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Review Article

EGFR Targeted Nanocarriers for Cancer Diagnosis and Therapy

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HIGHLIGHTS

- Highlights the potential of EGFR targeted nanocarriers for cancer diagnosis and therapy.
- Summarizes the role of EGFR targeting in cancer therapy.
- Describes various examples of recent researches on EGFR targeted nanocarriers.
- Explains illustrative examples of various ligands for EGFR targeting.

Keywords:
Cancer
EGFR
Ligand
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Targeting

ABSTRACT

Conventional cancer management is directly associated with many problems, including accurate therapeutic delivery to tumours and serious side effects of chemotherapeutics. A specific and efficient anticancer delivery to the tumour site without damaging normal tissues is the ultimate goal of all cancer treatment strategies. Nanomedicine has immense potential for cancer therapy that focuses on improving treatment efficacy, while reducing toxicity to normal tissues as well. However, the biodistribution and targeting capability of nanoparticles lacking targeting ligands rely solely on their physicochemical properties and the pathophysiological parameters of the body. Targeting is a promising strategy for selective and efficient therapeutic delivery to tumour cells with reduced detrimental side effects. Taking advantage of the fact that molecular markers and receptors over-express on the tumour cell surface as compared to a normal cell, the active targeting approach would be beneficial for cancer therapy. The epidermal growth factor receptors (EGFR), abnormally overexpressed in many epithelial tumours, have received much attention for molecular targeting in cancer diagnostics and therapeutics. This review presents the role of EGFR targeting in cancer imaging and therapy, and some recent researches on treatment of EGFR overexpressing cancers by using targeted nanoparticulate platforms. It also discusses illustrative examples of various ligands, including antibodies, antibody fragments, nanobodies, and peptides.

Introduction

Despite advances in early diagnosis, treatment, and survival, cancer has been among the main causes of morbidity and mortality worldwide (Bray et al., 2012). It is characterized by abnormal and uncontrolled cellular proliferation, and an absence of cell death that generates an abnormal cell mass or tumour. Cancer treatment has relied on four treatment modalities, namely cytotoxic chemotherapy, surgery, radiotherapy, and hormone therapy (Urruticoechea et al., 2010). Although radiation and anticancer agents are able to kill neoplastic cells, they suffer from severe side effects due to a lack in precision and non-specific damage of normal tissues and cells (Cao et al., 2014; Perez-Herrero and Fernandez-Medarde, 2015). Moreover, sometimes tumours fail to respond to the most commonly used treatment methods. Thus, there

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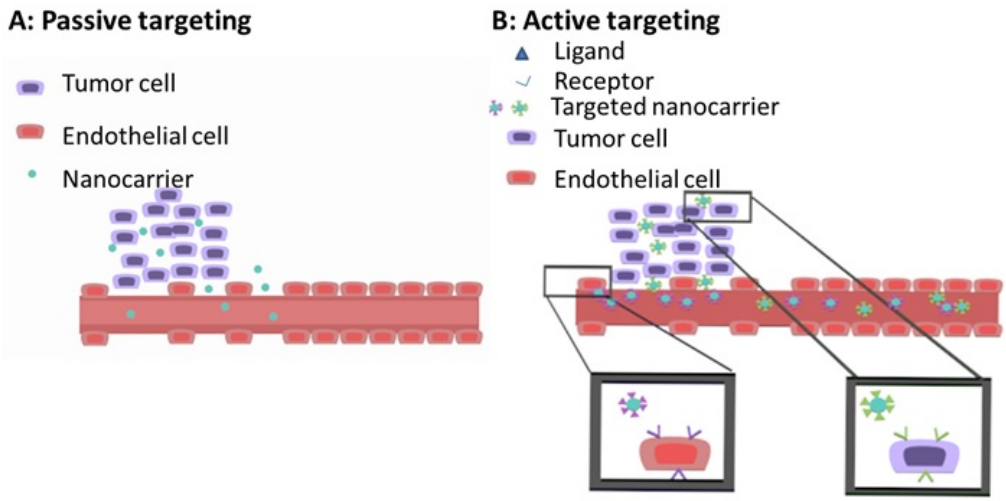

Figure 1. Schematic representation of passive and active tumor accumulation of nanoparticles due to the EPR effect.

is great demand for the development of novel methods to improve the current outcomes and also reduce toxic effects.

The systemic adverse effects of chemotherapeutic agents and treatment failures have led to research on the development of advanced delivery systems. Nanomedicine, the application of nanotechnology in medicine, aims for significant breakthroughs in cancer detection, treatment, and monitoring (Chow and Ho, 2013; Babu et al., 2014).

Nanoparticles are colloidal particles with an average particle size of 10 to 1000 nm. However, in cancer nanotechnology, average particle sizes of less than 200 nm are often used. Nanoparticles, led by liposomes, began to gain the focus of cancer research nearly three decades ago. Since then, biomedical researches on nanoparticles have been continuously proving them as being effective for drug and gene delivery (Kim et al., 2010; Wicki et al., 2015; Bregoli et al., 2016). Diverse nanoparticulate delivery platforms, including liposomes, polymeric nanoparticles, polymeric micelles, dendrimers, nanosuspensions, and inorganic nanoparticles (e.g., calcium phosphate, iron oxide, silica, and gold), highlight their promise for producing tailor-made cancer therapies and gain attention as being potential candidates to address the challenges of treating resistant tumours (Estanqueiro et al., 2015; Wicki et al., 2015).

Nanocarriers offer unique advantages such as nanoscale size, high surface-to-volume ratio, favourable physicochemical characteristics, the possibility to encapsulate hydrophilic and hydrophobic cargos, controlled released properties, and increased drug in vitro and in vivo stability. They have the potential to modulate pharmacokinetics, pharmacodynamics, blood circulation time, and tissue distribution of drugs, thereby enhancing their therapeutic index. Thus, in oncology, nanomedicines can alter the biodistribution of drugs by allowing the highest accumulation of cytotoxic agents preferably in the tumour tissue (Aslan et al., 2013; Petschauer et al., 2015). One of the most commonly cited reasons for the preferred accumulation at target site, is the nanocarriers' ability to exploit the enhanced permeability and retention (EPR) effect (Fig. 1). In EPR phenomenon, the combination of the leaky microvasculature and missing or tight lymphatic capillary system are involved. Deregulated angiogenesis as well as increased expression and activation of vascular permeability factors result in a porous vasculature. In addition to the formation of this discontinuous endothelial layer, dysfunctional lymphangiogenesis and compression of the lymphatic vessels by growing cancer cells lead to impaired lymphatic drainage in tumour tissue (Taurin et al., 2012; Nehoff et al., 2014). Moreover, the absence of large fenestrations in non-pathological tissues prevents the extravasation and deposition of nanoparticles in healthy tissue. Due to their large size (> 10 nm), anticancer loaded nanoparticles administered intravenously (IV) can escape renal clearance (Greish, 2010). These notable parameters improve treatment effectiveness and limit the side-effects associated with cytotoxic agents (Mattheolabakis et al., 2012; Bertrand et al., 2014).

Passive targeting depends on EPR facilitating efficient localization of small non-targeted particles in the interstitial space of tumours (Fig. 1). For successful passive targeting, a sufficiently long blood circulation time of the nanoparticles, prevention of premature release of the payload from the nanocarriers, and efficient extravasation through the walls of tumour site blood vessels are important issues. These goals can be achieved

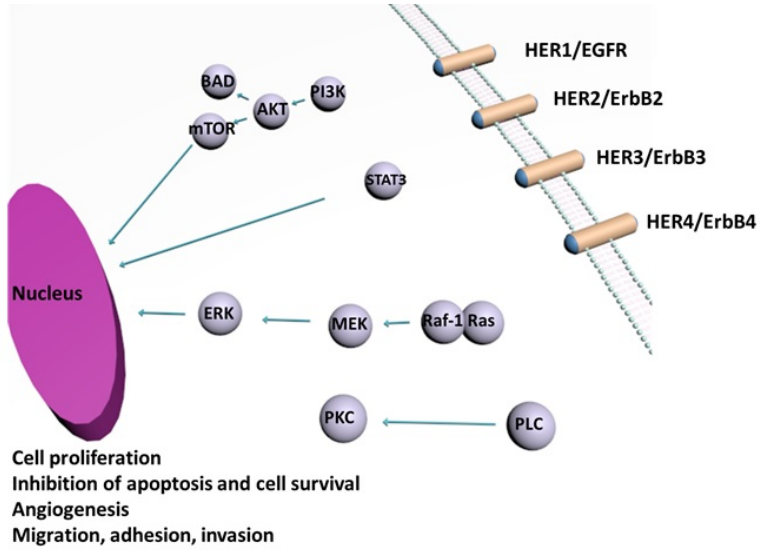

Figure 2. Schematic representation of cellular effects and signaling pathways regulated by the four HER family members.

through nanocarriers with appropriately small size, optimal stealth properties, typically by the incorporation of hydrophilic polymers like polyethylene glycol (PEG) into the nanomaterial shell, and controlled release properties (Lehner et al., 2013). However, passive targeting cannot further promote the uptake of nanoparticles by cancer cells and is restricted to some tumours. Nonvascularized sites and nascent tumours are unlikely to benefit from EPR mediated passive targeting. The EPR effect is also influenced by other factors, such as the amount of infiltration by macrophages, tumour location, the surrounding stroma, and patient characteristics (Sawant and Torchilin, 2012; Markman et al., 2013; Kraft et al., 2014).

This second step in uptake can be achieved by actively targeting nanoparticles towards internalization-prone cell-surface receptors or other surface membrane proteins overexpressed on the target sites (Fig. 1B). The addition of targeting ligands allows the delivery of drug-loaded nanoparticles to uniquely identifiable diseased organs, tissues, cells or subcellular domains, thereby reducing unwanted systemic exposure to cytotoxic agents. Therefore, the approach is aimed towards increasing specific interactions between nanoparticles and cells, and promoting internalization of the drugs by triggering receptor-mediated endocytosis without altering the overall biodistribution (Peer et al., 2007; Bertrand et al., 2014). The enhanced cellular internalization rather than an increased tumour accumulation is responsible for the antitumoral efficacy of actively targeted nanocarriers. Furthermore, active targeted nanocarriers have shown a potential to bypass P-glycoprotein-mediated drug efflux and suppress multidrug resistance (MDR) (Yu et al., 2010). Active targeting exploits specific modification of the nanoparticle surface with numerous targeting ligands selected on the basis of their selectivity or overexpression of their target in cancer cells or tumour vasculature, including antibodies, antibody fragments, nucleic acids (aptamers), peptides, whole proteins (e.g., transferrin), carbohydrates, and vitamins (Peer et al., 2007; Bertrand et al., 2014). The breakthrough of targeted nanoparticles has the potential to become the optimal delivery strategy and might shift the paradigm of cancer diagnostics and treatment.

EGFR: Signalling pathways and overexpression in cancer

The epidermal growth factor receptor (EGFR)/Her1/ErbB1 is one of the four transmembrane growth factor receptor proteins that belong to the ErbB family of receptor tyrosine kinase, which share similarities in structure and function. Other members of the ErbB group include HER2 (ErbB2 or Neu), HER3 (ErbB3) and HER4 (ErbB4) (Fig. 2) (Hynes and Lane, 2005).

EGFR is a 170 kDa glycoprotein and consists of an extracellular domain, a hydrophobic transmembrane region, a cytoplasmic tyrosine kinase-containing region, and several intracellular tyrosine residues. The extracellular domain provides a ligand-binding site for epidermal growth factor (EGF) and transforming growth factor-alpha (TGF-α), and the intracellular domain of EGFR is activated upon ligand binding that triggers the EGF-mediated tyrosine kinase signal transduction

Table 1. EGFR expression in various types of cancer (Rocha-Lima et al., 2007; Yewale et al., 2013).

Tumor type	Percentages of tumors overexpressing EGFR
Head and neck	80 - 100
Renal	50 - 90
Non-small cell lung	40 - 80
Breast	14 - 91
Colon	22 - 75
Ovarian	35 - 70
Prostate	39 - 47
Glioma	40 - 63
Pancreas	30 - 50
Bladder	31 - 48

pathway (Herbst, 2004; Vallbohmer and Lenz, 2005).

In normal cells, the expression of EGFR ranges from 4×10^4 to 1×10^5 receptors per cell and leads to cellular growth. Its signalling and overexpression can provide substantial advantages in tumour cells survival and may confer worse prognosis. EGFR is overexpressed in the majority of solid tumours, including head and neck, renal, breast, non–small cell lung (NSCLC), bladder, ovarian, and colon cancer (Table 1) (Herbst, 2004; Rocha-Lima et al., 2007; Yewale et al., 2013). For example, some tumour cells can express more than 2×10^6 receptors per cell (Herbst, 2004).

Multiple ligands bind to and activate the EGFR, including EGF, TGF-α, heparin-binding EGF, and amphiregulin. The activation of EGFR leads to multiple cell responses, inducing gene expression, cellular growth, differentiation, and migration. After ligand binding with EGFR, receptor homo- or heterodimerization occurs at the cell surface, followed by internalization of the dimerized receptor, intrinsic protein tyrosine kinase activation, and autophosphorylation of the intracytoplasmic EGFR tyrosine kinase domains. Phosphorylated tyrosine kinase residues serve as binding sites for the recruitment and phosphorylation of several intracellular substrates, which then stimulate an intracellular signal transduction cascade (Fig. 2) (Slichenmyer and Fry, 2001; Goffin and Zbuk, 2013).

Since their introduction by Mendelsohn and his co-researchers as a target for cancer therapy (Kawamoto et al., 1983; Sato et al., 1983), they have been receiving much attention in the last 20 years for designing therapeutic agents to target EGFR and these new treatment options have shown great benefits in several epithelial tumours malignancies.

EGFR inhibition can be achieved mostly by using two classes of drugs, small-molecule tyrosine kinase inhibitors and monoclonal antibodies (mAbs) (Dassonville et al., 2007). They share the same target, but display different EGFR specificity and mechanisms of action. Small-molecule tyrosine kinase inhibitors compete reversibly with adenosine 5′ triphosphate (ATP) binding to the EGFR tyrosine kinase domain of the receptor, and inhibit EGFR autophosphorylation and the downstream EGFR signalling pathways. The mAbs bind to the extracellular domain of EGFR, and compete with endogenous ligands and interfere with ligand-dependent receptor activation by blocking the ligand-binding region (Dassonville et al., 2007).

Great efforts have also been made for the molecular targeting of nanoparticles by EGFR pathway and this is the main focus of the current review paper.

EGFR targeting by ligand conjugated nanoparticles

EGFR targeting by antibodies decorated nanoparticles

Antibodies with specific binding affinity to cell-surface receptors enable the selective delivery of drug-loaded nanocarriers to target cells and may possess therapeutic effects as well (Zhong et al., 2014; Perez-Herrero and Fernandez-Medarde, 2015). EGFR is the first molecular target for cancer therapy against which mAbs have been developed. Till now, a number of mAbs such as cetuximab (Erbitux®), panitumumab (Vectibix™), necitumumab (TheraCIM®), and matuzumab have been developed for blocking EGFR activation (Friedlander et al., 2008; Pirker, 2013). Among them, cetuximab (Kaluzova et al., 2015; Tseng et al., 2015) and panitumumab (Li et al., 2012; Maya et al., 2013; Yook et al., 2015, 2016) have been conjugated to different nanocarriers to improve cancer imaging and therapy over non-targeted carriers (Tables 2-4).

Cetuximab has been conjugated to different nanoformulations, namely liposomes, nanoparticles, and micelles (Tables 2-4). Cetuximab (Erbitux®, C225) is a monoclonal chimeric human–murine IgG1 antibody that

Table 2. Selected examples of research on EGFR targeted liposomes.

Targeting ligand	Cargo	Cell line / Tumor	Observations	Investigation status	Ref.
Cetuximab	Celecoxib	Human colon cancer cell line	Celecoxib loaded anti-EGFR immunoliposomes improved toxicity compared to the non-targeted ones in EGFR-overexpressed cancer cells.	In vitro	(Limasale et al., 2015)
Cetuximab	Benzoporphyrin derivative monoacid A	Human epidermoid carcinoma cell line, human breast carcinoma cell line, human epithelial ovarian carcinoma cell line	The cetuximab targeted liposomes selectively bound to EGFR overexpressed cells. The inhibition of EGFR signaling by photo-immunoconjugate associating liposomes enhanced photodynamic therapy.	In vitro	(Mir et al., 2013)
Cetuximab	Indocyanine green	Human epidermoid carcinoma cell line	The binding of cetuximab targeted fluorescent labeled liposomes to A431 was greater compared with normal enterocytes expressing physiological EGFR levels, ensuring imaging abilities of the targeted nanocarrier.	In vitro	(Portnoy et al., 2011)
Cetuximab / cetuximab-Fab' fragments	Oxaliplatin	Colorectal cancer	EGFR targeted oxaliplatin liposomes were more efficient in tumor drug accumulation than free drug or non-targeted vesicles, which improved efficacy in mice inoculated with a tumor cell line overexpressing this receptor.	In vitro / in vivo	(Zalba et al., 2015)
Cetuximab-Fab' fragments	Doxorubicin		Large-scale, GMP-compliant production of anti-EGFR-targeted nanoparticles for clinical application was described.	In vitro	(Wicki et al., 2015)
EGFR antibody	Cisplatin	Non small cell lung cancer	Cisplatin loaded EGFR targeted liposomes showed higher efficacy than non-targeting nanocarriers both *in vitro* and *in vivo*. The targeted nanocarrier radiosensitized cells in a targeted manner without inducing nephrotoxic effects.	In vitro / in vivo	(Jung et al., 2015)
Anti-EGFR Fab'	siRNA	Hepatocellular carcinoma	Targeted nanocarriers possessed high siRNA entrapment and improved serum stability. Compared with non-targeted vesicles, targeted carriers showed enhanced EGFR targeting efficiency and achieved a superior gene silencing activity.	In vitro / in vivo	(Gao et al., 2012)
EGF	Cisplatin	Ovarian cancer	Sodium alginate-cisplatin conjugate was synthesized and incorporated into EGF-targeted liposomes. This targeted nanoparticle improved the antitumor efficacy *in vitro* as well as *in vivo*.	In vitro / in vivo	(Wang et al., 2014)
EGF	Oxaliplatin	Colorectal cancer	Targeted liposomes showed improved oxaliplatin cytotoxicity, higher tumor accumulation, and more homogeneous tumor distribution.	In vitro / in vivo	(Zalba et al., 2016)

Table 2. (continue).

Anti-human HB-EGF monoclonal antibody (IgG)	Doxorubicin	Breast cancer	Doxorubicin loaded anti-HB-EGF targeted liposomes caused strong suppression and regression of MDA-MB-231 tumors in mice.	In vitro / in vivo	(Nishikawa et al., 2012)
EGa1 nanobody	AG538: an anti-IGF-1R kinase inhibitora	Head and neck squamous cell cancer	AG538-loaded nanobody decorated liposomes blocked EGFR and IGF-1R activation, downregulated EGFR, and induced a strong inhibition of tumor cell proliferation.	in vivo	(van der Meel et al., 2012)
EGa1 nanobody	AG538: an anti-IGF-1R kinase inhibitor	Head and neck squamous cell, breast cancer	Tumors highly dependent on EGFR and IGF-1R signaling could be treated by combination therapy with kinase inhibitor-loaded nanobody decorated liposomes.	In vitro / in vivo	(van der Meel et al., 2013)
GE11	Doxorubicin	Non small cell lung cancer	GE11-conjugated liposomes showed higher accumulation and prolonged retention in tumor tissue.	In vitro / in vivo	(L. Cheng et al., 2014)

specifically targets the human EGFR with a 2-log higher affinity than the natural ligands, TGF-α and EGF (Kim et al., 2001; Capdevila et al., 2009). It inhibits signal transduction through binding to domain III of the EGFR extracellular region and, thereby blocks ligand binding. The binding of cetuximab to EGFR promotes receptor internalization and subsequent degradation, resulting in downregulation of the receptor. Cetuximab may also act via antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity. The cetuximab antitumor activity has been demonstrated in preclinical studies, including blockage of the cell cycle in G1, inhibition of proliferation and angiogenesis, induction of apoptosis, inhibition of DNA repair, and inhibition of tumour cell motility, invasion, and metastasis (Labianca et al., 2007; Vincenzi et al., 2010).

The US Food and Drug Administration (FDA) has approved cetuximab as a first-line treatment for EGFR-overexpressing *K-ras* wild-type metastatic colorectal cancer in combination with chemotherapy. Cetuximab is also indicated for the treatment of patients with recurrent and/or metastatic squamous cell carcinoma in combination with platinum-based therapy plus 5-florouracil (5-FU) and in combination with radiation therapy for locally advanced disease (Harding and Burtness, 2005; Vincenzi et al., 2010; Petrelli et al., 2014).

In a recent study, anti-EGFR mAbs (225) were conjugated to organic-inorganic hybrid liposomal cerasomes creating immunocerasomes for selective EGFR cancerous cell cargo delivery (Leung et al., 2014). mAbs were conjugated to cerasomes via maleimide–thiol coupling chemistry and a fluorescent lipid (NBD-DPPE) was incorporated into nanocarriers for imaging analysis of cellular binding, internalization, and intracellular fate of the lipidic nanovesicles in three cell lines differing in EGFR expression. By conjugation of about 60 anti-EGFR mAbs to each nanovesicle, receptor-mediated endocytosis of immunocerasomes was increased by 4.6-fold and 2.4-fold in A431 epidermoid carcinoma cells and DU145 prostate carcinoma cells, respectively. Immunocerasomes also inhibited the proliferation of A431 cells. Interestingly, the presence of serum in the cell culture medium increased the endocytosis of immunocerasomes, possibly by stimulating a variety of cell activities such as the recycling of internalized EGFR (Leung et al., 2014).

Panitumumab is a fully human IgG2 antibody. Similar to cetuximab, the induction of cell cycle arrest, promotion of apoptosis, and EGFR downregulation have been proposed as mechanisms of growth inhibition (Keating, 2010). In a study on panitumumab decorated liposomes, S. Li et al. (2012) proposed a new post-lumpectomy locoregional therapy using tumour targeted immunoliposomes through a technique of preparing liposomes carrying diagnostic technetium-99m (^{99m}Tc), and therapeutic beta-emitting (rhenium-186 (^{186}Re)/rhenium-188 (^{188}Re)) radionuclides for cancer therapy with the advantages of real time monitoring of pharmacokinetic and prediction of therapy effect. Panitumumab was conjugated to PEGylated lipids, and the antibody-modified PEGylated lipids were incorporated into liposomes comprising DSPC/DSPG/

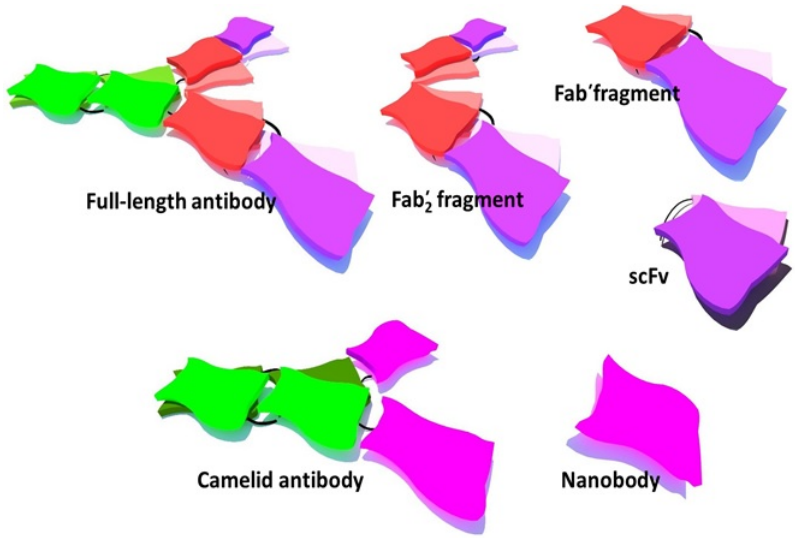

Figure 3. Schematic representation of antibody, antibody fragments, single-chain fragment variable (scFv) antibody and nanobody.

cholesterol. An enhanced green fluorescence protein-expressing MDA-MB-231 breast cancer orthotopic xenograft nude rat model was used for in vivo retention and biodistribution monitoring. The groups treated with panitumumab modified liposome had higher intracavitary retention as compared to the non-targeted vesicle administered groups. Beta-emitting radionuclide-carrying panitumumab targeted liposomes improved therapeutic potential as compared to non-targeted liposomes for use in enhanced post-lumpectomy focal radiotherapy to eradicate peripheral lymph node and locally remaining metastatic breast cancer cells with low systematic toxicity (Li et al., 2012).

EGFR targeting by Fab' decorated nanoparticles

Most mAbs that are used for targeted drug delivery belong to the IgG class of immunoglobulins (IgG1, IgG2a or IgG2b). Although the approach of ligand mediated targeting was initialized by mAbs, the trend has shifted at least partially towards the use of antibody fragments for targeting drug nanocarriers. It has been reported that mAbs have higher binding avidity due to the presence of two binding sites on the molecule (Fig. 3). However, linking the whole mAb to nanoparticles usually results in random orientation of this molecule on the carrier surface, which limits antigen or receptor binding site exposure and also yields exposure to Fc domains. The presence of the Fc domain can promote mAb binding to normal tissues through Fc receptors, particularly on macrophages. This can result in high liver and spleen uptake of the ligand decorated nanocarriers, faster clearance from circulation, and increased immunogenicity of the molecule (Allen, 2002).

$F(ab')_2$ and Fab′ fragments (Fig. 3) lack the Fc domain and the complement-activating region, and this might abolish uptake by the phagocytic system and reduce their immunogenicity. $F(ab')_2$ fragments have two binding regions linked by disulphide bonds and can be quite stable during storage. Under reducing conditions, the disulphide bonds are cleaved to yield two Fab′ fragments—each of these Fab′ fragments contains a thiol (-SH) group that is very useful for the conjugation of the fragments to the nanocarrier. Fab′ fragments, however, have only one binding site, which reduces their binding avidity. This disadvantage can be restored by coupling several fragments at the nanocarrier's surface (Allen, 2002; Cheng and Allen, 2008).

Fab′ fragments have been used to direct liposomes to EGFR-overexpressed cells (Zalba et al., 2015; Haeri et al., 2016). We prepared a novel multifunctional stimulus-triggered nanocarrier by a preparation of thermosensitive liposomes conjugated to Fab′ fragments of cetuximab to combine the tumour-targeting capability of EGFR specific ligands with on-demand drug release properties of heat sensitive liposomes (Haeri et al., 2016). Cetuximab-Fab′ fragments, comprising both variable heavy (V_H) and variable light (V_L) domains, have been reported to retain the antigen-binding affinity of cetuximab (cetuximab K_d ~ 2.7 nM; Fab′ K_d ~ 3.7 nM) (Kamat et al., 2008; Hur et al., 2010). The effect of ligand density on in vitro targeting efficiency was studied. The results revealed that a density of about 36-40 Fab′ fragments per vesicle was enough to enable receptor-ligand binding. The physicochemical characteristics of liposomes did

Table 3. Selected examples of research on EGFR targeted nanoparticles.

Nanocarrier	Targeting ligand	Cargo	Tumor	Observations	Investigation status	Ref.
Silk fibroin nanoparticle	iRGD–EGFR nanobody	Paclitaxel	Cervical cancer	Nanonody conjugated paclitaxel loaded nanocarriers showed superior antitumor efficacy and better in vitro and in vivo tumor targeting compared to unconjugated nanoparticles in EGFR overexpressing tumors.	In vitro / in vivo	(Bian et al., 2016)
Iron Oxide nanoparticle	ScFv from EGFR antibody		Breast cancer cell line	Protein-coated nanoconstructs as a promising new class of MRI contrast agents were prepared and easily functionalized with a single chain fragment from the antibody of EGFR.	In vitro / in vivo	(Huang et al., 2013)
Superparamagnetic iron oxide nanoparticles	EGFR-targeting peptide		Lung cancer	Results showed that EGFR targeting enhanced tumor retention of nanoparticles. Magnetic hyperthermia treatment using targeted nanoparticles resulted in significant inhibition of in vivo lung tumor growth.	In vitro / in vivo	(Sadhukha et al., 2013)
Polymeric nanoparticles	EGFR-specific monoclonal antibody	Gemcitabine	Human pancreatic cell line	Antibody-conjugated PLGA-PEG nanoparticles were prepared by direct covalent coupling of antibodies to vesicles using glutaraldehyde and showed specific targeting to EGFR-overexpressing cells.	In vitro	(Aggarwal et al., 2013)
Polymeric nanoparticles	EGFR targeting peptide	Tylocrebrine	Human epidermoid cancer	Encapsulation of the drug in polymeric nanoparticles significantly limited its CNS penetration and toxicity. Targeted nanoparticles enhanced tumor cell uptake, tumor tissue accumulation, and in vivo antitumor efficacy.	In vitro / in vivo	(Kirtane et al., 2015)
Gold nanoparticles	Panitumumab		Human breast cancer	Targeted gold nanoseeds as a novel neoadjuvant brachytherapy agent injected intratumorally were highly effective for inhibiting the tumor growth.	In vitro / in vivo	(Yook et al., 2016)
Gold nanoparticles	Cetuximab		Lung cancer	Cetuximab gold nanoparticles were prepared and radiolabeled with In-111. Elevated uptake of the targeted nanoparticles into the tumor was observed.	In vitro / in vivo	(Kao et al., 2014)
O-carboxymethyl chitosan nanoparticle	Cetuximab	Paclitaxel	Epidermoid cancer, lung cancer, and breast cancer cell lines	Spherical stable targeted nanoparticles were prepared. Enhanced cell death was observed in different EGFR positive cancer cell lines exposed to cetuximab targeted chitosan nanoparticles.	In vitro	(Maya et al., 2013)
Lipid nanoparticles	Fab' antibody against heparin-binding EGF-like growth factor	siRNA	Human breast cancer cell line	siRNA encapsulated in targeted nanoparticles induced obvious suppression of both target mRNA and protein levels in MDA-MB-231 cells resulting in effective gene silencing.	In vitro	(Okamoto et al., 2014)

Table 3. (continue).

Lipid based nanoparticles	EGF	Gemcitabine	Breast cancer	EGF conjugated nanoparticles accumulation in tumor cells was correlated to EGFR expression. In vivo, EGFR over-expressing tumors treated with the targeted nanoparticles grew significantly slower than tumors treated with untargeted vesicles.	In vitro / in vivo	(Sandoval et al., 2012)
Gelatin nanoparticles	EGF	Doxorubicin	Lung cancer	The targeted nanoparticles effectively internalized into and inhibited EGFR overexpressing lung cancer cells via a receptor-mediated endocytosis. The tumor growth remarkably suppressed in animals treated with targeted nanoparticles.	In vitro / in vivo	(Long et al., 2014)
Eudragit based hollow mesoporous silica nanoparticles	EGF	5-fluorouracil	Colorectal cancer cell line	Targeted 5-FU loaded highly dispersed mesoporous silica nanospheres were prepared and highly specific targeting to EGFR positive cells was achieved.	In vitro	(She et al., 2015)

not change significantly upon ligand conjugation. Fab´-functionalized thermosensitive liposomes can specifically and efficiently bind to the EGFR overexpressed cancer cells. Calcein labelled Fab´-conjugated thermosensitive liposomes showed adequate stability at 37°C in serum and a temperature dependent release at > 40°C. FACS analysis and live cell imaging revealed EGFR mediated cellular association as well as dramatic intracellular cargo release upon hyperthermia. Fab´-conjugation and induced hyperthermia enhanced tumour cell cytotoxicity of doxorubicin loaded liposomes. The relative cytotoxicity of Fab´-linked liposomes was also correlated to EGFR density on the tumour cells.

These results suggest that this new combinatory active targeting and triggering strategy may offer a promising approach for selective treatment of EGFR high-expressing tumours, while restricting drug delivery to the tumour site by localized hyperthermia (Haeri et al., 2016).

EGFR targeting by single-chain variable fragment (scFv) decorated nanoparticles

Small recombinant antibody fragments are being increasingly used as alternatives to mAbs for medical therapeutic and diagnostic applications. One of the most promising types of recombinant antibody fragments is scFv (Fig. 3) (Ahmad et al., 2012; van der Meel et al., 2013).

The scFv is the smallest unit of immunoglobulin molecule that functions in antigen-binding activities and consists of V_H and V_L chains, which are joined together by a flexible peptide linker. Peptide linkers usually vary from 10 to 25 amino acids in length, and typically include hydrophilic amino acids to avoid intercalation of the peptide within or between the variable domains throughout the protein folding. The length of the flexible is critical in yielding the correct folding of the polypeptide chain (Weisser and Hall, 2009; Ahmad et al., 2012).

Engineered scFvs conjugated to various nanoformulations are poised to provide the next wave of Ab-mediated targeted drug delivery platforms. These promising ligands have been utilized for EGFR targeting of tumours (Tables 2 and 3) (Peng et al., 2011; Huang et al., 2013).

Peng et al. (2011) developed a novel scFv conjugated-heparin nanoparticle for targeted delivery of cisplatin to EGFR-positive tumour cells. The nanocarrier was evaluated in terms of drug loading efficiency, sustained drug release profile, and in vitro cytotoxicity.

The results showed that the targeted nanoparticles can significantly increase intracellular concentrations of cisplatin in EGFR-expressing NSCLC H292 cells via an EGFR-mediated pathway. Compared to the free drug, systemic delivery of the nanoparticles significantly prolonged drug blood circulation time, and improved pharmacokinetics and biodistribution profiles. The new nanoparticle delivery system significantly enhanced cisplatin antitumor activity without weight loss or toxicity to the kidney and spleen in nude mice bearing H292 cell tumours (Peng et al., 2011).

Table 4. Selected examples of research on EGFR targeted micelles.

Targeting ligand	Cargo	Tumor	Observations	Investigation status	Ref
Cetuximab	Docetaxel	Breast cancer cell lines	Judged by IC50, therapeutic effects of docetaxel greatly enhanced by the formulation of cetuximab conjugated TPGS micelles. Targeted micelles showed successful delivery of docetaxel into the tumors.	In vitro / in vivo	(Kutty and Feng, 2013; Kutty et al., 2015;)
Cetuximab	Doxorubicin / superparamagnetic iron oxide	Human epidermoid carcinoma cell line	Immunomicelles showed specific interactions with EGFR-overexpressing tumor cells and higher cytotoxicity.	In vitro	(Liao et al., 2011)
GE11	Silicon phthalocyanine-4 photosensitizer	Head and neck cancer	EGFR-targeted photosensitizer nanoformulation showed improved uptake as well as significant cell-killing in EGFR-overexpressing cancer cells. EGFR-targeted nanoformulation resulted in significant intratumoral cargo uptake and subsequent enhanced photodynamic therapy response in vivo.	In vitro / In vitro	(Master et al., 2013)
GE11	Aminoflavone	Triple negative breast cancer	GE11 peptide targeted nanocarrier resulted in enhanced cellular uptake, strong growth inhibitory effects, and high plasma and tumor levels in triple negative breast cancer.	In vitro / In vitro	(Brinkman et al., 2016)
GE11	Coumarin-6 / paclitaxel	Human laryngeal cancer cell line	Paclitaxel loaded GE11-modified micelles efficiently bound to target cell and significantly inhibited cell proliferation.	In vitro	(Ren et al., 2015)
GE11	Doxorubicin	Liver cancer	GE11-modified micelles exhibited a much higher level of cargo in tumor tissue than nontargeted micelles.	In vitro /in vivo	(Fan et al., 2016)
LT6 hexapeptide	Doxorubicin / paclitaxel	Human ovarian carcinoma cell line human breast adenocarcinoma cell line	The peptide-conjugated micelles increased intracellular accumulation and cytotoxicity of anticancer drugs in EGFR high-expressed cells.	In vitro	(Lin and Kao, 2014)

EGFR targeting by nanobodies decorated nanoparticles

Notwithstanding the vast amount of testing and research on engineering Fab' fragment, variable fragment, and scFv to overcome the restrictions of full-length mAbs, their average activities are still suboptimal due to lower affinities and limited stability, especially in the case of scFv. Nanobodies, recombinant single-domain, variable fragments of camelid heavy chain-only antibodies (~95 kDa) (Fig. 3), which are able to bind selectively to a specific antigen, may address several of these concerns. Nanobodies, with approximate molecular weight of 12-15 kDa, are considered the smallest naturally derived antigen-binding fragment (Fig. 3).

The investigation of their structures revealed a prolate (rugby ball) shape of approximately 2.5 nm in diameter and 4.2 nm in length. Nanobody hallmarks include specificity and affinity, small size, high solubility, refolding capacity and stability, weak immunogenicity, ease of cloning with high yield as well as thermal and chemical resistance (Oliveira et al., 2013; Chakravarty

et al., 2014; Kijanka et al., 2015; Van Audenhove and Gettemans, 2016). With all the mentioned characteristics, nanobodies are very promising building blocks to function as novel targeting ligand for a wide variety of nanoparticulate platforms. Nanobody-linked nanoparticles have been used for enhanced EGFR specificity (Tables 2 and 3).

As an example, PEGylated extracellular vesicles were decorated with EGFR specific nanobodies. The nanobodies were conjugated to phospholipid DMPE-PEG derivatives to prepare nanobody-PEG-micelles.

By 'post-insertion' method, a temperature-dependent transfer of nanobody-PEG-lipids to the vesicle membranes was observed.

This process did not affect extracellular vesicles size distribution, morphology, or protein composition. Due to the shielding properties of PEG, cellular binding of PEG-conjugated control nanobodies to extracellular vesicles was compromised. However, EGFR-specific nanobodies dramatically increased specific binding to EGFR-overexpressing tumour cells. Moreover, the circulation time of nanobody-PEG-lipids increased at least six times as compared to unmodified extracellular vesicles (Kooijmans et al., 2016).

EGFR targeting by EGF decorated nanoparticles

EGF is an endogenous growth factor and the natural ligand for EGFR. It is a single-chain polypeptide comprising 53 amino acids with a molecular weight of approximately 6 kDa. EGF is a small stable peptide that undergoes receptor-mediated endocytosis and transports the ligand-receptor complex into the target cell (Bohl Kullberg et al., 2002). EGF offers unique advantages for EGFR targeting over EGFR antibody: Stronger binding affinity and more rapid clearance rates due to a smaller molecular weight as compared to the EGFR antibody (Diagaradjane et al., 2008; Ryu et al., 2013). However, its use has provided different results, leading to a controversy across experiments and authors (Bhirde et al., 2009; Bunuales et al., 2011). The binding of EGF to EGFR induces the phosphorylation of the receptor promoting the activation of an internal signalling pathway involved in several processes, including cell proliferation. It was reported that treatments with cetuximab and EGF led to the inhibition and the activation of cell proliferation, respectively. Interestingly, some authors reported that no changes were observed in the phosphorylation basal status at the receptor and the post-receptor levels after treatment with EGF-conjugated nanoparticles (Zalba et al., 2016).

In a recent study, hollow mesoporous silica nanoparticles (HMSNs) were functionalized with EGF in order to selectively target colorectal cancer cells that overexpressed EGFR. HMSNs are one of the promising carriers for drug delivery due to several advantages like large surface area and high volume for drug loading. However, the non-ionic surfactant templated HMSNs often have limitations such as a broad size distribution and a defective mesoporous structure. In this work, HMSNs with large internal cavities were prepared by utilizing the Eudragit nanoparticles as the core template and an assistant in the self-organization of surfactant micelles. The HMSNs have uniform pore sizes (2.5 nm) and small diameters (120 nm) that facilitate the effective encapsulation of 5-fluorouracil. EGF conjugated HMSNs can specifically and efficiently target cancer cells with overexpressed EGFR (She et al., 2015).

EGFR targeting by peptide decorated nanoparticles

Peptides as targeting ligands have numerous advantages, including large scale production by chemical methods, high specificity and affinity, and small size. Screening of peptide libraries produced by either phage display or chemical synthesis is the main strategy to select peptide sequences with increased affinities to a specific target. Phage display is more widely used to identify peptides that target a specific receptor and is adaptable to both in vitro and in vivo studies. Selected peptides have been used as molecular probes for imaging as well as therapeutics. Till date, various peptide ligands have been discovered for different types of receptors or cells, such as integrin receptors, tumour cells, thrombin receptors, and cardiomyocytes. Tumour-targeting peptides have been successfully conjugated to nanovesicles to deliver imaging agents, small-molecule drugs, and oligonucleotides to tumours (Ruoslahti, 2012; Zhang et al., 2012).

Recently, a novel 12 amino acids peptide, GE11 (sequence: YHWYGYTPQNVI), was reported as a potent EGFR ligand (L. Cheng et al., 2014). GE11 peptide specifically and efficiently binds to EGFR with a much lower mitogenic activity than that of EGF (Z. Li et al., 2005).We designed thermosensitive liposomes functionalized with anti-EGFR ligands for targeted delivery and localized triggered release of chemotherapy (Haeri et al., 2016). For targeting, an EGFR-specific peptide (GE11) was used. The prepared multifunctional nanoparticles were characterized with regard to cellular binding, uptake, and cytotoxicity studies using flow cytometry, live cell imaging, and cell viability assay on cell lines with different expressions of EGFR under normothermic and hyperthermic conditions. In our study, it has been shown that much lower number of cetuximab Fab' targeting moieties was required on the surface of thermosensitive liposomes as compared to GE11 to achieve similar EGFR binding (Haeri et al., 2016).

Recent investigations have shown that GE11peptide as a single molecule had no detectable binding affinity for EGFR and the attachment of several ligands was needed

for specific and measurable EGFR binding (Abourbeh et al., 2012). GE11 binding affinity for EGFR was reported to be approximately 10–20 folds lower than EGF (Z. Li et al., 2005).

Conclusion

In the past decade, rapid development has taken place in ligand-directed active tumour targeting of nanoparticles for cancer chemotherapy. EGFR-targeted nanoparticles can be used to deliver an imaging and therapeutic agent to EGFR-overexpressing tumour cells. In vitro and in vivo studies have demonstrated that EGFR specific ligand-decorated nanoparticles can enhance accumulation and retention of drugs in the tumour tissue, increase targeted cell uptake, improve therapeutic efficacy, and minimize systemic side effects. Together, these research results highlight EGFR-targeted nanoparticulate platforms as being an effective therapeutic option for EGFR-overexpressing tumours.

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