

Exosome and liposomes as drug delivery vesicles for treatment of cancer

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Cancer treatment drugs can have rather detrimental side effects. Researchers focus on drug delivery systems such as liposomes and exosomes in order to decrease the side effects. In this paper an overview of different preparation methods, formulations and cases showing the advantages of using liposomes as such systems are described. Also the isolation and effects of using exosomes as such systems are described. At the end the pros and cons of both systems are compared.

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Introduction

As modern world progresses in the 21st century by pushing the frontiers of science it still battles numerous deadly diseases. One of these diseases is cancer, which can occur in every part of our body. Unfortunately in 2015, cancer was responsible for 8.8 million deaths, which roughly can be translated that every 6th death was due to cancer.[1]

The cancer treatment centers of America have published an extensive list of 62 drugs for treatment of cancer. [2] Doxorubicin (DOX) (figure 1) and Paclitaxel (PAX) (figure 2) are among the drugs on this list and lot of research groups focus on these two as they serve as typical examples of hydrophilic and hydrophobic agents.

For the first time the hydrophilic molecule doxorubicin was isolated in the 1960s. One part of the molecule is the aglyconic moiety which consists of a tetracyclic ring with quinone-hydroquinone groups, a methoxy group and a short side chain with carbonyl group. The other part of the molecule is a sugar moiety named daunosamine and consists of a 3-amino-2,3,6-trideoxy-L-fucosyl.[3]

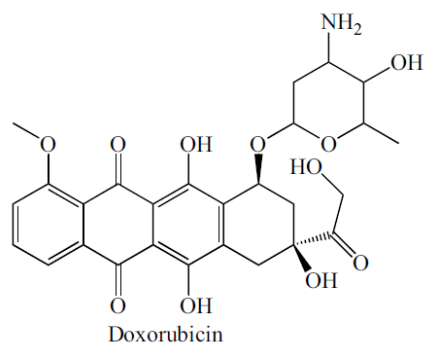


Figure 1 Structure of Doxorubicin[3]

The antitumor activity of doxorubicin is accredited to its ability to bind to proteins, involved in DNA replications and transcription; furthermore, it possesses the ability to intercalate into the DNA helix. Although it is used for many types of cancers, the usage of DOX has rather severe side effects: acute nausea, vomiting, gastrointestinal agitation, baldness, hallucinations, dizziness, fever, los of fertility and cumulative cardiotoxicity.[3,4].

Paclitaxel was isolated for the first time from the bark of *Taxus Brevifolia* by Mrs. Monroe E. Wall and Mansukh C. Wani. It has a rather complex structure with 10-deacetylbaccatin derivative as its core which contains carbonyl groups while it lacks hydroxy groups making the molecule hydrophobic. The mechanism of action by which the molecule functions is promoting and stabilizing microtubules as well as inhibiting the late G2 or M phases of the cell cycle.[5]

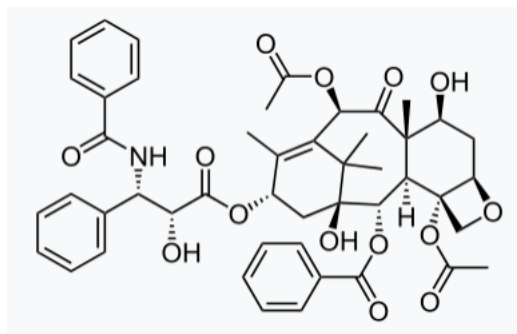


Figure 2 Structure of Paclitaxel [5]

This drug also has side effects. Some of them are: hair loss, low blood counts, pain in the joints, numbness of the extremities, nausea, vomiting, diarrhea and a sore mouth.[6]

Having in mind that some of the side effects of these and other anticancer agents are serious to a significant extent, researchers started to look for a drug delivery systems that could decrease the cytotoxicity of the drugs in the healthy organs. By encapsulation of the cancer drugs, the interaction between the drug and the healthy tissue can be limited to a minimum level thus decreasing the detrimental effects. Drug delivery system can use passive targeting which utilizes the enhanced permeability retention effect (EPR). EPR is present in tumor tissues. Retention and accumulation for macromolecules is highly improved. This is possible because of the leaky vasculature in tumors tissues allowing the accumulation of macromolecules while the small molecules are not retained due to diffusion (figure 3).[7]

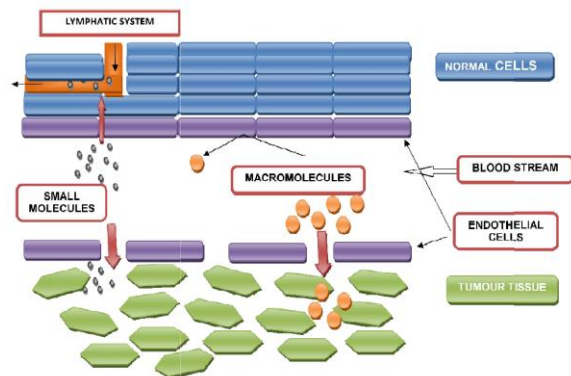


Figure 3 Structure of normal cell tissue and tumor tissue showing the leaky vasculature

In order to dissolve paclitaxel into the blood which is constituted of plasma (water, proteins, glucose, mineral ions and carbon dioxide) and blood cells, a surfactant is required in order to make them more soluble. [8] The surfactants contain long alkyl chain which gives them lipophilic properties and a hydrophilic group.

Alec D. Bangham using negatively stained phospholipids while testing a new electron microscope noticed lamellar concentric circles that turned out to be from the phospholipids.[9] This is considered to be the start of the liposomes. In the later years, research scientists started to try to utilize liposomes as drug delivery systems due to the convenience with which the liposomes can be prepared, as well as the capability to incorporate both hydrophobic and hydrophilic drug molecules.

Liposomes present a lipid bilayer in the shape of a sphere. Hydrophilic molecules can be trapped within the

aqueous core while the hydrophobic can be incorporated into the bilayer (figure 4).[10]

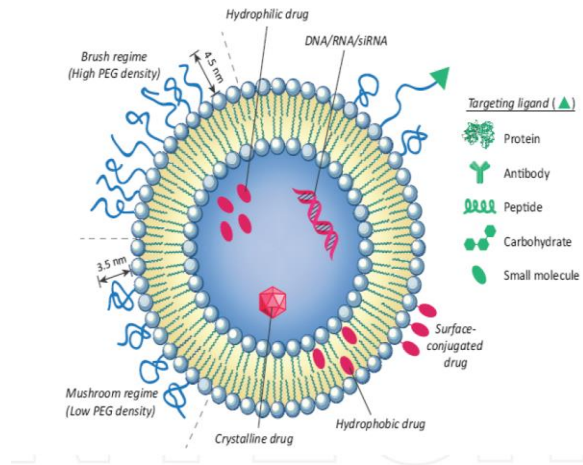


Figure 4 Structure of Liposomes.[9] Hydrophilic molecules such as small molecules, DNA, RNA and siRNA are in the aqueous core while hydrophobic agents are incorporated within the lipid bilayer. The outside of the lipid bilayer can have polyethylene glycol (PEG) as well as targeting ligands in the form of proteins, antibody, peptide, carbohydrate and small molecules.

Table 1 List of approved liposomal formulations[11].

Drug	Product name	Type	Lipid composition	Route of administration	Approved treatment
Amphotericin B	Ambisome	Liposome	HSPC, DSPG and cholesterol	Intravenous	Sever fungal infections
Doxorubicin	Myocet	Liposome	EPC and cholesterol	Intravenous	Metastatic breast cancer
	Doxil	PEGylated liposome	HSPC, cholesterol and DSPE-PEG ₂₀₀₀	Intravenous	Kaposi's sarcoma, ovarian and breast cancer
	Lipo-dox	PEGylated liposome	DSPC, cholesterol and DSPE-PEG ₂₀₀₀	Intravenous	Kaposi's sarcoma, ovarian and breast cancer
Daunorubicin	DaunoXome	Liposome	DSPC and cholesterol	Intravenous	Blood cancer
Verteporfin	Visudyne	Liposome	EPG and DMPC	Intravenous	Age-related molecular degeneration
Cytarabine	Depocyt	Liposome	DOPC, DPPG, cholesterol and triolein	Spinal	Neoplastic meningitis and lymphomatous meningitis
Morphine sulfate	DepoDur	Liposome	DOPC, DPPG, cholesterol and triolein	Epidural	Pain
Vincristine sulfate	Marqibo	Liposome	Egg sphingomyelin and cholesterol	Intravenous	Acute lymphoblastic leukemia

Table 2 Different liposomal formulations in clinical trials[11]

Drug	Product name	Lipid composition	Route of administration	Treatment under investigation	Trial phase
Paclitaxel	LEP-ETU	DOPC, cholesterol and cardiolipin	Intravenous	Ovarian, breast and lung cancers	I
	EndoTAG-1	DOTAP and DOPC	Intravenous	Anti-angiogenesis, breast and pancreatic caners	II
Doxorubicin	ThermoDox	DPPC, MSPC and DSPE-PEG ₂₀₀₀	Intravenous	Non-resectable hepatocellular carcinoma	III
Cisplatin and its analog	SPI-077	HSPC, cholesterol and DSPE-mPEG	Intravenous	Lung, head and neck cancers	I/II
	Lipoplatin	SPC, DPPG, cholesterol and DSPE-mPEG	Intravenous	Pancreatic cancer, head and neck cancer, mesothelioma, breast cancer, gastric cancer and non-small-cell lung cancer.	III
	Aroplatin	DMPC and DMPG	Intravenous/ intravenous	Malignant pleural mesothelioma and advanced colorectal carcinoma	II
Mitoxantrone	LEM-ETU	DOPC, cholesterol and cardiolipin	Intravenous	Leukemia, breast, stomach, liver and ovarian cancers	I
Topotecan	INX-0076	Egg sphingomyelin and cholesterol	Intravenous	Advanced solid tumors	I
Vinorelbine	INX-0125	Egg sphingomyelin and cholesterol	Intravenous	Breast, colon and lung cancers	I
Lurtotecan	OSI-211	HSPC and cholesterol	Intravenous	Ovarian, head and neck cancers	II
Amikacin	Arikace	DPPC and cholesterol	Inhaled as aerosol	Lung infection	III
BLP25 lipopeptide	Stimuvax	Monophosphoryl lipid A, cholesterol, DMPG and DPPC	Subcutaneous	Non-small-cell lung carcinoma	III
All-trans retinoic acid	Atragen	DMPC and soybean oil	Intravenous	Advanced renal cell carcinoma	I/II
Annamycin	Liposome-annamycin	DSPC, DSPG and tween	Intravenous	Breast cancer	I/II
Cytarabine and daunorubicin	CPX-351	DSPC, DSPG and cholesterol	Intravenous	Acute myeloid leukemia	II
Irinotecan HCL and floxuridine	CPX-1	DSPC, DSPG and cholesterol	Intravenous	Colorectal cancer	II

A proof that liposomes are conventional as drug delivery systems is the number of already available FDA approved and drug systems in clinical trials (tables 1 and 2).[11]

Preparation of liposomes and their usage as drug delivery vehicles

So far numerous different procedures for preparation of liposomes have been developed like sonication, extrusion, freeze-thawing, microemulsification and membrane extrusion. These methods can be divided in two groups, a “top-down” and “bottom-up” approach. Their preparation is important as it shows why they are convenient and the diversity in formulations.[12]

For the “bottom-up” approach, one of the techniques that can be used is freeze-thawing. This procedure is executed by first creating the SUVs from adding powdered lipids in organic solvent after which with evaporation of the solvent lipid films are formed. By hydration of the films SUVs can be obtained. The following step is the cycle of quick freezing (several cycles) of SUV (small unilamellar vesicles with size smaller than 100 nm). With this the SUV are fused together into LUV (large unilamellar vesicles, with size ranging between 100 nm and 500 nm). This

technique requires high concentration of phospholipids and the incorporation of the drug ranges between 20 % and 30%.[12]

Alternatively, solution-derived liposomes can be obtained. The liposomal vesicles are produced by dissolving phospholipids in ether/methanol or only ether, followed by evaporation of the solvent under vacuum. This leads to heterogeneous population, where the size of the vesicles ranges from 70 nm to 200 nm. A second way to produce the liposomes is by dissolving the phospholipids into ethanol which is then injected into excess amount of buffer. Again, the population is heterogeneous ranging from 30 nm to 110 nm. Beside these two techniques also it is possible to use reverse phase evaporation for which the solution of phospholipids is added to an aqueous buffer solution in which the evaporation of the organic phase leads to the formation of inverted micelles. Further evaporation leads to formation of gel. The extra phospholipids, who haven't formed or whose inverted micelle is disrupted are forming the second layer of the bilayer. Encapsulating the desired drug molecule is simple as adding it to the water phase, yielding up to 65 % of encapsulation. With this MLV (multilamellar vesicles) are obtained.[12]

The following step after obtaining the MLV is the “top-down” approach where the unilamellar vesicles with desired size can be obtained. Sonication is possibly the most used method for this kind of

preparation of SUV/LUV from MLV. The tube with MLV is put into a water bath sonicator which allows for control of the temperature. The negative side of this method is the possibility of low encapsulation of the drug, possible degradation of the phospholipids as well as degradation of the drug and the presence of MLVs in addition to the wanted SUVs. On the other side this procedure can be done in a sterile vessel thus protecting the starting material and the control of temperature is easy. Another “top-down” method is extrusion. It is performed by extruding the MLV through a small opening. The SUV obtained by this method are larger than the ones obtained by sonication but this method can't reach high temperatures as well as that the working volume is limited to 50 mL maximum.[12]

In 1995 FDA approved the first drug for cancer treatment that employed liposomes as drug delivery vehicles is Doxil[®] (table 1). Doxil[®] is a pegylated liposomal formulation in which the drug is loaded via transmembrane ammonium sulphate gradient. The composition of the liposomal formulation is hydrogenated soybean phosphatidylcholine (HSPC), cholesterol and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000] (DSPE-PEG₂₀₀₀) with a ratio of 3:1:1. High-sensitivity differential scanning calorimetry measurements were performed on Doxil[®]. The results showed lack of membrane-drug

interactions as well as no degradation due to temperature (up to 90 °C) and a low leakage of the drugs from the liposomes (less than 5 %). [12,13]

In 1997 Sharma *et al.* published a paper where the emphasis was on developing liposome formulation for drug delivery of PAX in order to decrease the negative effects of the drug. The first liposomal formulation (named ETL) was from a single phospholipid, egg PC with PAX: phospholipid molar ratio of 1:33. The second one (named TTL) employs 3 different phospholipids: di-elaidoyl PC, di-myristoyl PC and 1-stearyl-1, 2,-caproyl with ratio of 1:1:0.9. This formulation also had the same ratio in relation to paclitaxel. The liposomal formulations as well as the free paclitaxel showed a significant decrease in growth of ovarian tumors at the same dosage level. For the cancer to grow to 1000 mm³ with 10mg/kg dose by using Taxol, 77 days were required. With TTL-lyo, 75 days were required as for the ETL-lyo 79 days. The best results were obtained not by the formulations which were done by freeze-drying (lyophilisation) but from the TTL formulation from aqueous suspension (table 3).[15]

Table 3 ¹Dosage on 4, 6, 8 and 10 days after tumor injection. ² Required time for tumor to reach 1000 mm³. ³ Cure, defined as tumor not being palpable for a period of time. TTL-lyophilisated powder (TTL-lyo), TTL-liquid suspension (TTL-liq), ETL-lyophilisated[15]

Treatment	Dosage ¹ (mg/kg/inj.)	Total dose (mg/kg)	Time ² (days)	Cure ³	Number of mice
Taxol	5	20	45	0	7
	10	40	77	1	15
	25	100	>120	5	15
TTL-liq	10	40	>120	4	8
	25	100	>120	5	8
	5	20	43	1	7
TTL-lyo	10	40	75	1	15
	25	100	>120	5	15
	5	20	40	1	7
ETL-lyo	10	40	79	2	15
	25	100	>120	6	15
	0	0	29	0	13
Saline	0	0	30	0	13
Diluent 12	0	0	32	0	13
Plain liposomes	0	0	32	0	13

Although all treatments showed comparable efficacy the liposome formulations displayed a lower lethal toxicity for CDF1 mice in comparison to the free drug (table 4). In all 3 cases, for LD₁₀ (dose needed to kill 10 % of the test population), LD₅₀ (dose needed to kill 50 % of the test population) and LD₉₀ (dose needed to kill 90 % of the test population) the liposomal formulation had higher values for the lethal dosage. Also for BALB/c mice the liposomal formulations had lower toxicity. This study shows that one liposomal drug formulation can be used for more than one type of mice without the need to optimize the liposome formulation. In order to explore the cytotoxicity and negative effects, 200 mg/kg cumulative dosage was given to sample pools of mice. In the case of the TTL formulation 60 % of the mice died while for the ETL formulation 80 % of the mice died. This proves that although the lethal dosage of liposomal formulations is higher, it is still required to optimize the dosage in order to prevent any the negative effects that

might even result in death of patients.[15]

Table 4 Acute toxicity in female CDF1 Mice. ¹Diluent12 (Cremophor containing 50 % absolute ethanol) with an equal dose to that of Taxol[15]

Treatment	Paclitaxel dose (mg/kg)		
	LD ₁₀	LD ₅₀	LD ₉₀
Diluent 12 ¹	50	80	120
Taxol	8	16	33
TTL-liq	25	69	190
ETL-lyo	25	69	190

Suk *et al.* produced DOPE-PEG2000 liposomes with average size around 176 nm with dispersity less than 0.4. Also they investigated the effects of different concentration ratios of DOPE-PEG2000: oleic acid. In cases where the concentration of DOPE-PEG2000 was high, aggregation of the liposomes was noticed. In order to be certain that the liposomes were stable; a 30 days trial was conducted on the size of the liposomes at different ratios (figure 5). The trials proved that the liposomes were rather stable although a slight increase in the size of the liposomes was noticed.[16]

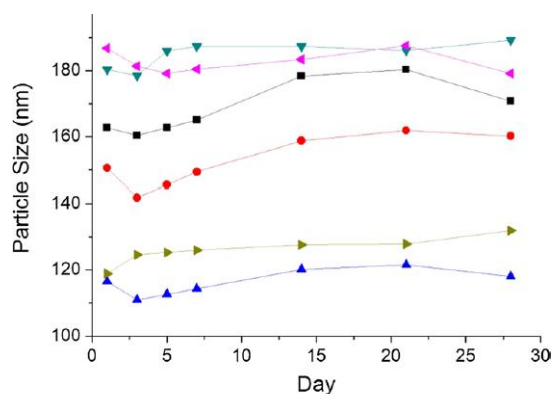


Figure 5 Particle size of DOPE-PEG2000:Oleic acid liposomes with ratio: a) 0:1 (filled squares), 0.1:1 (filled red circles), 0.02:1 (filled blue triangles), 0.04:1 (filled purple left angled triangles) and 0.005:1 (filled green right angled triangles)

angled triangles) for 30 days incubation in pH 8.5 at 30 °C[16]

The efficiency of encapsulating doxorubicin with this formulation was over 60 %. In vitro release study showed the function of DOPE-PEG2000 is the enhancement of permeability of liposome bilayer.[17] The optimal ratio for drug release was DOPE-PEG2000:oleic acid of 0.01:1. With this ratio, doxorubicin was slowly released where only 20 % of the encapsulated drug was released leading to a possible drug delivery system. [16]

Studies have shown that also the liposomes can be used in conjunction with high intensity focused ultrasound (HIFU). HIFU works by focusing ultrasounds and in the focal point the energy can be utilized in the form of thermal or mechanical effects. A study done by VanOsdol *et al.* showed that a liposomal drug delivery system is suitable for this. They devised two liposomal formulations. The first one was made of DPPC, MSCP and DSPE-mPEG2000 (with molar ratio of 85.3:9.7:5.0) named LTSL and the second one named NTLS; made from DPPC, cholesterol, DSPE-mPEG2000 (58.1:36.8:5.07). NTSL represents non-thermosensitive liposomes while the LTSL is low temperature sensitive liposomes.[18] In LTSL the DPPC (dipalmitoylphosphatidylcholine) has the main role in forming the lipid bilayer and the thermal sensitivity originates from this phospholipid having a melting transition of approximately 41.5 °C. MSCP (monopalmitoylphosphatidyl-

choline) can further affect the temperature at which the liposomes are affected and increase in percentage in the lipid bilayer leads to higher permeability and increase in drug release speed, while 4 mol % increases in DSPE-PEG2000 raises the melting temperature for 1 °C.[17] VanOsdol *et al.* loaded the formulations with doxorubicin and went on to investigate the release and the effect of the doxorubicin on tumor tissue in athymic nude mice bearing C26 adenocarcinoma. Also the liposomes have been further modified and were named ENTSL (echogenic encapsulated NTSL) and ELTSL (echogenic encapsulated LTSL). This was done by adding perfluoropentane (PFP, also used as contrast agent) which due to Laplace pressure creates a gas nanobubble form (figure 6). The combination of HIFU with the liposomal formulations ENTSL and ELTSL showed the most promising results. Furthermore, all four different types of liposomes had somewhat similar average size (table 5).[18]

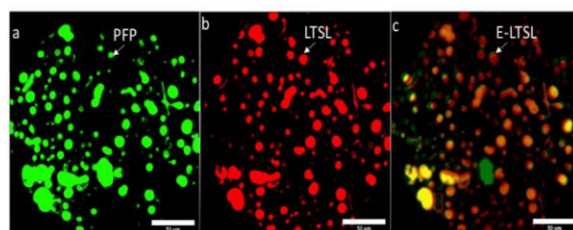


Figure 6 Confocal microscopy showing PFP(green encapsulated in the lipid bilayer(red). E-LTSL image shows that PFP is contained in LTSL. [17]

Table 5 Size, dispersity and zeta potential of liposomes [17]

Liposome	Size (nm)		Polydispersity		Zeta potential	
	Mean	SEM	Mean	SEM	Mean	SEM
NTSL	190.1	1.9	0.10	0.01	-26.7	3.6
ENTSL	193.8	1.2	0.11	0.04	-43.8	0.6
LTSL	176.8	0.5	0.11	0.01	-48.0	1.7
ELTSL	171.6	0.5	0.12	0.01	-28.3	1.1

The research also focused on the temperature required to obtain a high percentage of release of doxorubicin from the liposomes.

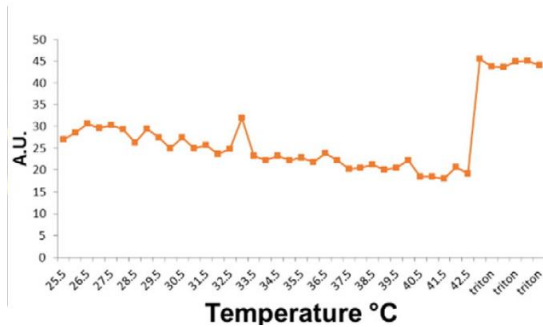


Figure 7 Thermal scan for ELTSL loaded with PKH labeled PFP. The baseline for fluorescence is from LTSL [17]

Figure 7 and 8 shows that the release of doxorubicin is around 40 °C for PBS and for FBS showing that at different buffer solution with different growing cell rate the properties of the liposomes aren't changed drastically but the release of doxorubicin is affected. For PBS at 40 °C the release of doxorubicin from ELTSL and ENTSL was roughly 20 % while in 10 % FBS the release of doxorubicin was approximately 90 %.

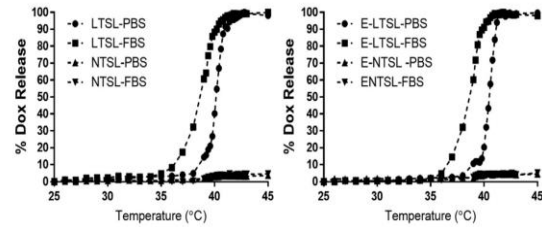


Figure 8 Release percentage of DOX in PBS and FBS as a function of temperature [17]

Then again, the ratio of DOX in tumor adjoining muscles heated with HIFU and unheated contralateral muscles for the echogenic (ELTSL and ENTSL) liposomal formulation is similar to that of the non-echogenic (LTSL and NTSL) (figure 9). [18]

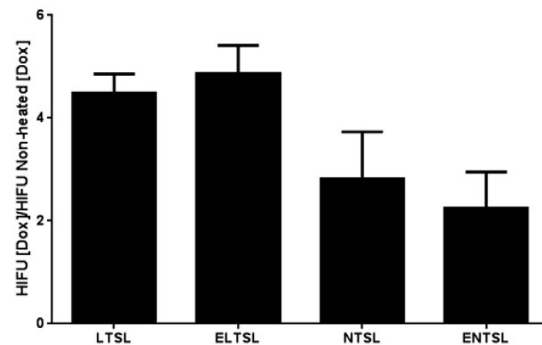


Figure 9 Ratio of doxorubicin in tumor adjoining muscle heated with HIFU and unheated contralateral muscle for different liposomal formulations

Most of these researches are done on tumors with high EPR but some tumors can have low EPR.

Theek *et al.* explored this topic in hope of finding a possible way as how to improve accumulation of liposomes in high cellular A431 epidermoid xenograft and highly stromal BxPC-3 pancreatic carcinoma xenografts. Both types of cancer have low EPR and present a good opportunity to investigate the possible ways to improve accumulation.

Sonoporation is a combination of ultrasound and microbubbles in order to increase the accumulation of the liposomes. Microbubbles are used in order to increase the permeabilization of the cell membranes as well as opening tight junctions in vascular endothelium through stable and inertial cavitation effects as microstreaming, jet formation and shock waves. For this, two different microbubbles were used in order to investigate the ways in which they affect the accumulation of the liposomes. The commercial MM (MicroMarker®) which has a soft phospholipid shell roughly 3 nm thick and are 1-6 µm in diameter and PBCA (Poly butyl cyanoacrylate) which has a smaller diameter and a hard shell with approximately 50 nm thickness. The sonoporation was done for 10 min. [19]

Table 6 Increase in fluorescent signal after sonoporation in comparison to before sonoporation with standard deviation in the brackets. [19]

Tumor model	A431		BxPC-3	
	PBCA	MM	PBCA	MM
4 h	+48 (±55) %	+113 (±54) %	+57 (±55) %	+56 (±50) %
24 h	+87 (±31) %	+63 (±60) %	+26 (±56) %	+54 (±22) %
48 h	+33 (±65) %	+90 (±51) %	+17 (±54) %	+33 (±40) %

Overall, the experiments showed that for two different models as well as for the two microbubble formulations with different properties, an increase in accumulation of liposomes in sonoporated tumor tissue was noticed in comparison to the untreated tumor tissue but the results vary due to

variability in EPR and tumor model (table 6).[19]

In order to prolong the circulating time of liposomes in the blood, Ishida *et al.* developed a method for producing a sterically stabilized immunoliposomal drugs by adding DSPE-mPEG2000 which prolonged the circulation time. By incorporating anti-CD19 into the bilayer, the binding of the liposomes to CD19⁺ human B lymphoma cell was three fold increased thus showing targeting of tumor tissue.[20]

Liposomes present a convenient way to introduce cancer drugs but they still can have some issues. Their functionalization sometimes can present a problem due to the fact it might require several chemical steps to change the surface of the lipid bilayer by introducing ligands, antibodies and proteins.[19, 20] The functionalization of the bilayer to some degree can be considered a drawback, so researchers started looking for alternate and more advanced drug delivery systems.

Isolation of Exosomes and their usage as drug delivery vehicles

One more advanced system of liposomes is exosomes. Exosomes have the same type of bilayer as liposomes. The outer surface of the bilayer contains

the same ligands, proteins and antibodies as the vesicles are derived from different types of cells from the human body. The positive thing about this is that the constituents on the surface of the bilayer of the exosomes are the same as the cell from which it originates meaning that it can hone in on the tumor cells from which it is derived. [23]

Isolation of the exosomes can be done by ultracentrifugation (figure 10). This technique is based on the fact that different constituents will sediment differently thus allowing for the extraction of purified exosomes. One of the characteristics of this method is its long term affordability requiring only an ultracentrifuge machine. Furthermore, it requires expertise in handling the equipment as well as cleaning step in order to rid of the larger bioparticles. There are two possibilities of how to execute this type of isolation. One is by differential ultracentrifugation. As a prework for this isolation of exosomes with this method, sometimes protease inhibitors are added whose role is to decrease degradation of exosomal proteins. The second way, density gradient ultracentrifugation utilizes a medium which contains different gradients of the solvents. Isolation here occurs on size, weight and density of exosomes. Unfortunately, the negative side of the second method is that it has lower processing capacity.[24] Market analysis shows that this procedure is used by more than 56 % of researchers in the field.[25]

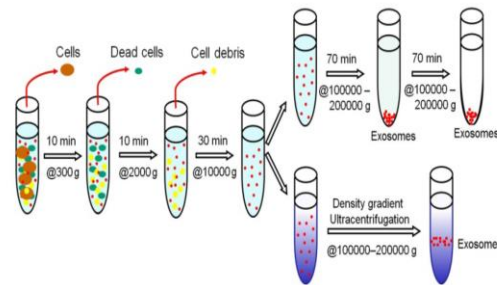


Figure 10 Steps in differential ultracentrifugation.[22] The first step is at 300g in order to remove cells. By increasing the centrifugal force to 2000g, 10000g or higher different constituents can be removed until only the exosomes are left as sediment.

Another way to isolate exosomes is to use segregation by size exclusion. This method uses membrane filtration which is based either on the exosome's size or weight and the need for sophisticated, specialized equipment is not necessary. Some companies have even developed a kit for ultrafiltration of exosomes. This kit is only appropriate to use for cell-free samples like urine, serum and cerebrospinal fluid.[22, 23]

Vykoukal and coworkers have developed a sequential filtration, which can be used to isolate exosomes. The procedure consists of 3 steps. The first step is using a normal filtration membrane with pore size of 100 nm. This membrane is to get rid of all the larger particles in the solution. Second step uses 500 kDa molecular weight filters that keeps the exosomes in the solution but lets smaller molecular weight components to pass thus making the solution more concentrated. For the last step a 100 nm track-etch filter is used after which only the exosomes, with smaller size than 100 nm are collected (figure 11).[26]

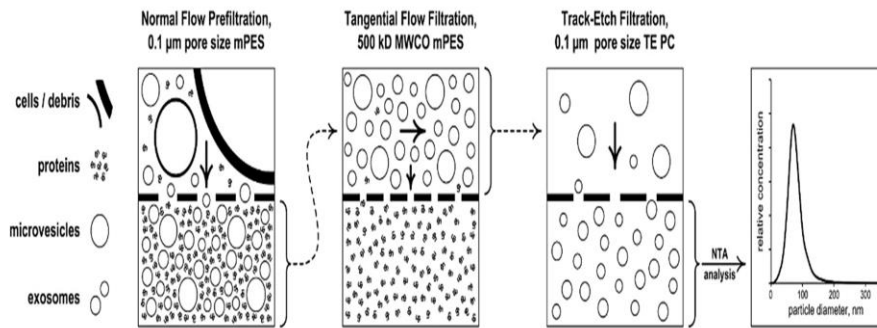


Figure 11 Schematic of 3 step procedure for sequential filtration [26]

A third procedure is to use size exclusion chromatography where a stationary porous phase can absorb molecules and particles with smaller diameter than the pores of the stationary phase. The molecules with higher hydrodynamic radii are eluted faster than the molecules with smaller hydrodynamic radii. [24]

Exosomes can be precipitated from the biological fluids by adding polyethylene glycol (PEG). It works by binding the water molecules and forcing the less soluble sample components to precipitate which can be collected by low-speed centrifugation or filtration. The drawback of this method is parallel co-precipitation of contaminants in the form of proteins and other debris. [22, 25]

Due to the fact the exosomes are cell derived entities the bilayers contain a multitude of proteins and receptors (figure 12). Immunoaffinity capture-based techniques utilize this, and use the interaction between the proteins and their antibodies as well as the receptor-ligand interactions.

For isolation of exosomes from serum, urine or plasma, ELISA can be employed. It stands for enzyme-linked immunosorbent assay. Also ELISA can be used to obtain exosome counts in the sample after careful calibration. Even though there are many techniques for isolation of exosomes, immunoaffinity was proven superior to ultracentrifugation. As a comparison, the extracted RNA from exosomes using immunoaffinity required only 400 μL of sample while ultracentrifugation requires 6-fold higher amount of sample. [24]

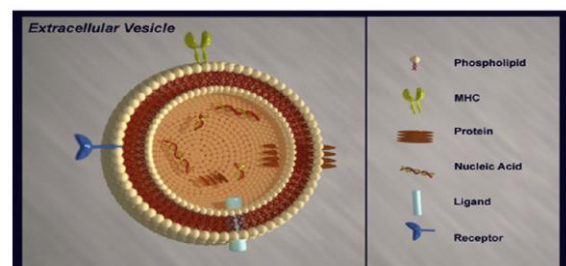


Figure 10 Exosome and its surface [28]

Yang *et al.* established primary brain cancer in zebrafish (*Danio rerio*) using U-87 MG cells administered by xenotransplantation to embryos two days post fertilization (dpf). At 4 dpf the embryos were treated with doxorubicin

in PBS, doxorubicin in exosomes derived from bEND.3 or phosphate buffered saline (PBS). High level of protein CD63 allowed bEND.3 derived exosomes to cross the BBB to deliver fluorescent Rhodamine 123 (red) (figure 13) as well as the cancer drugs. Experiments were conducted on the penetration into the brain tissue with fluorescent DOX and PAX loaded exosomes in comparison to only the cancer drug.[29]

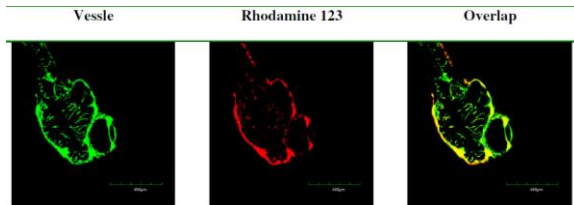


Figure 11 In vivo imaging of delivered rhodamine 123 and vessel as well as overlap of both shows that rhodamine 123 spreads through the vessel in the case where exosomes are not used. [29]

The in vivo images showed that the amount of drug in the vasculature was decreased while the drug concentration was increased in the tumor tissue (figure 14), showing that the exosomes with CD63 were efficient in reaching the target tumor tissue.

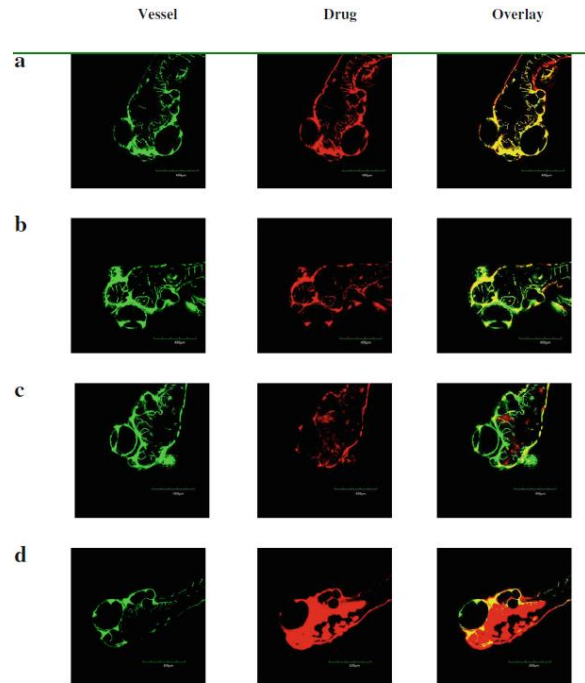


Figure 12 In vivo brain imaging of anticancer drugs in embryos. Doxorubicin (a) and fluorescence labelled paclitaxel (b) without exosomes. Doxorubicin (c) and paclitaxel (d) injected with exosomes from brain endothelial bEND.3 cell [27] The images here, clearly show that using DOX and PAX without exosomes leads to the drugs being in the vessels (a and b) while with exosomes the overlap shows that only a small amount of the drugs in the circulatory system and the rest is in the tumor tissue.

Figure 14 (a) and (b) shows a high overlap between the vessel and the drug while leading to the conclusion that the drug is in vasculature; in (c) and (d) the overlap is a lot smaller meaning the exosomes target the cancer cells and leave the vasculature thus decreasing their detrimental effects.[29]

Jang *et al.* developed exosome-mimetic nanovesicles by extrusion of U937 monocytic cells and Raw264.7 macrophages in order to investigate the targeting ability of the nanovesicles for cancer treatment. Tumor tissue expresses ICAM-1 in significantly higher

amount in comparison to healthy tissue (figure 15). Addition of antibody for ICAM-1 is important in delivering the doxorubicin to the tumor tissue.[30] Although these don't represent the classical exosomes they could still be considered as exosomes since they are derived from cells and have the same lipid bilayer as the cells of origin.

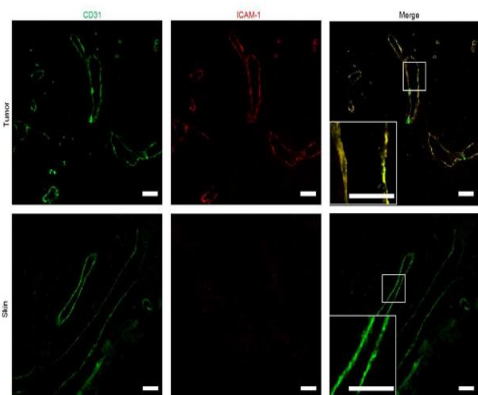


Figure 13 Green fluorescence signal of CD31 and red fluorescence signal of ICAM-1

Treatment with 60 μg free doxorubicin showed similar antitumor effect in comparison to $\text{Raw264.7.NV}_{\text{dox}}$ (Raw264.7 derived nanovesicles loaded with doxorubicin) but free doxorubicin also had detrimental impact on the white blood cell count as well as body weight. Furthermore, a comparison study on efficiency of nanovesicles and exosomes was conducted; both nanovesicles and exosomes showed similar performances but the upper hand of the nanovesicles is the 100-fold higher production than exosomes.[30]

Study on prostate cancer done by H. Saari *et al.* used paclitaxel as cancer treatment drug while the exosomes were derived from two prostate cancer cells

lines, PC-3 and LNCaP. Trypsin treatment of 20 kDa MV (Microvesicle with molecular weight of 20 kDa) and 110 kDa Exo (Exosomes with molecular weight of 110 kDa) didn't damage the vesicles while it removed the proteins from the lipid bilayer showing that further treatment of exosomes is possible without suffering any damage or efficiency. Although this might seem contradictory due to the fact that scientists are interested in the already bound ligands on the exosomes, this study shows that even after losing the ligands exosomes retain their function and can be used as drug delivery system. The best results were obtained by low concentration of the exosomes with high amount of paclitaxel encapsulated. Both, 20 kDa MV and 110 kDa Exo formulations showed similar effectiveness in increasing the cytotoxicity of paclitaxel. The mechanism by which they delivered the drug is different than non-encapsulated paclitaxel. While free paclitaxel is taken in from the surroundings of the cell, the exosome formulation released paclitaxel from within the cell. It can be concluded from the uptake mechanism that 110 kDa Exo and 20 kDa MV use is a safer way to deliver the drug as PAX isn't in contact with the surrounding healthy tissue. [31]

Experiments were done on the efficiency of doxorubicin using exosomes in relation to PC:Chol liposomes. The doxorubicin was spontaneously incorporated into the exosomes using high concentration (250 $\mu\text{g}/\text{mL}$). The

exosomes and liposomes are immediately taken by the liver and spleen when they are administered via IV but when injected intratumorally the exosomes associated to the tumor to a greater extent than the liposomes. A high a dosage of exosomes (400 μ g) was administered intravenously to a mouse that resulted in death. Imaging done on its internal organs showed accumulation in the lungs thus running the risk in case of a high dosage of exosomes, that it might lead to death of the patient (figure 16). The study proved that exosomes remain associated with the tumor tissue to a longer extent in comparison to the liposomal formulation. This can be ascribed to the protein profile as well as the unique lipid composition of the exosomes.[32]

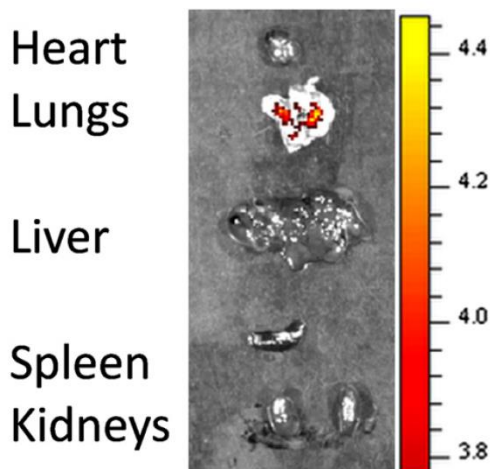


Figure 14 Organs of a Balb/c mouse injected with 400 μ g exosomes labeled with fluorescent tracer DIR [30]

Discussion

The usage of doxorubicin, paclitaxel and other cancer drugs can have rather harsh consequences on the human body. Due to these reasons, researchers started looking into drug delivery systems such as liposomes and exosomes. The need to utilize such systems comes from the necessity to decrease the negative effects of the cancer drugs on the healthy organs and to increase the cytotoxicity of the drug in the tumor tissue.

The amount of exosomes that can be obtained from isolated cells is approximately 0.1 μ g from million cells in 24h [33], although Jang *et al.* developed exosome-mimetic nano-vesicles from cells with 100 fold higher yield in comparison to the exosomes. In this context liposomes have the upper hand due to the fact that they have to possibility to be produced on a bigger, industrial scale. The convenience of liposomes is the tunability of the properties as a function of the composition of the bilayer. By controlling the amount of DOPE-PEG2000 in the bilayer it is possible to control the release of the cancer treatment drugs due to phospholipid bilayer permeability which results in longer exposing of the tumor tissue to the drugs. It is also possible to make them thermosensitive in which case HIFU can be used to activate the liposomes. [16,17] Furthermore, not only the composition of

the bilayer is tunable but also the surface of the bilayer can be tuned. By doing this it is possible to avoid liposomes being taken up by reticuloendothelial system (RES), whose macrophages and monocytes have the purpose to phagocytize bacteria, viruses and foreign particles in the tissues.[20] All surface modifications require several chemical steps which can be seen as somewhat of a drawback though the trade-off between couple of chemical steps for tuning of properties isn't a hefty price to pay as the possibilities to tune the properties are vast.

On the other hand, exosomes already have ligands on their surface. The type of ligands depends on the cells from which they are derived as they represent signalling agents and naturally associate with certain target cells. In this case the need for functionalization is not required. Clayton *et al.* worked on investigating the effects of CD-55, CD-59 proteins incorporated into the bilayers of the exosomes. The blocking of these two proteins lead to significant lysis which proves the reasoning that exosomes' circulation in the blood depends on the functional group present on the surface.[34] On the other hand, H. Saari *et al.* proved that even in the case when the ligands are removed from the surface it won't lead to damage of the extracellular vesicles (20 kDa MV and 110 kDa Exo) while still having an improvement to cytotoxicity of paclitaxel in tumor tissue.[31] This study is very interesting as it shows that if required the exosomes can be stripped from their

original ligands and different functional groups can be added. Although the two statements about the stability of the exosomes without the surface proteins and ligands are somewhat contradictory it can be explained rather easily. Clayton *et al.* and H. Saari did the experiments in vitro. After blocking CD-55 and CD-59 there was no lysis of the exosomes. Lysis was observed when antibody W6/32 was added to the human serum. [31, 34] The Clayton study not only does it show that the exosomes are stable without surface ligands but it also points out that results might vary depending whether they are conducted in vivo or in vitro.

Conclusion

Liposomes present a synthetic system while the exosomes are derived from human body. Both exosomes and liposomes have positive and negative side in relation to being used as drug delivery systems for cancer treatment. Liposomes have higher yield as it isn't required to isolate them from cells, also they are tunable which makes them a great candidate for targeted drug delivery. On the other hand exosomes don't need functionalization while it is still possible to do it; meaning if the exosomes are derived from tumor tissue then the exosomes would target those specific cells without additional chemical treatment. Since exosomes are derived

from cells they can be used in patient specific therapy (exosomes derived from the patient cells and administered to him/her only) while the liposomes formulations can be used for treating a bigger group of patients. Overall, the two systems present a very interesting possibility in cancer treatment with decreased negative side effects due to the drug but at the moment the liposomes have the upper hand. This can be proven by simply looking at the number of FDA approved drug formulation in comparison to the exosome formulations.

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