to measure the growth rate of cells. The doubling time of the Parental NS0 cells was approx. 23-24 hours in non-baffled shake flasks under standard cell culture conditions. The moderate hydromechanical stress model caused an increase in doubling time of approx. 15% for Parental NS0 cells in media with 0g/L BSA and 0.025g/L Pluronic F-68.

ii) Cell membrane damage assay (LDH Activity) after approx. 2 days in culture. This assay determines the extracellular LDH Activity which is a method of measuring the membrane damage experienced by cells.

![Figure 3.8: Conditions for the moderate hydromechanical stress model in baffled shake flasks.](image)

**Model 1: Moderate hydromechanical stress model**

Figure 3.8: Conditions for the moderate hydromechanical stress model in baffled shake flasks. This figure shows the conditions for exposing NS0 cells to moderate hydromechanical stress in 125ml baffled shake flasks.
3.4.6 NS0 cells experience rapid damage at high orbital shaker speeds in baffled shake flasks

Following on from the results in Section 3.4.3 showing that the growth of NS0 cells was reduced when the cells were exposed to moderate hydromechanical stress in baffled shake flasks, the next objective was to develop an extreme hydromechanical stress model in baffled shake flasks where exposure to the hydromechanical stress caused a reduction in the VCD. Based on the knowledge gathered in Section 3.4.3 showing that the VCD of the NS0 cells was reduced to $0 \times 10^6$ cells/ml in baffled shake flasks shaken at 150 RPM (after approx. 24 hours), the orbital shaker speed was increased further to expose the NS0 cells to more extreme hydromechanical stress. A number of orbital shaker speeds (110, 150, 175 and 200 RPM) were tested in 125ml baffled shake flasks for 60 minutes in media with 0.025g/L Pluronic F-68 and 0g/L BSA. The orbital shaker speed of 110 RPM was used as a positive control, as it had been shown previously in Section 3.4.3 that the NS0 cells were capable of growth under these conditions.

The NS0 cells shaken at 110 and 150 RPM had no significant reduction in VCD after 60 minutes exposure, whereas there was approx. 64% reduction in VCD for NS0 cells shaken at 175 RPM and approx. 72% reduction in VCD at 200 RPM, as shown in Figure 3.9A. The LDH Activity of the cells shaken at 175 RPM was approx. 11.5 times higher than the LDH Activity for cells shaken at 110 RPM (approx. 290 LU for 175 RPM and approx. 25 LU for 110 RPM). Previous results demonstrated that NS0 cells cultured at 150 RPM had a 100% reduction in VCD after approximately 24 hours to the hydromechanical stress (Figure 3.6B); therefore the exposure time to the hydromechanical stress is an important factor in determining
the damage experienced by NS0 cells and extended exposure to the hydromechanical stress at 150 RPM reduces the VCD of the NS0 cells.

Next, NS0 cells were exposed to orbital shaker speeds between 150 and 175 RPM in baffled shake flasks to identify if there was a critical point where short exposure (60 minutes) to the hydromechanical stress causes a reduction in VCD for the NS0 cells. There was a reduction in VCD at 155, 160, 165 and 175 RPM (Figure 3.9B), which suggests that there is a critical point between 150 and 155 RPM where the damage experienced by the cells causes a reduction in VCD. The membrane damage (LDH Activity) experienced by the NS0 cells under these conditions also supports this result – data shown in Figure 3.9B. This supports the hypothesis that the damage experienced by the NS0 cells is due to the power input into the cell culture with increasing orbital shaker speeds in the baffled shake flasks (with exposure time to the hydromechanical stress also being a factor).

It was observed that shaking baffled shake flasks at 175 RPM caused the formation of bubbles whereas no bubbles were formed at 135 RPM, as shown later in Section 4.6.3. The presence of bubbles in the baffled shake flasks shaken at 175 RPM may contribute to the extreme damage experienced by the cells as bubble bursting has been shown to be detrimental to cells (Trinh et al., 1994). Taking these results into consideration, 175 RPM was chosen as the orbital shaking speed for testing the protective effects of BSA on NS0 cells experiencing extreme hydromechanical stress in baffled shake flasks.
Figure 3.9: NS0 cells are susceptible to damage in baffled shake flasks at high orbital shaker speeds in media with low Pluronic F-68 concentrations. Parental NS0 cells were exposed to various orbital shaker speeds in media with 0.025g/L Pluronic F-68 and 0g/L BSA in 125ml baffled shake flasks with a working volume of 24ml. Figure 3.9A shows the results for cells that were exposed to 110, 150, 175 and 200 RPM for 60 minutes at an initial cell density of 1x10^6 cells/ml, 175 RPM \( p=0.00004 \) (****), 200 RPM \( p=0.00003 \) (****), student T-test, n=3. Dotted line indicates LDH Activity after 30 minutes exposure. Error bars show the standard deviation of three replicate flasks. Figure 3.9B shows the reduction in VCD for cells exposed to 150, 155, 160, 165 and 175 RPM. Dotted line indicates LDH Activity after 30 minutes exposure. Error bars show the standard deviation of two replicate flasks.
3.4.7 Exposure time and initial seeding density affect the damage experienced by NS0 cells under extreme hydromechanical stress in baffled shake flasks

After showing that the NS0 cells were susceptible to damage in baffled shake flasks shaken at 175 RPM in media with 0g/L BSA and 0.025g/L Pluronic F-68, the next experiment was designed to test the effects of exposure time and initial seeding density of the NS0 cells to the extreme hydromechanical stress. The effect of exposure time was tested by shaking the NS0 cells at 175 RPM in baffled shake flasks in media with 0g/L BSA and 0.025g/L Pluronic F-68 for 180 minutes with samples taken after 5, 30, 60, 90, 120 and 180 minutes.

Increasing exposure time to the damaging conditions caused a greater reduction in VCD for the NS0 cells, as shown in Figure 3.10A. The damage experienced by the NS0 cells increased in a linear manner, which supports the hypothesis that the damage experienced in baffled shake flasks at high orbital shaker speeds is due to the exposure of the cells to hydromechanical stress caused by greater power input into the cell culture (i.e. greater stress). The results show that the reduction in VCD increases from approx. 13% after 5 minutes exposure to approx. 90% after 180 minutes exposure. There was approx. a 56% reduction in VCD after 60 minutes exposure, therefore 60 minutes exposure was chosen as the exposure time for the extreme hydromechanical stress model.

The effect of initial seeding density was tested by exposing NS0 cells to the damage (175 RPM for 60 minutes) at a number of different initial seeding densities; 1, 4 and 8x10^6cells/ml, which are all typical cell densities which would be found in a bioreactor during the production of mAbs. The initial seeding density had an impact on the damage experienced by the NS0 cells, with cells seeded at 1x10^6cells/ml experiencing more damage than cells seeded at 4 and
8×10^6 cells/ml. The NS0 cells with an initial seeding density of 1×10^6 cells/ml have a reduction in VCD of approx. 60%, whereas the cells with initial seeding densities of 4 and 8×10^6 cells/ml had reductions in VCD of approx. 38% and 30% respectively, as shown Figure 3.10B. This result suggests that the hydromechanical stress experienced by each individual cell is greater with decreasing cell densities; therefore the percentage reduction in VCD is lower with increasing cell densities. From these results, an initial seeding density of 1×10^6 cells/ml was chosen for the extreme hydromechanical stress model as it was the seeding density which had the greatest reduction in VCD.

Figure 3.10: Exposure time and initial cell density affect the damage experienced by NS0 cells in baffled shake flasks at high orbital shaker speeds. Parental NS0 cells were cultured with 0.025g/L Pluronic F-68 and 0g/L BSA in 125ml baffled shake flasks with a working volume of 24ml. Figure 3.10A shows the reduction in VCD with increasing exposure time for cells exposed to 175 RPM at an initial seeding density of 1×10^6 cells/ml. Figure 3.10B shows the reduction in VCD for cells exposed to 175 RPM for 60 minutes at various seeding densities: 1, 4 and 8×10^6 cells/ml. Error bars show the standard deviation of three replicate flasks.
3.4.8 The damage experienced by NS0 cells at high orbital speeds is due to the presence of baffles in the shake flasks

The next investigation aimed to determine whether the damage experienced by the NS0 cells in the extreme hydromechanical stress model was due to the presence of baffles in the shake flasks rather than the increasing orbital shaker speeds. NS0 cells were exposed to the extreme hydromechanical stress conditions (175 RPM) in baffled and non-baffled shake flasks in media with 0.025g/L Pluronic F-68 and 0g/L BSA to assess whether the damage was due to the presence of the baffles in the shake flasks.

The NS0 cells experienced more damage in baffled shake flasks than non-baffled flasks. The NS0 cells in baffled shake flasks had a reduction in VCD of approx. 64% compared to approx. 11% for the non-baffled shake flasks, as shown in Figure 3.11A. The NS0 cells in baffled shake flasks also had a higher LDH activity (approx. 290 LU) compared to the non-baffled shake flasks (approx. 25 LU), as shown in Figure 3.11B.

It was concluded from these results that the damage to the NS0 cells was due to the hydromechanical stress caused by the presence of the baffles in the baffled shake flasks, which may be due to the increased power input into the liquid, the formation of bubbles (Büchs, 2001) or damage at the air-liquid interface due to the rapidly moving air-liquid surface (Chisti and Moo-Young, 1993a) in baffled shake flasks compared to non-baffled shake flasks.
Figure 3.11: Baffled shake flasks cause more damage to NS0 cells compared to non-baffled shake flasks under extreme hydromechanical stress conditions. Parental NS0 cells were exposed to extreme hydromechanical stress (175 RPM) in media with 0g/L BSA and 0.025g/L Pluronic F-68 in baffled and non-baffled shake flasks with working volumes of 24ml. Figure 3.11A shows the percentage reduction in VCD for cells exposed to 175 RPM for 60 minutes, p=0.01 (*), student T-test, n=3. Figure 3.11B shows the LDH Activity for cells exposed to 175 RPM for 30 minutes, p=0.001 (**), student T-test, n=3. Error bars show the standard deviation of three replicate flasks.
3.4.9 Model 2: Extreme hydromechanical stress

The objective of the extreme hydromechanical stress model was to expose the NS0 cells to damage where the cells were not capable of proliferation and the VCD of the cells was reduced after short exposure to the stress compared to cells shaken at the standard orbital shaker speed (110 RPM). This model could then be used to test whether BSA protects NS0 cells from extreme hydromechanical stress which causes a rapid reduction in VCD. This model was named “extreme” hydromechanical stress as the NS0 cells were not capable of proliferation and the VCD of the cells was rapidly reduced due to cell lysis under the conditions.

The conditions and readouts for the extreme hydromechanical stress model are summarised in Figure 3.12. The conditions for the extreme hydromechanical stress model were; 125ml baffled shake flasks with a working volume of 24ml, 175 RPM orbital shaker speed, 1x10^6 cells/ml seeding density and 60 minutes exposure to the hydromechanical stress. These conditions caused to formation of bubbles at the air-liquid interface which may contribute to the damage experienced by the cells when the bubbles burst. The damage experienced by the cells exposed to the extreme hydromechanical stress model was quantified using the following readouts;

i) The percentage reduction in VCD.

The percentage reduction in VCD was used as a readout for damage in the extreme hydromechanical stress model, as a reduction in VCD caused by extreme hydromechanical stress would have a negative effect on product titre in a bioreactor. In the extreme hydromechanical stress model Parental NS0 cells in media with 0g/L BSA and 0.025g/L Pluronic F-68 had a reduction in VCD of approx. 60-70% under these conditions.
ii) Cell membrane damage assay (LDH Activity).

This assay determines the extracellular LDH Activity which is a method of measuring the membrane damage experienced by cells.

Model 2: Extreme hydromechanical stress model

![Diagram showing cell culture conditions and readouts for extreme hydromechanical stress model]

Figure 3.12: Conditions for the extreme hydromechanical stress model in baffled shake flasks. This figure shows the conditions for exposing NS0 cells to extreme hydromechanical stress in 125ml baffled shake flasks.
3.5 Evaluation of the protective effect(s) of BSA on NS0 cells experiencing hydromechanical stress

3.5.1 BSA promotes NS0 cell growth under moderate hydromechanical stress in baffled shake flasks

After developing a model to show that the NS0 cell growth is reduced by moderate hydromechanical stress in baffled shake flasks in media with low concentrations of Pluronic F-68 (0.025g/L) and 0g/L BSA, the next objective was to determine whether BSA has a growth promoting effect for the NS0 cells under the moderate hydromechanical stress conditions. NS0 cells were cultured in baffled shake flasks using the moderate hydromechanical stress conditions (as described in Section 3.4.5) in media with varying concentrations of BSA (0, 0.1, 0.5, 1 and 2g/L) and 0.025g/L Pluronic F-68 to test the effects of BSA on NS0 cells experiencing moderate hydromechanical stress.

The presence of BSA in the media promoted NS0 cell growth in the moderate hydromechanical stress model. This growth promotion was seen by an increase in the maximum VCD (Figure 3.13A) and a reduction in doubling time (Figure 3.13B) for NS0 cells exposed to the moderate damage in media supplemented with BSA compared to media with 0g/L BSA. Although low concentrations of BSA (0.1g/L) did not have any significant effect on the growth of the NS0 cells, the maximum VCD increases dose dependently with increasing BSA concentrations, with the NS0 cells in media with 2g/L BSA having a maximum VCD of approx. 3.5x10^6 cells/ml compared to approx. 2.5x10^6 cells/ml for cells in media with 0g/L BSA, as shown in Figure 3.13A. The doubling time also decreases dose dependently with increasing BSA concentrations, with NS0 cells in media with 2g/L BSA having a doubling time of approx. 21.7 hours compared to 28.4 hours for cells
in media with 0g/L BSA, as shown in Figure 3.13B. The presence of 1g/L BSA in the media (which is the concentration used in the current NS0 cell culture process) increased the maximum VCD reached by approx. 22% (Figure 3.13A) and also decreased the doubling time by approx. 19% (Figure 3.13B) compared to cells in media with 0g/L BSA. These results show that the addition of 1g/L BSA protects the NS0 cells from the moderate hydromechanical damage restores the standard maximum VCD and standard doubling times for the Parental NS0 cells as outlined in Section 3.4.5. Therefore the protective effect afforded by BSA for NS0 cells exposed to moderate hydromechanical stress resulting in improved cell growth would increase mAb titre in media where the protective effect of Pluronic F-68 is limited.

The addition of BSA to the media also had a protective effect on the NS0 cells, as seen by a decrease in LDH Activity (Figure 3.13C) for cells cultured with BSA compared to cells cultured with 0g/L BSA. However, the results show that the reduction in LDH Activity was not dose dependent with increasing BSA concentrations, with the cells in media with 0.5g/L BSA having the lowest LDH Activity. This result suggests that the protective effect of BSA on the NS0 cell membrane is limited in this moderate hydromechanical stress model as increasing concentrations of BSA did not result in a lower LDH Activity.

Previous results showed that BSA did not cause a significant difference in the maximum VCD reached and the doubling times of NS0 cells shaken at 135RPM in media with 1g/L Pluronic F-68 (Figure 3.3A and Figure 3.3B respectively) and the NS0 cells under these conditions also had similar (low) LDH Activities (Figure 3.3B). Therefore, the results gathered here support the hypothesis that the effect of BSA is only evident for NS0 cells in media with limited protection from Pluronic F-68.
Figure 3.13: BSA protects NS0 cells experiencing moderate hydromechanical stress in baffled shake flasks in media with low Pluronic F-68 concentrations. Parental NS0 cells were cultured at 135 RPM in 125ml baffled shake flasks with a working volume of 24ml in media with 0.025g/L Pluronic F-68 and varying concentrations of BSA (0, 0.1, 0.5, 1 and 2g/L). Figure 3.13A shows the growth profiles for the NS0 cells over 4 days culture. Figure 3.13B shows the doubling times for the cells after approximately 3 days in culture, 0.5g/L BSA p=0.05 (*), 1g/L BSA p=0.05 (*), 2g/L BSA p=0.03 (*), student T-test, n=3. Figure 3.13C shows the LDH activity of the NS0 cells cultured after approximately 2 days of culture, 0.1g/L BSA p=0.002 (**), 0.5g/L BSA p=0.003 (**), 1g/L BSA p=0.001 (**), 2g/L BSA p=0.0005 (**), student T-test, n=3. Error bars denote the standard deviation of three replicate flasks.
3.5.2 BSA affords dose dependent protection for NS0 cells exposed to the extreme hydromechanical stress model

Following on from the results gathered showing that BSA promotes NS0 cell growth under moderate hydromechanical stress in baffled shake flasks, the next step was to test whether BSA protects the NS0 cells from damage in the extreme hydromechanical stress model in baffled shake flasks. NS0 cells were exposed to the extreme hydromechanical stress conditions (as described in Section 3.4.9) in baffled shake flasks in media with 0.025g/L Pluronic F-68 and varying concentrations of BSA (0, 0.1, 0.5, 1 and 2g/L).

The presence of BSA offered dose dependent protection to the NS0 cells exposed to the extreme hydromechanical stress, as seen by lower reduction in VCDs and lower LDH Activities with increasing BSA concentrations. The NS0 cells in media with 0g/L BSA had a reduction in VCD of approx. 73% compared to approx. 3% for cells in media with 2g/L BSA, as shown in Figure 3.14A. The NS0 cells in media with 0g/L BSA had approx. 4.5 times higher LDH Activity (approx. 270 LU) compared to cells in media with 2g/L BSA (approx. 60 LU), as shown in Figure 3.14B.

The protective effect of 1g/L BSA was also tested and compared to 0g/L BSA at various time points (5, 30, 60, 90, 120 and 180 minutes) and at a number of initial seeding densities (1, 4 and 8x10^6cells/ml) using the extreme hydromechanical conditions (as described in Section 3.4.9). The protective effect of BSA was present at every time point between 30 and 180 minutes exposure, as shown in Figure 3.15A. The protective effect of BSA was also evident at each cell density tested: 1, 4 and 8x10^6cells/ml, as shown in Figure 3.15B. As seen previously (in Section 3.4.7) the hydromechanical stress experienced the NS0 cells
decreases with increasing cell densities, which is likely due to there being more cells present to absorb the hydromechanical stress in the baffled shake flasks, therefore there is less damage experienced by each individual cell with increasing cell densities.

These results show that BSA protects NS0 cells from damage experienced in the extreme hydromechanical stress model in media with limited protection from Pluronic F-68. Therefore the protective effect of BSA would be favourable during mAb production in media where the protective effect of Pluronic F-68 is limited, as there would be less cells damaged by the extreme hydromechanical stress in the bioreactor which would improve the product titre.

Figure 3.14: BSA affords dose dependent protection for NS0 cells exposed to extreme hydromechanical stress conditions in media with low Pluronic F-68 concentrations. Parental NS0 cells were exposed to 175 RPM for 60 min in 125 ml baffled shake flasks at a seeding density of 1x10^6 cells/ml in media with 0.025 g/L Pluronic F-68 with various concentrations of BSA (0, 0.1, 0.5, 1 and 2 g/L). Figure 3.14A shows the reduction in VCD after 60 minutes exposure, 0.5 g/L BSA p=0.00005 (****), 1 g/L BSA p=0.002 (**), 2 g/L BSA p=0.000007 (****), student T-test, n=3. Figure 3.14B shows the LDH activity for samples taken after 30 minutes exposure, 0.1 g/L BSA p=0.0001 (**), 0.5 g/L BSA p=0.000004 (****), 1 g/L BSA p=0.000001 (****), 2 g/L BSA p=0.0001 (**), student T-test, n=3. Error bars denote standard deviation of three replicate flasks.
Figure 3.15: BSA protects NS0 cells against extreme hydromechanical stress at all exposure times and cell densities tested. Parental NS0 cells were exposed to 175 RPM in 125ml baffled shake flasks with a 24ml working volume in media with 0.025g/L Pluronic F-68 with either 0 or 1g/L BSA. Figure 3.15A shows the percentage reduction in VCD for NS0 cells seeded at 1x10^6 cells/ml with varying exposure times (5, 15, 30, 60, 90, 120 and 180 minutes), 30 min p=0.007 (**), 60 min p=0.0006 (**), 90 min p=0.009 (**), 120 min p=0.002 (**), 180min p=0.008 (**), student T-test, n=3. Figure 3.15B shows the percentage reduction in VCD for cells seeded at 1, 4 and 8x10^6 cells/ml after 60 minutes exposure, 1x10^6 cells/ml p=0.002 (**), 4x10^6 cells/ml p=0.002 (**), 8x10^6 cells/ml p=0.0007 (**), student T-test, n=3. Error bars depict the standard deviation of three replicate flasks.
The protective effect of BSA (1g/L) was tested for NS0 cells exposed to the extreme hydromechanical stress model (as described in Section 3.4.9) in media with various concentrations of Pluronic F-68 (0, 0.025, 0.05, 0.1 and 1g/L) to test the hypothesis that high concentrations of Pluronic F-68 cause the protective effect of BSA to be redundant.

Pluronic F-68 afforded dose dependent protection for NS0 cells experiencing extreme hydromechanical stress in baffled shake flasks, as shown by a lower reduction in VCD and a lower LDH Activity with increasing Pluronic F-68 concentrations, as shown in Figure 3.16A and Figure 3.16B respectively. The dose dependent protection afforded by Pluronic F-68 was evident in media with both 0 and 1g/L BSA, therefore Pluronic F-68 offers protection to the cells in the presence or absence of BSA.

The protective effect of BSA was only evident in media with concentrations of Pluronic F-68 ≤0.1g/L. The protection was shown by a lower reduction in VCD (Figure 3.16A) and a lower LDH Activity (Figure 3.16B) for NS0 cells in media with 1g/L BSA compared to cells in media with 0g/L BSA. The NS0 cells in media with 0g/L BSA and 0g/L Pluronic F-68 had a reduction in VCD of approx. 88% and an LDH Activity of approx. 220 LU compared to a reduction in VCD of approx. 52% and an LDH Activity of approx. 65 LU for cells in media with 1g/L BSA and 0g/L Pluronic F-68. Therefore, these results show that the protective effect of BSA is independent of Pluronic F-68.

The NS0 cells exposed to the extreme hydromechanical stress in media with 1g/L Pluronic F-68 were not damaged by the extreme hydromechanical stress in
media with 0 and 1g/L BSA. This was clear as there was no reduction in VCD for the cells in media with 1g/L Pluronic F-68 as shown in Figure 3.16A and the cells had similar LDH Activities (approx. 10 LU), as shown in Figure 3.16B. This result supports the hypothesis that high concentrations of Pluronic F-68 cause the protective effects of BSA on NS0 cells to be redundant. These results suggest that BSA and Pluronic F-68 independently protect the NS0 cells from the extreme hydromechanical stress in baffled shake flasks.

These results support the hypothesis that the protective effect of BSA is only evident in media with limited protection from Pluronic F-68. Therefore if BSA is removed from the media the only source of protection from hydromechanical damage in the bioreactor would be from Pluronic F-68. As there is increased pressure being placed on Pluronic F-68 in large-scale cell culture, it has been advised to have at least two active surfactants in media (Hu et al., 2008; Chalmers, 2015). This advice is supported by the fact there have been a number of reports describing batches of with limited protective effects in large-scale bioreactors (Peng et al., 2014; Apostolidis et al., 2015).
Figure 3.16: BSA protection is only evident at reduced concentrations of Pluronic F-68 for NS0 cells exposed to extreme hydromechanical stress. Parental NS0 cells were exposed to 175 RPM shaking conditions in 125ml baffled shake flasks with a 24ml working volume in media with 0 or 1g/L BSA and various concentrations of Pluronic F-68 (0, 0.025, 0.05, 0.1 and 1g/L). Figure 3.16A shows the percentage reduction in VCD for samples after 60 minutes exposure, 0g/L Pluronic F-68 p=0.00002 (**), 0.025g/L Pluronic F-68 p=0.0001 (**), 0.05g/L Pluronic F-68 p=0.0007 (**), 0.1g/L Pluronic F-68 p=0.02 (*) , student T-test, n=3. Figure 3.16B shows the LDH Activity results after 30 minutes exposure, 0g/L Pluronic F-68 p=0.001 (**), 0.025g/L Pluronic F-68 p=0.00002 (****), 0.05g/L Pluronic F-68 p=0.0007 (**), 0.1g/L Pluronic F-68 p=0.0005 (**), student T-test, n=3. Error bars denote standard deviation of three replicate flasks.
3.6 Confirming the protective effect of BSA on mAb-producing NS0 cells experiencing hydromechanical stress

3.6.1 BSA offers protection to mAb-producing NS0 cells in industrial media with reduced concentrations of Pluronic F-68

As the moderate and extreme hydromechanical stress models were developed using Parental NS0 cells, the next objective was to test whether BSA had the same protective effect on an industrial mAb-producing NS0 cell line. The mAb-producing NS0 cells were exposed to the moderate (as described in Section 3.4.5) and extreme (as described in Section 3.4.9) hydromechanical stress models in baffled shake flasks in media with 0.025 and 1g/L Pluronic F-68 and 0 or 1g/L BSA. The concentration of 0.025g/L Pluronic F-68 was chosen as this concentration of Pluronic F-68 was used for previous experiments with Parental NS0 cells.

The mAb-producing NS0 cells were susceptible to the moderate hydromechanical conditions in baffled shake flasks in media with 0g/L BSA and 0.025g/L Pluronic F-68, as there was no cell growth detected (Figure 3.17A). The presence of 1g/L BSA in the media promoted cell growth, as shown in Figure 3.17A. The presence of 1g/L BSA also protected the mAb-producing NS0 cells from the moderate hydromechanical stress, as seen by a decrease in LDH Activity for cells in media with 1g/L BSA (approx. 50 LU) compared to cells cultured in media with 0g/L BSA (approx. 375 LU), as shown in Figure 3.17B. This result suggests that the mAb-producing NS0 cell line is more sensitive to the moderate hydromechanical stress compared to the Parental NS0 cell line. Therefore, higher concentrations of Pluronic F-68 (>0.025g/L) may be required in the media for the mAb-producing NS0 cells for the same effect to be seen compared to the Parental NS0 cells. This increased sensitivity may also be due to the fact that the cells are producing and
secreting mAbs, which could cause the cell membrane to be more susceptible to damage. However, this theory was not further investigated due to restricted access to the mAb-producing NS0 cells.

The effect of BSA on the mAb-producing NS0 cells exposed to the moderate hydromechanical stress in media with 1g/L Pluronic F-68 was redundant, which is similar to results shown earlier using Parental NS0 cells (Section 3.3.3). This result is seen by the similar maximum VCDs for mAb-producing NS0 cells in media with 0g/L BSA (approx. 3.4x10^6 cells/ml) and 1g/L BSA (approx. 3.5x10^6 cells/ml), as shown in Figure 3.17A. The mAb-producing NS0 cells were also protected from damage in media with 1g/L Pluronic, as seen by the low LDH Activities in media with 0 and 1g/L BSA (16 LU and 20 LU, respectively), as shown in Figure 3.17B.

The protective effect observed for BSA on the mAb-producing NS0 cells exposed to extreme hydromechanical stress in media with 0.025g/L Pluronic F-68 was comparable with the protective effect for BSA on Parental NS0 experiencing the same stress. The protective effect of BSA on the mAb-producing NS0 cells in media with 0.025g/L Pluronic F-68 was shown by a lower reduction in VCD for NS0 in media with 1g/L BSA (approx. 24%) compared to 0g/L BSA (approx. 64%), as shown in Figure 3.18A. The LDH Activity for the cells exposed to the damage in media with 1g/L BSA (approx. 65 LU) was also lower compared to cells in media with 0g/L BSA (approx. 135 LU), as shown in Figure 3.18B. The protective effect of BSA was redundant for the mAb-producing NS0 cells in media with 1g/L Pluronic F-68 as shown by the similar percentage reduction in VCD and LDH Activities for the cells exposed to the damage with 0 and 1g/L BSA, as shown in Figure 3.18A and Figure 3.18B respectively. This result was similar to results seen
earlier for Parental NS0 cells exposed to the extreme hydromechanical stress in media with 1g/L Pluronic F-68 (Figure 3.16).

These results show that BSA offers protection to mAb-producing NS0 cells when the cells are exposed to the baffled shake flask moderate and extreme hydromechanical stress models in media with low concentrations of Pluronic F-68 (0.025g/L). Although the mAb-producing NS0 cells were more sensitive to the moderate hydromechanical stress conditions in media with 0g/L BSA and 0.025g/L Pluronic F-68 compared to the Parental NS0 cells, these results support the fact that Parental NS0 cells can be used to investigate the protective mechanism of action of BSA on mAb-producing NS0 cells. A recent study conducted by Peng et al. showed that two CHO cell lines did not experience the same damage in media with an ineffective lot of Pluronic F-68 (Peng et al., 2014). This agrees with the results gathered in this project showing that that the Parental and mAb-producing NS0 cell lines have different growth profiles in the moderate hydromechanical stress model.
Figure 3.17: BSA promotes the growth of mAb-producing NS0 cells experiencing moderate hydromechanical stress in media with low Pluronic F-68 concentrations. MAb-producing NS0 cells were exposed to moderate hydromechanical stress (135 RPM) in 125ml baffled shake flasks with a working volume of 24ml. Media with 0 and 1g/L BSA was tested with 0.025 and 1g/L Pluronic F-68. Error bars depict the standard deviation of three replicate flasks. Figure 3.17A shows the growth profiles for NS0 cells. Figure 3.17B shows the LDH Activities for NS0 cells after approximately 2 days in culture. Error bars depict the standard deviation of at least two replicate flasks.
**Figure 3.18:** BSA protects mAb-producing NS0 cells exposed to extreme hydromechanical stress in media with low Pluronic F-68 concentrations. MAb-producing NS0 cells were exposed to extreme hydromechanical stress (175 RPM) in 125ml baffled shake flasks with a working volume of 24ml. Media with 0 and 1g/L BSA was tested with 0.025 and 1g/L Pluronic F-68. Figure 3.18A shows the percentage reduction in VCD for NS0 cells, p=0.0003 (**), student T-test, n=3. Figure 3.18B shows the LDH Activity after 30 minutes exposure for NS0 cells, p=0.001 (**), student T-test, n=3. Error bars depict the standard deviation of three replicate flasks.
3.7 Discussion

In this chapter two hydromechanical stress models were developed in baffled shake flasks to test the protective effects of BSA on NS0 cells. Firstly it was shown that BSA has no effect on the growth of NS0 cells under various culture conditions (regular shake flasks, AMBR15 mini-bioreactor system and baffled shake flasks) in a typical industrial media supplemented with 1g/L Pluronic F-68. These results suggest that BSA does not have an essential role in the culture of NS0 cells in media with high concentrations of Pluronic F-68. It was hypothesised that BSA may only have an effect on NS0 cells when the protection afforded by Pluronic F-68 is limited. Therefore, the concentrations of Pluronic F-68 were reduced in the NS0 cell culture media to test the effects of BSA on NS0 cells experiencing damage in media with limited protection from Pluronic F-68.

Firstly, an AMBR15 mini-bioreactor was used to test the effects of BSA on NS0 cells in media with reduced concentrations of Pluronic F-68. The results suggested that BSA has a growth promoting effect for NS0 cells in media with reduced concentrations of Pluronic F-68; however the AMBR15 was not used for further investigations into the effects of BSA on NS0 cells due to the high cost of the disposable mini-bioreactors and limited equipment availability.

Two high throughput and cost-effective hydromechanical stress models were developed in baffled shake flasks to test the protective effect of BSA on NS0 cells in media with reduced concentrations of Pluronic F-68. The damage that the cells were exposed to in the hydromechanical stress models was controlled by altering the speed at which the baffled shake flasks were orbiting. The objective of the two models was to test the protective effect of BSA on NS0 cells experiencing different levels of hydromechanical stress which would potentially reduce the product titre in...
a large-scale bioreactor. Model 1 was a moderate hydromechanical stress model where the growth of the NS0 cells was reduced due to hydromechanical stress. Model 2 was an extreme hydromechanical stress model where the VCD of the NS0 cells was reduced after short exposure to the hydromechanical stress. It was shown that the damage experienced by the NS0 cells was due to the presence of the baffles in the shake flasks by exposing the cells to the same conditions in non-baffled shake flasks. The growth of the NS0 cells was significantly improved in the moderate hydromechanical stress model and there was a significantly lower reduction in VCD in the extreme hydromechanical stress model for NS0 cells exposed to the stress in non-baffled shake flasks compared to baffled shake flasks. The damage experienced by the NS0 cells in the baffled shake flasks compared to non-baffled shake flasks under the same conditions may be due to the greater power input into the cell culture (Peter, Suzuki, Rachinskiy et al., 2006; Peter, Suzuki and Büchs, 2006), the formation of bubbles (Büchs, 2001) or damage at the air-liquid interface due to the rapidly moving air-liquid surface (Chisti and Moo-Young, 1993a) in the baffled shake flasks.

BSA was shown to increase the growth of NS0 cells in the moderate hydromechanical stress in conditions with low concentrations of Pluronic F-68 (0.025g/L). BSA also offered dose dependent protection to the NS0 cells in the extreme hydromechanical stress model in media with low concentrations of Pluronic F-68 (≤0.1g/L). The effect of BSA against the moderate and extreme hydromechanical stress was also confirmed using mAb-producing NS0 cells, however the mAb-producing NS0 cells were not capable of proliferation in the moderate hydromechanical stress model in media with 0g/L BSA and 0.025g/L Pluronic F-68. These results suggest that the damage experienced in these
hydromechanical stress models is cell line dependent, which is in agreement with a report by Peng et al. who showed that a commercially available CHO cell line and a mAb-producing CHO cell line had different growth profiles when exposed to hydromechanical stress conditions in media with a lot of Pluronic F-68 which was known to have limited protection (Peng et al., 2014).

The results gathered in this chapter were in agreement with previous investigations showing that BSA offers protection to cells experiencing damage in culture (Hülscher et al., 1990). However, BSA was shown to have no effect on the cells in media with 1g/L Pluronic F-68 in the moderate and extreme hydromechanical stress models which supports the hypothesis that the effect of BSA is made redundant by the presence of high concentrations of Pluronic F-68 in the media. Therefore if BSA is removed from the NS0 cell culture media there would be increased pressure on Pluronic F-68 to protect the cells from the hydromechanical damage in the bioreactor. This could be problematic as the protective effect of Pluronic F-68 has been shown to be limited at high cell concentrations (Ma et al., 2004), and there have also been reports of batches of Pluronic F-68 with limited protection for cells in bioreactors (Peng et al., 2014; Apostolidis et al., 2015).

In summary, in this chapter it was shown that BSA is not essential for the growth of NS0 cells in various cell culture conditions (shake flask, sparged and agitated mini-bioreactor and baffled shake flasks) in media with 1g/L of an effective lot of Pluronic F-68. However, it was also shown that BSA protects NS0 cells from hydromechanical stress in media with limited protection from Pluronic F-68. The baffled shake flask hydromechanical stress models developed in this chapter will be used in Chapter 4 to investigate the protective mechanism(s) of action of BSA on NS0 cells in media with limited protection from Pluronic F-68.
Chapter 4:

Investigations into the protective mechanism(s) of action of BSA on NS0 cells
4.1 Abstract

BSA was shown to protect NS0 cells from moderate and extreme hydromechanical stress in baffled shake flasks; therefore these experiments were conducted to determine the protective mechanism(s) of action of BSA in media with limited protection from Pluronic F-68. A number of potential mechanisms of action for BSA were investigated, the first experiments focused on determining whether BSA aids nutrient provision for NS0 cells, however BSA was shown to have no effect on the levels of amino acids in the NS0 cell culture media. BSA was shown to bind cholesterol in the NS0 culture media. Although NS0 cells are auxotrophic for cholesterol, the protective effect of BSA was evident in media without cholesterol (CLC) supplementation, therefore the protective effect of BSA is not mediated by the presence of cholesterol in the media. The protective effect of BSA was shown to be afforded and lost rapidly for NS0 cells exposed to moderate and extreme hydromechanical stress in baffled shake flasks. Therefore, the protection afforded by BSA is due to a physical or fast-acting biological protective effect, however as BSA offered minimal protection for NS0 cells exposed to laminar shear the remaining experiments focused on physical or fast-acting biological protective mechanisms relevant to the damage experienced in baffled shake flasks. A number of physical protective mechanisms of action of BSA were investigated; BSA caused a dose dependent reduction in the surface tension of NS0 cell culture media with low concentrations of Pluronic F-68, however BSA had no effect on the surface tension of media supplemented with 1g/L Pluronic F-68. BSA and Pluronic F-68 had no effect on the dynamic viscosity of the NS0 cell culture media, however Pluronic F-68 and BSA (in media with low concentrations of Pluronic F-68) caused bubble stabilisation in the extreme hydromechanical stress model in baffled shake flasks.
Next, the potential fast-acting biological protective mechanisms of action of BSA were investigated; BSA associated with NS0 cells in culture in a dose dependent manner in media with low and high concentrations of Pluronic F-68. The association of BSA with NS0 cells was shown to be transient, as the association was interrupted by culturing the cells in media with 0g/L BSA for 1 hour. BSA caused a dose dependent reduction in the NS0 cell membrane hydrophobicity in media with 0 or 0.025g/L Pluronic F-68. Denatured BSA afforded increased protection for NS0 cells from extreme hydromechanical stress compared to native BSA, therefore the protective effect of BSA is not dependent on the protein secondary and tertiary structure. Denatured BSA caused a greater reduction in surface tension compared to native BSA. Therefore, it was concluded that the protective effect of BSA may be due to the protein acting as a surfactant, reducing the media surface tension and also coating the NS0 cells reducing the cell membrane hydrophobicity. Methyl cellulose, a synthetic surfactant, was shown to protect NS0 cells from the hydromechanical stress models (in media with reduced concentrations of Pluronic F-68), showing that the protective effect of BSA could be replaced by the addition of a synthetic surfactant to the media.
4.2 Introduction

The results gathered in Chapter 3 showed that BSA protects NS0 cells from hydromechanical stress in baffled shake flasks. Therefore the aims of the experiments carried out in this chapter were to identify the protective mechanism(s) of action of BSA for NS0 cells experiencing damage.

Serum has been historically added to cell culture media as a nutrient source for cells (hormones, growth factors etc.) and although BSA is added to some large-scale mammalian cell culture media, its exact function has not been clearly established (Francis, 2010). The addition of animal sourced materials to industrial cell culture for human therapeutics is not favourable mainly due to the increased risk of contamination of the cell culture media with animal sourced bacteria, viruses and prions (Merten, 2002). There are a number of cell lines which have been successfully cultured in large-scale bioreactors without the provision of serum or BSA; therefore biopharmaceutical companies are striving to remove all animal sourced materials (including BSA) from large-scale mammalian cell cultures used to produce human therapeutic proteins.

There have been a number of reports showing a concentration dependent protective effect for serum in aerated and bubble-free suspension cultures using various different mammalian cell types (Croughan et al., 1989; Handa-Corrigan et al., 1989; Ramirez and Mutharasan, 1990; Michaels et al., 1991; Martens et al., 1992; Ramirez and Mutharasan, 1992). The protective effect of serum has been shown to be physical or fast-acting biological in nature, due to the addition of serum having an immediate protective effect for cells exposed to damage (Michaels et al., 1991; Martens et al., 1992; van der Pol et al., 1992). There have also been reports suggesting that serum also affords biological protection which takes longer to elicit.
an effect (Michaels et al., 1991). Therefore the protective effect of serum may physical or biological in nature (or possibly both) and the protective mechanism may be cell line dependent.

Serum albumin is the most abundant protein in serum at approximately 50mg/ml, which makes up approximately 60% of the total protein content (Kaneko, 1997; Francis, 2010). The majority of studies show a protective effect for whole serum, and there have been a number of different protective mechanisms of action (both physical/fast-acting biological and biological) suggested for serum; however there are not many reports showing these effects for BSA. The addition of serum has been shown to increase the dynamic viscosity of cell culture media; however the increase in viscosity is negligible (Aunins and Henzler, 1993). Some reports associate the protective effect of serum with the turbulence dampening effect, due to the increase in viscosity (Croughan et al., 1989); however other reports have suggested that there is no relationship between the protective effect of serum and the increase in viscosity (Goldblum et al., 1990). Serum and BSA have also been shown to act as bubble stabilisers stabilise bubbles, which protects cells by allowing the cells to drain out of the bubble before the bubble ruptures (Michaels et al., 1995a; Michaels et al., 1995b; Chisti, 2000). Bubble stabilisation also allows the film formed by the bubble to become thinner, thus resulting in a less damaging bubble-burst event (Handa-Corrigan et al., 1989). It has been shown that the reduction of media surface tension (by the addition of surfactants) inhibits the association of cells with the air-liquid interface, therefore protecting the cells from potentially damaging bubble burst conditions (Chattopadhyay et al., 1995a; Chattopadhyay et al., 1995b), which may be a mechanism by which BSA (and serum) protects cells from damaging bubble burst conditions. The function of serum and BSA has also been
associated with nutrient provision, and it has been postulated that BSA may be involved in the provision of cholesterol for cholesterol deficient cell lines (e.g. NS0 cells), as serum albumin aids the transfer of cholesterol to lipoproteins (Zhao and Marcel, 1996).

A number of fast-acting biological protective mechanisms have been suggested for serum, including coating cells in culture, which has been linked to a protective effect due to the fact that damaging fluid eddies can no longer penetrate into the vicinity of the cell membrane (Croughan et al., 1989). Coating the cells with serum may also reduce the cell membrane hydrophobicity, which inhibits the adhesion of cells to bubbles and to the air-liquid interface in bioreactor culture, therefore protecting the cells from the damaging bubble burst event (Wu et al., 1997). Pluronic F-68 and serum have been shown to reduce the membrane hydrophobicity of cells (Wu et al., 1997; Ghebeh et al., 2002). Serum and Pluronic F-68 have also been shown to reduce the plasma membrane fluidity of a hybridoma cell line, therefore making the cells more resistant to damage (Ramírez and Mutharasan, 1990; Ramírez and Mutharasan, 1992).

Most of the published research focuses on the protective effects and mechanisms of action of serum for cells experiencing damage during culture. Therefore there is a gap in knowledge of the effects of BSA on cells in large-scale industrial cell culture conditions. Here, the objective was to investigate the potential nutritional, physical and fast-acting biological protective mechanisms of action of BSA on NS0 cells experiencing damage. Although there has been some research conducted in the past into the protective mechanisms of action of serum, the protective mechanism of action of BSA for cells exposed to damage is poorly
understood. Attempts were also made to replace the protective effect of BSA with a non-animal derived compound.
4.3 Investigations into BSA nutrient provision for NS0 cells

4.3.1 BSA does not affect the uptake or release of amino acids by NS0 cells

While the hydromechanical stress models described in Chapter 3 were under development, several experiments were carried out to test whether BSA plays a role in nutrient provision for the NS0 cells. The first experiment tested whether BSA improved the uptake of amino acids by NS0 cells from the cell culture media. NS0 cells were grown in non-baffled shake flasks for 14 days in media with 0 and 1g/L BSA (and 1g/L Pluronic F-68). The growth profiles for these flasks were shown previously in Section 3.3.1. The concentrations of amino acids in the media were analysed by NMR analysis after 1, 6, 9 and 14 days in culture.

The levels of amino acids in the NS0 cell culture media were not affected by the presence of BSA in the media, as shown in Figure 4.1. The concentrations of some of the amino acids increased over time (e.g. aspartate and proline) as they are metabolites released by the cells, whereas the concentrations of other amino acids decreased with time (e.g. arginine and isoleucine) as they are nutrients consumed by the cells. The results show that the levels of leucine and tryptophan are limiting in the media as they are completely depleted after 14 days. It was concluded from these results that BSA had no impact on the uptake or release of amino acids by NS0 cells.
Figure 4.1: BSA has no effect on the amino acid levels in media during NS0 cell culture. MAb-producing NS0 cells were cultured with 0 or 1g/L BSA in 250ml shaking flasks with a working volume of 100ml under standard growth conditions; 1g/L Pluronic F-68, 10% CO₂, 37°C and 110 RPM and 0.3x10⁶ cells/ml seeding density. Samples were taken on day 1, 6, 10 and 14 for NMR analysis to quantify the amino acid levels in the media.
4.3.2 BSA associates with cholesterol in NS0 cell culture media

Next, it was determined whether BSA associates with cholesterol in NS0 cell culture media after it was observed that media with 0g/L BSA became turbid (compared to media with 1g/L BSA) when cholesterol (CLC) was added during media formulation. It was hypothesised that CLC releases a proportion of its cholesterol from the cyclodextrin compounds when it is added to the media and that the presence of BSA may solubilise the released cholesterol and reduce the media turbidity. This reduction in turbidity was only quantified visually. Therefore this test was conducted to determine whether cholesterol binds to BSA in NS0 cell culture media supplemented with CLC. BSA was purified from NS0 cell culture media (supplemented with 2ml/L 1000X CLC) by IP using Agarose beads conjugated with anti-BSA antibody, as shown in Figure A.1 (Appendix A) and the immunoprecipitated BSA was tested for the presence of cholesterol using an Amplex Red Cholesterol Quantification Kit. Two control IPs were also carried out using; i) media with 0g/L BSA and 2ml/L CLC and ii) media with 1g/L BSA and 0ml/L CLC.

Cholesterol associated with BSA in media supplemented with CLC, as shown in Figure 4.2. The result in the first control sample with 0g/L BSA and 2ml/L CLC shows that there is a minimal amount of cholesterol purified by the IP process. The second control sample with 1g/L BSA and 0ml/L CLC shows that there is no cholesterol bound to BSA in media not supplemented with CLC. This result shows that BSA associates with cholesterol in NS0 cell culture media supplemented with CLC. Next, the extreme hydromechanical stress model developed in Chapter 3 was used to investigate whether cholesterol was required to be bound to BSA in order for BSA to elicit its protective effect.
Figure 4.2: BSA binds to cholesterol in NS0 culture media supplemented with CLC. This figure shows the cholesterol bound to BSA which was purified by IP from NS0 cell media supplemented with 1g/L BSA and 2ml/L 1000X CLC. Two control IPs were used in this experiment: i) Media with 0g/L BSA and 2ml/L CLC, and ii) Media with 1g/L BSA and 0ml/L CLC. The cholesterol content of the immunoprecipitated samples was quantified using an Amplex Red Cholesterol Quantification Kit.
4.3.3 BSA offers protection to NS0 cells in media not supplemented with CLC

Having determined that BSA associates with cholesterol in the NS0 cell culture media, the next objective was to test whether the protection afforded by BSA was dependent on the presence of CLC in the NS0 cell culture media. NS0 cells were exposed to extreme hydromechanical stress (as described in Section 3.4.9) in media with 0.025g/L Pluronic F-68 and 0 or 1g/L BSA in media supplemented with various concentrations of 1000X CLC (0, 2 and 4ml/L).

The presence of CLC in the media provided dose dependent protection for NS0 cells experiencing extreme hydromechanical stress, as shown in Figure 4.3. The protection afforded by CLC was seen by a lower reduction in VCD with increasing concentrations of CLC. This protective effect was evident for NS0 cells cultured in media with both 0 and 1g/L BSA, therefore it was concluded that the protective effect afforded by CLC is not dependent on the presence of BSA in the media.

NS0 cells exposed to extreme hydromechanical stress in media with 0g/L BSA and 0ml/L CLC were highly susceptible to damage with approximately 95% reduction in VCD, compared to 30% reduction for NS0 cells exposed in media with 1g/L BSA and 0ml/L CLC, as seen in Figure 4.3. Therefore, BSA offered protection to the NS0 cells in media supplemented with 0ml/L CLC, which shows that the protective effect of BSA is not dependent on the presence of CLC in the media.

The protective effect of BSA was not evident in media with high concentrations of CLC (4ml/L). Therefore, it was concluded that high concentrations of CLC may reduce the susceptibility of NS0 cells to damage caused by hydromechanical stress.
Figure 4.3: BSA protective effect is independent of CLC supplementation for NS0 cells exposed to extreme hydromechanical stress. Parental NS0 cells exposed to extreme hydromechanical stress conditions (175 RPM) in baffled shake flasks in media with various concentrations of 1000X CLC (0, 2 and 4ml/L) in media with 0.025g/L Pluronic F-68 with 0 and 1g/L BSA. The p-value for the cells exposed in media with 0ml/L CLC is p=0.0003 (**), and the p-value for the cells exposed in media with 2ml/L CLC is p=0.003(*), student T-test, n=3. Error bars denote the standard deviation of three replicate flasks.
4.3.4 Increased concentrations of Pluronic F-68 cause the protective effects of CLC for NS0 cells to be redundant

After showing that NS0 cells are highly susceptible to damage in media with low concentrations of Pluronic F-68 and 0g/L BSA and 0ml/L CLC, the next experiment was set up to determine whether increased concentrations of Pluronic F-68 could reduce the susceptibility of the NS0 cells to the hydromechanical stress in media with 0ml/L CLC. This experiment was therefore testing whether the protection afforded by CLC was due to its cholesterol provision capabilities, or due to another protective mechanism of action. The NS0 cells were exposed to the extreme hydromechanical stress conditions (as described in Section 3.4.9) in media with 0.025, 0.1 and 1g/L Pluronic F-68, 0g/L BSA and 0ml/L CLC.

High concentrations of Pluronic F-68 (1g/L) protected the NS0 cells from damage in media with 0ml/L CLC, this protective effect was evident as there was no reduction in VCD and low LDH Activity (approx. 25 LU) for NS0 cells exposed to extreme hydromechanical stress in media with 1g/L Pluronic F-68, as shown in Figure 4.4A and Figure 4.4B respectively. There was a reduction in the VCD and an increase in LDH Activity for NS0 cells exposed to the hydromechanical stress in media with 0.025g/L Pluronic F-68 (approx. 97% reduction in VCD and approx. 345 LU) and 0.1g/L Pluronic F-68 (approx. 44% reduction in VCD and approx. 165 LU) in media with 0ml/L CLC, as shown in Figure 4.4A and Figure 4.4B respectively.

Therefore, it was concluded that the protective effect of CLC is not evident in media supplemented with 1g/L Pluronic F-68. This suggests that the protective effect afforded by CLC against extreme hydromechanical stress for NS0 cells is not likely to be due to the provision of cholesterol to the cells and its protective effect can be substituted by the addition of Pluronic F-68.
Figure 4.4: High concentrations of Pluronic F-68 cause the protective effects of CLC on NS0 cells exposed to extreme hydromechanical stress to be redundant. Parental NS0 cells were exposed to extreme hydromechanical stress (175 RPM) in media without CLC supplementation. Media with 0g/L BSA and various concentrations of Pluronic F-68 (0.025, 0.1 and 1g/L) were tested. Figure 4.4A shows the percentage reduction in VCD after 60 minutes exposure. Figure 4.4B shows the LDH Activity results after 30 minutes exposure. Error bars denote standard deviation of three replicate flasks.
4.4 Determination of whether BSA protection is afforded rapidly for NS0 cells experiencing hydromechanical stress

4.4.1 BSA offers rapid protection for NS0 cells exposed to moderate hydromechanical stress

As the results gathered thus far suggested that the protective effect of BSA is not likely to be due to the provision of nutrients to the NS0 cells, the hydromechanical stress models in baffled shake flasks (developed in Chapter 3) were used to determine whether the protective effect of BSA is afforded rapidly or acquired by extended exposure during culture. NS0 cells were originally cultured in media with 0g/L BSA and 1g/L Pluronic F-68 and exposed to moderate hydromechanical stress conditions (as described in Section 3.4.5) in media with 0.025g/L Pluronic F-68 and 0 or 1g/L of BSA. Similarly, NS0 cells originally cultured in media with 1g/L BSA and 1g/L Pluronic F-68 were exposed to the moderate hydromechanical stress conditions in media with 0.025g/L Pluronic F-68 and 0 or 1g/L BSA. The cells in each experiment were washed twice with media (with 0g/L BSA and 0g/L Pluronic F-68) to avoid any BSA or Pluronic F-68 being carried over.

NS0 cells originally cultured in media with 0g/L BSA were less susceptible to the moderate hydromechanical stress in media with 1g/L BSA. This result is seen by an increase in the maximum VCD of approx. 33% (Figure 4.5A) and a reduction in the doubling time of approx. 14% (Figure 4.5B) for the NS0 cells in media with 1g/L BSA compared to cells exposed in media with 0g/L BSA. There was also a decrease in LDH Activity for NS0 cells in media with 1g/L BSA (approx. 85 LU) compared to 0g/L BSA (approx. 190 LU), as shown in Figure 4.5C, which suggests that the NS0 cells in media with 1g/L BSA are experiencing less membrane damage.
under the moderate hydromechanical stress compared to cells in media with 0g/L BSA.

NS0 cells originally cultured in media with 1g/L BSA were more susceptible to the moderate hydromechanical stress in media with 0g/L BSA. This result is seen by a reduction in the maximum VCD of approx. 45% (Figure 4.6A) and an increase in the doubling time of approx. 47% (Figure 4.6B) for the cells in media with 0g/L BSA compared to cells exposed in media with 1g/L BSA. There was also an increase in LDH Activity for the cells in media with 0g/L BSA (approx. 255 LU) compared to cells exposed in media with 1g/L BSA (approx. 90 LU), as shown in Figure 4.6C, which suggests that the NS0 cells in media with 1g/L BSA were experiencing less membrane damage than the cells in media with 0g/L BSA.

It was concluded from these results that the protective effect of BSA is afforded and lost rapidly for NS0 cells exposed to moderate hydromechanical stress. These results are in agreement with previous reports showing that serum has an immediate protective effect for cells experiencing damage (Michaels et al., 1991; Martens et al., 1992; van der Pol et al., 1992). Following on from these results tests were conducted to determine whether BSA offers rapid protection to NS0 cells exposed to the extreme hydromechanical stress model in baffled shake flasks.
Figure 4.5: BSA protection for NS0 cells exposed to moderate hydromechanical stress is afforded rapidly for cells originally cultured in media with 0g/L BSA. Parental NS0 cells were exposed to hydromechanical stress by shaking at 135 RPM in 125ml baffled shake flasks with a working volume of 24ml. Media with 0.025g/L Pluronic F-68 and 0 or 1g/L BSA was used in this experiment. NS0 cells originally cultured with 0g/L BSA were exposed to hydromechanical stress in media with 0 and 1g/L BSA. Figure 4.5A shows the growth profiles for NS0 cells. Figure 4.5B shows the doubling times for the NS0 cells after approximately three days in culture, p=0.0009 (**), student T-test, n=3. Figure 4.5C shows the LDH Activity for cells after approximately 2 days in culture, p=0.000002 (****), student T-test, n=3. Error bars denote standard deviation of three replicate flasks.
Figure 4.6: BSA protection for NS0 cells exposed to moderate hydromechanical stress is rapidly lost for cells originally cultured in media with 1g/L BSA. Parental NS0 cells were exposed to hydromechanical stress by shaking at 135 RPM in 125ml baffled shake flasks with a working volume of 24ml. Media with 0.025g/L Pluronic F-68 and 0 or 1g/L BSA was used in this experiment. NS0 cells originally cultured with 1g/L BSA were exposed to hydromechanical stress in media with 0 and 1g/L BSA. Figure 4.6A shows the growth profiles for NS0 cells. Figure 4.6B shows the doubling times for the NS0 cells after approximately three days in culture, p=0.0009 (**), student T-test, n=3. Figure 4.6C shows the LDH Activity for cells after approximately 2 days in culture, p=0.000004 (****), student T-test, n=3. Error bars denote standard deviation of three replicate flasks.
4.4.2 BSA offers rapid protection for NS0 cells exposed to extreme hydromechanical stress

As the protective effect of BSA was shown to be afforded rapidly to NS0 cells exposed to moderate hydromechanical stress, the next objective was to test whether the protective effect of BSA is also afforded rapidly for NS0 cells exposed to the extreme hydromechanical stress model in baffled shake flasks. NS0 cells were cultured in media with 0g/L BSA and 1g/L Pluronic F-68 from vial thaw and exposed to the extreme hydromechanical stress model (as described in Section 3.4.9) in media with 0.025g/L Pluronic F-68 and 0 or 1g/L BSA. Similarly, NS0 cells were cultured in media with 1g/L BSA and 1g/L Pluronic F-68 from vial thaw and exposed to extreme hydromechanical stress conditions in media with 0.025g/L Pluronic F-68 and 0 or 1g/L BSA.

The NS0 cells originally cultured in media with 0g/L BSA were less susceptible to the extreme hydromechanical stress in media with 1g/L BSA compared to cells in media with 0g/L BSA. This result is seen by a lower reduction in VCD for cells in media with 1g/L BSA (approx. 14% reduction in VCD) compared to cells exposed in media with 0g/L BSA (approx. 70% reduction in VCD), as shown in Figure 4.7A. The cells in media with 1g/L BSA also had a lower LDH Activity (approx. 70 LU) compared to cells in media with 0g/L BSA (approx. 165 LU), as shown in Figure 4.7B. Therefore, it was concluded that the protective effect of BSA was afforded rapidly against the extreme hydromechanical stress for NS0 cells originally cultured with 0g/L BSA.

The NS0 cells originally cultured in media with 1g/L BSA were more susceptible to the extreme hydromechanical stress in media with 0g/L BSA, as shown by a greater reduction in VCD for cells in media with 0g/L (approx. 48%
reduction in VCD) compared to cells exposed in media with 1g/L BSA (approx. 3% reduction in VCD), as shown in Figure 4.7C. The NS0 cells in media with 0g/L BSA also had a greater LDH Activity (approx. 145 LU) compared to cells in media with 1g/L BSA (approx. 50 LU), as shown in Figure 4.7D. It was concluded from these results that the protective effect of BSA is rapidly lost against the extreme hydromechanical stress for NS0 cells originally cultured with 1g/L BSA.

These results demonstrated that the protective effect of BSA is likely due to a physical or fast-acting biological protective mechanism of action for NS0 cells exposed to hydromechanical stress in baffled shake flasks. Physical protective mechanisms are due to the presence of BSA altering the hydromechanical stress conditions experienced by the cells, e.g. by increasing the viscosity of the media resulting in less damaging hydromechanical stress conditions (Cherry and Papoutsakis, 1988; Croughan et al., 1989; McQueen and Bailey, 1989) or reducing the surface tension of the media which inhibits the association of cells to damaging air-liquid interfaces (Chattopadhyay et al., 1995a; Chattopadhyay et al., 1995b) and also causes bubble stabilisation which allows cells to drain out of the bubbles before the bubble burst event occurs (Michaels et al., 1995a; Michaels et al., 1995b; Chisti, 2000). Fast-acting biological protective mechanisms are due to BSA affecting the cells in a rapid manner which reduces the susceptibility of the cells to damage, e.g. BSA coating the cells acting as a boundary between the damaging conditions and the cells (Croughan et al., 1989) or reducing the membrane hydrophobicity of the cells therefore inhibiting the association of BSA with damaging air-liquid interfaces (Wu et al., 1997; Ghebeh et al., 2002). These protective mechanisms of action are referred to as fast-acting biological protective mechanisms of action as they are afforded rapidly and do not require any cellular response (e.g. signal transduction).
The results gathered here are in agreement with previous reports showing that serum has a physical or fast-acting biological protective mechanism of action which is afforded rapidly for cells exposed to damaging conditions (Michaels *et al.*, 1991; Martens *et al.*, 1992; van der Pol *et al.*, 1992).
**Figure 4.7:** BSA protection for NS0 cells exposed to extreme hydromechanical stress is afforded and lost rapidly. Parental NS0 cells were exposed to hydromechanical stress by shaking at 175 RPM for 60 minutes in 125ml baffled shake flasks with a working volume of 24ml. Media with 0.025g/L Pluronic F-68 and 0 or 1g/L BSA was used in this experiment. NS0 cells originally cultured with 1g/L BSA were exposed to hydromechanical stress in media with 0 and 1g/L BSA. NS0 cells originally cultured with 0g/L BSA were exposed to hydromechanical stress in media with 0 and 1g/L BSA. Figure 4.7A shows the percentage reduction in VCD for cells originally cultured with 0g/L BSA, p=0.003 (**), student T-test, n=3. Figure 4.7B shows the LDH Activity for cells originally cultured with 0g/L BSA, p=0.002 (**), student T-test, n=3. Figure 4.7C shows the percentage reduction in VCD for cells originally cultured with 1g/L BSA, p=0.0006 (**), student T-test, n=3. Figure 4.7D shows the LDH Activity for cells originally cultured with 1g/L BSA, p=0.004 (**), student T-test, n=3. Error bars denote standard deviation of three replicate flasks.
4.5 Assessment of BSA protection against laminar shear stress

4.5.1 BSA offers limited protection for NS0 cells exposed to laminar shear stress

Laminar shear devices have been widely used to expose cells to well defined levels of shear (Michaels et al., 1991; Joshi et al., 2006). There are a number of limitations associated with the use of laminar shear devices, including the fact that the devices have limited oxygenation capabilities; therefore experiments must be restricted to short periods and there is difficulty correlating the results obtained in the laminar shear devices with the results seen in turbulent stress models (Joshi et al., 1996). However, laminar shear devices have been used to further our understanding of the effects and cellular responses involved when cells are exposed to shearing conditions (Schürch et al., 1988; Goldblum et al., 1990; Mardikar and Niranjan, 2000). A concentric cylinder laminar shear device was designed and fabricated by Michael Collins in Stokes Institute, University of Limerick to test the protective effects of BSA on NS0 cells exposed to defined magnitudes of laminar shear. The laminar shear device has no air-liquid interface and therefore exposes the NS0 cells to damage without the possibility of bubble-associated damage. The experimental conditions (shear stress magnitude, exposure time) were evaluated to test the protective effect of BSA on NS0 cells, as shown in Figure A.2 (Appendix A). The optimised conditions for testing the protective effect of BSA under laminar shear stress were: 2.5Pa of laminar shear and 3 hours exposure time. The NS0 cells were exposed to laminar shear in media with 0.025g/L Pluronic F-68.

BSA protected NS0 cells exposed to laminar shear, as seen by a lower reduction in TCD for NS0 cells exposed to the laminar shear in media with 1g/L BSA (approx. 49% reduction in TCD) compared to 0g/L BSA (approx. 58% reduction in TCD), as shown in Figure 4.8. BSA at 1g/L offered similar protection...
for the NS0 cells under all magnitudes of laminar shear stress and all exposure times, and increasing concentrations of BSA also offered increased protection to NS0 cells exposed to laminar shear as shown in Figure A.2 (Appendix A). The protective effect of BSA for NS0 cells exposed to laminar shear was also shown to be afforded rapidly as shown in Figure A.3 (Appendix A). Therefore, the protective effect of BSA is likely to be physical or fast-acting biological in nature for NS0 cells exposed to laminar shear. As there is no air-liquid interface in the laminar shear device, the protective effect of BSA against laminar shear is not likely to be due to the BSA causing a reduction in the NS0 cell membrane hydrophobicity or reducing the media surface tension. The protective effect of BSA on NS0 cells exposed to laminar shear is likely to be associated with membrane reinforcement due to BSA coating the cells (as shown in Section 4.7.1).

The conclusions drawn from these experiments were that BSA protects NS0 cells from laminar shear; however the protection afforded by BSA is not as effective under laminar shear compared to conditions of hydromechanical stress in baffled shake flasks. Therefore, the protective effect afforded by BSA in the baffled shake flask models may be due to protection from damaging conditions which are not experienced in the laminar shear device (e.g. damage caused at the air-liquid interface). Therefore investigations into the protective mechanism of action of BSA focused on mechanisms of action which involve damage at the air-liquid interface (e.g. media surface tension and viscosity and cell membrane hydrophobicity etc.).
Figure 4.8: BSA affords limited protection for NS0 cells experiencing laminar shear stress. Parental NS0 cells were exposed to 2.5Pa of laminar shear for 3 hours with an initial seeding density of 1x10^6 cells/ml. These results show the percentage reduction in TCD for NS0 cells exposed to the laminar shear in media with 0 and 1g/L BSA and 0.025g/L Pluronic F-68, p=0.013 (*), student T-test, n=3. Error bars denote standard deviation of three replicate tests.

4.5.2 Protection afforded by Pluronic F-68 is limited for NS0 cells exposed to laminar shear stress

NS0 cells were exposed to 2.5Pa of laminar shear for 3 hours in media with 0g/L BSA and varying concentrations of Pluronic F-68 (0, 0.025, 0.1, 0.5, 1 and 2g/L). The results in Figure 4.9 show that Pluronic F-68 affords protection to the NS0 cells; however the protective effect of Pluronic F-68 is exhausted at concentrations above 0.5g/L. The reduction in TCD was approx. 72% for cells exposed to laminar shear in media with 0g/L Pluronic F-68, whereas the reduction in TCD was approx. 25% for cells in media with 0.5, 1 and 2g/L Pluronic F-68. This is in contrast to the results seen for cells exposed to hydromechanical stress in baffled shake flasks, where 1g/L completely protected the NS0 cells from the damage. Therefore, it was concluded that the protective effect of Pluronic F-68 may have a number of
mechanisms of action and that the protective mechanism(s) of action of Pluronic F-68 may be different in laminar shear compared to the protective mechanism(s) of action against hydromechanical stress in baffled shake flasks.

Figure 4.9: Protection afforded by Pluronic F-68 is limited for NS0 cells exposed to laminar shear stress. Parental NS0 cells were exposed to 2.5Pa of laminar shear for 3 hours in media with 0g/L BSA and varying concentrations of Pluronic F-68 (0, 0.025, 0.1, 0.5, 1 and 2g/L). The results show the percentage reduction in TCD for the samples.
4.6 Investigating the potential physical protective mechanisms of BSA on NS0 cells

4.6.1 BSA and Pluronic F-68 do not affect the dynamic viscosity of NS0 cell culture media

Having determined that the protective effect for BSA was afforded (and lost) rapidly for NS0 cells exposed to hydromechanical stress, it was hypothesised that the protective effect of BSA was likely to be due to a physical or fast-acting biological protective mechanism of action. The results gathered in the laminar shear device also suggested that although BSA had a protective effect for NS0 cells exposed to laminar shear, the protective effect of BSA for NS0 cells exposed to hydromechanical stress in baffled shake flasks was greater. It was hypothesised from these results that the protective effect of BSA in the hydromechanical models is likely to be against damage which is not experienced in the laminar shear device (e.g. damage at the air-liquid interface). To test this hypothesis investigations were carried out into the potential physical protective mechanisms of action of BSA against damage at the air-liquid interface. The first test investigated whether BSA (and Pluronic F-68) affected the dynamic viscosity of the NS0 cell culture medium. The effects of different concentrations of BSA (0, 1, 2, 4g/L) were tested in media supplemented with 0 and 1g/L Pluronic F-68.

The addition of BSA to the NS0 cell culture media did not affect the dynamic viscosity of the media, as shown in Table 4.1. The addition of Pluronic F-68 also had no effect on the dynamic viscosity of the media (as shown in Table 4.1). It was concluded from these results that the protection afforded by BSA (and Pluronic F-68) is not likely to be due to an increase in the medium viscosity. This result is in agreement with previous findings suggesting that the protective effect of serum is not
due an increase in medium viscosity (Goldblum et al., 1990). Following on from these results, the next tests evaluated the effects of BSA on the surface tension and bubble stabilisation of the NS0 cell culture media.

<table>
<thead>
<tr>
<th>BSA Concentration</th>
<th>Dynamic Viscosity (mPa.s)</th>
<th>STDev (mPa.s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0g/L</td>
<td>1.10</td>
<td>±0.02</td>
</tr>
<tr>
<td>1g/L</td>
<td>1.12</td>
<td>±0.01</td>
</tr>
<tr>
<td>2g/L</td>
<td>1.11</td>
<td>±0.02</td>
</tr>
<tr>
<td>4g/L</td>
<td>1.10</td>
<td>±0.02</td>
</tr>
<tr>
<td>1g/L Pluronic F-68 (+ 1g/L BSA)</td>
<td>1.09</td>
<td>±0.02</td>
</tr>
</tbody>
</table>

Table 4.1: BSA and Pluronic F-68 do not affect the dynamic viscosity of NS0 cell culture media. The effect of different concentrations of BSA (0, 1, 2 and 4g/L) on the dynamic viscosity of NS0 cell culture media with 0g/L Pluronic F-68 is shown in this Table. The dynamic viscosity of media with 1g/L BSA and 1g/L Pluronic F-68 was also tested. The results shown are the average of seven replicate tests at 20°C.
4.6.2 BSA reduces the surface tension of NS0 cell culture media at low concentrations of Pluronic F-68

The next potential physical protective mechanism of action of BSA tested was the effect of BSA on the surface tension of the NS0 cell culture media. The effects of increasing concentrations of BSA (0, 0.1, 0.5, 1, 2, 3 and 4g/L) were tested on the surface tension of NS0 cell culture media. The effect of BSA on the surface tension of the NS0 cell culture media was also tested in media supplemented with various concentrations (0, 0.025 and 1g/L) of Pluronic F-68. There was no CLC added to these test media.

Media with 0g/L BSA and 0g/L Pluronic F-68 was shown to have approx. the same surface tension as purified water (72mN/m) at 20°C, as shown in Figure 4.10A. The presence of BSA in the media caused a dose dependent reduction in the surface tension of the NS0 cell culture media (with 0g/L Pluronic F-68) from approx. 72mN/m for media with 0g/L BSA to approx. 67.4mN/m for media with 4g/L BSA which is a reduction in surface tension of approx. 6%, as shown in Figure 4.10A. The presence of 1g/L BSA reduced the surface tension of media supplemented with 0.025g/L Pluronic F-68 by approx. 6%, as shown in Figure 4.10B. However, high concentrations of Pluronic F-68 (1g/L) reduced the surface tension of the NS0 cell culture media from approx. 72mN/m to approx. 55mN/m, and BSA had no effect in this media, as shown in Figure 4.10C.

These results show that BSA reduces the surface tension of media; however 1g/L of an effective lot of Pluronic F-68 reduces the surface tension of media to such an extent that the addition of BSA has no effect on the media surface tension. A recent report into Pluronic F-68 lot variations showed that the surface tension of an effective lot of Pluronic F-68 was approx. 8% lower than the surface tension of a lot
of Pluronic F-68 which was shown to have limited protection for cells exposed to hydromechanical damage (Apostolidis et al., 2015). This observation supports the hypothesis that the protective effect of BSA on NS0 cells in media with 0.025g/L Pluronic F-68 is associated with a reduction in media surface tension, as 1g/L BSA was shown to cause a reduction in surface tension of approx. 6% in this media. The result showing that BSA has no effect on the surface tension of media supplemented with 1g/L of an effective lot of Pluronic F-68 is in line with observations gathered in this project showing that the protective effect of BSA is only evident in media with limited protection from Pluronic F-68. It was concluded from these results that the protective effect of BSA in media with low concentrations of Pluronic F-68 may be due to the presence of BSA causing a reduction in the media surface tension. These results suggest that BSA acts as a surfactant in the NS0 cell culture media, therefore removing BSA from the media would add increased pressure on the protective effect of Pluronic F-68. For this reason it has been recommended to have more than one active surfactant added to media used for large-scale cell culture (Hu et al., 2008; Chalmers, 2015).

In another experiment CLC was shown to reduce the surface tension of NS0 cell culture media (supplemented with 0g/L BSA and 0g/L Pluronic F-68) from 72 to approx. 65mN/m (for both 2ml/L and 4ml/L of 1000X CLC), which may contribute to the protection seen in Section 4.3.3 by the addition of CLC to the media, this data is shown in Figure A.4 (Appendix A).
Figure 4.10: BSA reduces the surface tension of NS0 cell culture media at low concentrations of Pluronic F-68. Media with 0ml/L of 1000X CLC was used in this experiment. Figure 4.10A shows the surface tension of NS0 cell culture media with 0g/L Pluronic F-68 and various concentrations of BSA (0, 0.1, 0.5, 1, 2, 3 and 4g/L), 0.5g/L BSA p=0.04 (*), 1g/L BSA p=0.04 (*), 2g/L BSA p=0.03 (*), 3g/L BSA p=0.02 (*) and 4g/L BSA p=0.006 (**), student T-test, n≥3. Figure 4.10B shows the surface tension of NS0 cell culture media with 0.025g/L Pluronic F-68 and 0 or 1g/L BSA. Error bars denote standard deviation of at least three replicate tests.
4.6.3 BSA and Pluronic F-68 cause bubble stabilisation at high RPMs in baffled shake flasks

The final physical protective mechanism of action investigated was the effect of BSA (and Pluronic F-68) on bubble stabilisation in baffled and non-baffled shake flasks. The tests were carried out in baffled and non-baffled shake flasks containing 24ml of media with two concentrations of Pluronic F-68 (0.025 and 1g/L) and 0 or 1g/L BSA. The flasks were shaken at 110, 135, 150 and 175 RPM for 1 hour. The pictures were taken immediately after the orbital shaker was stopped.

No bubbles were formed in baffled and non-baffled shake flasks when media was shaken at 110 and 135 RPM, as shown in Figure 4.11; however there were bubbles formed at 150 and 175 RPM (Figure 4.12). The bubbles were present in media with 1g/L Pluronic F-68 for baffled shake flasks shaken at 150 and 175 RPM (in media with 0 and 1g/L BSA), as shown in Figure 4.12A and Figure 4.12C respectively. There were also bubbles present in media with 0.025g/L Pluronic and 1g/L BSA (Figure 4.12B), however there were no bubbles in the media with 0.025g/L Pluronic and 0g/L BSA (Figure 4.12D). After 4 minutes the bubbles had burst in all the flasks, as shown in Figure A.5 (Appendix A), therefore Pluronic F-68 and BSA act as bubble stabilizers in the NS0 cell culture media. This bubble stabilisation may be linked with the reduction in surface tension as BSA and Pluronic F-68 are were previously shown to reduce the surface tension of the NS0 cell culture media (Figure 4.10). There were minimal bubbles formed in the non-baffled shake flasks shaken at 175 RPM for 1 hour in media with 0 or 1g/L BSA and 1g/L Pluronic F-68 and 0.025g/L Pluronic F-68 as shown in Figure A.6 (Appendix A).
It was concluded that the protective effect of the BSA (and Pluronic F-68) may be associated with bubble stabilisation (due to the reduced surface tension of the media) in the extreme hydromechanical stress model (175 RPM) in baffled shake flasks. The fact that bubbles form when the baffled shake flasks are shaken at higher orbital shaker speeds may contribute to the damage experienced by the cells in this model which cause a reduction in the VCD of the cells. The results gathered here are in agreement with previous studies showing that Pluronic F-68, serum and BSA stabilise bubbles (Handa-Corrigan et al., 1989; Michaels et al., 1995a; Michaels et al., 1995b). These studies also associated the bubble stabilisation of the additives with a reduction in surface tension. However, it was hypothesised that there were potentially other protective mechanisms of action for BSA (and Pluronic F-68) protecting the NS0 cells from the moderate hydromechanical stress model (135 RPM) as there were no bubbles formed in baffled shake flasks at this orbital shaker speed.
Figure 4.11: Shaking NS0 cell culture media in baffled shake flasks at low orbital shaker speeds (110 and 135 RPM) does not result in bubble formation. This figure shows 24ml of NS0 cell culture media after 1 hour shaking at 110 and 135 RPM. Figure 4.11A shows media with 1g/L Pluronic F-68 and 0 and 1g/L BSA shaken at 110 RPM. Figure 4.11B shows media with 0.025g/L Pluronic F-68 and 0 and 1g/L BSA shaken at 110 RPM. Figure 4.11C shows media with 1g/L Pluronic F-68 and 0 and 1g/L BSA shaking at 135 RPM. Figure 4.11D shows media with 0.025g/L Pluronic F-68 and 0 and 1g/L BSA shaking at 135 RPM.
Figure 4.12: Pluronic F-68 and BSA stabilise bubbles formed in NS0 cell culture media shaken in baffled shake flasks at high orbital shaker speeds (150 and 175 RPM). This figure shows 24ml of NS0 cell culture media after 1 hour shaking at 150 and 175 RPM. Figure 4.12A shows media with 1g/L Pluronic F-68 and 0 and 1g/L BSA shaken at 150 RPM in baffled shake flasks. Figure 4.12B shows media with 0.025g/L Pluronic F-68 and 0 and 1g/L BSA shaken at 150 RPM in baffled shake flasks. Figure 4.12C shows media with 1g/L Pluronic F-68 and 0 and 1g/L BSA shaken at 175 RPM in baffled shake flasks. Figure 4.12D shows media with 0.025g/L Pluronic F-68 and 0 and 1g/L BSA shaking at 175 RPM in baffled shake flasks.
4.7 Testing the potential fast-acting biological protective mechanisms of BSA on NS0 cells

4.7.1 BSA associates with NS0 cells in culture in a dose dependent manner

Our next series of experiments focused on the possible fast-acting biological protective mechanisms of action of BSA. Firstly, investigations were conducted to determine whether BSA coats the NS0 cells in culture. NS0 cells originally grown with 0g/L BSA (and 1g/L Pluronic F-68) were incubated in media with various concentrations of BSA (0, 0.1, 0.5, 1 and 2g/L) and 0.025g/L Pluronic F-68 for one hour to investigate whether the BSA associates with the NS0 cells. The cells were washed twice (using media with 0g/L BSA and 0g/L Pluronic F-68) and lysed. The washes and lysates were run on an SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was then probed for BSA (using beta-actin as a loading control).

BSA was detected in a dose dependent manner in the first wash of the NS0 cells cultured in media supplemented with BSA, as shown in Figure 4.13A. There was no BSA detected in the second wash of any of the cells. Therefore, it was concluded that the BSA in the first wash may be BSA loosely associated with the NS0 cells or it could be BSA in the residual media surrounding the pellet of cells being washed. These results suggest that the washes successfully remove any loosely bound BSA from the NS0 cells. The lysates of the NS0 cells also showed the presence of BSA in a dose dependent manner corresponding with the increasing concentrations of BSA in the cell culture media, as shown in Figure 4.13B.

It was concluded from these results that BSA may coat the NS0 cells in culture in a fast-acting and dose dependent manner. Therefore, the coating of the NS0 cells with BSA could be a potential fast-acting biological protective mechanism.
of action of BSA, which was a proposed mechanism of protection for serum by Croughan et al. (Croughan et al., 1989). Following on from these results, the next objective was to test whether the presence of high concentrations of Pluronic F-68 affected the association of BSA with the NS0 cells. Investigations were also conducted to determine whether the BSA associates with the external part of the cell membrane by testing whether the presence of BSA in the lysates could be removed by incubating the cells in media with 0g/L BSA.
Figure 4.13: BSA associates with NS0 cells in a dose dependent manner during cell culture. Parental NS0 cells were cultured in media with 0g/L BSA and 1g/L Pluronic F-68. The cells were then washed and resuspended in media with 0, 0.1, 0.5, 1 and 2g/L BSA and 0.025g/L Pluronic F-68 and incubated for one hour. 1x10^6 cells were centrifuged and washed twice using 100µl of media (with 0g/L BSA and 0g/L Pluronic F-68). The NS0 cells were then lysed in 100µl of lysis buffer. Washes and lysates were run on 12% SDS-PAGE gels and transferred to nitrocellulose membranes and probed for BSA. Figure 4.13A shows the BSA in the cell washes. Figure 4.13B shows the BSA in the cell lysates. Beta-actin is used as a loading control in this experiment.
4.7.2 BSA association with NS0 cells is not interrupted by high concentrations of Pluronic F-68

Having shown that BSA associates with NS0 cells in media with 0.025g/L Pluronic F-68, the next experiment was conducted to determine whether BSA associates with NS0 cells in media with high concentrations of Pluronic F-68. NS0 cells originally grown in media with 0g/L BSA (and 1g/L Pluronic F-68) were incubated in media with 1g/L BSA and 0.025 or 1g/L Pluronic F-68 for one hour. The NS0 cells were centrifuged and washed twice. The cells were lysed and the washes and lysates were run on an SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was probed for BSA (using beta-actin as a loading control).

Similar amounts of BSA were seen in the first washes of the cells cultured with 0.025 and 1g/L Pluronic F-68, and no BSA was detected in the second washes as seen previously, data shown in Figure A.7 (Appendix A). Similar amounts of BSA were also detected in the NS0 cell lysates for cells cultured in media with 0.025 and 1g/L Pluronic F-68, as seen in Figure 4.14. It was concluded from these results that Pluronic F-68 does not have an effect on the association of BSA with the NS0 cells. This suggests that Pluronic F-68 should not interfere with the protective effect of BSA if the protection afforded by BSA is mediated by the protein coating the NS0 cells.
Figure 4.14: Pluronic F-68 does not affect the association of BSA with NS0 cells during cell culture. Parental NS0 cells were cultured in media with 0g/L BSA and 1g/L Pluronic F-68 from thaw. The cells were then resuspended in media with 1g/L BSA and either 0.025 or 1g/L Pluronic F-68 and incubated for one hour. 1x10^6 cells were centrifuged and washed twice using 100µl of media (with 0g/L BSA and 0g/L Pluronic F-68). The NS0 cells were then lysed in 100µl of lysis buffer. Washes and lysates were run on 12% SDS-PAGE gels and transferred to nitrocellulose membranes and probed for BSA. These results show BSA in the cell lysates. Beta-actin is used as a loading control in this experiment.
4.7.3 BSA association with NS0 cells is transient in nature

Previous results (Section 4.4.1 and Section 4.4.2) showed that the protective effect of BSA is lost when NS0 cells are cultured in media with 0g/L BSA, therefore it was hypothesised that the association of BSA with NS0 cells may be removed by culturing the cells in media with 0g/L BSA. NS0 cells cultured in media with 1g/L BSA (and 1g/L Pluronic F-68) from vial thaw were centrifuged and washed twice and resuspended in media with 0g/L BSA (with 0.025g/L Pluronic F-68) and incubated for one hour. Similarly, NS0 cells cultured in media with 0g/L BSA (and 1g/L Pluronic F-68) from vial thaw were centrifuged and washed twice and resuspended in media with 1g/L BSA (and 0.025g/L Pluronic F-68) and cultured for one hour. After 1 hour in culture the cells were centrifuged and washed twice. The cells were lysed and the washes and lysates were run on an SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was then probed for BSA (using beta-actin as a loading control).

There was no BSA detected in the first or second wash for the NS0 cells originally cultured with 1g/L BSA and resuspended in media with 0g/L BSA for 1 hour, as shown in Figure 4.15A. There was also no BSA detected in the lysates for these cells (Figure 4.15B). It was concluded from this result that the association of BSA with the NS0 cells can be removed by culturing the NS0 cells in shake flasks in media with 0g/L BSA for 1 hour. These results suggest that BSA is likely to associate with the external fraction of the NS0 cell membrane, as there is no BSA present in the washes or lysates for cells cultured with 1g/L BSA 1 hour culture in media with 0g/L BSA.
BSA was detected in the first wash (Figure 4.15A) and in the lysate (Figure 4.15B) for NS0 cells originally cultured with 0g/L BSA and resuspended in media with 1g/L BSA. This result was expected, as it was shown previously in Figure 4.13.

These results support the hypothesis that BSA coating the NS0 cells may be involved in the protective mechanism of action of BSA against hydromechanical stress, as the association of BSA with the NS0 cells BSA is fast-acting and the association can be removed by culturing the cells in media with 0g/L BSA and previous results have shown that the protective effect of BSA is also fast-acting and can also be removed by exposing the NS0 cells to hydromechanical stress in media with 0g/L BSA. The protection afforded by BSA coating the NS0 cells may be due to BSA acting as a barrier for the NS0 cells against the damage, as suggested by Croughan et al. (Croughan et al., 1989) or it may be associated with BSA reducing the cell membrane hydrophobicity of the NS0 cells (Wu et al., 1997; Ghebeh et al., 2002).
Figure 4.15: BSA association with NS0 cells is removed by culturing the cells in media with 0g/L BSA for one hour. Parental NS0 cells were cultured in media with 0 and 1g/L BSA. The cells cultured in media with 0g/L BSA were resuspended in media with 1g/L BSA and incubated for one hour. The cells cultured in media with 1g/L BSA were resuspended in media with 0g/L BSA and incubated for one hour. 1x10^6 cells were centrifuged and washed twice using 100µl of 0g/L BSA media. The NS0 cells were then lysed in 100µl of lysis buffer. Washes and lysates were run on 12% SDS-PAGE gels and transferred to nitrocellulose membranes and probed for BSA. Figure 4.15A shows the BSA in the cell washes. Figure 4.15B shows the BSA in the cell lysates. Beta-actin is used as a loading control in this experiment.
4.7.4 BSA reduces NS0 cell membrane hydrophobicity in media with low concentrations of Pluronic F-68

As BSA was shown to associate with the NS0 cells in culture, the next experiment was designed to test whether BSA causes a reduction in the NS0 cell membrane hydrophobicity. The relative membrane hydrophobicity was determined by measuring the association of the NS0 cells with a hydrophobic liquid, hexadecane. The effect of various concentrations of BSA (0, 0.1, 0.5, 1 and 2g/L) were tested in media with 0, 0.025 and 1g/L Pluronic F-68. NS0 cells were cultured in media with 0g/L BSA and 1g/L Pluronic F-68 and washed in media with 0g/L BSA and 0g/L Pluronic F-68 before being resuspended in the test media.

Increasing concentrations of BSA reduced the cell membrane hydrophobicity of NS0 cells in a dose dependent manner in media with 0 and 0.025g/L Pluronic F-68. NS0 cells in media with 0g/L BSA and 0g/L Pluronic F-68 had a relative hydrophobicity of approx. 100%, whereas cells in media with 2g/L BSA and 0g/L Pluronic F-68 had a relative hydrophobicity of approx. 17%, as shown in Figure 4.16A. NS0 cells in media with 0g/L BSA and 0.025g/L Pluronic F-68 had a relative hydrophobicity of approx. 97%, whereas cells in media with 2g/L BSA and 0.025g/L Pluronic F-68 had a relative hydrophobicity of approx. 41%, as shown in Figure 4.16B. The reduction in cell membrane hydrophobicity caused by BSA was rapid, as the NS0 cells had been previously cultured with 0g/L BSA. Therefore, the reduction in membrane hydrophobicity may be a protective method of action of BSA for NS0 cells experiencing hydromechanical stress in baffled shake flasks. However, BSA was shown to have no effect on the NS0 cell membrane hydrophobicity in media with 1g/L Pluronic F-68, with the cells having a relative hydrophobicity of approx. 0% in media with 1g/L Pluronic F-68 and 0 or 1g/L BSA, as shown in Figure 4.16C.
The error bars around 0% relative hydrophobicity for the samples in media with 1g/L Pluronic F-68 are due to the formation of an emulsion between the cell culture media and the hexadecane, therefore causing an increase in the cell density in the sample which gives a relative hydrophobicity of <0%. The reduction in cell membrane hydrophobicity may be associated with the ability of BSA to coat the NS0 cells in culture as shown earlier in Figure 4.13.

It was concluded from these results that the reduction in cell membrane hydrophobicity may be a protective mechanism of action of BSA on NS0 cells experiencing hydromechanical stress in media with low concentrations of Pluronic F-68, however high concentrations of Pluronic F-68 cause the effect of BSA on the membrane hydrophobicity of NS0 cells to be redundant. These results are in parallel with previous observations by Wu et al. who showed that serum reduces the cell membrane hydrophobicity of insect cells (Wu et al., 1997), however these results have shown that BSA (a component of serum) alone reduces the NS0 cell membrane hydrophobicity.
Figure 4.16: BSA causes a dose dependent reduction in NS0 cell membrane hydrophobicity in media with low Pluronic F-68 concentrations. Figure 4.16A shows the effects of various concentrations of BSA (0, 0.1, 0.5, 1 and 2g/L) on the NS0 cell membrane hydrophobicity in media with 0g/L Pluronic F-68, 0.5g/L BSA p=0.004 (**), 1g/L BSA p=0.006 (**), 2g/L BSA p=0.0098 (**), student T-test, n=3. Figure 4.16B shows the effect of various concentrations of BSA (0, 0.1, 0.5, 1 and 2g/L) on NS0 cell hydrophobicity in media with 0.025g/L Pluronic F-68, 0.5g/L BSA p=0.01 (*), 1g/L BSA p=0.005 (**), 2g/L BSA p=0.007 (**), student T-test, n=3. Figure 4.16C shows the effect of 0 and 1g/L BSA on NS0 cell hydrophobicity in media with 1g/L Pluronic F-68. Error bars denote the standard deviation of three replicate tests.
4.8 Assessing the protective effect of denatured BSA on NS0 cells

4.8.1 Denatured BSA offers increased protection for NS0 cells experiencing hydromechanical stress

Having established that the protective effect of BSA is afforded (and lost) rapidly for NS0 cells exposed to damage and having proposed a number of physical and fast-acting biological protective mechanisms of action of BSA, the next objective was to determine whether the secondary and tertiary structures of BSA are required for its protective effect. BSA was denatured by heat treatment to assess whether denatured BSA protects NS0 cells similar to native BSA. BSA was denatured by heating a 40g/L solution of BSA (in purified water) at 100°C for 30 minutes. The denaturation of BSA was confirmed using Circular Dichroism and native PAGE as shown in Figure A.8 and Figure A.9 respectively (Appendix A). The protective effect of 1g/L of denatured BSA was compared to 1g/L of native BSA for NS0 cells exposed to extreme hydromechanical stress (as described in Section 3.4.9) in media with 0.025g/L Pluronic F-68.

Denatured BSA offered protection to the NS0 cells exposed to extreme hydromechanical stress, as shown by a lower reduction in VCD for cells in media with 1g/L denatured BSA (approx. 17% reduction in VCD) compared to cells in media with 0g/L BSA (approx. 59% reduction in VCD), as shown in Figure 4.17. These results suggest that the secondary and tertiary structures of BSA are not required for BSA to protect NS0 cells from extreme hydromechanical stress.

The NS0 cells exposed to extreme hydromechanical stress in media with 1g/L denatured BSA had approx. 17% reduction in VCD compared to a reduction of approx. 42% for 1g/L native BSA, as shown in Figure 4.17. This result suggests that the protection afforded by denatured BSA is greater than native BSA. The NS0 cells
in media with 1g/L denatured BSA also had a lower LDH Activity (approx. 55 LU) compared to cells in media with 1g/L native BSA (approx. 85 LU) as shown in Figure 4.17B, which also supported the result that denatured BSA offered increased protection to the extreme hydromechanical stress compared to native BSA,

Denatured proteins have been shown to cause a greater reduction on the surface tension of liquids compared to native proteins (Tomczynska-Mleko et al., 2014). Therefore the effect of native and denatured BSA on the surface tension of the NS0 cell culture media will be investigated in Section 4.8.4. Following on from the results gathered in this experiment, the effects of denatured BSA and native BSA were compared for NS0 cells exposed to laminar shear.
Figure 4.1: Denatured BSA offers improved protection to NS0 cells exposed to extreme hydromechanical stress in media with low Pluronic F-68 concentrations. BSA was denatured at 100°C for 30 minutes. Parental NS0 cells were exposed to hydromechanical stress (175 RPM in 125ml baffled shake flasks) in media with 0g/L BSA and 1g/L of native or denatured BSA and 0.025g/L Pluronic F-68. These results show the percentage reduction in VCD after 60 minutes exposure to extreme hydromechanical stress, 1g/L denatured BSA vs 0g/L BSA p=0.0009 (**), 1g/L native BSA vs 1g/L denatured BSA p=0.005 (**) student T-test, n=3. Figure 4.17B shows the LDH Activity of the samples after 30 minutes exposure to extreme hydromechanical stress p=0.008 (**) student T-test, n=3. Error bars denote the standard deviation of three replicate flasks.
4.8.2 Denatured BSA does not offer increased protection for NS0 cells exposed to laminar shear stress

As denatured BSA was shown to offer greater protection to NS0 cells exposed to extreme hydromechanical stress in baffled shake flasks (in Section 4.8.1), the next test was conducted to determine whether denatured BSA offered greater protection to NS0 cells exposed to laminar shear stress. BSA was denatured at 100°C for 30 minutes, as described in Section 2.2.16. The NS0 cells were exposed to 2.5Pa of laminar shear for 3 hours in media with 1g/L of native and denatured BSA (and 0g/L Pluronic F-68).

Denatured BSA retained its protective effect for NS0 cells exposed to laminar shear stress, however denatured BSA did not have a greater protective effect for NS0 cells exposed to laminar shear, as shown in Figure 4.18. Therefore it was hypothesised that the greater protection afforded by denatured BSA for NS0 cells exposed to the extreme hydromechanical stress in baffled shake flasks is likely due to protective effects which would not affect the cells under laminar shear, i.e. greater reduction in media surface tension or further reduction in membrane hydrophobicity.
Figure 4.18: Denatured BSA protects NS0 cells from laminar shear stress. Parental NS0 cells were exposed to 2.5Pa of laminar shear for 3 hours in media with 0g/L Pluronic F-68 and either 0 or 1g/L BSA (native or denatured at 100°C for 30 minutes). These results show the percentage reduction in TCD for the samples.
4.8.3 Denatured BSA associates with NS0 cells in culture and reduces NS0 cell membrane hydrophobicity similar to native BSA

After showing that denatured BSA offers increased protection to NS0 cells from extreme hydromechanical stress in baffled shake flasks, the next investigation aimed to determine whether denatured BSA coats NS0 cells and reduces the cell membrane hydrophobicity similar to native BSA. NS0 cells grown in media with 0g/L BSA (and 1g/L Pluronic F-68) were incubated in media with 1g/L of native and denatured BSA (heat denatured at 100°C as described in Section 2.2.16) (with 0.025g/L Pluronic F-68) for one hour. The NS0 cells were then centrifuged and washed twice. The NS0 cells were lysed and the washes and lysates were run on an SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was then probed for BSA (using beta-actin as a loading control).

BSA was detected in the first washes of the cells cultured with native and denatured BSA, and no BSA was detected in the second washes, as shown in Figure A.10 (Appendix A). Both native and denatured BSA were detected in the lysates of the NS0 cells, as shown in Figure 4.19A. Therefore, it was concluded from these results that the secondary and tertiary structures of BSA are not required for the protein to associate with NS0 cells in culture.

The effect of 1g/L native and denatured BSA on the cell membrane hydrophobicity of NS0 cells was tested in media with 0g/L Pluronic F-68 using NS0 cells originally grown in media with 0g/L BSA. Denatured BSA reduced the relative cell membrane hydrophobicity to approx. 58%, whereas native BSA reduced the relative cell membrane hydrophobicity to approx. 63%, as shown in Figure 4.19B. The difference between the effect of denatured and native BSA on the relative cell membrane hydrophobicity was not shown to be statistically significant, as shown in
Figure 4.19B. These results showed that the secondary and tertiary structures of BSA are not required for the BSA to reduce the cell membrane hydrophobicity of NS0 cells. However, it was concluded that the improved protective effect of denatured BSA is not likely to be due to reduced cell membrane hydrophobicity as denatured BSA reduced the NS0 cell membrane hydrophobicity similar to native BSA.
Figure 4.19: Denatured BSA associates with NS0 cells in culture and reduces the NS0 cell membrane hydrophobicity similar to native BSA. Parental NS0 cells originally cultured in media with 0g/L BSA were incubated in media with 1g/L of native or denatured BSA for one hour. 1x10^6 cells were centrifuged and washed twice. The NS0 cells were then lysed in 100µl of lysis buffer. Washes and lysates were run on 12% SDS-PAGE gels and transferred to nitrocellulose membranes and probed for BSA. Figure 4.19A shows the BSA in the cell lysates. Beta-actin is used as a loading control in this experiment. Figure 4.19B shows the effects of 1g/L native and denatured BSA (heat denatured at 100°C for 30 minutes) on NS0 cell membrane hydrophobicity in media with 0g/L Pluronic F-68, Native BSA p=0.005 (**), Denatured BSA p=0.003 (**), student T-test, n=3. Error bars denote the standard deviation of three replicate tests.
Denatured BSA reduces the surface tension of NS0 culture media to a greater extent compared to native BSA

Having shown that denatured BSA does not reduce the NS0 cell membrane hydrophobicity of NS0 cells to a greater extent than native BSA; the next objective was to compare the effects of denatured and native BSA on the surface tension of the NS0 cell culture media. The effects of 1g/L native and denatured BSA on the surface tension of NS0 cell culture media supplemented with 0.025g/L Pluronic F-68.

Media supplemented with 1g/L denatured BSA had a surface tension which was approx. 5% lower than media supplemented with 1g/L native BSA, as shown in Figure 4.20. Media with 1g/L denatured BSA had a surface tension of approx. 60mN/m, whereas media supplemented with native BSA had a surface tension of approx. 63mN/m, as shown in Figure 4.20. As described earlier, a recent report into Pluronic F-68 lot variations showed that the surface tension of an effective lot of Pluronic F-68 was approx. 8% lower than the surface tension of a lot of Pluronic F-68 which was shown to have limited protection for cells exposed to hydromechanical damage (Apostolidis et al., 2015). Therefore this supports the hypothesis that the increased protection afforded by denatured BSA is due to the fact that 1g/L denatured BSA caused a 5% reduction in the surface tension of the NS0 cell culture compared to 1g/L native BSA.

These results also support the hypothesis that the protective effect of BSA is associated with its ability to reduce the media surface tension. These results are in agreement with previous observations that denatured proteins have a greater reducing effect on the surface tension of liquids compared to native proteins (Tomczynska-Mleko et al., 2014).
Figure 4.20: Denatured BSA causes a greater reduction in the surface tension of NS0 culture media compared to native BSA. This figure shows the surface tension of NS0 cell culture media with 0.025g/L Pluronic F-68 and 2ml/L CLC supplemented with native or denatured BSA (heat denatured at 100°C for 30 minutes), p=0.0004 (**), student T-test, n=5. Error bars denote standard deviation of five replicate tests.
4.9 Substitution of the protective effect of BSA on NS0 cells with a non-animal derived surfactant

4.9.1 Methyl cellulose protects NS0 cells from moderate and extreme hydromechanical stress in baffled flasks similar to BSA

As the results gathered in this project showed that the protective effect of BSA on NS0 cells exposed to hydromechanical stress may be due to BSA acting as a surfactant in the media, the final set of experiments tested whether the protective effect of a BSA could be substituted by a non-animal derived surfactant. There are a number of different non-animal sourced surfactants which could potentially be used in conjunction with Pluronic F-68 in replacement for BSA, including different Pluronic polyols (e.g. Pluronic F-38 and Pluronic F-108) (Murhammer and Goochee, 1990b), derivatised celluloses (e.g. methyl cellulose) (Chattopadhyay et al., 1995a) and small molecule surfactants (e.g. maltopyranosides) (Hu et al., 2008). Methyl cellulose was the chosen surfactant to be tested, as it has previously been shown to protect cells from damage (Chattopadhyay et al., 1995a). NS0 cells were exposed to the moderate (as described in Section 3.4.5) and extreme (as described in Section 3.4.9) hydromechanical stress models in baffled shake flasks in media with 0.025g/L Pluronic F-68. Media with 0g/L BSA and 0 or 0.1g/L methyl cellulose was used.

Low concentrations of methyl cellulose (0.1g/L) protected the NS0 cells from moderate hydromechanical stress in baffled shake flasks, as seen by an increase in the maximum VCD of approx. 55% (Figure 4.21A) and decrease in doubling time of approx. 24% (Figure 4.21B) for NS0 cells cultured in media with 0.1g/L methyl cellulose compared to 0g/L methyl cellulose. These results also show that the protective effect of 0.1g/L methyl cellulose in media with 0.025g/L is similar in magnitude to 1g/L BSA for NS0 cells exposed to moderate hydromechanical stress.
in baffled flasks, as seen previously in Figure 3.13 (shown on next page). Methyl cellulose was shown to have no effect on the growth profiles of NS0 cells cultured in non-baffled shake flasks as shown in Figure A.11 (Appendix A); this was to be expected as it was previously shown that the cells did not experience damage in the non-baffled shake flasks.

Low concentrations of methyl cellulose (0.1g/L) also protected the NS0 cells from extreme hydromechanical stress in baffled shake flasks, as seen by a lower reduction in VCD (Figure 4.21C) and the lower LDH Activity (Figure 4.21D) for the NS0 cells exposed to the extreme hydromechanical stress in media with 0.1g/L methyl cellulose compared to media with 0g/L methyl cellulose. NS0 cells exposed to the extreme hydromechanical stress in media with 0g/L methyl cellulose had a reduction in VCD of approx. 51% and an LDH Activity of approx. 195 LU compared to a reduction in VCD of approx. 12% and an LDH Activity of approx. 80 LU for cells in media with 0.1g/L methyl cellulose.

The protective effect of methyl cellulose was afforded rapidly, similar to the protection afforded by BSA, as the cells used in these experiments were originally cultured in media with 0g/L methyl cellulose (and 0g/L BSA). This suggests that the protective mechanism of action of methyl cellulose is also physical or fast-acting biological in nature. These results show that the protective effect of 0.1g/L methyl cellulose in media with 0.025g/L is similar in magnitude to 1g/L BSA for NS0 cells exposed to extreme hydromechanical stress in baffled flasks, as seen previously in Figure 3.14 (shown on next page).

It was concluded from these results that the protective effect of BSA on NS0 cells experiencing moderate and extreme hydromechanical stress may be substituted by a non-animal derived surfactant.
Figure 3.13: BSA protects NS0 cells experiencing moderate hydromechanical stress in baffled shake flasks in media with low Pluronic F-68 concentrations. Parental NS0 cells were cultured at 135 RPM in 125ml baffled shake flasks with a working volume of 24ml in media with 0.025g/L Pluronic F-68 and 0 or 1g/L BSA. Figure 3.13A shows the growth profiles for the NS0 cells over 4 days culture. Figure 3.13C shows the LDH activity of the NS0 cells cultured after approximately 2 days of culture, 1g/L BSA p=0.001 (**), student T-test, n=3. Error bars denote the standard deviation of three replicate flasks.

Figure 3.14: BSA affords dose dependent protection for NS0 cells exposed to extreme hydromechanical stress conditions in media with low Pluronic F-68 concentrations. Parental NS0 cells were exposed to 175 RPM for 60min in 125ml baffled shake flasks at a seeding density of 1x10^6cells/ml in media with 0.025g/L Pluronic F-68 with 0 and 1g/L BSA. Figure 3.14A shows the reduction in VCD after 60 minutes exposure p=0.002 (**), student T-test, n=3. Figure 3.14B shows the LDH activity for samples taken after 30 minutes exposure, 1g/L BSA p=0.000001 (****), student T-test, n=3. Error bars denote standard deviation of three replicate flasks.
Figure 4.21: Methyl cellulose protects NS0 cells from moderate and extreme hydromechanical stress in baffled shake flasks in media with low Pluronic F-68 concentrations. Parental NS0 cells were exposed to moderate hydromechanical stress (135 RPM for 4 days) and extreme hydromechanical stress (175 RPM for 60 minutes) in 125ml baffled shake flasks. The cells were exposed in media with 0g/L BSA and 0.025g/L Pluronic F-68 with 0 or 0.1g/L methyl cellulose. Figure 4.21A shows the growth profiles for the NS0 cells exposed to moderate hydromechanical stress. Figure 4.21B shows the doubling times for the NS0 cells exposed to the moderate hydromechanical stress p=0.03 (*), student T-test, n=3. Figure 4.21C shows the percentage reduction in VCD for cells exposed to the extreme hydromechanical stress, p=0.0002 (**), student T-test, n=3. Figure 4.21D shows the LDH activity for samples taken after 30 minutes exposure to the extreme hydromechanical stress, p=0.000006 (****), student T-test, n=3. Error bars denote standard deviation of three replicate flasks.
4.9.2 Methyl cellulose reduces the surface tension of NS0 cell culture media and stabilises bubbles formed in baffled shake flasks

Having determined that 0.1g/L methyl cellulose offers rapid protection to NS0 cells, the effect of 0.1g/L methyl cellulose on the dynamic viscosity and surface tension of the NS0 cell culture media was tested. The effect of 0.1g/L methyl cellulose on the cell membrane hydrophobicity of the NS0 cells was also tested.

Methyl cellulose (0.1g/L) did not increase the dynamic viscosity of the NS0 cell culture media (Table 4.2) and also did not reduce the NS0 cell membrane hydrophobicity (Figure 4.22). However, 0.1g/L methyl cellulose reduced the surface tension of NS0 cell culture media with 0.025g/L Pluronic F-68 by approx. 12%, as shown in Figure 4.23A. Methyl cellulose also stabilised the bubbles formed in the extreme hydromechanical stress model in baffled shake flasks, as shown in Figure 4.23B, similar to BSA and Pluronic F-68. The bubbles formed had completely burst after 4 minutes as shown in Figure A.12 (Appendix A), which was similar to results seen previously for media with 0 and 1g/L BSA Figure A.5 (Appendix A). These results are in agreement with previous studies showing that bubble stabilisation is linked with a reduction in media surface tension (Michaels *et al.*, 1995a; Michaels *et al.*, 1995b).

Therefore, it was concluded from these results that the protective effect of methyl cellulose is likely due to the reduction in media surface tension and stabilisation of bubbles. This result supports the hypothesis that the protective effect of BSA for NS0 cells may is due to BSA acting as a surfactant and that the protective effect of BSA could potentially be replaced by a non-animal derived surfactant.
### Table 4.2: Methyl cellulose does not affect the dynamic viscosity of NS0 cell culture media.

This table shows the effect of 0 and 0.1g/L methyl cellulose on the dynamic viscosity of NS0 cell culture media with 0g/L Pluronic F-68. The results shown are the average of seven replicate tests at 20°C.

<table>
<thead>
<tr>
<th>Methyl cellulose Concentration</th>
<th>0g/L</th>
<th>0.1g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dynamic Viscosity (mPa.s)</td>
<td>1.09</td>
<td>1.12</td>
</tr>
<tr>
<td>STDev</td>
<td>±0.02</td>
<td>±0.01</td>
</tr>
</tbody>
</table>

### Figure 4.22: Methyl cellulose has no effect on NS0 cell membrane hydrophobicity.

This figure shows the relative membrane hydrophobicity for NS0 cells in media with 0.025g/L Pluronic F-68 and 0 and 1g/L methyl cellulose. Error bars denote the standard deviation of three replicate tests.
Figure 4.23: Methyl cellulose reduces the dynamic surface tension of NS0 cell culture media and stabilises bubbles in baffled shake flasks. Figure 4.23A shows the dynamic surface tension of NS0 cell culture media with 0.025g/L Pluronic F-68, 0g/L BSA and 0ml/L 1000X CLC supplemented with 0 and 0.1g/L methyl cellulose, p=0.000003 (****), student T-test, n=5. Error bars denote the standard deviation of at least three replicate tests. Figure 4.23B shows the bubbles formed in media with 0g/L BSA and 0.025g/L Pluronic F-68 supplemented with 0 or 0.1g/L methyl cellulose.
4.10 Discussion

The results in Chapter 3 showed that BSA protects the NS0 cells from hydromechanical stress in baffled shake flasks, therefore the experiments in this chapter focused on determining the protective mechanisms of action of BSA on the NS0 cells.

The first set of experiments in this chapter showed that the protective effect of BSA is not likely to be due to the provision of amino acids or cholesterol to the NS0 cells. Therefore it was hypothesised that the protective effect of BSA was due to a physical or fast-acting biological protective mechanism of action. The moderate and extreme hydromechanical stress models developed in Chapter 3 were used to show that the protective effect of BSA was afforded (and lost) rapidly for NS0 cells exposed to hydromechanical stress, which agrees with previous reports showing that serum has a physical or fast-acting biological protective mechanism of action for cells experiencing damage (Michaels et al., 1991; Martens et al., 1992; van der Pol et al., 1992). Therefore, the main focus of the remaining experiments was to determine the potential physical or fast-acting biological protective mechanisms of action of BSA for NS0 cells.

Although BSA afforded protection to NS0 cells experiencing laminar shear, the protective effect of BSA was greater for cells exposed to hydromechanical stress in baffled shake flasks. This suggests that the protective mechanism of action of BSA in the baffled shake flasks is likely to be against damage which the cells do not experience in the laminar shear device, e.g. damage at the air-liquid interface. The dynamic viscosity of the NS0 cell culture media was not affected by the addition of BSA or Pluronic F-68; therefore it was concluded that the protective effect of BSA is
not likely to be due to the increase in the dynamic viscosity of the NS0 cell culture media.

A number of potential physical protective mechanisms of action were shown for BSA, including reducing the media surface tension and stabilising bubbles. Reducing the surface tension of media has been suggested to be a physical mechanism of protection for cells exposed to bubble damage (Chattopadhyay et al., 1995a; Chattopadhyay et al., 1995b). The surface tension of the NS0 cell culture media was reduced by the addition of BSA in media with 0 or 0.025g/L Pluronic F-68. However, BSA had no effect on the surface tension of media supplemented with 1g/L Pluronic F-68. Therefore, it was concluded that the protective effect of BSA on NS0 cells may be due to its ability to reduce the surface tension of media if the effect of Pluronic F-68 on the surface tension is limited. BSA was also shown stabilise bubbles in baffled shake flasks, which has been proposed as a physical protective mechanism of action of serum and BSA (Handa-Corrigan et al., 1989; Michaels et al., 1995a; Michaels et al., 1995b; Chisti, 2000). The bubble stabilisation effect of BSA has been associated with the reduction in surface tension of the media (Michaels et al., 1995a; Michaels et al., 1995b).

Some fast-acting biological protective mechanisms of action for BSA were also shown in this chapter. BSA associated with the NS0 cells in a dose dependent manner and this association was shown to be removed by culturing the cells in media with 0g/L BSA for 1 hour. This suggests that the BSA coats the external cell membrane of the NS0 cells. Therefore, the BSA associating with the cell membrane may be involved with the protective mechanism of action of the protein on NS0 cells experiencing hydromechanical stress, this mechanism of protection has been a suggested mechanism for serum by Croughan et al. (Croughan et al., 1989). BSA
also reduced the cell membrane hydrophobicity of the NS0 cells in a dose dependent manner in media with 0 and 0.025g/L Pluronic F-68. However, BSA did not have an effect on the cell membrane hydrophobicity of NS0 cells in media with 1g/L Pluronic F-68. The reduction of the cell membrane hydrophobicity of the NS0 cells may be due to the BSA associating the NS0 cells and with the air liquid interface, as suggested by Wu et al. (Wu et al., 1997).

The secondary and tertiary structures of BSA were not required for BSA to protect NS0 cells from extreme hydromechanical stress. In fact, denatured BSA had improved protection for NS0 cells, which may be due to the denatured BSA reducing the surface tension of NS0 cell culture media to a greater extent than native BSA. This has been shown previously for other proteins (Tomczynska-Mleko et al., 2014).

The final investigations carried out during this project were designed to determine whether the protective effect of BSA could be replaced by a non-animal derived surfactant. Low concentrations (0.1g/L) of methyl cellulose protected the NS0 cells exposed to the moderate and extreme hydromechanical stress models in baffled shake flasks. Investigations into the protective mechanism of action of methyl cellulose showed that the surfactant had no effect on the dynamic viscosity of the NS0 cells culture media. Although, methyl cellulose has previously been shown to reduce the cell membrane hydrophobicity of insect cells (Wu et al., 1997), the results in this project showed that methyl cellulose did not reduce the NS0 cell membrane hydrophobicity in media with 0.025g/L Pluronic F-68. Methyl cellulose was shown to significantly reduce the surface tension of the NS0 cell culture media, which suggests that the protective effect of methyl cellulose may be due to the reduction in media surface tension and stabilise the bubbles formed in the extreme hydromechanical stress model in baffled shake flasks. These results indicate that the
protective effect of BSA can be replaced by the addition of low concentrations of methyl cellulose (a non-animal derived surfactant) into the NS0 cell culture media.
Chapter 5:

Discussion
There are large-scale biopharmaceutical cell culture processes for the production of therapeutic mAbs in which BSA is added to the cell culture media; however the exact function of BSA in the media is not fully understood. It is favourable to remove animal-sourced raw materials used in the production of human therapeutic proteins as it reduces the risk of contamination of the cell culture and ultimately improves patient safety; however it is difficult to remove BSA from the cell culture media as its function has not been adequately defined. There have been a number of different functions proposed for BSA in large-scale cell culture, including; protecting cells from damage in the bioreactor, acting as an antioxidant and aiding nutrient transport (Merten, 2002). Therefore, the objective of this project was to carry out investigations to determine the function of BSA in the culture of NS0 cells. The results compiled during this project show that BSA protects NS0 cells from hydromechanical stress (in media with limited protection from Pluronic F-68) with a number of potential mechanisms of action. The protective effect of BSA was also shown to be potentially replaced by the addition of a non-animal sourced surfactant. Therefore the results gathered in this project have improved the prospect of removing BSA from NS0 cell culture media for large-scale mAb production.

Prior to this project there was no data published on the effects of BSA on NS0 cells. Therefore, this is the first report investigating the function of BSA in the culture of NS0 cells.

5.1 NS0 cell growth is not impacted by the presence of BSA in non-damaging conditions

The first set of results in this project showed that BSA has no effect on the growth of NS0 cells under various culturing conditions (non-baffled shake flasks, sparged and
agitated reactors and baffled shake flasks) in media supplemented with 1g/L Pluronic F-68. It was concluded from these results that BSA is not an essential component for NS0 cell growth under non-damaging culture conditions (in the presence of sufficient concentrations of Pluronic F-68), as BSA had no effect on the NS0 cell growth. Therefore, it was hypothesised that the effect of BSA may only be evident under damaging conditions and that the presence of 1g/L of an effective lot of Pluronic F-68 in the media may render any effect that BSA might be having on NS0 cells to be redundant.

5.2 NS0 cells do not require Pluronic F-68 for normal cell growth under non-damaging shake flask conditions

The protective effect of Pluronic F-68 against damaging cell culture conditions has been known for many years (Murhammer and Goochee, 1988; Handa-Corrigan et al., 1989; Murhammer and Goochee, 1990a; Murhammer and Goochee, 1990b; Michaels et al., 1991). However, there have been a number of limitations reported which may reduce the protective effects of Pluronic F-68, including; batches of Pluronic F-68 with reduced protective effect (Peng et al., 2014; Apostolidis et al., 2015), limited protective effect of Pluronic F-68 at high cell densities (Ma et al., 2004) and reduced concentrations of Pluronic F-68 in the media over time due to endocytosis (Gigout et al., 2008). Therefore, it has been recommended to have more than one active surfactant added to media used for large-scale cell culture due to the reliance that industry is placing on Pluronic F-68 to protect cells from damage in the bioreactor (Hu et al., 2008; Chalmers, 2015).

The concentration of Pluronic F-68 was reduced in the NS0 cell culture media during this study to mimic conditions where the protective effect afforded by
Pluronic F-68 is reduced due to the limitations outlined above. The reduced concentrations of Pluronic F-68 were shown to have no effect on the growth of NS0 cells in non-damaging cell culture conditions in non-baffled shake flasks, therefore Pluronic F-68 is not essential for NS0 cell growth in non-damaging conditions.

5.3 Development of lab-scale hydromechanical stress models in baffled shake flasks

The use of baffled shake flasks in this study was adapted from a recent study carried out by Peng et al. where baffled shake flasks were used to identify batches of Pluronic F-68 which have limited protective capabilities for cells experiencing damage (Peng et al., 2014). Peng et al. showed that baffled shake flasks were capable of replicating the reduced protective effect of certain lots of Pluronic F-68 seen in large-scale and lab-scale bioreactor. The baffled shake flasks have several advantages over lab-scale bioreactors, e.g. they are user friendly and can easily be used for high-throughput screening (Peng et al., 2014). Therefore baffled shake flasks were used in this project to test whether BSA has a protective effect on NS0 cells experiencing damage in culture.

It has been shown that the power input into the cell culture media in baffled shake flasks increases at higher orbital shaking speeds (RPM) and with increasing orbital shaker diameter (Peter, Suzuki, Rachinskiy et al., 2006; Peter, Suzuki and Büchs, 2006). Increasing the orbital shaker diameter was shown to improve the separation of the ineffective lots of Pluronic F-68 in the study by Peng et al. which may be due to the increased power input (Peng et al., 2014). In this project the power input into the cell culture was increased in baffled shake flasks by increasing the orbital shaker speed (RPM).
The NS0 cells in this study were shown to be susceptible to damage in baffled shake flasks with increasing RPMs in media with low concentrations of Pluronic F-68 ($\leq 0.1\text{g/L}$). This result shows that increasing the orbital shaking speed of baffled shake flasks causes more damage to the cells, which agrees with the observations by Peter et al. that increasing orbital shaker speeds result in increased power input into the cell culture (Peter, Suzuki, Rachinskiy et al., 2006). Increasing the orbital shaker speeds in baffled shake flasks also causes the formation of bubbles (Büchs, 2001) and can cause damage to cells at the air-liquid interface due to the rapidly moving air-liquid surface (Chisti and Moo-Young, 1993a) However, the presence of $1\text{g/L}$ of an effective lot of Pluronic F-68 completely protected the NS0 cells from hydromechanical stress in baffled shake flasks irrespective of the presence or absence of BSA in the media. This result is in agreement with concentrations of Pluronic F-68 which have been previously suggested to protect cells from damage in large-scale cell culture (Handa-Corrigan et al., 1989; Velez-Suberbie et al., 2013).

It was observed that the orbital shaker speed and exposure time to the hydromechanical stress in the baffled shake flasks were important factors when quantifying the damage experienced by the NS0 cells. Therefore, two different models were developed in the baffled shake flasks.

- Model 1: Moderate hydromechanical stress

The first model was a moderate hydromechanical stress model where the cells were shaken at a 135 RPM for approx. 4 days and the cells were capable of proliferation; however the growth of the cells was reduced due to the hydromechanical stress. This hydromechanical stress model was termed “moderate” as the NS0 cells were capable of proliferation under the conditions. There were no bubbles formed in this hydromechanical stress...
model. The readouts for this model were the effects on the growth of the cells (maximum VCD reached and doubling time) and the membrane damage (LDH Activity) experienced by the cells. This model was used to test whether BSA promotes NS0 cell growth under conditions of moderate hydromechanical stress which would potentially cause a reduction in mAb titre in large-scale bioreactors.

- Model 2: Extreme hydromechanical stress

The second model was an extreme hydromechanical stress model where the NS0 cells were shaken at 175 RPM for 60 minutes. This model was termed “extreme” hydromechanical stress as the NS0 cells were not capable of proliferation under the conditions and the VCD of the NS0 cells was reduced after short exposure to the stress. The conditions for the extreme hydromechanical stress model caused the formation of bubbles in the baffled shake flasks. The readouts for this model were the percentage reduction in VCD of the cells and the membrane damage experienced by the cells (LDH Activity). This model was used to test whether BSA protects NS0 cells from hydromechanical stress which causes a reduction in VCD due to cell lysis (e.g. damage due to bubble burst), which would cause a reduction in the mAb titre.

The damage experienced by cells in the two hydromechanical stress models was shown to be cell line dependent, which is in agreement with a report by Peng et al. showing that two different CHO cell lines had different growth profiles when exposed to hydromechanical stress conditions in media with a lot of Pluronic F-68 which was known to have limited protection (Peng et al., 2014).
5.4 BSA protects NS0 cells from hydromechanical stress in baffled shake flasks

The presence of BSA was shown to protect the NS0 cells exposed to the moderate and extreme hydromechanical stress in baffled shake flasks in media with reduced concentrations (≤0.1g/L) of Pluronic F-68. The presence of BSA in the media caused an increase in the cell growth (greater maximum VCD and decreased doubling time) and a decrease in LDH Activity for NS0 cells exposed to the moderate hydromechanical stress. The protective effect of BSA was evident in the extreme hydromechanical stress model, as the presence of BSA in the media caused a lower reduction in VCD and a lower LDH Activity for NS0 cells. These results are in agreement with previous observations showing that serum and BSA offer protection to various cell types experiencing damage (Hülscher et al., 1990; Michaels et al., 1991; Martens et al., 1992; Michaels et al., 1995a; Michaels et al., 1995b; Hesse et al., 2003).

It was concluded from these results that NS0 cells are susceptible to hydromechanical stress in baffled shake flasks and that the presence of 1g/L of an effective lot of Pluronic F-68 completely protects the NS0 cells from the damage, thus causing the protective effect of BSA to be redundant. These results are summarised in Figure 5.1.

The hydromechanical stress models (moderate and extreme, in baffled shake flasks) were used to test whether BSA has a protective effect on a mAb-producing NS0 cell line. Although BSA offered protection to the mAb-producing NS0 cells exposed to moderate hydromechanical stress in media with 0.025g/L Pluronic F-68, the mAb-producing NS0 cells were more sensitive to the moderate hydromechanical stress in media with 0g/L BSA. This could be due to the mAb-producing cell line having a more sensitive cell membrane due to the fact that the cells are producing
and secreting high concentrations of mAbs. BSA had a similar protective effect for the cells exposed to the extreme hydromechanical stress in media with 0.025g/L Pluronic F-68. Similar to the Parental NS0 cells, BSA had no effect on the damage experienced by the mAb-producing NS0 cells in media with 1g/L of an effective lot of Pluronic F-68. Therefore, it was concluded that the protective effect of BSA is similar for Parental and mAb-producing NS0 cells.

**Figure 5.1:** Schematic showing the protective effect of BSA is only evident in media with limited protection from Pluronic F-68.

Flask (a) depicts media supplemented with BSA and an effective lot of Pluronic F-68. Flask (b) depicts media supplemented with an effective lot of Pluronic F-68. Flask (c) depicts media with limited protection from Pluronic F-68 supplemented with BSA. Flask (d) depicts media with limited protection from Pluronic F-68. Flask (e) depicts media supplemented with BSA. Flask (f) depicts media not supplemented with BSA or Pluronic F-68.

**5.5 BSA protection is physical or fast-acting biological in nature**

The results gathered during this project indicate that BSA does not play a role in the provision of nutrients (amino acids and cholesterol) to NS0 cells. It was shown that
although BSA binds to cholesterol in media (supplemented with CLC), the NS0 cells were protected from damage by BSA in the absence of CLC supplementation. Therefore the protective mechanism of action of BSA is not likely to be involved with cholesterol provision, which has been a suggested function of BSA in the culture of NS0 cells (Francis, 2010).

It has been shown previously that serum has a physical or fast-acting biological protective effect for cells exposed to damage (Michaels et al., 1991; Martens et al., 1992; van der Pol et al., 1992), however there was also reports of a biological protective mechanism of action for serum which takes longer to have an effect (Michaels et al., 1991; Martens et al., 1992). Prior to this project there was no report showing the physical or biological protective effect for BSA on cells experiencing damage. It was shown that BSA has a physical or fast-acting biological protective mechanism of action on NS0 cells, as the protective effect of BSA was afforded (and lost) rapidly for NS0 cells exposed to damage. These observations are in agreement with the results from Michaels et al., Martens et al. and van der Pol et al. showing that serum has a physical protective mechanism of action (Michaels et al., 1991; Martens et al., 1992; van der Pol et al., 1992). However the results gathered in this project showed no indication of a biological protective mechanism for BSA which is in contrast to the results reported by Michaels et al. and Martens et al. who showed that serum may also have a biological protective mechanism of action (Michaels et al., 1991; Martens et al., 1992). Therefore the biological protective mechanism of serum may have been due to the presence of other components in the serum (e.g. hormones, growth factors, cholesterol etc.) which are not present in purified BSA.
Heat denatured BSA was also shown to retain its protective effect for the NS0 cells. Therefore the secondary and tertiary structures of BSA are not required for the BSA to elicit its protective effect on NS0 cells. This result supports the hypothesis that the protective effect of BSA is physical or fast-acting biological in nature, rather than providing nutrients (e.g. hormones, growth factors, cytokines etc.), as the nutrients associated with BSA would likely be denatured during the heat treatment and therefore would be rendered ineffective. Interestingly, the protective effect of denatured BSA was shown to be more effective than native BSA, however this was attributed to the denatured BSA causing a greater reduction of the media surface tension. The fact that heat denatured BSA protects the cells from damage is promising, as there are now guidelines in place from the CHMP which state that all serum derived products must be gamma-irradiated before use unless otherwise justified (CHMP, December 2013). The function of the gamma-irradiation of the serum products is to deactivate potential viruses, however it has been known for many years that gamma-irradiation also causes protein denaturation (Barron and Dickman, 1949; Ciešla et al., 2000). Therefore, the protective effect of BSA may not be affected by denaturation due to gamma-irradiation. However, irradiation of proteins has been shown to cause fragmentation (Audette-Stuart et al., 2005) and aggregation which may reduce the protective efficacy of BSA.

5.6 Potential physical or fast-acting biological protective mechanisms of action of BSA

Laminar shear devices have been used previously to test the protective effect of media additives against shear stress (Goldblum et al., 1990; Ramirez and Mutharasan, 1990; Michaels et al., 1991) and also to assess the cellular responses to
shear stress (Schürch et al., 1988; Born et al., 1992; Mardikar and Niranjan, 2000; Graf et al., 2003). In this project the protective effect of BSA was tested on NS0 cells experiencing laminar shear (in the absence of a potentially damaging air-liquid interface) using a concentric cylinder laminar shear device designed and fabricated in the University of Limerick by Michael Collins. The results showed that there was minimal protection afforded to the NS0 cells in media with 1g/L BSA under all magnitudes of laminar shear stress tested (0.5, 1.5 and 2.5Pa) and for all exposure times (1, 2 and 3 hours), however higher concentrations of BSA (>1g/L) offered greater protection for NS0 cells experiencing laminar shear. Therefore, it was clear that BSA offers protection to NS0 cells experiencing laminar shear, however the protective effect is minimal for media supplemented with 1g/L BSA. This highlighted that the protective mechanism(s) of action of BSA in baffled shake flasks may be associated with protection from damage which is not present in the laminar shear device (i.e. damage caused at the air-liquid interface). The protective effect of BSA for NS0 cells experiencing laminar shear may be due to BSA coating the NS0 cell membrane, therefore acting as a protective layer against the damage. A similar protective mechanism of action of serum has been suggested for protecting cells against damage in turbulent flow (Croughan et al., 1989).

Having shown that BSA offers rapid protection to NS0 cells, a number of possible physical protective mechanisms of action of BSA were investigated. It was shown that the protective effect of BSA was not due to BSA increasing the dynamic viscosity of the NS0 cell culture medium (and thus reducing the damage experienced by the cells), which was a suggested physical protective mechanism of action of serum (Cherry and Papoutsakis, 1988; Croughan et al., 1989; McQueen and Bailey, 1989). The surface tension of the NS0 cell culture medium was shown to be reduced
by the addition of BSA to media supplemented with 0 and 0.025g/L Pluronic F-68, however BSA had no effect on the surface tension of media supplemented with 1g/L Pluronic F-68. Reducing the surface tension of the medium was a physical mechanism of protection proposed by Chattopadhyay et al. as media with lower surface tension inhibits the association of cells with bubbles and the air-liquid interface (Chattopadhyay et al., 1995a; Chattopadhyay et al., 1995b). BSA was also shown to stabilise the bubbles formed in baffled shake flasks in media with low concentrations of Pluronic F-68 (0.025g/L), which is a suggested physical mechanism of protection as it allows the cells to drain from the bubbles before the damaging bursting event occurs (Michaels et al., 1995a; Michaels et al., 1995b; Chisti, 2000) and it also results in a thinner bubble film, which leads to less damaging bubble burst event (Handa-Corrigan et al., 1989). High concentrations of Pluronic F-68 (1g/L) were also shown to stabilise the bubbles in the presence and absence of BSA. These results are in agreement with previous reports showing that serum and BSA stabilise bubbles (Handa-Corrigan et al., 1989; Michaels et al., 1995a; Michaels et al., 1995b).

Investigations were also carried out into a number of possible fast-acting biological protective mechanisms of action for BSA on NS0 cells during this project. BSA was shown to coat the NS0 cells in a dose dependent manner, which was a protective mechanism of action of serum proposed by Croughan et al., as it may avoid damaging fluid eddies coming into contact with the cell membrane (Croughan et al., 1989). BSA also caused a reduction in the NS0 cell membrane hydrophobicity, which was a proposed protective mechanism of action of serum by Wu et al. as it also interrupts the association of the cells with the bubbles and air-liquid interface (Wu et al., 1997). These protective mechanisms of action have been previously
suggested for serum in the large-scale culture of cells in a bioreactor, and the protective mechanisms mainly rely on the interruption of the cell-bubble interaction. However, these mechanisms of action have not been definitively shown for BSA before this project was conducted. These physical and fast-acting biological protective mechanisms of action of BSA (and Pluronic F-68) rely on the association of the compounds with the cell membrane and with the air-liquid interface. The protective mechanisms of action of BSA suggested in this project are depicted in Figure 5.2 for media with different concentrations of Pluronic F-68 (0.025 and 1g/L). As outlined earlier, Pluronic F-68 has a molecular weight of approximately 8400 Da (Chisti, 2000) and BSA has a molecular weight of approximately 66,000 Da (Hirayama et al., 1990). Therefore in media with 1g/L Pluronic F-68 and 1g/L BSA the ratio of Pluronic F-68 to BSA is approximately 8:1, whereas in media with 0.025g/L Pluronic F-68 and 1g/L BSA the ratio of Pluronic F-68 to BSA is 1:5 (as depicted in Figure 5.2).

Taking all the results into consideration, it was concluded that BSA may be protecting the cells in a similar way as a surfactant in the NS0 cell culture media, and therefore this may explain why the protective effect of BSA may only be evident when the protection afforded by the surfactant Pluronic F-68 is limited. The physical and fast-acting biological protective mechanisms of action of BSA on NS0 cells outlined in this project are summarised in Figure 5.3.
Figure 5.2: Schematic of the association of BSA and Pluronic F-68 with the air-liquid interface and with the NS0 cell membrane. Figure 5.2A depicts NS0 cells cultured in media with 0.025g/L Pluronic F-68 and 0g/L BSA. The red arrows indicate the affinity of the NS0 cells with the air-liquid interface, causing the cells to be highly susceptible to damage. Figure 5.2B depicts NS0 cells in media supplemented with 0.025g/L Pluronic F-68 and 1g/L BSA. Media with 0.025g/L Pluronic F-68 and 1g/L BSA gives a ratio of Pluronic F-68 molecule to BSA molecule of approximately 1:5 as depicted in this figure. BSA and Pluronic F-68 have an affinity for the NS0 cell membrane and for the air-liquid interface. The red indicates a reduced affinity of the cells with the air-liquid interface, which causes a reduced susceptibility to damage. Figure 5.2C depicts NS0 cells in media supplemented with 1g/L Pluronic F-68 and 0 or 1g/L BSA. Media with 1g/L Pluronic F-68 and 1g/L BSA gives a ratio of Pluronic F-68 molecule to BSA molecule of approximately 8:1 as depicted in this figure. The red arrows indicate the reduced association of the NS0 cells with the air liquid interface, which results in low susceptibility to damage. The presence of BSA does not affect the damage experienced by the NS0 cells in media with 1g/L Pluronic F-68.
Figure 5.3: Overview of the protective effect of BSA on NS0 cells. This figure shows the physical and fast-acting biological protective mechanisms of action of BSA (native or denatured) on NS0 cells. It also shows that the protective effect of BSA may be replaced by a functioning surfactant (e.g. Pluronic F-68).

5.7 Replacing BSA protection by a synthetic surfactant

The results gathered in this project suggest that the protection offered to NS0 cells by BSA is due to BSA acting as a surfactant in the NS0 cell culture media. The protective effect of BSA was shown to be redundant in media with sufficient concentrations of an effective lot of the surfactant Pluronic F-68. It was also shown that when the protective effect of Pluronic F-68 is limited, the protective effect of BSA can be replaced by another non-animal derived surfactant for NS0 cells exposed to moderate and extreme hydromechanical stress in baffled shake flasks. Therefore, the protective effect of BSA on NS0 cells in large-scale cell culture could potentially be replaced by a synthetic surfactant.
Although the results in this project suggest that the function of BSA is redundant in media with sufficient quantities of Pluronic F-68, there are a number of concerns associated with removing BSA from NS0 cell culture media. With regards to gaining regulatory approval the easiest method to remove BSA from the media used for NS0 cell culture for the production of therapeutic mAbs would be to remove the BSA and not replace it another surfactant. However, if BSA was removed from the media and not replaced by a non-animal derived surfactant it would add increased pressure to Pluronic F-68 to protect the cells from damaging cell culture conditions. It is worth noting here that it has been recommended to have more than one active surfactant in media used for large-scale cell culture (Hu et al., 2008; Chalmers, 2015). The concentration of Pluronic F-68 could also be increased in the media if BSA is removed to try to compensate for the loss of the protective effect of BSA, this option is supported by a recent report showing that increasing the concentrations of Pluronic F-68 in media used for mAb production had no effect on the mAb product quality (Tharmalingam and Goudar, 2015). If BSA was removed and replaced by a non-animal derived surfactant (other than Pluronic F-68) a lot more data and potentially further clinical trials would be required by regulatory bodies to prove that the “new process” produces the exact same mAb as the process which was originally approved. Therefore there is a balance between the advantages and disadvantages of removing BSA from the media used to produce therapeutic mAbs; however the advantages of removing BSA outweigh the disadvantages as long as the protective effect of Pluronic F-68 is not limited.

Some companies are have started to test the protective efficacy of new batches of Pluronic F-68 before the new batch of Pluronic F-68 is used in media for the production on mAbs in large-scale bioreactors to avoid an ineffective lot of
Pluronic F-68 being used (Peng et al., 2014). Peng et al. reported that baffled shake flask models are used in quality control laboratories to test the protective efficacy of new batches of Pluronic F-68 (Peng et al., 2014), however other methods of identifying ineffective lots of Pluronic F-68 could also be conducted, e.g. comparing the infrared spectroscopy or mass spectrometry profiles of batches of Pluronic F-68 which have been shown to be effective with the profiles of new batches.

5.8 Final Conclusions

The results in this project have extended our knowledge of the effects of BSA on NS0 cells. These experiments showed that BSA offers physical protection for NS0 cells experiencing damage; however this protection is not required when the cells are sufficiently protected by Pluronic F-68. Significant steps have been taken towards understanding the role BSA plays in the large-scale culture of NS0 cells. The findings in this project could aid the removal of BSA from industrial cell culture media in the future. The main conclusions drawn during this project are as follows:

- BSA has no effect on NS0 cells in media supplemented with high concentrations of an effective batch of the surfactant Pluronic F-68; however BSA protects NS0 cells from damaging cell culture conditions in media with limited protection from Pluronic F-68. Therefore, the protective effect of BSA on NS0 cells is redundant in media with sufficient protection from Pluronic F-68.
- Native and denatured BSA offer physical or fast-acting biological protection for NS0 cells as their protective effect is afforded (and lost) rapidly (in media with reduced protection from Pluronic F-68). The fact that denatured BSA
retains its protective effect indicates that the secondary and tertiary structures of BSA are not required for BSA to elicit its protective effect.

- The protective effect of BSA is not due to nutrient provision (including provision of cholesterol), and the results showed that BSA acts as a surfactant in the NS0 cell culture media. Several physical and fast-acting biological protective mechanisms of action for BSA were shown (in media with reduced protection from Pluronic F-68), including; reducing media surface tension, acting as a bubble stabiliser, coating the NS0 cells and reducing the cell membrane hydrophobicity.

- The protection afforded by BSA on NS0 cells could potentially be replaced by a non-animal derived surfactant. If BSA is removed from the NS0 cell culture media, then there is added pressure placed on Pluronic F-68 to provide sufficient protection for the NS0 cells against the hydromechanical damage in bioreactors. Therefore it would be beneficial to determine the efficacy of batches of Pluronic F-68 before they are added to media used in large-scale bioreactors. The protective effect of BSA on NS0 cells could also be replaced by another non-animal derived surfactant; however this could cause complications with regulatory bodies.

5.9 Future Work

The work carried out during this project has greatly increased our understanding of the effect of BSA on NS0 cells in culture. As with all projects, the results gathered here have left a number of areas where further research could be conducted, the main areas which could be expanded upon are detailed in the following paragraphs.
Firstly, investigations into the hydromechanical stress models in baffled shake flasks could be carried out to further understand the damage experienced by the cells as the RPM of the orbital shaker is increased. Although the power input into the cell culture has been shown to be greater in baffled shake flasks compared to non-baffled flasks, CFD could be used to quantify the power input (or EDR) in the baffled shake flasks hydromechanical stress models (moderate and extreme) compared to the non-baffled shake flasks. It would also allow the critical power input where the NS0 cells experience damage to be determined, which would therefore give a greater understanding of the conditions where media additives (including Pluronic F-68 and BSA) offer protection to the NS0 cells. Although previous research has been conducted into the power input of non-baffled shake flasks (Büchs and Zoels, 2001), there is currently a lack of understanding of the conditions experienced in baffled shake flasks compared to non-baffled flasks. The results from this study would give a better understanding of the damage experienced in the baffled shake flasks; however the results would not likely aid the removal of BSA from the NS0 cell culture media. A high speed camera could also be used to visualise the formation of bubbles in the baffled shake flasks in the extreme hydromechanical stress model, and the size and frequency of formation and bursting of the bubbles could be estimated. Although the results gathered in this project suggest that BSA interrupts the association of NS0 cells with bubbles and the air-liquid interface (due to BSA reducing the media surface tension and reducing the NS0 cell membrane hydrophobicity), it could be visually determined if the addition of BSA interrupts the association of NS0 cells with bubbles using a high definition camera, similar to experiments carried out by Chalmers and Bavarian (Chalmers and Bavarian, 1991).
Further experimentation could also be carried out into the interaction of BSA with the NS0 cell membrane. Although it was shown that the association of BSA with the NS0 cell membrane is rapid and transient in nature, there is no evidence of the mechanism of association of BSA with the NS0 cell membrane. It is likely that BSA may associate with the NS0 cell membrane due to hydrophobic interaction, however further investigations would be required to determine the mechanism of association. If the association of BSA is due to a sub section of the protein, then there is the possibility that short peptides (containing amino acids from the BSA sequence) which also associate with the NS0 cell membrane could be synthetically produced and supplemented into the medium used for NS0 cell culture. These peptides would also need to cause a reduction in surface tension and membrane hydrophobicity similar to the whole BSA molecule.

As mentioned earlier, serum has been shown to reduce the plasma membrane fluidity of cells resulting in protection from damage. However, there are no reports of BSA affecting the plasma membrane fluidity of cells. Therefore, investigations could be carried out to test the effect of BSA on the plasma membrane fluidity of NS0 cells. The effect of serum on the cell membrane fluidity may be due to components of serum other than BSA integrating with the cell membrane (e.g. cholesterol, lipids and fatty acids); therefore the protective effect of BSA may not be due to reducing the plasma membrane fluidity of the NS0 cells as it was shown that BSA coats the cells, but does not integrate into the membrane.

Our hypothesis is that the protective effect of BSA is only evident when the protective effect of Pluronic F-68 is limited, therefore it would be beneficial to test the protective effect of BSA for NS0 cells exposed to hydromechanical stress in media supplemented with 1g/L of an ineffective batch of Pluronic F-68. An
ineffective batch of Pluronic F-68 was requested from the vendors to test this hypothesis; however it was not possible for the vendors to send a sample of such batches of Pluronic F-68.

It was observed that mAb-producing NS0 cells were more susceptible to moderate hydromechanical stress than Parental NS0 cells. Therefore it could be of industrial interest to investigate why the mAb-producing NS0 cells experienced more damage; potentially due to the mAb-producing NS0 cells having a more sensitive cell membrane due to the fact that the cells are producing and secreting high concentrations of mAbs. These investigations were not possible in this project, due to restricted access to the mAb-producing NS0 cells.

It was also hypothesised that the protective effect of BSA may be replaced by a surfactant other than Pluronic F-68. There are a number of different surfactants which could be added to the NS0 cell culture media to replace the function of BSA, e.g. other Pluronic polyols (Pluronic F-38 and Pluronic F-108) (Murhammer and Goochee, 1990b), derivatised celluloses (e.g. methyl cellulose) (Chattopadhyay et al., 1995a) and small molecule surfactants (e.g. maltopyranosides) (Hu et al., 2008). However, investigations would be required to identify a surfactant which has no effect on the NS0 cell growth, as well as not affecting the structure of the mAb produced. Therefore removing BSA and not replacing it with another compound would require much less experimental support for the regulatory bodies; however it is advised to have more than one active surfactant present in media used for large-scale cell culture (Hu et al., 2008).
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Appendix A:

Supplementary Information
BSA immunoprecipitation

Several different methods of purifying BSA from NS0 cell culture media were tested to determine the optimum BSA purification mechanism for quantifying the cholesterol bound to BSA in media. BSA was purified using i) ProG beads, ii) ProG beads conjugated with anti-BSA antibodies and iii) Agarose beads conjugated with anti-BSA antibodies. The BSA was removed from the beads and run on an SDS-PAGE gel and stained with coomassie blue.

All methods of purification were successful in purifying BSA from NS0 cell culture media, as shown by the presence of BSA in the IPs in Figure A.1. Agarose beads conjugated with anti-BSA antibodies were the chosen method of purification for identifying whether BSA binds cholesterol in media, as this mechanism purified the highest concentration of BSA from the NS0 cell culture media.
Figure A.1: Immunoprecipitation of BSA from NS0 cell culture media. BSA was purified from monoclonal antibody producing NS0 cell culture media supplemented with 1g/L BSA using a number of different immunoprecipitating methods: ProG beads, ProG beads with anti-BSA antibody and Agarose beads with anti-BSA antibody. Control IPs were carried out using media from NS0 cells cultured with 0g/L BSA. Samples were run on a 12% SDS-PAGE gel and stained with coomassie blue. M: protein marker, S: Sample pre-IP, ProG: ProG beads, ProG+mAb: ProG beads and Rabbit anti-BSA antibody, Agarose+mAb: Agarose beads and Rabbit anti-BSA antibody.
Optimisation of laminar shear conditions to test the protective effect of BSA on NS0 cells

NS0 cells were exposed to laminar shear in a novel concentric cylinder laminar shear device designed by Michael Collins in the Stokes Institute, University of Limerick, Ireland. The device was designed to expose suspension cells to extremely accurate levels of laminar shear. This device was used to test the protective effect of BSA on NS0 cells exposed to laminar shear. The following experiments were conducted by Michael Collins; however I provided intellectual input into the design of the experiments and provided the raw materials (cells and media).

Once the device was designed and built, several tests were carried out to optimise the laminar shear conditions to test the protective effect of BSA on NS0 cells. The main conditions to be optimised were shear magnitude and shear exposure time. The results in Figure A.2A show that the NS0 cells are damaged by laminar shear; this is seen by a reduction in TCD after exposure to laminar shear. The results show that the damage experienced by the NS0 cells is greater with increasing shear magnitude. The results also show that BSA offers protection for NS0 cells exposed to laminar shear stress. This protection is seen by a lower reduction in TCD for cells exposed to laminar shear with 1g/L BSA; however this reduction in TCD is only approx. 8.5%. The results in Figure A.2B show that NS0 cells experience greater damage with increasing exposure time to laminar shear. The results also show that the protective effect of BSA is seen at all exposure times to laminar shear. The results in Figure A.2C show that BSA offers a dose dependent protection for NS0 cells exposed to laminar shear stress (2.5Pa for 3 hours). Higher concentrations of BSA are required to see similar protective effects compared to hydromechanical stress in baffled shake flasks; therefore it was concluded that the protective effect of
BSA on NS0 cells is most prevalent when the cells are exposed to damaging conditions that are not present in the laminar shear device (i.e. damage caused at the air-liquid interface).

Figure A.2: BSA offers dose dependent protection for NS0 cells exposed to laminar shear stress in media with low Pluronic F-68 concentrations. Parental NS0 cells were exposed to various laminar shear conditions in media with 0.025g/L Pluronic F-68 and various concentrations of BSA. The results show the percentage reduction in TCD after exposure to the shear. Figure A.2A shows the results of NS0 cells exposed to 0.5, 1.5 and 2.5Pa of laminar shear for 3 hours in media with 0 or 1g/L BSA. Figure A.2B shows the results of NS0 cells exposed to 2.5Pa of laminar shear stress for 1, 2 and 3 hours in media with 0 or 1g/L BSA. Figure A.2C shows the results of NS0 cells exposed to 2.5Pa of laminar shear stress for 3 hours in media with 0, 1, 2 and 4g/L BSA. Error bars denote the standard deviation of two replicate tests.
Figure A.3: BSA protection is afforded rapidly for NS0 cells exposed to laminar shear stress. Parental NS0 cells were exposed to 2.5Pa of laminar shear for 3 hours in media with 0g/L Pluronic F-68 and 0 or 4g/L BSA. The NS0 cells were cultured in the BSA for 10 minutes or 24 hours before being exposed to the laminar shear stress. These results show the percentage reduction in TCD for the samples.
Figure A.4: CLC reduces the surface tension of NS0 cell culture media. This figure shows the surface tension of NS0 cell culture media with 0g/L Pluronic F-68 and 0g/L BSA supplemented with various concentrations of 1000X CLC (0, 2 and 4ml/L). Error bars denote standard deviation of at least three replicate tests.
Figure A.5: Bubbles formed in NS0 cell culture media in baffled shake flasks at 175 RPM burst after 4 minutes. This figure shows 24ml of NS0 cell culture media shaken for 1 hour at 175 RPM. The pictures were taken 4 minutes after the shaking had stopped. Figure A.5A shows media with 1g/L Pluronic F-68 and 0 and 1g/L BSA. Figure A.5B shows media with 0.025g/L Pluronic F-68 and 0 and 1g/L BSA shaken at 175 RPM.
Figure A.6: Non-baffled flasks do not cause bubble formation at 175 RPM. Figure A.6A shows media with 1g/L Pluronic F-68 and 0 and 1g/L BSA shaken at 175 RPM in non-baffled shake flasks. Figure A.6B shows media with 0.025g/L Pluronic F-68 and 0 and 1g/L BSA shaken at 175 RPM for in non-baffled shake flasks.
Figure A.7: Pluronic F-68 does not affect the amount of BSA in the first washes of NS0 cells. Parental NS0 cells were cultured in media with 0g/L BSA and 1g/L Pluronic F-68. The cells were then resuspended in media with 1g/L BSA and either 0.025 or 1g/L Pluronic F-68 and cultured for one hour. 1x10^6 cells were centrifuged and washed twice using 100µl of media (with 0g/L BSA and 0g/L Pluronic F-68). The NS0 cells were then lysed in 100µl of lysis buffer. Washes and lysates were run on 12% SDS-PAGE gels and transferred to nitrocellulose membranes and probed for BSA. This figure shows the BSA detected in the first and second washes of the cells.
Confirmation of BSA denaturation by heat treatment

The near UV spectrum in Circular Dichroism is sensitive to certain aspects of the tertiary structure of a protein; therefore the two different absorbance profiles for native and denatured BSA in Figure A.8A showed that there was a change in the overall tertiary structure of denatured BSA compared to native BSA. The far UV spectrum in Circular Dichroism is used to determine changes in the secondary structure of proteins; therefore the two different absorbance profiles for native and denatured BSA in Figure A.8B showed that the secondary structure of the denatured BSA is also altered by heat denaturation. The alpha helix percentage was calculated to reduce from approx. 50% in the native BSA sample to approx. 28% in the denatured BSA sample (calculated using equations in Section 2.2.17). The denaturation of BSA was also confirmed using native PAGE, as two distinct bands were detected for the native and denatured BSA, as shown in Figure A.9. The denatured BSA moved through the gel at a slower rate, suggesting that the structure of denatured BSA was larger than the native BSA (due to denaturation or polymerisation).

It was concluded that the secondary and tertiary structures of BSA were altered by the heat denaturation process. This denatured BSA could be used to test whether denatured BSA protects NS0 cells similarly to native BSA.
Figure A.8: Confirmation of BSA denaturation by Circular Dichroism. Figure A.8A shows the near UV results for native and denatured BSA measured using Circular Dichroism. Figure A.8B shows the far UV results for native and denatured BSA measured using Circular Dichroism.
Figure A.9: Confirmation of BSA denaturation using Native PAGE. This figure shows native and denatured BSA separated by Native PAGE using a 4%/8% stacking acrylamide gel with no SDS added to any of the reagents.
Figure A.1: Native and denatured BSA are detected in the first washes of NS0 cells. Parental NS0 cells originally cultured in media with 0g/L BSA were cultured in media with 1g/L of native or denatured BSA and for one hour. 1x10^6 cells were centrifuged and washed twice. The NS0 cells were then lysed in 100µl of lysis buffer. Washes and lysates were run on 12% SDS-PAGE gels and transferred to nitrocellulose membranes and probed for BSA. This figure shows the BSA detected in the first and second washes of the cells.
Figure A.11: Methyl cellulose has no effect on NS0 cells growth in non-baffled shake flasks. Parental NS0 cells were cultured in non-baffled shake flasks using the moderate hydromechanical stress model (135 RPM in 125ml non-baffled shake flasks) The cells were cultured in media with 0g/L BSA and 0.025g/L Pluronic F-68 with 0 or 0.1g/L methyl cellulose. This figure shows the growth profiles of the NS0 cells. Error bars denote standard deviation of at least three replicate flasks.
Figure A.12: Bubbles formed in NS0 cell culture media supplemented with methyl cellulose burst after 4 minutes. This figure shows 24ml of NS0 cell culture media shaken for 1 hour at 175 RPM. The picture was taken 4 minutes after the shaking stopped. Media with 0.025g/L Pluronic F-68 and 0g/L BSA was supplemented with 0 or 0.1g/L methylcellulose.