Review Article

The Chemistry of Bioconjugation in Nanoparticles-Based Drug Delivery System

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Nanomedicine is, generally, the application of nanotechnology to medicine. The term nanomedicine includes monitoring, construction of novel drug delivery systems, and any possible future applications of nanotechnology and nanovaccinology. In this review, the most important ligand-nanocarrier and drug-nanocarrier bioconjugations are described. The detailed characterizations of covalently formed bonds between targeted ligand and nanocarrier, including amide, thioether, disulfide, acetyl-hydrazone and polycyclic groups, are described. Also, the coupling of small elements and heteroatoms in the form of R-X-R the “click chemistry” groups is shown. Physical adsorption and chemical bonding of drug to nanocarrier surface involving drug on the internal or external surfaces of nanocarriers are described throughout possibility of the formation of the above-mentioned functionalities. Moreover, the most popular nanostructures (liposomes, micelles, polymeric nanoparticles, dendrimers, carbon nanotubes, and nanohorns) are characterized as nanocarriers. Building of modern drug carrier is a new method which could be effectively applied in targeted anticancer therapy.

1. Introduction: Nanotechnology and Nanomedicine

Nanotechnology has led to a junction of different fields including chemistry, biology, applied physics, optics, computational analysis and modeling, and materials science. Recent advances in the physical sciences have provided the ability to analyze and manipulate structures at nanometer scales, and this has been accompanied by advances in molecular modeling and computational science. Using them one can predict by modeling and simulation the behavior of biological structures in (the most important for human) aqueous solution [1]. The purpose of nanomedicine is the same as that of medicine: to diagnose as accurately and early as possible and to treat as effectively as possible. It means that side effects should be avoided [2]. Human health-care nanotechnology research can definitely result in immense health benefits [3].

Therefore, there is increasing funding for nanotechnology all over the world [4]. Estimates of the impact from advances emerging from nanotechnology developments over the next 15 to 20 years have been estimated to be approximately \$1 trillion by studies conducted at the National Science Foundation [3].

Generally, nanomedicine may be defined as the monitoring, reparation, construction, and control of human biological systems at the molecular level. To do this nanodevices and nanostructures are applied [3]. The prefix “nano-” means the use of materials of which at least one of their dimensions is in the scale range 1–100 nm [5].

There are numerous methods for drugs delivering into organisms. They include oral, transdermal, transepithelial, and intravenous delivery. All of these methods could be fulfilled using nanostructures as drug containers. Different forms of drug nanocontainers as polymeric nanoparticles,

FIGURE 1: The mechanism of drug delivery in classical and targeted therapy (the figure is based on [12, 199]).

liposomes, magnetic nanoparticles, and carbon nanostructures are extensively studied [4–10]. All these DDSs have been reported to have huge biomedical potential.

Nanomedicine is a rapidly developing research area in biological and medical science and the development of nanomedicine research requires collaboration among different scientists: physicians, engineers, molecular biologists, material scientists, chemists, and so forth [8]. Applications of nanotechnology in medicine are especially promising and areas such as disease diagnosis, drug delivery targeted at specific sites in the body (see the next paragraph), and molecular imaging are intensively investigated and some products are undergoing clinical trials. Nanotechnology plays an important role in therapies of the future as “nanomedicine” by lowering doses required for efficacy as well as increasing the therapeutic indices and safety profiles of new therapeutics [9]. The therapeutics are delivery systems in the nanometer size range containing encapsulated, dispersed, adsorbed, or conjugated drugs and imaging agents. Selected nanoscale systems including liposomes, micelles, nanoemulsions, nanoparticulate systems (drug nanoparticles, polymer-, lipid-, carbon-, and ceramic-based, albumin, and nanogels), and dendrimers are used for drug delivery and imaging [9].

Within the next 5 to 10 years, nanomedicine will address many important medical problems by using nanoscale-structured materials and simple nanodevices that can be manufactured today. Many approaches to nanomedicine being carried out today are already close enough to fruition that it is fair to say that their successful development is almost inevitable and their subsequent incorporation into valuable medical diagnostics or clinical therapeutics is highly likely and may occur very soon. Among them, Freitas Jr. [4] mentioned immunoisolation, gated nanosieves, ultrafast DNA sequencing, fullerene-based pharmaceuticals, nanoshells, single-virus detectors, tectodendrimers, radio-controlled biomolecules, and biologic robots [4]. Flynn and Wei [10] mentioned also the importance of commercialization of nanomedicine.

In the next chapter, we present short review on one of the practical applications of nanotechnology in medicine. We focus on the application of nanoparticles as DDS.

2. Targeted Anticancer Therapy

A deeper understanding of the molecular events leading to tumor formation, invasion, angiogenesis, and metastasis has provided a new mechanistic basis for drug discovery: targeted anticancer therapy. By specifically blocking the molecular pathways implicated in the pathogenesis of cancer, targeted anticancer agents are expected to alter the natural course of the disease and, at the same time, to offer an enhanced therapeutic index over traditional cytotoxic agents.

Targeted anticancer therapy must fulfill few requests. Anticancer drugs should be delivered to the cancer cells with minimal activity loss. The drug should kill selectively the cancer cells. The release of drug active form must be, of course, controlled [11]. Simultaneously, only the minimal doses of chemotherapeutic agent should be administered during the targeted therapy (doses should be smaller than those in the traditional chemotherapy). The therapy should also minimize different possible side effects [11, 12].

Currently, drugs are usually delivered inside or outside of nanocarriers *via* the passive or selective mechanism in conventional or targeted therapy, respectively [12] (Figure 1). The traditional mechanism is connected with a drug aggregation process (in form of carrier drug) inside cancer tissues *via* enhanced permeability and retention effect (EPR) [13–15]. This method is based on the abnormal structure of blood vessels near tumor. Thus, the drug reaches easily the tissues near cancer cells [9, 14]. The most popular drugs used during conventional treatment are doxorubicin [16], paclitaxel [17], methotrexate [18], hexamethylmelamine [19], and gemcitabine [20] and drugs based on platinum [21] like cisplatin (DDP) or carboplatin (Figure 2). The linking of drugs to nanocarriers is described in this paper, together with progressive activity after linking with antibody.

FIGURE 2: Chemical structures of drugs: (a) paclitaxel, (b) doxorubicin, (c) methotrexate, (d) gemcitabine, (e) carboplatin, (f) cisplatin, and (g) hexamethylmelamine.

The targeted anticancer therapy uses systems composed of the ligands connected to the carriers of chemotherapeutic agent. Thus, constructed structure facilitates the bioconjugation with appropriate receptors of cancer cells. The targeted anticancer therapy, in contrast to the conventional treatment, supports the overexpression of cancer cell receptors and the affinity of ligand to receptor [12, 14, 15]. The main advantage of targeted treatment is the delivery of chemotherapeutic agents to the most resistant cancer cells and longtime circulation inside them. Application of this method guarantees high concentration of drug inside the tumor. Moreover, drug cannot be released back to the blood. The main factor determining the type of used ligand is immunogenicity [13].

3. Nanoparticles as Drug Delivery System

3.1. The Importance of Proper Drug Delivery: The Role of Nanoparticles. The major practical purpose of nanotechnology in medicine is the application of nanoparticles in DDS [22]. The reason of this is that during the past two decades

researchers involved in the development of pharmaceuticals have understood that drug delivery is a fundamental part of drug development, and a wide range of DDS has thus been designed [3]. It is also very important that, at present, 95% of all new potential therapeutics have poor pharmacokinetics and biopharmaceutical properties [9]. The therapeutic index of nearly all drugs currently being used would be improved if they were more efficiently delivered to their biological targets through appropriate application of nanotechnologies. Some drugs that have previously failed clinical trials might also be reexamined using nanotechnological approaches [22].

What are the requirements for an effective and safe drug? For example, in anticancer therapy there must be an adequate drug concentration in the body to allow for an effective dose at the tumor site. The target must be strongly inhibited, with the function essential for tumor cell viability. The drug must have a high toxicity toward the tumor or a favorable therapeutic window. Many authors have delivered a variety of drugs such as hydrophilic and hydrophobic drugs, proteins, vaccines, and biological macromolecules using nanoparticles

as carriers. For the delivery of antigens for vaccination [3], three main types of gene delivery systems have been described: viral vectors, nonviral vectors (in the form of particles such as nanoparticles, liposomes, or dendrimers), and the direct injection of genetic materials into tissues using so-called gene guns [3]. Nanostructured architectures are promising candidates that will enable targeted delivery of novel drug compounds. Nanoscale drug delivery mechanism has effects on continuous drug release and intracellular entry capability. Moreover, it minimizes side effects and allows for the direct treatment of the cause of the disease rather than the symptoms of the illness. Generally nanoparticles [14, 23, 24]

- (i) have advantage over larger microparticles, because they are better suited for intravenous delivery,
- (ii) have been highly exploited for controlled drug release and site-specific drug targeting,
- (iii) have shown promising results in the case of site-specific drug targeting for treating various diseases including cancer, human immunodeficiency virus infection, and central nervous system disorders,
- (iv) have a higher surface to volume ratio as compared with bulk material, and therefore the dose and frequency of administration would be reduced hence increasing patient compliance; recently, it was shown that solid lipid doxorubicin loaded nanoparticles have potential to acne,
- (v) have the additional advantage of prolonged circulation in the blood, which would facilitate extravasation and passive targeting (nanoparticles made with hydrophilic polymers),
- (vi) avoid opsonization with particle size less than 100 nm (hydrophilic nanoparticles). These systems prolong the duration of action as well as increasing the targeting of the drug to specific sites [25].

Multifunctional nanodelivery systems could combine targeting, diagnostic, and therapeutic actions. There are already an astonishing number of emerging applications. These purposes either take advantage of the unique properties of nanoparticles as drugs or components of drugs *per se* or are designed for new approaches to controlled release, drug targeting, and salvage of drugs with low bioavailability [3].

As mentioned above, the success of a therapy depends on the drug delivery method. Its importance is exemplified by the presence of more than 300 companies based on the United States involved with developing drug delivery platforms. In addition to the commonly used oral and injection routes, drugs can also be administered through other means, including transdermal, transmucosal, ocular, pulmonary, and implantation delivery. The mechanisms used to achieve alternative drug delivery typically incorporate one or more of the following materials: biologics, polymers, silicon-based materials, carbon-based materials, or metals [3].

In this paragraph, we will focus mainly on carbon nanomaterials in drug delivery reporting the results of new findings. The applications of biologic structures, polymers, dendrimers, silicon-based structures, and some carbon

materials in drug delivery were reviewed in [24]. In [26], the review on nanoshells, carbon nanotubes, dendrimers, superparamagnetic nanoparticles, and liposomes applied in cancer therapeutics is presented. All of these nanotechnology platforms can be multifunctional, and so they are frequently named “smart” or “intelligent.” The authors raise awareness of the physiological challenges for the application of these therapeutic nanotechnologies, in light of some recent advances in our understanding of tumor biology [26].

When drugs and imaging agents are associated with nanoscale carriers, their volumes of distribution are reduced. Nanoscale DDS also has the ability to improve the pharmacokinetics and increase biodistribution of therapeutic agents to target organs, which will result in improved efficiency. Drug toxicity is reduced as a consequence of preferential accumulation at target sites and lower concentration in healthy tissues. Nanocarriers have been designed to target tumors and inflammation sites that have permeable vasculature. Targeting and reduced clearance increase therapeutic index and lower the dose required for efficacy. Delivery systems have been shown to increase the stability of a wide variety of therapeutic agents such as small hydrophobic molecules, peptides, and oligonucleotides. Nanocarriers composed of biocompatible materials are investigated as safe alternatives to existing vehicles that may cause hypersensitivity reactions and peripheral neuropathy [9]. A number of additional obstacles can be overcome with various novel applications of nanodrug delivery. Many drugs are not very soluble, making it difficult to administer therapeutic doses. These compounds can be “solubilized” by formulating them into crystalline nanosuspensions that are stabilized by surfactants or by combining them with organic or lipid nanoparticles that keep them in circulation for longer periods. If an efficacious compound has a short half-life in the circulation, its stability can be increased tremendously by encasing it within, for example, nanosized liposome as a drug carrier. In the case of cancers, for example, of central nervous system, many drugs have difficulty in crossing the blood-brain barrier to attack the tumor. Drug-loaded nanoparticles are able to penetrate this barrier and have been shown to greatly increase therapeutic concentrations of anticancer drugs in brain tumors. The best way to increase the efficiency and to reduce the toxicity of a drug is to direct it into its target and maintain its concentration at the site for a sufficient time for therapeutic action to take effect [22]. The majority of solid tumors exhibit a vascular pore cutoff size between 380 and 780 nm [9], although tumor vasculature organization may differ depending on the tumor type, its growth rate, and microenvironment. Therefore, particles need to be of a size much smaller than the cutoff pore diameter to reach to the target tumor sites. By contrast, normal vasculature is impermeable to drug associated carriers larger than 2 to 4 nm compared to free, unassociated drug molecules. This nanosized window offers the opportunity to increase drug accumulation and local concentration in target sites such as tumor or inflamed sites by extravasations and significantly to reduce drug distribution and toxicity to normal tissues [9].

Ideal DDS can be achieved by creation of materials undergoing no chemical changes and satisfying the demands

of biodegradability and biocompatibility of the nanoparticles carrier, the rate of biodegradation of the carrier, and the release dynamics of the drug [15, 27]. For example, for so-called passive targeting to be successful, the nanocarriers need to circulate in the blood for extended times so that there will be multiple possibilities for the nanocarriers to pass by the target site. Nanoparticulates usually have short circulation half-lives due to natural defense mechanisms of the body to eliminate them after opsonization by the mononuclear phagocytic system (also known as reticuloendothelial system). Therefore, the particle surfaces need to be modified to be “invisible” to opsonization. A hydrophilic polymer such as polyethylene glycol (PEG) is commonly used for this purpose because it has desirable attributes such as low degree of immunogenicity and antigenicity, chemical inertness of the polymer backbone, and availability of the terminal primary hydroxyl groups for derivatization. PEG-grafted liposomes, in the size range of 70 to 200 nm, containing 3 to 7 mol% methoxy-PEG-2000 grafted to distearoyl phosphatidylethanolamine (DSPE) or dipalmitoyl phosphatidylethanolamine, showed extended circulation half-lives of 15 to 24 hours in rodents and up to 45 hours in humans, whereas non-PEGylated liposomes had half-lives of 2 hours [15, 27].

Nanocarriers typically consist of macromolecular materials with the active principle either dissolved within a polymeric matrix, entrapped inside lipid, encapsulated, or adsorbed onto surfaces of particles. Accordingly, they can be classified into mainly two types: nanocapsules and nanospheres. The former are vesicular systems in which drug molecules are surrounded by a membrane, whereas the latter are matrix systems with the drug molecules dispersing throughout [2]. Though the technology is still young, more than 1000 nanopharmaceutical patents have been issued by the U.S. Patent and Trademark Office (U.S. PTO) during the period 1999–2008 [2]. Nanotechnology-based methods of synthesis are most commonly developed on the basis of two rational designs: either top-down or bottom-up engineering of individual components. The top-down process involves starting with a larger object and breaking it up into nanostructures through etching, grinding, or ball milling. The process can be accelerated by addition of chemicals or using laser. Microscale or macroscale manufacturing, like silicon microfabrication and photolithography, is often accomplished as top-down process. However, the method is time-consuming and frequently generates considerably broader particle size distribution. The bottom-up technique refers to synthesis based on atom-by-atom or molecule-by-molecule arrangement in a controlled manner. The process takes place through controlled chemical reactions, in either gas or liquid phase, resulting in nucleation and growth of nanoparticles. Bottom-up techniques (like supercritical fluid antisolvent techniques, precipitation methods, etc.) create heavily clustered masses of particles that do not break up on reconstitution [2]. All preparation methods: high-pressure homogenization, complex coacervation, coprecipitation, salting-out, nanoprecipitation, solvent emulsification-diffusion, supercritical fluid, rapid expansion of supercritical solutions, supercritical antisolvent precipitation, and

self-assembly methods, were in detail described in [2]. Among nanoparticles they describe polymeric nanoparticles, solid lipid nanoparticles, magnetic nanoparticles, metal and inorganic nanoparticles, quantum dots, polymeric micelles (PMs), phospholipid micelles, and colloidal nanoliposomes.

3.2. Drug Delivery Systems. The examples of recent application of nanoscale systems for drug delivery are shown in Figure 3. We focus on solid nanomaterials most often used as nanocarriers. These carriers, after joining some ligands and/or drugs, can be used in designing of the systems for targeted therapies as described in the following sections.

Liposomes and lipids have been used as DDS since 1960. Liposomes are defined as vesicles in which an aqueous volume is entirely surrounded by a phospholipid membrane. Liposome size can vary from 30 nm up to several micrometers and can be uni- or multilamellar [9]. Recently it was shown that solid lipid doxorubicin loaded nanoparticles have potential to serve as a useful therapeutic approach to overcome the chemoresistance of Adriamycin-resistant breast cancer. Koren et al. [28], Koshkaryev et al. [29], and Etzerodt et al. [30] showed that the entrapment of some chemical compounds inside modified liposomes (resp. by antibody [28, 30] and transferring [29]) causes an increase of apoptosis of cancer cells. Moreover, the solid lipid nanoparticle system could be generally applied for the delivery of many chemotherapeutic agents in chemotherapy-resistant cancers [31].

Micelles are self-assemblies of amphiphiles that form supramolecular core-shell structures in the aqueous environment. Hydrophobic interactions are the predominant driving force in the assembly of the amphiphiles in the aqueous medium when their concentrations exceed the critical micelle concentration. Phospholipid, Pluronic, poly(L-amino acid), and polyester micelles are most often applied. In [32] authors summarize advances related to targeted anticancer drug delivery to tumor sites using PMs via active and passive mechanisms (see below). PMs can be conjugated with diverse ligands such as antibodies fragments, epidermal growth factors, α -2-glycoprotein, transferrin, and folic acid.

Over the past few decades, there have been repeated attempt to develop an ideal DDS that selectively acts against diseased cells but is not harmful to healthy cells. PMs are one of the nanocarriers that can do this. Also, as most of the anticancer drugs are poorly water-soluble, PMs are convenient drug carriers for carrying as well as targeting such drugs to tumors. Collectively, all these studies suggest that drugs encapsulated in micelles show enhanced therapeutic index in solid tumors, correlating to their passive targeting, taking advantage of tumor characteristics as well as active targeting using various mechanisms and fewer side effects in comparison with conventional drug formulations. Among these, a few PM formulations have been successfully developed and a few more are at preclinical stage. There is a dire need to translate these proven experimental advantageous concepts into clinical practice to diminish the death rate from cancers and increase hope in cancer chemotherapy [32].

Besides micelles, there is the other group of nanomaterials forming self-assembling structure, known as cell-penetrating

FIGURE 3: Schematic representation of nanocarriers: (a) liposome, (b) micelle, (c) polymeric nanoparticle, (d) dendrimer, (e) carbon nanotube, and (f) carbon nanohorn.

peptides (CPPs). These peptides could be applied to identify hydrophobic anticancer drugs and intracellular delivery of biomolecules such as nucleic acids (siRNA, pDNA). The application of CPPs in novel delivery systems is favorable because of numerous advantages: biocompatibility, low toxicity, easy preparation, and stability of the structure [33–36]. Arukuusk et al. [33] proved that the addition of hydrophobic moieties to CPPs improves their properties during application as the nucleic acid delivery systems. Deshayes et al. [37] and Hou et al. [38] described the creation of siRNA delivery system based on the CPPs. The process of peptides self-assembly proceeds spontaneously during the contact with siRNA. A stable structure was formed mainly due to both electrostatic and hydrophobic interactions. Those complexes easily penetrate the cell and could be applied to successfully primary cell lines.

Polymeric Nanoparticles. Many methods have been developed for preparing polymeric nanoparticles. These methods can be classified into two main categories according to whether the formulation requires a polymerization reaction or is achieved directly from a macromolecule or preformed

polymer. Polymerization methods can be further classified into emulsion and interfacial polymerization, and there are two types of emulsion polymerization: organic and aqueous, depending on the continuous phase. Nanoparticles can be also prepared directly from preformed synthetic or natural polymers and by desolvation of macromolecules. Recently these polymeric systems have been prepared by nebulization techniques. In [39] authors present all these methods including their detailed procedures and technological advantages, as well as providing several examples of encapsulants that are entrapped into or adsorbed to these particles. The evolution of nanoparticle preparation methods has been marked by three aspects: need for less toxic reagents, simplification of the procedure to allow economic scale-up, and optimization to improve yield and entrapment efficiency. Efficient drug entrapment and transition to large scale are of highest importance to industrial applicability. Depending on the physicochemical characteristics of a drug, it is now possible to choose the best method of preparation and the best polymer to achieve an efficient entrapment of the drug. Nevertheless, there are several problems remaining to be solved. The process is not suitable to all drugs. In addition, the

postpreparative steps, such as purification and preservation, particularly important for nanocapsules, and residual solvent analysis must be extensively investigated. Other difficulties such as the formation of an incomplete or of discontinuous film with inadequate stability of certain active components, no reproducible or predictable release characteristics, causes that the final product is economically unfeasible [39]. In [40] authors discuss possibilities of the polymeric nanoparticle-based technique of targeted drug delivery through the blood-brain barrier. The biodistribution of novel nanoparticles showed two orders of magnitude greater efficiency in comparison to other known drug carriers [40].

Dendrimers. The role of dendrimers in delivery of different compounds (e.g., 5-fluorouracil, primaquine phosphate, doxorubicin, artemether, tamsulosin, indomethacin, tropicamide, and pilocarpine) is presented in [41]. Authors discuss the methods of intravenous, transdermal, ophthalmic, and oral delivery. There are different results that prove the versatility of dendrimers, and some very important *in vitro* studies with *in vivo* potential further endorse this versatility. More detailed studies on the routes already investigated and studies on other routes for dendrimer-mediated drug delivery are required; yet the existing data emphasized the potential of dendrimers as drug carriers *via* various routes. However, the toxicological status of candidate dendrimers must be established conclusively before drawing any final conclusions in this regard [41]. Interesting review describing dendrimers is presented by Wen et al. [42].

Carbon Nanotubes. Basic properties and application of carbon nanotubes in drug delivery were presented in [43–47]. Different studies [48–54] describe the application of carbon nanotubes in DDS. Firme III and Bandaru [45] describe the most popular strategies applied to increase the solubility of nanotubes. The role of defects is also discussed and finally it is concluded that the lack of centralized toxicity database limits makes the comparison between research results impossible. Jain et al. [55] present a novel cascade of chemical functionalization of multiwalled carbon nanotubes (MWCNTs) through chemical modification by a carbohydrate as D-galactose. Galactose-conjugated MWCNTs were synthesized involving the sequential steps of carboxylation, acylation, amine modification, and finally galactose conjugation. The modification of MWCNTs with galactose was investigated by different methods at every sequential step of functionalization. Size and surface characteristics of chemically modified MWCNTs were monitored. That galactosylation improved dispersibility of MWCNTs in aqueous solvents was confirmed by investigation of their dispersion characteristics at different pH values. Thus, the galactosylated MWCNTs could be used for delivery of different bioactive(s) as well as active ligand (galactose) based targeting to hepatic tissue [55].

Carbon nanohorns belong to a new class of carbon materials, similar to carbon nanotubes. Single-walled carbon nanohorn (SWNH) aggregates, composed of thousands of graphitic tubules (similar in structure to single-walled CNTs) having wide diameters of 2–5 nm, have a spherical structure with a diameter of 50–100 nm. On the basis of their

morphology, they were classified into dahlia, bud, and seed types. SWNHs contain no metal catalyst because they are produced by laser ablation of a pure graphite target. This means that the effects of metal impurities can be excluded when determining toxic responses, enabling investigation of the pure toxicological effects of nanometer-sized graphitic structures. To avoid potential health hazards caused by occupational exposure to SWNHs and to promote industrial and biomedical applications of SWNHs, the toxicity of SWNHs should be proactively investigated from various aspects. Comprehensively investigated *in vivo* and *in vitro* toxicities of as-grown SWNHs lead to conclusions that carbon nanohorns are nontoxic [56].

There are much more important materials that can be used as drug carriers: quantum dots [57, 58], Pluronic [59, 60], mesoporous silica [61, 62], nanoemulsions [63, 64], drug nanocrystals [65, 66], ceramic-based nanoparticles [67], albumin nanoparticles [68, 69], nanogels [70, 71], magnetic nanoparticles [72, 73], and so forth; however, in this review we focus on (in our opinion) the most important ones. Also, proteins are promising delivery agents. They could be bioconjugated with drugs, as albumin-bound paclitaxel, forming Abraxane nanoparticle. The formation of albumin-paclitaxel linking is prepared *via* homogenization process at high pressure [74]. Abraxane is used as Cremophor EL-free formulation. Thanks to this, the system is more effective and less toxic than conventional drugs. Chemotherapeutic agent is released from Abraxane nanoparticle *via* the albumin receptor in tumor blood vessel [74, 75]. Albumin-paclitaxel conjugation was successfully applied against gastric [75], lung [74], and metastatic breast [76] cancer.

4. Covalent Bond Formation between Targeted Ligand and Nanocarrier

Various methods have been employed to link ligands with reactive groups of the surface of the nanocarriers, and the methods can be classified into covalent and noncovalent conjugations [77]. Common covalent coupling among the other methods involve conjugation of

- (i) 2 thiol groups,
- (ii) 2 primary amines,
- (iii) a carboxylic acid and primary amine,
- (iv) maleimide and thiol,
- (v) hydrazide and aldehyde,
- (vi) a primary amine and aldehyde.

On the other hand, the noncovalent bonding proceeding by physical association of targeted ligands to the nanocarrier surface has the advantage due to avoiding of rigorous, destructive reaction agents. However, there are some problems, such as weak bonding and low control of reaction. Also, the ligands may not be in the desired orientation after the decoration process [9].

4.1. Amide Group. The formation of amide bond proceeds by two stages. During the first one, carboxylic acid groups

SCHEME 1

on the carrier surface are activated by EDC 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide. This reagent is usually applied as carbodiimide which can form different chemical structures [77]. EDC has good solubility in water. This property enables direct application of EDC in aqueous solutions without addition of any organic compounds. These conditions are suitable for the attachment of bioactive molecules to the carrier surface. During the reaction of EDC with carboxylic group, the active form of intermediate product O-acylisourea ester is formed. The latter reacts with primary amine forming amide bond (Scheme 1).

The main advantage of this method is that applied ligand does not need any preliminary modifications usually causing the loss of its activity [78, 79]. The possibility of activation of carboxylic groups derived from peptide could be some inconvenience in the case of amino acids. These carboxylic groups should have been locked, for example, by NHS (N-hydroxysuccinimide). Ishida et al. [80] presented the addition of transferrin to liposome decorated by polyethylene glycol. They employed DSPE-PEG-COOH system which was the source of required carboxylic acid groups. The product in the form of TF-PEG-liposome has interesting properties: long time of biodistribution and large accumulation in brain tumor. Also, Maruyama [81, 82] or Blume [83] et al. described the formation of amide bond between ligand and liposome surface decorated with PEG-COOH. Zeng's group [84] reported the attachment of EGF with carboxylic group of micelle. The product was employed in the targeted delivery of drug. Also the formation of amide bond formed on the carbon nanotube surface was reported. Ou et al. [85] reported the SWCNTs modification by PEG and antibody (mAb), which was selectively encapsulated by integrin receptor of cancer cells. This process is schematically shown in Scheme 2.

The SWNT-PEG-mAb is characterized by a high stability, low toxicity, and high dispersion in water environment. The product was effectively captured by receptors of cancer cells [85]. Zhang et al. [86] modified oxidized MWCNT surface by biocompatible polyamidoamine dendrimer (PAMAM). The PAMAM chains were successfully conjugated into the surface of carbon nanotubes. This was confirmed by TEM images. The MWCNTs-PAMAM has very good dispersion and stability in aqueous solution. Some extra tests performed by authors confirmed the efficiency of application in gene therapy [86]. Dvir et al. [87] showed the amide conjugation between targeted ligand and liposome. This system during *in vitro* tests was effectively used against cardiac cells [87]. Chou et al. [88] described the amide conjugation between antibody and functionalized multiwalled carbon nanotubes. This system was applied successfully in targeted photothermal therapy. Authors confirmed the better photothermal effect

of the f-MWCNTs connected with antibody for cancer cell destruction.

The above described information was connected with the activation of the carboxylic group on the surface of nanocarrier. Alternative method is associated with the activation of primary amine of nanocarrier as shown in Scheme 3.

This process takes place when homobifunctional dithio-bis(succinimidyl propionate) (DSP) is used [77]. The DSP was used in the synthesis of drug carrier which was applied in the targeted breast cancer therapy [89]. The DSP activates amine groups of carriers. Additionally, application of NHS allows the formation of ester during the reaction with monoclonal antibody (trastuzumab). The methodology was very efficient and selective throughout cancer gene therapy.

4.2. Thioether Group. Thioether bond is formed during the reaction between thiol group and C_1 carbon of maleimide which is attached to the R_2 carrier (see Scheme 4). The reaction runs quickly and under mild conditions: at the room temperature and in aqueous solution [12]. Formed thioether bond is stable within 24 hours in human serum even in the presence of reduction agent, for example, DTT [77, 78, 90]. Unfortunately, the selectivity towards the thioether group formation is quite low in aqueous solution, due to the side reactions, such as intermolecular rearrangement or formation of disulfides. The efficiency of reaction could be increased through addition of activating agents, for example, SPDP N-succinimidyl-3-(2 pyridyldithio) propionate or SATA N-succinimidyl-S-acetylthioacetate. Application of the systems containing the thioether bonds guarantees the high selectivity of delivery and the long time of distribution [12, 78].

Kirpotin et al. [91] described the synthesis of a selective DDS based on liposome, cholesterol, and PEG modified DSPE. Antibodies were conjugated to the nanocarrier through free thiol group. The synthesis is based on two ideas. First is the coupling of antibody with double layer of liposome. Alternatively, the second provides a simple conjugation of ligand to the distal end of PEG chains. Authors demonstrated that the uptake of anti-HER2-immunoliposome correlated with density of cancer cell surface and with the effect of tumor reduction. The efficiency of the process has been increasing with increasing amount of encapsulated antibody Fab. Similar techniques with the use of different antibodies were applied successfully by, for example, Maruyama et al. [81], Allen et al. [92], Hansen et al. [93], Zalipsky et al. [94], Park et al. [95], and Ren et al. [96]. Produced systems were effectively used in drug delivery, especially for doxorubicin.

Anhorn et al. [97] were pioneers in the synthesis of DDS based on monoclonal antibody trastuzumab and nanoparticle doped with doxorubicin. The conjugation had a nature of thioether bond. The covalent bonding took place between thiol group of ligand and maleimide fragment of a carrier (Scheme 5). Initially, the carrier was modified by molecules of doxorubicin. Nanoparticles were activated through poly(ethylene glycol)-R-maleimide- ω -NHS ester. Applied ligand enabled effective uptake of anticancer system by HER2 receptor of breast cancer cells. The drug is characterized by a long time of circulation in blood without

SCHEME 2

any side effects. Nanoparticles had higher loading capacity of doxorubicin comparing with liposomes.

The bioconjugation of protein to nanocarrier by thioether bond was described by Gindy's group [98]. The carrier EG-*b*-PCL was modified by maleimide. The BSA was used as targeted ligand containing SH fragments. The process occurred in aqueous solution. The covalent bioconjugation

between albumin and nanocarrier was confirmed by the analysis of the nanocarrier volume before and after the process. Higher concentration of protein in solution during the reaction caused increase in the number of BSA-nanoparticle bonds. The targeted nanoparticles modified by thioether bond between ligands, that is, aptamers and nanocarrier, were shown by Farokhzad et al. [99] and Xiao et al. [100].

SCHEME 3

SCHEME 4

The nanoparticle-aptamers system was successfully applied in anticancer therapy. The therapeutic effect was enhanced. Also, Lu et al. [101] successfully applied thioether bond between folic acid and functionalized carbon nanotubes. This new system was characterized in special properties to the application in targeted delivery of DOX against cancer treatment.

Alternative way is the coupling of thiol groups of the nanocarrier with maleimide fragments from the ligand. The thiol groups could be activated via amines or carboxylic acid groups (Scheme 6).

The amide and thioether are the main bond types used to form the ligand-nanocarrier systems. Other connection types are only the derivatives [12].

4.3. Disulfide Group. The disulfide bond is formed by the conjugation of two thiol groups (Scheme 7). The first group originates from a nanocarrier while the other from ligand.

The reduction of disulfide functionalities [102] or the application of suitable agents as SATA or SPDP [103] can form thiol groups of a ligand. In order to form the thiol groups on nanocarrier surface, one should modify it with PDP-PE, PDP-SA, and PDP-PEG-DSPE, where PDP fragment is a source of thiol groups [12].

In [104] the formation of disulfide bond between liposomes and monoclonal antibody anti-My9 was described. The carrier surface was decorated with PDP-SA. The ligand was modified with SPDP and kept its own immunoreactive level after the modification. The obtained system in this way acts strongly against human HL-60 promyelocytic leukemia cells [104]. The formation of disulfide groups between targeted ligands (nanobodies) and micelles was shown by Talelli et al. [105].

4.4. Acetyl-Hydrazone Group. The conjugation of hydrazide groups to the nanocarrier surface occurs with aldehyde

groups of the ligands (Scheme 8) [77, 78]. As generally ligands do not possess the aldehyde structure, the latter must be formed *via* oxidation of hydroxyl groups. The oxidative agents are usually sodium periodate [106] and galactose oxidase [107]. The major advantage of this method is the rigorous control of the ligand modification [108]. However, the yield of the process in this technique is very poor [93].

Harding et al. [109] described the linking of antibodies C225 with liposome through acetyl-hydrazone group formation. The *in vivo* study showed that the activity of antibody in produced immunoliposome was fully kept. The system was characterized by long time of distribution in blood and high immunological level. Using this methodology, one can easily control immunoliposome-structure synthesis.

4.5. Polycyclic Group. The Diels-Alder reaction (Scheme 9) is the cycloaddition reaction between a diene and a dienophile. As a result, bicyclic compound is formed. The coupling of ligands and nanocarriers is beneficial because of high yield (close to 100%) and easy synthesis under mild conditions [77].

The system of ligand-nanocarrier formed in the DA reaction causes specific bonds creation between ligand and cancer cells [12]. Shi et al. [110] used the DA reaction to conjugate antibody anti-HER2 with the polymeric nanocarrier (Scheme 10). The carrier was synthesized *via* a soft templating method. The furan group on the external surface of carrier was the diene while maleimide group of antibody was the dienophile. Authors demonstrated the ability of this technique in creation of bioactive immunonanoparticles and concluded that the versatility of the nanoparticle system can be extended to create multiple functional delivery vehicles. Formed immunosystem was tested against breast cancer [111, 112].

4.6. The “Click Chemistry”. The term “click chemistry” (CC) was developed by Kolb’s group [113] and describes the coupling of small elements and heteroatoms in the form of R-X-R. Reactions belonging to “click chemistry” group are characterized by high efficiency, stereospecificity, and harmless side products. These processes need (i) mild conditions, (ii) readily available reagents, and (iii) easily removed solvent, for example, water [77].

Formed product should be stable under physiological conditions and should be easily isolated. The main method of the purification of final product is crystallization or distillation [113]. Within the CC, one can find four major types [113, 114]:

- (i) Cycloadditions, for example, Huisgen catalytic cycloaddition (Scheme 11);
- (ii) nucleophilic substitution chemistry, for example, ring opening of heterocyclic electrophiles (Scheme 12);
- (iii) carbonyl chemistry of the “nonaldol” type, for example, formation of ureas, thioureas, and hydrazones (Schemes 13-14);

SCHEME 5

SCHEME 6

SCHEME 7

SCHEME 8

SCHEME 9

- (iv) additions to carbon-carbon multiple bonds, for example, epoxidation and dihydroxylation (Schemes 15-16).

The major type of “click chemistry” reactions is the Huisgen cycloaddition. This process creates 1,2,3-triazole by the 1,3-dipolar cycloaddition between azides and terminal alkynes in the presence of the catalyst, Cu (I) [12, 77, 115]. The most probable mechanism of the reaction is shown in Scheme 17 [114].

Huisgen's cycloaddition requires reagents tolerating aqueous solution with broad range of pH level and biological molecules [114]. The effective ligand-liposome conjugation

based on Huisgen's cycloaddition was described by Hassane et al. [116]. The ligand was α -D-mannose derivative with azide group. The liposome possessed groups with triple bonds. Reported studies confirmed the essential role of addition of Cu (I) in order to increase efficiency. The synthesized product was almost perfect selective immunosystem [116].

The De et al. [117] results confirmed the possibility of “click chemistry” application in targeted anticancer therapy in order to conjugate the folic acid to the micellar nanocarrier. Also, Lu et al. [118] decorated nanoparticles by selective ligands using Huisgen's cycloaddition. The process was effective and almost 400 peptide molecules, modified coumarin, were attached to each nanoparticle. Fluorescence intensity of

SCHEME 10

SCHEME 11

coumarin confirmed the efficiency of the reaction between peptide connecting alkyne group and nanoparticle with azide group [118]. Carbon nanotubes can also be modified by Huisgen's cycloaddition. Carbon nanotubes functionalized by Huisgen's cycloaddition were used in delivery of methotrexate to breast cancer cells [18].

We described here only the most popular covalent ligand-nanocarrier bonds which are often used in practice. However, up to date, much more types of covalent bonds are

known. The other, covalent link, carbamate bond, between targeted ligand and liposomes was shown by Sawant and Torchilin [119]. An alternative and effective functionalization method is the formation of amide bonds between azides and triphosphine known as Staudinger's reaction. Generally, it occurs at room temperature and in aqueous solution and does not require any catalyst. Zhang et al. [120] attached glycoliposome with lipid containing triphosphine fragments by Staudinger's reaction. This method is applicable to water soluble molecules.

Another type of covalent bond is the immobilization of primary amine with free aldehyde group of ligand by formation of Schiff's base [78]. This technique was used during the linking of the transferrin (TF) with poly(lactic acid) surrounded by cholesterol chains containing indomethacin molecules. The TF-nanocarrier was studied against glioma cell. Experiment confirmed the bioactivity of transferring [121].

The main covalent interactions and essential conditions of the processes are shown in Table 1.

5. Physical and Chemical Bonding of Drug to Nanocarrier Surface

Drugs applied in targeted anticancer therapy could be accumulated outside and/or inside of nanocarriers (Figure 4). The couplings can be nonspecific (e.g., adsorption) or covalent [122].

The entrapment of a drug to nanocarrier is possible during the synthesis of nanocarrier or after the carrier formation. The loading of chemotherapeutic agent depends on its solubility in the nanoparticle matrix, the mass of the molecule, the interaction of drug nanocarrier, and the type of functional groups present on a nanocarrier surface [123]. A nanocarrier should be chemically resistant and should possess high purity. These properties are essential to control delivery of drug [124].

Nanocarriers are often modified before the entrapment of drugs. The most popular method of functionalization employs polymers. The medical practice uses both natural and synthetic polymers, for example, poly(ethylene glycol), poly(lactic acid), N-(2-hydroxypropyl)methacrylamide copolymer, poly(L-glutamic acid), and poly(D,L-lactic-co-glycolic acid) [124]. The bioconjugation of drug with polymer leads to the extension of chemotherapeutic circulation and controlled drug release in targeted place [125–129]. PEG has high solubility in water. Moreover, it is a good “candidate” for application in medicine because of its biochemical properties: biodegradation, minimal toxicity, and controlled mechanical properties [125, 130]. Generally, it is important to apply the polymeric phase because it improves the drug accumulation in targeted place and generates the barrier between nanocarrier and cancer cell [126, 129].

5.1. Drug on the Internal Surfaces Nanocarriers. The internal walls of a nanocarrier (e.g., of CNT) in contrast to the external walls reveal high interaction energy with adsorbed molecules. The entrapped drug molecules inside the nanocarrier are

TABLE 1: Types of covalent ligand-carrier bindings.

Name	Functional group of nanocarrier	Functional group of ligand	Conditions of process	Literature
Amide group	Carboxylic	Primary amine	EDC, NHS, pH 7.5–8.5, 2–24 h, 4°C lub RT	[98, 102–108]
Thioether group	Maleimide	Thiol	pH > 7, 4–24 h, RT	[102, 111–117]
Acetyl-hydrazone group	Hydrazide	Hydroxylic	24 h, 5–6°C	[124]
Disulfide group	Thiol	Thiol	pH 8.0, 2–24 h, 4°C or RT	[120]
Diels-Alder	Furan	Maleimide	pH 5.5, 2–6 h, 37°C	[125, 126]
“Click chemistry” (HDC)	Azide	Alkyne	Cu (I), RT, 2–3 h	[131–134]
Staudinger	Azide	Triphosphine	PBS, pH 7.4, 6 h, RT	[135]
Schiff's base	Primary amine	Aldehyde	pH 9.2, RT	[138]

Nucleophilic substitution chemistry

SCHEME 12

“Nonaldol” carbonyl chemistry

SCHEME 13

SCHEME 14

FIGURE 4: The drug conjugation to surface of a carrier and an entrapment process (the figure is based on [122]).

isolated from external environment and they are protected against early activation and degradation process. Thus, the interaction of chemotherapeutic with healthy tissue is impossible [131, 132].

Drug molecules can be entrapped by nanoextraction and/or nanocondensation process (Figure 5). Nanoextraction process occurs in liquid phase and at the room temperature. The idea is based on the selection of solvent adequate to the guest molecule. Entrapped drugs should have poor affinity to the solvent and high affinity to the nanocarrier. Weak solubility of guest molecules in the solvent enables formation of the suspension in liquid phase and the gradual diffusion inside the nanocarrier [133].

Nanocondensation, similarly as nanoextraction process, is performed in liquid phase also at the room temperature.

This process is relatively easy and quick. Guest molecules should demonstrate high affinity to the solvent and to the nanocarrier. Generally, nanocondensation process is based on the adsorption of solvent molecules on the external and internal walls of nanocarriers. Guest molecules migrate through the thin layer of a solvent and are adsorbed in the most active centers *via* the van der Waals interactions [133].

Nanoextraction process was utilized by Ren and Pastorin [19]. Authors loaded the drug, hexamethylmelamine, inside

Carbon multiple bond addition

SCHEME 15

SCHEME 16

SCHEME 17

CNTs. C60-fullerenes were used as caps of open CNTs ends. In the first step, CNTs were treated by a mixture of acids (HNO_3 , H_2SO_4). During this process, CNTs were opened and functional groups (mainly carboxylic) were generated. Carboxylic fragments created the barrier for other molecules compared to the drug ones. Authors proved that suitably functionalized CNTs are good candidates for drug nanocarriers. The stable structures of CNTs and C60 protected drug molecules from circulation in blood before they were released at specific places [19].

Studies concerning the cisplatin loaded inside SWCNTs were reported by Tripisciano et al. [134]. The system of SWCNTs-cisplatin was used against colon cancer. Authors

utilized a mixture of HNO_3 and H_2SO_4 acids in order to open SWCNTs. The drug was loaded inside SWCNTs by nanocondensation process. Carbon material was dispersed in dimethylformamide (DMF) containing cisplatin (DDP). Carbon nanotubes limited the drug precipitation to aqueous solution and its degradation. Analysis of FTIR, Raman spectroscopy, HR-TEM, and EDX results confirmed the presence of cisplatin inside SWCNTs structure. Authors observed the proportional correlation between the anticancer properties and the amount of SWCNTs-DDP. The new DDS caused similar effect on the cancer cells as free drug. However, the novel system minimized the presence of side effects. Another work of Tripisciano et al. [135] reported the nanocondensation

TABLE 2: Effectiveness of DDP delivery by SWCNTs [134] and MWCNTs [135].

	SWCNTs	MWCNTs
The amount of DDP inside CNTs	21 $\mu\text{g}/100 \mu\text{g}$ of CNTs	13.6 $\mu\text{g}/100 \mu\text{g}$ of CNTs
The amount of released DDP	68%	95%
The rate of DDP release	Slower	Faster
The maximum release of DDP	72 h	48 h

FIGURE 5: Scheme of the nanoextraction and nanocondensation processes (the figure is based on [133]).

process used to the loading of cisplatin inside the MWCNTs. The method of entrapment process was similar to SWCNTs. Table 2 summarizes the results for SWCNTs and MWCNTs.

It is interesting that almost all drug molecules were released from MWCNTs. This confirms the fact that carbon nanotubes can be successfully used as drug containers and therefore they could serve as DDS. Moreover, according to the authors, inside DDP-MWCNT systems the specific interaction did not occur, in contrast to DDP-SWCNTs. Those interactions are probably responsible for slower and smaller percentage release of DDP from SWCNTs. Likewise, Li et al. [136] and Sui et al. [137] described the encapsulated DDP inside carbon nanotubes. Authors confirmed the improved efficiency of anticancer therapy.

Another interesting approach is the entrapment of DDP inside the nanoparticle covered with copolymer. Gryparis et al. [138] used this container against colon cancer. The

drug kept its activity after loading inside the nanocarrier. It was delivered selectively to cancer cell. The therapeutic effect was better comparing with the traditional chemotherapy. The DDP-nanoparticle system was safe for human organism. Cisplatin can be also adsorbed within PMs [139, 140] or liposomes [141, 142] or carbon nanohorns [143, 144]. The *in vivo* and *in vitro* studies were aimed against lymphoma (J6456) [141], colon (C26) [141, 142], lunge [142–144], and stomach (MKN 45) [140] cancer cell. Analysis confirmed that the DDP internalization inside nanocarriers improved the drug activity and minimized side effects [139–142].

Hampel et al. [145] used the entrapment process of carboplatin inside carbon nanotubes. They were opened and next the drug was placed inside the structure using wet impregnation method. Authors confirmed the presence of carboplatin inside the structure which protects the drug against the environment. Carboplatin inhibited the growth of blood cancer

FIGURE 6: Schematic diagram of DOX-PLGA-Au (the is figure based on [149]).

cells. The results confirm efficiency of carbon nanotubes as chemotherapeutic nanocarriers [145]. In another interesting work [146], an encapsulated Pt(IV) prodrug inside polymeric nanoparticle was described. Additionally, the targeted ligands were conjugated to the surface of nanoparticle. This DDS inhibited growth of breast as well as prostate cancer cells. This fact was connected with the protection of prodrug structure by nanoparticles. Moreover, the biodistribution of novel system was better than that in the case for cisplatin. Mitra et al. [147] reported the DDS based on doxorubicin inside nanoparticle (diameter ca. 100 nm). Drug was linked with additional remedy, dextran. The conjugation limited side effects of doxorubicin. The nanoparticle-DOX system improved therapeutic effect of drug. Authors observed the reduction of tumor volume after 4 weeks. Doxorubicin time circulation in blood and amount inside cancer cell were better in comparison to the traditional chemotherapy.

Lince et al. [148] and Park et al. [149] described process of doxorubicin entrapment inside nanoparticle. Lince et al. [148] showed nanoparticle based on biodegradable and biocompatible copolymer. The polymeric nanocarrier improved activity of drug which was placed inside and limited its potential side effects. Thus, the comfort level and quality of patient's life increased. Park et al. [149] as well as Lince et al. [148] described the encapsulation of doxorubicin inside polymeric nanoparticle. The polymer nanocarrier was covered with the extra gold layer (Figure 6). The DOX-PLGA-Au system combined traditional chemotherapy and phototherapy. The efficiency was examined against cervical carcinoma. The novel therapy reveals high therapeutic efficiency and short time of treatment. The DOX-PLGA-Au system delivered selectively drug to cancer cell and thus caused rise of temperature, which destroys cancer tissues. The warmth was generated as a result of absorption of NIR radiation by gold layer.

Doxorubicin in targeted anticancer therapy was used successfully within liposomes by Matsumura et al. [150] or Gabizon [151] and within micelles by Kataoka et al. [152], Perche et al. [153], Ebrahim Attia et al. [154], or Cambón et al. [155]. Paclitaxel similarly as cisplatin or doxorubicin can be adsorbed inside the nanocarrier. Koziara et al. [156] used this drug inside nanoparticle *in vitro* against brain cancer cells U-118 and HCT-15. Authors proved the rise in cytotoxic

FIGURE 7: Schematic structure of NK105 (the is figure based on [160]).

activity of drug and the limitation of the transmembrane pump action. Thus, the uptake of a drug by cancer cell was facilitated. The new system penetrated through blood-brain barrier. The stability of PTX was maintained during the entrapment process. Ruan and Feng [157] proved that the release from nanoparticle is easy when the nanoparticle is covered with copolymer PLA-PEG-PLA. Probably this effect occurs because hydrophilic fragments of PEG inside hydrophobic fragments of PLA increase the porosity [157]. Fonseca et al. [158] and Chan et al. [159] also described the immobilization of paclitaxel inside polymer nanoparticles. Authors proved the efficiency of this method [158]. Hamaguchi et al. [160] proposed the new form of PTX-NK105, namely, the drug inside micelle (Figure 7).

The delivery of NK105 to organism runs without Cremophor EL and ethanol in contrast to the traditional anticancer therapy. The NK105 shows lower toxicity against nervous system and better activity [160]. Similarly, Kim et al. [161] and Wang et al. [162] located paclitaxel within polymer micelle. They confirmed the efficiency of novel form.

5.2. Drug on the External Surfaces of Nanocarriers. To construct the delivery system with a drug on external surface of a nanocarrier, the chemical or physical conjugation is applied. This method is based on chemical properties and high surface area of a nanocarrier [132]. Drug molecule is linked with functional groups of nanocarrier or polymer covering nanocarrier by covalent bonds forming ester, amide, or acetyl-hydrazone groups. This type of coupling has usually low stability [163] and shows pH sensibility [164]. Drugs with plane or aromatic structure can be adsorbed on the surface of nanocarriers by π - π interactions [163].

5.2.1. Ester Group. The ester bond formation between, for example, doxorubicin and nanocarrier, is possible as a result of

- (i) the bioconjugation of drug's primary hydroxylic group ($-C=OCH_2OH$) with polymer's carboxylic group, for example, PLGA surrounding nanocarrier (Scheme 18);
- (ii) the bioconjugation of drug's primary amine group with polymer's hydroxylic group (Scheme 19).

SCHEME 18

Yoo et al. [127, 128] described the formation of ester bond between DOX and nanocarrier covered with PLGA. The conjugation takes place between hydroxylic and carboxylic groups [127] and between amine and hydroxylic groups [128]. The novel systems were *in vivo* and *in vitro* tested against cancer cell line HepG2. For both of the cases, drug delivered on the external surface was better absorbed by cancer cell than the drug placed inside nanocarrier. Authors showed that the release of drug from nanocarrier is correlated with the molecular mass of polymer placed on the surface of nanocarrier [127].

Another anticancer drug as, for example, paclitaxel, can be conjugated to nanocarrier by ester bond. Li et al. [165] showed the bioconjugation of paclitaxel with polymeric nanocarrier covered by poly(L-glutamic acid) (PG). The therapeutic activity of drug against OCa-1 cancer cells was higher than during traditional chemotherapy. Authors proved that

the PTX-PG conjugation has lower toxicity than free PTX. The novel system was better bonded to cancer cells and its circulation time was longer. It was confirmed that labile ester bond formation between polymeric nanocarrier and chemotherapeutic agent is useful method for the designing of targeted DDS. Milas et al. [166] used the same idea to link paclitaxel with polymeric nanocarrier and confirmed the efficiency of the method.

5.2.2. Acetyl-Hydrazone Group. The hydrazone bond can be formed *via* the conjugation of carbonyl group with hydrazide one. This nanocarrier-drug linking has high cytotoxicity level against selected cancer cell lines [167]. Doxorubicin possesses the carbonyl group which can combine with hydrazide group on the surface of nanocarrier covering polymer [168]. The most popular polymers described in the literature are poly(allyl glycidyl ether) [169] (Scheme 20),

SCHEME 19

N-(2-hydroxypropyl)methacrylamide copolymer [167], and poly(ethylene oxide)-block-poly(allyl glycidyl ether) [168].

Vetvicka et al. [169] proposed micellar arrangement based on amphiphilic, diblock copolymer, and doxorubicin. This system shows 20-time lower toxicity and longer time of circulation than the free drug. The therapeutic activity against lymphoma EL-4 T was promising, and 75% of mice population was cured completely and showed specific resistance against new cancer cells [169].

Hrubý et al. [168] as well as Liu et al. [170] described the formation of acetyl-hydrazone group between doxorubicin and nanocarrier covering poly(ethylene oxide)-block-poly(allyl glycidyl ether) [168] and poly(ethyleneimine)-polyethylene glycol [170], respectively. Drug release was pH-sensitive: it was faster in pH similar to that of endosomes (pH 5.0) than in pH of blood plasma (pH 7.4). Probably, this effect is related to the shift of equilibrium between free and bonded molecules of doxorubicin to the direction of drug release in lower pH [168]. Yoo et al. [171] showed the hydrazone bond between doxorubicin and polymeric micelles. Drug was linked to the terminal hydrazide groups on the nanocarrier, poly(lactic acid) and methoxy-polyethylene glycol.

Doxorubicin was delivered quickly to the cancer cell and possessed high cytotoxicity [171].

Platinum drugs structural properties allow the formation of the hydrazone bond between drug and nanocarrier. Aryal et al. [172] described the bioconjugation of copolymer PEG-PLA with the hydrazide moieties PEG-PLA-NH-NH₂ with levulinic acid modified with Pt(IV) cisplatin analogue. The amount of the associated drug molecules was controlled. Polymeric nanoparticles increased the cytotoxicity properties against ovarian cancer cells. The drug loss during nanocarrier circulation in the blood was low in natural pH [172].

The hydrazone bond is used for the bioconjugation of paclitaxel with dendrimeric nanocarrier. It was claimed that also polyamidoamine dendrimers (PAMAM) could be treated as nanocarrier of chemotherapeutic agents. The core is built up with alkyl-diamine with tertiary amine branches. Amine groups allow the easy functionalization of dendrimer with the drug. The modification of the nanocarrier surface by paclitaxel was used against ovarian cancer cells. The targeted ligand (protein LXW7) was also attached to the nanocarrier. The method was effective and eliminated cancer cells. The drug-nanocarrier bond was broken at low pH

SCHEME 20

level and the drug was released [173]. Rodrigues et al. [174] demonstrated the bioconjugation of paclitaxel with polymeric nanocarrier, polyethylene glycol. The reaction required the preliminary modification of chemotherapeutic agent. The maleimide derivatives of paclitaxel were formed by bonding the maleimide fragments with drug molecule. At the first step, the synthesis of drug ester derivative in position C-2'-OH by using 4-acetylbenzoic acid occurred. The intermediate product is a donor of carboxylic group which was essential to the attachment of maleimide derivative containing hydrazide fragment. Authors proved the efficiency of the method. The structure of drug was protected and its activity was improved via *in vitro* and *in vivo* studies.

5.2.3. Amide Group. Amide bond is formed during the reaction of carboxylic group with a nucleophile containing the primary amine. Feazell et al. [175] described the formation of amide bonds between platinum compound and SWCNTs. Functionalized SWCNTs were covered by phospholipid containing amine groups. The compound *c,c,t*-[Pt(NH₃)₂Cl₂(OEt)(O₂CCH₂CH₂CO₂H)] as prodrug was bonded to the modified carbon nanotubes. The novel system was taken up effectively by endosomes, where pH level is low, and as a consequence cisplatin (DDP) which was released as active compound. This method enhances weak circulation and distribution of drug in blood and minimizes the DDP toxicity against healthy cells [175].

Liu et al. [48] described the conjugations between paclitaxel and SWCNTs by the amide bond. The structure of SWCNTs was functionalized by polyethylene glycol with amine fragment (PEG-NH₂). The carboxylic group was introduced to the drug structure in C-2'-OH position. Drug showed better solubility than clinical form of paclitaxel, Taxol. Additionally, the SWCNTs-PTX system showed longer time distribution in the blood, the 10-time larger uptake by cancer cells, and stronger therapeutic effect in comparison to Taxol. The new form of paclitaxel minimized the tumor

volume at drug doses as small as 5 mg/kg. The side effects were lower than those during traditional therapy [48].

Doxorubicin was attached to the nanocarrier using the amide bond in [166, 176]. Lai et al. [177] discussed the bioconjugation of drug to the dendrimer. The efficiency of novel system was examined against gingival cancer cell line Ca9-22. The cytotoxicity was improved comparing with free drug.

5.2.4. Disulfide Group. Disulfide bond between drug and nanocarrier is created in order to obtain the systems for selective delivery and accumulation of and large amount of drugs. Most of the drugs and nanocarriers do not possess thiol groups in the structure and thus the method requests preliminary modification. The chemotherapeutic structures possess usually amine and carboxylic groups and these groups may be linked with peptides, the source of the thiol groups. This makes the formation of disulfide bond possible [164].

Paclitaxel bonded by disulfide coupling to SWCNTs was used against leukemia line L1210FR. The disulfide linkage was attached in C-2' position of the drug. SWCNTs were modified by oxidation, conjugation of amide fragments, and functionalization by amine groups. Biotin was used as the targeted ligand. The new DDS successfully destroyed cancer cells. The chemotherapeutic agent was released when the PTX-SWCNTs were entrapped inside cancer cells [178].

5.2.5. Adsorption. Chemotherapeutic agents can be accumulated on the external surface of nanocarriers also by physical adsorption (e.g., by electrostatic interactions between nanocarrier surface and biomolecule) [179–190].

Kataoka et al. [180] described physical adsorption of doxorubicin on the surface of micellar nanocarrier (poly(ethylene glycol)-poly(L-benzyl-L-aspartate)) (PEG-PBLA). The drug-nanocarrier system was formed by the π - π interactions between anthracycline groups of DOX and benzyl residues of PBLA segments. Drug on the nanocarrier revealed greater activity comparing with the free drug. Micelles allowed

longer circulation time of doxorubicin in the blood. Greish et al. [181] proved 13-time higher drug concentration inside tumor S-180 when the doxorubicin was loaded on the surface of polymeric micelle. The new system showed better therapeutic properties and lower side effects than what is observed in traditional therapy [180, 181].

Another study [182] reported the noncovalent attachment of doxorubicin to carbon nanotubes. MWCNTs were covered by triblock copolymer Pluronic F127. Authors proved the rise of DOX cytotoxicity against breast cancer cells line MCF7 in comparison to standard therapy. The use of MWCNTs as drug nanocarrier improved the activity of drug and its uptake by cancer cells. The efficiency of this MWCNTs-DOX complex was determined by effective release of drug in suitable time and place [182]. Heister et al. [183] presented a noncovalent complex of doxorubicin with single-walled carbon nanotubes. As targeted, ligand monoclonal antibody was used. Authors proved that SWCNTs can be applied in order to improve the DDS. High activity of DOX adsorbed on the surface of oxidized carbon nanotubes or modified by polycarbohydate, PEG, PEG44-PPS20 copolymer, and poly(acrylic acid) was proved by Wang's [191], Zhang's [184], Liu's [185], Di Crescenzo's [186], and Lu's [101] groups, respectively.

Paclitaxel can be adsorbed on the modified surface of nanocarrier. Tian et al. [187] adsorbed PTX on carbon nanotubes by π - π interactions. MWCNTs were covered with polyethyleneimine. Next the reaction was performed with folic acid as targeted ligand. Drug showed the rise of solubility and was selectively taken up by cancer cells. The activity *in vitro* of paclitaxel loaded on the surface of carbon nanotubes was greater in comparison to free drug used during traditional therapy [187].

Adsorption of platinum complex is determined by the stability of nitrogen ligand and the mobility of chloride ion. The positively charged platinum aqua-complex is strongly adsorbed on nanocarriers. Adsorption of cisplatin is determined by electrostatic interactions [188]. Barroug et al. [189] studied cisplatin adsorption on nanocarrier and showed the rise of drug adsorption and the rate of drug release with the rise in temperature. The cytotoxicity was tested *in vitro* against K8 clonal murine osteosarcoma cell line. Electrostatic interactions between cisplatin and the surface of nanoparticle did not change the drug activity. Cisplatin analogue, that is, carboplatin, was adsorbed on the surface of carbon nanotubes by Arlt et al. [190]. Adsorbed drug stopped the growth of tumor, and the efficiency of loaded chemotherapeutic agent was greater than observed for free carboplatin.

Bonding by electrostatic interactions as the method of the drug and nanocarrier bioconjugation is less popular in comparison to covalent linking. The covalent couplings are preferred due to the possibility of controlling the drug amount and the orientation during synthesis [179].

We described only selected drug accumulation possibilities. Ionic drug complexes for example cisplatin with nanoparticles based on hyaluronic acid [192] or doxorubicin with ionic complex [193] are also discussed in the literature. The covalent functionalization by 1,3-dipolar cycloaddition is also widely used for the bioconjugation of drugs and nanocarrier. Terminal amine groups on the nanocarrier

surface are ideal and reactive centers for the coupling of biomolecules [45]. Pastorin et al. [194] discussed the functionalization of MWCNTs via 1,3-dipolar cycloaddition. Active groups formed on the surface of carbon nanotubes were attached with active carboxylic fragments of drug, methotrexate. This coupling minimized the intracellular uptake barrier and caused the increase in the efficiency.

Next interesting attempt is the attachment of two anticancer drugs to one nanocarrier. Few nanocarriers (e.g., polymeric nanoparticle and liposomes) have characteristic properties which are essential during the coupling of different chemotherapeutic agents. Drugs attached together should be easily hydrolyzed, because they are independently aggregated inside cancer cells [20]. This methodology minimizes the drug resistance of cancer cells and releases essential drug dose [195].

Aryal et al. [20] showed the accumulation of paclitaxel and gemcitabine hydrochloride on the nanoparticle surface. The cytotoxicity of drugs comparing with their free analogs was greater against human pancreatic cancer cells XPA3 [20]. Similarly, Zhang et al. presented the linking of doxorubicin with docetaxel [196].

6. Summary

Synthesis of a novel DDS, by application of nanomedicine recipes, is very important in anticancer therapy. Application of nanocarriers can improve the activity of drugs. Nanocarriers isolate drug molecules from biological environment and consequently minimize the enzymatic degradation of chemotherapeutic agent [125]. Additionally, nanocarriers cause the rise in solubility and prolong the time of distribution in the blood. Those novel systems break the biological barriers, for example, blood brain. As a consequence, a drug is delivered into the targeted cells, hardly available during standard chemotherapy. The new DDS as targeted ligand-nanocarrier drug fulfills all requirements of the effective and safe anticancer therapy: (i) adequate concentration, (ii) effective dose, and (iii) high cytotoxicity of chemotherapeutic agents [195]. The delivery of drug by the application of nanocarriers opens the way to the treatment of diseases showing so-called multidrug resistance [122]. Drugs can be aggregated on the external or internal surface of a nanocarrier. Suitable modification of nanocarrier facilitates the chemical bioconjugation of drug or targeted ligand *via* the formation of the functionalities as amide, ester, disulfide or acetyl-hydrazone groups. The most important factor is the structure of drug or ligand determining the type of reaction.

The results of recent *in vitro* and *in vivo* studies [195–198] suggest that described novel DDS should be more effective and successful against cancer cells in comparison to traditional chemotherapy. Application of nanovehicles in targeted active treatment seems to be very promising direction in current material science and medicine.

Abbreviations

BSA: Bovine serum albumin
CC: Click chemistry

CNT:	Carbon nanotube
DA:	Diels-Alder reaction
DDP:	Cisplatin
DDS:	Drug delivery system
DMAP:	N,N-Dimethylaminopyridine
DMF:	Dimethylformamide
DOX:	Doxorubicin
DSP:	Dithiobis(succinimidyl propionate)
DSPE:	Phosphatidylethanolamine
DTT:	Dithiothreitol
EDC:	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide
EDX:	Energy-dispersive X-ray spectroscopy
EGF:	Epidermal growth factor
EPR:	Enhanced permeability and retention effect
Fmoc-Osu:	9-Fluorenylmethoxycarbonyl-N-hydroxysuccinimide
FTIR:	Fourier transform infrared spectroscopy
HR-TEM:	High-resolution transmission electron microscopy
MWCNT:	Multiwalled carbon nanotube
NHS:	N-Hydroxysuccinimide
PAGE:	Poly(allyl glycidyl ether)
PAMAM:	Polyamidoamine dendrimer
PCL:	Poly(ϵ -caprolactone)
PBLA:	Poly(β -benzyl-L-aspartate)
PDP:	Pyridyldithiopropionate
PDP-PE:	N-[3-(2-Pyridyldithio)propionyl]phosphatidylethanolamine
PDP-SA:	N-[3-(2-Pyridylthio)propionyl]-stearylamine
PEG:	Polyethylene glycol
PEG-b-PCL:	Poly(ethylene glycol)-b-poly(ϵ -caprolactone)
PEO-PAGE:	Poly(ethylene oxide)-block-poly(allyl glycidyl ether)
PEG-PBLA:	Poly(ethylene glycol)-poly(b-benzyl-L-aspartate)
PG:	poly(L-glutamic acid)
PLA:	Poly(lactic acid)
PLGA:	Poly(D,L-lactic-co-glycolic acid)
PM:	Polymeric micelle
PPS:	Poly(propylene sulfide)
PTX:	Paclitaxel
PyBroP:	Bromo- <i>tris</i> -pyrrolidino-phosphonium hexafluorophosphate
RT:	Room temperature
SATA:	N-Succinimidyl-S-acetylthioacetate
SPDP:	N-Succinimidyl-3-(2-pyridyldithio)propionate
SWCNT:	Single-walled carbon nanotube
SWNH:	Single-walled carbon nanohorn
TEA:	Triethylamine
TEM:	Transmission electron microscopy
TF:	Transferrin.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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