# ‘The Immune Bomb’ – Investigating immunotherapy combinations for use in ultrasound triggered liposomes in targeted treatment of cancer

Morgan Elsmore | Trinity College | Institute of Biomedical Engineering, University of Oxford

## Outline

The purpose of this project is to identify possible combinations of immune checkpoint inhibitors and immune stimulators with additive or synergistic effects in treating cancer, and then to analyse the properties of these therapies when inserted into cavitation sensitive, ultrasound triggered liposomes. This project aims to identify combinations of immune modulators, encapsulate these modulators within ultrasound sensitive liposomes and then gather data on the properties of these liposomes using immunochemical techniques.

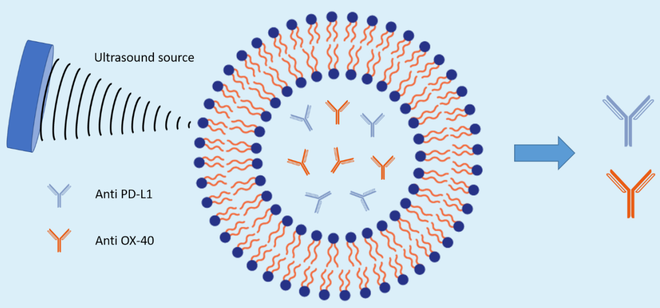



Figure 1: Graphical abstract

## Immune modulators

Checkpoint blockage takes advantage of the fact that the immune system already ‘knows’ how to fight cancer. T-cells have different surface receptors, some act as an accelerator to promote a response and others as a brake to lessen an immune response. Checkpoint inhibitors overcome these ‘brake’ signals which many cancers hijack by producing a molecule to reduce the immune response. Immune stimulators act by inducing activation or increasing activity of immune system components.

This project investigated the use of the immune checkpoint inhibitor Anti-PDL1 and the immune stimulant Anti OX-40.

The two immune agents, anti PD-L1 antagonist and anti OX-40 agonist monoclonal antibodies were chosen since the site of action for both antibodies is found at the tumour site on tumour infiltrating lymphocytes for anti OX40 and on the tumour cells themselves for anti PD-L1. These two antibodies were chosen to be tested together due to potentially complementary effects, size and likely compatibility with encapsulation.

## Materials

The following immune agents were sourced from ThermoFisher Scientific: PD-L1 Monoclonal Antibody MIH1 (mouse), CD134 (OX40) Monoclonal Antibody, Functional Grade (rat). Lipids for liposome preparation were sourced from Avanti Polar Lipids, with cholesterol from Sigma Chemical Company. For use in ELISAs for quantifying antibody concentrations, HRP conjugated highly cross adsorbed antibody (rat and mouse) were sourced from ThermoFisher Scientific. ELISAs were carried out in Nunc MaxiSorp™ 96 well plates and developed using 1-Step™ Ultra TMB-ELISA Substrate Solution. Separation of unincorporated antibody from the liposome preparations was attempted using Pall Nanosep centrifugal device with Omega membrane MWCO 300 kDa.

## Liposome formulation

Liposomes were prepared using the thin film hydration method with preparations shown below.

The first formulation is designed to be a control formulation and not release under ultrasound exposure. The second formulation contains DOPE, shown to exhibit release of contents upon exposure to low doses of ultrasound.

| Lipid | 1) ‘Doxil like’ | 2) DOPE US Sensitive |
|---|---|---|
| DSPE-PEG | 5% | 10% |
| DSPC | 56% | 0% |
| DOPE | 0% | 65% |
| Cholesterol | 39% | 25% |

## Procedure

- Thin film hydration was completed as per protocol from Avanti Polar Lipids and extruded using the Avanti Mini Extruder and 400nm / 200nm polycarbonate membranes.
- Lipids were suspended in 2:1 chloroform-methanol and evaporated above the lipid transition temperature. Following evaporation, lipid films were dried overnight using a high vacuum pump.
- Hydration was then completed using a solution of antibodies in phosphate buffered saline, with swelling for 1 hour.
- Lipids were then extruded to form liposomes above the lipid transition temperature with 11 passes through a 400nm membrane, followed by 11 passes through a 200nm membrane to yield liposomes of approximately 200nm.

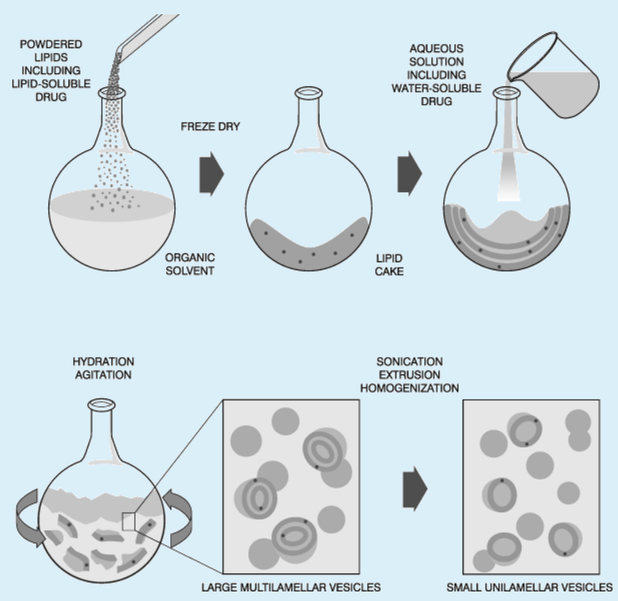



Figure 2: Preparation of liposomes through thin layer hydration (Avanti Polar Lipids)

## Analysis

Size distribution of extruded liposomes was found using dynamic light scattering (Malvern Zetasizer). Post extrusion liposomes were found to be homogenous with mean size of 197nm (±11%) for formulation 1 and 177nm (±14%) for formulation 2.

Analysis of antibody encapsulation was completed using enzyme linked immunosorbent assays (ELISAs). This allowed quantification of antibody concentrations before and after filtration, simulated release using heat or sonication, and release following ultrasound exposure.

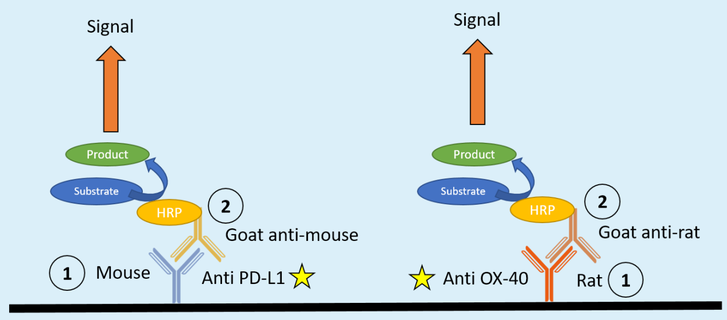



Figure 3: ELISA set up for quantifying antibody concentrations

## Purification

Following hydration of the lipid film, antibody not incorporated into liposomes was removed using centrifugal filters with membrane size 300kDa. Samples to be purified were split into 100ul samples and centrifuged for 5min at 10,000g. Following each centrifugation, approximately 50ul of flow through was removed and the retentate resuspended in 50ul PBS. This was repeated for three cycles. Each 100ul fraction was then transferred to a new filter and this was repeated to give a total of three filters passed through.

## Ultrasound exposure

In order to test liposome formulations for sensitivity to ultrasound exposure, each formulation was added to an ultrasound test chamber. Ultrasound was administered to 300ul of liposome sample diluted to 750ul in PBS. 50ul of microbubbles (large gas filled lipid bubbles, ≈1-10μm) were also added to the ultrasound chamber to improve release. The ultrasound test system delivered approximately 1-1.5 MPa pressure for 20s.

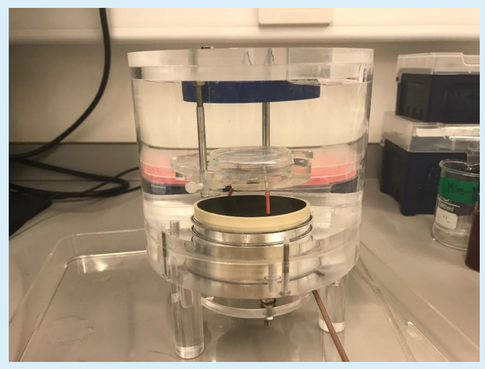
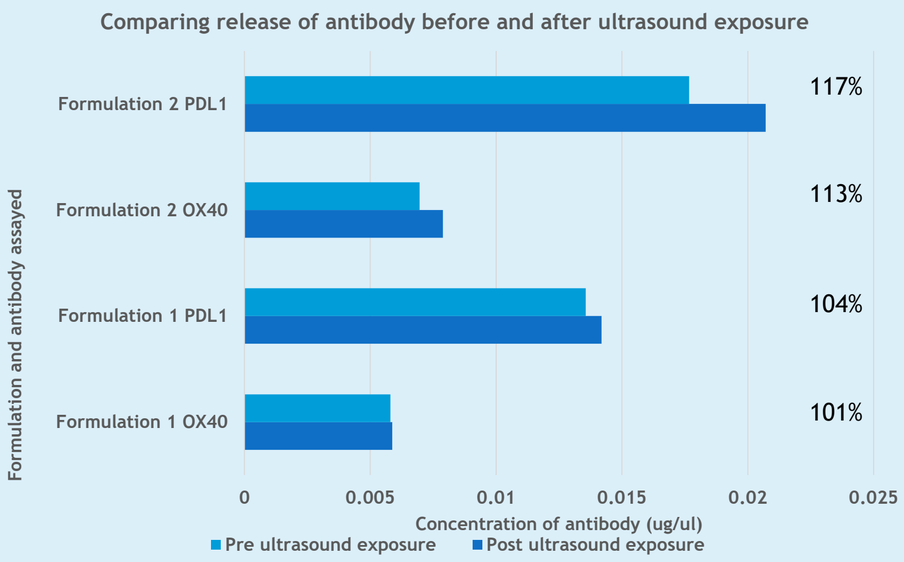


Figure 2: Ultrasound exposure chamber

## Results

Triggered release appears to have taken place in response to ultrasound exposure for the formulation designed to be ultrasound sensitive (formulation 2, DOPE). This release however is modest with post ultrasound exposure antibody concentrations ≈115% of levels prior to exposure. This lesser than expected release may be due to a high background signal as a result of non-optimal purification.

Release from control formulation 1 (‘Doxil’ like) appears to be minimal, suggesting these liposomes are stable when exposed to ultrasound and are a suitable control for determining ultrasound mediated release.




This project was completed thanks to the Laidlaw Undergraduate Research & Leadership Programme at the University of Oxford. Thank you to my supervisor Robert Carlisle for his support throughout this project.