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Review

An overview of active and passive targeting strategies to improve the nanocarriers efficiency to tumour sites

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Abstract

Objectives This review highlights both the physicochemical characteristics of the nanocarriers (NCs) and the physiological features of tumour microenvironment (TME) to outline what strategies undertaken to deliver the molecules of interest specifically to certain lesions. This review discusses these properties describing the convenient choice between passive and active targeting mechanisms with details, illustrated with examples of targeting agents up to preclinical research or clinical advances.

Key findings Targeted delivery approaches for anticancers have shown a steep rise over the past few decades. Though many successful preclinical trials, only few passive targeted nanocarriers are approved for clinical use and none of the active targeted nanoparticles. Herein, we review the principles and for both processes and the correlation with the tumour microenvironment. We also focus on the limitation and advantages of each systems regarding laboratory and industrial scale.

Summary The current literature discusses how the NCs and the enhanced permeation and retention effect impact the passive targeting. Whereas the active targeting relies on the ligand-receptor binding, which improves selective accumulation to targeted sites and thus discriminates between the diseased and healthy tissues. The latter could be achieved by targeting the endothelial cells, tumour cells, the acidic environment of cancers and nucleus.

Introduction

Any dosage form cannot carry a therapeutic activity if the administered biologically active molecule is not able to cross the biological barriers which separate the site of administration from the site of action. The barriers to be crossed are very complex systems composed of several elements (epithelium, endothelium, cellular membrane) and several components (mechanical or physicochemical barriers and enzymatic barriers). Certain molecules are ineffective because they do not diffuse spontaneously into the cell whereas their therapeutic target is with intracellular localization. The specific delivery of therapeutic agents to an organ, a tissue or a type of cells currently constitutes a major challenge for the treatment of the human diseases, in particular infectious, cancerous and genetic diseases.[1]

Most of the APIs are often prone to display low bioavailability, poor water solubility, biological degradation and inadvertent intrinsic side effects. To overcome such drawbacks, the design of novel drug carrier systems is necessary because of their efficient applicability through different administration routes such as oral, parenteral, topical and pulmonary. To achieve these goals, targeted delivery systems bearing genes/drugs to specific tissues/cells have been widely investigated.

Introducing the nanotechnology into medicine field termed as 'nanomedicines' makes it possible today to present the concept of 'vectorization', also called drug targeting. Based on new physicochemical concepts and new materials,

nanomedicines serve in envisage submicron systems of administration of the drugs. Nanocarriers are able to improve drug properties in various ways: by encapsulating hydrophilic or hydrophobic molecules in their cores, controlling release and distribution, enhancing drug absorption by mucosa or cells and through protecting the drug from degradation. Some nanocarriers allowed the development of new treatments with improved specificity.

Researchers worldwide have sought to develop submicron particles (i.e. nanoparticles and liposomes, as in Table 1) for transport of drugs. After intravascular administration, the carriers are opsonized,[2,3] that is covered with proteins and recognized by the macrophages of the liver and spleen. This controlled biodistribution improves the targeting and the experimental treatment of pathologies such as hepatic metastasizes and also can lead to significant reduction of the drug concentrations in the undesired locations, thus decreasing the toxicity of certain anticancer drugs.

Though some formulations have already been developed in the market during the last decade,[4] there still no universal platform is suitable for the delivery of all kinds of drugs, that is to say the theory 'one size fits all' does not apply. Nonetheless, current nanotechnologies have potential limitations such as:

1. Poor drug loading that is usually <5% (weight% of the transported drug vs the carrier composition). In consequence, either the quantity of the drug administered is not sufficient to reach a pharmacologically effective concentration in the body or the amount of the carrier material that is administered is too high, leading to adverse effects.
2. Rapid release, sometimes called 'burst release', of the encapsulated drug after administration, generally resulting from the release of a proportion of the drug fraction which is simply adsorbed (or anchored) at the surface of the nanocarrier. As a result, a significant fraction of the drug will be released before reaching the pharmacological target in the body, leading to low activity and toxicity issues.
3. The difficulty of designing an efficient nanodelivery system gathering low toxicity, high immunogenicity and biodegradability, and accumulation on desired cells/tissues. This could be attributed to non-controlling physicochemical features and non-targeted nanoparticles. For that, careful designed nanoparticles, including composition, size, shape, surface charge, and functionalization, are necessary to overcome existing issues. Herein, we mention briefly different types of nanocarriers which have been developed for tumour via passive and active targeting. Thus, we discuss the relationship between targeting mechanisms and physicochemical factors of nanocarriers as well as pathophysiological characteristics of the tumour microenvironment (TME).

In this review, we highlight the active and passive targeting processes to enable such nanoparticles to be targeted to desired bindings, particularly tumours, efficiently. To realize the two strategies, we demonstrated in brief different kinds of nanoparticles and their physicochemical properties and how they interact with the TME. We will focus on the enhanced permeation and retention (EPR) phenomenon and how it influenced by the targeted nanoparticles introduced into the body. Therefore, we will discuss some of the

Table 1 Advantages and limitations of various amphiphilic-based drug delivery nanosystems

Nanosystems	Advantages	Limitations
Micelles	Easy and non-costly production	Disassembly upon dilution; too fast drug release; only suitable for lipophilic drugs
Cubosomes and hexasomes	Very ordered; high encapsulation efficiency; suitable for oral administration	Extremely high viscosity; short release duration
Liposomes	Biocompatible, biodegradable; extremely versatile; high-throughput synthesis, lyophilization; surface modifications; new generation hybrid systems	Limited shelflife (in solution); too slow drug release; hydrophilic drug leakage
Lipid nanoparticles	Biocompatible; high drug loading; batch-to-batch reproducibility; easy to scale-up and sterilize; long shelflife	Drug loading is limited by its solubility in lipid melt; risk of drug expulsion after polymeric transition
Nanoemulsions	Kinetically stable; high drug loading capacity; biocompatible; slow and controlled drug release; low cost of industrial production compared a many other colloidal systems	Fragile nanoparticles, most of them are suspensions not in a solid form
Polymer-based self-assemblies	Possible 'smart' drug release (pH, temperature, redox sensitive); adaptable chemistry	Costly synthesis; safety and biodegradability concerns
Macrocycle self-assemblies	Multi-dimensional hierarchical self-assemblies; novel topological structures	High cost of production; poor water solubility; low biocompatibility

NCs parameters (i.e. size, chemical composition, surface functionalization, etc.). We will also demonstrate the possible routes to improve the active targeting through cellular, vascular, nuclear and the acidity of the TME. Eventually, we will offer many targeted models used in both preclinical and clinical phases.

Nanocarriers as Drug Delivery Systems

The key challenges in diagnosis/treatment of cancer lie in engineering drug/gene delivery systems capable of specifically targeting the diseased cells without affecting the normal healthy cells/tissues. This might be achievable by efficient delivery of anticancer agents into TME and thereby tumour cells.[5,6] On the other hand, the formulated NPs must pass through several physiological and biological barriers. Their use as delivering systems imposes requirements to their size, biocompatibility and surface chemistry for preventing unspecific interactions and introducing specific binding to their targets.

These nanocarriers must be able (1) to remain stable in the blood as long as they reach TME, (2) to escape from the reticuloendothelial system (RES) clearance and not captured by mononuclear phagocyte system (MPS). Both points have been attained by PEGylating the NPs surface, for improving their hydrophilic properties and conferring stealth characteristics, to delay their recognition by immune system and to increase the chance to target the desired tissues/cells. (3) To accumulate in TME through irregular tumour vasculature, (4) to penetrate into the tumour interstitial fluid of TME with high pressure and (5) to reach the active site and interact with the targeted cells exclusively.[7,8] Active/passive targeting is the ideal solution to promote NPs' accumulation in the location of interest. The main factors that control the drug targeting by NPs are their surface functionalization, their physicochemical properties and the pathophysiological characteristics of the TME. These factors will be discussed in the following sections.

Development of nanocarriers is crucial to prevent the cargos molecules from degradation or release before reaching their targets causing long-term toxicity issues. NPs are also excellent candidates for increasing the payload efficiency of the APIs through covalent binding or by encapsulation. Nanoparticles can be made from a variety of materials such as lipids, compositing polymers, proteins, metals or semiconductors. Numerous nanoparticles with well-defined shapes such as solid spheres, rods, tubes and others have been recently developed. Current nanoparticle platforms for tumours can be classified into three major categories including organic-based NPs (e.g. liposomes, dendrimers, polymeric NPs, micelles and solid lipid nanoparticles (SLNPs)), inorganic-based NPs (e.g. iron oxide nanoparticles IONPs, gold nanoparticles AuNPs, ceramic nanoparticles, semiconductor nanocrystals and carbon nanotubes CNTs) and hybrid nanoparticles. The latter is synthesized from two or more types of nanomaterials (NMs) and are generally formed with a metallic or polymeric core covered with a single or multiple lipid layers to increase the biocompatibility of the system. This type of NPs can be utilized in both diagnostic and therapeutic applications. Table 1 shows some benefits and drawbacks of such nanocarriers.

An alternative design of nanoparticles may also contain intrinsic thermal, electrical, optical or magnetic properties that can be served in imaging or therapeutic purposes. Interestingly, AuNPs are used as efficient imaging agents for X-ray micro-CT thanks to their high absorption coefficient. Also, they are used in photothermal therapy (PTT)[9] as they are able to absorb photons at specific wavelength and immediately convert them into heat destroying the cancer cells. IONPs can be used for magnetic resonance imaging for potential detecting small lesions and very sensitive to image brain tumours. Meanwhile, because of their magnetic properties, they could be targeted to specific cells/tissues. After injection of IONPs and applying external magnetic field will raise the particles temperature, a phenomenon called 'hyperthermia'. Quantum dots can act as photodynamic therapeutic agents (PDT) to induce cytotoxicity by generating reactive oxygen species (ROS) under light.

Tumour-Targeted Drug Delivery Systems

Targeted drug delivery systems (DDS) have several advantages including (1) protection of healthy cells from the cytotoxic compounds, (2) reduction of the dose-limiting adverse effects and (3) combating the drug-resistant cancerous cells. As matter of fact, the nucleus is ultimately the final target for many therapeutics treating various disorders including cancers, brain disorders and heart dysfunction. Because of their specific cell uptake and trafficking mechanisms, NPs allow the delivery of sensitive therapeutics to their targeted lesions in active form, in sufficient concentration and decrease the amounts that accumulate in undesired organs/tissues. However, it has become increasingly obvious that cytosolic internalization of a drug molecule does not entail its interaction with its subcellular target and hence careful nanoparticle design and optimization is necessary to enable cellular/nuclear targeting.

Passive targeting

It is now a well-established fact that under certain conditions (inflammation/hypoxia, which is typical for tumours), the endothelium of blood vessels becomes more

permeable than in the healthy state.[10] Upon hypoxia, rapidly growing tumours recruit new vessels or engulf existing blood vessels. These newly formed leaky vessels allow selective enhanced permeation of macromolecules larger than 40 kDa and nanosystems to the tumour stroma.

Furthermore, the absence of normal lymphatic drainage in tumour contributes to the NPs retention. This unique feature, however, is not applicable to small molecule drugs which have almost short circulation time and fast washout from the tumour. Thus, the encapsulation of small-molecule drugs in nanosized drug carriers enhances their pharmacokinetics (prolonged systemic circulation), provides some tumour selectivity and decreases side effects. This type of tumour targeting termed 'passive' relies on carrier characteristics (size, circulation time) and tumour biology (vascularity, leakiness etc.), but does not possess a ligand for specific tissue or organ binding.[11,12] A general scheme illustrating this phenomenon along with active targeting discussed below is proposed in Figure 1.

Since the discovery of the EPR effect in 1980s by Maeda *et al.*,[13] a lot of efforts were done to understand the significance of this phenomenon in tumour targeting and develop appropriate DDS. Some of these nanocarriers, such as the marketed Doxil© and Caelyx©, are now successfully used in clinics and EPR effect became a golden standard in the design of passive tumour-targeted systems.[14]

However, EPR effect provides rather modest tumour specificity with 20–30% in delivery increase compared to normal organs. The EPR effect is highly dependent on the intrinsic tumour biology and in particular: (1) the degree of angiogenesis and lymphangiogenesis, (2) the degree of perivascular tumour growth and the density of the stromal response and (3) intratumour pressure.[15] All of these factors, together with the physicochemical characteristics of nanocarriers, will determine its drug delivery efficiency.

Though the leakiness of newly formed tumour vessels influences the nanomedicine permeation, it contributes to the high interstitial pressure, which in contrast, is able to inhibit the accumulation of drug carriers in tumour.[16] Moreover, due to the disproportion of pro- and anti-angiogenic signalling inside of different parts of the tumour, vessels are abnormal with dilated, tortuous and saccular channels, disorganized patterns of interconnection and branching.

Due to such heterogeneous blood supply, tumour cells also grow irregularly – those that are near blood vessels proliferate faster than those that are in the tumour core and receive less nutrients and oxygen. This explains hypoxic/necrotic areas in the cores of large tumours (i.e. 1–2 cm in diameter in mice) and often impossibility for nanomedicines to reach these areas. Moreover, blood vessels in the central area of the tumours do not leak as much as one could expect due to the high interstitial pressure. Such phenomenon was observed in various kinds of murine and human tumours. High interstitial pressure not only inhibits drug delivery by convection but also compresses newly formed blood vessels. In this way, blood is conducted away from the centre of the tumours towards the periphery.[12,14]

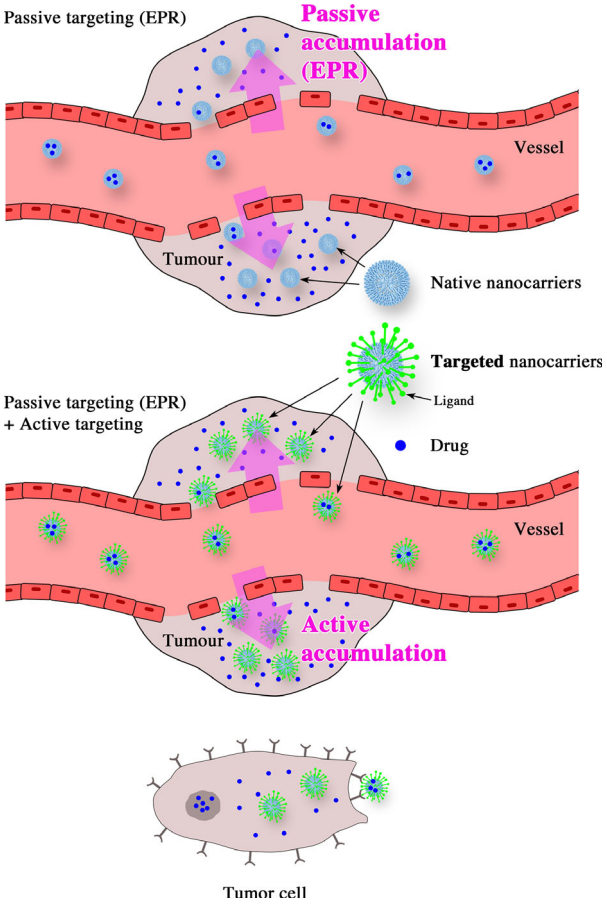

Figure 1 Scheme illustrating the passive targeting (EPR) and the active targeting into a tumour. [Colour figure can be viewed at wileyonlinelibrary.com]

However, it is possible to modulate EPR effect chemically or mechanically to achieve vascular normalization leading to higher accumulation of nanocarriers. Among chemical EPR enhancers, one could find bradykinin (kinin), nitric oxide, peroxynitrite, prostaglandins, vascular permeability factor (VPF)/vascular endothelial growth factor (VEGF) and other cytokines.[11,12] These molecules induce hypertension or vascular normalization, which could temporary enhance tumour perfusion. Other approaches utilize ultrasound, radiation, hyperthermia or photo-immunotherapy to modulate tumours vasculature and increase nanosystems permeation. Nevertheless, all described methods have limitations and

contra-indications and thus require careful consideration.[12,14,16]

Nanocarriers characteristics affect passive targeting strategy

Biodistribution, pharmacokinetics and the toxicity profiles are influenced either by the physicochemical properties of the developed nanocarrier or by the pathophysiological properties of TME. Globally, it has been extensively demonstrated that particle size and surface charge affect the efficiency and the pathway of cellular uptake for liposomes,[17] quantum dots,[18] polymeric NPs,[19,20] AuNPs[21] and silica NPs[22] by influencing the adhesion of the particles and their interaction with cells.[23]

Other factors influencing EPR-based tumour targeting are nanometric size and circulation time. Size is important for the permeation and retention in the tumour and thus is limited by the fenestrations in tumour vessels (200–800 nm).[10,17] On the other hand, nanomedicine diameter influences their renal excretion (less than 6 nm) or through Reticuloendothelial System (RES) (more than 500 nm). Thus, as already mentioned, the optimal size range is around 20–200 nm.[14]

Surface chemistry and charge also play vital role in the circulation time – too hydrophobic or charged systems are rapidly opsonized by the MPS. Hence, it is preferred to make nanoparticle surface 'look like water' – hydrophilic and neutral or slightly anionic. For this purpose, water-soluble polymers (generally PEGs) are grafted on the nanocarrier surface.[10,24] Moreover, PEGylation prevents nanoparticle aggregation and non-specific interactions by changing surface charge and hydration.[25] The optimal loading of PEG-modified lipids in the liposome has been shown to be around 5–9 mol% of classically utilized DSPE-PEG2000. At this concentration each polymer chain adopts a mushroom-like globular structure with slight overlay between distinct polymers and ensures a complete 'stealth' nanoparticle surface coverage.[25]

However, the first injection of PEGylated liposomes was shown to induce PEGs-specific IgM and as a consequence, rapid elimination and enhanced hepatic uptake of a second dose of PEGylated liposomes. This is known as an accelerated blood clearance phenomenon and represents an important obstacle to the pharmacokinetics and pharmacodynamics of PEGylated liposomes and particles. In addition, PEG corona could also be a steric hindrance preventing efficient internalization of nanosystems into tumour cells. This issue is termed in literature as 'PEG dilemma'.[10,12] Thus, in a design of a drug delivery carrier one should find an appropriate compromise between prolonged circulation time and better intracellular trafficking. The possible solutions could be shorter PEG chains (i.e. Mw < 1000), PEG attachment by enzyme-cleavable bound or utilization of specific tumour targeting ligands.[12]

Nevertheless, PEGylation is a clinically accepted tool to control nanoparticle surface properties and produce 'stealth' drug delivery carriers. Moreover, it offers a possibility to chemically bound a targeting ligand on its surface and therefore enhances intracellular uptake.[25] This so-called 'active targeting' approach that will be discussed in the following section. The chemical structure of the molecules also impacts the accumulation of NPs into specific organs. As has been demonstrated in one of our reports[26] for developing new X-ray CT contrast agents based on iodinated nano-emulsion platforms, the results of the *iv* injections of the two nanoemulsions (NEs) loading different molecular structures (vitamin E and castor oil) revealed different accumulation sites.

Active targeting (tuning surface functionality)

It is noteworthy that the active targeting is essential for the delivery of drugs, genes and theranostics to the location of interest avoiding the normal tissues and thereby enhances the therapeutic efficiency and limits the side effects. Active targeting is able to significantly increase the quantity of drug delivered to the target cell compared to free drug or passively targeted nanosystems.

After accumulation in the tumour region, the drug efficiency can be even increased by the so-called active targeting. This is achieved through the decoration of the nanocarrier surfaces with ligands binding to receptors overexpressed onto the tumour cells (illustrated in Figure 1). This strategy will improve the affinities of the nanocarriers for the surface of cancer cell and thus enhance the drug penetration. The first evidence of this phenomenon was proposed in 1980 with antibodies grafted in the surface of liposomes,[27] followed by other various kinds of ligands like for instance peptides, nucleic acids, aptamers.[28,29]

Among the classical targets, we can cite the transferrin receptors (TfR) or nicotinic acetylcholine receptors that allow the reach the environment of brain tumours. In this case, the mechanism concerns targeting if endothelial cells, that is vascular targeting. Applied to target glioma, for drug delivery or biomedical imaging, transferrin ligands were grafted on solid lipid nanoparticles (SLNPs),[30] micelles,[31] dendrimers[32,33] and superparamagnetic iron oxide NPs (SPIONPs).[34] In addition, literature reports examples in which central nervous system (CNS) and glioblastoma have been reached through the targeting of nicotinic acetylcholine with micelles.[35–37]

A vast number of receptors have been recognized as well as their antibodies were successfully synthesized and investigated *in vitro* and *in vivo*. Inducing very strong

ligand/receptor binding, consequently serving as potential models to promote active targeting technology. It has been found that RGD peptide binds to $\alpha_V\beta3$ integrin. These receptors are highly presented on both the glioma cells and on the vasculature of TME.[38] F3 peptide was found to bind to nucleolin receptor expressed on angiogenic endothelial cells in the TME.[39] Likewise, aminopeptidase N (CD 13) has been identified as potential receptor in the TME[40] and has been shown to be targeted by a tri-peptide (Asn-Gly-Arg (NGR) peptide).[15] Among the classical examples of ligands, we can cite the folic acid (FA) that specifically binds to the folate receptor (FAR) as well as present in TME. In that case, different strategies have been reported, through synthesis of FA-drug conjugates and through FA-grafting onto nanocarriers promoting their endocytosis in cancer cells. Examples of commonly targeting ligands are presented in Table 2. To summarize, the active targeting of tumours can be performed by directly targeting tumour cells, targeting the mildly acidic TME, targeting the vascularization of TME and targeting the tumour nucleus as described in the following sections.

Tumour cell targeting

The majority of tumour targeting is performed by the tumour cell targeting in general by nanocarriers (see Figure 1) that improves their cell penetration.

It has been demonstrated that folic acid-conjugated to silica NPs slightly improved the tumour pressure compared to non-conjugated silica NPs.[41] Similar results were observed in FA-conjugated polymer-based DDS.[42] These incoherent results oppose the rules of active targeting and this could be attributed to four possibilities: (1) not all tumour cells overexpress receptors all the time, thus receptors density on the cell surfaces are varied accordingly. The ligand/receptor interaction occurs only at the high-density receptors and meantime NPs pass by, therefore enhancing cell penetration. (2) On the other hand, it is noteworthy that surface density of ligand could have an importance on the nanocarrier specific cell binding. The higher the density, the higher the targeting efficiency.[43] However, some simulations[44] argued that ligand-functionalized NPs enhance their interactions with leaky vessels to the detriment of deeper tumour tissues. Besides, if the density is too high, the opposite effect was observed and resulting of their own steric hindrance.[45] It follows therefrom that a beforehand work on whole process is necessary. (3) The third phenomenon to be taken into account is the potential increase of the nanocarrier opsonisation due to the ligands.[46] (4) Finally, the specific affinity between folic acid and liver could induce a premature hepatic uptake of the FA-decorated nanocarriers, after i.v. administration.[47]

To conclude, when designing ligand-functionalized targeted DDS, the essential parameters of the ligand itself should be considered, encompassing molecular weight

Table 2 Examples of commonly used targeting moiety[21]

Class	Ligand	Targets	Advantages	Limitations	Clinical approve
Antibodies	a-Herceptin Rituxan b-CD19	– HER2 – CD20 – CD19 antigen	High affinity and strong binding; already in clinical trials; therapeutic potential	High production cost; pharmacokinetics; 'binding site barrier effect'; potential immunogenicity	a-Approved as antimetastatic breast cancer (i.e. trastuzumab).[105] b-Approved as HIV Medicines.[106]
Peptides	a-RGD b-NGR	– $\alpha_v\beta_3$ integrins, Aminopeptidase N	High affinity	Reduced circulation half-life	a-[^{18}F]Galacto-RGD is approved as RGD PET tracer in human.[107] b-NGR-hTNF/DOX as vascular targeting agent is in phase 1b.[108]
Proteins	a-Transferrin LHRH	– Transferrin receptor – LHRH receptor	Already in clinical trials	High production cost	a-SGT-53, a scFv anti-TfR1 liposome complex is in Phase I and II.[109]
Aptamers	a-Pegaptanib	– VEGF receptor	Possible to develop for any target	High production cost	a-Approved as Macugen (Pegaptanib Sodium) Injection.[110]
Small molecules	a-Folate b-Galactose	– Folate receptor – Asialoglyco-protein receptor	Low production cost, low molecular weight; simple chemistry	Could reduce circulation time	a-Phase II.[111] b-Not approved yet

(MW), targeting affinity, valence and biocompatibility. The latter is a critical parameter due to many active targeting DDS are often very efficient *in vitro*,[48] while they do not always enhance drug accumulation in tumours when studied *in vivo*.[49]

Vascular targeting (endothelial cells)

Another potential alternative strategy is to target angiogenic endothelial cells, which are adjacent to tumour cells and have intimate contact with blood vessels as described in Figure 1. This will reduce blood supply to the tumour and deprive cancer cells from oxygen and nutrients with subsequent hypoxia and necrosis.[50] The integrins (α2bβ3, αvβ3 and α5β1) and aminopeptidase N (CD13) are the most common targets for tumour neovasculature. They are recognized by cyclic and linear derivatives of the peptide RGD (arginylglycylaspartic acid) and NGR (asparaginyl-glycyl-argininic acid), respectively.[51] Unlike the EPR effect, an important advantage of vascular targeting lies in the fact that its efficiency is not correlated to the specific blood vessel permeability or cell uptake.[52,53] Vascular targeting is able to limit poor delivery of drugs and the drug resistance and can be more adapted to the tumour heterogeneity or to various different sorts of tumours.[54]

Earlier work on vascular targeting were reported in the 1920s,[55] it was only in 1993 that researchers proved the real potential of this approach with a successful tumour eradication *in vivo*[56] Then several literature reports extended the concept using ligands like vascular endothelial growth factor or RGD peptides, grafted on nanoparticles or nanocarriers like nanotubes,[57] nanographene oxide[52] or QDs.[58]

Targeting the mildly acidic tumour microenvironment

It has been found that tumour tissues are more acidic (pH 6.5–7.0) than normal ones (pH 7.4)[59] and pH dropping came from the rapid growth rate of tumour cells.

In the mid-twentieth century, Otto Warburg[60] described a switch of the cancerous cellular metabolism into glycolysis with the formation of lactic acid as an endpoint to this glycolytic metabolism.[61] The lactic acid if accumulated intracellularly would lead to cell death.[62] The cancerous cells cope with this by overexpression of proton pumps and transporters to remove the protons from the cytosol to the extracellular milieu.[63,64] This phenomenon, now known as Warburg effect, leads to the acidification of tumour extracellular environment. Therefore, pH-sensitive DDSs based on liposomes, polymers, etc. have been deeply investigated, aiming at tumour-targeted delivery.

Since then, considerable amount of research was carried out to exploit the acidic pH of the tumorous extracellular fluids.[65] For example, pH-sensitive liposomes were recently used to increase the therapeutic window of doxorubicin in treating breast cancer.[66] It has been shown that estrone-anchored pH-sensitive liposomes (ES-pH-sensitive-SL) were significantly more cytotoxic than free doxorubicin or non-pH-sensitive estrone-anchored liposomes (ES-SL) vis-à-vis MCF-7 cell line. Furthermore, cardiotoxicity, the foremost clinical side effect of doxorubicin, of ES-pH-sensitive-SL was lower than free doxorubicin. Indeed, ES-pH-sensitive-SL displayed higher accumulation in tumour and less take up by heart, liver and kidney comparing to ES-SL or free doxorubicin. Also, ES-pH-sensitive-SL showed better inhibition of tumour growth than ES-SL and free doxorubicin when tested on breast tumour animal model.[67] On the other hand, imidazole groups-[68,69] or poly(β-amino ester)-based[70] polymers responsive to tumoral low pH as well as polymers having pH-sensitive chemical linkages, like acetal,[71] hydrazine,[72,73] vinyl and ortho esters,[74] pH-sensitive cell-penetrating peptides and cationic polymers undergoing pH-dependent protonation have been studied to employ the pH component along the endocytic pathway for intracellular drug delivery.

Nuclear targeting

Beside the drug delivery to the TME or more precisely to the tumour cells, some treatments need an even more precise level which is the drug delivery at organelle level, for example, nucleus, lysosomes, mitochondria or endoplasmic reticulum. In that way, the therapeutic response will be maximized, and their toxic side effects minimized. In the case of delivery of therapeutic genes, the target is the cell nucleus to exert their effects in correcting dysfunctional or missing genes. On the other hand, cancer cell nucleus can be targeted for a destroying effect. Indeed, the mechanism of action of most anticancer drugs, for example doxorubicin, involves oxidative DNA damage and topoisomerase II inhibition within the nucleus.[75] However, the effect of such anticancer drugs can be dramatically reduced if they are not specifically targeted to enter the cell as well as to the cell nucleus.[75] Nucleus targeting was investigated with different imaging probes like magnetic nanoparticles,[76] AuNPs,[77–79] AgNPs[80,81] and QDs.[82] Biologically, to deliver the payload drug targeted NPs to the nucleus, they are subjected to bypass a number of barriers such as (1) the cell membrane, (2) avoid the entrapment and degradation in endosomes/lysosomes, (3) cytoplasmic trafficking and finally (4) nuclear entry. Actually, the nuclear targeting should mostly be performed using nano-scaled carrier, that are able to cross physiological barriers and to specifically

deliver active ingredient or imaging probe to intracellular regions.

Therefore, specific nucleus delivery has become a challenging task that should take into account the nanocarrier entering the cytoplasm and then in the nucleus membrane. The nuclear pore complex (NPC) drastically controls the communication between the cytosol and nucleus.

One interesting example of nanoparticles used for nucleus targeting is the gold nanoparticles. Besides their facile synthesis, their controllable and very small size allows to reach dimension below the one of the NPC, and their chemical nature simplifies their surface functionalization.[83] The NPs size is even more important since below 9 nm AuNPs present a high nucleus penetration along with a fast blood clearance. On the other hand, for higher sizes from 20 to 200 nm, it is the opposite, and the blood circulation time is higher but the nucleus entering is low.[84] Drug delivery solutions to nucleus were proposed by, for instance, gold nanostars functionalized with nucleolin-specific aptamers.[85] AuNPs sizing at 30 nm, decorated with PEG (for increasing the NPs circulation in bloodstream) and peptides (RGD and nuclear-targeted peptide) were shown to selectively disturb the division of cancer cells, resulting in cytokinesis arrest and resulting in apoptosis.[83]

Additionally, NPs made with cationic polymers like poly (ethyleneimine) (PEI) and poly-(L-lysine) were showed to be able to enter the nucleus efficiently. These polymers follow the microtubule cytoskeleton up to the nucleus.[86] Similar results are also obtained with cationic liposome-plasmid DNA complexes that successfully brought plasmid DNA into the nucleus.[87]

Investigations on ligands promoting the nuclear penetration shown that nuclear location sequence (NLS) decorating NPs significantly promotes their nucleus targeting.[88] Literature provided some other examples in that sense using NLS (CGGGPKKKRKVGG)-functionalized PLGA NPs (sizing about 72 nm) and NLS-functionalized QD-conjugated PLGA NPs (168 nm), that had been shown to target and enter the nucleus of HeLa cells.[89] Transactivator of transcription (TAT) peptide (from HIV-1) has been shown efficient for the same purpose,[90] especially functionalizing ultrasmall mesoporous silica nanoparticles.[91,92] Table 3 lists different nanocarriers following active or passive targeting strategy.

Examples of Targeted Nanocarriers

For the delivery of nucleic acids, active targeting could be an extremely useful approach.[49] As they are large, polyanionic molecules are not able to penetrate the cells because of electrostatic repulsions from negatively charged cellular membrane. In addition, DNA and siRNA site of action is inside the cells and their unselective uptake might provoke additional side effects. In contrast, the active targeting is able to enhance nucleic acid cellular internalization and at the same time limit off-target side effects. Moreover, cationic lipids classically used for nucleic acid delivery could induce *in-vivo* toxicity.[93]

Recent study using double-targeted photolabile-caged cell-penetrating peptide (pcCPP) ()/NGR liposomes encapsulating siRNA showed efficacy in c-myc gene silencing *in vitro* and *in vivo*.[94] pcCPP/NGR liposomes demonstrated enhanced uptake and endosomal escape in HT1080 cell line. After systemic administration in mice, pcCPP/NGR liposomes were preferentially accumulated in the tumour and delayed tumour growth via RNA interference.

The first evidence of RNA interference in humans was shown with targeted nanoparticles.[95] On the other hand, cyclodextrin-based polymer functionalized with free PEG and PEG-transferrin conjugates. siRNA was designed to silence the expression of the M2 subunit of ribonucleotide reductase (RRM2). Systemic administration of nanoparticles revealed their dose-dependent accumulation in tumours and the decrease in RRM2 protein and corresponding mRNA. This study demonstrates that RNA interference could occur in humans after systemic administration of siRNA in targeted nanoparticulate carrier and thus could be utilized as gene-specific therapeutics (Table 4).

Preclinical trials and subsequent clinical translation were shown for targeted polymeric micelles encapsulated docetaxel (BIND-014).[96] The micelles combine passive targeting via the EPR with active targeting provided by peptide derivative S,S-2-[3-[5-amino-1-carboxypentyl]-ureido]-pentanedioic acid (ACUPA), a PSMA (prostate specific membrane antigen) substrate analogue inhibitor. Administration in mice, rats and non-human primates showed prolonged circulation time (compared to free drug), minimal accumulation in the liver and controlled drug release. The same pharmacokinetics was observed in first phase I human trials.

Antibodies (Ab) and their fragments are so far one of the most studied targeting agents in preclinical and clinical trials. For instance, HER2-targeted PEGylated liposomal doxorubicin formulation was developed to reduce unspecific cardiotoxicity of anthracyclines and enhance the drug therapeutic potential.[97] The successful results obtained in preclinical studies in mice and embryonic stem cells derived cardiomyocytes brought the formulation to phase I clinical trials (MM-302) in patients with advanced breast cancer. Another immunoliposome doxorubicin formulation (MCC-465) undergoing phase I clinical trials showed no specific cardiac toxicity and pharmacokinetics comparable to Doxil©.[98,99] Here a F(ab′)2 fragment of the human monoclonal antibody GAH (recognizes >90% of stomach cancer tissues) was conjugated to standard PEGylated

Table 3 Selected studies have been reported showing the active or passive targeted nanocarriers bearing molecules of interest either therapeutic agents or contrast agent to specific cells

Carrier	Ligand (coating shell)	Active/Passive targeting	Imaging or therapeutic agents	Application	Ref.
Nanoemulsions	PEGylated hydrophilic molecules (Kolliphore ELP)	Passive	Iodinated mono glyceride and iodinated castor oil contrast agents	Blood pool imaging agents, accumulated particularly in Liver or spleen, and imaged by X-ray CT	[26]
Dendrimers	PEG-RGD peptide	Active	AuNPs and Gd^{3+} chelate imaging agents	Dual-mode nanoprobe for targeted CT/MR imaging of different types of αvβ3 integrin-overexpressing cancer.	[112]
Dendrimers	(PEG) monomethyl ether, and PEGylated Folic acid	Active	AuNPs and Gd^{3+} complexes imaging probes	CT/MR imaging of folate receptors (FAR) of cancer cells	[113]
Polyethylene-imines (PEIs)	(PEG), non-covalent complexes with siRNA	Active	Therapeutic siRNAs	RNAi-mediated gene targeting, especially to lung	[114]
Liposomes	Decorated by cyclic RGD peptide conjugated to tandem peptide R8 to develop a multifunctional peptide R8-RGD	Active	Paclitaxel	Targeting for brain tissues and selectively accumulated in the glioma foci	[115]
PEI-AuNPs	PEI- PEG monomethyl ether-FA	Active	AuNPs contrast agent	FA-Au-PENPs for targeted tumour CT imaging of FAR	[116]
Polymeric NPs (PLGA-PEG) NPs	PEG-cyclic pentapeptide c(RGDfK)	Active	Cisplatin Pt(IV) Prodrug	Targeted to the αvβ3 integrin (prostate and breast cancer epithelial cells)	[117]
Polymeric Micelles NPs	Transferrin-Modified PEG-phosphatidyl-ethanolamine (Tf-mPEG-PE)	Active	R547 drug (a potent and selective ATP-competitive cyclin-dependent kinase (CDK) inhibitor)	Targeted to A2780 ovarian carcinoma cells-overexpressing transferrin receptors (TfR)	[118]
Albumin NPs	–	Passive	Tacrolimus (TAC)	TAC-loaded HSA-NPs Targets inflamed joints of rheumatoid arthritis tissues	[119]
Gold NPs	Anti-EGFR-PEG-AuNPs and Anti-IgG-PEG-AuNPs	Active	AuNPs imaging probes	Targeting the human squamous cell carcinoma head and neck cancer.	[120]
Polymeric NPs	C18PMH-PEG	Passive	Fe_3O_4 contrast agent and DOX drug	Magnetically control drug delivery, and serving as a contrast agent in T2-weighted MR imaging (theranostics)	[121]
Gold NPs	Gum Arabic-FA	Active	Both epirubicinDrug and AuNPs contrast agent	Targeted delivery of epirubicin to A549 lung cancer cells.	[122]
Quantum Dots	PEGylated molecules	Active	F3 peptide and siRNA	F3/siRNA-QDs NPs produce significant knockdown of EGFP signal, and used as bioimaging probes by fluorescent imaging	[123]
Lipid Nano capsules (LNCs)	Polysaccharides lipochitosan (LC) and lipodextran (LD)	Passive	DiD fluorescent dye	Selected to HEK293(β3) cells-bearing mice and detected by fluorescent imaging	[124]

doxorubicin formulation. Other targeted nanosystems undergoing clinical trials based on liposomes and polymeric nanocarriers are presented in Table 4.

Small natural molecules such as sugars and vitamins[25,51] could also represent interesting alternatives to antibodies and peptides. Moreover, they are easily metabolized and should not induce toxicity or side effects. One of the successful examples in small-molecule targeting are galactosylated solid lipid nanoparticles with encapsulated doxorubicin that showed enhanced cellular uptake and

Table 4 Examples of targeted nanomedicine formulations in clinical trials [90,111].

Nanoplatform	Drug	Ligand	Target	Cancer type	Phase
Liposomes	Oxaliplatin (MBP-426)	Transferrin (tr)	tr receptor	Advanced/metastatic solid tumours	I/II
Liposomes	Doxorubicin (MCC-465)	F(ab')2 from GAH		Metastatic stomach cancer	I
Liposomes	p53 gene (SGT53-01)	scFv	tr receptor	Solid tumours	Ib
Liposomes	RB94 plasmid DNA (SGT-94)	scFv	tr receptor	Solid tumours	I
Liposomes	Doxorubicin (MM-302)	scFv	ErbB2 (HER2)	Advanced breast cancer	I
Liposomes	Melanoma Ag and IFN (Lipovaxin-MM)	Single domain Ab fragment	DC-SIGN	Melanoma vaccine	I
Polymers	Docetaxel (BIND-014)	ACUPA peptide	PSMA	Solid tumours	I
Polymers	RRM2 siRNA (CALAA-01)	tr	tr receptor	Solid tumours	I

similar biodistribution *in vivo* compared to non-targeted.[30]

Despite the advantages that active targeting could provide and a lot of efforts that were put by the scientific community over the last 20 years for its development, clinical outcomes stay quite modest. This is often due to discrepancies between animal models and human primary tumours, tumour heterogeneity in target expression and rapid blood clearance. When designing targeted systems, a careful attention should be pointed at ligand properties, target expression profile and nanoparticle surface chemistry. It is however important to point out that active targeting strategies are still highly needed for the delivery of fragile bioactive molecules such as peptides, proteins and especially nucleic acids. Thus, novel targets and ligands could represent a major interest in this field. In the past few years, many actively targeted therapeutic models have entered phase I and phase II clinical trials. As an example, PEG-glutaminase combined with a glutamine antimetabolite 6-diazo-5-oxo-norleucine (DON) phase (I/II),[100] liposomal irinotecan HCl: floxuridine mixture (CPX-1) (NCT00361842)[101] and PSMA-targeted liposomal docetaxel (BIND-014) for solid tumours (NCT01812746, NCT01792479, NCT01300533) (phase II).[102] EC90 (keyhole-limpet haemocyanin fluorescein isothiocyanate conjugate) and EC 17 (folate-fluorescein isothiocyanate conjugate) vaccine (NCT00485563)[103] and probiotics[104] are currently under investigation. These examples reflect progress in the development of chemotherapeutics that have improved performances.

Conclusion and Future Perspective

The choice between active or passive tumour targeting should firstly rely on characteristics of the tumour cells as well as of the chemical nature of the drug. For the drugs that do not have issues with cell penetration, such as doxorubicin, the simple encapsulation in 'stealth' nanosystem (that reach the target passively) is sufficient. The best efficient result will be obtained by encapsulating these drugs in stealth nanocarriers with high blood half-life, thus, increasing their chance to accumulate specifically within the tumorous tissues, and consequently decreasing the drug toxicity towards highly perfused organs like heart, kidneys and liver. On the other hand, for the therapeutic molecules that have difficulties to cross-cell membrane and could induce severe damage to normal cells, active targeting should be a preferential strategy. This involves the nanocarrier decoration with ligands specific to the receptors overexpressed on the surface of cancer cells. However, even if the result is more efficient, the technology for surface decoration with ligands can be a complex chemistry (especially for nanoemulsions), as a result, in some cases, the global strategy can orientate the choice of the drug privileging only the simple EPR effect without active targeting.

Described earlier, nucleic acids are an excellent example of drugs that require the development of ligand-targeted systems. While cationic non-targeted nanocarriers could induce the interactions with cell membrane, they are non-selective and toxic. On the other hand, nanocarriers decorated with ligands will stimulate receptor-mediated endocytosis and therefore the delivery of the drugs to their site of action, cytoplasm or even nucleus. This will also minimize off-target side effects and general toxicity. A combination of active or passive drug carrier with an imaging or a diagnostic agent will generate 'intelligent' theranostics system able to monitor disease progression and evaluate therapeutic efficacy of the drug in real time. The development of such systems relies on the careful consideration of tumour biology as well as on the exploration of new targets potential and original drug carriers. Despite the large number of published reports demonstrating their therapeutic potential in preclinical models, only 15 passively targeted NCs have been approved for clinical use and none of the actively targeted NCs have advanced clinical trials. This limited success arisen from the challenges presented by physiological barriers (i.e. tumour heterogeneity, penetration, hypoxia and endosomal escape). In addition to the regulatory hurdles and the relatively complex scale-up of the manufacturing

process of actively targeted NCs. This beside other reasons pointed out above. Accordingly, much efforts and new strategies are necessary to develop NCs with controllable/predictable biological identity for accelerating the clinical translation of NCs.

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