Optimising the Functionalisation of Alginate Hydrogels for Three-dimensional Astrocyte Culture Systems

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2 Abstract

Astrocytes are glial cells in the central nervous system that contribute significantly to brain function and are involved in several neurological disorders. Despite the widespread impact of these disorders, current treatments often fail to address their underlying causes. Magnetomechanical stimulation (MMS) has emerged as a promising non-invasive technique, using magnetic fields and functionalised particles to stimulate astrocyte activity. This technology offers a new way to study astrocytes and explore potential therapeutic applications.

This study focuses on improving the functionalisation of alginate hydrogels to support astrocyte growth in 3D culture models, which are essential for MMS research. Using carbodiimide chemistry, various ratios of EDC and NHS were tested to optimise ligand attachment to alginate scaffolds. The success of functionalisation was measured with the Bradford assay, which quantified the amount of ligand bound to the scaffold.

Results showed that 0.084 mg/mL of EDC produced the highest ligand attachment efficiency, though differences between concentrations were small. This research provides a foundation for future work aimed at refining 3D astrocyte models and advancing the potential of MMS in developing new treatments for neurological disorders.

3 Introduction

Astrocytes are a type of glial cell in the central nervous system, with a star-shaped structure and widespread presence throughout the brain. Positioned strategically between neurons and blood vessels, they provide metabolic and structural support to neurons and regulate neural circuit activity (Gradisnik and Velnar, 2023). Their functions are vast and complex, contributing significantly to brain health and playing key roles in various neurological disorders, including neurodegenerative diseases, mood disorders, epilepsy, and stroke (Yu *et al.*, 2022).

Neurological disorders, impacting over 3 billion individuals globally, pose a major challenge to public health (*World Health Organisation*, 2024). Despite their prevalence, our understanding of these disorders remains constrained by the complexity of brain function and the intricacies of its diseases. Existing treatments tend to prioritise alleviating symptoms rather than tackling the underlying causes or slowing the disease progression (Ricci, 2024). This gap highlights the critical need for deeper investigation into the underlying mechanisms driving these conditions.

In this context, research into astrocytes becomes important. Targeting astrocytes in therapeutic interventions not only advances our knowledge of these cells in both healthy and

diseased states but also opens new possibilities for treatment. By focusing on astrocyte-specific mechanisms, researchers have begun to explore more effective ways to modify disease progression rather than simply treating symptoms.

Emerging technologies like optogenetics and chemogenetics offer promising avenues for astrocyte manipulation. These methods have been tested in the context of epilepsy, addictive behaviours, and neurodegenerative diseases (Yu, Nagai and Khakh, 2020). Despite their potential, both techniques have notable drawbacks. Optogenetics requires invasive procedures, such as implanting optic fibres into the brain, while chemogenetics relies on systemically administered drugs that are slow to take effect, making it less suitable for applications where quick and precise responses are needed (Yu *et al.*, 2022).

Recent progress, however, has led to the introduction of magnetomechanical stimulation (MMS), which leverages the endogenous mechanosensitivity of astrocytes. This technology allows for non-invasive, targeted stimulation of astrocytes without the need for genetic modifications. MMS uses magnetic fields and antibody-functionalised magnetic particles to remotely stimulate astrocytic activity, such as calcium signalling and ATP release. This method not only provides a powerful tool for studying astrocyte function but also shows potential for clinical applications due to its simplicity and non-invasive nature (Yu *et al.*, 2022).

As MMS research progresses, the development of 3D culture models becomes essential. Traditional two-dimensional (2D) models limit cell growth and may alter the mechanosensory properties of astrocytes, potentially skewing experimental results. Implementing 3D models offers a more realistic environment to study astrocyte behaviour, enabling researchers to better understand their roles in the brain's cellular microenvironment and to refine therapeutic strategies (Frampton *et al.*, 2011).

Creating 3D systems involves several steps, including functionalisation of the hydrogel scaffolds, where ligands are added to promote astrocyte growth and survival (Frampton *et al.*, 2011). This study evaluates different ratios of EDC and NHS to optimise ligand attachment to alginate scaffolds that ultimately would support astrocyte adhesion and growth under MMS. The findings aim to enhance 3D models for studying astrocyte responses to mechanical stimuli, with broader implications for therapeutic applications in neurological disorders.

4 Background

4.1 Approaches to creating three-dimensional culture models for astrocyte research

Given the significance of 3D culture models in accurately mimicking astrocyte behaviour in MMS research, it is essential to explore the different approaches for creating these models. Two primary techniques are employed: scaffold-free and scaffold-based systems. Scaffold-free methods rely on cell self-assembly and de novo formation to generate multicellular structures like spheroids and organoids, while scaffold-based approaches use biomaterials to

guide the formation of 3D cell networks. The latter more closely replicates the organised interaction between cells and the extracellular matrix, providing a more reliable environment for nutrient and oxygen diffusion (Castillo Ransanz *et al.*, 2022).

Among the diverse biomaterials used in scaffold-based systems, hydrogels—composed of crosslinked polymer networks—have been proven useful for supporting cell growth and tissue development. They can be derived from natural polymers such as agarose, chitosan, collagen, hyaluronan, and alginate (Frampton *et al.*, 2011).

4.2 Alginate as a hydrogel scaffold for neural cell studies

Alginate, a polysaccharide sourced from the cell walls of brown algae, consists of β-D-mannuronic acid and α-L-guluronic acid residues. When exposed to divalent cations like Ca^{2+} and Ba^{2+}, alginate easily undergoes crosslinking (Frampton *et al.*, 2011). For astrocyte studies, alginate offers several key advantages as a hydrogel scaffold due to its biocompatibility. The choice of a suitable scaffold depends on the polymer's chemical nature and the sensitivity of the cells involved. Neural cells are highly sensitive to oxidative damage due to their low levels of antioxidant defences (Raps *et al.*, 1989). Thus, materials that avoid free radical-driven polymerisation and do not release harmful reactive by-products during degradation are ideal for neural tissue engineering. Alginate is well-suited in this regard, as it fulfils these criteria (Frampton *et al.*, 2011).

Additionally, the properties of alginate scaffolds, such as concentration, structure, porosity and cell adhesion, can be tailored to fit the specific requirements of different cell culture systems. For example, alginate hydrogels can be engineered to closely mimic the mechanical properties of living tissue, making them an ideal substrate for supporting and examining astrocytes (Drury, Dennis and Mooney, 2004).

Moreover, it has been shown that alginate scaffolds in 3D culture systems effectively support neural cells by maintaining both their viability and function. Notably, astrocytes cultured within these 3D alginate scaffolds show distinct morphologies compared to those grown on 2D substrates. In 2D cultures, astrocytes take on an epithelial-like shape, whereas in 3D alginate constructs, they develop a stellate form, more closely resembling their natural structure in brain tissue (Frampton *et al.*, 2011).

4.3 Cell entrapment and functionalisation of alginate

Cell entrapment involves immobilising the cells of interest within a hydrogel by crosslinking or polymerising the hydrogel around suspended cells in a 3D scaffold. However, for anchorage-dependent cells, such as neurons, hydrogels must also provide attachment points to ensure cell survival and promote growth. To achieve this, hydrogels can be functionalised

by covalently attaching peptide sequences or proteins, which enhance cell adhesion and growth (Frampton *et al.*, 2011). Alginate, with its carboxylate groups on the polysaccharide chains, is particularly well-suited for functionalisation. These groups allow the covalent bonding of peptides and proteins that support cell attachment (Rowley, Madlambayan and Mooney, 1999). This functionalisation is typically carried out using carbodiimide chemistry, a widely used technique that modifies the carboxylic acid groups on alginate monomers to form amide bonds. As a result, alginate becomes a versatile scaffold, capable of supporting the adhesion and growth of cells, including neurons, in 3D culture systems.

4.4 Carbodiimide Chemistry for Alginate Functionalisation

The functionalization of alginate via carbodiimide chemistry involves activating the carboxyl groups of alginate with a water-soluble carbodiimide, typically 1-ethyl-(3-dimethylaminopropyl) carbodiimide (EDC). First, an MES buffer is used to create an acidic environment. When EDC is added, it reacts with the carboxyl group to form a highly reactive intermediate called O-acylisourea. However, this intermediate is unstable and hydrolyses quickly, reducing the efficiency of the reaction. To stabilise the intermediate and improve yield, N-hydroxysuccinimide (NHS) or its water-soluble version, sulfo-NHS, is often added. NHS reacts with O-acylisourea, forming a more stable ester, which prolongs the reaction and increases the likelihood of a successful bond with a primary amine. This stabilised ester, created by the combination of EDC and NHS, allows for more efficient functionalisation of alginate, enabling the covalent attachment of bioactive molecules such as peptides (Saeger Mitchell, 2016).

4.5 Quantifying protein concentrations with the Bradford Assay

The Bradford assay is a widely recognised method for quantifying protein concentration in solutions. This assay operates on the principle of Coomassie Brilliant Blue dye binding to proteins, resulting in a detectable colour shift that corresponds to the protein concentration. By analysing the absorbance at a designated wavelength, researchers can accurately assess protein levels in a sample. To do so, a standard curve is first established by preparing a series of dilutions with known protein concentrations. These samples are measured for their absorbance, which produces a linear curve where the absorbance increases proportionally with the protein concentration. Once this curve is generated, it serves as a reference (Kielkopf, Bauer and Urbatsch, 2020).

In the context of functionalising alginate scaffolds, the Bradford assay can be an effective tool for assessing how successful the functionalisation process was. By measuring the absorbance of the sample with an unknown protein concentration (i.e., the peptide attached to the scaffold) and then comparing this value against the standard curve generated from known protein concentrations. The unknown concentration is then calculated by finding the

corresponding point on the standard curve, where the absorbance of the sample intersects the linear relationship between absorbance and protein concentration. This allows researchers to accurately quantify how much protein has bound to the scaffold, providing a reliable measure of the functionalisation efficiency.

5 Methods

5.1 Bradford assay standard curve of concentration versus absorbance

The Bradford assay was used to establish the standard curve for jellagen, collagen, and poly-D-lysine (PDL). The methods followed were adapted from the study by Yu et al. (2022).

To prepare the standard curves, all three samples were serially diluted, starting from a concentration of 1 mg/mL and halving through successive dilutions to achieve final concentrations of 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL, and 0 mg/mL.

For each sample, 5 µL of the test solution was added to 250 µL of the Bradford reagent (Coomassie Brilliant Blue) and vortexed to ensure thorough mixing. The reagent was modified to contain 0.035 mg/mL sodium dodecyl sulphate (SDS) to enhance the slope of the collagen standard curve, thus improving its sensitivity to small differences in concentration. The samples were incubated at room temperature and two incubation times—10 minutes and 30 minutes—were tested in separate experiments to determine which produced the most accurate standard curve.

Following incubation, the samples were vortexed again, and 50 µL of each sample was transferred to a 384-well microplate. Absorbance was measured at 570 nm and 595 nm using a microplate reader (Multiskan FC, Thermo Scientific).

The absorbance readings were plotted against the known protein concentrations to generate a standard curve. A linear regression analysis was performed to calculate the best-fit line, which allowed the determination of the relationship between absorbance and protein concentration. This linear equation was then used to quantify the concentration of ligands in unknown samples by comparing their absorbance to the standard curve.

5.2 Creating the alginate hydrogels

The method for fabricating the alginate hydrogels was adapted from the research conducted by Frampton et al. (2011). Alginate was dissolved at a concentration of 1.0% w/v in 0.1 M 2-morpholinoethanesulfonic acid (MES) buffer, containing 0.3 M sodium chloride (NaCl). The

solution was left to stir overnight to ensure complete dissolution of the alginate in the buffer solution.

5.3 Assessing functionalisation efficiency with different EDC/NHS ratios

The methodology and calculations for assessing the functionalization efficiency of alginate scaffolds were adapted from the work by Saeger (2016) and Frampton et al. (2011). The objective was to evaluate how varying ratios of EDC and NHS impacted the binding of poly-D-lysine (PDL) to the alginate scaffolds.

A mixture of 0.152 mg of EDC and 0.161 mg of NHS were added to 350 µL of alginate. A control sample, consisting of 350 µL of alginate without EDC or NHS, was also prepared**.** PDL (553.5 µL) was added to all samples, which were then incubated overnight in a thermomixer (Eppendorf ThermoMixer).

On the following day, 100 µL aliquots were taken in triplicate from each mixture to allow for three independent readings per sample. To each aliquot, 20.9 µL of 200 mM $CaCl_2$ was added, followed by vortexing. The samples were then left to undergo gelation for 45 minutes. After gelation, 353.5 µL of water was added to each sample, and they were incubated overnight in the thermomixer.

The next day, 5 µL of the water layer, formed above the alginate hydrogel, was extracted and mixed with 250 µL of Coomassie Brilliant Blue reagent for the Bradford assay (absorbance measured at 570 nm and 595 nm). The assay, with a 30-minute incubation time, was used to quantify the unbound ligand, allowing for an assessment of how much ligand remained unattached to the scaffold. This provided a basis for evaluating the efficiency of the functionalisation process.

The absorbance values were applied to a regression equation derived from the PDL standard curve, allowing the calculation of ligand concentrations in the functionalised samples. These concentrations were then used to calculate the coupling efficiency, defined as:

$$\frac{(maximum\ ligang\ concentration - actual\ ligand\ concentration)}{maximum\ ligand\ concentration}\ x\ 100$$

Subsequent experiments examined whether a 2-fold change in EDC and NHS amounts would affect coupling efficiency. These experiments followed the same procedure described above, with the only variable being the adjusted concentrations of EDC and NHS.

Finally, bar charts were made to examine how varying EDC concentrations influenced the coupling efficiency of PDL to the alginate scaffold. The focus was placed on EDC

concentrations because EDC plays a direct role in activating the carboxyl groups on the alginate scaffold, facilitating the formation of amide bonds.

6 Results

6.1 Bradford Assay and Standard Curve

The standard curve for PDL after 30 minutes of incubation exhibited the highest linearity and precision, compared to the curves generated from collagen and jellagen. These standard curves were used to calculate unknown PDL concentrations in the subsequent experiments. Figures 1 and 2 display the standard curves for absorbance readings at 570 nm and 595 nm, respectively.

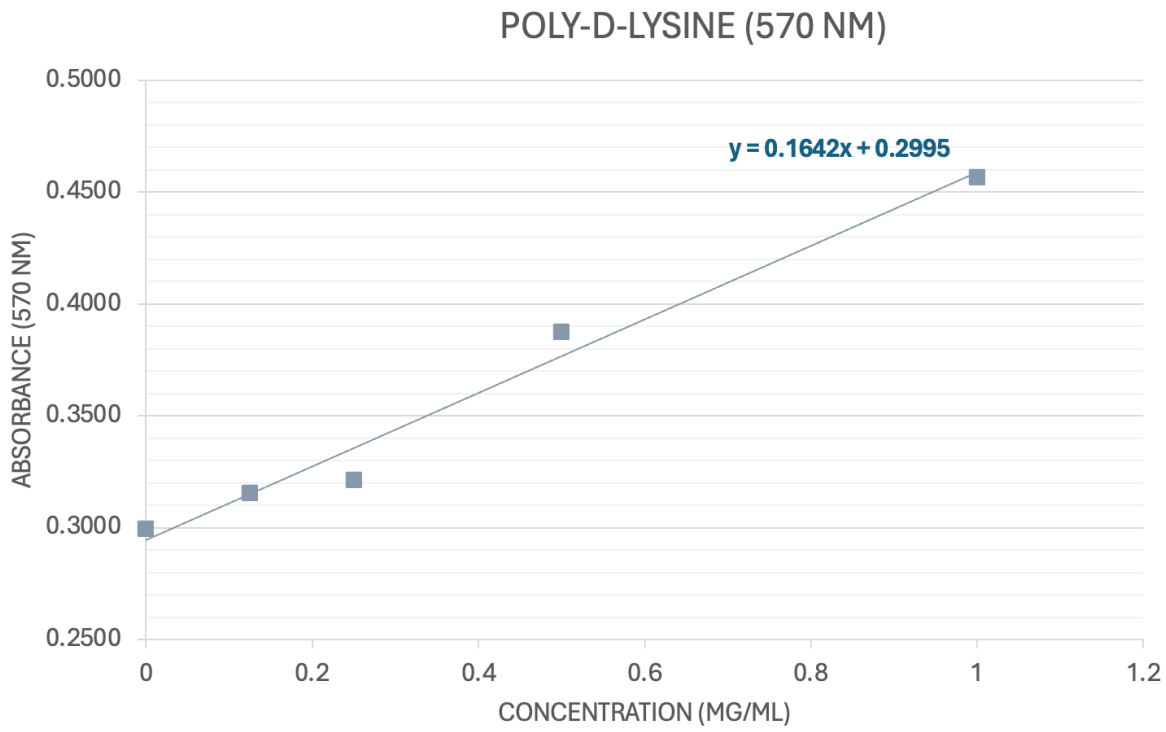

Figure 1: *Standard curve for PDL at 570 nm, with the linear regression equation (y = 0.1642x + 0.2995) representing the relationship between absorbance and concentration.*

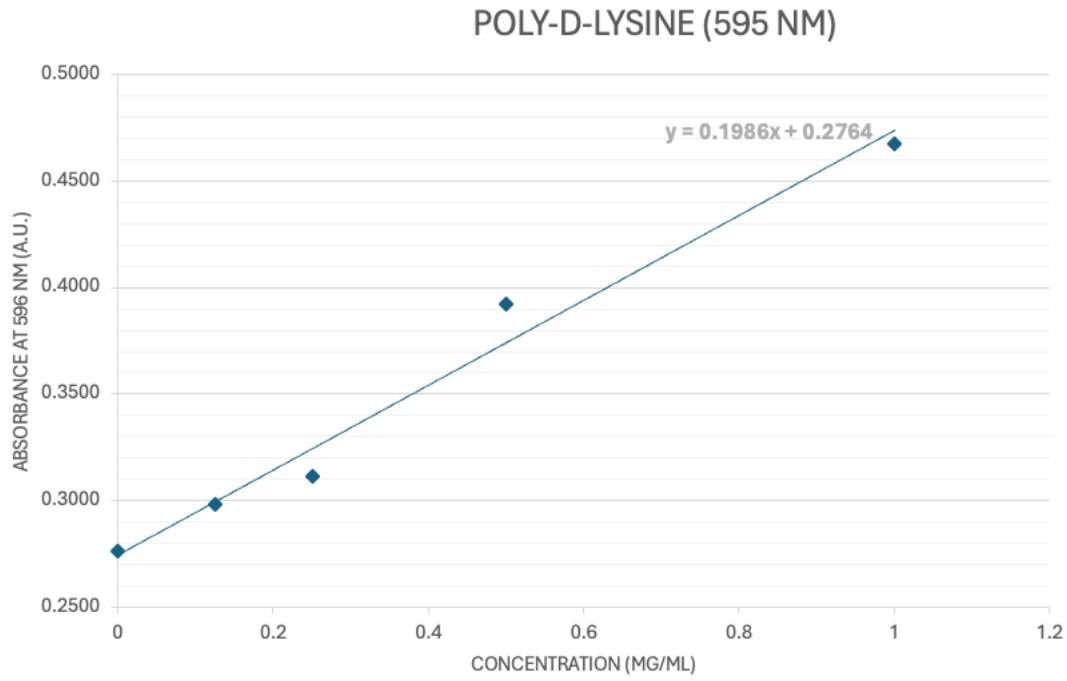

Figure 2: *Standard curve for PDL at 595 nm, with the linear regression equation (y = 0.1986x + 0.2764) representing the relationship between absorbance and concentration.*

6.2 Functionalisation Efficiency with Different EDC and NHS Ratios

The coupling efficiency of PDL was evaluated by adjusting the ratios of EDC and NHS. Although both EDC and NHS amounts were varied, the analysis focused on EDC concentrations, as EDC directly facilitates the activation of carboxyl groups on the alginate scaffold. The coupling efficiency reflects how much ligand successfully bound to the scaffold, with higher coupling efficiency indicating more effective ligand attachment.

The results were measured at two wavelengths (570 nm and 595 nm), and the corresponding bar charts display the mean coupling efficiency for each EDC concentration.

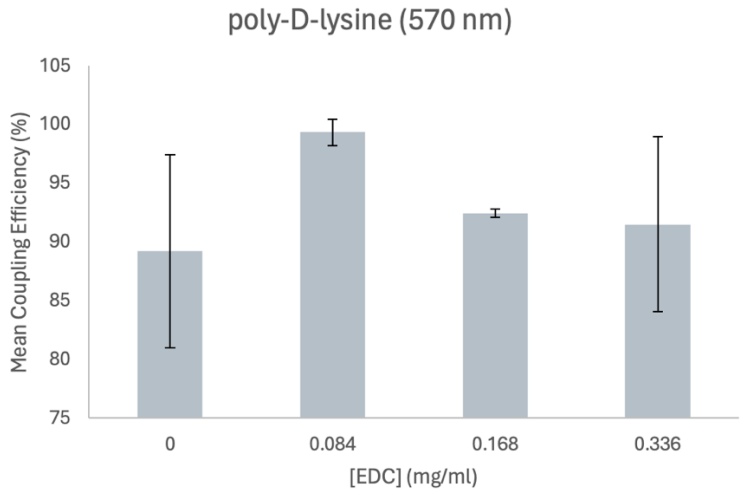

Figure 3: *Mean coupling efficiency of poly-D-lysine at 570 nm for varying EDC concentrations (0, 0.084, 0.168, and 0.336 mg/mL). NHS concentrations were also varied, but EDC concentration was the key variable. Error bars represent standard deviation.*

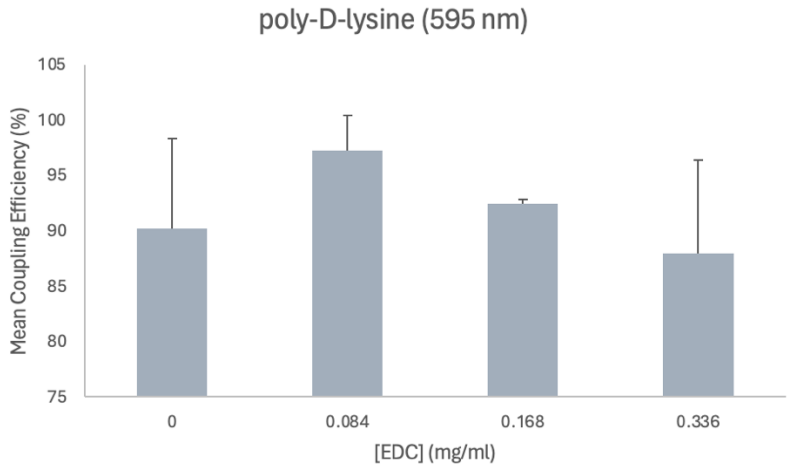

Figure 4: *Mean coupling efficiency of poly-D-lysine at 595 nm for varying EDC concentrations (0, 0.084, 0.168, and 0.336 mg/mL). NHS concentrations were also varied, but EDC concentration was the primary focus. Error bars represent standard deviation.*

7 Discussion and conclusion

The results of these experiments did not yield a definitive answer regarding the optimal ratio of EDC and NHS for enhancing alginate functionalisation. As shown in figure 3 and 4, the changes in coupling efficiency with varying EDC concentrations were relatively small. While 0.084 mg/mL of EDC appeared to achieve the highest coupling efficiency, the overall differences between the concentrations were not substantial. This suggests that additional variables may influence the functionalisation process, or that the balance between EDC and NHS is more complex than expected. Additionally, the variability in the error bars reflects inconsistencies across samples, making it difficult to identify an ideal ratio. Despite this, the experiments provided valuable foundational insights by offering a basis for future research. Several improvements could be made to refine the experimental process and potentially yield clearer results in subsequent studies.

Firstly, the method used to weigh out the small masses of EDC and NHS (in milligrams) may have introduced inaccuracies. Because such small quantities were measured, it is possible that the masses were not as precise as required. To improve accuracy in future experiments, stock solutions of known concentrations for EDC and NHS could be prepared, allowing for more precise control over the amounts used in each trial.

Secondly, the functionalisation experiments were conducted at different times, with the 2-fold increase and decrease experiments carried out after the initial trials. This necessitated the preparation of a new alginate solution. Although the same protocol was followed, slight variability could have been introduced, affecting the consistency of the results. To minimise variability in future experiments, it would be beneficial to prepare all samples and conduct all trials at the same time, using a single batch of alginate solution.

Furthermore, the initial Bradford assay did not yield linear or precise standard curves for jellagen and collagen, preventing their use in the subsequent functionalisation experiments. Optimising the standard curves for these proteins in future experiments would be beneficial. This would allow for assessing how functionalisation influences the coupling efficiency of different proteins to the alginate scaffold, as their interactions may vary.

Lastly, future experiments could explore the effects of increasing the ligand concentration while reducing the alginate volume. By increasing the ligand concentration, the likelihood of ligand binding to the functionalised sites on the scaffold would be enhanced, allowing for a more accurate assessment of functionalisation efficiency. This approach would ensure that there are enough ligands available to bind to the scaffold, making it easier to determine the effectiveness of the functionalisation process.

8 Reflections on Skills Gained and Insights Acquired

Throughout this project, I gained hands-on experience in various lab techniques, including splitting and counting the human colorectal adenocarcinoma cell line (SW1222). Additionally, I had the opportunity to shadow a postgraduate student, which provided valuable insights into the creation of 3D culture systems with alginate and the use of bioluminescence to measure cell viability. This experience broadened my understanding of advanced cell culture methods and how they can be applied in a research context.

More importantly, I developed a deeper appreciation for the complexity of research design, planning, and execution. From designing experiments that account for multiple variables, to ensuring the accuracy and reproducibility of results, I learned how much thought and detail goes into crafting a well-structured research project. Working through troubleshooting, refining protocols, and interpreting data showed me just how important it is to be flexible and think critically throughout the research process.

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