Lipase-based *in vitro* release assays for pharmaceutical lipid formulations

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Abstract

The purpose of this project was to develop and test lipase-based *in vitro* release (IVR) assays for model and active pharmaceutical ingredients (API) entrapped in lipid formulations based on mixtures of phosphatidylcholine (PC) and glycerol dioleate (GDO).

Two dyes, Fluorescein disodium salt and Patent Blue V sodium salt, functioning as model drug compounds, were investigated separately in the PC/GDO formulation. As the lipid formulation containing one of the two dyes was injected *in vitro* into a buffered saline solution, the lipids self-assembled into liquid crystal (LC) phases which trap the dye inside a nearly spherical LC depot. The dye was then released slowly over time. In the presence of lipases, the dye release was accelerated by biodegradation of the lipids. The IVR rate was followed by spectrophotometric measurements of the dye concentration in the aqueous release medium.

This thesis describes the investigation of many parameters such as the suitability of the two dyes as model APIs, the effect of two different triacyl glycerol lipases (TGL) and a phospholipase, different concentrations of lipases as well as mixtures of different lipases, different lipid formulation types, the difference between a single vial experimental setup and a 96 well plate setup, as well as an overall evaluation of the project.

It was shown that the addition of lipases dramatically increases the IVR rate, even at low concentrations. The effect on IVR was much higher for the phospholipase than for the TGLs.

This research is of interest to the research- and development based pharmaceutical company Camurus AB in Lund, Sweden, currently developing pharmaceutical products based on the lipid liquid crystal formulation technology. The results will be used in the assessment and development of *in vitro in vivo* correlation (IVIVC) assays which are useful for formulation development and for regulatory purposes in conjunction with post registration changes related to Chemistry, Manufacturing and Controls (CMC).
Acknowledgements

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I would also like to thank my wife and children for being the light of my life.
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1. Introduction

1.1 Controlled drug release with a lipid formulation

There are many ways to take a medicine. Two common examples are a simple tablet taken orally or a drug in solution being injected intravenously or subcutaneously. In all of these cases, particularly the latter, a high amount of drug enters the bloodstream fast. Sometimes this is desirable and sometimes not. Some drug compounds may have a narrow span (or therapeutic window) between the therapeutic blood concentration and the toxic concentration and perhaps the drug concentration in the blood needs to be more or less constant in a chronic treatment setting. With an immediate release formulation, such as the pill or (simple) injection mentioned above, the medications would have to be administered often and at low doses to avoid high peaks and valleys of drug concentration in the blood, depending on the therapeutic window. To comply with such a medical treatment, the patient needs to be very careful not to miss a dose, potentially thus causing serious health issues.[1]

The solution for many of these compliance problems is controlled release. It means a drug formulation that does not immediately release all of the drug compound at once. It releases the drug at a desired place, rate or time. There is a wide array of controlled release products on the market. Some controlled release formulations may delay release until it reaches a certain part of the gastrointestinal (GI) tract, some will last for a few hours, and some medical applications sustain a steady release for several years (e.g., some implants). The indications where sustained release products are used are also very diverse, ranging from treatment of diabetes to contraceptives. [2][3][4][5]

The research- and development-based pharmaceutical company Camurus AB in Lund, Sweden, has developed a subcutaneous sustained release technology called “FluidCrystal® Injection depot” and it is described on their web page as “...a liquid solution that transforms into a controlled release liquid crystal gel matrix in situ on contact with minute quantities of aqueous fluid at site of injection.” On the same web page there is also a scheme illustrating the functionality aspects of the technology, see Figure 1.[6]

![Figure 1](image)

**Figure 1.** On the Camurus website this image can be found along with the following explanation:

1. Subcutaneous injection of lipid-based formulation
2. Formation of liquid crystal gel by absorption of water (W)
3. Slow release of drug (D), biodegradation of depot”[6]

The formulation is a mixture of lipids that can self-assemble into nano-structured liquid crystal (LC) phases when in aqueous solutions. The lipid-based formulation is a mixture of the two lipids, a phospholipid (e.g., phosphatidylcholine (PC)) and a diglyceride (or diacylglycerol) in combination with small amounts of pharmaceutically acceptable co-solvents (e.g., ethanol). PC and the diglyceride glycerol dioleate (GDO) are shown in Figures 2 and 3 respectively. PC and GDO belong to the lipid class called glycerolipids made up of a glycerol backbone with one or more fatty acids which have long
hydrophobic hydrocarbon chains and a polar head group. The fatty acids are covalently bound to the glycerol backbone with ester bonds. Soybean phosphatidylcholine (SPC) is a phospholipid derived from soybean and has a natural distribution of fatty acid chain lengths and unsaturation, comprising mainly linoleoyl (C18:2) acyl chains, with a choline group attached to it at position three as shown in Figure 2. GDO has two hydrophobic oleic acids on the glycerol backbone, as shown in Figure 3.

![Figure 2. Phosphatidyl choline (DioleoylPC), one of the two main components of the lipid formulation under study.](image)

![Figure 3. Glycerol dioleate (GDO), the other of the two main components of the lipid formulation under study. This is the regio-isomer 1,2-GDO. 1,3-GDO also exists. Both are present in the formulation.](image)

The self-assembly of the lipids into LCs is driven by hydrophobic interactions minimizing the contact between the hydrocarbon chains of the lipids and water and also interactions between the polar head groups and the aqueous fluids surrounding the lipids after injection and upon water absorption.[8] Different LC phases, with specific nanostructures that can entrap the active pharmaceutical ingredient (API) inside, will form depending on lipid composition and this is the key to achieving controlled long-acting release. Figure 4 shows how the LC formation has started at the interface between the saline solution and the lipid solution. The LC formation will progress inwards as water is absorbed until equilibrium has been reached.[7]

![Figure 4. A PC/GDO lipid solution injected into a saline solution will form liquid crystals by absorbing water. The self-assembly process will start at the interface and progress inwards. This polarized light microscopy image illustrates the process. It was taken after 2 minutes. The image has a magnification of 80 times and has 10 µm between the scale bars.[7]](image)

The release rate of the API from the depot is determined by a combination of parameters, most importantly the geometric structure of the nano-domains, drug diffusion out from the depot and also biodegradation of the depot. A phase diagram is shown in Figure 5[9] and in Figure 6[10] there is an image showing what the phases look like on a microscopic
level. The phases and structures found in the depot will affect its ability to retain drug compounds entrapped or dissolved therein. The diffusion rate depends on the permeability of the drug molecule which can differ widely between small molecules and larger biomolecules such as peptides. Enzymatic biodegradation by various lipases found in the body is believed to play an important role in the clearance of the depot and in controlling the release of entrapped APIs. Different types of lipases are found everywhere in the body, not only in the GI tract.[11] With certain formulations, diffusion is hindered by poor API permeability and good encapsulation properties and biodegradation then becomes the rate determining release mechanism.[10] Hence, physiologically relevant in vitro release models may potentially be developed utilizing lipases. Knowledge gained from such in vitro studies may also increase the mechanistic understanding of the in vivo fate of injected lipid-based drug depot formulations.

Figure 5. Phase diagram with diacylglycerol (DAG), deuterated water (2H2O) and soy phosphatidyl choline. From top to bottom the different phases are: L2, reversed micellar solution phase; I2 (called I2 in Figure 6), cubic liquid crystalline phase; HII (called H2 in Figure 6), reversed hexagonal phase and Lα, lamellar phase. Phase diagrams are used to understand what phases different lipid formulations are likely to form.[9]
1.2 Project objectives

As outlined in the introductory part, physiologically relevant in vitro release models may potentially be developed utilizing lipases. Such in vitro studies may also increase the mechanistic understanding of the in vivo fate of injected lipid-based drug depot formulations. Accordingly, the goals of this project were to assess and develop lipase based in vitro release (IVR) assays. It was mainly an experimental study of release behavior of model drugs (dyes) from lipid formulations in the presence lipases. The following parameters were to be studied:

1. The effect of lipase type on in vitro release kinetics of dyes from the lipid formulation.
2. The effect of different concentrations of those lipases.
3. The effect of combining different lipases.
4. Comparison of different experimental setups.

The methods will be tested on different formulations denoted Formulation A, Formulation B, etc. The results of this work will increase the understanding of the effect of lipases on the lipid formulations. As lipases play a role in in vivo drug release it may further be possible to use lipase-based IVR assays as a basis for an in vitro in vivo correlation (IVIVC). IVIVC assays are useful tools for formulation development and for regulatory purposes in conjunction with post registration changes related to Chemistry, Manufacturing and Controls (CMC).

1.3 Lipase based in vitro release

The experimental part of the project revolved around the in vitro release (IVR) from liquid crystal depots injected into a buffered saline water solution with or without addition of different lipases and different concentrations of lipases. Figure 7 shows what this in vitro release may look like visually. The concentration of a molecule released from the depot into the aqueous solution was measured over time. Instead of an actual active pharmaceutical ingredient, two dyes were used as “model APIs” for convenience. The dyes would be safer to handle being non-toxic, easy to detect using visual range spectroscopy even in low quantities, cheap and readily available. Initially, the dye Fluorescein sodium salt (Fluorescein for short) was used, but it was found to be less ideal and was replaced by Patent Blue V sodium salt (Patent Blue for short), which was then used for all subsequent experiments. Figure 8 shows the molecular structures of the two dyes mentioned.
At pH 7.5, Fluorescein is mostly deprotonated, has a molecular weight of 330.29 g/mol and log P = -0.08.[12] At pH 7.4, Patent Blue has a molar weight of 559.67 and log P = -2.98,[13] Patent Blue is thus predicted to be less permeable and less soluble in the formulation than Fluorescein and therefore is expected to be released at a lower rate from the liquid crystals. As they are both very soluble in water, sink condition will apply, meaning that the dye concentration at maximum release is far below the solubility limit of the respective dye in the release medium.

Lipases are enzymes that remove fatty acids from the glycerol backbone by catalyzing hydrolysis of the ester bonds. In this work three enzymes were used: Two triacyl glyceride lipases (TGL), *Thermomyces lanuginosus* lipase (henceforth called TLL) and *Candida antarctica* lipase B (called CALB from now on) and a phospholipase A1 from *Thermomyces lanuginosus* simply called PLA1 in this report. The TGLs have similar functions, they hydrolyze the ester bonds in GDO, producing first glycerol mono oleate (GMO) and a free oleic acid, and later it may cleave the second ester bond, producing a free glycerol molecule and another free oleic acid. This is illustrated in Figure 9. PLA1 hydrolysers the bond shown in Figure 10.[14][15]
Figure 9. Hydrolysis of 1,2-GDO. The product if one bond is hydrolysed is GMO and a free fatty acid. Further hydrolysis will result in a total of two free fatty acid and a free glycerol molecule. TGLs catalyze hydrolysis of both bonds, though it was not within the scope of this project to verify if or to what extent hydrolysis of the second ester bond takes place.

Figure 10. Phospholipases catalyze hydrolysis of the ester bonds of phospholipids. In this project SPC was used so R1 and R2 are primarily linoleic acids. A type A1 phospholipase (PLA1) was used, which hydrolyses the ester bond at position 1. The hydrolyzed products are then a free linoleic acid and a lyso-phosphatidyl choline (lyso-PC).[10]
2. Materials and Methods

2.1 Clarification of this chapter

When reading chapter 2 and trying to make sense of everything, it may be helpful to keep in mind the hands-on nature of the project. Some vital changes were done: Fluorescein was exchanged with Patent Blue. phosphate buffered saline (PBS) was replaced by Tris buffered saline (TBS) and the use of the surfactant Triton X-100 was discontinued in favor of the solvent ethanol (EtOH) for solubilizing lipids in the samples taken out for analysis by spectroscopy.

The next section describes how the formulations and buffers were prepared and what chemicals were used. After that the single vial method is be described along with the two analysis methods, both the so called “Fluorescein assay” and the “Patent Blue assay”. The 96 well plate setup will also be explained. As the methods were developing, the results were taken into account when adapting the steps and therefore some results were included in this chapter.

2.2 Materials and formulation preparation

The lipid formulations in the project were made out of mixtures of PC, GDO, EtOH and a dye. Ratios are always expressed as wt/wt ratios, unless stated otherwise. Lipids/EtOH ratio is in every formulation 90/10 and the dye content is always 0.5 wt%, i.e., about 5 mg/mL. For confidentiality reasons, exact descriptions of the formulations will not be disclosed and the formulations will be denoted “Formulation A”, “Formulation B” etc.

Initially it was planned that Fluorescein should be used all throughout the project, but after some initial experiments, it was replaced by Patent Blue. The main two reasons were that Fluorescein was found to have stability issues, and that Patent Blue was found to be released very slowly in the absence of lipases, i.e., having a very low background release. Low background release is desirable as the observed release then can be attributed to the effect of lipases only. Additionally, a dye with low passive release is a better model for peptide drugs, as peptides also show very low IVR rates in the absence of lipases or other accelerating agent.

The lipids had been stored in freezers at -18 °C and before opening the containers, they were left at room temperature for at least one hour to avoid condensation inside the containers. The lipid formulation was made by adding the three components to a glass vial on the balance (Precisa 303A) and mixed overnight on a roller mixer (Stuart® roller mixer SRT9). The day after the lipids and EtOH seemed to be well mixed. Fluorescein was added and was left to mix on a roller mixer for one more day. The Patent Blue formulations were made in the same way.

Two saline buffers were used. For the Fluorescein experiments Phosphate Buffered Saline (PBS) solutions were prepared by adding one tablet of PBS salts, to 200 g of sterile Water for Injection (WFI). Adding one PBS-tablet to 200 g of WFI results in a 10 mM phosphate buffer also containing 2.7 mM potassium chloride and 137 mM sodium chloride and having a pH of 7.4. The buffer strength of PBS proved too weak to maintain a constant pH over the course of the lipase induced release experiments. As fatty acids were hydrolyzed, the fatty acid concentration got high enough to lower the pH. Therefore a buffer with higher buffering capacity was needed. Previous work at Camurus had been done using Tris Buffered Saline (TBS) buffers so for the sake of consistency it was decided to change to a strong TBS buffer instead of using a stronger PBS buffer. The TBS buffer was prepared by adding Trizma® Base and NaCl to WFI to get a Trizma concentration of 100 mM and a NaCl concentration of 150 mM. The pH was adjusted by adding enough HCl to reach 7.5 pH (pH-meter: MeterLab® PHM290) with a silver chloride electrode.

Three fungal lipases were selected for the study. The fungal lipases that were chosen are readily available compared to mammalian/human lipases. The TGLs and PLA1 enzymes are assumed to have similar functions and mechanisms to the lipases found in the human body and therefore should be relevant for the in vivo situation. The lipases chosen were:

- Triacylglycerol lipase from Thermomyces lanuginosus (Novozymes® Lipolase 100L) (TLL).
- Triacylglycerol lipase from from Candida antarctica (recombinant, expressed in Aspergillus niger) (Novozymes® CALB L).
- Phospholipase A1 (PLA1) from Thermomyces lanuginosus (Novozymes® Lecitase™ Ultra).

The TGL from T. lanuginosus is in this report called TLL, the TGL from C. antarctica is called CALB and the Phospholipase A1 from T. lanuginosus is called PLA1. The lipases were delivered from the supplier in solution form and throughout this report, the amount of lipase added in an experiment is always mentioned as absolute volume amount of the original lipase solutions, even when they were diluted. For instance, when it says that 0.05 µl of PLA1 has been added to
a vial with lipid formulation containing Fluorescein it is understood that it had been diluted with PBS. The same goes for the Patent Blue experiments, except that they had been diluted with TBS. According to specifications the lipase solutions had the following lipase activity (LU)/g solution and composition:

- **TLL:** 130000 LU/g solution (73% (w/v) water, 25% (w/v) propylene glycol, 2% (w/v) lipase, and 0.5% calcium chloride; density 1.2 g/mL)
- **CALB:** 6031 LU/g solution (solution composition unknown; density 1.2 g/mL)
- **PLA1:** 11000 LU/g solution (solution composition unknown; density 1.2 g/mL)

To measure the dye concentration in solution over the course of the IVR experiments, small volume samples were taken out and put in a cuvette and measured in a spectrophotometer. Measurements could have been done directly had it not been for an effect of the lipase reaction that caused formation of turbid emulsions. To solve this problem the small sample taken out for analysis was mixed with a solution of Triton X-100 in PBS. Two solutions of Triton X-100 in PBS were made, one with 10 wt% Triton X-100 and one with 20 wt% Triton X-100 (their uses are explained later). A calibration curve was created with the solution of 10% Triton X-100 in PBS for the Fluorescein experiments by creating a dilution series. This is explained in section 6.1 of the appendix.

Even though dilution of the samples with Triton X-100 solutions successfully managed to make the turbidity disappear and make the samples clear and acceptable for spectrophotometric measurements, it was not the ideal solution due to foaming and a relatively high viscosity making pipetting difficult. Later in the study, the Triton X-100 was replaced by EtOH which was also able to solubilize turbid samples though much easier to work with. Diluting the sample with EtOH was always done with ratio of Aqueous sample:EtOH 1:5 so that the diluted sample contained 83.3 vol% EtOH. A flask with a mixture of 16.67 vol% TBS and 83.33 vol% EtOH was prepared so that any sample needing further dilution would retain the same TBS/Ethanol ratio. The contents of that flask, with 16.67 vol% TBS and 83.33 vol% EtOH was also used for a calibration curve by creating a dilution series with Patent Blue. This is explained in section 6.1 of the appendix.

### 2.3 Experimental procedure

The experimental procedure for both the single vial method and the 96 well plate method can be thus summarized:

1. Lipid dye formulation is injected into PBS/TBS.
2. Lipases are added to the vials/wells.
3. Samples are taken out and analyzed in a spectrophotometer.

The following subsections contain detailed explanations to how this was done for the singe vial setup and the 96 well plate setup.

#### 2.3.1 Experimental procedure

The single vial experimental procedure is now described. It was the same for formulations containing Fluorescein and Patent Blue, however the analysis was gone about differently. Samples were done in triplicate.

1. **2 ml PBS or TBS is added to a 6R glass vial.**
   
   PBS for Fluorescein experiments and TBS for Patent Blue experiments.
2. **100 mg of lipids/dye formulation is injected into the buffer solution.**
Injection was done using a 1 ml Norm-Ject® latex- and silicone oil free syringe and a BD Microlance™3 18Gx1½” 1.2 mm x 40 mm needle.

3. **The vial is placed for 0 – 78 h at 37 °C in an incubated shaker for the formulation to fully hydrate/equilibrerate.**
The vials were placed on an incubated shaker overnight (Heidolph Instruments Titramax 1000 with the add-on Heidolph Instruments Incubator 1000) at 37 °C with the rotating speed of 150 rpm. A calibrated thermometer (Testo 174T) was also placed in the incubator, and it would consistently show 36.9 °C.

4. **The lipase solutions are injected into the vials and then returned to the incubated shaker 37 °C.**
If a small absolute volume was to be added, a larger quantity was diluted first to make it easier to add the correct amount. After the change from Fluorescein to Patent Blue, even larger volumes of lipases were mixed will buffer solution before injection.

5. **50 µl from every vial is taken out for analysis and placed in a 3 ml Eppendorf tube.**
Normally it was simple to take out 50 µl of aqueous solution with a pipette. However, in many of the vials with PLA1, the depot had started to disintegrate. It was important to be careful not to get any floating lipid droplets, depot fragments or small flecks of suspended liquid crystals in the pipette. This was difficult and resulted in bigger variation in the results of those experiments. After the 50 µl sample was taken out, the vial was returned to the incubated shaker. The experiments lasted around a week. Samples were taken three times daily in the beginning, then twice daily and in the final days once daily.
6. The samples are prepared for the spectrophotometer. The samples were prepared for spectrophotometric analysis. This is described in the next two subsections.

2.3.2 Analysis – The Fluorescein assay

The spectrophotometric analysis was done in two different ways. They are called the Fluorescein assay and the Patent Blue assay, respectively. Continuing from step 6 above, this is the Fluorescein assay:

7. Adding 50 µl of 20 wt% Triton X-100 solution to the 50 µl sample. As mentioned before, the purpose of adding a Triton X-100 solution was to dissolve any contaminant causing turbidity. Adding 50 µl of a 20 wt% solution would result in a net concentration of 10 wt% Triton X-100 in the Eppendorf tube. Further dilution with the 10 wt% Triton X-100 solution would not change the PBS:Triton X-100 ratio.

8. Dilution with 10 wt% Triton X-100. The cuvette needed at least 300 µl to give accurate results so at least 200 µl of 10 wt% Triton X-100 solution was added to the Eppendorf tube now.

9. Mixing by inverting the Eppendorf tube gently 10 times. This was done gently to avoid foaming that could be caused by the surfactant Triton X-100.
10. **Transfer to a cuvette for spectrophotometric measurements.** The cuvettes used were Brand® 70 µl disposable micro cuvettes with a light path of 10 mm.

11. **Place in spectrophotometer and measure the range 350 nm – 600 nm.** The cuvettes were placed in the spectrophotometer, in the cuvette holder raised up as high as possible. The spectrophotometer used was a Perkin Elmer® Lambda 25, controlled by the software UV-WinLab ES V. 6.0.4. Measurements were made at the range 350 nm – 600 nm. Scanning a range would reveal additional information than just reading the absorbance at one fix wavelength \( \lambda \), e.g. changes in peak shape or drifting peaks but this was never observed. \( \lambda_{\text{max}} \) was always at 492 nm.

12. **If absorbance was too high, steps 8 – 12 were repeated for those samples until acceptable absorbance was achieved.** The calibration curves had the absorbance range 0 – 1. If a measurement gave \( A > 1 \) it would be re-measured after additional dilution. Steps 8 – 12 were repeated.

13. **Processing results using computer software.** The results were processed using the computer programs UV-WinLab Data Processor and Viewer version 1.00.00 and Microsoft Excel.

### 2.3.3 Analysis – The Patent Blue assay

The Patent Blue assay was slightly different. Continuing from step 6 above, this is how it was done:
7. **Diluting the 50 µl sample with 250 µl EtOH.**
The 50 µl sample was diluted with 250 µl EtOH. This is enough to solubilize the possible lipid contaminants in the sample. This would in a solution of 16.67 vol% sample from the vial and 83.33 vol% EtOH.

8. **When necessary, additional dilution with mixture containing 83.33 vol% EtOH and 16.67 vol% TBS.**
Diluting with this mixture would maintain the EtOH/TBS ratio.

9. **Mixing by inverting the Eppendorf tube 10 times.**

10. **Transfer to a cuvette for spectrophotometric measurements.**
11. Measuring the range 550 nm – 700 nm.
This is the same as in the Fluorescein assay, except that measurements were made at the range 550 nm – 700 nm. A range was scanned in order to see drifting peaks or changes in peak shape but this was never observed. $\lambda_{\text{max}}$ was always at 633 nm.

12. If absorbance was too high, steps 8 – 12 were repeated for those samples until acceptable absorbance was achieved.
The calibration curves had the absorbance range 0 – 1. If a measurement gave $A > 1$ it would be re-measured after additional dilution. Steps 8 – 12 were repeated.

13. Processing results using computer software.
The results were processed using the computer programs UV-WinLab Data Processor and Viewer version 1.00.00 and Microsoft Excel.

2.3.4 96 well plate method
The 96 well plate method is essentially the same as the single vial setup but much more efficient. Handling the vials and analyzing them one at a time takes much longer than using a well plate instead. Also less chemicals is used per well.

1. Injection of 50 µl formulation at the bottom of the well.
50 mg of the lipid formulations were injected at the bottoms of the 1 ml wells of a Deep well PP Plate, natural, RNase/DNase – free. The well plate would be positioned on the balance for easy recording of the weight of added formulation and TBS. The needles and syringes were the same as in the single vial setup. The formulation would settle on the bottom and a flat surface would be exposed to the air. To limit evaporation of EtOH from the formulation, only up to 10 wells at a time were injected with formulation before TBS was added.
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<tbody>
<tr>
<td>2.</td>
<td>Gentle addition of 1 ml TBS on top of the formulation. Gentle pipetting would not disrupt the flat formulation surface.</td>
</tr>
<tr>
<td>3.</td>
<td>Waiting 0 – 72 h for the formulation to hydrate in an incubated shaker at 37 °C.</td>
</tr>
<tr>
<td>4.</td>
<td>The lipases are injected into the TBS and the well plate is again placed in the incubated shaker. The lipases were always mixed with TBS before injection.</td>
</tr>
<tr>
<td>5.</td>
<td>25 µl samples are taken thrice, twice or once daily for about a week…</td>
</tr>
</tbody>
</table>
6. …and placed in an analysis well plate.

7. 125 µl EtOH is added to each well containing a 25 µl sample.

8. The plate is placed in the Elisa reader and shaken for 20 seconds and read at 620 nm.
The plate for analysis was placed in the Elisa reader, BioTek® EL808. Using the software Gen5™ 1.11.5 the Elisa reader was programmed to shake the plate for 20 seconds at medium speed to dissolve lipids, liquid crystal particles, etc. After shaking, the plate would be read with the following settings: Detection method = Absorbance, Read type = End point, Read speed = Normal, Wavelength = 620.

9. Processing results using computer software, comparing it to a calibration curve.
The data was exported from Gen5 to and processed with Microsoft Excel.

2.4 Data processing and calculations

The dye concentration in the vials increased over time as more and more was released from the depot which in turn increased absorbance. Beer-Lambert's law states that:

\[ A = \varepsilon \cdot l \cdot C \]

A is the absorbance, \( \varepsilon \) is the molar absorptivity, \( l \) is the distance the light has to pass through the solution containing the analyte, \( C \) is the concentration. The law says that there is a linear relationship between \( A \) and \( C \). In practical work however,
background absorption must be taken into account. In principle, by plotting $A$ versus $C$ and performing linear regression, a straight line going through zero (0,0). However, due to imperfect correction of background absorbance and some scattering in the absorbance measurements, the data obtained in this work were better described by the following equation:

$$A = \varepsilon \cdot l \cdot C + b = a \cdot C + b$$

$$\Leftrightarrow C = \frac{(A - b)}{a}$$

Here, $b$ is the value where the linear regression line crosses the y-axis. With these equations the absorbance measured in the spectrophotometer or Elisa reader can be used to calculate the concentration in the cuvettes. Since the cuvettes contained diluted liquids, the dilution factor was used to get the concentration in the vials. The concentration in the vial would increase when released from the depot after injection and theoretically go from 0% to 100% release when all of it was released from the depot. Release in % was calculated by taking the calculated vial concentration and dividing it with the maximum possible dye concentration, meaning what the concentration would have been had all of the dye that was injected with the lipids into the vial been released from the depot into the aqueous solution:

$$Release(%) = \frac{Concentration \text{ in cuvettes} \cdot \text{Dilution factor}}{Total \text{ dye content in vial} / \text{Volume of liquids in vial}} \cdot 100$$

After the release was calculated for each vial at the measured time points, the result was plotted in curves that had release in % on the y-axis with standard deviation included as vertical thin bars and the time in hours on the x-axis. Unless otherwise pointed out, the plots all have the same size and scales and share a common system of colors and symbols to make them easier to understand at a glance. Note that the term equilibrated means that the vials/well plate were left overnight or longer in an incubated shaker before they had lipases added to them.

**Table 1. Symbols and colors used in the charts in chapter 3.**

<table>
<thead>
<tr>
<th>Item</th>
<th>Equilibrated</th>
<th>Not equilibrated</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLA1</td>
<td></td>
<td></td>
<td>Red with diamond symbol</td>
</tr>
<tr>
<td>PLA1 TLL mixture</td>
<td></td>
<td></td>
<td>Purple with x in a box</td>
</tr>
<tr>
<td>TLL</td>
<td></td>
<td></td>
<td>Blue with triangle symbol</td>
</tr>
<tr>
<td>CALB</td>
<td></td>
<td></td>
<td>Green with box symbol</td>
</tr>
<tr>
<td>No Lipase</td>
<td></td>
<td></td>
<td>Gray with circle symbol</td>
</tr>
<tr>
<td>Relative lipase concentration</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Highest concentration</td>
<td>Solid line</td>
<td></td>
<td>Solid line</td>
</tr>
<tr>
<td>A little lower</td>
<td>Long dash</td>
<td></td>
<td>Long dash</td>
</tr>
<tr>
<td>A little lower</td>
<td>Long dash dot</td>
<td></td>
<td>Long dash dot</td>
</tr>
<tr>
<td>A little lower</td>
<td>Short dash</td>
<td></td>
<td>Short dash</td>
</tr>
<tr>
<td>A little lower</td>
<td>Short dash dot</td>
<td></td>
<td>Short dash dot</td>
</tr>
<tr>
<td>Lowest concentration</td>
<td>Dashed line</td>
<td></td>
<td>Dashed line</td>
</tr>
</tbody>
</table>
3. Results and discussion

3.1 Effect of lipases on \textit{in vitro} release

3.1.1 Effect of TLL and CALB on \textit{in vitro} release

The experiments in this section were made with the single vial setup and the Fluorescein assay was used for analysis. 20 µl CALB and 5 µl TLL was added. All experiments were made with Formulation E containing Fluorescein and all samples are triplicates.

In Figure 11 above, the \textit{in vitro} release results CALB and TLL are found. The IVR of Fluorescein is significantly higher in the samples with TLL and CALB than in the samples without lipases. After a week, at 187 h the background release (samples with No Lipase) was 11.2 % while CALB had released 24.7% and TLL 25.0%. As two TGLs are hydrolyzing GDO the three component phase diagram shown in Figure 5 can no longer be used to describe the phases in the liquid crystal depot. GMO, free fatty acids and possibly glycerol (if GMO is hydrolyzed) are also introduced. This composition change may be what results in higher release in the lipase samples than in the lipase free samples.

Looking at the curves in Figure 11, TLL and CALB are both TGLs so they are expected to behave similarly and act upon the GDO in a similar way, though it is striking that the curves followed each other so closely. The TLL solution has according to specifications 130000 activity units/g (LU/g) and CALB has 6031 LU/g. Both solutions have the same density. Adding 5 µl of TLL should give much higher enzyme activity and potentially higher release rate than 20 µl CALB. Why they appear to have almost the exact same effect is unknown.

![Figure 11. In vitro release of Fluorescein from Formulation E in the presence and absence of the enzymes TLL and CALB. Lipid formulation was injected into PBS at t = -24 h. Lipases were added at t = 0 h.](image-url)
Images of what these experiments looked like after 187 hours can be seen in Figure 12. The depot in the vial without lipases is brighter because it contains more Fluorescein. The two depots which have had lipases in their vials are clearer. One could also comment on the fact that though new components are introduced, the depots are still ordered enough to retain their structure intact.

3.1.2 Effect of PLA1 on in vitro release

The experiments in this section were made with the single vial setup and the Fluorescein assay was used for analysis. 5, 2 and 0.5 µl PLA1 was added. All experiments were made with Formulation E containing Fluorescein and all samples are triplicates.

As can be seen in Figure 13 the IVR for the three different concentrations is very similar for the first 60 hours or so of the experiment after which something odd is observed in the results. The Fluorescein concentration appears to be decreasing which may indicate that there is an issue with Fluorescein stability. This is one of the reasons why Fluorescein was abandoned. Another reason is the background release rate. After one week 11.2 % was released. Compared to the release
of certain drugs, for instance for certain peptide APIs, over 10% release in PBS after 1 week is high. Therefore, the decision was taken to start over with another model molecule, Patent Blue.

In the specifications provided by the lipase supplier the activity of TLL had 130000 LU/g solution which is 11.8 times higher than 11000 LU/g solution for PLA1. The experiments showed that PLA1 had much greater effect on the release rate than TLL. PLA1 targets PC and hydrolysis results in lyso-PC and free fatty acids being introduced to the system as PC is lost. Lyso-PC is known to be a very effective surfactant with powerful fragmentation (or lipid solubilizing) properties[10] and hence this may be the reason why PLA1 is much more effective than TLL in accelerating the IVR rate.

![Fluorescein IVR experiment vials 187 h after lipase injection. The lipase used here is PLA1. The depot has been completely destroyed. Some remains that may be liquid crystal fragments or structures are stuck to the wall](image)

Figure 14. Fluorescein IVR experiment vials 187 h after lipase injection. The lipase used here is PLA1. The depot has been completely destroyed. Some remains that may be liquid crystal fragments or structures are stuck to the wall

Figure 14 shows the effect of PLA1 187 h after lipase injection. The depot was completely destroyed and depot fragments were floating at the aqueous surface and some stuck to the walls.
3.2 Effect of different lipase concentrations on *in vitro* release

These release experiments were made with vials containing TBS instead of PBS with Formulation E and Patent Blue instead of Fluorescein. Some plots in this subsection will both be presented in with normal y-axis for comparison with other plots and zoomed in for visibility.

3.2.1 Effect of different concentrations of TLL on *in vitro* release

![Graph](image)

**Figure 15.** *In vitro* release of Patent Blue from Formulation E with the enzyme TLL. Lipids were injected into TBS at t = -24 h. Lipases were added at t = 0 h. Both images are the same, except for the y-axis.

*In vitro* release of patent blue is as expected much lower than Fluorescein. After 140 hours the vials with 50 µl Lipase solution had released 5.4% which is one third of the 15.8% Fluorescein released with 5 µl TLL. Background release is also very low. The first day close to 1% was released and after that it does not increase much at all. That release takes place as soon as the formulation reaches the TBS before liquid crystals had been formed at the surface. Another thing we notice is how similar the results between all of them are. Relatively the release of in the 50 µl vials is much greater but in absolute terms very little has been released in any of the vials.

TGLs are not very effective in biodegrading the depots. Probably because they are used in digesting dietary fats, and fats stored in. The reason Patent Blue releases slower both in the vials with lipases and those without compared to Fluorescein, is due to the solubility and permeability being lower. The Patent Blue has a harder time escaping from the depot once it is entrapped.
3.2.2 Effect of different concentrations of CALB on \textit{in vitro} release

Again we notice how very different amounts of lipases yield pretty much the same result, though the lipase induced release is still significantly higher than background release. Again CALB is less effective than TLL.

\textbf{Figure 16.} \textit{In vitro} release of Patent Blue from Formulation E with the enzyme CALB. Lipids were injected into TBS at $t = -24$ h. Lipases were added at $t = 0$ h. Both images are the same, except for the $y$-axis.
3.2.3 Effect of different concentrations of PLA1 on *in vitro* release

One can see that the release of Patent Blue is again much higher for PLA1 than for TLL and CALB. Another thing is that in contrast to TLL and CALB, it is possible to control the release rate by controlling the amount of lipase. Different amounts of lipases do give different release rates.

![Graph showing release vs time for different PLA1 concentrations](image1)

*Figure 17. In vitro release of Patent Blue from Formulation E with PLA1. Lipids were injected into TBS at t = -20 h. Lipases were added at t = 0 h. Both plots are the same, except for the y-axis. Note that the 5 µl sample was not left to equilibrate overnight. The 0.5 µl, 0.05 and 0.005 µl were not done in triplicate.*

One can see that the release of Patent Blue is again much higher for PLA1 than for TLL and CALB. Another thing is that in contrast to TLL and CALB, it is possible to control the release rate by controlling the amount of lipase. Different amounts of lipases do give different release rates.

![Images showing vials with Patent Blue](image2)

*Figure 18. Selection of vials showing IVR of Patent Blue from Formulation E. The vial to the left contains 2 µl CALB solution, the one in the middle contains 50 µl TLL solution the one to the right contains 5 µl PLA1 solution. Photos were taken three days into the experiment.*
Figure 18 shows vials from release experiments with three different lipases in the third day of the experiment. They are comparable to the same experiments with Fluorescein, except for that the release is much slower and one cannot observe that the depot is getting clearer with time as in the Fluorescein experiments. TLL and CALB again leave the depot intact while PLA1 is again causing the depot to fragment and break up.

In Figure 19 are two vials with 5 and 0.5 µl PLA1. These vials are from the triplicate vials that were used in the experiment presented in Figure 16. It just happened that the 5 µl samples did not equilibrate before lipase addition. The clean appearance of the vial to the left was very different to the messy appearance of the vial on the right, and also very different from the 5 µl PLA1 sample with Fluorescein (Figure 13). All the lipids were at the surface of the aqueous solution and they were clear, not blue, in contrast to the vials that had been left to equilibrate, that had blue colored lipids all over the inside walls of the glass vials. Not only did this sample look “cleaner”, it was the first one to yield a release close to 100%. The student believed that this was connected, that only up to about 60% release was achieved in the other cases both for Fluorescein and Patent Blue.

Another thing to comment is how it is possible for some samples to have a release of dye much higher than 100%. Two things can contribute to this: 1. The calculation of “Release (%)” compares the dye concentration in the cuvette with the highest possible dye concentration in the vial. If a piece of liquid crystal got into the pipette it may give a higher concentration in the cuvette than is possible in the vial. 2. As the experiment progresses, 50 µl of the aqueous solution is taken out for each data point. After many samples have been taken out, the effects may be noticeable in the results as more dye is released into less aqueous media.

3.3 Effect of PLA1 on in vitro release using the 96 well plate setup

The results from 96 well plate experiments will be shown here. Every sample is done in quintuplicate. The first experiment is presented in five plots, all for Formulation E and in the following order of descending concentration of PLA1 lipase; 25 µl, 5 µl, 2.5 µl, 0.5 µl, 0.25 µl, 0.05 µl and no lipase. There was an idea that there would be a difference in release between formulations that had been left to equilibrate and those that had not. Because of that, the five following plots will have one line for equilibrated and one for non-equilibrated samples. Important note: As mentioned previously, there was an experiment where the contents of some wells would contaminate other wells. Even though many wells were excluded the results may still be untrustworthy. As this project revolves around assay development, one comment on the 96 well plate setup is that it impossible to assure that the wells don’t contaminate each other even when much is taken in the handling of the plate and wiping clean the plate and lid at the time of sampling. The wells are filled to the brim. Scaling down the experiments is not a good solution to the over-filled wells problem as a relatively large volume is needed for spectrophotometric measurements, or else the concentration of analyte will be under the limit of detection. This is the case for dyes which are readily detected at low concentrations in a spectrometer. The results are shown in figures 20 -26 and are compiled in figures 27 and 28.
**Figure 20.** *In vitro* release of Patent Blue from Formulation E with 25 µl PLA1 solution. TBS was pipetted on top of Lipids at t = -115 h. Lipases were added at t = 0 h.

**Figure 21.** *In vitro* release of Patent Blue from Formulation E with 5 µl PLA1 solution. TBS was pipetted on top of Lipids at t = -65 h. Lipases were added at t = 0 h.

**Figure 22.** *In vitro* release of Patent Blue from Formulation E with 2.5 µl PLA1 solution. TBS was pipetted on top of Lipids at t = -65 h. Lipases were added at t = 0 h.
Figure 23. *In vitro* release of Patent Blue from Formulation E with 0.5 µl PLA1 solution. TBS was pipetted on top of Lipids at t = -115 h. Lipases were added at t = 0 h.

Figure 24. *In vitro* release of Patent Blue from Formulation E with 0.25 µl PLA1 solution. TBS was pipetted on top of Lipids at t = -65 h. Lipases were added at t = 0 h.

Figure 25. *In vitro* release of Patent Blue from Formulation E with 0.05 µl PLA1 solution. TBS was pipetted on top of Lipids at t = -65 h. Lipases were added at t = 0 h.
**Figure 26.** *In vitro* release of Patent Blue from Formulation E with No Lipases. TBS was pipetted on top of Lipids at t = -65 h. Lipases were added at t = 0 h.

**Figure 27.** Compilation of Figures 20 – 26, showing the *in vitro* release of dyes from the lipid formulation depots that equilibrated before addition of PLA1.

**Figure 28.** Compilation of Figures 20 – 26, showing the *in vitro* release of dyes from the lipid formulation depots that did not equilibrate before addition of PLA1.
As seen comparing Figure 17 and Figures 20 – 28 the PLA1 induced IVR in the 96 well plate setup are very similar to the single vial setup and that the results are similar. The depots are very different geometrically. In the single vial setup the depots have the geometry of a sphere and are floating in the aqueous media. In the 96 well plate setup the depots are half-spheres stuck at the bottom of the well with a flat surface (the bottoms of the wells are half-spheres). Also that there is in most plots no difference in release mechanism between the equilibrated samples and the non-equilibrated ones. The only exception being the 25 µl and the 5 µl experiments where the release from the non-equilibrated depots is much higher.

Looking at the wells in that plate there was a difference between equilibrated and non-equilibrated wells, see Figure 29. This is not clearly reflected in the data, however.

The background release of dye shown in Figure 25 is 3.9 %. In the singe vial experiment the release under the same conditions was around 0.8 % at the same time. This is much higher. It may be because of the different geometry, etc. though it is also possible that the wells were contaminated. As stated before it is impossible to know for sure that they were not.

Another comment for the assay development is that during the experiments with PLA1 there would be many liquid crystal fragments in the wells that were hard to avoid with the pipette, which would increase variability and yield higher dye concentration in the analysis wells after dilution with EtOH. Figure 30 shows this this. Future work might include a way to prevent this, for example with some kind of dialysis membrane setup.
Figure 30. What three 25 µl samples looked like before (top) and after dilution with EtOH and shaking in the Eliza reader (bottom). The three wells are from the same experiment and should look exactly the same. The flecks or fragments were unavoidable by any means using a pipette. They cause greater variation.

3.4 Effect of formulation type on *in vitro* release

The following five plots show the *in vitro* results from 96 well plate experiments with 2.5, 0.25 µl and no PLA1 acting upon Formulation C, D, E, F and G.

![Graph](image)

Figure 31. *In vitro* release of Patent Blue from Formulation C. TBS was pipetted on top of Lipids at t = -115 h. 2.5 or 0.25 µl of PLA1 solution was added at t = 0 h.

![Graph](image)

Figure 32. *In vitro* release of Patent Blue from Formulation D. TBS was pipetted on top of Lipids at t = -115 h. 2.5 or 0.25 µl of PLA1 solution was added at t = 0 h.
Figure 33. *In vitro* release of Patent Blue from Formulation E. TBS was pipetted on top of Lipids at $t = -115$ h. 2.5 or 0.25 µl of PLA1 solution was added at $t = 0$ h.

Figure 34. *In vitro* release of Patent Blue from Formulation F. TBS was pipetted on top of Lipids at $t = -115$ h. 2.5 or 0.25 µl of PLA1 solution was added at $t = 0$ h.

Figure 35. *In vitro* release of Patent Blue from Formulation G. TBS was pipetted on top of Lipids at $t = -115$ h. 2.5 or 0.25 µl of PLA1 solution was added at $t = 0$ h.
The Figures 31 – 35 show the Formulations are affected differently by PLA1. The background release is expected to be the lowest for Formulation E. This is not the case and cross-contamination from other wells may be the cause. PLA1 effects Formulation C and D less and it affects Formulation F and G more. This is probably due to the nature of the formulation, the phases formed and the reaction products from the lipase hydrolysis.

3.5 Effect of lipase combinations on *in vitro* release

These experiments also used the 96 well plate setup. The purpose was to see how a combination of lipases would affect the IVR of depots with Formulations C, D, E, F and G.

**Figure 36.** *In vitro* release from Formulation C with 2.5 µl PLA1, a combination of 50 µl TLL and 2.5 µl PLA1 and 50 µl TLL compared to background release.

**Figure 37.** *In vitro* release from Formulation D with 2.5 µl PLA1, a combination of 50 µl TLL and 2.5 µl PLA1 and 50 µl TLL compared to background release.
Figure 38. *In vitro* release from Formulation E with 2.5 µl PLA1, a combination of 50 µl TLL and 2.5 µl PLA1 and 50 µl TLL compared to background release.

Figure 39. *In vitro* release from Formulation F with 2.5 µl PLA1, a combination of 50 µl TLL and 2.5 µl PLA1 and 50 µl TLL compared to background release.

Figure 40. *In vitro* release from Formulation G with 2.5 µl PLA1, a combination of 50 µl TLL and 2.5 µl PLA1 and 50 µl TLL compared to background release.
In all of the five plots one can see that samples with TLL has much lower release than PLA1, though in Formulation F and G it also has a fragmenting effect (the depot is unable to remain intact at the bottom of the well) resulting in higher variation.

The “No Lipase” in Figure 40 clearly shows a relatively high release occurring before the formation of liquid crystals throughout the depot has managed to entrap the dyes and reduce the release rate.

The combination of TLL and PLA1 do not give significantly higher release than just having PLA1. Possibly in Formulation C and D. This may be due to the nature of those formulation.

3.6 Discussion on assay development and future work.

The lipases have been proved here to have a big impact on IVR and the lipases present \textit{in vivo} may be important for biodegradation of lipid subcutaneous injection depots and the release of drugs from them. Lipase based IVR is a candidate to the \textit{in vitro} part of the IVIVC instead of a common dissolution test.

PLA1 was found to be important in the biodegradation of the lipid depot \textit{in vitro}. The phospholipase PLA1 is suggested to be used in a lipase based IVIVC assay and not the TGLs TLL or CALB. It may be interesting to compare it to other phospholipases, e.g. PLA2 or PLB. No activity optimization in regards to pH or co-factors was done due to time constraints and also because the system is more physiologically relevant at pH 7.5 and with the other conditions mentioned in section 2.2.

The single vial method was very time consuming when running experiments with many flasks but one big advantage to the 96 well plate setup is that the vials do not share the same lid and do not contaminate each other. This was unavoidable with the 96 well plate setup. The advantage with the 96 well plate setup is that it saves time and is convenient to handle. This is an advantage in an exploratory study such as in this project or in other similar cases, when many samples need to be run simultaneously.

Another thing that can be worth looking at is an alternative to spectrophotometric measurements. Relatively large volumes were taken out over the course of the experiments. An alternative method could be one that requires a lower volume, for instance chromatography. Chromatography often does not require large sample volumes. The disadvantage though is that it takes a lot longer to run samples in a chromatographic column than it does to quickly measure a sample in a cuvette. In this study dyes were used which are readily detected even at low concentrations with a spectrophotometer. This does not have to be the case with the actual API in the final IVIVC assay. As such, alternatives to spectroscopy could be looked at.

One issue with the results from the PLA1 measurements in this report is that they have a high variation and several data points had to be excluded as they were obvious outliers and contaminated, as shown in Figure 28. A setup with a dialysis membrane to make sure lipid depot fragments are not taken up in the sampling volume would be an option.

During the experiments in this project lipase stability and activity was not monitored or measured due to time constraints. In a regulatory situation lipase activity needs to be known for reproducibility and control.
4. Conclusions

Lipase based *in vitro* release assays were developed and tested. The experiments showed that lipases speed up the release rate of dyes from the lipid based pharmaceutical formulation. In the case of the dye Patent Blue, the background release rate was so low that almost all of the dye released after the liquid crystals had been formed could be attributed to the lipases. Details about assay development and future work is discussed in section 3.6.

The phospholipase PLA1 was found to have a dramatic effect on the liquid crystal lipid depot. Even small volumes of PLA1 solutions fragmented and destroyed the depot and most dye could be released within a few days after lipase injection. This leads to the conclusion that phospholipases are likely to have a major role in the biodegradation of the lipid depot *in vivo*. Triacyl glycerol lipases TLL and CALB were not as effective. Adding those lipases would only speed up the release rate moderately, even when relatively large amounts were added. TGLs are thus believed to play a less important role in the *in vivo* biodegradation of the lipid depots. In the development of an *in vitro in vivo* correlation assay, it appears as PLA1 would be the better candidate.

The different lipid formulations were affected differently by the lipases. The lipase-induced dye release from Formulations C and D was not as big as the lipase-induced dye release from Formulations F and G. This is likely due to the liquid crystal phases formed by these formulations and the effect of the lipid hydrolysis products on these phases.

A combination of lipases showed that they did not have a synergistic or even additive effect on dye release. Wells only having PLA1 added to them had very similar release kinetics to wells with a combination of PLA1 and TLL.

The results between the single vial setup and the 96 well setup are comparable and both are alternatives for an IVR assay.
5. References


6. Appendices

6.1 Calibration curves

Before the start of the release experiments, calibration curves were made. One was made for the Fluorescein formulation. Two different calibration curves were made for the Patent Blue experiments; one for the singe vial experimental setup and one for the 96 well plate setup.

Fluorescein & Triton X-100 in PBS

The calibration curve for the Fluorescein experiments was made with a dilution series of Fluorescein in a solution of 10 wt% Triton X-100 in PBS. There were samples with higher concentrations that were not included due to absorbance values above 1. The data point with concentration 0 µg is simply 10 wt% Triton X-100 in PBS.

Table 2. Measured absorbance for calibration curve for the lipid formulations containing Fluorescein. Used in the singe vial setup.

<table>
<thead>
<tr>
<th>Fluorescein concentration (µg / g solution of 10 wt% Triton X-100 in PBS)</th>
<th>Absorbance (A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.969</td>
<td>0.821</td>
</tr>
<tr>
<td>4.619</td>
<td>0.579</td>
</tr>
<tr>
<td>2.302</td>
<td>0.321</td>
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<tr>
<td>1.540</td>
<td>0.241</td>
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<tr>
<td>0.755</td>
<td>0.153</td>
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<tr>
<td>0.515</td>
<td>0.131</td>
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<tr>
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<tr>
<td>0.172</td>
<td>0.088</td>
</tr>
<tr>
<td>0</td>
<td>0.080</td>
</tr>
</tbody>
</table>

Figure 41. Calibration curve and linear regression from a dilution series of Fluorescein disodium salt in a solution of 10 wt% Triton X-100 in PBS. This is used for calculation of Fluorescein concentration during the singe vial IVR experiments.

Patent Blue in TBS & EtOH for the singe vial setup

The calibration curve for the Patent Blue singe vial experiments was made with a dilution series of Patent Blue in a solution of 16.67 vol% TBS and 83.33 vol% EtOH. There were samples with higher concentrations that were not included due to absorbance values above 1. The data point with concentration 0 µg is simply a solution of 16.67 vol% TBS and 83.33 vol% EtOH.
Table 3. Measured absorbance for calibration curve for the lipid formulations containing Patent Blue. Used in the single vial setup.

<table>
<thead>
<tr>
<th>Patent Blue concentration (µg / ml solution of 16.67 vol% TBS &amp; 83.33 vol% EtOH)</th>
<th>Absorbance (A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.617</td>
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</tr>
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<td>2.309</td>
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</tr>
<tr>
<td>1.539</td>
<td>0.352</td>
</tr>
<tr>
<td>0.770</td>
<td>0.216</td>
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<tr>
<td>0.513</td>
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<td>0.257</td>
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<td>0.124</td>
</tr>
<tr>
<td>0</td>
<td>0.096</td>
</tr>
</tbody>
</table>

Figure 42. Calibration curve and linear regression from a dilution series of Patent Blue in a solution of 16.67 vol% TBS and 83.33 vol% EtOH. This is used for calculation of Patent Blue concentration during the single vial IVR experiment.

Patent Blue in TBS & EtOH for the 96 well plate setup

Table 4. Measured absorbance for calibration curve for the lipid formulations containing Patent Blue. Used in the 96 well plate setup.

<table>
<thead>
<tr>
<th>Patent Blue concentration (µg / ml solution of 16.67 vol% TBS &amp; 83.33 vol% EtOH)</th>
<th>Absorbance (A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>41.5556</td>
<td>*2.218182</td>
</tr>
<tr>
<td>20.7778</td>
<td>*1.146591</td>
</tr>
<tr>
<td>4.15556</td>
<td>0.268318</td>
</tr>
<tr>
<td>2.07778</td>
<td>0.154727</td>
</tr>
<tr>
<td>0.41556</td>
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</tr>
<tr>
<td>0.20778</td>
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<tr>
<td>0.04156</td>
<td>0.038273</td>
</tr>
<tr>
<td>0</td>
<td>0.036</td>
</tr>
</tbody>
</table>

* Note that A < 3 is acceptable with this Elisa reader. Often, and especially with older spectrophotometers A < 1 is outside the limit of linearity. Many modern spectrophotometers correct for this.
6.2 How to make turbid samples clear

Solutions of Triton X-100 can be used to solubilize.

Before the actual release experiments could commence, there needed to be a good solution for dealing with the turbid samples that would appear. Solutions with different concentrations of Triton X-100 in TBS were prepared and formulation (without dye) was added in different amounts to see if it was possible to completely solubilize all the lipids. The first experiment showed that it was indeed possible to use Triton X-100.

It was decided that when taking out samples for analysis, 50 µl would be taken out, mixed with a Triton X-100 solution, transferred to a cuvette and placed in a spectrophotometer. When determining the final concentration of Triton X-100 needed in the cuvettes for solubilizing even the most contaminated samples, 10 wt% Triton X-100 was found to be sufficient.

![Figure 44](image)

*Figure 44.* Three vials showing how it is possible to solubilize lipids and liquid crystals in a vial containing PBS using Triton X-100. Notice how the liquid in the rightmost vial is clear unlike the rest.

The Triton X-100 solutions were used to dilute samples from the IVR experiments with formulation containing Fluorescein. As Fluorescein was abandoned in favor of Patent Blue, so was Triton X-100 in favor of EtOH.
Dilution with EtOH also removes turbidity

Instead of diluting samples with Triton X-100 solutions, EtOH was tried and found to work. EtOH doesn’t foam and is less viscous than a Triton X-100 solution and so it is easier to pipette, which is important when handling many samples, especially as many as there are on a 96 well plate. An experiment was done to determine how much ethanol would be needed to make a worst-case-scenario-sample clear. It was found that adding 250 µl EtOH to 50 µl of such a sample was enough. This was also more than the minimum amount of liquid that was needed in the cuvettes and so it was decided that the 50 µl samples would be diluted with 250 µl EtOH.

Figure 45. Three vials with increasing of ratios EtOH:Mixture of PBS/Formulation from left to right. It is possible to solubilize lipids and liquid crystals in a vial of PBS using EtOH. Notice how the liquid in Vial nr. 5 is completely clear unlike 3 and 4.