Liposomal Delivery Systems

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Contributor: Katarzyna Juszkiewicz

The versatility of liposomal carriers does not just simply rely on their capability to encapsulate various types of therapeutic substances, but also on the large array of components used for constructing liposome-based nanoformulations that allow for a straightforward control over targeting and the release of the encapsulated contents. This leads to a wide array of design strategies which can be easily adapted to any desired theraupetic effect, rendering liposomes one of the most promising systems for drug delivery.

Keywords: liposomes ; liposomal formulations ; drug delivery ; liposomal systems ; thermosensitive liposomes ; drug release ; triggered release ; pegylation

1. Three Main Features of Liposomal Delivery Systems

Liposomal systems can be characterized by three main features that influence their physicochemical properties, which have a direct impact on the pharmacokinetics and pharmacodynamics of the therapeutic compounds upon administration into the bloodstream:

- Lipid composition: The diversity and molar ratio of lipids present in the bilayer directly impact membrane fluidity, permeability, and surface charge, as well as the loading capacity of drugs.
- Drug loading and release: The nature of the encapsulated drug, which can be either hydrophilic or lipophilic. The inclusion of stimuli-sensitive lipids or other components allows for a triggered drug release under specific conditions.
- Targeting methods: Active targeting by the attachment of ligands/molecules on the vesicle surface, which are preferentially (or exclusively) recognizable by target cells/tissues, and passive targeting through usage of the enhanced permeability and retention effect (EPR) effect. The vast majority of liposomal drug formulations contain “PEGylated lipids” (lipids with attached polyethylene glycol (PEG) chains) that affect the clearance of liposomes.

One of the most well-known liposomal formulations available in clinical practice is Doxil, which was created to overcome the cardiotoxicity of doxorubicin and clearly shows reduced cytotoxicity when compared to the free drug. At the same time, Myocet, which is another liposomal formulation of doxorubicin, displays vastly different pharmacokinetics in comparison with Doxil, which may be partly due to the lack of PEGylated lipids in the liposomal shell. For those reasons, these two formulations are used in treatment of different types of cancer, despite encapsulating the same type of drug (see Table 1) [1].

Table 1. List of liposomal drug products for injection clinically approved by European Medicines Agency (EMA) and Food and Drug Administration (FDA).

Drug	Product Name	Route of Administration	Lipid Composition (Molar Ratio [1])	Treatment	Ref.
Amphotericin B	Abelcet	Intravenous	DMPC, DMPG (7:3)	Systemic fungal infections	[2]
	Ambisome	Intravenous	HSPC, DSPG, cholesterol (2:0.8:0.4)	Systemic fungal infections	[3]
Bupivacaine	Exparel	Supraperiosteal Injection	DEPC, DPPG, cholesterol, tricaprylin	Postsurgical local analgesia	[4]
	Nocita	Supraperiosteal Injection	DEPC, DPPG, cholesterol, tricaprylin	Postsurgical local analgesia (for dogs only)	[5]

Drug	Product Name	Route of Administration	Lipid Composition (Molar Ratio [1])	Treatment	Ref.
Cytarabine	Depocyt	Spinal	DOPC, DPPG, cholesterol, triolein (7:1:11:1)	Lymphomatous meningitis	[6]
Daunorubicin	DaunoXome	Intravenous	DSPC, cholesterol (2:1)	Kaposi's sarcoma	[7]
Doxorubicin	Doxil/Caelyx [2]	Intravenous	HSPC, cholesterol, DSPE-PEG (2000) (56:39:5)	Kaposi's sarcoma	[8]
	Lipodox	Intravenous	DSPC, cholesterol, DSPE-PEG (2000) (56:39:5)	Kaposi's sarcoma, ovarian/breast cancer	[9]
	Myocet liposomal [3]	Intravenous	EPC, cholesterol (55:45)	Metastatic breast cancer	[10]
Inactivated hepatitis A virus	Epaxal	Intramuscular	DOPC, DOPE (75:25)	Hepatitis A	[11]
Inactivated hemagglutinin of influenza virus strains A and B	Inflexal V	Intramuscular	DOPC, DOPE (75:25)	Influenza	[12]
Irinotecan	Onivyde	Intravenous	DSPC, MPEG-2000-DSPE	metastatic adenocarcinoma of the pancreas	[13]
Mifamurtide	Mepact [2]	Intravenous	POPC, DOPS (7:3)	High-grade non-metastatic osteosarcoma	[14]
Morphine sulfate	DepoDur	Epidural	DOPC, DPPG, cholesterol, triolein (7:1:11:1)	Pain management	[15]
Verteporfin	Visudyne	Intravenous	DMPC, EPG (5:3)	Age-related macular degeneration, pathologic myopia, ocular histoplasmosis	[16]
Vincristine sulfate	Marqibo	Intravenous	Sphingomyelin, cholesterol (6:4)	Acute lymphoblastic leukaemia	[17]

HSPC—hydrogenated soya bean phosphatidylcholine; DSPG—1,2-distearoyl-sn-glycero-3-phosphoglycerol; DEPC—1,2-dierucoyl-sn-glycero-3-phosphocholine; DSPE-PEG(2000)—1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000]; EPC—egg phosphatidylcholine; MPEG-2000-DSPE—1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[biotinyl(polyethylene glycol)-2000]; DOPS—1,2-dioleoyl-sn-glycero-3-phospho-L-serine; EPG—egg phosphatidylglycerol. [1] If available. [2] Outside the United States, Doxil is known as Caelyx. [3] These formulations are only approved by EMA and not by FDA.

2. Drug Loading and Release

There are many ways to encapsulate drugs into liposomes, but all these methods generally fall under one of two categories. Passive loading is carried out during the formation of the liposomes where either the dry lipid film is formed in the presence of a hydrophobic drug or the lipid film is rehydrated with the use of a hydrophilic drug solution. Unfortunately, the encapsulation efficacy of hydrophilic drugs is usually low. This method can also cause a rapid, uncontrolled release of entrapped contents from the liposomes [18]. Active loading often depends on either an ion or a pH gradient across the membrane of already preformed liposomes. The properties of an encapsulated drug make a major difference in their liposome-modulated bioavailability. For example, the release rate of the hydrophobic drug dibucaine is much lower than that of the hydrophilic 5-fluorouracil when encapsulated within multilamellar liposomes formed with egg phosphatidylcholine and cholesterol. This difference was further increased in the case of negatively charged liposomes where the hydrophobic drug release was inhibited due to the charge on the liposomal surface [19]. It is possible to achieve tighter control over the release of the liposomal cargo through the use of stimulus-responsive liposomes, which become metastable under certain conditions, such as pH, redox potential or temperature [20]. These aspects are unique to a disease condition and pathological state of tissues, because inflammation is always accompanied by local hyperthermia. For the design of thermosensitive liposomes lipids such as 1-myristoyl-2-palmitoyl-sn-glycero-3-phosphocholine (MPPC)

(Tm = 35 °C) and 1-myristoyl-2-stearoyl-sn-glycero-3-phosphocholine (MSPC) (Tm = 40 °C) are commonly used [21]. The Tm of these lipids is around the physiological temperature, rendering the lipid bilayer more permeable during the phase transition temperature, which occurs in the tissue in which the inflammation takes place [22]. Local hyperthermia can also be artificially induced by near-infrared (NIR) radiation that is able to penetrate into deep tissues. This was the case with thermosensitive liposomes carrying both the NIR-absorbing dye indocyanine green and the anticancer drug doxorubicin. This synergic solution allowed for the effective release of encapsulated contents into the cancer cells [23]. Yet another way of facilitating drug release from thermosensitive liposomes is with the use of radiofrequency ablation (RFA). This procedure involves high-frequency electrical pulses which pass through an electrode, creating a small region of heat in a selected area. A phase III clinical study was conducted on a combination of lyso-thermosensitive liposomal doxorubicin (ThermoDox) with RFA. ThermoDox contains a lysolipid monostearoyl-phosphatidylcholine (MoSPC) that forms defects above its Tm (40 °C) that aid the release of encapsulated contents [24]. An animal study was conducted in order to optimize heating time and then this combinational therapy was tested on patients with hepatocellular carcinoma, and was found to significantly improve their overall survival [25][26][27]. Chen et al. developed a thermoresponsive liposomal system for extracellular delivery of doxorubicin. Its key component, ammonium bicarbonate, which is used in generating a transmembrane gradient for the encapsulation of the drug, decomposes to carbon dioxide bubbles upon heating. This process generates defects in the lipid bilayer, leading to the quick release of the encapsulated doxorubicin. These liposomes are also more stable in blood plasma and have a longer circulation time when compared to lysolipid liposomes, such as ThermoDox [28][29]. In solid tumors, the intratumoral pH value is slightly lower than the pH of blood and surrounding tissues, which is taken into consideration when composing pH-sensitive liposomal formulations. Those liposomes enter the tumor tissue and quickly become destabilized while releasing the encapsulated contents. However, pH values differ in endosomes and in the tumoral environment. All these elements must be taken into account when designing a pH-sensitive liposomal formulation and choosing lipids with the desired Tm [30][31]. DOPE is the most popular choice as thanks to its cone shape it forms hexagonal phases. However, due to this, DOPE cannot form lipid bilayers by itself in neutral pH and requires the presence of weakly acidic amphiphilic lipids such as cholesteryl hemisuccinate or cylindrically shaped lipids such as PC [32][30][33]. Redox potential can also be used as a stimulus, as in the case of liposomes designed for the treatment of human osteosarcoma. The surface of liposomes was coated with chitooligosaccharides via disulfide bonds. The intracellular environment (especially in cancer tissues) is much more reductive than the extracellular environment. This means that liposomes did not show any unwanted drug leakage in physiological conditions but were destabilized by reducing agents such as dithiothreitol or glutathione [34].

3. Targeting and Clearance

Liposomes can be the subject of active and passive targeting. The latter depends on a phenomenon called the enhanced permeability and retention effect (EPR) in the environment of tumors. Rapid tumor vascularization leads to the formation of immature tumor vessels inside the tumoral mass characterized by high permeability, which leads to the accumulation of nanoparticles smaller than 150 nm which are able to cross these vessels. Moreover, due to the abrupt ending of these vessels, there is a lack of functional lymphatic drainage, so the clearance of any accumulated particles is hindered (Figure 1) [35]. The EPR effect does not occur only in tumors, as inflamed tissues are also characterized by enhanced vascular permeability, as for example in the case of rheumatoid arthritis. Jia et al. tested a liposomal formulation of the hydrophobic drug dexamethasone on an adjuvant-induced arthritis (AIA) rat model. While the free drug showed a decrease in inflammation, it also led rats to develop hyperglycemia. The liposomes seemed not to have such a strong side effect and also showed better accumulation in inflamed tissues [36]. Targeting via size is also effective when the target is a part of the RES. Particles in the range of 100 nm to 150 nm are preferentially taken up by phagocytes and accumulate in the liver [37]. Liposomes that extend beyond 150 nm are characterized by rapid uptake by the mononuclear phagocytic system (MPS), which matters in the treatment of such diseases as leukemia and rheumatoid arthritis [38].

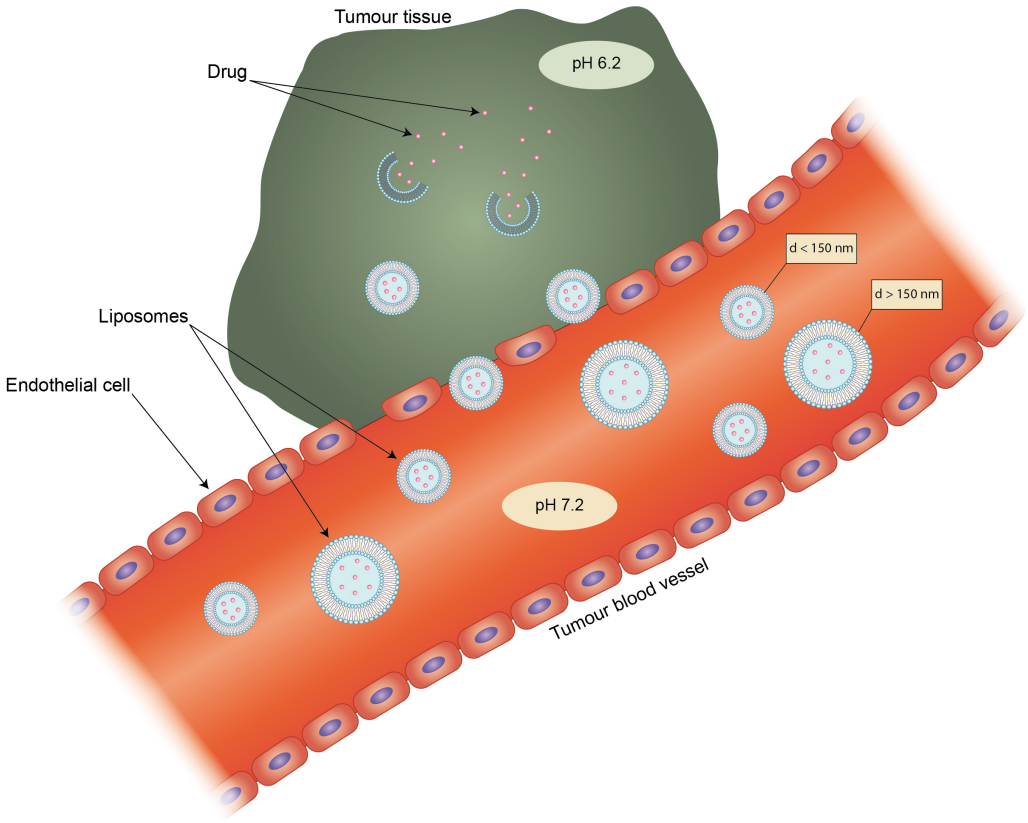

Figure 1. The complementary effect of enhanced permeability and retention effect (EPR) and pH sensitivity of liposomes. Only liposomes with a diameter (d) smaller than 150 nm are able to pass through leaky endothelium cells in blood capillaries into tumor tissue. Blind-ended tumor blood vessels lack lymphatic drainage, thus increasing the accumulation of liposomes.

Effective targeting is one of the major aims in designing an efficient liposomal formulation for drug delivery. Another important element to take into consideration is the level of clearance of liposomes. In order to reduce the MPS uptake, several physico-chemical properties of the bilayer that forms liposomes can be modified [39]. Denser packing of lipids in the bilayer means reduced absorption of opsonins, which can be achieved by the incorporation of cholesterol, as mentioned before. It must be noted that while smaller liposomes evade RES more easily, their aqueous internal compartment has a smaller volume, meaning less available space for hydrophilic drugs [40]. The blood circulation time of the liposomes decreases with increasing size of the particles and net charge density [41]. The rigidity of the membrane can be increased by incorporating lipids with high Tm, such as DSPC (Tm = 55 °C), which results in the reduction in the MPS uptake [33]. The most prevalent way of decreasing the uptake by the RES is grafting PEG chains to the liposomal surface. This happens due to the fact that PEG establishes a steric barrier on the liposomal surface, which reduces opsonization by the serum components in vivo, thus positively influencing pharmacokinetics of liposomes [39][42]. The conformation of PEG chains depends on their grafting density. At higher densities, PEG is found in a brush-like conformation, while mushroom-like PEGs dominate in lower densities (Figure 2a). These conformations yield different degrees of hydrophobic shielding, as this effect is greater in the case of brush-like PEG chains. Longer PEG chains provide better protection against plasma proteins than shorter ones [43][44]. For instance, Doxil consists of “stealth” liposomes, because PEGylation reduces their RES uptake, greatly increasing their circulation time [45]. On the other hand, repeated injections of PEGylated formulations may lead to the production of anti-PEG antibodies that absorb to the liposomal surface [46]. At the same time, some alternatives to PEG are also being tested so as to combat the problem of PEG immunogenicity. Some hydrophilic polymers (such as polyglycerols and polyoxazolines) and zwitterionic polymers have shown a comparable or improved results in producing stealth formulations[47].

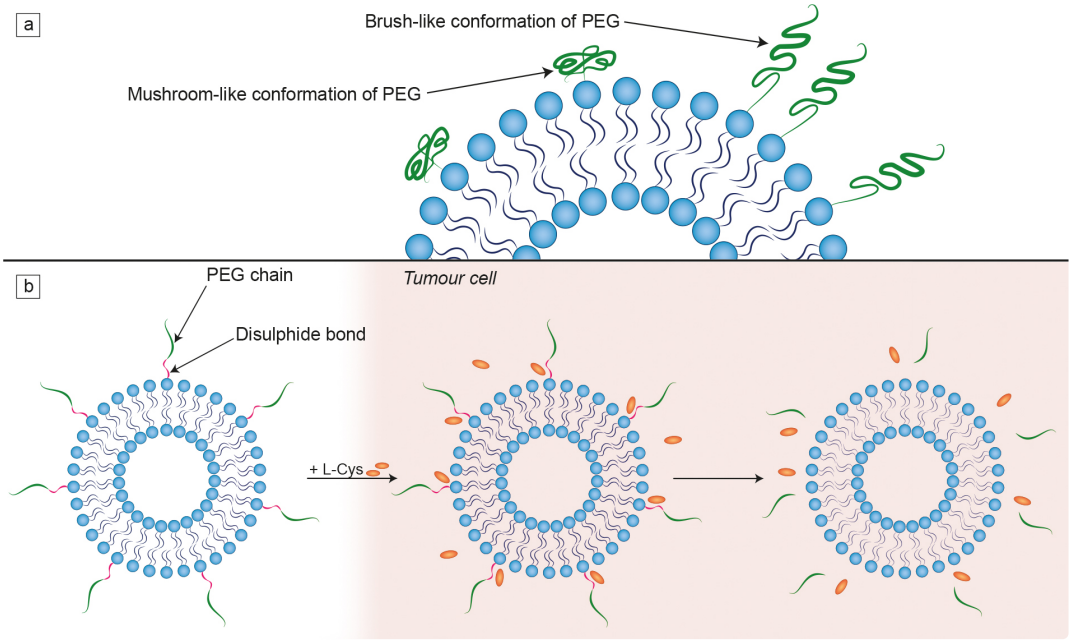

Figure 2. Characteristics of PEG chains on the liposomal surface. (**a**) The dependence of the PEG chain conformation on its surface density. When PEG chains are engrafted at a higher density, the brush-like conformation is favoured; (**b**) the mechanism of detachment of PEG chains from a liposome by the L-cysteine (L-Cys) that is found at an elevated concentration in tumor cells. This illustration was developed based on a procedure described in a study by Kuai et al. [48]

Nevertheless, the binding of opsonins to therapeutic nanoparticles can also work in favor of targeted delivery. Such is the case of the formulation designed by Zhang et al. for treatment of hereditary transthyretin-mediated (hATTR) amyloidosis. After administration into the bloodstream, liposomes (called “lipid nanoparticles”) are opsonized by apolipoprotein E and taken to the liver, where they bind to the surface of hepatocytes due to the presence of apolipoprotein E receptors. This phenomenon allows them to effectively deliver encapsulated siRNA into hepatocytes and silence a mutated version of the TTR gene, which was tested on patients suffering from hATTR amyloidosis[49].

Active targeting consists of conjugating various types of ligands to the liposomal surfaces such as antibodies, sugars, lectins and proteins, as reviewed by Toporkiewicz et al. [50]. Targeting agents may be bound directly to lipid anchors on the liposomal bilayer or attached by a linker such as PEG. The second option is preferred because adjacent PEG chains, which are included in the formulation for the RES evasion, may sterically inhibit the binding of ligands (if found closer on the liposome surface) to the target cells [51]. This is especially significant to antibodies, as those directly attached to the liposomal bilayer surface have their antigen binding abilities partly inhibited by PEG chains of a molecular weight of 5000 Da [52]. Moreover, PEG chains on the liposomal surface can prevent endosomal escape of a drug. It may both suppress electrostatic interactions required for effective cellular uptake and interfere with the fusion of the endosomal membranes with those of liposomes [53]. Thus, in order to overcome these limitations, a biochemical approach to detaching PEG from the lipid anchor under specific conditions was developed [20][54]. It happens by means of attachment of PEG chains to lipid anchors via a disulfide bond that is easily cleavable by exogenous L-Cys found in tumor tissue (Figure 2b) [48]. There are many other potential liposomal components sensitive to various stimuli—for instance, hydrazone bonds break in an acidic environment, which is characteristic for pathological tissues [55]. Attaching ligands without using PEG is possible using other types of linkages containing various groups binding, e.g., tagged recombinant proteins. For example, synthetic lipid 1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (DGS-NTA(Ni)) binds to polyhistidine tags. This was used in an experiment where a His-tagged p24 was bound to nanovesicles with surface-chelated nickel. The effectiveness of this liposomal formulation was confirmed both in vitro and in vivo in mouse models [56].

The liposomal bilayer provides a flexible platform for possible targeting. The ability of attaching multiple surface ligands allows for even more specific targeting [57]. It has been shown than both PEG chains and antibodies can be attached to form “stealth” immunoliposomes that both bind to the targeted ligands and evade RES uptake [58]. Nonetheless, such liposomes show reduced cellular uptake in comparison to non-PEGylated immunoliposomes. Some formulations take advantage of both active targeting and combined therapy. Lv et al. designed and tested a formulation consisting of lysolipid-containing thermosensitive liposomes. They contained both marimastat and paclitaxel, and had hyaluronic acid grafted on the surface, thus showing a strong affinity for CD44 receptors, which are overexpressed in cancer tissues. Marimastat is a strong inhibitor of metastasis, but it is not sufficient to eliminate cancer cells, so an effective cytostatic drug, paclitaxel, was also included. Those liposomes crossed into the tumor microenvironment successfully, releasing the encapsulated contents due to the local hyperthermia [59]. On the other hand, Lakkadwala and Singh grafted transferrin for

targeting to the liposomal surface but also used the cell-penetrating protein FVYLI (to increase cellular uptake) attached by the PEG chain to DSPE. These dual surface-functionalized liposomes were supposed to serve as a treatment for glioma, as they are able to cross the BBB. The successful drug release of both doxorubicin and erlotinib was tested on glioblastoma (U87) cells that served as an in vitro brain tumor model [60]. This is a large step towards the preparation of multifunctional nanocarriers for cancer therapy.

4. Individual Blocks That Make the Difference

One of the main reasons why liposomes are such an attractive drug delivery system is the modularity/interchangeability of their components. It is quite a simple task to replace just one or two components in order to generate a new formulation targeted towards different types of cells. This is why a graphical analogy to building blocks is used in Figure 3 where we summarize the results of our recent studies discussed below.

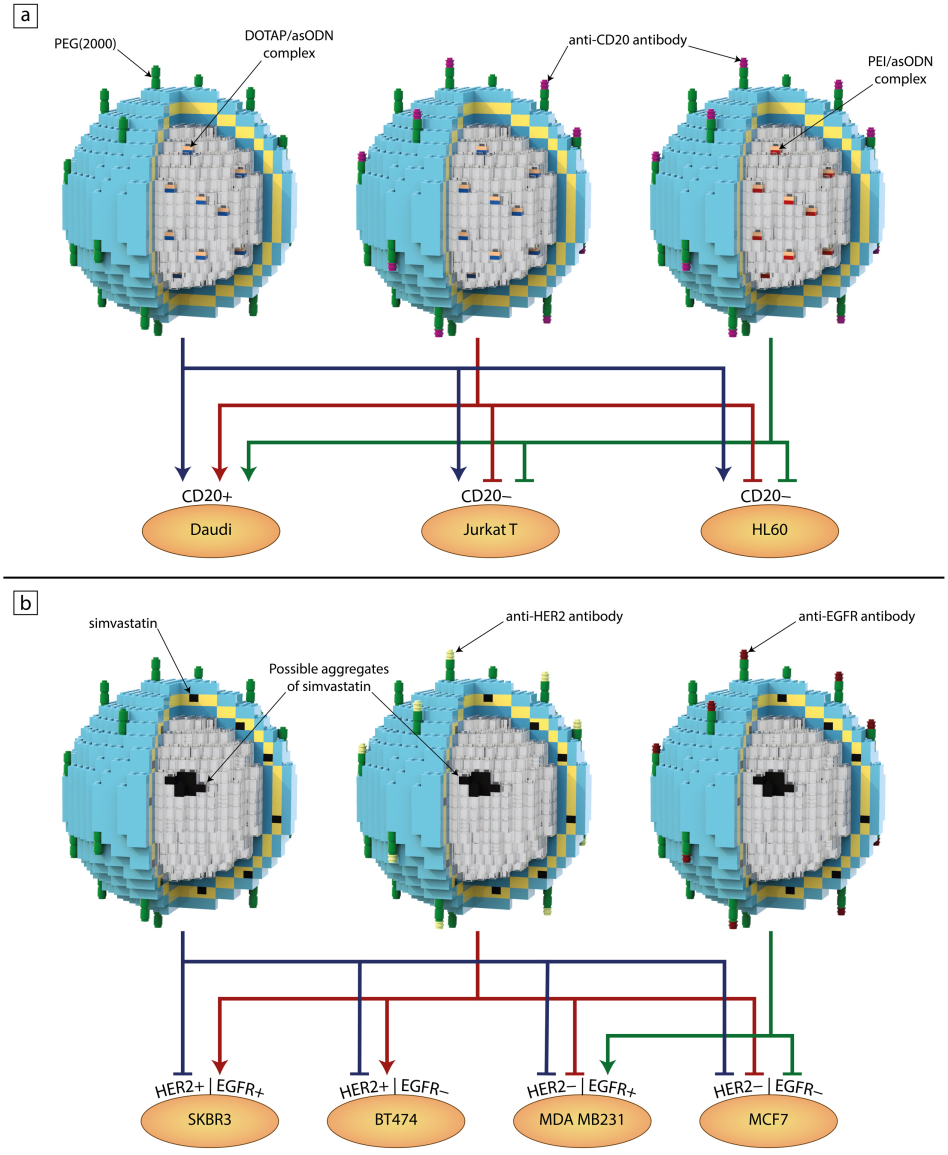

Figure 3. The interchangeability of individual blocks resulting in different or synergistic effects on individual cell lines after treatment with liposomal formulations containing antisense oligonucleotide (asODN) complexes (**a**) [61][62] and simvastatin (**b**) [63][64]. Arrows show a noticeable decrease in cell viability and/or interactions with cells, while blind-ended arrows indicate a lack of a significant effect after the addition of liposomes to the cell culture medium.

Wyrozumska et al. designed an untargeted liposome-coated lipoplex (L-cl) containing antisense oligonucleotides (asODNs) against the *BCL-2* gene. The liposomal bilayer consisted of hydrogenated egg phosphatidylcholine (HEPC), 3b-(N-[dimethylaminoethane]carbamoyl)cholesterol) (DC-Chol), DOPE and DSPE-PEG. The negatively charged asODN molecules were complexed with a positively charged lipid, DOTAP. In vitro tests proved that L-cl significantly decreased Bcl-2 protein expression in Jurkat T and Daudi cells. In addition, L-cl reduced the survival rates of Jurkat T, HL60, Daudi and white blood cells isolated from patients with acute myeloid leukemia. Liposomes accumulated in the livers and

spleens of NOD/SCID mice, in which Daudi Burkitt's lymphoma was engrafted, and were detectable in the bloodstream up to 24 h after injection. This experiment shows that passive targeting alone could produce satisfactory results both in vitro and in vivo [61].

Meissner et al. also focused on liposomal lipoplexes carrying anti-*BCL-2* asODNs composed of similar lipid composition, but they attached rituximab (a therapeutic anti-CD20 antibody) to the liposomes via a maleimide PEG derivative (DSPE-PEG-Mal). Moreover, they tested two formulations with varying DNA complexing factors. One contained asODNs complexed with DOTAP (L-D) and the other had asODNs complexed with PEI (L-P). The non-specific toxicity was tested on cell lines that do not overexpress CD20 (Jurkat T, HL60, HEL) and white blood cells isolated from the peripheral blood of healthy volunteers. As expected, both targeted formulations showed toxicity only towards CD20-expressing Daudi cells, which was manifested by a reduced Bcl-2 protein level and induction of a substantial level of apoptosis [62].

Another example is in the research of Matusewicz et al., who proposed immunoliposomes targeted against breast cancer cells overexpressing human epidermal growth factor receptor 2 (HER2) encapsulating a highly lipophilic drug, simvastatin. The final composition consisted of hydrogenated soya bean phosphatidylcholine (HSPC), DSPC, cholesterol, DSPE-PEG and DSPE-PEG-Mal. The FDA-approved humanized monoclonal antibodies (trastuzumab/Herceptin) were attached using the same procedure as described in the previously mentioned study [62]. Targeted and non-targeted liposomal formulations were tested on cell lines that overexpressed HER2 (SKBR3 and BT474) and on those that did not (MDA MB 231). As expected, neither formulation showed a decrease in the viability of cells without HER2 overexpression, while immunoliposomes induced apoptosis and inhibited the signaling pathway involving Akt and Erk in the HER2-overexpressing SKBR3 cell line [63]. In another study, Matusewicz et al. took their previously established liposomal composition and exchanged trastuzumab for an anti-EGFR therapeutic antibody (cetuximab) with the hope of treating triple-negative breast cancers, as about half of them overexpress the epidermal growth factor receptor (EGFR). Viability tests were carried out on a cell line overexpressing EGFR (MDA MB 231) as well as on MCF7 cells that had low EGFR expression. Immunoliposomes proved to be far less toxic for the MCF7 cells, while inducing apoptosis and inhibiting the Akt signaling pathway in the MDA MB 231 cell line [64].

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