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Radiosensitising tumour cells

Optimisations *in vitro* and *in vivo*

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Abstract

Radiosensitising tumour cells: Optimisations *in vitro* and *in vivo*

Sara Lundsten

One in three people in Sweden will sometime during their life be diagnosed with cancer. The most important therapies used today to treat these patients are surgery, radiation and chemotherapy. Small stapled peptides targeting the inhibition of tumour suppressor protein p53 have great potential of becoming radiosensitising drugs and increase efficacy of radiation therapy. This project aimed to test two modifications of stapled peptides PM2 and PM3 to gain further knowledge about their properties and optimise specificity and delivery of them. Radioiodination was optimised for labelling yield, oxidative stress and purification. Final product was quality tested on tumour cells to assess whether the modification affected the biological efficacy of the peptides. Results showed no reduction of efficacy. The compound was injected in tumour-bearing nude mice, resulting in tumour tissue absorption and low risk for toxicity in critical organs. Lipid bilayer disks (lipodisks) were used as a potential delivery system. The structure has previously been tested as carrier for amphiphilic peptides and shows great potential. Furthermore, it is non-toxic and biocompatible. Association of PM2 and PM3 to disks was measured with the use of fluorescence emission shift and QCM-D. Results showed that PM3 has high affinity to the disks. The study conducted here show that a drug delivery system consisting of tumour-targeting lipodisks carrying radiosensitising peptides have great potential. By adding a therapeutic radionuclide, the system can also be used for targeted radionuclide therapy.

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Populärvetenskaplig sammanfattning

En av tre i Sverige kommer någon gång under sin livstid bli diagnostiserad med cancer och efter diagnosen väntar ofta tuffa behandlingar som ibland orsakar kraftiga biverkningar. Att använda strålning i cancerterapi är vanligt, där man utnyttjar cancercellens nedsatta förmåga att reparera skador på arvsmassan. Det används ofta i kombination med kirurgi och cytostatika.

Strålsensitiverande mediciner har förmågan att göra en cell mer känslig för strålning, vilket potentiellt kan förbättra effekten av behandlingen utan att behöva öka dosen. Det har vid Uppsala universitet utförts cellförsök på två molekyler som har stor potential att bli strålsensitiverande medicin, men de behöver undersökas närmare.

För det första är det svårt att veta var i kroppen dessa molekyler hamnar vid en injektion. Därför ägnades första delen av detta projekt åt att märka dem med en radioaktiv ”flagga”. Genom denna modifikation blev det möjligt att till exempel spåra molekylerna när de injicerades i möss. Det visar sig att de kan ta sig in i en tumör om de injiceras under huden strax ovanför tumören och det sker inte någon ackumulering inte i njurarna, vilket kan vara en risk med molekyler av den här typen. Märkningen påverkade heller inte molekylernas förmåga att öka cancercellernas känslighet för strålning, vilket var väldigt positivt.

Ett annat problem med dessa molekyler är att de skulle kunna orsaka biverkningar, eftersom de inte är specifika mot cancerceller. Därför testades ett leveranssystem, där strålsensitiverarna laddades på en stor disk-formad fettpartikel som själv kan leta upp cancerceller med hjälp av receptorer som finns på cellerna. Det visade sig att denna struktur kan bära ett stort antal strålsensitiverare på ytan.

Resultaten från detta projekt kan användas för att designa en ny sorts medicin som är specifik mot cancerceller och gör dem extra känsliga mot strålning utan att orsaka allvarliga biverkningar. Genom att sätta på en speciell sorts radioaktiv ”flagga” skulle molekylerna dessutom själva kunna leverera strålning direkt till tumören samtidigt som de fungerar som strålsensitiverare.

Table of contents

List of abbreviations

CAT – chloramine T. Oxidising agent used in radioiodination.

cps – counts per second.

DLS – dynamic light scattering. Used to determine size of small particles.

DSPC – distearoyl phosphatidylcholine. Used in lipodisk and liposome production.

DSPE-PEG-2000 - PEG-linked distearoyl phosphatidylethanolamine-2000. Used in lipodisk and liposome production.

DSPE-PEG-2000-amine – functionalised DSPE-PEG-2000 with amine. See above.

EGF – epidermal growth factor.

EGFR – epidermal growth factor receptor.

ITLC – instant thin layer chromatography. Used to determine purity of radiolabelled compounds.

MDM2 – mouse double minute 2 protein which controls degradation of p53.

MDM4 – mouse double minute 4 protein structurally similar to MDM2.

NBS – sodium metabisulfite. Reducing agent often used in radioiodination.

NHS-PEG-3400 – PEG functionalised with NHS.

OG – octyl glucoside. Detergent used in lipodisk production.

p53 – tumour suppressor protein involved in (among many pathways) stress response.

PBS – phosphorous buffered saline.

PM2 & PM3 – stapled peptides binding to p53-binding pocket of MDM2 and MDM4.

PEG – polyethylene glycol.

QCM-D – quartz crystal microbalance with dissipation monitoring. Used for interaction studies.

Rpm – revolutions per minute.

1 Introduction

1.1 The evolvement of cancer therapy

One in three people in Sweden will sometime during their life be diagnosed with cancer. The most important therapies used today to treat these patients are surgery, radiation and chemotherapy. Statistics show that the number of cured patients have steadily increased since the 1970s, but some cancer forms are still associated with high mortality (Johansson 2013).

Tumour development is a multistep process and a number of mutations are required for a cell to become malignant. One of the most important characteristics of a cancer cell is therefore genomic instability, where the cell no longer can respond properly to mutations. One central protein involved in this process is p53, which this projects very much evolves around (Hanahan & Weinberg 2011).

Even though mutations can be beneficial for the survival of a cancer cell, they can also be used to attack it. Ionising radiation has the ability to induce damage to biomolecules, mainly DNA, and the cell membrane (Eriksson *et al.* 2008). While a healthy cell has the ability to repair the damage to some extent, a tumour cell has lost many of the signalling pathways responsible for this mechanism and is therefore more sensitive to radiation (Stenerlöw *et al.* 2008). There are different kinds of radiation treatment available in the clinic, but they all rely on this mechanism (Carlsson *et al.* 2008).

When it comes to chemotherapy, an interesting development has been going on over the last few decades. From using unspecific cytotoxic drugs discovered with the help of screening techniques, the focus has shifted. More and more work focuses on identifying and investigating genes and pathways that are involved in cancer formation and progression which serve as possible targets for cancer drugs. This mechanism-based drug discovery process has been aided tremendously by techniques such as whole-genome sequencing and X-ray crystallography. A number of mechanism-based drugs have been successful in the clinic and by understanding the underlying mechanisms behind a drug response, it is possible to predict whether a patient is likely to benefit from a certain treatment (Workman & Collins 2008).

1.2 p53 and MDM2 are important for cancer development

The tumour suppressor protein p53 plays an essential part in protecting the genome of a cell from stress such as radiation or oncogenic stress (Khoo *et al.* 2014). As a sequence-specific transcription factor, it controls a large number of pathways such as apoptosis, senescence and cell cycle arrest (Michael & Oren 2003). More than 50% of tumours have a mutation in the p53 gene (Vogelstein *et al.* 2000, Liu *et al.* 2015). If there is no mutation in the gene, the p53 pathway is often interrupted in another way (Brown *et al.* 2009). It is very much involved in the cellular response after radiation therapy (Eriksson *et al.* 2008).

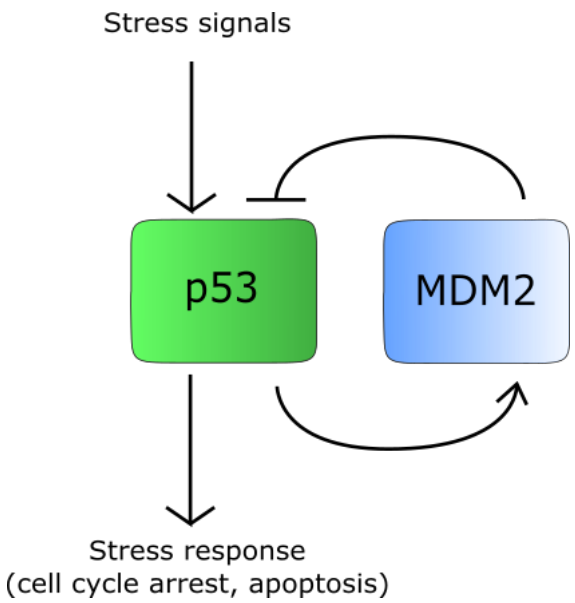

Figure 1. The autoregulatory negative feedback loop connecting p53 and MDM2. Stress signals stimulate expression of p53, which as a transcription factor induces transcription of a number of downstream targets, including MDM2. MDM2 can then induce degradation of p53, resulting in decreased p53 activity.

The interaction between p53 and its repressor MDM2 is considered a very important aspect of p53 regulation (Michael & Oren 2003, Lane *et al.* 2010). The two proteins are connected through an autoregulatory negative feedback loop (Figure 1). p53 can induce MDM2 transcription by binding to elements present on the MDM2 gene (Michael & Oren 2003).

The MDM2 protein can in turn promote degradation of p53 through a number of mechanisms. MDM2 in its role as an oncogenic E3 ligase has the ability to mark p53 for degradation through ubiquitination. This is a well-established model of MDM2 function (Michael & Oren 2003, Lane *et al.* 2010, Khoo *et al.* 2014). It has also been proposed that MDM2 is involved in p53 export from the nucleus where p53 acts. Both of these mechanisms are depended on physical interaction between MDM2 and p53 (Michael & Oren 2003). By binding directly to p53, MDM2 can also prevent p53 from interacting with DNA (Michael & Oren 2003, Lane *et al.* 2010, Khoo *et al.* 2014).

MDM2 has a small hydrophobic pocket which is responsible for the interaction with p53 (Michael & Oren 2003, Lane *et al.* 2010, Khoo *et al.* 2014). After determining the structure of MDM2 with X-ray crystallography and discovering this pocket the idea of targeting this pocket with drugs emerged. By interrupting the interaction between the two proteins, p53 can be protected from degradation (Lane *et al.* 2010).

1.3 PM2 and PM3 – two potential radiosensitisers

In 2013 Brown *et al.* presented a number of small peptides with high affinity for both MDM2 and MDM4. These molecules are about 10-15 amino acids long and have a stabilizing linker (a staple) between two non-adjacent amino acids (Khoo *et al.* 2014). The stapled peptides have the ability to increase levels of wildtype p53 in tumour cells (Figure 2, unpublished data).

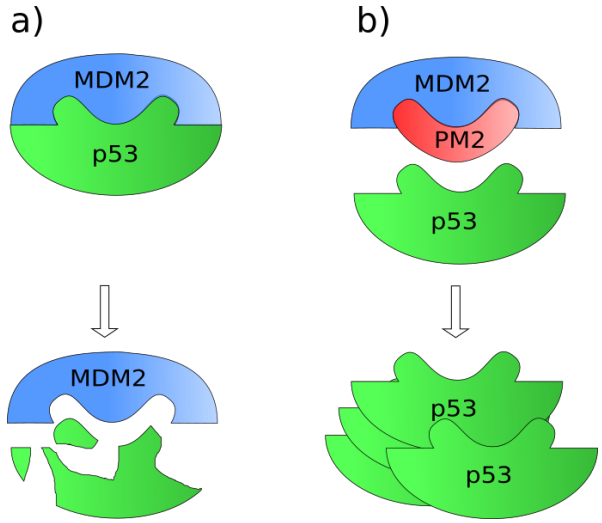

Figure 2. The proposed mechanism of action of PM2 and PM3. (a) In a normal setting, MDM2 can interact with p53 and induce degradation. (a) If PM2 or PM3 are present, MDM2 is hindered from binding to p53 and can no longer induce degradation.

As mentioned earlier, p53 is involved in activating pathways involved in apoptosis when the cell is exposed to radiation. If the cellular levels of p53 are low due to overexpression of MDM2, as is the case with about 7% of human cancers, radiation might not be able to induce p53-mediated cell cycle arrest or apoptosis (Eriksson *et al.* 2008, Zhao *et al.* 2015). PM2 and PM3 interrupt MDM2-mediated degradation of p53 according to a proposed model. Since ionising radiation has been shown to induce p53-dependent transcription of MDM2, targeting the p53-MDM2 interaction in this way could further sensitise tumour cells against radiation (Impicciatore *et al.* 2010).

There are some limitations using the stapled peptides. Because of their hydrophobicity, the solubility in water or blood is probably very low. Unpublished data suggests that the peptides aggregate with each other and proteins present in the circulation system, which could mean lower efficacy of delivery and function. The potential off-target effects are also a problem. There is no specificity of the peptides function; they have the potential ability to increase p53 levels in all kinds of cells and tissues. In order to protect healthy tissue and target tumour cells exclusively, a more specific delivery of the drugs would be optimal for future clinical applications.

1.4 Aim of the project

PM2 and PM3 have great potential to become radiosensitising drugs used for cancer therapy, but they need to be assessed further. First of all, it is of interest to be able to trace the molecules both *in vitro* and *in vivo* in order to gain knowledge of their properties. Secondly, it is also of interest to investigate possible ways to incorporate PM2 and PM3 into a drug delivery system, to avoid possible problems with insolubility and off-target effects of the drugs.

Therefore, this project aimed to investigate two possible modifications of the stapled peptides PM2 and PM3. The first part of the project assesses the possibility to use direct radioiodination to label the two molecules with ^{125}I and, if such a modification was possible, investigate how the iodination could be used as a tracer in different applications *in vitro* and *in vivo*. The potential therapeutic effect of delivering radioactivity to the cell through a radiosensitising drug would also be assessed. The aim of the second part was to investigate whether PM2 and PM3 could be incorporated into lipid bilayer disks (lipodisks) that target tumour cells.

2 Radioiodination of PM2 and PM3

2.1 Introduction

Labelling a peptide or protein with a radionuclide can be used for a number of different applications, for example as a tracer in biodistributions studies or as a therapeutic in radionuclide therapy (Bailey 1994, Sugiura *et al.* 2014). The choice of peptide, radionuclide and labelling method is crucial in order to obtain a product which suits the application (Tolmachev 2008). There are a number of different radionuclides suitable for therapy, imaging or detection in a scintillator for example. The labelling technique needs to be optimal in order to obtain a stable compound with retained specificity (Tolmachev 2008, Sugiura *et al.* 2014).

Since PM2 and PM3 each have one tyrosine residue, Chloramine-T (CAT) mediated direct radioiodination was the first choice of labelling technique. The technique is straightforward and fast, where CAT oxidises iodine and the iodine attacks a tyrosine residue on the peptide. A substitution of hydrogen to iodine takes place via electrophilic substitution. The reaction is stopped by adding a reducing agent that neutralises CAT (Greenwood *et al.* 1963).

Since the application at this stage mainly is to use the radiolabel as a tracer, ^{125}I was the choice of radionuclide. The radiation emitted from the nuclide is absorbed in heavy materials such as lead which reduces risk of exposure during lab work if shielded properly (Bergman *et al.* 1994). It also has a long half-life, which together with its emission properties makes it suitable for *in vitro* assays and *in vivo* biodistribution studies with mice (Sugiura *et al.* 2014).

Iodination could potentially impair the biological function of the labelled peptide. The effect of CAT may cause oxidative damage of the peptide (Bailey 1994, Fani & Maecke 2012). Therefore, it is very important to investigate whether the peptide retains their biological function after labelling. Furthermore, if the peptides are internalised and processed the bond between ^{125}I and tyrosine acid might break. In an *in vivo* environment, this could cause accumulation of iodine in the thyroid. When using a more potent radionuclide this could lead to radiation toxicity (Sugiura *et al.* 2014).

2.2 Material and methods

2.2.1 Optimisation of radioiodination and purification

CAT-mediated labelling of PM2 with ^{125}I was optimised to obtain the highest labelling yield with minimal oxidative stress. 2.5 nmol PM2 was mixed with 1-10 MBq ^{125}I. The reaction was initiated by adding 4 µL CAT with concentrations ranging from 1-16 mg/mL. The reaction vial was kept on ice for 0.5-5 minutes and the reaction was stopped with 8 µL sodium metabilsulfite (NBS). Incubation of peptide, ^{125}I and non-radioactive NaI prior to labelling was also tested.

The labelling yield was analysed with Instant Thin Layer Chromatography (ITLC) using either water, 0-100% aceton solutions or 0.9% NaCl as a mobile phase. The ITLC spectra was visualised and quantified with PhosphoImaging. The final protocol was also tested on PM3 to assess whether further optimisation for this compound was required. Purification of the labelled product was optimised using a size exclusion column. Different ratios of ethanol and water were tested as a mobile phase.

2.2.2 Viability assay

Tumour cells (UM-SCC-74B cancer cell line) were seeded in a 96-well plate with a cell count of 1500 cells/well. Plates were incubated at 37°C in an atmosphere containing 5% CO_2 for 72 hours prior to treatment.

25 MBq ^{125}I, 125 nmol PM2 or PM3 solved in DMSO and 125 nmol NaI solved in phosphorous buffered saline (PBS) was incubated 20 minutes in room temperature. Labelling was performed on ice for 60 seconds with 100 µL 2 mg/mL CAT and stopped with 200 µL 2 mg/mL NBS. The labelling yield was analysed with ITLC using 0.9% NaCl as mobile phase. Purification of labelling was performed on a size exclusion column with 100% ethanol as mobile phase. The pure fractions containing PM2 or PM3 were pooled and concentrated by evaporation of ethanol. Purified and unpurified ^{125}I-PM2 and ^{125}I-PM3 as well as unlabelled peptides were added to cells with a final concentration of 20 µM. A manual cell count was performed 24-72 hours after treatment to determine the number of viable cells in each well.

2.2.3 Biodistribution of ^{125}I-PM2 in tumour bearing nude mice

An ethical permit (C 33/16) for the animal study was approved by the local Ethics Committee for Animal Research. BALB/c nu/nu mice were injected with approximately 10^6 HCT-116 cells subcutaneously on the right flank and left to grow for roughly 2 weeks. Labelling and purification of PM2 was performed according to protocol in section 2.2.2. Labelled product was diluted in PBS to a final concentration of 130 µM (approx. 3-5.5% ethanol). A 100 µL subcutaneous injection of ^{125}I-PM2 right above the tumour was performed. Mice were sacrificed after 6, 24 and 48 hours post injection, organs were collected and radioactivity was measured in a gamma counter.

2.3 Results

2.3.1 Labelling was optimized for labelling yield, oxidising environment and purification

When optimising the labelling process, the aim was to obtain a high labelling yield with little or no impact on biological function. The amount of CAT was therefore minimised due to its oxidising properties. For 2.5 nmol of PM2, 4 µL 8 mg/mL CAT gave a labelling yield of 20-25%. A decrease to 1 mg/mL did not significantly affect the labelling efficiency.

Increased amounts of ^{125}I and longer reaction time did not have a great impact on the labelling yield. However, when non-radioactive iodine was incubated with ^{125}I and PM2 for 20 minutes

prior to reaction the yield increased to approximately 50%. This was sufficient for continuing with optimising the purification process.

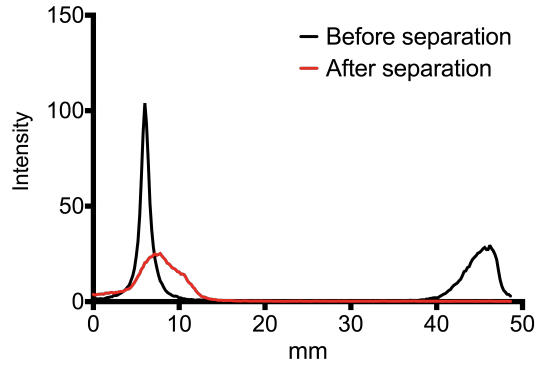

Figure 3. Purity of radiolabelled fractions before (black line) and after (red line) separation. The left peaks represent peptide and the right peak represents free ^{125}I.

In order to further assess the effect of radiolabelled products, it would have been beneficial to eliminate free ^{125}I and the chemicals used in the labelling process since they may disturb future assays. Using size exclusion chromatography with pure ethanol as a mobile phase it was possible to purify ^{125}I-PM2 and ^{125}I-PM3 from free ^{125}I. By evaporating the majority of the ethanol, solutions could then be concentrated. The labelling was stable for at least 48 hours (data not shown).

ITLC was used to assess the labelling yield of the reaction and the purity of chromatography fractions. Using 0.9% NaCl as a mobile phase, the free iodine moved with the mobile phase while the peptides retained close to the application point. The ITLC strips were analysed with Phosphoimaging which could visualise and quantify the radioactive fractions (See Figure 3).

2.3.2 Radioiodination does not interfere with the peptides biological function

The labelling process did not, despite being a rather harsh modification, interfere with the peptides ability to decrease tumour cell viability. In Figure 4, the results show that labelling of PM3 increases the efficacy of the compound while PM2 have a similar efficacy before and after labelling. The increased efficacy of ^{125}I-PM3 is not due to the fact that it is solved in ethanol, which oddly enough seems to be able to protect the cells from unlabelled PM2 and PM3.

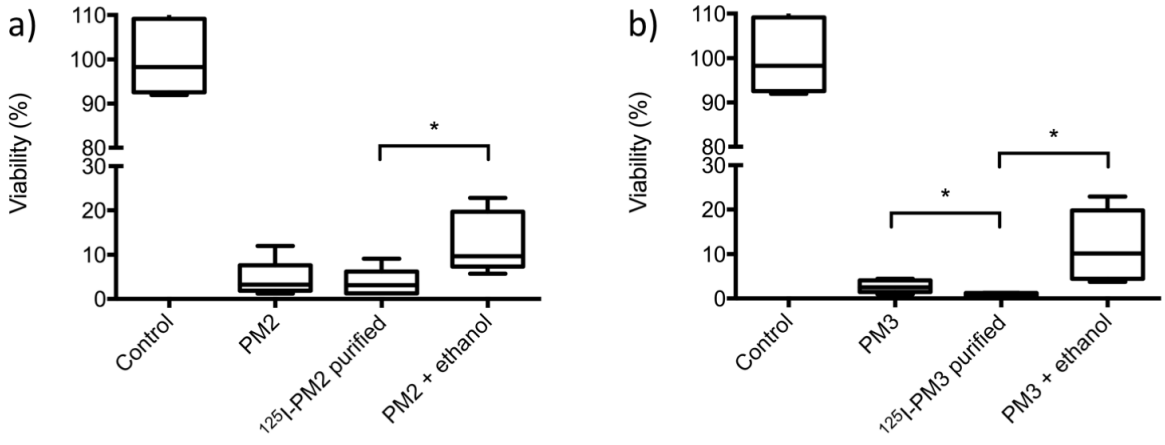

Figure 4. Viability assay of PM2 (a) and PM3 (b). The effect on viability off 20 μM peptide, labelled peptide (solved in ethanol) and unlabelled peptide together with ethanol corresponding to the ethanol amount added with labelled products. n=5, whiskers include all data points. The viability of the treatments was significantly lower than the control group ($p<0.0001$). * $p<0.05$.

2.3.3 Subcutaneous injection of ^{125}I-PM2 is followed by absorption in tumour tissue

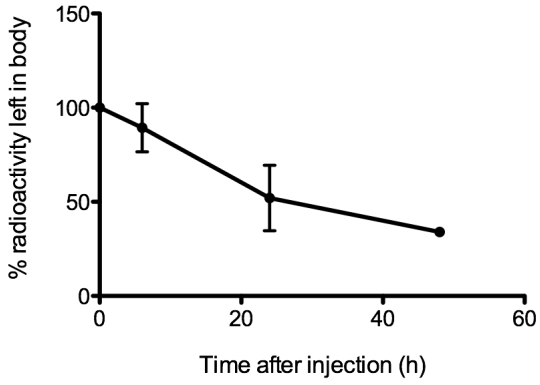

Figure 5. Clearance of radioactivity from body over time. n=3 for t=6 and 24 hours and n=1 for t=48 hours. Error bars show 95% CI.

The biodistribution data (shown in Figures 5 and 6) demonstrated that the injection of ^{125}I-PM2 was followed by absorption of radioactivity in the tumour. About 10-15% of the radioactivity, which corresponded to a maximum of 1.95 nmol of PM2, was situated in the tumour after 24 hours and stayed in the tissue for an additional 24 hours. A rather high percent of the initial activity was situated in the skin around the injection site after 6 hours, but decreased with time. Kidneys and thyroid, which are two sensitive organs, did not seem to accumulate any radioactivity. The liver and intestine were exposed to the radioactivity during a short period of time. The peptide was cleared from the body with 34% of the radioactivity left in the body after 48 hours.

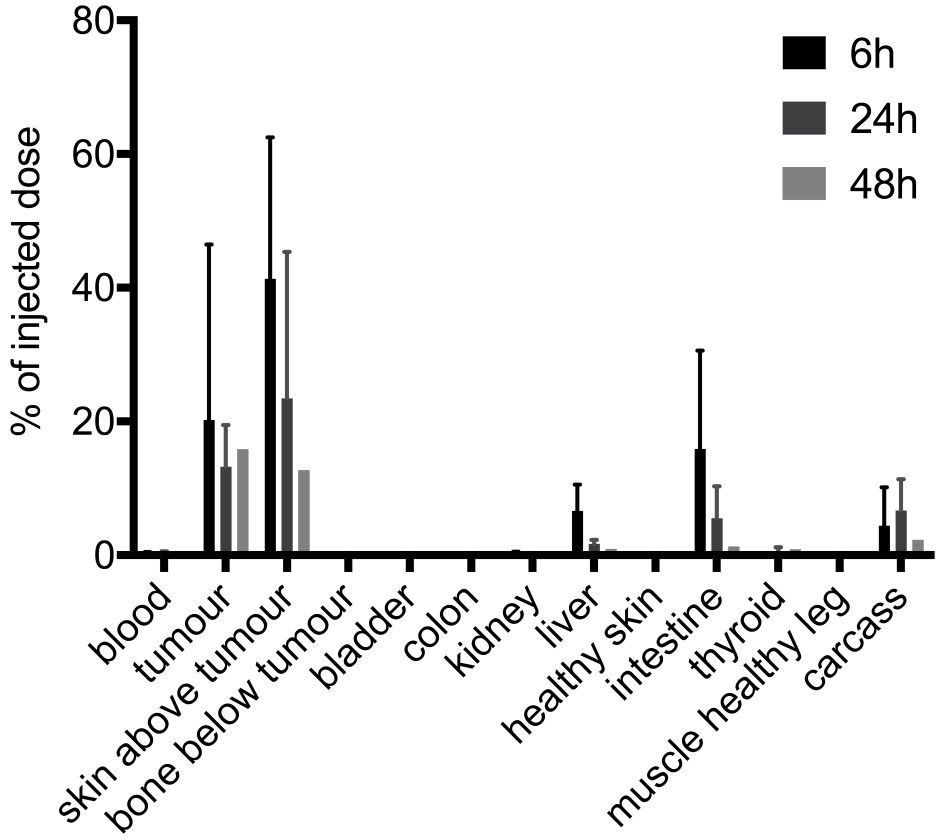

Figure 6. Biodistribution of ^{125}I-PM2. The amount of radioactivity per organ, normalised against the injected dose. Error bars show 95% CI. For t=6 and 24 hours, n=3. For t=48 hours, n=1.

2.4 Discussion

The first part of the project was successful. A labelling and purification protocol has been established which results in high labelling yield, efficient purification and minimal impact on peptide integrity. The established protocol can be used in a number of different applications. The effect on tumour cell viability was investigated and show positive results. Further *in vitro* analysis should aim to further determine the impact of radiolabelling on the biological efficacy of the peptides. It is of importance to assess the physical interaction between peptides and MDM2 as well as continuing with more detailed cell assays. It would also be interesting to assess whether a therapeutic radionuclide, ^{131}I for example, could be used to obtain a stronger effect.

When injecting ^{125}I-PM2 into mice, a number of interesting facts could be stated. With small peptides, there is always a risk that they are cleared from circulation rapidly and accumulate in the kidney. This can cause off-target radiation toxicity. Further, if the peptide is internalised and processed there is a probability that free iodine accumulates in the thyroid. One can see a small amount of radioactivity in both organs, which means that peptides reached the circulation system, but there is little risk for renal or thyroid toxicity in this experimental setting.

Since the injection is followed by absorption of radioactivity in the tumour, the results can be used as basis for future *in vivo* therapy studies. It is possible to assess the therapeutic properties of PM2 *in vivo* with prior knowledge about the approximate dose delivered to the tumour when using subcutaneous injection. It is important to state that this type of administration is usually not an option in the clinic, where oral or intravenous administration is more suitable. The subcutaneous injection serves as a first step in investigating the therapeutic effects of PM2 and PM3 in an *in vivo* setting. If intravenous injection was used, there would be no guarantee that the peptides reach the tumour and it would be hard to draw any conclusions about the therapeutic effect of the molecules.

One way to avoid this problem would be to increase tumour tissue accumulation with some sort of delivery system, where the drug is actively delivered to tumour cells. Furthermore, potential off-target effects could be avoided with increased specificity. Hence, the remainder of the project was spent on assessing a possible delivery system.

3 Optimising drug delivery with lipid bilayer disks

3.1 Introduction

It has been shown that PM2 has high affinity to MDM2 and the structurally similar protein MDM4 (Xiong Tan *et al.* 2015). Although the affinity is high, it has also been indicated that PM2 have difficulties penetrating the cell membrane (Xiong Tan *et al.* 2015). Therefore, it is of importance to investigate how delivery of the drug to the cell can be optimised. Controlled delivery of PM2 and PM3 is also crucial in order to avoid off-target effects since changes in p53 levels can potentially induce toxicity in healthy tissue (Khoo *et al.* 2014).

Lipid bilayer disks (lipodisks) were first described in 1997. They consist of phospholipids and polyethylene glycol-conjugated lipids (PEG-lipids) which under certain conditions can form flat, circular bilayer structures (Edwards *et al.* 1997). A schematic cross-section of the structure can be seen in Figure 7. Disks of different sizes can be produced to suit the desired application and it has been proposed that lipodisks can be a tool for drug delivery (Zetterberg *et al.* 2011, Zetterberg *et al.* 2016). The disks were discovered 20 years ago, but it is only during the last couple of years that they have been studied in detail. There is much left to learn about their interaction with other structures and potential applications. However, *in vivo* studies have already shown that they are biocompatible and non-toxic (Zhang *et al.* 2014). Focus lies much in developing targeted drug delivery systems for cancer therapy (Gao *et al.* 2016, Reijmar *et al.* 2016).

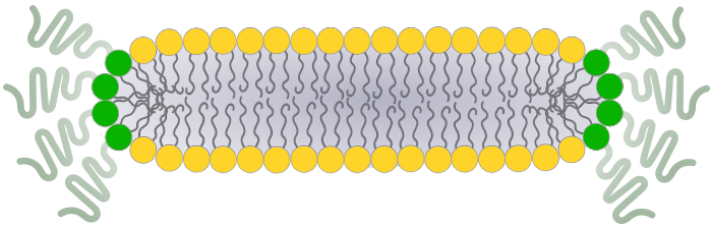

Figure 7. Cross-section of a disk. The yellow flat circular bilayer is surrounded by a rim consisting of green PEGylated lipids.

In order for the lipodisks to be specific towards cancer cells, a targeting agent such as an antibody or signalling peptide that actively binds to tumour cells can be attached. One such targeting molecule is the epidermal growth factor (EGF) which binds to the EGF receptor (EGFR). EGF is overexpressed in many cancers (Sebastian *et al.* 2006). It has been used in targeted tumour therapy with lipid-based drug delivery systems (Brannon-Peppas & Blanchette 2004, Noble *et al.* 2014). It is currently being tested *in vitro* with lipodisks at Uppsala University (unpublished data).

One more commonly known lipid-based drug delivery system is the liposome. It is sphere-shaped and has a hydrophilic core (Allen & Cullis 2013). The liposomal delivery system has undergone great development over the last few decades and there are now available liposomal cancer drugs in the clinic, but further optimisations can be made (Allen & Cullis 2004, Noble

et al. 2014). The hydrophilic inner core of the liposome is perfect for delivery of water-soluble drugs, but it has been, and still is, very difficult to use hydrophobic compounds (Allen & Cullis 2013). Studies have shown that it is possible to load amphiphilic peptides onto the rim of the lipodisks (Zetterberg *et al.* 2011). Lipodisks may therefore be an alternative to liposomes when it comes to delivery of hydrophobic peptides.

During the project, it was investigated whether PM2 and/or PM3 had the ability to associate to different kinds of lipodisks. PM2 and PM3 were tested with different kinds of lipodisks, both targeted and untargeted. Two different methods, fluorescence emission shift and quartz crystal microbalance with dissipation monitoring (QCM-D), were used to measure the association between peptides and disks.

3.2 Material and methods

3.2.1 Production of lipid bilayer disks and liposomes

There are a number of different methods available for lipodisk production. The choice of method together with the lipid composition will determine the size and homogeneity of the resulting lipodisks. In this project, detergent depletion and extrusion were used. The lipid composition for each production together with a more detailed description of each method can be found in appendix 1.

Lipids were initially weighed, mixed in a glass vial and dissolved in chloroform. The solvent was then evaporated under a flow of N_2, which caused a lipid film to form on the glass surface. The film was kept in vacuum over night to ensure full removal of chloroform.

For detergent depletion, the lipid film was hydrated in a mix of Hepes buffer and a detergent. By adding Biobeads, the detergent was removed from the solution and the lipids formed disks. When using extrusion, a lipid film hydrated in PBS was mixed by vortexing and freeze-thawing. The solution was filtered back and forth across a membrane in order to form small, homogenous disks. This method was also used to produce liposomes.

3.2.2 Measure binding with fluorescence

The emission spectrum changes when the polarity of the environment of a peptide containing fluorescent amino acids, mainly tryptophan, changes. This shift can be monitored and has been used to describe the interaction between peptides and disks (Agmo Hernández *et al.* 2013). Since PM2 and PM3 both have a tryptophan residue this was the initial method of choice.

A 20 μM solution of PM2 or PM3 in PBS was prepared in a quartz cuvette. Using a double spectrofluorometer, excitation wavelength was set to 280 nm and emission spectra were collected while stepwise adding 10-50 μL lipodisks. The ratio between emission fluorescence intensities at 325 and 355 nm were calculated to monitor the shift as more and more peptides bound to the disks. The ratio could in this way describe the fraction of free peptide, assuming

the experimental setup allows the fraction of free peptide to go from 0 to 100%. The effective peptide to lipid ratio (R_{eff}) could be determined for a certain lipid concentration with the following equation:

$$\frac{(\alpha * R_i)}{(1-\alpha) * [P]_{tot}} = \frac{R_{eff}}{[P]_{aq}} \quad (1)$$

where α is the fraction of peptide that is bound to the disk, R_i is the mixing ratio between peptide and lipid, $[P]_{tot}$ is the total concentration of peptide and $[P]_{aq}$ is the free peptide concentration in the buffer. R_{eff} is defined as

$$R_{eff} = \frac{[P]_{lip}}{[lipid]} \quad (2)$$

where $[P]_{lip}$ is the concentration of peptide that is associated to the membrane and $[lipid]$ is the lipid concentration. R_{eff} plotted against the concentration of free peptide can describe the binding of peptide to disk (Figure 8).

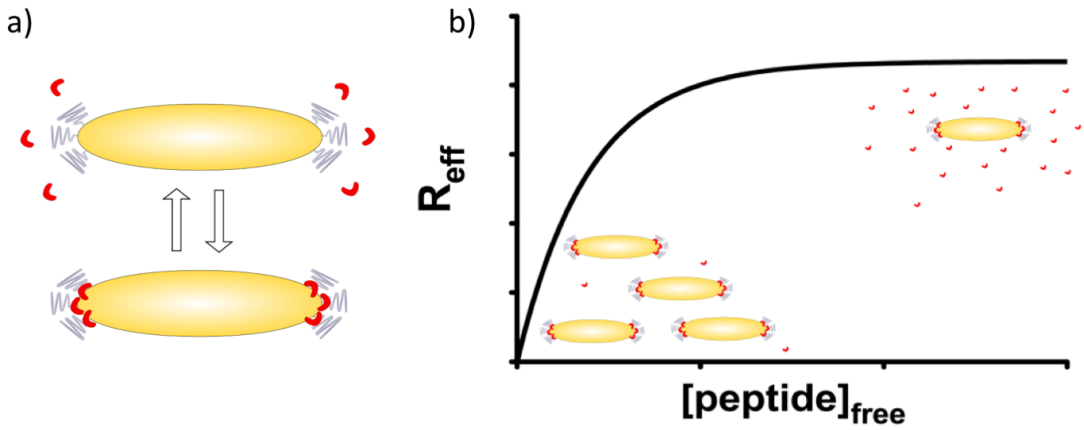

Figure 8. Obtaining a binding isotherm. (a) When the peptides (in red) bind to disks (yellow), the amount of free peptide decreases. (b) In the fluorometric measurement assay, the concentration of free peptide is initially high, but as more and more disks are added the amount of free peptide decreases (moving from right to left on the curve). When there is an overflow of disks, the effective peptide to lipid ratio (R_{eff}) decreases.

3.2.3 Troubleshooting the fluorometric method

For some reason, using fluorescence to characterise the binding of the stapled peptides to lipodisks was not functioning properly. Therefore, a period of troubleshooting followed the initial experiments.

First, it was tested if residual Biobeads in the lipodisk solution after production could influence binding and/or signal. Lipodisk solutions were filtered two times through filters with pore sizes 0.2 and 0.1 µm and the binding assay was repeated after each filtration. Dynamic Light Scattering (DLS) (see appendix 2) was used to characterise the lipodisk solution before and after filtration.

Secondly, the solubility of PM3 was assessed. Due to the high fraction of hydrophobic residues of PM3, the peptides might form aggregates in water or PBS. This also applies for PM2. The aggregation could give a shift in emission signal since the amino acids no longer would be surrounded by polar water molecules. PM3 was dissolved in PBS with a concentration of 60-750 μM. The sample was spun down at approximately 13000 revolutions per minute (rpm) for 10 minutes in order to remove precipitated peptide. Supernatant was collected, diluted 20-75 times in ethanol and transferred to a quartz cuvette. Using an excitation wavelength of 280 nm, the emission intensity at 353 nm was measured. 1-5 μL of a standard peptide solution with concentration ranging from 1-10 mM was added and fluorescence measurements were repeated for each addition. The assay was repeated multiple times.

Apart from the standard addition method, a standard curve approach was also tested to determine the solubility of PM3. A 4.4 μM solution of PM3 in PBS:ethanol 1:49 was prepared in a cuvette. 1 μL 1 mM PM3 was added stepwise and the emission at 353 nm was collected for each addition. A saturated PM3 solution was then diluted in ethanol and measured separately.

Lastly, the photobleaching effect was investigated. The fluorescent signal from the peptide could be unstable and decrease over time, which can influence calculations. A solution containing 8 μM PM3 was placed in the spectrometer and the emission intensity at 353 nm was measured every second for 15 minutes, keeping the excitation wavelength at 280 nm.

3.2.4 Binding assay with QCM-D

Quartz Crystal Microbalance with Dissipation monitoring (QCM-D) is a nano-scale technique to measure adsorption and interactions on surfaces. This includes interactions between small molecules and lipodisks. A quartz crystal is placed between two electrodes and is excited to oscillate at its resonance frequency. When something adsorbs to the surface of the crystal, it oscillates slower due to the mass change. For rigid surfaces the frequency correlates to the mass of the crystal, but for softer films the relationship is more complicated. Therefore, dissipation monitoring is also used. The electrical current is frequently shut off and sensor oscillates freely until the movement stops. The damping pattern of the oscillation correlates to the viscosity of the film. The frequency shift together with the dissipation can together describe the binding of PM2 or PM3 to a crystal surface covered with lipodisks (Biolin Scientific).

Lipodisks functionalised with DSPE-PEG-2000-amine were produced with extrusion according to the protocol in section 3.2.1. A QCM-D gold sensor was prepared and mounted in the instrument according to the protocol developed by Agmo *et al.* (2013). The surface was activated with 1:1 0.1 M NHS:0.4 M EDC for 15 minutes. A few minutes of rinsing with PBS was followed by a solution of functionalised lipodisks. The system was rinsed with PBS to remove unbound disks. The surface was deactivated using 1 M ethanolamine. Solutions of PM3 with concentrations of 0.025-2 μM were flushed over the surface with a flow rate of 50-

150 µL/min, rinsing with PBS in between. The oscillation frequency and dissipation was automatically collected.

The shift in frequency (*Δf*) and dissipation (*ΔD*) at different overtones were fitted to the equation

$$\frac{\Delta f}{n} = -\frac{m_d f_0}{t_q \rho_q} + \frac{\pi \eta_1 (f_0)^2}{\mu_1}(n\Delta D) \qquad (3)$$

where n is the overtone number, m_d is the adsorbed mass surface density, f_0 is the fundamental oscillation frequency, t_q and ρ_q are the thickness and density of the quartz crystal, η_1 is the viscosity and μ_1 is elastic modulus of the absorbed layer. A plot of Δfn^{-1} vs $n\Delta D$ should give a linear curve with the intercept at $-\frac{m_d f_0}{t_q \rho_q}$, which corresponds to the frequency shift expected from a rigid film with the same mass surface density. The relationship between frequency and mass then becomes much simpler:

$$\Delta m = C * \Delta f \qquad (4)$$

where C is a constant determined by the properties of the crystal. The bound mass was converted to amount of peptide and R_{eff} could be calculated for each concentration of PM3.

In order to see if PM3 had the ability to interact with the flat part of the disk, a QCM-D assay with a lipid bilayer instead of lipodisks was prepared. Liposomes was prepared using extrusion and rinsed over a silica sensor. The flow was stopped and a steady increase in temperature over 2.5 hours caused the liposomes to burst and form a bilayer over the sensor surface. The temperature was lowered to 21°C and the system was rinsed with PBS. A 0.25 µM PM3 solution was flushed to the sensor.

3.3 Results

3.3.1 Fluorometric measurements were not reliable

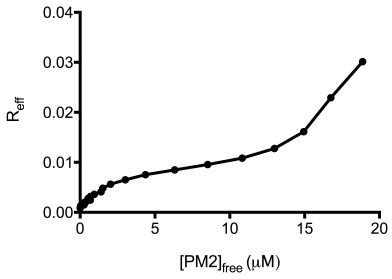

Figure 9. Binding isotherm obtained from fluorometric measurements.

Initial fluorescence based measurements of PM2-disk interaction, shown in Figure 9, could not describe the interaction properly. The binding isotherm did not reach a maximum R_{eff}, which meant there was no visible saturation of the disks. It was also possible to see a cloudy peptide solution become clear after addition of lipodisks, which indicated some sort of interaction.

DLS showed that bigger particles were present in the lipodisk solution after production, which could interact with peptide and/or lipodisks (Figure 10). These particles could be removed with a two-step

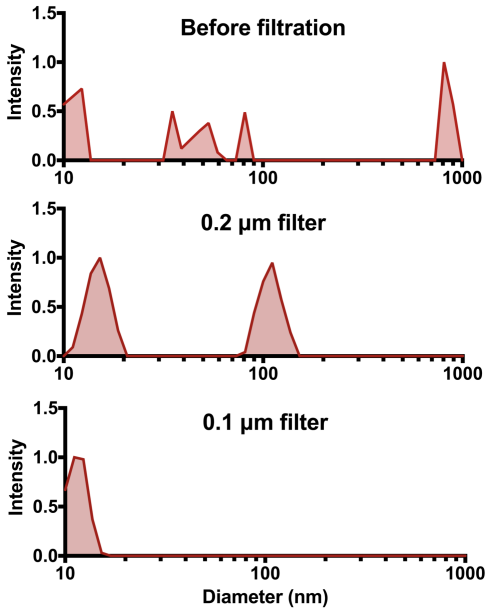

Figure 10. Particle population before filtration (top), after filtration using 0.2 µm (middle) and 0.1 µm pore size (bottom). The x-axis shows the hypothetical hydrodynamic diameter.

filtration using 0.2 and 0.1 µm filters. Despite this, the binding measurements were still inconsistent (data not shown). The conclusion was that the particles could potentially disturb the measurements, but they were not the main problem with the current experimental setup.

Solutions of peptide in PBS were cloudy, which indicated low solubility. Since aggregated peptides could disturb the fluorescence shift, it was important to conduct the measurements within a concentration interval where no aggregation occurred. Multiple experiments were performed, but the solubility varied massively between runs. Even when using a sample with a known concentration, the measured concentration was almost two times lower than expected. A measurement of a solution with PM3 showed that the signal faded with time, which verified that photobleaching takes place (Figure 11). This explains why it was so hard to determine the solubility of the peptides when using fluorescence methods.

The photobleaching process could possibly be compensated for, but this in combination with the unknown solubility of the peptides complicated the experimental setup. Therefore, it was decided that a change of method was the best option.

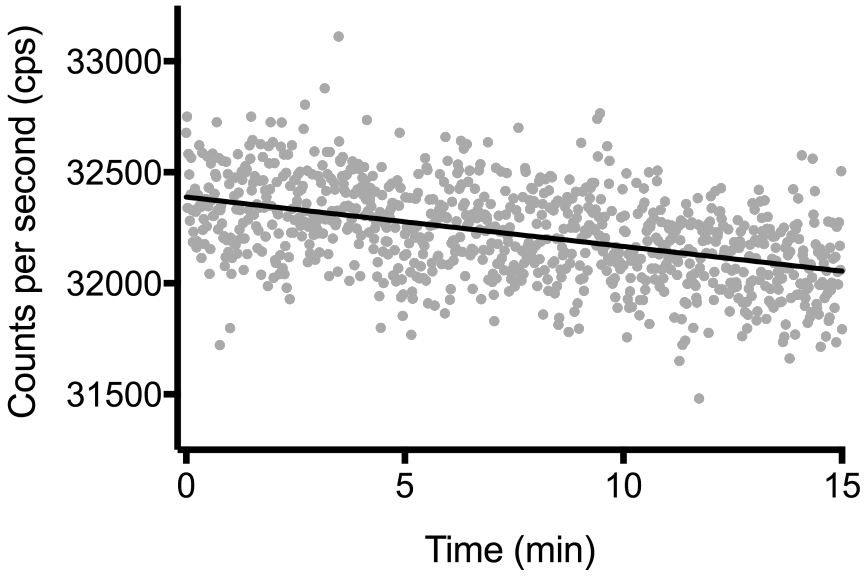

Figure 11. The change in emission intensity at 353 nm over time for a 8 µM solution of PM3.

3.3.2 QCM-D shows strong association

The association between PM3 and lipodisks could be characterised with QCM-D and a binding isotherm was obtained (Figure 12). PM3 was able to bind at low concentrations and did not initially release from the disk when rinsing with PBS, which is a sign of a strong association. The disks became saturated when the concentration of free peptide was around 0.25 μM and the peptides started to release to some extent during rinsing steps. The calculated isotherm showed a maximum peptide/lipid ratio of approximately 0.17.

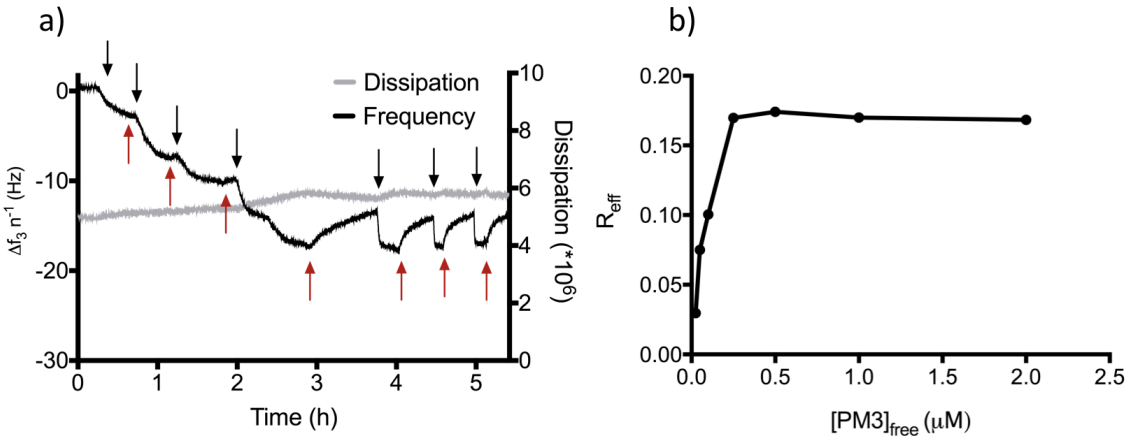

Figure 12. (a) QCM-D experiment with lipodisks and PM3. Black arrows indicate addition of PM3 with increasing concentration (0.025, 0.05, 0.1, 0.25, 0.5, 1, 2 μM) and red arrows indicate rinsing with PBS. (b) The obtained binding isotherm from the QCM-D data.

For the lipid membrane assay, the results were inconclusive (data not shown). When PM3 is added, the frequency decreases. This indicates binding. After a few minutes, the frequency increases and the sensor becomes lighter than prior to PM3 addition.

3.4 Discussion

The second part of the project was partly successful, where QCM-D data indicated that lipodisks are a suitable delivery system for PM2 and PM3. It was shown that PM3 can interact with a planar lipid bilayer which represents the flat part of the disk, but there is more work needed to characterise the binding further. The experiment conducted here raised quite a few questions, but the results indicated that there is an interaction between peptide and the flat part of the disk. During the assays conducted here the peptide spontaneously associates with the disk, but it may also be possible to incorporate peptides into the lipid membrane of the disk. It would have been interesting to test the system on tumour cells, but more optimisation is required.

The complications of using fluorescence to measure the interaction were time-consuming, but raised the very important question of how the solubility and potential aggregates can disturb measurements. Since it is a useful method suitable for a number of applications, it may be of interest to further characterise and possibly compensate for the photobleaching. If a solution

to this problem could be achieved it would be easier to investigate the solubility of the peptides. Knowledge about this would be extremely valuable when continuing with further studies.

As mentioned in the introduction, it is interesting to use targeting lipodisks in order to increase specificity towards cancer cells. EGF was briefly tested in this project with fluorescence-based measurements, but was never properly tested with cells. Using EGF as a targeting molecule has been successful when using lipodisks, but it was hard to find cancer cell lines with both wildtype p53 (a requirement for PM2- and PM3-dependent radiosensitisation) and overexpression of EGFR. The lack of available cell lines could indicate that these two properties rarely are associated in a clinical setting, which would make EGFR-targeting disks loaded with PM2 or PM3 less useful as a therapeutic agent. Therefore, the possibilities of using another targeting agent should be investigated. The choice of using EGF here was simply because an established protocol for production of EGF-conjugated lipodisks was already in place.

4 Conclusions

This project has resulted in an established and optimised protocol for CAT-mediated ^{125}I-labelling of PM2 and PM3. The labelled products have been tested *in vitro* and *in vivo*. The protocol can be used to assess cellular uptake, internalisation and other kinetical properties of the peptides. It has also been shown that PM3 can associate with lipid bilayer disks. By further assessing and optimising the interaction, targeted delivery of PM2 and PM3 using lipodisks could be obtained. Together, this could in the future lead to a new therapeutic scheme where a radiolabelled and radiosensitising peptide can be specifically delivered to a tumour with targeting lipodisks.

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Appendix 1 – Production of lipodisks and liposomes

All methods started with weighing and mixing of lipids in a glass tube. Chloroform was added and then removed under a flow of nitrogen gas in order, causing a lipid film to form on the glass surface. The film was kept in vacuum over night to ensure full removal of chloroform. The used lipids were distearoyl phosphatidylcholine (DSPC), PEG-linked distearoyl phosphatidylethanolamine-2000 (DSPE-PEG-2000), functionalised PEG with NHS (NHS-PEG-3400) and functionalised DSPE-PEG-2000 with amine (DSPE-PEG-2000-amine).

Lipodisks for fluorescent based binding assays using detergent depletion: DSPC:DSPE-PEG-2000:NHS-PEG-3400 with a molar ratio of 7:2:1 were used. Lipid films were resolved in Hepes buffer together with octyl glucoside (OG, final concentration 39.5 mM) in 60°C. In order to obtain targeted disks, NHS-PEG-3400 was conjugated to EGF by incubating lipid and EGF for 12 hours while stirring. EGF-lipid was separated from free EGF with a size exclusion column using Hepes buffer as a mobile phase. OG was added to the solution with a final concentration of 39.5 mM. The lipid solution was then mixed with the hydrated lipid film. For untargeted disks, the functionalised lipid was dissolved in Hepes and OG prior to mixing with hydrated lipid films. The final lipid solutions were incubated at 60°C an additional 15 minutes and cooled to room temperature before incubation with de-gased Biobeads under rotation for 2 hours. Biobeads eliminated the detergent from the solution and caused lipids to form disks. Supernatant containing lipodisks were separated from Biobeads using a syringe. Sample was spun down to fully eliminate fragments. The lipid concentration was determined with phosphorous analysis (see appendix 3).

Extruded disks for QCM-D: lipid films consisting of DSPC, DSPE-PEG-2000 and DSPE-PEG-2000-amin with a molar ratio of 80:16:4 were resolved in 1 mL PBS. The solution was heated to 70°C for 30 minutes with vortex every five minutes. Sample was freeze-thawed 8 times (liquid nitrogen – water bath 70°C) and extruded 15 times at 70°C through a polycarbonate filter with a pore size of 100 nm.

Extruded liposomes produced for QCM-D: Lipid film consisting of 2 mg DSPC was resolved in 1 mL PBS, 15 cycles of freeze-thawing (liquid nitrogen - water bath 60°C) and 31 cycles of extrusions at 60°C were performed.

Appendix 2 – Dynamic Light Scattering (DLS)

When particles are suspended in a fluid, they randomly move around. The movement depends on the size of the molecules; a larger molecule moves slower than a small one. When light hits a solution with particles, the light will be scattered. The intensity of the scattered light will fluctuate around an average value, where the level of fluctuation correlates with the velocity and therefore the size of the particle. The fluctuation of intensity can be used to determine the translation diffusion coefficient (D). D relates to the hydrodynamic radius R_h of the particles according to the Stokes-Einstein equation

$$D = \frac{k_B T}{6\pi\eta R_h} \tag{5}$$

where k_B is the Boltzmann constant, T is the absolute temperature and η is the viscosity of the sample.

It is important to state that the hydrodynamic radius is not equal to the actual radius of the particle. The hydrodynamic radius represents the radius of a hard sphere which moves with the same speed as the particle and should only be used to state the indicative size of the particle. If the solution contains particles of different sizes, it is hard to correctly determine the size of the smaller particles, since bigger particles scatter more light and hide signals from smaller structures (Bhattacharjee 2016).

Appendix 3 – Determination of phosphorous content

This is a method used to determine the total amount of phosphorous in a sample.

50 µl of lipodisk solution in a glass bottle was placed in a heating oven (550°C) over night. After cooling to room temperature, the sample were dissolved in 4 mL water. The following two reagents were mixed 7:3

- 1 part $K(SbO)C_4H_4O_6 \bullet 5H_2O$ 0.6855 g/250 mL, 3 parts $(NH_4)Mo_7O_{24} \bullet 4H_2O$ 2g/50 mL, 10 parts 2.5 M H_2SO_4
- 0.1 M ascorbic acid

and 1 mL was added to each sample. Standard solutions with a known phosphorous concentration were dissolved in 4 mL water and 1 mL of the reagent solution. Solutions were incubated at room temperature for 20 minutes and the absorbance at 882 nm was measured. A standard curve was obtained with the standard solutions and the absorbance of each sample could be correlated to a certain lipid concentration.