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Intracellular processing of liposomal targeting agents

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Abstract

The cellular fate of targeted EGF-liposomes has been studied in human A431 carcinoma and U343 glioma cells. This was done by investigating the specificity, uptake, internalisation and retention of ^{125}I-EGF-liposomes and EGF-^{3}H-liposomes. A specific binding of the EGF-liposomes to the EGF receptors was found as the specific binding could be inhibited with excess of non-radiolabelled EGF. The EGF-liposome uptake was found to increase with incubation time. A431 cells displayed a higher EGF-liposome uptake than U343 cells. A majority of the EGF-liposomes were internalised in both cell lines, and the liposomes displayed long retention times, while the ^{125}I attached to EGF was quickly released from the cells. These are promising results for future liposome targeting to the EGFR for both diagnosis and therapy of cancer.

Keywords
Epidermal Growth Factor, EGFR, Tumour Targeting, Liposomes, Carcinoma, Glioma

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Intracellular processing of liposomal targeting agents

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Sammanfattning

När kroppens celler ska växa och dela sig behövs en tillväxtfaktor, EGF (Epidermal Growth Factor). Därför producerar cellen EGF-receptorer som kan fånga upp EGF på cellens yta. Många tumörceller överproducerar EGF-receptorer, vilket kan utnyttjas i modern cancerforskning. En applikation är att använda EGF som en målsökare. EGF-proteinet fästs då på ytan av det som ska transporteras, t.ex. en liposom fylld med en celldödande substans. Eftersom cancercellerna överproducerar EGF-receptorer kommer EGF-liposomer att fästa till dessa, och tumörcellerna kan dödas. I det här examensarbetet undersöktes vad som sker när EGF-liposomen når EGF-receptorn. Detta gjordes bl.a. genom att studera om EGF-liposomen fäster specifikt till EGF-receptorn, om EGF-liposomerna sitter kvar på cellytan eller tas in i cellen, och hur länge de EGF-liposomer som tagits in i cellen stannar kvar. Försöken visade att EGF-liposomen fäster specifikt till EGF-receptorn och att merparten tas in i cellen och stannar kvar där i flera dagar. Dessa resultat är mycket lovande för för framtida liposom-målsökning mot EGF-receptorn för både diagnostisering och terapi av cancer.

Examensarbete 20 p i Molekylär bioteknikprogrammet

Uppsala universitet januari 2002

2 Introduction

2.1 Conquering cancer

Humans have been plagued by diseases throughout the history of civilisation. Medical science has conquered many of those threats, but even in the 21:st century, cancer is still considered the worst killer disease of non-epidemic kind. Although the risk of a few types of cancer has declined in developed countries in the last century, the incidence of the most significant forms has increased. Cancers of the lung, breast, prostate, colon and rectum have all become more frequent in countries where risk factors such as cigarette smoking, dietary habits, old age and exposure to dangerous chemicals are now more common[1]. Cancer can attack almost any tissue in any part of the body, and as cancer cells grow and divide out of control, nearby tissues and organs will be damaged. Furthermore, cancer cells can break away from a malignant tumour and enter the bloodstream or lymphatic system and form secondary tumours in other parts of the body (metastasis)[2]. Cancer treatments in the present day are mainly based on surgery, chemotherapy and external radiotherapy[3]. These treatments can be unsatisfactory, since they offer no efficient methods for locating or treating metastases and might harm surrounding tissues. One method of finding more resourceful cancer treatments is to concentrate on the differences between normal and malignant cells. By targeting those differences, science may find more sophisticated and efficient methods of treating cancer.

Such differences can be found in many of the vital functions of our bodies, such as growth and nutrition of living cells. Besides nutrients like sugar and fat, the cell also needs substances such as vitamins and hormones that it cannot produce itself. The cellular uptake of these substances is dependent on receptor proteins with specific interactions. In malignant cells, the need for proteins such as growth factor receptors often increases dramatically, as the cell needs constant stimulation of cell growth. This means that in many malignant cell types there are profound differences in the function and quantity of growth factor receptors in normal and malignant cells[4]. These differences, combined with the fact that these receptors usually are exposed at the cell surface, make growth factor receptors a promising tool for targeted anticancer drug delivery.

2.2 Two-step targeting

The two step targeting principle, described in Figure 1, provides a new and interesting approach to targeted tumour diagnostics and therapy. The principle is the same as that of a Trojan horse; in step one the toxic agents are delivered in a liposome directly to the tumour cell. By attaching to a tumour specific receptor, the liposome is endocytosed and possibly degraded by the cell. This will cause the toxic agents to become released, thus triggering step two; binding of the toxin to the DNA. The two

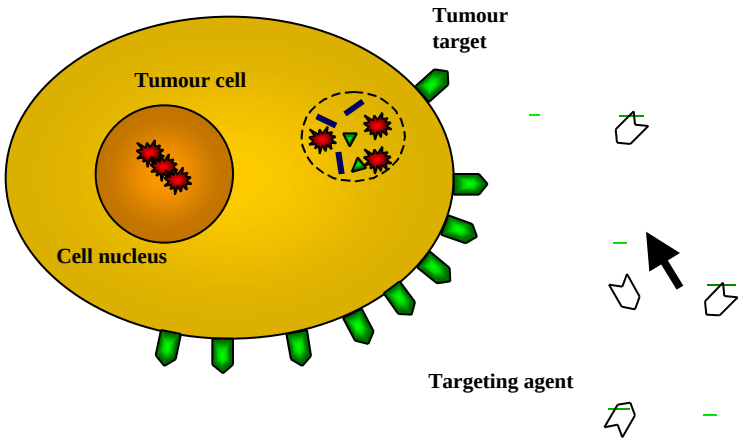

Figure 1. The principle of two-step targeting. In step one the toxic agents are delivered in a targeted liposome directly to the tumour cell. In step two the toxin binds to the DNA.

step targeting leads to a more effective treatment where non-malignant cells are less exposed to the toxic agents, since the targeted liposome will actively seek out the tumour cells. Also, normal tissue will be exposed to lower radiation doses than in conventional treatments. These are factors that will all contribute to enhancing the therapeutical index[5]. A best case scenario of this approach is a dramatic increase of the specificity and a possible treatment of several metastasised tumour sites within the body at the same time[6]. Important components for this kind of therapy are the specificity, uptake, internalisation and retention of the liposomes in the tumour tissue, all studied in this project work.

2.3 Liposomes

Liposomes have been of interest as delivery vehicles for tumour therapy for over 25 years[7], but only in recent years have they become really attractive, as the problem of circulation stability has been solved[8]. They are vesicular structures generally composed of one or more enclosed phospholipid-bilayer membranes. The phospholipid sphere contains an aquatic environment filled with the substance of interest. By producing the liposome from natural lipid components of biomembranes, the liposomes are compatible with living tissues. Liposomes are able to deliver large amounts of drug in one cellular uptake, and are known to gather in tumours and inflammation sites even without a targeting ligand due to the leaky vasculature[9-14]. A targeting ligand can further improve the tumour targeting, and a PEG (poly ethylene glycol) chain causes the liposome to become sterically stabilised. The PEG coating also prolongs the blood circulation time of the liposome, resulting in low blood-clearance rates[8]. The composition of a targeted liposome can be seen in Figure 2. In this degree project we have chosen the epidermal growth factor receptor (EGFR) as a target, and the ligand EGF as a targeting agent. Enclosed within the liposome is the boronated compound WSA[15], which can be used for boron neutron capture therapy (BNCT).

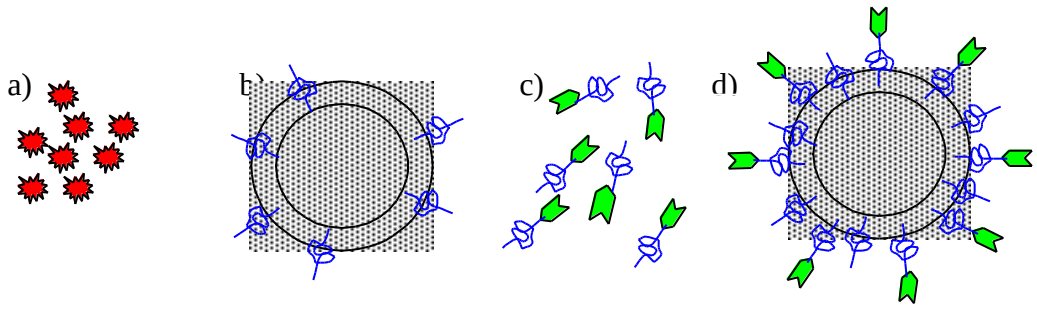

Figure 2. A targeted liposome consists of a) a toxin, b) one or more phospholipid-bilayer membranes, and c) a targeting ligand, often coupled to a stabilising polymer. The complete targeted liposome will be composed as in d).

2.4 Boron neutron capture therapy (BNCT)

In boron neutron capture therapy, a boronated compound is implanted in a tumour cell followed by the application of thermal neutrons. When ^{10}B captures thermal neutrons, it produces high energy helium and lithium ions, as seen in Figure 3, and is highly cytotoxic within a short range. The ions produced have a range smaller than a cell diameter, resulting in low damage to surrounding tissues. The probability of ^{10}B to capture a neutron much exceeds that of other atoms in the body, thus further minimising the risk of unwanted side effects[16]. For BNCT to be successful, about 10^9

WSA molecules, resulting in 10^5-10^6 borons per liposome[18]. Thus between 10^3 and 10^4 liposomes needs to be internalised into each cell.

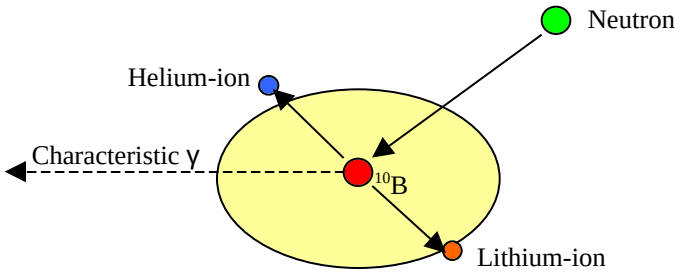

Figure 3. The principle of BNCT. When ^{10}B captures thermal neutrons, it produces high energy helium and lithium ions and is highly cytotoxic within a short range[16].

2.5 The Epidermal growth factor receptor

One suitable receptor for targeted drug delivery is the epidermal growth factor receptor (the EGF receptor), a 170 kDa transmembrane protein with an extracellular domain responsible for EGF binding. A schematic representation of the EGF receptor structures can be seen in Figure 4. Once EGF has attached to the receptor, a dimerisation of the receptor-ligand complex occurs. The dimers are then autophosphorylised and internalised, which eventually induces cell proliferation and DNA replication[19]. Malignant cell types that have been shown to overexpress EGF receptors are tumour cells of brain[20, 21], bladder[22], breast[23] and lung[24]. One such cell can express between 10^5 - 10^6 EGF receptors per cell, compared to a normal cell expression of 0 – 10^4 receptors per cell[25].

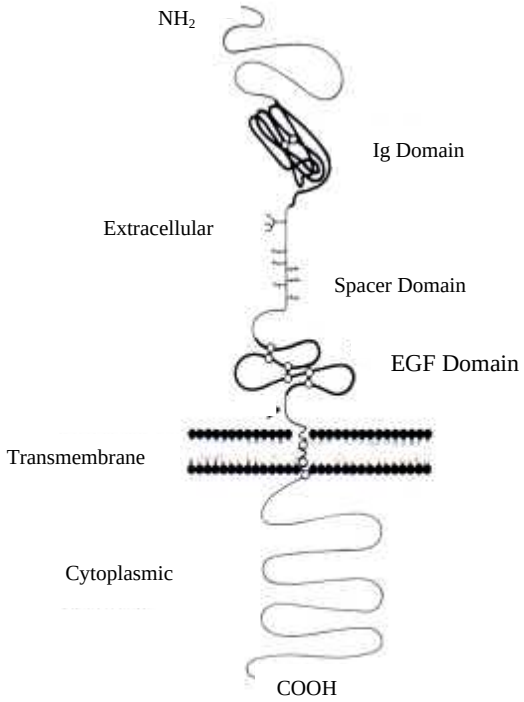

Figure 4. Schematic representation of the structures of the EGF receptor[2].

2.6 The EGF protein

EGF is a polypeptide consisting of 53 amino acids, as seen in Figure 5, and is thought to be involved in growth and differentiation of a variety of cells[19, 26]. It is a relatively low molecular weight protein of 6 kDa whose many β structures contribute to a very stable and dense structure. Three disulphide bonds create a triple-loop necessary for binding to the EGF receptor[19]. The receptor-ligand complex is normally internalized via receptor

then further transported to the lysosomes where the complex is degraded[28]. Mouse EGF can easily be labelled with ^{125}I and attached to a lipid without interference with the binding of EGF to the EGF receptor.

2.7 Tumour cell lines

Two different human tumour cell lines, U343 MGa C12:6, (glioblastoma cell line), and A431 (squamous carcinoma cell line), were used in this project work. They differ in the amount of EGF receptors overexpressed on the cell surface. A431 cells have a large amount of EGF receptors (about $2.6 * 10^6$ receptors per cell[29]), whereas U343 cells have a somewhat smaller amount (about $1.8 * 10^5$ receptors per cell[30]). From this follows that A431 cells should respond the most to the EGF labelled liposomes. Also, the A431 cells are bigger, and unlike the U343 cells they recycle some of the EGF receptors[31]. This means that after EGF uptake, the receptor is internalised and separated from the EGF. The receptor is then sent back to the cell surface instead of being broken down together with the EGF in the lysosome. This causes a growing pool of receptors as the number of cells grows.

2.8 Aim of study

The cellular fate of the targeted liposomes was investigated in this degree project. The aim was to find out what happens to the liposome and its content when allowed to interact with tumour cells. The *specificity* of liposome binding was studied with displacement tests with different cell lines, where the EGR receptors were first blocked with different concentrations of EGF. The results were then confirmed by using cells with blocked EGF receptors as a negative control in the following studies. The cellular *uptake* was studied with incubation test where the results of different incubation times as well as the results from different cell lines were analyzed. The amount of *internalised* or *membrane bound* liposomes was measured on different cell lines with internalisation studies, in which the results of differently labelled liposomes were compared. The *intracellular fate* of the liposomes was investigated by studying the retention of differently labelled liposomes in different cell lines. By studying the specificity, uptake, internalisation and retention of the liposomes we hoped to achieve an overall understanding of the fate of both the EGF-lipid and the liposome after receptor attachment.

3 Materials and methods

3.1 Production of ^{125}I-EGF–liposome conjugates

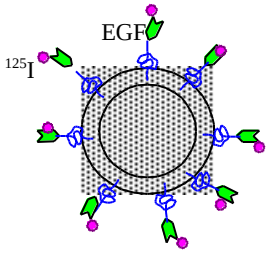

Figure 6. Schematic picture of a complete ^{125}I-EGF-liposome. The ^{125}I labelling can be seen in purple.

25 µg murine epidermal growth factor (EGF) obtained from Chemicon International (USA) was labelled with 50 MBq ^{125}I from Amersham Pharmacia Biotech, Uppsala, Sweden. The reaction was catalysed by 0.02 µg Chloramine T for 1 minute, and interrupted by 0.05 µg Sodium metabisulphite. The sample was then separated on a NAP-5 column purchased from Amersham Pharmacia Biotech, Uppsala, Sweden. The ^{125}I-EGF fractions were pooled together, and 500 µg 2-Iminothiolane (Trauts reagent) from Sigma, USA, was added. This was allowed to react under an argon atmosphere for one hour in order to form a ^{125}I-EGF-SH complex. The sample was then separated on a NAP-5 column, and the ^{125}I-EGF-SH fractions were pooled together. 300 µg Mal-PEG-DSPE lipid was added to the sample and allowed to react for 24 hours in room temperature. The sample was then separated on a PD-10 column purchased from Amersham Pharmacia Biotech (Uppsala, Sweden) filled with Sephadex G-150 gel. After separation the ^{125}I-EGF-lipid fractions were pooled together. Liposomes, made at the Department of Physical Chemistry, Uppsala University, Sweden, were added to the sample with 33 times lipid molar excess and the ^{125}I-EGF-lipids were incorporated into the liposomes during 1 hour at 60°C. The sample was then separated on a PD-10 column (Sepharose CL-4B gel) and the conjugate fractions were pooled together and stored in 4°C. A schematic picture of a complete ^{125}I-EGF-liposome can be seen in Figure 6.

3.2 Production of EGF-^{3}H-liposome conjugates

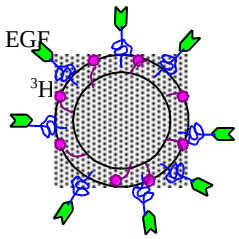

Figure 7. Schematic picture of the complete EGF-^{3}H-liposome. The ^{3}H labelling can be seen in purple.

2 MBq ^{3}H-cholesterol (0.03 mol%) was evaporated and added to 4 µmol lipid. The sample was evaporated and freeze-dried over night. 2 ml citrate buffer (300 mM citric acid) was added, and the sample was freeze-thawed (heated in 60°C and frozen in liquid nitrogen) 8 times in order to form liposomes. The sample was then extruded 20 times in order to produce the 100 nm liposomes. The liposomes were loaded with WSA (0.2:1) by obtaining a pH gradient with citrate buffer. The gradient (pH 4 on the inside and pH 8 on the outside) causes the WSA compounds to enter the liposomes and traps them by protonation. The loaded liposomes were stored in 4°C. The EGF-lipids were produced without the ^{125}I labelling step, and incorporated into the previously formed ^{3}H-liposomes as described above. The conjugate fractions were pooled together and stored in 4°C. Figure 7 shows a schematic picture of a complete EGF-^{3}H-liposome.

3.3 Tumour cell lines

The tumour cells were grown in complete medium, (i.e. Ham's F-10 medium (1x) supplemented with 10% foetal calf serum, L-glutamine and PEST) at 37°C in humidified air containing 5% CO_2. Before every study, 150000 cells in 2 ml complete medium were added to each dish and grown at 37°C in humidified air containing 5%

3.4 Displacement studies

The displacement studies were made with both ^{125}I-EGF-liposomes and ^{125}I-EGF. The ^{125}I-EGF was produced by labelling 2.5 µg EGF with 5 MBq ^{125}I. The reaction was catalysed by 0.02 µg Chloramine T for 1 minute, and interrupted by 0.05 µg Sodium metabisulphite. The sample was then separated on a NAP-5 column and the ^{125}I-EGF fractions were pooled together. ^{125}I-EGF-liposomes and ^{125}I-EGF were then diluted with complete medium to a volume of 50 ml. Non radio-labelled EGF was diluted with complete medium to 9 different concentrations (0 - 10000 ng/ml).
Each cell dish was washed with 1 ml Ham's F-10 medium, and then incubated for 4 hours with 0.5 ml of one of the EGF-dilutions and 0.5 ml of either ^{125}I-EGF-liposomes (Figure 8a) or ^{125}I-EGF (Figure 8b). The dishes were washed 6 times with 1 ml Ham's F-10 medium and the cells were detached with 0.5 ml trypsin in 37°C. After that, the cells were resuspended with 1 ml complete medium. 1 ml of the suspension was used for radioactivity measurements in a gamma counter, and 0.5 ml was used for cell counting.

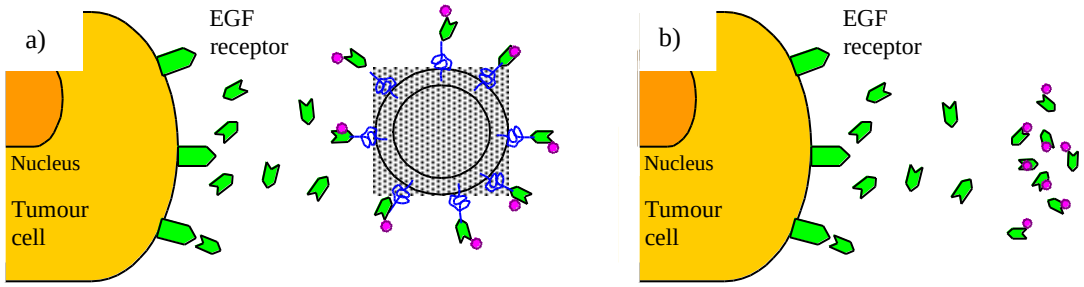

Figure 8. Different outlines of displacement studies. 0 - 10000 ng/ml non radiolabelled EGF was first added to the tumour cells in order to block the EGF receptors in capacity of its concentration. Then either a) ^{125}I-EGF-liposomes or b) ^{125}I-EGF was added to the cells.

3.5 Incubation studies

The cells grown in dishes were washed with 1 ml Ham's F-10 medium. 0.5 ml EGF (1 µg/ml) was added to half of the dishes (Figure 9a and 9c), and to the rest 0.5 ml complete medium was added (Figure 9b and 9d). 0.5 ml of the conjugate was then added to all dishes. The conjugate consisted of either ^{125}I-EGF-liposomes (Figure 9a and 9b) or ^{3}H-EGF-liposomes (Figure 9c and 9d), diluted with complete media. The dishes were then incubated for 1 - 24 hours. The incubation medium was then collected, and the dishes were washed 6 times with 1 ml Ham's F-10 medium. The cells were detached with 0.5 ml trypsin in 37°C, and resuspended with 1 ml complete medium. Measurements of ^{125}I were made on 1 ml of the cell suspension in a gamma counter, and 0.5 ml of the cell suspension was used for cell counting. In the ^{3}H study, measurements of ^{3}H were made on 1 ml of the cell suspension added to 9 ml scintillation liquid in a scintillator, and 0.5 ml of the cell suspension was used for cell counting.

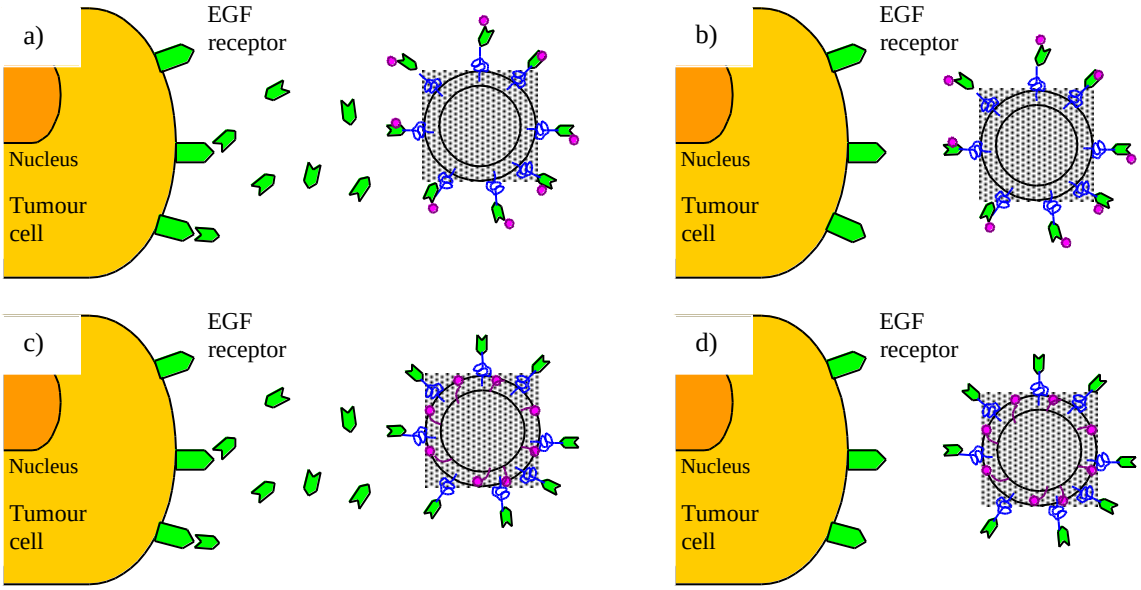

Figure 9. Different outlines of the incubation and internalisation studies. a) EGF was added to block the EGF receptors before the ^{125}I-EGF-liposome conjugates were added. b) ^{125}I-EGF-liposome conjugates were added to the tumour cells without receptor blocking with EGF. c) EGF was added to block the EGF receptors before the EGF-^{3}H-liposome conjugates were added. d) EGF-^{3}H-liposome conjugates were added to the tumour cells without receptor blocking with EGF.

3.6 Internalisation studies

The cells grown in dishes were washed with 1 ml Ham's F-10 medium. 0.5 ml complete medium (Figure 9b and 9d) or 1ug/ml EGF (Figure 9a and 9c) and 0.5 ml conjugate was added. The conjugate consisted of either ^{125}I-EGF-liposomes (Figure 9a and 9b) or ^{3}H-EGF-liposomes (Figure 9c and 9d), diluted in complete media. The cells were incubated for 1 - 24 hours. After incubation the cells were washed 6 times with 1 ml Ham's F-10 medium. 0.5 ml glycin-HCl buffer (33% 0.1M HCl, 67% 0.1M glycin) pH 2.5 was added to the dishes, and allowed to incubate in 4°C for 6 minutes. The fluid was collected, and another 0.5 ml glycin-HCl buffer was added and collected from the dishes. The membrane associated ^{125}I activity of the collected fractions was measured in a gamma counter, and the membrane associated ^{3}H activity was added to 9 ml scintillation liquid and measured in a scintillator. 0.5 ml NaOH was then added to the dishes and allowed to incubate for 60 minutes in 37°C. Another 0.5

ml NaOH was added and the fluid was collected two times. The internalised ^{125}I radioactivity of the collected fractions was measured in a gamma counter, and the internalised ^{3}H radioactivity was added to 9 ml scintillation liquid and measured in a scintillator.

3.7 Retention studies

The cells grown in dishes were washed with 1 ml Ham's F-10 medium. Then 0.5 ml complete medium and 0.5 ml conjugate was added. The conjugate consisted of either ^{125}I-EGF-liposomes (Figure 10a) or ^{3}H-EGF-liposomes (Figure 10b) diluted with complete media. The dishes were then incubated for 24 hours and thereafter washed 6 times with 1 ml Ham's F-10 medium. 1 ml complete medium was then added to the dishes, and the cells were further incubated for 0 - 48 hours. After incubation, the dishes were washed 6 times with 1 ml Ham's F-10 medium. The cells were detached with 0.5 ml trypsin in 37°C, and then resuspended with 1 ml complete medium. The radioactivity of the cells exposed to the ^{125}I-EGF-liposomes was measured on the entire 1.5 ml in a gamma counter. In the ^{3}H study, measurements of ^{3}H were made on 1 ml of the cell suspension added to 9 ml scintillation liquid in a scintillator.

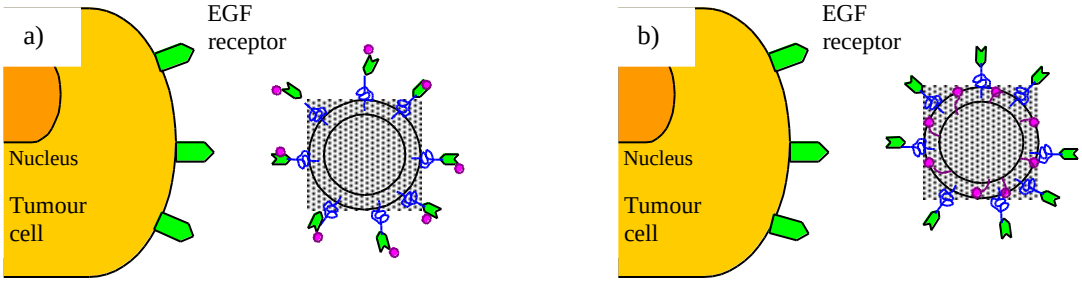

Figure 10. Different outlines of the retention studies. a) ^{125}I-EGF labelled liposomes were added to tumour cells. b) ^{3}H labelled EGF-liposomes were added to tumour cells.

4 Results and discussion

4.1 Production of ^{125}I-EGF-liposome and EGF-^{3}H-liposome conjugates

The incorporation of ^{125}I-EGF-lipids into the liposomes resulted in 8 to 15 incorporated ^{125}I-EGF-lipids per liposome. The 4°C storage of the ^{125}I-EGF-liposome is acceptable, since a stability study (methods not shown) showed that even after two weeks of 4°C storage, 90% of the conjugates were still intact (see Figure 11). The amount of incorporated EGF-lipid into ^{3}H liposomes was not investigated, as well as the stability of the EGF-^{3}H-liposome conjugates. For now, we assume that the incorporation and stability of the two conjugates are about the same, but this needs to be verified in future studies.

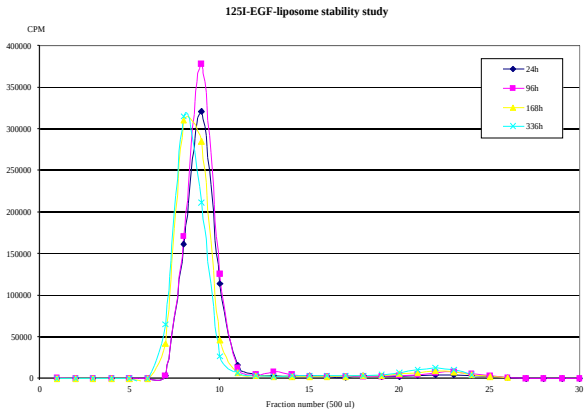

Figure 11. Stability study of ^{125}I-EGF-liposomes stored in 4°C. N = 1.

4.2 Displacement studies

4.2.1 ^{125}I-EGF-liposomes

The specificity of the targeted liposomes is demonstrated in Figure 12. The uptake of liposomes in both cell lines was inhibited by adding EGF that effectively blocked the EGF receptors of the tumour cells. When 1 µg / ml EGF was added, the liposome uptake in A431 cells and U343 cells was reduced to 4% and 13% respectively of the liposome uptake when no EGF was added. A431 cells showed the highest uptake of liposomes, and this is well in agreement with theory. Since A431 cells have the highest amount of EGF receptors they should be the most receptive to EGF-liposome targeting. U343 cells also overexpress the EGF receptor, although not in the same quantities, which is also coherent with the results.

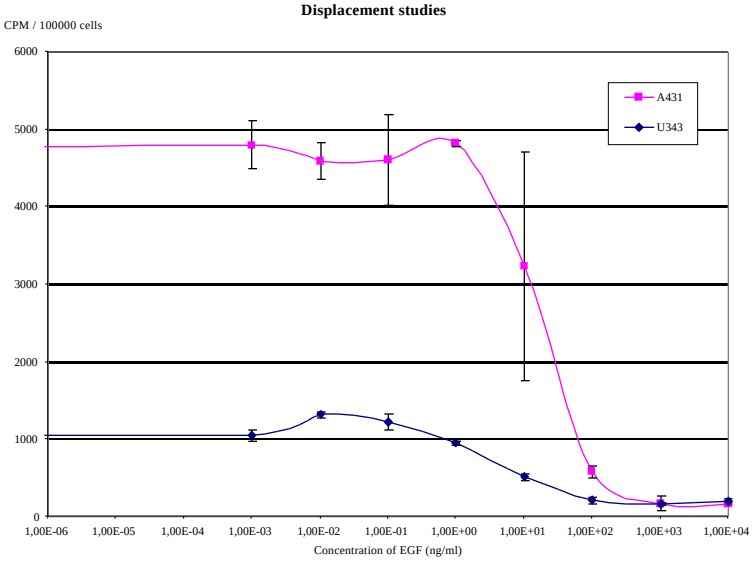

Figure 12. Displacement study using ^{125}I-EGF-liposomes and cell lines A431 and U343. When 1 µg / ml EGF was added, the liposome uptake in A431 cells and U343 cells was reduced to 4% and 13% respectively of the liposome uptake when no EGF was added. N = 3. Error bars represent maximal errors.

4.2.2 ^{125}I-EGF

Figure 13 shows the cellular uptake of ^{125}I-EGF as a function of added amount of non radiolabelled EGF. When 1 µg / ml EGF was added, the ^{125}I-EGF uptake in A431 cells and U343 cells was reduced to 3% (in both cell lines) of the liposome uptake when no EGF was added. The tumour cells showed the same displacement pattern with ^{125}I labelled EGF as with ^{125}I-EGF-liposomes, further confirming the specificity of the ^{125}I-EGF-liposomes. Again, the A431 cells showed the largest response to EGF targeting of the two cell lines.

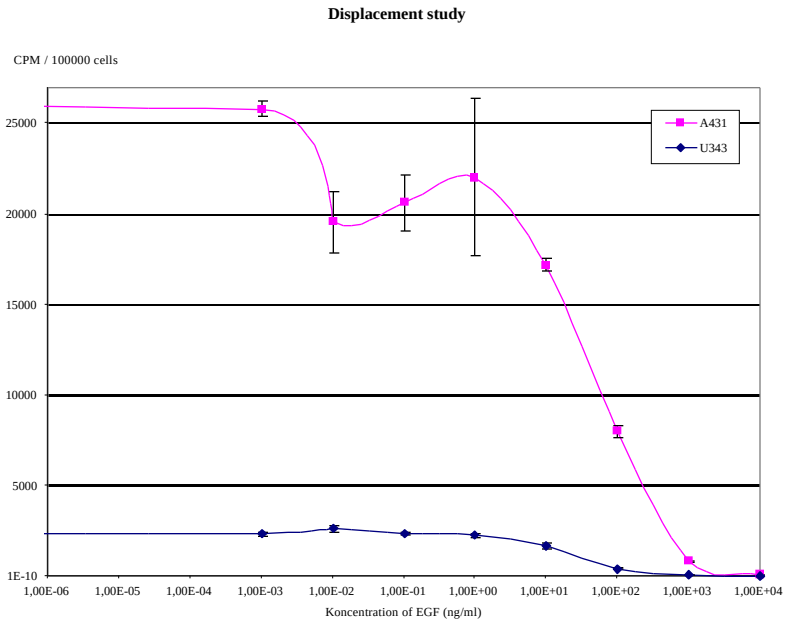

Figure 13. Displacement study using ^{125}I labelled EGF with cell lines A431 and U343. When 1 µg / ml EGF was added, the ^{125}I-EGF uptake in A431 cells and U343 cells was reduced to 3% of the liposome uptake when no EGF was added. N = 3. Error bars represent maximal errors.

4.3 Incubation studies

4.3.1 U343

4.3.1.1 U343 cells exposed to ^{125}I-EGF-liposomes

The incubation study results of U343 cells exposed to ^{125}I-EGF-liposomes can be seen in Figure 14. The specific binding (i.e. the binding to cells with unblocked receptors) of the liposomes increased with incubation time, having increased its value threefold from 1 to 24 hours. The unspecific binding of U343 cells, i.e. the binding of liposomes to cells with blocked EGF receptors increased slightly during the first 8 hours, but kept a value around 13% of the specific binding. At the 24 hours measuring point the unspecific binding had increased to 27% of the specific binding.

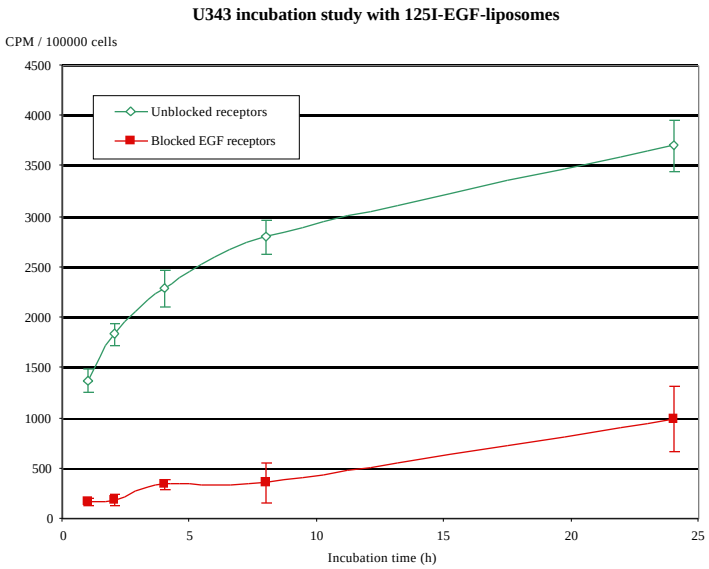

Figure 14. Incubation study of U343 cells exposed to ^{125}I-EGF-liposomes. The specific binding increased with time and was significantly higher than the unspecific binding. N = 6. Error bars represent maximal

4.3.1.2 U343 cells exposed to EGF-^{3}H-liposomes

The incubation study of U343 cells exposed to EGF-^{3}H-liposomes can be seen in Figure 15. The specific binding of the liposomes increased with incubation time, with a 9-fold increase in uptake from 1 hour to 24 hours. The unspecific binding in the ^{3}H labelled liposome study reached a value of 22% of the specific binding after 24 hours.

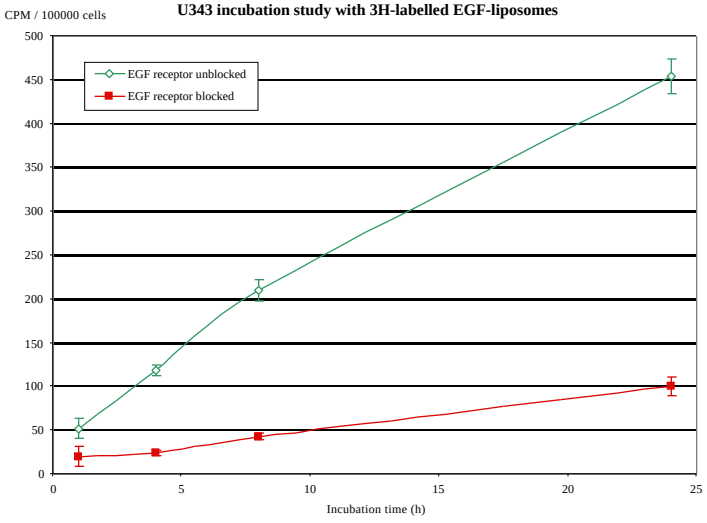

Figure 15. Incubation study of U343 cells exposed to EGF-^{3}H-liposomes. The specific binding increased with time and was significantly higher than the unspecific binding. N = 3. Error bars represent maximal errors.

4.3.2 A431

4.3.2.1 A431 cells exposed to ^{125}I-EGF-liposomes

The incubation study of A431 cells exposed to ^{125}I-EGF-liposomes can be seen in Figure 16. The specific binding of the liposomes increased with incubation time with a maximum of ^{125}I activity at the 8 hours measuring point (with a 2.6-fold increase in liposome uptake). The total ^{125}I activity then appears to somewhat decrease after 8 hours. The unspecific binding remained at a low level (around 9% of specific binding) until the 24 hours measuring point, where a striking increase of the binding can be seen (51% of specific binding). It can be speculated that some of this increase could be due to the fact that A431 cells, unlike U343 cells, recycles a number of its EGF receptors as described in the introduction. This means that after EGF uptake, the receptor is sent back to the cell surface instead of being broken down by the cell. The recycled receptors are then ready to bind new EGF, creating a larger receptor pool to be blocked by the added EGF. Chances are that the amount of EGF might not be enough to block all receptors during 24 hours, resulting in some liposomes bound to the EGF receptors in the ”blocked” cells.

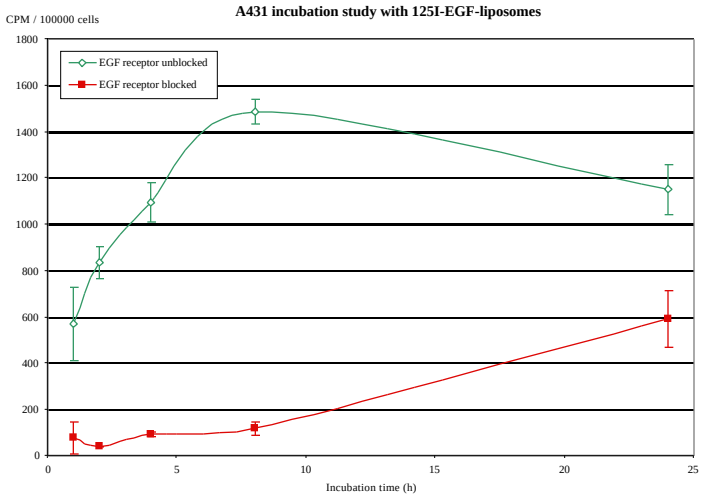

Figure 16. Incubation study of A431 cells exposed to ^{125}I-EGF-liposomes. The specific binding increased with time and was significantly higher than the unspecific binding. N = 3. Error bars represent maximal errors.

4.3.2.2 A431 cells exposed to EGF-^{3}H-liposomes

The incubation study of A431 cells exposed to EGF-^{3}H-liposomes can be seen in Figure 17. The unspecific binding remained at around 23% of the specific binding until the 24 hours measuring point where it increased to 38% of the specific binding. Some background at the 24 hours measurement might be accounted to the fact that A431 cells recycles its receptors as described above. The specific binding of liposomes increased with incubation time, showing a 4-fold increase in liposome uptake from 1 to 24 hours. Thus the ^{3}H activities in the 8 and 24 hours measurements differ from the activities obtained in the incubation study of A431 cells exposed to ^{125}I-EGF-liposomes (where the 24 hours measurement displayed a lower value, see Figure 17). Since no data has been collected between the 8 and 24 hours measurements, no certain conclusions can be drawn from this, although one speculation could be that the A431 cells are degrading the ^{125}I-EGF and releasing some of the ^{125}I from the cells whereas the liposome or at least the ^{3}H-cholesterol is retained. Thus, at the 24 hours measurement the rate of ^{125}I release is greater than the rate of the ^{125}I-EGF-liposome uptake.

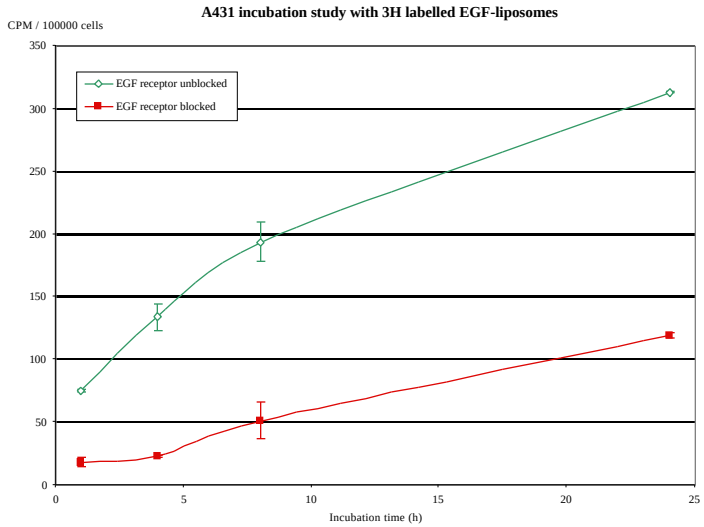

Figure 17. Incubation study of A431 cells exposed to EGF-^{3}H-liposomes. The specific binding increased with time and was significantly higher than the unspecific binding. N = 3. Error bars represent maximal errors.

4.3.3 Comparison

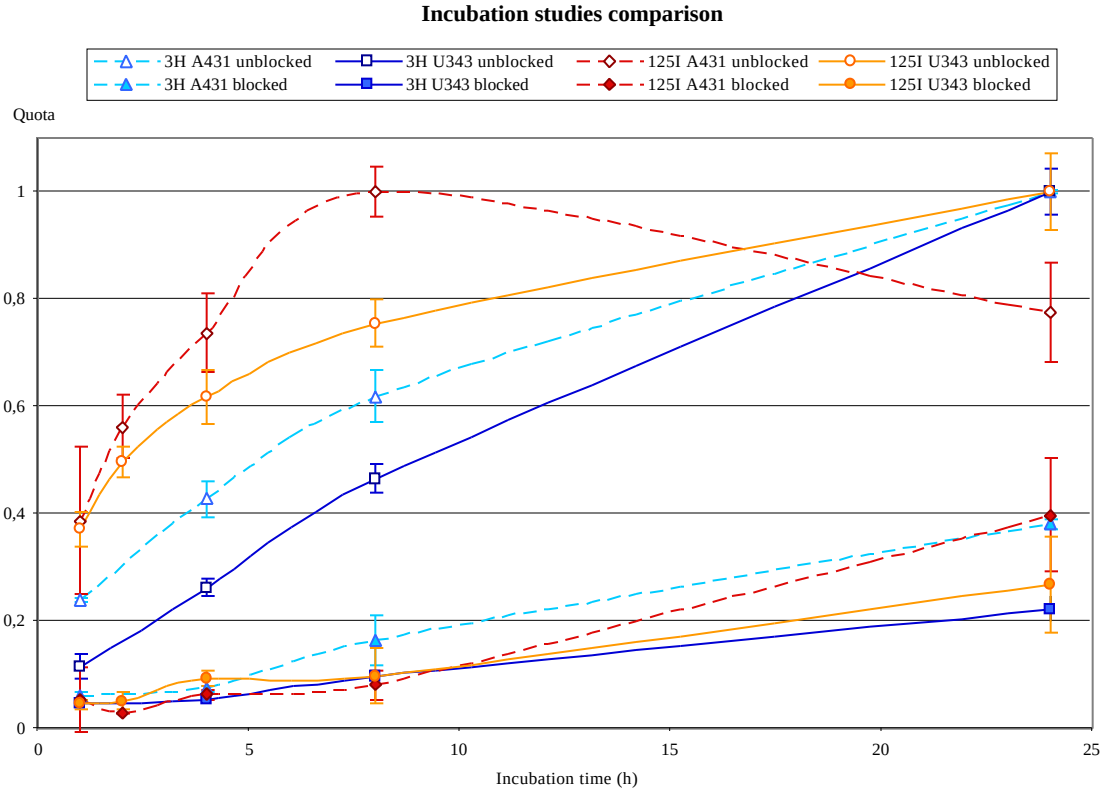

Figure 18. Comparison of four different incubation studies. Y values represent the quota, i.e. the value divided with the maximum value of that study. Error bars represent maximal errors. For all studies, N = 3, except the ^{125}I U343 study where N = 6.

The incubation studies are summarised in Figure 18. All studies show that the binding of targeted liposomes is specific and increases with incubation time. The fact that the EGF-liposomes show specificity for both EGF-overexpressing cell lines in all studies is very promising for future studies. It can also be seen that U343 cells generally

specific binding curves, a difference between the ^{125}I and the ^{3}H studies can be noticed. The ^{125}I curves generally display a larger tendency to decline or reach a plateau with time than their ^{3}H counterparts. One explanation could be that the EGF is degraded in the lysosome, causing some of the ^{125}I to be released from the cells, whereas the liposome or at least the ^{3}H-cholesterole is remaining inside. This idea is also supported by the retention results obtained in this project (Chapter 4.5).

4.4 Internalisation studies

4.4.1 U343

4.4.1.1 U343 cells exposed to ^{125}I-EGF-liposomes

Figure 19 shows the amount of membrane bound and internalised ^{125}I-EGF-liposomes in blocked and unblocked cells. It can clearly be seen that the vast majority (85-90%) of the liposomes incubated in 37°C have been internalised in the unblocked cells even after only one hour of incubation. The membrane bound liposomes accounted for an average of merely 12% of the total specific binding of the liposomes incubated in 37°C. The total unspecific binding was low, only 6% of the total liposome binding in 37°C. It can also be seen that the unspecific binding mostly consists of internalised liposomes (74-100% of the total unspecific binding in 37°C). The 24 hour sample on ice displayed a different pattern. The membrane bound liposomes in unblocked cells accounted here for 53% of the total specific binding. This was not surprising, since ice is expected to inhibit the receptor mediated endocytosis. It should be noted that the 24 hour samples on ice seemed to have lost many living cells, which could explain the low activity in that measurement. Overall, these results point to a high internalisation rate of the liposomes, and a low unspecific background.

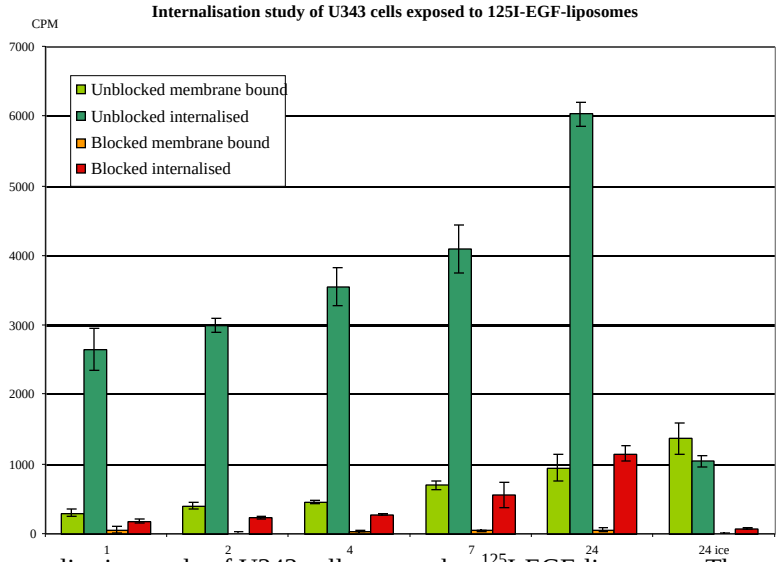

Figure 19. Internalisation study of U343 cells exposed to ^{125}I-EGF-liposomes. The vast majority (85-90%) of the liposomes incubated in 37°C have been internalised in the unblocked cells and the unspecific binding was low. The sample on ice displayed a majority of membrane-bound liposomes. N = 3. Error bars represent maximal errors.

4.4.1.2 U343 cells exposed to ^{3}H labelled EGF-liposomes

Figure 20 shows the amount of membrane bound and internalised ^{3}H labelled EGF-liposomes in blocked and unblocked cells. The majority (90-95%) of the liposomes incubated in 37°C with unblocked cells have been internalised even after one hour of incubation. The membrane bound liposomes accounted only for a small part (6.7%) of the total specific binding of the liposomes incubated in 37°C. It can also be seen that

the total unspecific binding is low, with an average of 9.7% of the total binding in 37°C, and mostly consists of internalised liposomes (75-95% of the total unspecific binding in 37°C). As expected, the 24 hours sample on ice displayed an opposite pattern, with the membrane bound liposomes in unblocked cells accounting for 80.6% of the total specific binding. The overall high internalisation and specific binding of the liposomes to the U343 cells is well in agreement with the results obtained for the internalisation study for U343 cells exposed to ^{125}I-EGF-liposomes, and are very promising results for future liposome targeted radiotherapy.

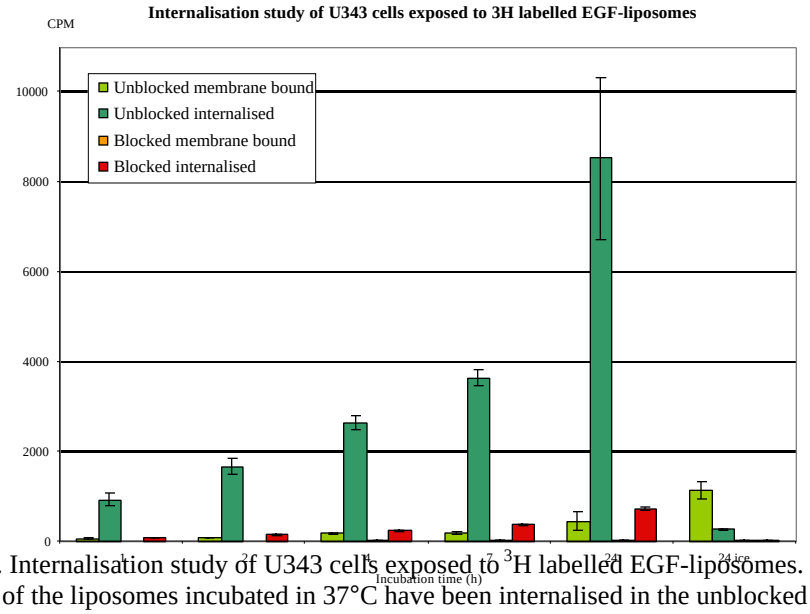

Figure 20. Internalisation study of U343 cells exposed to ^{3}H labelled EGF-liposomes. The vast majority (90-95%) of the liposomes incubated in 37°C have been internalised in the unblocked cells and the unspecific binding was low. The sample on ice displayed a majority of membrane-bound liposomes. N = 3. Error bars represent maximal errors.

4.4.2 A431

4.4.2.1 A431 cells exposed to ^{125}I-EGF-liposomes

Figure 21 shows the amount of membrane bound or internalised ^{125}I-EGF-liposomes in blocked and unblocked cells. The A431 cells have internalised 60-70% of the liposomes incubated with unblocked cells in 37°C. Similarly to the results of the U343 incubation study, the 24 hours sample on ice displayed an opposite pattern with 75% of the liposomes membrane bound to the unblocked cells. The fact that the 24 hours sample on ice displayed a larger CPM value than the 24 hours sample in 37°C might be due to the fact that some of the liposomes are degraded and disposed of over time, and that process is likely to be inhibited on ice. The background is low, with an unspecific binding of 10.8% of the total liposome binding in 37°C.

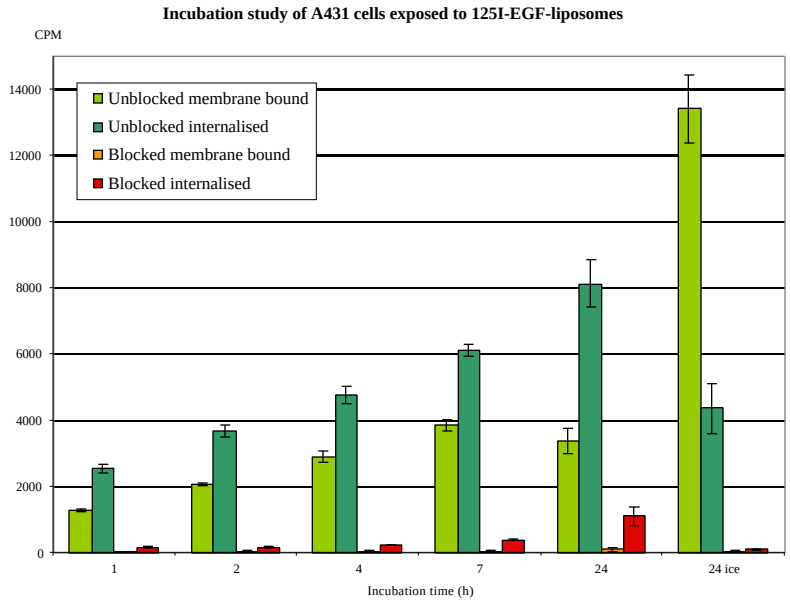

Figure 21. Internalisation study of A431 cells exposed to ^{125}I-EGF-liposomes. The majority (60-70%) of the liposomes incubated in 37°C have been internalised in the unblocked cells and the unspecific binding was low. The sample on ice displayed a majority of membrane-bound liposomes.
N = 3. Error bars represent maximal errors.

4.4.2.2 A431 cells exposed to ^{3}H labelled EGF-liposomes

Figure 22 shows the amount of membrane bound and internalised ^{3}H labelled EGF-liposomes in blocked and unblocked cells. The A431 cells have internalised the majority (51-83%) of the liposomes incubated in 37°C, even after only one hour of incubation. The membrane bound liposomes accounted for an average of 37% of the total specific binding of the liposomes incubated in 37°C. Figure 23 also shows that the unspecific binding is low, with an average of 14.4% of the total binding in 37°C, and mostly consists of internalised liposomes (60-90% of the total unspecific binding). The A431 cells incubated for 24 hours on ice on the other hand have, as expected, only internalised 20.6% of the total specific liposome binding. The overall high internalisation and specific binding of the liposomes to the A431 cells is coherent with the results obtained for the internalisation study for A431 cells exposed to ^{125}I-EGF-liposomes described above, and are positive results for future liposome targeted radiotherapy

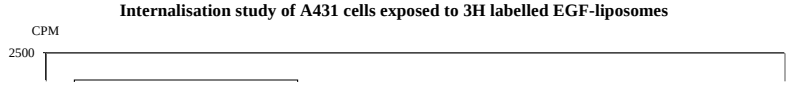

Figure 22. Internalisation study of A431 cells exposed to ^{3}H labelled EGF-liposomes. The majority (51-83%) of the liposomes incubated in 37°C have been internalised in the unblocked cells and the unspecific binding was low. The sample on ice displayed a majority of membrane-bound liposomes. N = 3. Error bars represent maximal errors.

4.5 Retention studies

4.5.1 ^{125}I-EGF-liposomes

The retention study of A431 and U343 cells exposed to ^{125}I-EGF-liposomes can be seen in Figure 23. After 24 hours of incubation with the conjugate, both U343 and A431 cells showed a clear disposing of ^{125}I containing material. When the incubation media was removed after 24 hours, both cell lines reduced the ^{125}I activity to half its value in the first two hours (A431 reduced the ^{125}I activity to 45.5% and U343 to 53.5% of the original ^{125}I activity). After 48 hours the activity had declined to 16% and 22% respectively of the original value for A431 and U343 cells. These results were confirmed in another study where ^{125}I-EGF-liposomes were incubated with tumour cells in 10 cm dishes (data not shown).

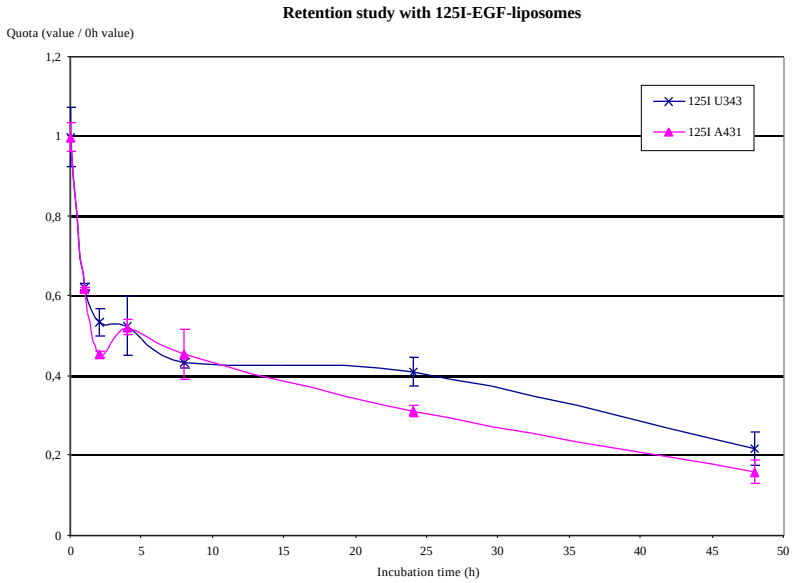

Figure 23. Retention study of A431 and U343 cells exposed to ^{125}I-EGF-liposomes. Y values represent the quota, i.e. the value divided with the starting value. Both cell lines reduced the ^{125}I activity to half its value in the first two hours. N = 3. Error bars represent maximal errors.

4.5.2 EGF-^{3}H-liposomes

The retention study of A431 and U343 cells exposed to EGF-^{3}H-liposomes can be seen in Figure 24. The tumour cells displayed a slow release of ^{3}H containing material. Even after 48 hours the U343 cells contained 89%, and A431 cells contained 52% of original activity.

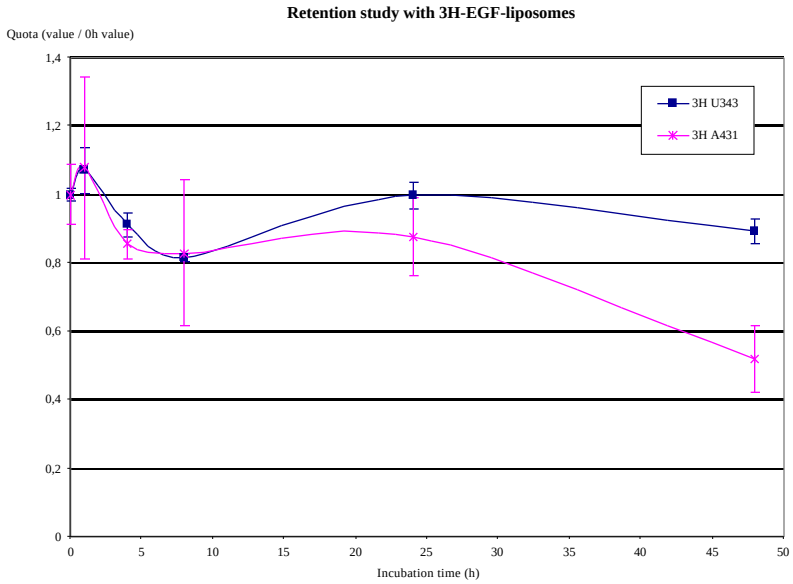

Figure 24. Retention study of A431 and U343 cells exposed to EGF-^{3}H-liposomes. Y values represent the quota, i.e. the value divided with the starting value. The tumour cells displayed a slow release of ^{3}H containing material. Even after 48 hours the U343 cells contained 89%, and A431 cells contained 52% of the original activity N = 3. Error bars represent maximal errors.

4.5.3 Comparison

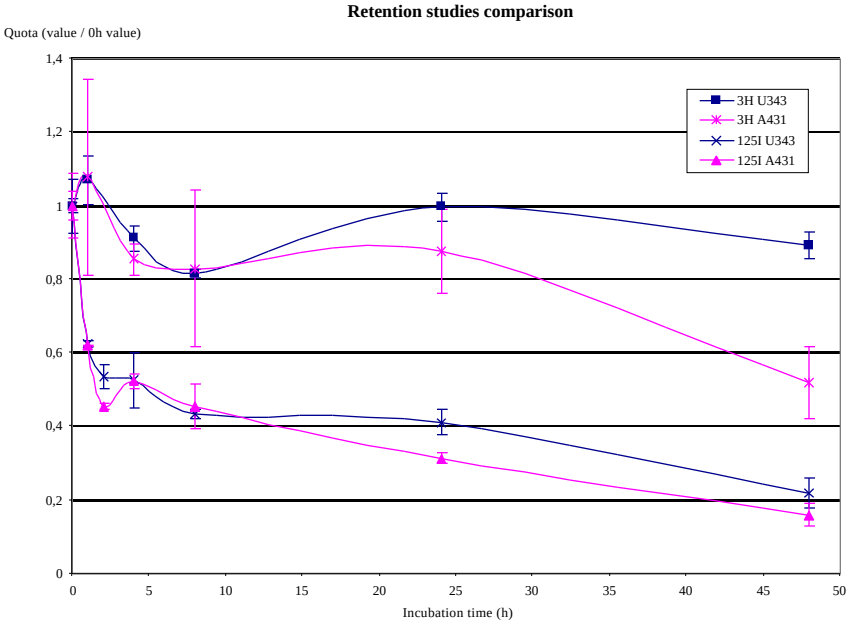

Figure 25. Comparison of the retention studies. Y values represent the quota, i.e. the value divided with the starting value. Both A431 and U343 cells displayed a much slower release of the EGF-^{3}H-liposomes than the ^{125}I-EGF labelled liposomes. N = 3. Error bars represent maximal errors.

A comparison of the retention studies can be seen in Figure 25. The results obtained with ^{125}I-EGF-liposomes differ from the results obtained with EGF-^{3}H-liposomes. When the liposomes themselves were labelled with ^{3}H, results showed a much better retention of radioactivity. Thus, the poor retention obtained with ^{125}I labelled lipids might indicate that it's not the liposomes themselves that are disposed of, but the ^{125}I-EGF-lipid, or parts of it. This could mean that the cells are degrading the EGF complex, releasing ^{125}I but leaving the liposome or liposome parts within the cell. If this is the case, the slow retention of the liposomes is a promising ground for liposome targeting.

5 Conclusions

There is a specific binding of the targeted EGF-liposome to the EGF receptor. The amount of liposomes binding to the EGF receptors increases with incubation time. The specific binding of the targeted liposomes to cell lines A431 and U343 can be inhibited by adding 1ug/ml EGF although results indicate that it might not be enough when the liposomes are incubated for 24 hours. Highly EGF-receptor overexpressing cells displayed a higher liposome uptake than other cells. A majority of the liposomes are internalised into the cells, and the liposome itself has a long retention time inside the cell, while ^{125}I attached to EGF is quickly released from the cells.

The fact that the unspecific binding is kept at an approximate 10% level for both EGF overexpressing cell lines in all studies is a promising result for further studies. However, the background levels have not been consistent throughout the studies, and seem to vary with labelling, incubation time, cell line and experiment. At the present, no satisfactory explanation for this has been provided.

6 Future work

- A retention study investigating the intracellular fate of the boron carried inside the targeted liposome has already been undertaken, although the measurements have not yet been conducted.
- Experimental therapy on WSA-loaded liposomes using a neutron beam at Studsvik has been initiated and will be further developed.
- In the near future, research will also investigate targeting against a similar receptor, HER-2, a receptor overexpressed in both breast and colon cancer.

7 Acknowledgements

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