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Review article

Multifunctional and stimuli-sensitive pharmaceutical nanocarriers

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ABSTRACT

Currently used pharmaceutical nanocarriers, such as liposomes, micelles, and polymeric nanoparticles, demonstrate a broad variety of useful properties, such as longevity in the body; specific targeting to certain disease sites; enhanced intracellular penetration; contrast properties allowing for direct carrier visualization *in vivo*; stimuli-sensitivity, and others. Some of those pharmaceutical carriers have already made their way into clinic, while others are still under preclinical development. In certain cases, the pharmaceutical nanocarriers combine several of the listed properties. Long-circulating immunoliposomes capable of prolonged residence in the blood and specific target recognition represent one of the examples of this kind. The engineering of multifunctional pharmaceutical nanocarriers combining several useful properties in one particle can significantly enhance the efficacy of many therapeutic and diagnostic protocols. This paper considers the current status and possible future directions in the emerging area of multifunctional nanocarriers with primary attention on the combination of such properties as longevity, targetability, intracellular penetration, contrast loading, and stimuli-sensitivity.

1. Introduction

Various pharmaceutical carriers, including nanocarriers, such as nanospheres, nanocapsules, liposomes, micelles, cell ghosts, lipoproteins and many others are widely used for experimental and clinical delivery of therapeutic and diagnostic agents to enhance the *in vivo* efficiency of many drugs and drug administration protocols [1–3]. Various modifications of these carriers are often used to control their *in vivo* properties in a desirable fashion, for example, to increase the longevity and stability of the carrier in the circulation, achieve targeting effect specifically to pathological organ or tissue, impart sensitivity to certain stimuli characteristic of pathological area or applied from the outside of the body, provide visual information regarding carrier clearance and body distribution, etc. An increasing number of publications in the field of drug delivery systems (DDS) describe DDS that simultaneously demonstrate more than one useful function by combining, for example, longevity and targetability, targetability and stimuli sensitivity, or longevity, targetability and contrast properties. Ideally, DDS could be engineered, which can simultaneously or sequentially demonstrate the following set of properties: (1) Stay (circulate) long in the body; (2) specifically target the site of the disease; (3) respond local stimuli characteristic of the pathological site, such as intrinsically abnormal pH values or temperature, or externally applied heat, magnetic field, or ultrasound, by releasing an entrapped drug or changing some other properties; (4) provide an enhanced intracellular delivery of drugs and genes as required; (5) carry a reporter (contrast) component supplying a real time information about the DDS biodistribution and target accumulation. Although a certain work in this direction is already done, see for example the reviews in [4–6], the development of DDS like this is still in its early stage.

2. Longevity and targetability of pharmaceutical nanocarriers

Making long-circulating and targeted DDS is clearly the most developed approach. The longevity of drug carriers allows for maintaining a required level of a pharmaceutical agent in the blood for extended time intervals. In addition, long-circulating drug-containing nanocarriers can slowly accumulate (via the enhanced permeability and retention – EPR – effect, see [7,8]) in pathological sites with affected and leaky vasculature (tumors, inflammations, and infarcts), and can facilitate the drug delivery in those areas [7–9]. In addition, the prolonged circulation can help to achieve a better targeting effect for targeted (specific ligand-modified) drugs and drug carriers allowing for more time for their interaction with the target [10]. Naturally, long-circulating pharmaceuticals and pharmaceutical carriers represent an important and still growing area of biomedical research [10–15] .

The most frequent way to impart the *in vivo* longevity to drug carriers is to modify their surface with certain synthetic polymers, such as poly(ethylene glycol) or PEG, as was initially suggested for liposomes [16–20]. Coating nanoparticles with PEG results in the formation of the polymeric layer over the particle surface, which

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is impermeable for other solutes even at relatively low polymer concentrations [21,22] and sterically hinders the interaction and binding of blood components with their surface [18,23–27], preventing thus drug carrier opsonization and capture by RES [28]. Currently, there exist many chemical approaches to synthesize activated derivatives of PEG and to couple these derivatives with a variety of drugs and drug carriers, see reviews in [29–31]. Although PEG is clearly the most popular for the preparation of long-circulating DDS, some other biocompatible, soluble, and hydrophilic polymers have also been suggested as steric protectors for pharmaceutical nanocarriers, such as single terminus lipid-modified poly(acryl amide) and poly(vinyl pyrrolidone) [26,27], poly(acryloyl morpholine) [32–34], phospholipid(PE)-modified poly(2-methyl-2-oxazoline) or poly(2-ethyl-2-oxazoline) [35], phosphatidyl polyglycerols [36], and polyvinyl alcohol [37].

Although the PEGylation procedure seems to be the most developed to prepare long-circulating liposomes, there are many examples of absorbing/attaching PEG on the surface of various hydrophobic polymeric nanoparticles, which considerably influences their body residence time and biodistribution [38]. Long-circulating polymeric nanoparticles with insoluble core and water-soluble shell covalently linked to the core can also be prepared from block-copolymers of PEG and polylactide-glycolide (PEG-PLA-GA) [39–41]. Grafting PEG onto the surface of gold particles via mercaptosilanes expectedly resulted in decreased protein adsorption onto modified particles and in less platelet adhesion [42].

The most significant biological consequence of nanocarrier modification with PEG and similar polymers is a sharp increase in its circulation time and decrease in their RES accumulation [11,16,22]. Various long-circulating nanocarriers have been shown to effectively accumulate in many tumors via the EPR effect [7–9,43]. Long-circulating liposomes were prepared containing various anticancer agents, such as doxorubicin, arabinofuranosylcytosine, adriamycin, and vincristin [44–47]. PEG-liposome-incorporated doxorubicine (Doxil®) has already demonstrated very good clinical results [9,48,49].

The idea to add the property of the specific target recognition to the carrier's ability to circulate long seems quite natural and was thoroughly investigated. Targeting of nanoparticular DDS with the aid of specific ligands selective to certain cell-surface components/receptors allows for the selective drug delivery to those namely cells. There are, however, some issues to be considered when designing such systems: (1) The ligand (antibody, another protein, peptide or carbohydrate) attached to the carrier surface may increase the rate of its uptake by the RES despite the presence of a sterically protecting grafts; see for example [50]; (2) Ligand-bearing long-circulating nanocarriers could facilitate the development of an unwanted immune response (as was shown with the raise of anti-liposome antibodies), the extent of which depends on the type of the ligand (small peptides or Fv fragments are less immunogenic than a complete IgG molecule) and the liposome composition [51–53]; (3) The amount of ligand attached to the carrier may be critical to ensure successful binding with the target, while maintaining the extended circulation of the carrier.

To achieve selective targeting by long-circulating PEGylated nanoparticulates, targeting ligands were attached to nanocarriers via the PEG spacer arm, so that the ligand is extended outside of the dense PEG coat, which allows for its unhindered binding to the target receptors (see the scheme on Fig. 1). Ligands were attached to the activated water-exposed ends of liposome-grafted polymeric chain [54,55]. For this purpose, several types of end-group functionalized lipopolymers of general formula X-PEG-PE [29,56], where X represents a reactive functional group-containing moiety. Most of the end-group functionalized PEG-lipids were synthesized from heterobifunctional PEG derivatives containing hydroxyl and carboxyl or amino groups. Another amphiphilic reactive PEG derivative, p-nitrophenylcarbonyl-PEG-PE (pNP-PEG-PE), was also introduced [55,57,58], which easily adsorbs on hydrophobic nanoparticles or incorporates into liposomes and micelles via its phospholipid residue, and readily binds amino group-containing compound via its water-exposed pNP group.

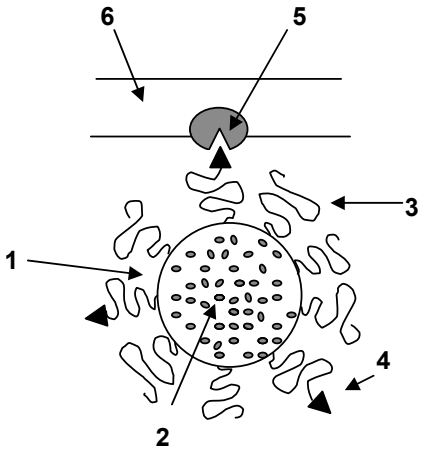

Fig. 1. The schematic structure of long-circulating targeted nanocarrier: (1) nanocarrier; (2) drug; (3) sterically protecting polymer (usually, PEG) grafted on the surface of the nanocarrier; (4) targeting ligand (antibody, folate, transferring) chemically coupled with distant tips of some of the protecting polymer grafted chains; (5) specific receptor targeted with the attached ligand; (6) cell membrane.

Several strategies have been suggested to prepare targeted PEGylated nanocarriers, first of all, liposomes [56,59]. The first approach involves the modification of preformed PEGylated nanocarriers, containing a certain number of reactive groups exposed into the aqueous surroundings. In the second approach, ligand-PEG-lipid conjugate is mixed with other liposome-forming components, and then made into unilamellar vesicles [59–62]. According to the third approach, a ligand-modified with the reactive PEG-PE is post-inserted into preformed liposomes [59,63].

The majority of research in this area relates to cancer targeting, which utilizes a variety of monoclonal antibodies. Thus, it was suggested to target HER2-overexpressing tumors using anti-HER2 long-circulating liposomes [51]. Antibody CC52 against rat colon adenocarcinoma CC531 attached to PEGylated liposomes provided specific accumulation of liposomes in rat model of metastatic CC531 [64]. A nucleosome-specific monoclonal antibody (mAb 2C5) capable of recognition of various tumor cells via the tumor cell surface-bound nucleosomes significantly improved Doxil® targeting to tumor cells and increased its cytotoxicity [65] both *in vitro* and *in vivo* in different test systems including intracranial human brain U-87 tumor xenograft in nude mice [66]. The same antibody was also used to effectively target long-circulating PEG-liposomes loaded with an agent for tumor photo-dynamic therapy (PDT) both to multiple cancer cells *in vitro* and to experimental tumors *in vivo* and provided a significantly enhanced tumor cell killing under the conditions of PDT [67].

Combination of immunoliposome and endosome-disruptive peptide improves the cytosolic delivery of the liposomal drug, increases cytotoxicity, and opens a new approach to constructing targeted liposomal systems as shown with diphtheria toxin A chain incorporated together with pH-dependent fusogenic peptide diINF-7 into liposomes specific towards ovarian carcinoma [68].

Surface modification with antibodies was also applied to make targeted and long-circulating non-liposomal pharmaceutical nanocarriers, see [69] for review. Nanoparticles made of poly(lactic acid) were surface modified with PEG and with anti-transferrin receptor monoclonal antibody to produce PEGylated immunoparti-

cles with size of about 120 nm and containing ca. 65 bound antibody molecules per single particle [70]. Mammalian cells (NIH3T3, 32D, Ba/F3, hybridoma 9E10) were surface modified with distal terminus-activated oleyl-PEG, and various proteins (streptavidin, EGFP, and antibody) were successfully attached to the activated PEG termini [71] producing potentially interesting multifunctional (long-circulating and targeted) drug delivery system.

Similar combination of longevity and targetability can be also achieved by using some other specific ligands attached to long-circulating preparations. Thus, since transferrin (Tf) receptor (TfR) is over-expressed on the surface of many tumor cells, antibodies against TfR as well as Tf itself are among the popular ligands for targeting various nanoparticular DDS including liposomes to tumors and inside tumor cells [72]. The recent studies involve the coupling of Tf to PEG on PEGylated liposomes in order to combine longevity and targetability [73]. Targeting tumors with folate-modified nanocarriers also represent a popular approach, since folate receptor (FR) expression is frequently over-expressed in many tumor cells [74–77]. Folate was attached to the surface of cyanoacrylate-based nanoparticles via activated PEG blocks [78]. Similarly, PEG-polycaprolactone-based particles were surface modified with folate and, after loading with paclitaxel, demonstrated an increased cytotoxicity [79]. Other specific ligands attached to long-circulating nanocarriers have also been used. Thus, hyaluronan-modified long-circulating liposomes loaded with mitomycin C are active against tumors overexpress hyaluronan receptors [80]. Vasoactive intestinal peptide (VIP) was attached to PEG-liposomes with radionuclides to target them to VIP-receptors of the tumor, which resulted in an enhanced breast cancer inhibition in rats [81]. PEG-liposomes were targeted by RGD peptides to integrins of tumor vasculature and, being loaded with doxorubicin, demonstrated an increased efficiency against C26 colon carcinoma in mice [82].

3. Long-circulating, targeted and stimuli-sensitive nanocarriers

Further development of the "multifunctional approach" involved the addition of certain stimuli-sensitive functions to long-circulating and targeted pharmaceutical nanocarriers. The idea was that certain stimuli intrinsically characteristic of the pathological zone or applied to this zone from the outside of the body could beneficially modify the properties of the drug-in-nanocarrier system, for example, providing enhanced or controlled drug release, improving the cellular drug uptake, controlling the intracellular drug fate or even allowing for certain physical action on the surrounding pathological tissue. The stimuli, which are currently utilized to modify the behavior of drug delivery systems inside the target are summarized in Table 1. One can see that stimuli typical for a pathological tissues themselves include pH and redox conditions; temperature can serve as a local stimulus both within the tissue (inflammation is always accompanied with a local hyperthermia) and from the outside; ultrasound and (electro) magnetic field could be applied only "artificially" and mainly from the outside. For example, intratumoral pH value in solid tumors may drop to 6.5, i.e. one pH unit lower than in normal blood (7.4) because of hypoxia and massive cell death inside the tumor [83] [84], and drops still further inside cells, especially, inside endosomes (5.5 and even below) [85]. At the same time, intracellular concentration of glutathione (i.e. redox potential) in cancer cells is significantly (100-fold) higher than normal extracellular level of glutathione [86]. All these stimuli have been successfully utilized for specific drug targeting (see Fig. 2).

3.1. pH-sensitive systems

Historically, pH-sensitivity was the first example of stimuli-sensitivity used to modify in a desired way drug/DDS behavior in the pathological areas with the decreased pH value, such as tumors, infarcts, and inflammations. With this in mind, pH-sensitive/responsive components were incorporated/attached to nanocarriers. First studies relate to pH-sensitive liposomes, which have been made to

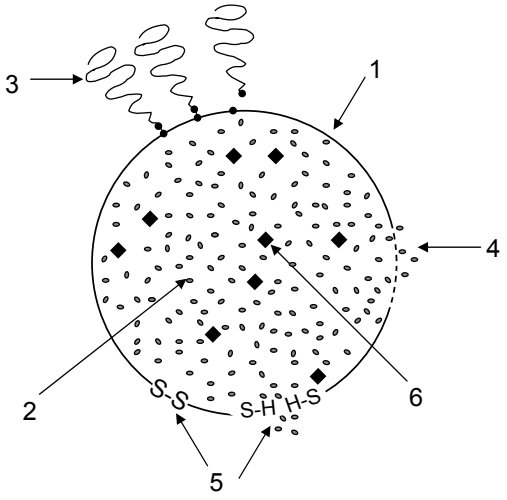

Fig. 2. Schematic picture of different stimuli acting on the stimuli-sensitive nanocarrier and expected responses: (1) nanocarrier; (2) drug; (3) protectine polymeric coating attached to the surface of the nanocarrier via pH-sensitive bonds could be detached and removed by the action of lowered pH in certain pathological areas (tumors) or inside cellular compartments (cytoplasm, endosome); (4) temperature-sensitive coating or components of the carrier, which can be influenced by the heat (hyperthermia in certain pathological areas or the heat brought upon by an external source) to destabilize the carrier and allow for drug release; (5), redox-sensitive coating or components of the carrier, which can be influenced by changing redox conditions (increased glutathione), for example by transforming –S-S-bonds into thiol groups, and allow for drug release; (6), particles of magneto-sensitive material (SPION), which can allow the whole nanocarrier to be transported to required site under the action of an external magnetic field.

Table 1
Stimuli that can be utilized to control the behavior and properties of drug delivery systems.

Stimuli		Stimuli origin
pH	Internal	Decreased pH in pathological areas, such as tumors, infarcts, and inflammations, because of hypoxia and massive cell death; decreased pH in cell cytoplasm, endosomes, and lysosomes
Redox potential	Internal	Increased concentration of glutathione inside many pathological cells compared to its extracellular concentration
Temperature	Internal	hyperthermia associated with inflammation
Temperature	External	Can be caused inside target tissues by locally applied ultrasound or by locally applied high frequency causing the oscillation of target-accumulated magneto-sensitive nanoparticles with heat release
Magnetic field	External	Magnetic field of different gradients and profiles applied to the body can concentrate magneto-sensitive DDS in required areas
Ultrasound	External	Sonication can be applied to the body to get a diagnostic signal from echogenic contrast agents and can also facilitate DDS penetration into cells and drug/gene release rom ultrasound-sensitive DDS

destabilize and release the incorporated drug/DNA at lowered pH values. Such liposomes contained phospholipids such as phosphatidylethanolamine variants with unsaturated acyl chains, capable of protonation or formation of non-bilayered structures at decreased pH and destabilizing liposomal or liposomal and endosomal membranes with the subsequent drug/DNA release from liposomes or from liposomes and endosomes [87–90].

Different methods of liposomal content delivery into the cytoplasm have been elaborated by adding the pH-sensitivity function to liposomal preparations, which can already bear some other functions, such as longevity and targetability [91,92]. It was believed that such pH-sensitive carriers would destabilize the endosomal membrane, when the inside endosomes liberated the entrapped drug into the cytoplasm. For example, according to one of these methods, the liposome is made of pH-sensitive components and, after being endocytosed in the intact form, it fuses with the endovacuolar membrane under the action of lowered pH inside the endosome and destabilizes it, releasing its content into the cytoplasm [93]. Thus, namely endosomes become the gates from the outside into the cell cytoplasm [94]. Cellular drug delivery mediated by pH-sensitive liposomes is not a simple intracellular leakage from the lipid vesicle, since the drug has to cross also the endosomal membrane [95]. The presence of fusogenic lipids in the liposome composition, such as unsaturated DOPE, is usually required to render pH-sensitivity to liposomes [96]. Multifunctional long-circulating PEGylated DOPE-containing pH-sensitive liposomes, although have a decreased pH-sensitivity, still effectively deliver their contents into the cytoplasm (recent review in [97]). Antisense oligonucleotides were delivered into cells by anionic pH-sensitive PE-containing liposomes, which are stable in the blood; however, they undergo phase transition at acidic endosomal pH and facilitate oligo release into cell cytoplasm (recent review in [98]). Serum-stable, long-circulating PEGylated pH-sensitive liposomes were also prepared using, on the same liposome, the combination of PEG and pH-sensitive terminally alkylated copolymer of *N*-isopropylacrylamide and methacrylic acid [99]. Combination of liposome pH-sensitivity and specific ligand targeting for cytosolic drug delivery utilizing decreased endosomal pH values was described for both folate and Tf-targeted liposomes [100]. Additional modification of pH-sensitive liposomes with an antibody results in pH-sensitive immunoliposomes. A successful application of pH-sensitive immunoliposomes has been demonstrated for the delivery of a variety of molecules including fluorescent dyes, antitumor drugs, proteins and DNA [101]. In addition to membrane-destabilizing lipid components, there exists a large family of membrane-destabilizing anionic polymers that also can enhance the endosomal escape of various drugs and biomacromolecules [102]. This family includes various carboxylated polymers, copolymers of acrylic and methacrylic acids, copolymers of maleic acid, polymers and copolymers of N-isopropylacrylamide, which demonstrate lower critical solution (solubility/insolubility switch) at physiological temperatures and when precipitated, destabilize the biomembranes that they are interacting with [103]. Such polymers can be attached to the surface of drug/DNA-loaded nanocarriers allowing for endosome destabilization and cytoplasmic escape.

In the case of polyplexes, which cannot directly destabilize the endosomal membrane, the mechanism of DNA escape from the endosomes is associated with the ability of polymers, such as PEI, which strongly protonate under the acidic pH inside endosome and create a charge gradient eventually provoking a water influx and endosomal swelling and disintegration [104]. In the both cases, however, DNA-containing complexes, when released into the cytosol, dissociate allowing for nuclear entry of free DNA. Nuclear translocation of the plasmid DNA is relatively inefficient because of the barrier function of the nuclear membrane and small size of the nuclear pores (ca. 25 nm), in addition DNA degrades rather fast under the action of cytoplasmic nucleases [105], and only 0.1% of plasmids undergo nuclear translocation from the cytosol [106]. The attachment of nuclear localization sequences to plasmid DNA may enhance its nuclear translocation and transfection efficiency [107]. New approaches in using multifunctional carriers for DNA delivery include the application of bimetallic nanorods that can simultaneously bind compacted DNA plasmid and targeting ligands in a spatially defined manner [108].

Polymeric micelles can also demonstrate pH-sensitivity and ability to escape from the endosomes. Thus, micelles prepared from PEG-poly(aspartate hydrazone adriamycin) easily release an active drug at lowered pH values typical for endosomes and facilitate its cytoplasmic delivery and toxicity against cancer cells [109]. Alternatively, micelles for intracellular delivery of antisense oligonucleotides (ODN) were prepared from ODN-PEG conjugates complexed with a cationic fusogenic peptide, KALA, and provided much higher intracellular delivery of the ODN that could be achieved with free ODN [110]. One could also enhance an intracellular delivery of drug-loaded micelles by adding to their composition lipid components used in membrane-destabilizing Lipofectin®. The compensation of the negative charge of PEG-lipid micelles [111] by the addition of positively charged lipids to PEG-PE micelles could improve the uptake by cancer cells of drug-loaded mixed PEG-PE/positively charged lipid micelles. After the enhanced endocytosis, such micelles could escape from the endosomes and enter the cytoplasm of cancer cells. This approach was used to increase an intracellular delivery and, thus, the anticancer activity of the micellar paclitaxel by preparing paclitaxel-containing micelles from the mixture of PEG-PE and positively charged lipids [112]. Multifunctional polymeric micelles capable of pH-dependent dissociation and drug release when loaded with doxorubicin and supplemented with biotin as cancer cell-interacting ligand were also described in [113]. Paclitaxel was loaded into mixed micelles, which could undergo dissociation into unimers and drug liberation even above the CMC value because of the ionization of their components at certain pH values [114]. Poly-L-histidine-containing micelles also demonstrate pH-sensitivity because of its pH-dependent endosome-destabilizing property (via the imidazole residue) [115,116]. Block copolymers containing 2-*N*-(morpholino)ethyl methacrylate (MEMA) component can also form pH-sensitive micelles [117].

3.2. pH-sensitive systems with detachable coatings

A special case of stimuli sensitivity relates to long-circulating PEGylated pharmaceutical carriers. In this case, the chemistry is used, which allows for the detachment of protecting polymer (PEG) chains under the action of decreased pH value or increased temperature. The matter is that the stability of PEGylated nanocarriers may not always be favorable for the drug delivery. In particular, if drug-containing nanocarriers accumulate inside the tumor, they may be unable to easily release the drug to kill the tumor cells. Likewise, if the carrier has to be taken up by a cell via an endocytic pathway, the presence of the PEG coat on its surface may preclude the contents from escaping the endosome and being delivered in the cytoplasm. In order to solve these problems, for example, in the case of long-circulating liposomes, the chemistry was developed to detach PEG from the lipid anchor in the desired conditions. Labile linkage that would degrade only in the acidic conditions characteristic of the endocytic vacuole or the acidotic tumor mass can be based on the diorto esters [118], vinyl esters [119], cystein-cleavable lipopolymers [120], double esters and hydrazones that are quite stable at pH around 7.5 but hydrolyzed relatively fast at pH values of 6 and below [118,121,122]. When the PEG brush is cleaved (for example, from the liposome surface), the membrane destabilization should occur, and the liposome con-

tents would be delivered to its target (e.g., by escaping from the primary endosome into the cell cytoplasm).

Polymeric components with pH-sensitive (pH-cleavable) bonds are used to produce stimuli-responsive DDS that are stable in the circulation or in normal tissues; however, they acquire the ability to degrade and release the entrapped drugs in body areas or cell compartments with lowered pH, such as tumors, infarcts, and inflammation zones or cell cytoplasm or endosomes [97,99,123]. A variety of liposomes [124,125] and polymeric micelles [115,126,127] have been described that include the components with acid-labile bonds as well as variety of drug conjugates capable of releasing such drugs as adriamycin [128], paclitaxel [129], doxorubicin [130], and DNA [131–133] in acidic cell compartments (endosomes) and pathological body areas under acidosis. Serum-stable, long-circulating PEGylated pH-sensitive liposomes were also prepared using the combination of PEG and pH-sensitive terminally alkylated copolymer of *N*-isopropylacrylamide and methacrylic acid [99] on the same liposome, since the attachment of the pH-sensitive polymer to the surface of liposomes might facilitate liposome destabilization and drug release in compartments with decreased pH values.

Combination of liposome pH-sensitivity and specific ligand targeting for cytosolic drug delivery utilizing decreased endosomal pH values was described for folate- and Tf-targeted liposomes [134–136].

An interesting example of novel pH-sensitive polymers is the pH-sensitive poly(β-amino ester), which rapidly dissolves at pH below 6.5, i.e. inside tumors, for example, and releases the drug incorporated into nanoparticles made of this polymers [137–140].

Dendrimeric systems derived from diaminobutane poly(propylene imine) with surface-attached PEG and loaded with various drugs demonstrated acid-sensitivity and were capable of releasing incorporated drugs, when titrated with acids followed by the addition of sodium chloride solution [141]. Doxorubicin was attached to the synthetic dendritic polyester based on 2,2-bis(hydroxymethyl)propanoic acid via pH-sensitive linkages and was released from the carrier as pH value was lowered [142]. PEG-dendrimer combinations were used to build pH-sensitive micelles capable of releasing micelle-incorporated doxorubicin at acidic pH values [143].

The stimuli sensitivity of PEG coats can also allow for the preparation of multifunctional drug delivery systems with temporarily "hidden" functions, which under normal circumstances are "shielded" by the protective PEG coat; however, they become exposed after PEG detaches (see the next paragraph on Intracellular Drug Delivery). Such systems require that multiple functions attached to the surface of the nanocarrier should function in a certain coordinated way. For the above system, the following requirements have to be met: (1) the life of the carrier in the circulation should be long enough to fit EPR effect or targeted delivery requirements (i.e. PEG coat mediating the longevity function or specific ligand mediating the targeting function should not be lost by the nanocarrier when in the circulation), and (2) the internalization of the carrier within the target cells should proceed fast not to allow for the carrier degradation and drug loss in the interstitial space (i.e. local stimuli-dependent removal of the protective function and the exposure of the temporarily hidden second function should proceed fast).

3.3. Temperature-sensitive systems

The idea of using temperature-sensitive nanocarriers naturally came from the fact that many pathological areas demonstrate distinct hyperthermia. Additionally, there exist various means to heat the required area in the body. The finding that a significantly greater fraction of the intravenously administered liposomes and other nanocarriers accumulated in the tumor mass upon heating to 42 °C in human ovarian carcinoma xenograft model and a higher concentration and effectiveness was observed for doxorubicin delivered into tumors in temperature-sensitive liposomes clearly demonstrated the feasibility of this approach [144] [145].

Temperature-sensitive liposomes frequently include dipalmitoylphosphatidylcholine (DPPC) as the key component, since liposomes usually become leaky at a gel-to-liquid crystalline phase transition and this transition for DPPC takes place at 41 °C [146]. Liposomes can also be made temperature sensitive via the incorporation of grafting of certain polymers, which display a lower critical solution temperature (LCST) slightly above the physiological one [147,148]. Because these polymers are soluble below LCST and precipitate when the temperature increases above the LCST, they can damage the liposomal membrane during precipitation and allow for drug release [149]. The most usual representative of this class of polymers is poly(N-isopropylacrylamide) (NIPAM) [150]. A review on thermo-responsive polymer-modified liposomes can be found in [147].

Similarly, polymeric micelles can be made temperature-sensitive by assembling them with amphiphilic copolymers, in which one of the blocks demonstrates properties similar to NIPAM [151]. Exact properties of such micelles can be adjusted by chemical modifications of both hydrophobic and hydrophilic blocks in such a way that the micelle can destabilize at temperatures above LCST and release the drug dissolved in its hydrophobic core [152].

3.4. Redox potential-sensitive systems

High redox potential difference, which exists between the reducing intracellular space and oxidizing extracellular space, can also be utilized for the construction of stimuli-sensitive DDS [86]. With this in mind, drug or DNA can be loaded into the nanocarrier, whose structure is maintained under normal condition by disulfide bonds. As soon as those bonds are reduced to thiol groups due to the presence of high glutathione inside the cells, the integrity of the carrier is compromised and drug or DNA can release. Thus, the authors of [153] have used polymers that are positively charged and thiol groups incorporated into the polymer structure to complex DNA (via positive charge) and to form polymeric network (via disulfide bridges formed from groups). When reduced, disulfide bridges convert back to thiols, polymeric carrier disintegrates and facilitates DNA release. Intracellular delivery of plasmid DNA was also performed using thiolated gelatin nanoparticles [154,155]. The transfection efficacy was also enhanced by using DNA condensed with thiolated polyethyleneimine [156]. Redox-responsive liposomes have also been prepared from the standard phospholipids with the addition of a small quantity of a lipid, in which head and tail are linked by the disulfide bond [157]. Long-circulating redox-responsive liposomes with detachable PEG coat were described in [158].

3.5. Magnetically sensitive systems

In still another approach, drug carriers, such as microcapsules, can be loaded not only with the drug alone, but also with magnetic nanoparticles allowing for the manipulation of such capsules in the magnetic field or with metallic nanoparticles, which can respond to the external electromagnetic field and control the rate of drug release by oscillating or heating the carrier [159]. In nanomedicine, iron oxide nanoparticles namely magnemite (γ-Fe_2O_3) or magnetite (Fe_3O_4), with particle size ca. 4–10 nm have drawn a special interest as clearly follows from several recent reviews on biomedical applications of these nanoparticles, see for example [160–163]. Due to their superparamagnetic properties and small size, they are referred to as superparamagnetic iron oxide nanoparticles (SPIONs). The use of SPION was suggested for drug targeting,

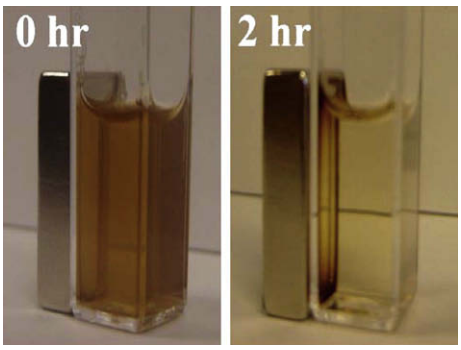

Fig. 3. Magneto-sensitive nanoparticles. PEG-PE micelles loaded with SPION concentrate in the vicinity of externally applied magnet.

bioseparation (cell sorting), magnetic resonance imaging, magnetic hyperthermia, magnetic transfection, etc. Several chemical methods can be used to prepare SPION. Most commonly used methods are controlled precipitation of iron salts in the presence of an alkali [164,165] or thermal decomposition method [166–168]. Irrespective of the method of synthesis, "plain" SPION are not stable at physiological conditions and aggregates because of the hydrophobic nature of these particles. In order to prevent aggregation or destabilization of SPION, it was suggested to coat the surface of these particles with certain compounds so that the particles can form homogenous suspensions into suitable solvents. Various substances, such as citric acid [169], dextran [170], poly(D,L-lactide-co-glycolide) [171], polyethyleneglycol-polycaprolactone block copolymer [172], organic silanes [173], and unsaturated fatty acids [166], have been used. Some early attempts to incorporate SPION into the polymeric micelles have also been described [174]; see also Fig. 3.

The concept of magnetic targeting or guiding magnetically susceptible particles towards the intended pathology site under the influence of external magnets has received increased attention. If drugs molecules are somehow conjugated to such particles then such a system will offer an increased therapeutic activity at lower doses and reduced undesired side-effect. Magnetic drug targeting (MDT) concept introduced by Widder et al. [175] in 1979 has recently received an increased attention with advances in nanotechnology. Gang et al. [176] have demonstrated targeting of magnetic poly ε-caprolactone nanoparticles loaded with gemcitabine in pancreatic cancer xenograft mouse model using external magnets. Cinteza et al. have also reported co-loading polymeric micelles of diacylphospholipid-poly(ethylene glycol) with the photosensitizer drug 2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-a, and magnetic SPION for magnetic drug targeting *in vitro*. Alexiou et al. have used mitoxantrone-loaded SPION and targeted them to VX2 squamous cell carcinoma in rabbits by using external magnets [177,178]. MDT has also been used to improve localized drug delivery to interstitial tumor targets. In particular, MDT is now being developed to improve drug delivery to tumor vessels. Thus, magnetite (MAG-C) was loaded into cationic liposomes together with etoposide and dacarbazine. It was noted that at lower concentrations MAG-C did not alter the efficiency of drug loading, but at higher concentrations (2.5 mg/ml), drug incorporation decreased [179]. It is very important that co-incorporation of SPION and drugs into the same nanocarriers only to a minimal extent influences the efficacy of drug loading [174].

Another interesting observation is that increasing local temperatures by using SPIONs in an alternating magnetic field has the potential to either directly kill tumors or make them more susceptible in combination with radiation or chemotherapy. The temperature increase achieved in this way depends on the size, shape and accumulation of the nanoparticles in the intended site and on the applied alternating magnetic field.

Using magnetic fluid depots, the concept of using magnetic hyperthermia has been illustrated by Johannsen et al. [180]. Wust et al. have carried out feasibility and tolerance studies for testing applicability of whole body magnetic field applicators and iron oxide nanoparticles in human patients. The study illustrated a great potential of the SPION-based hyperthermia [181]. "Magnetic thermal ablation" approach was examined under *in vivo* animal conditions [182]. The method is based on tumor accumulation of SPION and the exposure of the tumor to an alternating magnetic field, whereby the tumor is eliminated by heat developed by oscillating SPION.

With increased understanding of the process at the macromolecular level and identifications of ligands and targets, there have been a considerable progress in designing targeted systems. Boutry et al. have studied the targeting effect of E-selectin-specific ligand-modified SPION in mice with induced hepatitis and found that the SPIONs were retained extracellularly by the interaction with E-selectin over-expressed on the vascular endothelium [183]. Zhang et al. have designed $\alpha_v\beta_3$ integrin-angiogenisis-targeted SPION to noninvasively assess the angiogenic profile of tumors *in vivo* in nude mice grafted with subcutaneous tumor cells with different expression levels of $\alpha_v\beta_3$ integrin–positive vessels using 1.5 T MRI scanner [184].

3.6. Ultrasound-sensitive systems

The application of the external ultrasound to control drug delivery and to release from nanocarriers is a relatively novel approach, although some publications on this subject go back to 1998, when acoustically active lipospheres have been described containing paclitaxel [185]. The whole concept is based on the making of DDS, which upon accumulation in required areas can be made leaky by the locally applied external ultrasound, and can liberate incorporated drugs or genes. There already exist the whole set of promising data on drug and gene delivery by ultrasound-sensitive drug carriers, some of which are reviewed in [186–188]. Acoustically active liposomes, containing a small quantity of a certaing gas (air) or perfluorated hydrocarbon and initially developed as ultrasound contrast agent, can be loaded with various drugs and can release these drugs after being damaged by applied ultrasound [189,190]. Polymeric micelles have also been prepared, which can incorporate various drugs, such as doxorubicin, and release them after ultrasonication, which can also assist in delivering such DDS inside cells [191,192]. Similar approach was also used for the local release of thrombolytic enzymes, such as tissue plasminogen activator, from echogenic liposomes in the area of clot formation [193]. In this case, specific binding of plasminogen activator with fibrin additionally facilitated drug accumulation in the target zone providing a promising multifunctionality – contrast properties, targeting ability and thrombolytic drug release. Other examples of using ultrasound-sensitive carriers for targeting cardio-vascular pathologies are reviewed in [194]. When making DDS sensitive towards ultrasound, an important task appears for analyzing and estimating the destruction thresholds of echogenic carriers with clinical ultrasound [195]. Studies on ultrasound-sensitive formulations and the mechanisms controlling drug release from such formulations represent an important part of research on stimuli-sensitive nanocarriers [196,197].

4. Intracellular drug delivery by multifunctional nanocarriers

Intracellular transport of biologically active preparations, including various large molecules (proteins, enzymes, and

antibodies) and even drug-loaded pharmaceutical nanocarriers, is one of the key problems in the drug delivery. Many pharmaceutical agents need to be delivered intracellularly to exert their therapeutic action inside cytoplasm or onto nucleus or other specific organelles, such as lysosomes, mitochondria or endoplasmic reticulum. This group includes preparations for gene and antisense therapy, which have to reach cell nuclei; pro-apoptotic drugs, which target mitochondria; lysosomal enzymes, which have to reach lysosomal compartment; and some others. However, the cell membrane prevents various soluble small molecules as well as big molecules such as peptides, proteins and DNA from spontaneously entering cells unless there is an active transport mechanism as in the case of some short peptides. Even if molecules/particles enter cell via the endocytic pathway, they become entrapped into the endosomes and eventually end in lysosomes, where active degradation processes proceed under the action of the lysosomal enzymes. In addition, drugs inside the cells still should find their way to specific organelles, where they are expected to utilize their therapeutic potential. This is especially important in the case of gene delivery. Viral vectors for DNA delivery suffer from non-specificity and inherent risks of virus-induced complications. Non-viral delivery systems, first of all, cationic lipids/liposomes [198], also have certain drawbacks, such as same non-specificity, low efficiency, and cytotoxic reactions [199,200], though new cationic lipid derivatives with decreased toxicity are currently under development [201]. Still, the traditional routes of internalization of DNA carriers by endocytosis or pinocytosis with subsequent degradation of the delivered DNA by lysosomal nucleases strongly limit the efficacy of transfection [202].

The addition of the positive charge to the nanocarrier can significantly enhance its uptake by cells, and the use of cationic lipids and cationic polymers as transfection vectors for intracellular delivery of DNA was suggested about 20 years ago [202–204]. Currently, this is a well-developed field (see one of the recent reviews in ref. [205]). Complexes between cationic lipids (such as Lipofectin®, an equimolar mixture of *N*-(1-(2,3-dioleyloxy)propyl)-*N*,*N*,*N*-trimethylammonium chloride – DOTMA and dioleoyl phosphatidylethanolamine - DOPE) and DNA (lipoplexes) and complexes between cationic polymers, such as polyethyleneimine (PEI) [206], and DNA (polyplexes) are formed because of strong electrostatic interactions between the positively charged carrier and negatively charged DNA. A slight net positive charge of already formed lipoplexes and polyplexes is believed to facilitate their interaction with negatively charged cells and to improve transfection efficiency [207]. Endocytosis (including the receptor-mediated endocytosis) was repeatedly confirmed as the main mechanism of lipoplex/polyplex internalization by cells [208]. Of special importance is the fact that despite the endocytosis-mediated uptake of lipolexes and polyplexes, DNA does not end in lysosomes but releases in the cytoplasm due to the destabilization of the endosomal membrane provoked by the positively charged lipid or polymeric component of the complexes. Since the application of stimuli-sensitive (pH sensitive) pharmaceutical nanocarriers for intracellular was already discussed in the previous section, we will consider here other possibilities.

Relatively recent approach in intracellular drug delivery is based on the modification of drugs and drug carriers with certain proteins and peptides demonstrating a unique ability to penetrate cells (“transduction” phenomenon). This function can be added on top of the longevity, targetability and stimuli sensitivity of the pharmaceutical drug-loaded nanocarriers. Thus, the trans-activating transcriptional activator (TAT) protein from HIV-1 enters various cells when added to the surrounding media [209]. The same is true about several other cell-penetrating proteins and peptides (CPPs) [210]. For example, TAT peptide (TATp) includes a cluster of basic amino acids 47–57 (11-mer; Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg), which represents the minimal protein transduction domain (PTD) [211,212]. The minimal PTD of Antp, called penetratin, is the 16-mer peptide (43–58 residues) [213]. Other CPPs that can be used for the modification of nanocarriers include VP22; transportan, a 27 amino acid-long chimeric CPP [214]; 18-mer amphipathic model peptide with the sequence KLALKLALKALKAALKLA [215]. Current data assume more than one mechanism for CPPs and CPP-mediated intracellular delivery of various molecules and particles. CPP-mediated intracellular delivery of large molecules and nanoparticles was proved to proceed via the energy-dependent macropinocytosis with subsequent enhanced escape from endosome into the cell cytoplasm [216], while individual CPPs or CPP-conjugated small molecules penetrate the cells via electrostatic interactions and hydrogen bonding and do not seem to depend on the energy [217].

It was shown that CPPs could internalize nanosized particles into the cells [218]. Superparamagnetic iron oxide nanoparticles (SPIONs) conjugated with TATp and fluorescein isothiocyanate were taken up quickly by T cells, B cells and macrophages followed by migration of the conjugate primarily to the cytoplasm, which could be tracked readily by MRI [219]. A biocompatible dextran-coated SPION derivatized with TATp were internalized into lymphocytes by over 100-fold more efficiently than non-modified particles. The characterization on the number of TATp molecules required for an efficient delivery of magnetic nanoparticles revealed that higher numbers of TATp molecules (above 10 per single SPION) enhanced the intracellular accumulation of such particles by 100-fold [220]. The combination of longevity, magnetic properties and ability to penetrate the cells results in pharmaceutical nanopreparations with new and unique properties including contrast properties allowing for MR visualization and tracing cells taking up such particles.

Even relatively large particles, such as liposomes, could be delivered into various cells by multiple TATp or other CPP molecules attached to their surface [57,221,222]. The translocation of TATp-liposomes (both plain and PEGylated) into cells required the direct interaction of the liposomal TATp with the cell surface [57,223]. Complexes of TATp-liposomes with a plasmid (plasmid pEGFP-N1 encoding for the Green Fluorescence Protein, GFP) were used for successful *in vitro* transfection of various tumor and normal cells as well as for *in vivo* transfection of tumor cells in mice bearing Lewis lung carcinoma [224] (the combination of positive charge for DNA complexation and cell-penetrating functions). Antp and TATp coupled to small unilamellar liposomes were accumulated within tumor cells and dendritic cells more effectively than unmodified control liposomes [225]. Coupling of TATp to the outer surface of liposomes was also described, which resulted in an enhanced binding and endocytosis of the liposomes in ovarian carcinoma cells [226]. Antp-liposomes have also been considered as a carrier system for an enhanced cell-specific delivery of liposome-entrapped molecules [225]. Octamer of arginin (R8) attached to the surface of siRNA-loaded liposomes provided their effective intracellular delivery and silencing of the targeted gene [227].

Cell-penetrating function could be beneficially combined with the stimuli sensitivity that was discussed earlier. Thus, talking about the multifunctionality, one would like a nanoparticular DDS to be able to (1) specifically accumulate in the required organ or tissue, and then (2) penetrate the target cells delivering its load (drug or DNA) intracellularly. Organ or tissue (tumor, infarct) accumulation could be achieved by the passive targeting via the enhanced permeability and retention (EPR) effect [7,228] assisted by prolonged circulation of such nanocarrier (for example, as a result of its coating with protecting polymer such as PEG); or by the antibody-mediated active targeting [229,230], while the intracellular delivery could be mediated by certain internalizable ligands (folate, transferrin) [77,231] or by CPPs [232,233].

Evidently, such DDS should simultaneously carry on its surface various active moieties, i.e. be multifunctional, and possess the ability to "switch on" certain functions (such as intracellular penetration) only when necessary, for example, under the action of local stimuli characteristic of the target pathological zone (first of all, increased temperature or lowered pH values characteristic of inflamed, ischemic, and neoplastic tissues). These "smart" DDS should be built in such a way that during the first phase of delivery, a non-specific cell-penetrating function is shielded by the function providing organ/tissue-specific delivery (sterically protecting polymer or antibody). Upon accumulating in the target, protecting polymer or antibody attached to the surface of the DDS via the stimuli-sensitive bond should detach under the action of local pathological conditions (abnormal pH or temperature) and expose the previously hidden second function allowing for the subsequent delivery of the carrier and its cargo inside cells (see the general scheme in Fig. 4A). This is especially important for CPP-bearing nanocarriers, since all CPPs are highly non-selective and can lead their cargo to any cells including many non-target ones.

We have recently suggested and prepared targeted long-circulating PEGylated liposomes and PEG-phosphatidylethanolamine (PEG-PE)-based micelles possessing several functionalities [234,235]. First, such systems are capable of targeting a specific cell or organ by attaching the monoclonal antibody (infarct-specific antimyosin antibody 2G4 or cancer-specific anti-nucleosome antibody 2C5) to their surface. Second, these nanocarriers were additionally modified with TATp moieties attached to the surface of the nanocarrier via the short PEG spacer. PEG-PE used for liposome surface modification or for micelle preparation was made degradable by inserting the pH-sensitive hydrazone bond between PEG and PE (PEG-Hz-PE). Under normal pH values, TATp functions on the surface of nanocarriers were "shielded" by long protecting PEG-chains (pH-degradable PEG_{2000}-PE or PEG_{5000}-PE) or by long pNP-PEG-PE moieties used to attach antibodies to the nanocarrier (non-pH-degradable PEG_{3400}-PE or PEG_{5000}-PE). At pH 7.5–8.0, both liposomes and micelles demonstrated a high specific binding with antibody substrates, but very limited internalization by cells. However, upon brief incubation at lower pH values (pH 5.0–6.0), nanocarriers lose their protective PEG shell because of acidic hydrolysis of PEG-Hz-PE and are effectively internalized by cells via TATp moieties (Fig. 4B).

In vivo, TATp-modified pGFP-loaded liposomal preparations have been administered intratumorarly in tumor-bearing mice and the efficacy of tumor cell transfection was followed after 72 h. The administration of pGFP-TATp-liposomes with non-pH-sensitive PEG coating has resulted in only minimal transfection of tumor cells because of steric hindrances for the liposome-to-cell interaction created by the PEG coat. Contrarily, the administration of pGFP-TATp-liposomes with the low pH-detachable PEG resulted in the highly efficient transfection, since the removal of PEG under the action of the decreased intratumoral pH leads to the exposure of the liposome-attached TATp residues, enhanced penetration of the liposomes inside tumor cells and to effective intracellular delivery of the pGFP [236].

Interesting multifunctional envelope-type devices have been recently described for the cytoplasmic delivery of proteins, DNA and oligonucleotides [237]. Nanoparticles have been formed by the condensation of the substances to be delivered inside cells with lipid derivatives of CPPs, such as polyarginine, and were efficiently internalized by the cells and released their cargo into the cytosol.

5. Multifunctional nanocarriers for image-guided drug delivery and diagnostics

To use pharmaceutical nanocarriers for diagnostic/imaging purposes simultaneously with their therapeutic use and to allow for following their real-time biodistribution and target accumulation, the contrast reporter moieties can be added to multifunctionalized

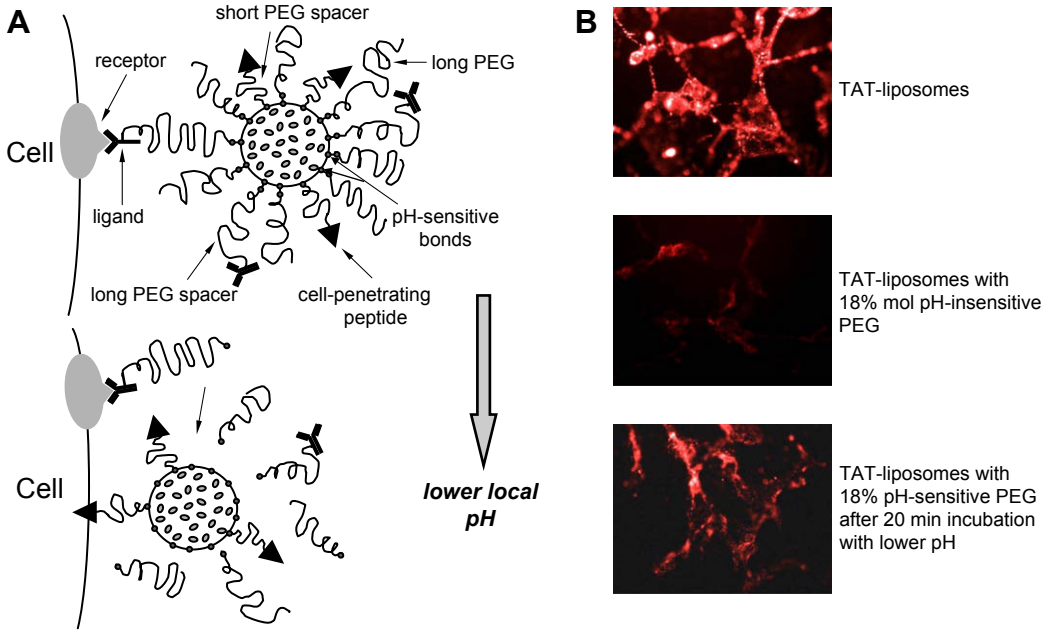

Fig. 4. (A), Schematic structure of a double-targeted "smart" nanocarrier with temporarily "hidden" function, for example cell-penetrating peptide, and "shielding" polymeric coat (with or without targeting antibody attached to it) providing longevity in the blood and specific target (tumor) accumulation and preventing the hidden function from the premature interaction with target cells. Polymeric chains are attached to the carrier surface via low pH-degradable bonds. After the accumulation in the tumor due to PEG (longevity) and/or antibody (specific targeting), pH-dependent de-shielding of the temporarily hidden cell-penetrating function allow for carrier penetration inside tumor cells. (B) Interaction of "smart" TAT peptide-modified liposomes. Rhodamin-labeled TAT-liposomes are effectively taken by cells. The attachment of PEG-chains to the liposome surface (18% mol) sterically shields TAT finction and TAT-mediated liposome uptake is almost completely blocked. If, however, PEG is attached to the liposome surface via pH-sensitive bonds, its brief incubation at the lowered pH results in the elimination of PEG-chains from the liposome surface, de-blocking TAT function and good TAT-mediated uptake of the liposomes by cells. Modified from [234–236].

nanocarriers. Among nanocarriers for contrast agents, liposomes, micelles, and later dendrimers draw much attention. In the case of liposomes, for example, two general approaches are used to prepare liposome-based contrasts for gamma- and MR-imaging, when heavy metal atoms are used as contrast moieties. The reporter metal could be chelated into a soluble chelator (such as diethylene triamine pentaacetic acid, DTPA), and then incorporated into the interior of a liposome [238]. Alternatively, DTPA or a similar chelating compound could be chemically modified with a hydrophobic group, which can anchor the chelating moiety onto the liposome surface during or after liposome preparation [239]. Different chelators and different hydrophobic anchors were tried for the preparation of ^{111}In, ^{99m}Tc, Mn-, and Gd-loaded liposomes [240–247]. In the case of MR-imaging, for a better MR signal, all reporter atoms should be freely exposed for interaction with water as in the case of membranotropic chelating agents - such as DTPA-stearylamine (DTPA-SA) [242] or DTPA-phosphatidyl ethanolamine (DTPA-PE) [239], which results in better relaxivity of the final preparation when compared with liposome-encapsulated paramagnetic ions [248–251]. The amphiphilic chelating probes (paramagnetic Gd-DTPA-PE and radioactive ^{111}In-DTPA-SA) can also be incorporated into PEG(5 kDa)-PE micelles and used for *in vivo* MR and scintigraphy imaging [252].

To still further increase the liposome load with diagnostic moieties, polychelating amphiphilic polymers (PAPs) were synthesized consisting of the main chain with multiple side chelating groups capable of firm binding many reporter metal atoms and hydrophobic terminal group allowing for polymer adsorption onto hydrophobic nanoparticles or incorporation into hydrophobic domains of liposomes or micelles [253]. Such surface modification of nanocarriers allows for a sharp increase in the number of bound reporter metal atoms per particle and image signal intensity. In the case of MR, metal atoms chelated into polymer side groups are directly exposed to the water environment that enhances the relaxivity of the paramagnetic ions and leads to the corresponding enhancement of the vesicle contrast properties [244,254,255]. Such PAP-nanoparticles were used for *in vivo* MR-imaging of lymphatic system components with Gd-loaded nanocarriers. Liposomes and micelles have been studied as delivery vehicles to the lymphatic [256,257]. Liposomes loaded with chelated paramagnetic ions (Gd, Dy, Mn, Fe) could serve as MRI contrast agents mostly for the visualization of the macrophage-rich tissues such as organs of the reticuloendothelial system [258]. The overall performance of Gd-PAP-liposomes or -micelles could be further improved in case of the co-incorporation of amphiphilic PEG onto the liposome membrane or micelle surface, which can be explained by increased relaxivity of PEG-Gd-liposomes because of the presence of increased amount of PEG-associated water protons in the close vicinity of chelated Gd ions [259,260]. Multifunctional approach certainly is important here, since in addition to the enhanced relaxivity, the coating of liposome surface with PEG polymer can help in avoiding the contrast agent uptake in the site of injection by resident phagocytic cells. In the case of multifunctional nanocarriers additionally loaded with a drug, the presence of a contrast moiety allows for the real-time control of drug accumulation in the target. All said is also true for other nanoparticulate carriers including polymeric micelles [261,262]. Both PAP-bearing liposomes and micelles additionally containing PEG on their surface can also serve as long-circulating contrast agents for the blood pool gamma- or MR-imaging. Gd-PAP-PEG-liposomes additionally modified with the cancer-specific monoclonal antibody demonstrated fast and specific tumor accumulation and could serve as effective contrast agents for tumor MRI [263].

The combination of drug loading, longevity, targetability, and contrast properties results in multifunctional nanopharmaceuticals of new generation. Thus, long-circulating PEGylated liposomes loaded with doxorubicin and additionally decorated with a tumor-specific antibody and contrast moieties [264–266] demonstrated an increased therapeutic activity *in vivo*, and their target accumulation coud be easily followed by gamma-scintigraphy (see Fig. 5) or MRI. Multifunctional nanocarriers for image-guided drug delivery, which combine therapeutic and imaging agents merged in one preparation, have also been described in [267] and [191], the last one of these studies combining the ultrasonic tumor imaging with targeted therapy by doxorubicin.

Summing up, the development of a broad variety of multifunctional and stimuli-sensitive pharmaceutical nanocarriers represents now an important area of DDS research, and eventually could allow for combined therapeutic and diagnostic systems with dramatically enhanced efficacy.

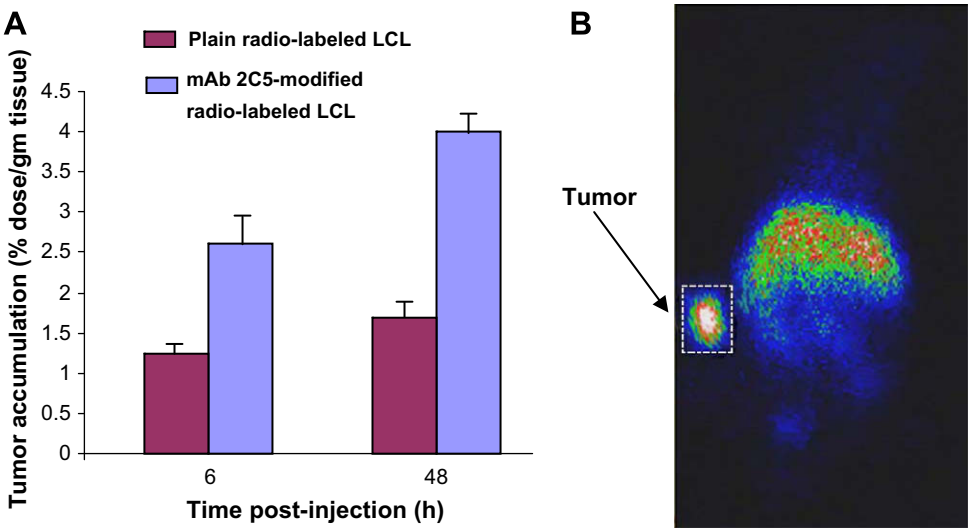

Fig. 5. Combination of the longevity, targetability, and contrast function. Radiolabeled (^{111}In) long-circulating (PEG) liposomes (LCL) modified with a tumor-targeted ligand (cancer-specific monoclonal antibody 2C5) demonstrate an enhanced tumor accumulation, A. This can be used for the fast and specific tumor visualization by gamma-scintigraphy (mice), B. Modified from [264].

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