Evaluation of different copolymers for purification of Glucose transporter 4 (GLUT4) from adipose cells.

Student: *Emily Strickland,* ***Supervisors:*** *Dr Francoise Koumanov & Dr Paul Whitley, The University of Bath, Department for Health, and Department of life sciences.*

Background and aims of the project:

Amphipathic co-polymers such as Styrene-maleic acid (SMA) have been utilised for extraction of endogenous integral membrane proteins in nanodiscs known as SMALPS[1] . This mode of extraction has been successfully used in structural and functional studies of membrane proteins including high resolution Cryo-EM[2]. The Koumanov/ Whitley labs are developing a pipeline combining SMA extraction with affinity purification using amylose/MaltoseBindingProtein-proteinA (MBP-pA) method, with the immediate aim of pulling down the integral membrane protein Glucose transporter 4 (GLUT4) from adipocyte (3T3-L1) cells for downstream functional studies (Fig.1). However, the MBP-pA fusion protein dissociates from amylose resin in the presence of even low concentrations of SMA, making the pipeline unworkable.

The goal of this 6-week studentship was to screen a range of other co-polymers for suitability of use in the pipeline (Fig1).

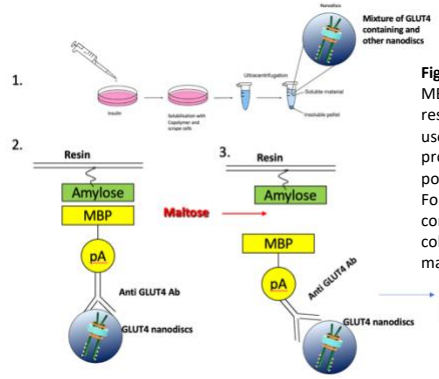


Figure 1- Graphical abstract. MBP-pA bound to Amylose resin and specific antibody is used to immunoprecipitate protein from soluble fraction of polymer extracted cell lysate. Following bead washes complexes are eluted from column using competition with maltose.

Materials and Methods:

Materials. Co-polymers including DIBMA screening kit (HEPES) & Ultrasolute Amphipol-17[3] were purchased from CubeBiotech. SMA-2000 was purchased from Cray valley and hydrolysed to form SMA following the protocol described by [4]. MBP-pA/amylose beads were prepared following protocol described in [5].

Assay to test MBP-pA binding to Amylose beads. 700µl of co-polymer solution in phosphate buffered saline (PBS) was incubated with 100µl of MBP-pA/amylose beads for 1 hour with rotation at room temperature. Beads were pelleted by centrifugation at 3000xg for 2 min and the supernatant was removed. Beads were washed in column buffer (10mM Tris, 150 mM NaCl, pH 7.4). The amount of MBP-pA remaining associated with the beads was assessed by SDS-PAGE, immunoblotting and densitometry.

Polymer extraction of proteins from mammalian cells. Confluent 3T3-L1 fibroblast were washed with PBS and 300µl co-polymer solutions containing protease inhibitors was applied to each well. Cells/debris were scraped from the dishes, placed in microfuge tubes, and incubated for 1 h at room temperature. Samples were separated into 'soluble' and 'insoluble fractions' by ultracentrifugation at 540,000xg for 20 mins. Fractions were further analysed by SDS-PAGE and immunoblotting to identify proteins present in the different fractions. The same protocol was used to extract proteins from insulin-stimulated fully differentiated adipocytes (10 day differentiation protocol followed as per[6])

Immunoprecipitation of GLUT4. MBP-pA Amylose beads were loaded with anti-GLUT4 antibody and washed in column buffer prior to incubation with co-polymer extracts (soluble fractions). Beads were washed to remove unbound material and samples eluted with 40 mM maltose in PBS. Note: These are very mild elution conditions in order to maintain the structural integrity of eluted proteins.

Transmission electron microscopy TEM - Samples were placed onto chromium coater-glow discharged, 200 Mesh copper grids. Samples were washed with distilled water and incubated with 4% Uranyl acetate before transmission electron microscopy was carried out.

Results and outcomes of studentship, including any relevant figures

Initial experiments confirmed that SMA-2000 significantly interferes with binding of MBP-pA to amylose resin (Fig2 and data not shown). As our immunoprecipitation pipeline requires MBP-pA to remain bound to resin different co-polymers were screened (Fig2).

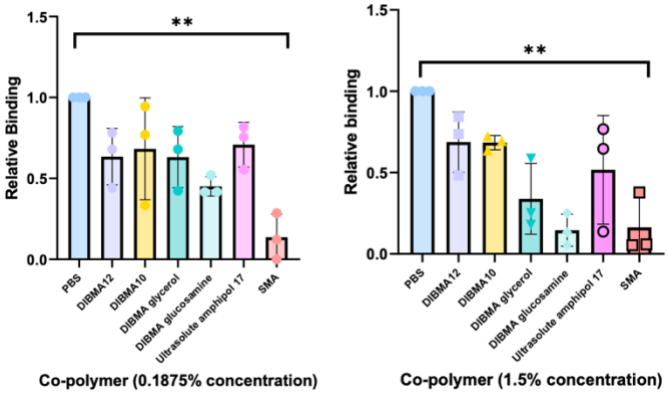


Figure 2- Co-polymer amylose/ MBP-Pa interference assays. Relative binding of MBP-pA to amylose beads incubated with 0.1875% or 1.5% co-polymer dissolved in PBS. MBP-pA bound to the amylose beads was quantified after SDS-PAGE and immunoblotting. Results were expressed as relative binding compared to the control condition (PBS). Mean ± SD (n=3). Comparisons between conditions with two-way Anova ** $p < 0.01$.

The results revealed that all the tested co-polymers perform more favourably than SMA in maintaining interaction between MBP-pA and the amylose resin at low concentrations. DIBMA 12, DIBMA 10 and Amphipol-17 allowed significant binding even at higher (1.5%) concentrations. We therefore concluded that DIBMA 12, DIBMA 10 and Amphipol-17 were worthy of further investigation.

For our pipeline to work the polymer should not only allow the MBP-pA/Amylose interaction but should be efficient at extracting membrane proteins from mammalian cells. We therefore tested efficiency of extraction of selected membrane proteins from 3T3-L1 cells using the chosen polymers (Fig3).

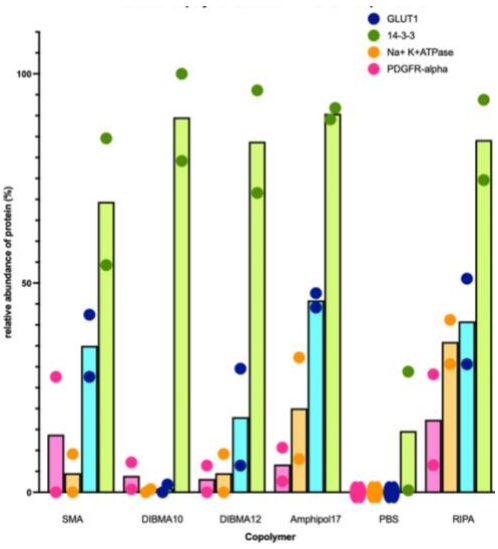


Figure 3. Testing the extraction efficiency of different polymers on fibroblasts. 3T3-L1 fibroblasts were treated with indicated polymers, detergent (RIPA) or PBS controls . Soluble and insoluble cell contents were separated by ultracentrifugation. These fractions were separated by SDS-PAGE and immunoblotted for selected proteins (see key) and quantified. Relative abundance of the protein of interest in the soluble fraction was calculated as a percentage of the total amount in the extract.

It is apparent that DIBMA10 and DIBMA12 have relatively low efficiency of membrane protein extraction as shown by the low amounts of GLUT1, Na^+/ K^+ ATPase and PDGFR-α in the supernatants

following centrifugation. Amphipol-17 extracted GLUT1 and Na^+/K^+ ATPase with similar efficiency to RIPA (detergent control) although PDGFR-α extraction was quite low.

As our initial aim was to develop a pipeline for purification of GLUT4 we performed extraction from 3T3L1 cells that had been differentiated into adipocytes (Fig.4). 3T3-L1 fibroblasts do not express GLUT4 whereas adipocytes do.

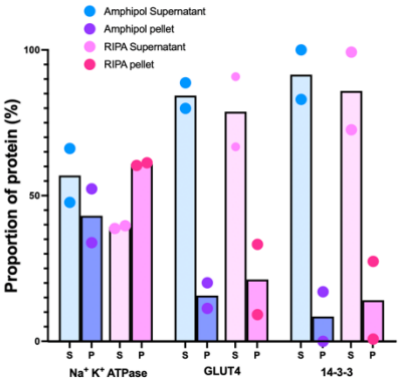


Figure 4. Testing the extraction efficiency of amphipol 17 on adipocytes. 3T3-L1 adipocytes were treated with 0.5% amphipol-17 or detergent (RIPA).soluble (S) and insoluble cell contents (P) were prepared by ultracentrifugation. These fractions were separated by SDS-PAGE and immunoblotted for selected proteins (see key) and quantified. Proportion (%) of selected protein present in the supernatants (soluble fraction) and pellets (insoluble fraction) is plotted on graph n=2).

Evidently, Amphipol-17 extracts GLUT4, our target with similar efficiency as RIPA detergent, (Fig4). Therefore, we concluded that amphipol-17 solubilised cell fractions are suitable for immunoprecipitation experiments (Fig5).

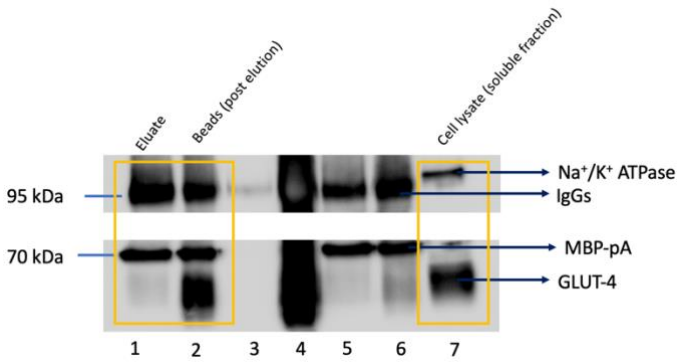


Figure 5. Figure 5. Affinity isolation of GLUT4 solubilised with Amphipol-17. Adipocytes were treated with Amphipol-17 and soluble cell fraction was prepared (lane 7). This fraction was incubated with amylose MBP-pA anti-GLUT4 beads. Beads were washed and elution was performed using maltose. Eluate (lane 1) and proteins remaining on the beads post elution (lane 2) were analysed. Lanes 3 to 6 – irrelevant samples.

The results (Fig5) indicate that GLUT4 has been pulled down/enriched on the amylose beads. Encouragingly there is no evidence of Na^+/K^+ ATPase bound to the beads suggesting that there is selectivity for GLUT4 in the immunoprecipitation. However, the amount of GLUT4 eluted from the beads with maltose was very low.

Despite the low yield of GLUT4 eluted from the beads we decided to image the eluate using TEM (Fig6) (along with other fractions from the extraction and controls (not shown).

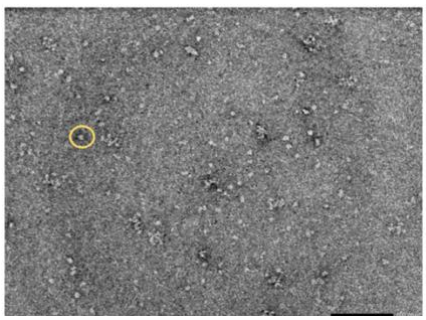


Figure 6- Transmission electron microscopy micrograph of amphipol-17 adipocyte extraction. Scale bar 100 nm. Yellow circle represents single nanodisc

While the results of the TEM are very preliminary, we were encouraged that we could see dense particles of a size consistent with being nanodiscs in the sample similar to those seen in [2].

Future directions in which the project could be taken

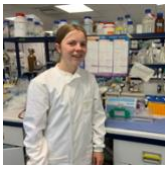
Purification of proteins needs to be scaled up. Elution from the amylose column needs to be optimised, this may be through the use of column rather than batch purification, as immunoblotting shows this recovers the highest abundance of GLUT4 (data not shown). Mass spectrometry proteomics would then be carried out on the purified material to assess the purity of the GLUT4 preparation prior to any cryo-EM. This may identify known GLUT4 binding proteins such as TUG [7], which will encourage us to develop methodology to study membrane protein interactions that are disrupted during conventional detergent extraction.

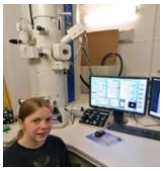
Departures from original project plan

As the lab had pre-existing stocks of recombinant MBP-pA the planned bacterial expression for week 1 was not necessary and I started testing the co-polymer interference in week 1 and 2. This allowed me to go much further in the characterisation of new co-polymers.
I carried out polymer extraction in fibroblast cells (3T3-L1) as well as in adipocytes, this allowed more repeats of successful polymers to be carried out once 10-day differentiation of adipocytes was complete. This efficient use of my time early in the project meant that I could learn and perform TEM imaging of nanodiscs, and maltose elution's extracted from adipocytes and fibroblasts, which was not originally planned.

Value of studentship to the student, focusing on skills developed (technical and transferable) and contribution to future career plans

During this unique and exciting opportunity, I have gained invaluable transferable skills such as organisation, time keeping, experimental design, and critical analysis which has allowed me to shape the direction of the project and achieve beyond the proposed timescale of the project. These skills alongside the numerous new technical skills such as SDS-PAGE, western blotting, transmission electron microscopy and cell culture will be greatly beneficial to take into my last two years at university in my M. Biochemistry degree and beyond, hopefully into a research career, which I realise now is something I wish to pursue as a result of this fantastic experience.





Pictures- *Top left- Emily in cell biology lab, Top right- Emily observing Transmission electron microscopy of eluate samples, Bottom image- lab group photo (L to R- Jariya Buniam, Francoise Koumanov, Paul Whitley, Linston Stanley, Emily Strickland, Alisha Spencer)*

Value of studentship to the research group

From our perspective as supervisors Emily's summer studentship was invaluable. Without Emily this work would not have been performed so promptly. Emily has really pushed the project along and achieved more than we had anticipated in a 6-week period. Amphipol-17 has been identified as a polymer that efficiently solubilises membrane proteins in mammalian cells and is compatible with our pipeline for the affinity purification of GLUT4. When examined using TEM the affinity purified GLUT4 sample looks to contain particles of a size and shape consistent with nanodiscs. We will definitely be exploring the use of Amphipol-17 in a continuation of this, and other studies and we are considering including these results in a research article.

References

1. Morrison, K.A., et al., *Membrane extraction with styrene-maleic acid copolymer results in insulin receptor autophosphorylation in the absence of ligand.* Sci Rep, 2022. **12**(1): p. 3532.
2. Sun, C. and R.B. Gennis, *Single-particle cryo-EM studies of transmembrane proteins in SMA copolymer nanodiscs.* Chem Phys Lipids, 2019. **221**: p. 114-119.
3. Higgins, A.J., et al., *Cycloalkane-modified amphiphilic polymers provide direct extraction of membrane proteins for CryoEM analysis.* Commun Biol, 2021. **4**(1): p. 1337.
4. Koumanov, F., et al., *Insulin signaling meets vesicle traffic of GLUT4 at a plasma-membrane-activated fusion step.* Cell Metab, 2005. **2**(3): p. 179-89.
5. Hall, S.C.L., et al., *An acid-compatible co-polymer for the solubilization of membranes and proteins into lipid bilayer-containing nanoparticles.* Nanoscale, 2018. **10**(22): p. 10609-10619.
6. Frost, S.C. and M.D. Lane, *Evidence for the involvement of vicinal sulfhydryl groups in insulin-activated hexose transport by 3T3-L1 adipocytes.* J Biol Chem, 1985. **260**(5): p. 2646-52.
7. Yu, C., et al., *The glucose transporter 4-regulating protein TUG is essential for highly insulin-responsive glucose uptake in 3T3-L1 adipocytes.* J Biol Chem, 2007. **282**(10): p. 7710-22.