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Review

Targeting nanoparticles to cancer

M. Wang a, M. Thanou a, b, *

a Imperial College London, Department of Chemistry, United Kingdom
b King’s College London Pharmaceutical Sciences Division, United Kingdom

ABSTRACT

Nanotechnology applications in medicine, termed as nanomedicine, have introduced a number of nanoparticles of variable chemistry and architecture for cancer imaging and treatment. Nanotechnology involves engineering multifunctional devices with dimensions at the nanoscale, similar dimensions as those of large biological vesicles or molecules in our body. These devices typically have features just tens to hundred nanometers across and they can carry one or two detection signals and/or therapeutic cargo(s). One unique class of nanoparticles is designed to do both, providing this way the theragnostic nanoparticles (therapy and diagnosis). Being inspired by physiologically existing nanomachines, nanoparticles are designed to safely reach their target and specifically release their cargo at the site of the disease, this way increasing the drug’s tissue bioavailability. Nanoparticles have the advantage of targeting cancer by simply being accumulated and entrapped in tumours (passive targeting). The phenomenon is called the enhanced permeation and retention effect, caused by leaky angiogenetic vessels and poor lymphatic drainage and has been used to explain why macromolecules and nanoparticles are found at higher ratios in tumours compared to normal tissues. Although accumulation in tumours is observed cell uptake and intracellular drug release have been questioned. Polyethyleneglycol (PEG) is used to protect the nanoparticles from the Reticulo-Endothelial System (RES), however, it prevents cell uptake and the required intracellular drug release. Grafting biorecognition molecules (ligands) onto the nanoparticles refers to active targeting and aims to increase specific cell uptake. Nanoparticles bearing these ligands are recognised by cell surface receptors and this leads to receptor-mediated endocytosis. Several materials are suggested for the design of nanoparticles for cancer. Polymers, linear and dendrimers, are associated with the drug in a covalent or non-covalent way and have been used with or without a targeting ligand. Stealth liposomes are suggested to carry the drug in the aqueous core, and they are usually decorated by recognition molecules, being widely studied and applied. Inorganic nanoparticles such as gold and iron oxide are usually coupled to the drug, PEG and the targeting ligand. It appears that the PEG coating and ligand decoration are common constituents in most types of nanoparticles for cancer. There are several examples of successful cancer diagnostic and therapeutic nanoparticles and many of them have rapidly moved to clinical trials. Nevertheless there is still a room for optimisation in the area of the nanoparticle kinetics such as improving their plasma circulation and tumour bioavailability and understanding the effect of targeting ligands on their efficiency to treat cancer. The need to develop novel and efficient ligands has never been greater, and the use of proper conjugation chemistry is mandatory.

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* Corresponding author at: 150 stamford st, London, United Kingdom.
E-mail address: maya.thanou@kcl.ac.uk (M. Thanou).

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1. Introduction

Nanomedicine, the application of nanotechnology to medicine, aims to overcome problems, related to diseases, at the nanoscale where most of the biological molecules exist and operate. Particularly nanotechnology application to cancer aims to bring significant breakthroughs in diagnosis, treatment and monitoring of cancer. Nanoparticles which are molecular assemblies of functional chemistries will be able to overcome biological barriers (bio-barriers), accumulate preferentially in tumours and specifically recognise single cancer cells for detection and treatment.

Today, there is a strong focus on nanotechnology application to cancer. Cancer Nanotechnology is a new field of interdisciplinary research cutting across biology, chemistry, engineering and medicine aiming to lead major advances in cancer detection diagnosis and treatment [1–4]. The field has received strong support especially in US where several centres for nanotechnology for cancer have been launched and are operating since 2004. There is no better definition and overview of this field, other than the one given in http://nano.cancer.gov/, which is termed as the NCI (National Cancer Institute, US) alliance for nanotechnology for cancer. This alliance aims to bring several teams together in a surprisingly multidisciplinary approach to explore solutions for cancer detection imaging and diagnosis [5]. In Europe a number of academic groups focus on research in this field. However it is only until recently Europe FPVII programs have announced calls for multidisciplinary team projects in nanomedicine for cancer. In the UK the major cancer research organisation (Cancer Research, UK) appears hesitant on supporting the field, possibly due to potential risks of non-tested nanomaterials. In a recent report “Roadmaps in Nanomedicine towards 2020” from Nanomedicine European technology platform (http://www.etc-nanomedicine.eu/public) specialists foresee that oncology imaging and therapy will be the main area of application of various “designer’s” type of Nanoparticles. The targeted therapies in oncology are predicted as a 30bn € global market in 2015.

The opportunity lies in the fact that for the first time we are able to tackle cancer management needs and individualise therapies by developing personalised treatments. Ideally, a clinical lab will be using nanotechnology based assays and detect tumour markers of each patient while at the same time scientists from this lab will formulate the nanoparticles using the same biomarkers as the ones found in the patient’s tumour, carrying the specific genetic drug (i.e. siRNA) designed to knock down the biomarker protein related to that tumour. Cancer biomarkers include a variety of molecules such as mutant genes, RNAs, proteins, lipids, carbohydrate and small metabolite molecules. Their altered presentation or expression is related to a biological change (expressed as neoplasia) and a clinical outcome. Molecular profiling studies can discover cancer biomarkers based on the relation between a molecular signature and cancer behaviour. By defining the interrelationships among these cancer biomarkers it could be possible to diagnose the patient’s cancer molecular profile, leading to personalised and predictive medicine [3]. A unique molecular profile can be used to predict the tumour’s invasive characteristics and metastatic potential. Molecular profiling was first reported by Golub et al. [6] who showed that gene expression patterns could classify tumours, providing information on the stage, grade, clinical course and response to treatment. Gene expression can demonstrate that the molecular signature of each metastatic tumour is a result of the combined tumoural, stromal, and inflammatory factors of the original heterogeneous tumour [7,8]. Further, cancer molecular profiling can combine cDNA microarrays with tissue microarrays for biomarker discovery and tissue immunohistochemical validation [9].

The identification of such biomarkers is very important for individualised therapy and treatment of cancer [10,11]. For example Herceptin is a monoclonal antibody targeting amplified and over-expressed Erbb2 (HER2) is a tyrosin kinase receptor found in 25–30% of breast cancers. In the process of drug approval, the FDA required diagnostic tests to detect HER2 over-expression indicating that individualised therapy for cancer is required to achieve cancer treatment without the unwanted effects. In vitro diagnostics for HER 2 now include an immunohistochemistry assay and a nucleic acid fluorescence in situ hybridisation (FISH) test to guide Herceptin treatment decisions. The design of methods that can detect in vivo the expression of such markers and monitor them during treatment is a real challenge. For the first time nanotechnology can be applied for the design of multifunctional nanoparticles that will be able to (a) detect, (b) image tumours and their metastases, (c) treat and (d) monitor treatment progression. The application and efficiency of these nanoparticles in vivo will help enormously the pre- and post-operative cancer treatment. The design of such nanoparticles is not trivial as several factors have to be taken into consideration. Primarily, the chemistry of the core and the layers needs to be done with consideration of the structural integrity and stability of the particles in biological fluids. Further, similar to the process of product drug development, nanoparticle development needs to consider physicochemical issues related to the properties of the nanoparticles, biopharmaceutical issues related to the properties of the “bio-barriers” and pharmacological issues related to the site, time and duration of nanoparticle’s action. These nanoparticles have to be considered differently to small and large molecular drugs introducing novel parameters for their design. FDA states that ADME (Administration, Distribution, Metabolism and Excretion) studies need to be redesigned in the case of nanoparticles to take under consideration their aggregation and surface chemical characteristics [12].

Most nanoparticles are expected to accumulate in tumours due to the enhanced permeation and retention (EPR) effect—a tumour characteristic that was identified by Maeda as a means to target anticancer agents to tumours [13]. Nanoparticle tumour-accumulation is deemed possible due to the highly permeable blood vessels of the tumours as a result of rapid and defective angiogenesis. In addition tumours are characterised by dysfunctional lymphatic drainage that helps the retention of nanoparticles in tumour long enough to allow local nanoparticle disintegration and release of the drug in the vicinity of tumour cells. The phenomenon has been used widely to explain the efficiency of nanoparticle and macromolecular drug accumulation in tumours [14].

Our knowledge of nanoparticle biokinetics, metabolism and clearance is poor as very few nanoparticle products have been clinically tested. Doxil® and Abraxane® are two nanoparticles for cancer therapy that have gone through preclinical and clinical evaluation and FDA approval. Doxil® is a liposomal system for doxorubicin delivery and treatment of ovarian carcinoma and Abraxane® is an albumin nanoparticle taxol conjugate for the treatment of metastatic breast cancer.

2. FDA-approved therapeutic nanoparticles for cancer

The first nanoparticles used to deliver cancer chemotherapy were the liposomes. Liposomes are usually sized at the nanoscale and consist of a lipid bilayer surrounding a water core hosting the drug. The first studies to report the efficiency of liposomes as nanoparticles focused on the improvement of pharmacokinet-ics and biodistribution of the anthracycline drug doxorubicin. The nature of doxorubicin, unfortunately also induces cardiotoxicity and this can limit the administered dose. To avoid this, doxorubicin was encapsulated in anionic liposomes, after which studies
showed that liposomal doxorubicin improved accumulation in tumours and had increased antitumour activity while cardiotoxicity was diminished [15,16]. Liposomal doxorubicin has been shown to be safe and efficient clinically, in ovarian and breast cancer [17,18]. Doxil®, the pegylated liposomal doxorubicin shows high efficiency due to improved pharmaco kinetics as it has been shown to escape the reticulo-endothelial system (RES)—an important barrier in nanoparticle systemic circulation [19–21]. Polyethylene- leneglycol (PEG) is used with a number of nanoparticles as it improves colloidal stability and prevents uptake by the RES. PEG is usually added on the surface of nanoparticles to create the so-called “steric stabilisation” effect where the PEG molecules form a protective hydrophilic layer on the surface of nanoparticles that prevents interaction with each other (aggregation) and with blood components. As a result, grafting of PEG on the surface of nanoparticles reduces uptake by the macrophages of the mononuclear phagocyte system (MPS) and prolongs the blood circulation times [22,23].

The second nanoparticle approved for cancer therapy was an albumin Taxol® conjugate, Abraxane® was designed to overcome Taxol® insolubility related issues, through binding of the drug to 130 nm albumin nanoparticles. Also known as nab-paclitaxel, Abraxane® was designed to avoid the use of Cremophor EL® solvent (polyethoxylated castor oil) that is used to solubilise Taxol® [24–26] and is the first albumin nanoparticle approved for human use by FDA. Albumin is a natural carrier of endogenous hydrophobic molecules which are bound onto it through non-covalent interactions, a type of binding interaction that is critical for pharma cokinetics (protein binding). More importantly, albumin assists endothelial transcytosis of protein bound and unbound plasma constituents principally through binding to a cell surface, a 60-kDA glycoprotein receptor gp60. The receptor then binds to caveolin-1 with subsequent formation of trancytotic vesicles (caveolae) [27]. In addition, albumin binds onto osteonectin, secreted protein acid rich in cystein (SPARC) which is present on breast lung and prostate cancer and that way albumin is accumulated in tumours [26,28]. Currently there are more than 50 clinical trials ongoing using nanoparticles for cancer. The majority of these nanoparticles are nab type (nanoparticle albumin bound) tested for the treatment of various cancer types (http://clinicaltrials.gov; search for nanoparticles and cancer on December 2009). The clinical trials also include a nanoliposomal irinotecan [29], a SPIO (superparamagnetic iron oxide) to diagnose pre-operative stage of pancreatic cancer [30] a transferrin targeted cyclodextrin polymer based nanoparticle for siRNA delivery CALAA-01 [31]. Importantly the clinical trials include studies of designed nanoparticles such as the lyso-thermosensitive liposomal doxorubicin (Thermodox®) as a novel activated therapy using radiofrequency ablation [32,33].

2.1. Cancer nanoparticles types, functionalities and modalities

During the last years a number of studies have been presented that show smartly designed nanoparticles for tumour targeting, imaging and therapy where reviews have attempted to describe and categorise these nanoparticles [34–41]. Most of the researchers agree that nanocarriers or nanovectors are nanosized materials that can carry multiple drugs and/or imaging agents [1,34]. Particularly, they consist of a scaffold (polymer or lipids), where drugs and contrast agents are attached, a corona of polymeric material that improves biokinetics and biodistribution and a ligand that adds specificity for cancer biomarker molecular recognition and attachment to cancer cells [34–36,42].

Therapeutic nanoparticles with a highly defined lipid scaffold are mainly the liposomes, discovered 40 years ago by Bangham [43,44]. Liposomes’ size is ranging from a minimal diameter of 30 nm to several microns [45]. Liposomes may vary in size, lipid composition, method of preparation and particularly surface chemistry. Liposomes have evolved through the years to a versatile carrier adapted each time to have a different functionality and serve a certain drug delivery purpose [46,47].

A progressing type of lipid based nanoparticle emerging in the therapeutic field is the Solid Lipid nanoparticles (SLN). Since their first description by Müller et al. [48,49] SLNs have attracted increasing attention as an efficient and non-toxic alternative to lipophilic colloidal drug carrier prepared either with physiologic lipids or lipid molecules used as common pharmaceutical excipients. Two production techniques have been reported: the high-pressure homogenisation described by Müller and Lucks and the microemulsion-based technique by Gasco [49,50]. In contrast to the preparation method of most polymeric microsphere and nanoparticle systems, SLN production techniques do not need to employ potentially toxic organic solvents, which may also have deleterious effect on protein drugs. Furthermore, under optimised conditions they can be produced to carry lipophilic or hydrophilic drugs and seem to fulfil the requirements for an optimum particulate carrier system. Their colloidal dimensions and their controlled release behaviour enable drug protection through administration by parenteral and non-parenteral routes thus emphasising the versatility of this nanoparticulate carrier [51,52].

Dendrimers are the main polymeric architectures that follow in the category of nanoparticles. They are a unique class of repeatedly branched polymeric macromolecules with a nearly perfect 3D geometric pattern. They can be synthesised with either divergent methods (outward from the core) or convergent methods (inward towards the core). Tomalia was the first to synthesise the 3D PAMAM (polyamidoamine) dendrimers using divergent methods [53]. These dendrimers contain tertiary amines that allow the binding of a number of molecules. The method of convergent dendrimer synthesis has been established by Frechet [54] and a dendrimer is characterised by the generation of monomers (G) added to a main core. The size of these dendrimers varies between 1.9 nm for G1 and 4.4 nm for G4, dendrimers, being the smallest nanocarriers developed which has promoted their suggestion for a number of pharmaceutical applications [55]. They have been extensively studied in the area of therapeutics and diagnostics for cancer [56] as well as for photodynamic therapy (activation therapies) [56], boron neutron capture therapy [57] and hyperthermia therapies using gold nanoparticles [58]. Dendrimers are versatile particles regarding their size and functionality and their chemistry allows for several modifications for certain imaging modalities. Gadolinium has been complexed with dendrimers and it was found that this complex enhanced conventional MR images in a dendrimer molecular weight dependent way and substantially better, compared with conventional diethylenetriaminepentaacetic acid Gd (III) chelates [59]. As drug delivery agents, dendrimers can carry drugs as complexes or as conjugates although one limitation lies in the effort of controlling the rate of drug release. The encapsulated, complexed, drugs tend to be released rapidly (before reaching the target site) and in the dendrimer–drug conjugates, it is the chemical linkage that controls the drug release. However, dendrimers offer several advantages as drug carriers targeting cancer. One major advantage is their surface functionality providing the selective coupling of imaging agents, targeting ligands and/or other components to increase tumour specificity.

The abovementioned nanoparticles either based on lipids or on polymers are generally “soft” and flexible nanoparticles. Their size ranges from 30 nm to slightly more than 100 nm. However, they can penetrate biological membranes due to their flexibility and biophysical interaction with cellular membrane components.
The inorganic nanoparticles represent a different class of nanoparticles that are usually much smaller, 5–40 nm and they do not have the flexibility observed in liposomes and polymeric nanoparticles. Inorganic nanoparticles have made their appearance in cancer therapy during the last decades in a number of applications [60]. The main type of inorganic nanoparticles—the iron oxide nanoparticles, has been used for imaging tumours [61]. The main advantage of magnetic nanoparticles is their ability to be visualised by Magnetic Resonance (MR) imaging. Additionally, iron oxide nanoparticles can be guided to target sites (i.e. tumour) using external magnetic field and they can be also heated to provide hyperthermia for cancer therapy [62]. Yu et al. reported thermally cross-linked superparamagnetic iron oxide nanoparticles that could carry a Cy5.5 near infra-red probe (dual imaging) and doxorubicin for the imaging and treatment of cancer. The nanoparticles substantially diminished tumour size and provided the proof of concept that they can combine several modalities for maximum antitumour effect [62]. Magnetic nanoparticles have been used in the development of dual purpose probes for the in vivo transfection of siRNA [63,64]. The iron nanoparticles used in that study delivered siRNA at the same time as imaging their own accumulation in tumour sites. The iron nanoparticles used, were coated with dextran on which Cy5.5 and siRNA were chemically coupled [64].

Dextran coated iron oxide nanoparticles are already in clinical practice. Ferumoxtran-10® is a commercially available ultrasmall superparamagnetic iron oxide particle (USPIO) product [65,66]. After i.v. injection the particles collect in lymph nodes, liver, spleen, or brain tissue where they can be seen using MRI. In a lymph node with proper architecture and function (healthy) macrophages take up a substantial amount of Ferumoxtran-10. This uptake results in a marked reduction in signal intensity and turns the lymph nodes dark when seen by MRI. Infiltration of lymph nodes with malignant cells replaces the macrophages and changes the architecture of the lymph nodes. In malignant lymph nodes there is no ferumoxtran-10 macrophage uptake and they can retain the high signal intensity or display heterogeneous signal intensity if micrometastases are involved. This way the grade of tumours and prognosis can be assessed as micrometastases are important for this assessment [67].

Gold nanoparticles appear as another type of inorganic metal particle used in targeting tumours. Metal nanoshells are a class of nanoparticles with tunable optical resonances. Metal nanoshells consist of a spherical dielectric core nanoparticle, in this case silica, which is surrounded by a thin metal shell, such as gold [68]. These particles possess a highly tunable plasmon resonance, a resonant phenomenon whereby light induces collective oscillations of conductive metal electrons at the nanoshell surface. When studied in vivo in mice the nanoshells were “stealthed” with PEG, systemically injected, and were shown to accumulate preferably at the tumour site due to the highly permeable, poorly organized vascular networks common in neo-plastic tumours. Then, NIR (Near Infra-red) laser treatment of the bulk tissue selectively heats and destroys the nanoshell-laden tumour regions within the tissue, while leaving surrounding tissue intact [69]. Nanoshells are currently evaluated in a number of clinical settings after a 5-year period of intensive preclinical development [70]. Such development of nanoshells included the combination of nanoshells with cancer antibodies. Anti-HER2 antibody was conjugated onto nanoshells providing the potential of combining antibody therapy with imaging and hyperthermia [71].

As gold nanoparticles have evolved other gold structures have also been suggested. Nanorods, with the appropriate PEG stealth layer, are being developed as an improved means of hyperthermia. After injection in xenograft bearing mice, aided by computational studies, nanorods were shown to eradicate all irradiated tumours. The nanorods were compared to nanoshells for their laser induced heat. It was found that the PEG–Nanorods solutions exhibited heat that was generated 6 times faster compared to the heat generated by nanoshells under the same conditions [72].

Overall, during the last decade nanoparticles for in vivo application targeting cancer appeared with different sizes structures and tuneable properties. However, there are 2 functional components that are required for their biological applications for cancer targeting: The stealth layer and the ligand for cancer receptors.

3. Nanoparticle surface constituents

PEG layers are added onto any nanoparticle that is aimed to be administered intravenously for accumulation into tumours. Whether this is a liposome, a dendrimer or an inorganic nanoparticle such a layer (corona or halo) provides long circulation as it inhibits the accumulation of opsonins and their uptake by the macrophages. Although, currently there are no clear rules regarding the organization and the type of PEG on the surface of nanoparticles, there are some trends, derived from the extensive work on liposomes for cancer targeting. These trends indicate that the PEG’s size and density on the nanoparticles are key features that will control the circulation times and accumulation in tumours. Such PEG layers can interfere with targeting ligands and inhibit them from interaction with the corresponding receptors. In the following two sections PEG and cancer targeting ligands will be discussed with emphasis on liposomes as these nanoparticles have been widely studied in vivo in tumour animal models.

3.1. Nanoparticle biological stability stealth polymers

Most nanoparticles require colloidal stability in vitro (in buffers i.e. for storage) and in vivo (biological stability). A protective layer can therefore be formed by the incorporation of a hydrophilic polymer layer. The FDA-approved PEG polymer is the mostly used material. PEG is commonly adopted for the stealth function, due to their enhanced hydrophilicity and flexibility [73]. PEG enhances the nanoparticle’s lifetime in circulation, by preventing their interaction with plasma proteins [74–77]. Particularly for liposomal nanoparticle systems their protective PEG layer can be achieved through different methods. The main technique is the inclusion of PEG-lipid conjugates (Fig. 1) into the lipid film of the liposomal formulation. Upon hydration, the liposomes are formed with PEG polymers exposed on the surfaces [78]. The other techniques are based on the formation of the liposomal platform before the addition of PEG polymers. The method of post-conjugation, includes the covalent attachment of functionalised PEG to the pre-formed nanoparticles. Post-insertion, on the other hand, is performed by incubating the pre-formed liposomes with PEG-lipid conjugates in aqueous solution [79]. The amphiphilic nature of the PEG-lipids renders them into micellar structures, and their insertion into liposomal surfaces is perceived as a method of relieving micellar strain.

However, the method of preparation, the configuration and the effect of the PEG layer is dependent on the PEG chain length and their surface densities. The effect of such a layer depends on its optimisation of the organisation of the PEG chains [80–83]. Although the inclusion of PEG on liposomal surfaces improves their efficiencies for transport to the tumour sites, it is important to remember that their presence may hinder their binding to and uptake by cancer cells.

The incorporated PEG-lipid conjugates need to be able to render steric stability of the nanoparticles and be able to prevent surface adsorption of opsonin proteins. Higher coverage of liposomal surfaces is achieved by introducing higher densities of PEG–lipids in liposomal systems [84]. However, in the case of liposomes, there is
a maximum molar percentage of the PEG-lipids that can be incorporated into the lipid formulation before they become detrimental to systemic delivery. A liposomal membrane is held together by non-covalent interactions, therefore the PEG-lipid conjugates are susceptible to dissociation from the liposomes if the PEG density is sub-optimal.

When presented on a liposomal surface, individual PEG chains exhibit a Flory dimension, \( R_f \), which represents the volume that each flexible PEG cloud occupies. Longer polymer chains have larger \( R_f \), although studies have shown that the PEG density on a liposomal surface is more important that the size of the polymer [85]. The \( R_f \) of a PEG\(_{2000}\) chain is approximately 5.6 nm, and in conjunction with grafting density, affects the resultant conformation of the PEG chains. Increasing the amount of PEG-lipids within a liposomal formulation increases the PEG density, which ultimately reduces the distance, \( D \), between each PEG molecule on the nanoparticle surface. When \( D > R_f \), the PEG chains will self-assemble into a random-coil like mushroom cloud. When \( D < R_f \), the lateral pressure between the overcrowded PEG mushroom clouds will force the extension of the PEG chains into a brush configuration (Fig. 2) [86]. The brush regime results from the increased lateral pressure between the PEG chains, which forces the extension of the polymers away from the surface, into more linear conformations.

What prolongs a nanoparticle’s retention time in circulation is its ability to avoid interaction with plasma proteins [87]. The arrangement of surface PEGs into brush configurations generates greater protein repulsion, and generally enhances a liposome’s lifetime in circulation [88–90]. Another phenomenon related to protein adsorption is the recognition of PEGylated liposomes by anti-PEG antibodies [91–93]. This effect has been reported to occur between 2 and 4 days after the first administration of PEG-liposomes, and leads to the event of accelerated blood clearance of further PEG-liposomes administered at repeat doses [94–96]. There are yet no data on other pegylated nanoparticles induced PEG–antibodies and such phenomenon should be critically investigated in the preclinical development of nanoparticles. What is left is therefore the optimisation of a delivery system with an optimal amount of PEG-lipids, where too little does not provide enough coverage to evade protein adsorption, and too much results in removal of the PEG-lipids.

Inorganic nanoparticles require different methods of coating or introducing the biocompatibility and colloidal stability layers. Generally this is performed by the co-precipitation of the particles with various types of polymers or cross-linked polymers [97] that can improve the particle monodispersity, an important parameter regarding their biological applications. Dextran, albumin and PEG (Mw 5000) are all types of molecules that have been studies as coating materials for providing biocompatibility to iron oxide nanoparticles [98]. PEG with a Mw of 5000 was added as a coating layer on iron oxide nanoparticles to improve biocompatibility [99].

Gold nanoparticles provide a more suitable surface for PEG-grafting. Thiol–PEG or bifunctional PEG can be added on the surface to provide colloidal stability and biocompatible gold nanoparticles [100]. Sulfydrylated PEG has been added to gold nanoparticles to provide 38 nm nanoparticle that gave a blood–pool contrast agent for tumour vascular structures [101]. Recently, PEG bidentate ligands (PEG-thioctic acid and PEG-dihydrolipoic acid) were introduced onto quantum dots and gold nanoparticles to provide substantially improved stability in biological media [102].

In contrast to liposome based nanoparticles inorganic nanoparticles form much smaller structures even when coated with a biocompatibility layer. It is still unknown the effect of the PEG size and grafting density on all nanoparticle kinetics and accumulation in tumours. For liposomes some studies have indicated the important role of PEG, however there is minimum work done on the role of PEG on the kinetics of inorganic nanoparticles. Further dendrimers and biodegradable polymers’ Pegylation is too variable to observe trends. On biodegradable polymer nanoparticles PEG is introduced as part of the copolymer and in most cases a micelle is formed [103].

3.2. Nanoparticle cancer recognition-targeting ligands

The addition of a targeting moiety onto the surface of nanoparticles (Fig. 3) aims to increase selective cellular binding and internalisation through receptor-mediated endocytosis. Without the incorporation of targeting ligands, nanoparticles rely on non-

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**Fig. 1.** Structures of PEG-lipid conjugates used in preparing stealth liposomes. Both lipids shown are formed with a PEG chain of 45 monomers, correlating to a molecular weight of approximately 2000 Da. PEG units in both lipids are capped at the distal end with a methoxy group, and conjugated to a DSPE lipid (a), or a DPPE lipid (b).

**Fig. 2.** Representation of different PEG conformations, formed through their incorporation onto surfaces at different densities. When the distance between the PEG-lipids, \( D \), is greater than the Flory diameter of the PEG (\( D > R_f \)), the polymer will collapse into a mushroom configuration. When \( D < R_f \), the PEG chains will be extended from the surface to form a brush-like conformation.
specific interactions with cell membranes, which can be low when covered in a layer of PEG polymers. The targeting effect of the surface ligands is dependent not only on the nature of the ligand itself, but also on a variety of factors that require cooperative optimisation. Discussed in this section are examples of ligand-targeted nanoparticle systems with emphasis on liposomes. These examples differ from each other in many ways, including liposomal lipid content, type of ligand, method of ligand loading, degree of PEGylation and ligand density. With attention to the physical and biological effects of differently prepared nanoparticles, patterns that lead to enhanced drug delivery to cancers, through the effect of targeting ligands, can be derived.

One challenge of targeting cancers and tumours is that the defective cells are often very similar in characteristics to its surrounding healthy tissue. To differentiate such cells, the ligands can be designed to have specificity for receptors that are over-expressed on cancerous cells, but are normally or minimally expressed on normal, healthy cells. These molecules should have high affinity to their cognate receptors, plus have innate abilities to induce receptor-mediated endocytosis. The targeting layer poses as the outermost exterior of the nanoparticle delivery system, where targeting ligands are generally presented on top of the stealth layer [104]. Structures such as antibodies, antibody fragments, proteins, small molecules, aptamers and peptides have all demonstrated abilities to induce nanoparticle-targeting to cancer cells.

Antibodies against the HER-2 receptor, the transferrin receptor (TR) and the prostate specific antigen receptor are both common examples of receptor targets, due to their over-expression of such receptors on cancer cells [105–108]. These antibodies generally exhibit strong interactions with corresponding receptors, with dissociation constants in the nanomolar range. Antibody fragments, consisting of only the Fab binding regions, have also been studied as targeting ligands [109,110]. The advantages of such structures is that they are smaller, and do not contain the Fc region of the antibody which can induce immunogenicity and antigenicity when present on liposome surfaces [111–113]. Like antibodies and antibody fragments, the use of whole proteins is also commonly considered as targeting ligands for their increased affinity for target receptors. For example, the natural ligand for TR, transferrin, binds to its receptor with a dissociation constant of around $K_d = 40 \text{nM}$. On overcoming systemic barriers to arrive in tumour vicinities, targeted liposomes have greater opportunities for binding to cancer cells if the ligand has naturally high affinities for the corresponding receptor. Another protein that has shown potential for cancer targeting is the urokinase plasminogen activator, uPA, a natural ligand for the urokinase plasminogen activator receptor. Examples have used protein fragments, containing only the binding region of uPA for targeting the over-expressed receptors on colon cancers and breast cancers [114].

Peptide sequences pose as even shorter and smaller versions of antibody and protein fragments. Derived from phage display assays and X-ray crystallography studies, short peptide sequences with receptor-specificity have also been used for nanoparticle-targeting. For uPAR, peptides such as the U11 peptide were identified from examination of the binding region of uPA [115,116]. Our work has focused on promoting this peptide for targeting prostate and breast cancer cells [117]. Another prominent example of peptide ligands is the RGD peptide (Fig. 4), identified through phage display to have high affinity to $\alpha_v \beta_3$ integrin receptors over-expressed on angiogenic vasculatures [118–120]. Other types of shorter ligands include small molecules such as anisamide, a ligand for sigma receptors over-expressed on lung cancer cells [121,122], and folic acid (Fig. 4), specific for the folate receptors on ovarian cells [123–125], are more examples of targeting moieties for nanoparticle-targeting.

Ligands chosen for receptor-targeting should have the function of inducing receptor-mediated endocytosis. Depending on the type of ligand–receptor interaction, the rate of cellular internalisation would differ. This is an important factor as rates of internalisation could affect the accumulation of nanoparticle in tumour sites. Some ligand species, such as folate, have been shown to have
fast internalisation as well as cell surface recycling rates in cancer cells [126]. On binding, ovarian cells, characterised to express 3–18 × 10^6 folate receptors/cell, can internalise 3 × 10^5 molecules of folic acid/h. This inherent property of the ligand–receptor conjugate could enhance accumulation of nanoparticles by faster clearance from the tumour interstitium through faster internalisation of ligand-conjugated nanoparticles. Creating greater diffusion gradients would encourage faster diffusion of nanoparticle through the tumour vasculature. The ligands also need to be conjugated onto nanoparticles in an optimal fashion, as to maintain their affinities for their corresponding receptors. For example, the measured dissociation constants for optimally conjugated HER-2 antibodies were similar to free antibodies, around 12-15 nM, depending on the surface PEG density [112,127]. The targeting layer is presented as the ultimate exterior of the nanoparticle, the surface that interacts primarily with cell membranes. Irrespective of their nature, the ligand must have the right conformation, high affinities for corresponding receptors, and be able to exhibit high rates of cellular internalisation. Furthermore, the loading and presentation of the ligand on nanoparticle surfaces must be cooperative with the stealth layer.

It is important to incorporate PEG chains at densities that allow optimal coverage, which means inducing brush conformations on nanoparticle surfaces, as discussed earlier. As the brush conformation allows the extension of the PEG chains away from the nanoparticle surface, presenting targeting ligands on such a platform should allow increased interactions with corresponding receptors. The use of antibodies as targeting ligands has been widely studied due to their high affinities to cognate receptors. A prominent example has been the use of HER-2 antibodies, which target the HER-2 receptor that are over-expressed on cancer cells [128–130]. Incorporation of anti-HER2 antibodies onto the surfaces of PEGylated liposomes has indeed shown greater efficiency for drug delivery compared to non-targeted PEG-liposomes [131]. Treatment of tumour models with doxorubicin-encapsulated liposomes enabled complete depletion in subcutaneous tumours, when the antibodies are conjugated onto a liposomal platform of optimal PEG_{2000} density (6 mol%). In this case, and in most examples of targeted nanoparticles, increased therapeutic effect of targeted nanoparticles is a result of increased tumour cell uptake, and not tumour-accumulation (within tumour interstitium). Both targeted and non-targeted nanoparticles arrive at the tumour vicinity via the EPR effect, after which the mechanism of tumour cell internalisation is enhanced by the presence of surface ligands.

Although the presentation of ligands on a PEG-brush surface enhances their binding to receptors, their improved exposure can decrease the nanoparticle lifetimes in circulation. Using an RGD-targeted stealth system, nanoparticles carrying doxorubicin were found to accumulate faster and in higher concentrations in the liver and the spleen [132]. Faster clearance from circulation generally corresponds to a higher and faster accumulation into the liver and the spleen (RES). In this example, the total amount of PEG_{2000} content was included at optimal brush densities, at 6.5 mol%. The ligands are incorporated as RGD-PEG-lipid conjugates, which indicates their extension from the nanoparticle surface as a consequence of the brush-like state. Presentation of the ligand in a PEG-brush fashion reveals the targeting molecules increasingly to plasma proteins, hence their faster clearance from circulation could be a result of fc-mediated RES uptake into liver and spleen-associated macrophages [133,134]. When ligands are loaded onto liposomes of ambient PEG_{2000} density (5–7 mol%), what is generally observed is a faster clearance from circulation, accompanied by a higher accumulation and internalisation into cancer cells [131,135–137].

Most nanoparticle examples that describe successful receptor-targeting, use targeting ligands that are loaded onto nanoparticle surfaces via a PEG spacer. The rationale for such an action lies in the extension of the ligand from the nanoparticle platform, enhancing its flexibility and interaction with receptors. Our own work has examined the effects of presenting a ligand without a PEG spacer [117]. Through the synthesis of a peptide–lipid conjugate, we were able to functionalise the surfaces of liposomes with peptide ligands specific for the urokinase plasminogen activator receptor. These targeted systems were able to induce enhanced cell-binding and gene delivery compared to non-targeted nanoparticles. However, these ligands are exposed at reduced proximities to nanoparticle surfaces, an event that becomes problematic when long PEG chains are included.

Addition of PEG_{2000} molecules at optimal densities for in vivo delivery is essential, but results in the sterically shielding of the short peptide-lipids, hence preventing their interaction with cellular receptors. Similar studies, using a RGD-type peptide for targeting α5β3 integrins, also describe this finding. This report showed that short peptide-targeted nanoparticles exhibited lower cell-binding abilities when higher mol% of PEG_{2000} was included into the formulation [106,138]. As a sufficient PEG coating is essential for avoiding recognition by the RES, ligands should be extended away from nanoparticle surfaces, to avoid shielding by the polymer chains.

The chain length of the PEG spacer between the ligand and the nanoparticle surface also requires consideration. When the chain length of the PEG spacer is longer than the other PEG chains involved in the PEG-brush, the conjugated ligand can become buried within the brush layer [139]. The extra units of a longer PEG spacer are subject to mushroom-like folding, which results again in limited exposure of the ligand. Implications of this effect have been described with a folate-targeted liposome. Conjugation of the folate through a PEG_{3000} spacer, when the rest of the stealth content is comprised of PEG_{2000}, the effect of the ligand is not apparent. The folate-targeted nanoparticles were unable to increase tumour-accumulation, plus they did not exhibit short circulation lifetimes, a consequence commonly accompanying an effectively exposed ligand [134]. The ligand-conjugated PEG spacer therefore needs to be at the same length as the stealth constituent on the surface of the nanoparticle.

Concentration of surface ligands is another parameter that affects the ligands’ targeting effect [140]. Higher ligand densities are envisioned as a method of increasing the probability of nanoparticle interactions with cell receptors (multivalency). However, the presence of increased non-PEG-like material on nanoparticle surfaces can be more detrimental than advantageous to delivery. A study using aptamers as a targeting ligand for prostate cancer-specific antigens (PSMA) demonstrated that higher densities of surface ligands resulted in greater accumulation of nanoparticles into the liver and the spleen [141]. In that study Gu et al. used poly (ε-lactide-coglycolyde) [PLGA] and PEG triblock copolymer based nanoparticles. The nanoparticles were composed by PLGA-b-PEG and PLGA-b-PEG-b-Apt (aptamer for PSMA) [141]. Localization of such nanoparticles in tumours was also at lower concentrations compared to nanoparticles functionalized with lower densities of aptamers. This suggests that higher coverage of the PEGylated nanoparticles’ surfaces with targeting ligands further shields the effect of the PEG layer, hence resulting in greater recognition by tumour and spleen-associated macrophages.

Later studies using a HER-2 targeting system enhanced this argument, where heavy-ligand loading led to higher clearance rates from circulation [142]. High surface coverage of ligands therefore renders the PEG layer obsolete, an occurrence that needs to be avoided in order to reduce interactions with plasma proteins. Although the physical implications of ligand loading were not studied, the biological effects of antibody-targeted nanoparti-
cles demonstrate the importance of optimising ligand densities for maximising their targeting abilities [143]. Examples of targeted nanoparticle drug delivery systems have shown faster and higher concentration accumulation in tumours, compared to non-targeted systems [133,144]. Generally it is believed that the introduction of targeting ligands does not enhance nanoparticle accumulation into tumours, but shows higher efficacy by enhancing internalisation into tumour cells. However, in some cases of antibody and folate-targeted nanoparticles accumulation into tumours was faster than compared to non-targeted nanoparticles [133,144–146]. This occurrence is envisioned as a consequence of recognition of the nanoparticles by tumour-associated macrophages (TAM) [134,147–150]. In response to this hypothesis, folate receptors have been identified to be over-expressed on TAMS. This effect of TAM-recognition is pronounced in the future to preclinical development for cancer imaging and therapy. From all these nanoparticles, we would like to highlight the potential value of theragnostic nanoparticles. These nanoparticles are designed to do both imaging (diagnosis) and therapy (i.e. chemotherapy or genetic therapy). A particular version of these nanoparticles is the “activation”-theranostic nanoparticles. In this case nanoparticles are monitored for their kinetics in tumours. When imaging (i.e. MRI) shows maximum nanoparticle accumulation in tumours a physical source is applied to change the status of the nanoparticles and burst-release the drug. Such source can be the NIR laser, induced heat or the ultrasound induced heat or light for therapy in tumours a physical source is applied to change the status of nanoparticles for their kinetics in tumours. Here, we present an overview of the clinically used and tested nanoparticles for imaging and treatment of cancer. Currently, both clinical and preclinical studies show a variety of the type of nanoparticles developed for cancer. There are more types of nanoparticles currently at an early design step that may progress in the future to preclinical development for cancer imaging and therapy. From all these nanoparticles, we would like to highlight the potential value of theragnostic nanoparticles. These nanoparticles are designed to do both imaging (diagnosis) and therapy (i.e. chemotherapy or genetic therapy). A particular version of these nanoparticles is the “activation”-theranostic nanoparticles. In this case nanoparticles are monitored for their kinetics in tumours. When imaging (i.e. MRI) shows maximum nanoparticle accumulation in tumours a physical source is applied to change the status of the nanoparticles and burst-release the drug. Such source can be the NIR laser, induced heat or the ultrasound induced heat or light for photosensitive components. The changes induced on the nanoparticles can be nanoparticle decomposition, or “melting” releasing rapidly and locally the active (i.e. chemotherapy). The combination of hyperthermia and locally delivered anticancer agents can lead to very specific tumour only cell killing in a short period of time. This way surgery and/or chronic chemotherapy treatments can be avoided.

We have also highlighted important factors that affect the performance of PEG-coated nanoparticles, the PEG size and its grafting density, based on the liposome nanoparticle example. The coupling of PEG and ligands on the nanoparticles, needs to be designed and studied for optimum circulation times, binding and tumour cell uptake. It is an important fact that targeting ligands lead to macrophage recognition and faster clearance compared to the non-targeted nanoparticles. In the future nanoparticles’ design should introduce cleavable masking of the ligands till reaching the tumour cells. A thorough study of the effect of PEG size and density on nanoparticle kinetics, is needed. As well as more studies are required on the clearance mechanism, the metabolism and excretion of nanoparticles and their components.

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