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REVIEW

NEW PHARMACEUTICAL DOSAGE FORMS USED IN THE TREATMENT OF BREAST CANCER. LIPOSOMES

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Abstract

In order to obtain antineoplastic compounds and innovative formulations, new technologies and testing methods are continuously being developed. Unfortunately, besides cancer cells, chemotherapy also affects normal cells. An option to avoid toxicity is represented by the targeted cancer treatment using novel pharmaceutical dosage forms.
Liposomes represent a relatively new pharmaceutical dosage form, used for their many advantages. In this article, the methods of liposomal preparation are mentioned, along with the classification and the latest improvements involving this pharmaceutical form. The bioavailability of conventional liposomes is currently improved by developing photodynamic liposomes, pH or temperature sensitive liposomes and targeted liposomes.

Keywords: *breast cancer, nanomaterials, liposomes*

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Introduction

Throughout recorded history, humans and animals have had cancer. Initially, the source of medical treatment were represented by the herbal medicines, which are still used in developing countries. Currently, surgery, chemotherapy, hormonal therapy, radiation therapy and immunotherapy are used as cancer treatment methods (1) Unfortunately, because they affect the normal cells also, chemotherapy agents have many side effects. A targeted cancer treatment using novel formulations is an option to avoid their toxicity (2) Furthermore, new technologies (3) are being developed to obtain anticancer products, in order to use them in innovative formulations (4,5).

Nanotechnology - applications in cancer therapy

Small colloidal systems (10-1000nm), also known as nanocarriers, are used in many medical fields as: cancer treatment, HIV and AIDS treatment, nutraceutical delivery and diagnostics. The basic components used to obtain nanocarriers are different types of materials with special features, called nanomaterials (6).

Compared to conventional therapy, nanocarriers have many advantages, which lead to an improved bioavailability:

- higher water solubility (with the posibility of incorporating insoluble drugs);
- posibility of modeling the release kinetics leading to controlled release drugs (using pH, ultrasound or high temperature);
- posibility of obtaining targeted release drugs, with minimal side effects (7).

To achieve optimal results, several clinical trials are testing various combinations of treatment using multifunctional nanocarriers with aptamer sequences, antigenic proteins, molecular components and imaging agents (8). The drug is loaded into the nanocarrier by two methods: incorporation and adsorption/absorption. Furthermore, the therapeutic agents (active substance, protein, gene or vaccine) can be attached to the nanocarriers by: dissolution, absorption, covalent or electrostatic bonding (9,10,14).

Raw material - Nanomaterials

In 2011, the EU adopted a nanomaterial's definition (2011/696/EU): „*A natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm. By derogation from the above, fullerenes, graphene flakes and single wall carbon nanotubes with one or more external dimensions below 1 nm should be considered as nanomaterials*" (11).

Lately, the number of new emerging nanomaterials is surprisingly increasing. Compared to conventional micron size materials, nanomaterials used in medicine field have unique features. Usually, medical nanomaterials may be included in the following categories:

- Nanoscale organic materials:
  - Polymers:
    - Natural polymers: chitosan;
    - Synthetic polymers: PEG and its derivatives, PLA and its derivatives, PVP and its derivatives, amphiphilic copolimers;
  - Lipids:
    - Neutral and anionic lipids: Phosphatidylcholine (PC), 1,2-dioctanoyl-sn-glycerol (DG), L-α-phosphatidic acid (PA), phosphoethanolamine (PE), L-α-phosphatidylglycerol (PG) and their derivatives;
    - Cationic lipids: ethyl phosphocholine (ethyl PC), 1,2-di-O-octadecenyl-3-trimethyl-ammonium propane (DOTMA), cationic cholesterol;
  - Proteins, peptides and nucleic acids: collagen, gellatin, albumin, DNA origami;

- Saccharides: cyclodextrins (CDs);
- Nanoscale inorganic materials: silica, gold, carbon, iron, calcium;
- Hybrid materials - organic and inorganic nanomaterials (12).

Classification of nanocarriers

There are different criteria of classification (2,13,14,15,16), such as:

- Based on the spatial arrangement:
  - Single dimension nanoparticles (monolayer nanoparticles);
  - Two dimensions nanoparticles (carbon nanotubes);
  - Three dimensions nanoparticles (Quantum Dots, Dendrimers, Fullerenes) (15).
- Based on the composition:
  - Inorganic nanocarriers: gold nanoparticles (AuNPs), quantum dots nanoparticles (QDNPs), lanthanide ions, superparamagnetic iron oxide nanoparticles (SPIO NPs). By having magnetic, photochemical and photothermal properties, they are suitable for diagnostic purpose;
  - Organic carriers: micelles, liposomes, nanogels, dendrimers. They have a versatile chemistry of the surface and core, a high biodegradability and an effective endocytosis (17).

Current state of nanotechnology used in cancer therapy

In the last decade, cancer has benefited of the newest methods of treatment. Both traditional chemotherapy drugs and new active molecules were used to generate conventional or innovative formulations. The most common were: doxorubicin (18), paclitaxel (19), docetaxel (20), cis-diamminedichloroplatinum (II) (cisplatin) (21), cytarabine (22), vincristine (23), vinblastine, vinorelbine (24), camptothecin (25), lurtotecan (26) and irinotecan (27).

Between 1993 – 2013, Food and Drug Administration (FDA) approved different types of nanoparticles for cancer therapy (28) (Figure 1), amongst which:

- Doxil (liposomal doxorubicin),
- Abraxane (albumin bound paclitaxel),
- DaunoXome (liposomal daunorubicin),
- Feridex (superparamagnetic iron nanoparticles+dextran),
- Mylotarg (gemtuzumab ozogamicin+monoclonal antibody),
- Zevalin (ibritumomab tiuxetan = MsIgG1+tiuxetan+radioactiv isotope Yttrium-90 or indium-111),
- Oncaspar (modified L-asparaginase enzyme), and
- Ontak (denileukin diftitox) (29).

In June 2010, after a clinical trial, Mylotarg was withdrawn from market, because the drug showed high levels in patient death. It also showed no benefit compared to conventional cancer therapies. After further investigations, it was re-introduced into the US market in 2017 (29).

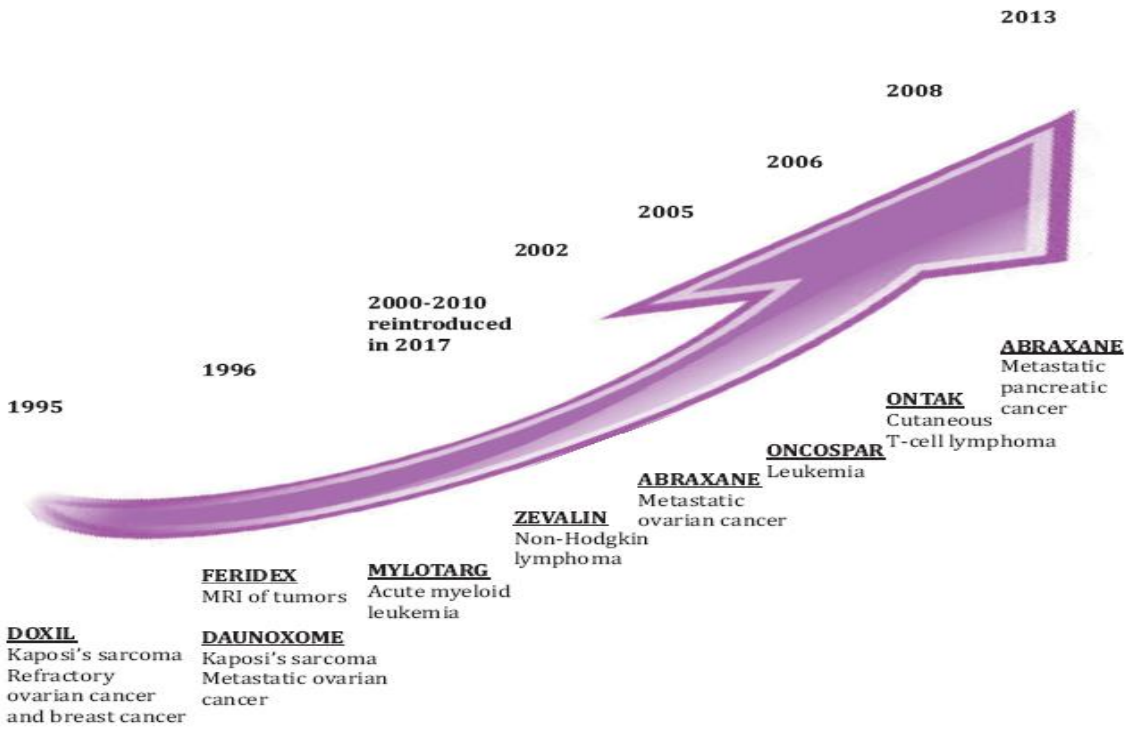

Figure 1 Examples of nanoparticles approved by Food and Drug Administration (29)

Doxil and Caelyx, two PEGylated liposomal formulation of doxorubicin, currently terminated the clinical trials concerning ovarian cancer, epithelial ovarian cancer, fallopian tube carcinoma, primary peritoneal carcinoma, lymphoma and myeloma (30). Doxil has been proven to be effective as first-line agent for the treatment of metastatic breast cancer (31).

Compared to the conventional paclitaxel, Abraxane (nanoalbumin encapsulated paclitaxel) resulted in higher overall response rate, longer median time for progression and longer median overall survival in the phase 3 clinical studies for the treatment of metastatic breast cancer (32). In 2005, Abraxane was approved by FDA for breast cancer treatment and in 2013 it was approved for pancreas cancer (33).

Tumor-Specific Targeting

In contrast to the conventional chemotherapy, nanotherapy offers the advantage of minimizing the toxicity of the tratment by distinguishing the cancerous cells from the normal cells. Furthermore, nanotherapy may benefit from a specific passive or active targeting.

Nanocarriers accumulate at tumor sites by passive targeting, due to their pharmacokinetic and physico-chemical properties, patterned according to the microenvironment (porous blood vessel network, pH, temperature).

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Active targeting involves the attachment of ligands on the surface of the nanocarrier. On the tumor cell surface, the ligands are capable to identify the uniquely overexpressed molecules. The following were used as ligands:

- antibodies: anti-Her2, anti-CD44, anti-CD24,
- proteins:transferrins,
- small bioactive molecules: galactose, mannose, glucose, biotin and folate,
- peptides: L-arginine-glycine-L-aspartic acid and
- oligonucleotides: aptamers (13)

Nanocarriers that deliver different combinations of drug-siRNA or drug-miRNA were also formulated (34). Endocytosis and tumor suppression were improved by using multifunctional cationic micelles of Paclitaxel combined with siRNA against VEGF and PIK-1 (35). A multipronged effect was achieved by targeting different metabolic pathways in the tumor cells with combination of active substances and negatively-charged siRNA or DNA (36).

Another method of aproach is by using the temperature-sensitive complexes such as pentablock pluronic copolymer that simultaneously deliver plasmid DNA and paclitaxel to cancer cells line, demonstrating synergistic and sustained delivery of both agents (37).

Pharmaceutical technology of liposomes

Background of liposomes. Advantages and disadvantages

Liposomes represent a relatively new pharmaceutical dosage form, used for their many advantages. Liposomes are versatile lipid nanosystems of transport and targeted delivery. They are spherical vesicles, with a diameter ranging from 20 nm to over 100 nm.

These vesicles are composed of several layers that separate an intravezicular environment (useful to encapsulate hydrophilic substances) from an external environment (useful to encapsulate lipophilic substances) as seen in Figure 2. Sphingomyelin, phosphatidylcholine and glycerophospholipids are the most common constituents of the phospholipidic bilayer (38).

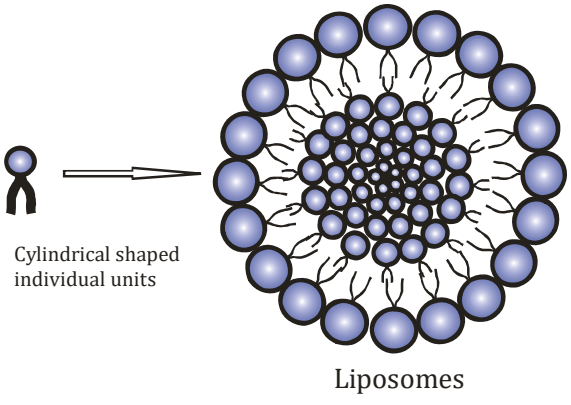

Figure 2 Liposome structure (38)

The formulation of active substances as liposomes has many advantages, from which few are mentioned in the Table 1.

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Some disadvantages may also be mentionned: physico-chemical stability, difficult sterilization, oxidation of phospholopidic bilayer, overcoming resistance and high production cost.

Classification of liposomes

Similar to other pharmaceutical dosage forms, for liposomes also there are several criteria of classification (39,40,41) The most important are: size, number of layers and mechanism of drug delivery (Table 2).

The liposomes have sizes between 0.025 μm-2.5 μm. Liposomal membrane may be siglelayer or bilayer. The circulation half life is influenced by the size of the vehicles, while the encapsulation rate is influenced by both the size and the number of bilayers number.

In the Figure 3, the structures of the four types of liposomes are plotted, based on the delivery mechanism criteria.

No. crt	Advantages of liposome encapsulation	Examples of active substances
1	Improves the solubility of lipophilic and amphiphilic active substances	Amphotericin B, Minoxidil, Anthraciclines, Doxorubicin, Acyclovir
2	Offers a passive targeting	Vaccines, Immunomodulators, Amphotericin B
3	Ensures a sustained release	Doxorubicin, Cortisones, Cytosine arabinoside, Vasopressin
4	Provides a specific targeting	Antineoplastics, Antiinflamatories, Antimicrobials
5	Improves the transfer of hydrophilic charged molecules	Genes, Plasmids, Antibiotics
6	Enhances the tissue penetration	Insulin, Anesthetics, Corticostroids

Tabel 1 Advantages of liposomal encapsulation (39)

No crt.	Criteria	Type of liposomes	
1	Number of bilayers and size	Unilamellar vesicles	Small unilamellar vesicles (SUV) 20-100nm
			Medium unilamellar vesicles (MUV) ≈ 100nm
			Large unilamellar vesicles (LUV) >100nm
			Giant unilamellar vesicles (GUV) >1μm
		Multilamellar vesicles (MLV) >0,5μm	
		Multivesicular vesicles (MV) >0,5μm	
		Multivesicular vesicles (MV) >1μm	
		Oligolamellar vesicles (OLV) >0,1-1μm	
2	Delivery mechanism	Conventional liposomes	
		PEGylated liposomes	
		Ligand-targeted liposomes	
		Theranostic liposomes	

Table 2 Liposomes classification (39,42,43,44)

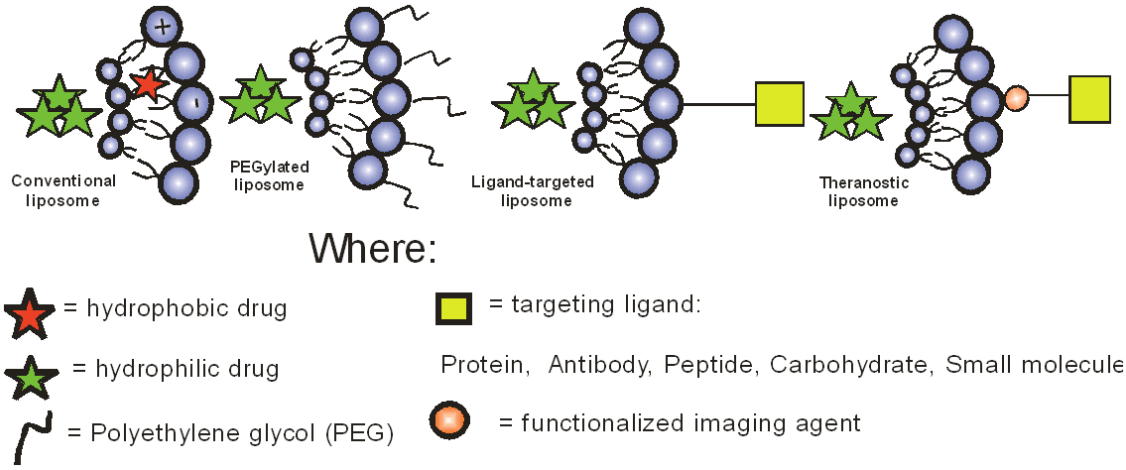

Figure 3 Types of liposomes (63)

Methods of preparation

Generally, the methodology for obtaining liposomes has two phases: vesicle preparation and drug loading. These two phases may be carried out successively (passive drug loading - after liposome formation) or concurrently (passive drug loading - during liposome formation). For example, passive encapsulation of hydrophobic drugs (e.g. amphotericin B, taxol, annamycin) is achieved by a direct combination of the drug into liposome, during vesicle formation.

Beside that, hydrophilic drugs with protonizable amine functions can be actively loaded into liposomes by using pH gradients. In fact, all the methods of liposome preparation involve four basic steps: drying the lipids from an organic solution, dispersing the lipids into an aqueous media (liposome formation), liposome purification and final analysis (45).

Conventional methods

1. Passive loading techniques

- Mechanical dispersion
  - Sonication – is the most used method to prepare SUV. MLVs are sonicated with a probe in bath sonicator in a passive atmosphere. Disadvantages: low internal volume of encapsulation, high degradation rate and MLV impurification.
  - Extrusion French pressure cell - the extrusion of MLV is made through a very small orifice. Compared to the sonicated SUVs, the extrusion french liposomes are larger. Disadvantage: high temperature is needed.
  - Freeze-thawed liposomes - the proceduse involves a rapid freeze of SUVs and then a slow defrost, leading to SUV fusion and unilamellar vesicle formation.
  - Microemulsification;
  - Membrane extrusion;
  - Lipid film hydration;

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- Dried reconstituted vesicles;

- Solvent dispersion
  - Solvent vaporization (ethanol or ether injection) - A solution of lipids in ethanol or diethyl ether/ether-methanol is injected into a drug aqueous solution. Aftewards, the solvent is removed, leading to the creation of liposomes: ether is removed under vacuum, but ethanol is more difficult to remove, because of the azeotrope formation. Disadvantage: the population of liposomes is heterogeneous.
  - Reverse phase evaporation - inverted micelles are involved. The micelles are created by sonication using a mixture of aqueous phase that was previously buffered (containing the drug) and organic phase (containing the amphiphilic molecules). The organic solvent slowly eliminates and the inverted micelles convert into viscous state, forming a gel. The gel collapses at a critical point and the excess of phospholipids creates a full bilayer embedding the residual micelles and converting them into liposomes.
  - Double emulsion vesicles;
  - Stable plurilamellar vesicles;

- Detergent removal (cholate, alkyl glicoside, Triton X100):
  - Dyalisis - dialysis bags are used.
  - Column chromatography - the following columns are used: Sepharose 2B-6B, Sephadex G-50 and Sephacryl S200-S1000;
  - Absorption;
  - Dilution;

2. Active loading technique – certain types of compounds with ionisable or amphiphilic groups are introduced into liposomes after the intact vesicles are obtained. Weak bases can be incorpotated using this method (e.g. doxorubicin, adryamicin, vincristine) (39,46,47).

Innovative methods

1. Double emulsions freeze drying method

The lipids and the water-soluble components are dissolved in a mixture of tert-butyl alcohol and water. Freeze drying of this system results in cakes of isotropic monophasic solution. When water is added, it spontaneously forms a MLVs dispersion. If necessary, the MLVs may be downsized by extrusion (45,48)

2. Membrane contactor method

This is a modified version of the ethanol injection method. An ethanolic solution of phospholipids is extruded by a membrane contactor into an aqueous phase. As membrane contractor, tubular Shirazu porous glass and polypropylene hollow fibres may be used. This method offers a greater control on liposomal dimensions and an augmented encapsulation rate (45,49).

3. Hydration of phospholipids which have been previously deposited on nanostructured materials

Electrospun amphiphilic nanofibres made of hydrophilic polymer polyvinyl pyrrolidone and soybean lecithin are used. When they are added to water, the phosphatidyl choline spontaneously self-assembly. (45,50)

4. Curvature-tuning method

Spontaneous vesiculation my happen by rapid change of pH. A mixture of lyso-palmitoylphosphatidylcholine and charged/zwitterionic lipids is subjected to a rapid pH change. The liposomes are obtained by directly adding the phospholipids to an aqueous buffer (40,41,45,51).

Liposomal drug delivery – new methods to improve bioavailability of breast cancer medication

As a drug delivery system, liposomes present several advantages of which: biocompatibility, increased bioavailability, self-assembly capacity, large encapsulation ability, mouldable physicochemical and biophysical properties (52) Even if they come with many advantages, there is always space for improvement. Therefore, several methods are used to improve the bioavailability of liposome drug delivery (39,40,41,45).

New agents entrapped in liposomes

The effectiveness of anticancer treatment can be increased by targeting several metabolic pathways of tumor cells (53). This may be achieved by using combinations of hydrophilic/lipophilic drugs and negatively charged siRNA or DNA. Cationic liposomes represent a specific type of liposomes that have been studied for gene delivery (54). They are a special category of liposomes that self-assemble in the presence of nucleic acids, forming “lipoplexes”. This process is possible because of the electrostatic interaction between the DNA/RNA’s negatively charged phosphate groups and the positively charged lipids (14). Their superiority has been demonstrated to the classical liposomes (e.g liposomes loaded with paclitaxel and plasmid DNA encoding IL-12 or siRNA against Bcl-255, or thermosensitive liposomes loaded with paclitaxel and plasmid DNA) (56).

PEGylated liposomes

One major limiting factor of liposomes relates to circulation time *in vivo*. The increasing size results in a faster uptake by the reticuloendothelial system (RES) and a decreased probability of accumulation within tumor tissue (for the intended cytotoxic effect). By reducing the size of the nanocarrier, this factor is overcome, but with a disadvantage of a lower loading capacity.

Another method to overcome both previous obstacles (circulation time and loading capacity) is the surface coating of liposomes with polymers, particularly with polyethylene glycol (PEG). This procedure generates dosage forms that are able to

escape the immune system, so called "*Stealth liposomes*". One example of PEGylated approved drug is Doxil. The limitation of these delivery systems is the steric barrier created by the PEG coating itself. The success of these liposomes could be ensured by modeling their ability to release the chemotherapeutic agent to the target site prior to cellular uptake (39,40,41,45).

Thermosensitive liposomes

Future strategies of drug delivery enhacement, like stimuli-responsive liposomes (temperature, pH, magnetic field, enzymes, redox potential ultrasound) are currently investigated.

The microenvironment of a tumor has special features. A local increase in temperature has been observed in breast tumors. Thermosensitive liposomes have proven to be effective in treating breast cancer (57). They are designed to be stable at the normal body temperature, but they destabilize above the body temperature (58) At 37°C, the phospholipids are found in a solid gel phase, with low permeability to hydrophilic drugs. Beyond phase transition temperature, the phospholipids pass in a liquid-crystalline phase, with increased permeability to hydrophilic molecules. Membrane pores are the specific mechanism of drug release from lysolipid liposomes (58).

Examples of thermosensitive liposomes:

- Doxorubicin was entrapped in thermosensitive liposomes made of dipalmitoylphosphatidylcholine(DPPC)/ monostearoylphosphatidylcholine(MSPC)/ distearoylphosphatidylethanolamine (DSPE) - PEG 2000. These liposomes were clinically investigated for treatment of breast cancer.59 One specific example is ThermoDox, which is in Phase II clinical trials for the treatment of recurrent chest wall breast cancer (60).
- In 2015, Idarubicin was incorporated in DPPC/distearoyl-phosphatidylcholine (DSPC)/DSPE-PEG thermosensitive liposomes by Lu et al. (61).
- In 2011, thermosoensitive liposomes were also formulated using Brij78 (a nonionic surfactant) by Tagami et al. (62).

Targeted Liposomes

The therapeutic index of chemotherapeutic agents incorporated into liposomes may also be increased by providing a targeted tumor therapy. For this, ligand insertion on the liposomal surface of various breast cancer overexpressed receptors (antiHER2, antitransferrin, antiER, antilectins) is used (40). As targeting ligands, the following may be used: proteins, antibodies, peptides, carbohydrates and small molecules (39,40,41,45,63). The most recently reported chemotherapeutic targeted

liposomes used in breast cancer treatment are mentionned in Table 3.

Target	Targeting ligand	Encapsulated agent
HER2	Anti-HER2 Fab	PE38KDEL64
Transferrin receptor	Antitransferrin receptor antibody	HER2 siRNA (65)
MCF07 cell specific	Peptide	PRDM14 siRNA
Estrogen receptor	17β estradiol / Estrone	Anticancer gene / Doxorubicin (66,67)
Lectins	Selectin ligand	Melphalan (68)

Table 3 Recently reported chemotherapeutic targeted liposomes used in breast cancer treatment (40)

Electrostatically Crosslinked Polymer Liposomes

To improve the stability of pH sensitive liposomes, a new method of manufacturing using electrostatic interactions was studied. Thus, liposomes were prepared using methoxy - poly (ethyleneglycol) - block-poly- (methacrylic acid) - cholesterol (mPEG-bP(MAAc) - chol) and cross-linking reagent poly(ethyleneglycol) end-capped with lysine. A satisfactory liposomal stability was found around pH 7.4, the acidic pH (typical of the tumor medium, reproduced by MDA-MB-231 breast cancer cells) lead to liposomal disintegration (69).

Photodynamic Liposome Therapy

Breast cancer cell apoptosis can also be triggered by obtaining oxygen species (ROS), which in turn can be produced by using photodynamic therapy (PDT). Methylene blue is one of the agents that, by exposure to light, produces ROS. Its disadvantage of having a poor cellular permeability can be solved by incorporating it into photodynamically active nanostructures. For this purpose, poly(12-(metacryloyloxy)dodecylphosphorylcholine) based liposomes were obtained. The tests demonstrated the superiority of this new pharmaceutical dosage form, compared to free methylene blue, when administered to mice (70).

Conclusions

The design of chemotherapeutics aims to obtain pharmaceutical dosage forms with high stability, but which release the active agent only when the target is reached.

In order to overcome the dimension obstacle, one option is to diminish the size of pharmaceutical dosage forms. The nanosized carriers ensure a better penetratation in the tumor microenvironment. Yet, a too small nanocarrier can create problems in providing an efficient dose. From this point of view, other emerging possibilities to improve the release rate of liposomes are also to be considered, such as: photodynamic liposomes, pH or temperature sensitive liposomes, targeted liposomes.

Although breast cancer benefits from very modern treatments, researchers try to develop nanomedicines that are specifically

tailored for this disease. Their objectives are to achieve significant improvements in antitumorigenic and antimetastatic properties, cancer specificity and drug resistance.

Conflict of interest

The authors declare no conflict of interest.

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