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Nanomedicines functionalized with anti-EGFR ligands for active targeting in cancer therapy: biological strategy, design and quality control

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Abstract

Recently, active targeting using nanocarriers with biological ligands has emerged as a novel strategy for improving the delivery of therapeutic and/or imaging agents to tumor cells. The presence of active targeting moieties on the surface of nanomedicines has been shown to play an important role in enhancing their accumulation in tumoral cells and tissues versus healthy ones. This property not only helps to increase the therapeutic index but also to minimize possible side effects of the designed nanocarriers. Since the overexpression of epidermal growth factor receptors (EGFR) is a common occurrence linked to the progression of a broad variety of cancers, the potential application of anti-EGFR immunotherapy and EGFR-targeting ligands in active targeting nanomedicines is getting increasing attention. Henceforth, the EGFR-targeted nanomedicines were extensively studied *in vitro* and *in vivo* but exhibited both satisfactory and disappointing results, depending on used protocols. This review is designed to give an overview of a variety of EGFR-targeting ligands available for nanomedicines, how to conjugate them onto the surface of nanoparticles, and the main analytical methods to confirm this successful conjugation.

Keywords: EGFR; nanomedicine; active targeting; cancer therapy; biological ligands

1. Epidermal Growth Factor Receptor (EGFR) and its role in tumor development

The epidermal growth factor receptor (EGFR/ErbB1/HER1) is a 170 kDa transmembrane glycoprotein of the tyrosine kinase family, which comprises HER1; HER2; HER3 and HER4. This protein is a single polypeptide chain of 1186 amino acid residues and a substantial amount of N-linked oligosaccharides, which divide an EGFR into three principal domains including an extracellular ligand-binding domain, a single hydrophobic transmembrane domain and a cytoplasmic tyrosine kinase (Fig. 1). On one side, the intracellular domain is highly conserved, typical for the family and helps distinguish EGFRs from other receptors in the same family. On the other hand, the extracellular domain is less conserved and allows EGFRs to bind to different ligands. The extracellular domain consists of four smaller segments from DI to DIV. DI and DIII are essential in the ligand-binding process, whereas DII and DIV constitute the disulfide bond (De Luca *et al.*, 2008; Grapa *et al.*, 2019; Sasaki *et al.*, 2013).

The activation of an EGFR occurs when its ligand binds to its extracellular domain. There are several natural mammalian ligands for EGFRs including the epidermal growth factor protein (EGF), the transforming growth factor- α (TGF- α), epiregulin (EPR), betacellulin (BTC), and EGFR-like growth factors. As shown in the Fig. 1, once the ligand binds to an EGFR, the dimerization and the phosphorylation of the cytoplasmic domain will occur and lead to a cascade of subsequent downstream processes including the phosphatidylinositol-3-kinase (PI3K) pathway, the signal transducer and activator of transcription 3 (STAT3) pathway, and the Ras-Raf-MAPK pathway (Grapa *et al.*, 2019; Sasaki *et al.*, 2013).

In normal cells, EGFRs play an important role in two main signal pathways of cell proliferation. Their expression is tightly regulated to ensure that the kinetics of cell proliferation corresponds to the tissues' requirement for homeostasis. On the contrary, in cancer cells, EGFRs are often perpetually stimulated. The EGFR overexpression has been found to be correlated to multiple cancer-related signaling pathways and contributes significantly to chemotherapy/radiotherapy resistance, angiogenesis, and apoptosis (Grapa *et al.*, 2019; Sasaki *et al.*, 2013). The expression level of EGFRs in ordinary and cancer cells is defined at $4.0-10.0 \times 10^4$ and over 10^6 EGFRs, respectively (Akbarzadeh Khiavi *et al.*, 2020). To determine the EGFR expression level, several methods can be used such as the analysis of DNA, RNA, the protein level or the assessment of the degree of receptors by analyzing the receptor activation or downstream markers. Among these methods, immunohistochemistry (IHC) is the most widely used method for clinical samples (Ciardiello and Tortora, 2003). The EGFR overexpression has been found to be related to poor prognosis in a variety of human solid tumors such as non-small cell lung, head and neck, colorectal, pancreas, breast, ovary, and bladder and kidney cancers. Table 1 gives information on the expression rate of EGFRs in these cancers and the EGFR-overexpressing cancer cell lines that are usually used in research.

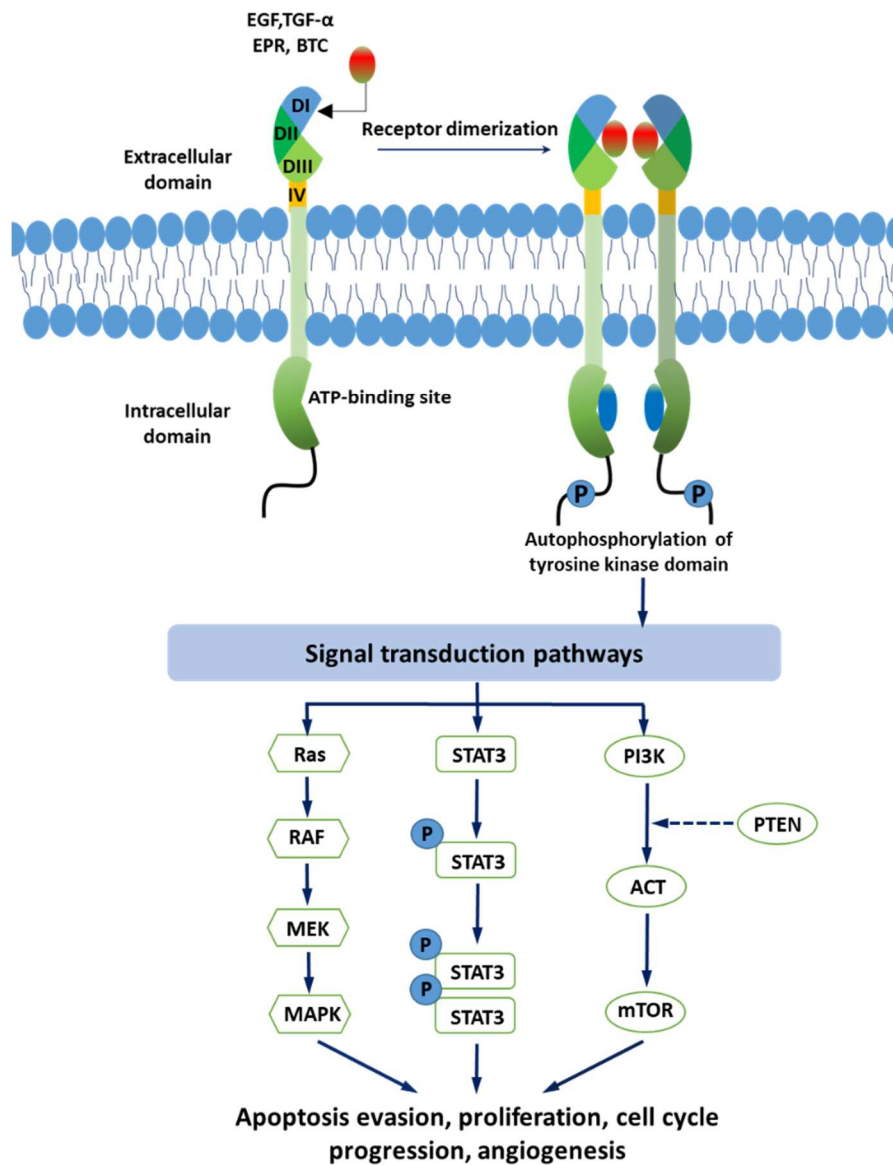


Fig. 1. EGFR structure and EGFR signaling pathways

Table 1. Types of cancer with EGFR overexpression and cancer cell lines used in preclinical studies

Type of cancer	Cancer cell line used in research	EGFR overexpression rate (%)	References
Non-small cell lung cancer	A549; H322; H358; H460; H1299; H1975	40-80	(Jorge et al., 2014; Master and Sen Gupta, 2012; Yoon et al., 2016)
Squamous-cell carcinoma of head and neck cancer	UM-SCC-14C, UM-SCC-22A, SCCVII	80-100	(Altintas et al., 2013; Master and Sen Gupta, 2012; ping et al., 2010; Zimmermann et al., 2006)
Colorectal cancer	Caco-2, HT-29, SW480, SW403, SW620, HCT116, HCT-8	25-77	(Krasinskas, 2011; Li et al., 2019; Master and Sen Gupta, 2012; Z. Zhang et al., 2018)
Pancreatic cancer	Pan-1, Capan-1	30-89	(Du et al., 2018; Grapa et al., 2019; Xu et al., 2013)
Breast cancer	MDA-MB-468, MDA-MB-231, MCF-7	15-45	(Brinkman et al., 2016; Changavi et al., 2015; Jin et al., 2017; Kim et al., 2017)
Ovarian cancer	SKOV-3	4-100	(Chen et al., 2017; Teplinsky and Muggia, 2015)
Bladder and kidney cancer	MBT-2	31-46	(Li et al., 2018)
Gastric cancer	MKN45	27-64	(Arienti et al., 2019; Tsai et al., 2018)

Considering the involvement of EGFRs in diverse cancer cellular processes, EGFRs appear as an interesting target for therapeutic intervention. Thus, a number of strategies have been developed to target and/or inhibit the EGFR expression. As therapeutic agents, monoclonal antibodies (mAb) and tyrosine kinase inhibitors (TKI) are being employed to inhibit EGFR expression. Anti-EGFR mAbs such as cetuximab bind to the extracellular domain and prevent the dimerization process, whereas TKIs including gefitinib, erlotinib, lapatinib block the proliferation signaling by targeting the intracellular TK domain. However, the use of anti-EGFR agents as therapeutic agents has demonstrated both satisfactory and disappointing results in clinical trials (Akbarzadeh Khiavi et al., 2020).

On the other hand, nanoparticles (NPs) functionalized with active targeting ligands are more and more developed and have emerged as one of the most promising strategies for cancer treatment. Not surprisingly, EGFRs appear among the most targeted receptors in active targeting nanomedicines. The following sections will be specifically focused on the application of EGFR-targeting molecules not as therapeutic agents but as targeting ligands for nanoscale-targeted systems.

2. EGFR-targeting ligands for active targeting nanomedicine

Since its discovery, the active targeting in nanomedicines has proved its potency to achieve high targeting specificity, drug delivery efficiency, and to avoid nonspecific binding. These properties not only help improve the therapeutic index due to a higher tumor accumulation of encapsulated therapeutic agents but also attenuate severe side effects that may improve the tolerance of patients with anti-cancer adjuvants' treatment. As a result, several targeting moieties have been developed and can be classified into four main groups including proteins (mainly mAbs and mAbs fragments), peptides, nucleic acids (aptamers), and small molecules (Yu *et al.*, 2012). This section is focused on different EGFR-targeting moieties used for active targeting with nanoparticles (NPs) as well as their potential benefits and drawbacks.

2.1. Epidermal growth factor protein and its derivatives

The epidermal growth factor (EGF) protein is the natural molecule that innately binds to EGFRs. The EGF-EGFR binding leads to receptor internalization via the clathrin-mediated endocytosis pathway. Significant efforts have been carried out to investigate the application of EGF-EGFR recognition to target cancer cells for diagnostic and therapeutic purposes with nanomedicines (Master and Sen Gupta, 2012).

2.1.1. EGF protein

The EGF protein was firstly isolated from the parotid gland of male mice and then from the urine for the human EGF. This protein is considered as a "urogastron" due to its ability to inhibit gastric acid secretion in human. The biological activity of the human EGF overlaps with that of the murine EGF (Nakagawa *et al.*, 1985; Sandoval *et al.*, 2012). It is reported that EGF proteins can prompt abundant effects on both cells and epithelial tissues. Moreover, EGF proteins have various effects on cell regeneration processes including the stimulation of proliferation, the migration of keratinocytes, the formation of granulation tissues, and the stimulation of fibroblast motility.

Structurally, an EGF is a single polypeptide consisting of 53 amino acids with a molecular weight (MW) of 6 kDa (Table 2). An EGF protein possesses six cysteine residues forming three internal disulfides, which might be used for further conjugation with NPs. Its binding affinity to EGFRs as reflected by the dissociation constant (K_d) is determined to be from 1.0 to 2.0 nM. Nowadays, several methods can be used to produce EGF proteins in industry such as recombinant DNA methods in animals, bacteria, yeasts and adenoviral vectors (Negahdari *et al.*, 2016).

As targeting ligands, the EGF protein is one of the most widely used ligands for EGFR-targeting. The functionalization of NPs with EGFs offers numerous advantages such as: i) a smaller size than that of antibodies (6 kDa vs 150 kDa), ii) a low risk of cytotoxicity, iii) easy conjugation thanks to the three internal disulfides, and iv) good stability at physiological conditions and neutral pH (Grapa *et al.*, 2019; Silva *et al.*, 2016). Many studies have confirmed the benefits of EGF-conjugated NPs in increasing the cell uptake and in the tumor accumulation that yield better anti-tumor activities and reduced systemic toxicity.

Venugopal *et al* carried out a standard example for EGF-conjugated NPs. The authors showed that by conjugating EGFs onto their co-polymeric PLGA-PEG NPs, a significant reduction of breast cancer cells (MDA-MB-468) viability (around 10.6 %) and a 93-fold higher paclitaxel accumulation had been achieved in the corresponding tumor group compared to the group treated with non-targeted NPs (Venugopal *et al.*, 2018). In another effort to improve the radiotherapy efficiency in esophageal cancer, Gill *et al* constructed PLGA NPs functionalized with EGF proteins for a specific delivery of a

therapeutic radionuclide. The finalized NPs presented a better cellular uptake and a greater radiotoxicity in EGFR-overexpressed cancer cells (OE21) compared to normally EGFR-expressed cancer cells or healthy cells. This specific delivery reduced significantly the toxicity of the encapsulated radionuclide on healthy cells and might lead to fewer side effects for further clinical applications (Gill *et al.*, 2018).

In addition to targeting properties, when an EGF protein is introduced to highly charged NPs such as dendrimers, it neutralizes the highly positive charges of these NPs and may minimize their toxicity. This phenomenon was observed in the study of Li *et al.* In fact, by conjugating EGF protein onto positive dendrimers for a better gene delivery, the authors were successful to not only enhance the *in vivo* EGFR-positive breast cancer (MDA-MB231) tumor accumulation of the EGF-dendriplexes but also decrease the cytotoxicity of dendrimers compared to conventional dendrimers (Li *et al.*, 2015a).

All the aforementioned studies have demonstrated the attractive potency of EGF proteins as EGFR active targeting ligand in cancers. Nevertheless, its clinical application remains challenged by several issues. On one side, murine EGF proteins are prone to immunogenicity problems. On the other side, EGF proteins from human sources or recombinant ones are relatively complex and expensive, that may explain why its application is limited to the laboratory scale.

2.1.2. Anti-EGFR affibodies

Affibodies (Afbs) are scaffold proteins with a 3-helix bundle structure and are composed of 58 amino acids. Anti-EGFR affibodies (Table 2) are engineered to have i) high affinity and specificity to EGFRs with a K_d around 2.8 nM, ii) a low molecular weight and size (7 kD and 1-2 nm), iii) high stability and easy conjugation via its terminal cysteine, iv) lower immunogenicity than traditional antibodies (Jokerst *et al.*, 2011; Lucky *et al.*, 2016; Yoo *et al.*, 2019; Y. Zhang *et al.*, 2018).

The presence of anti-EGFR affibodies on the surface can help NPs better internalize (faster and higher) into EGFR-positive cancer cells, deliver drug more efficiently into tumor sites (higher concentration and longer retention), and attenuate cytotoxicity on normal cells. As an example, Lucky and co-workers functionalized their upconversion NPs with an anti-EGFR affibody for a better photodynamic therapy (PDT) against oral cancer. The affibody-decorated NPs internalized more rapidly and efficiently in EGFR-overexpressed oral cancer cells (OSCC cells) than non-targeted NPs (3.8 folds), or low EGFR expressing cancer cells (MCF-7 and HepG2 cells). Thanks to this specificity, higher PDT toxicity in EGFR positive cells (A431 and OSCC cells) than EGFR negative MCF-7 cells (35 % vs 0 % respectively) was achieved. Furthermore, this study also pointed out that the interaction between the grafted anti-EGFR affibodies on the NPs and the cell-surface EGFRs was a prerequisite for an enhancement in the cellular uptake (Lucky *et al.*, 2016). Kwon *et al* developed protein/gold core/shell NPs for targeted and nanotoxicity-free cancer therapy. To be precise, the core of these NPs was made of hepatitis B virus capsids decorated with anti-EGFR affibodies for active targeting and dotted with many small gold NPs for the photothermal activity (PTT). The affibodies present on the surface helped the targeted NPs to be effectively delivered to EGFR-overexpressed breast cancer cells (MDA-MB-468) and increased the tumor cell necrosis with a remarkable tumor size reduction compared to the bare AuNPs. In addition, the affibody-free AuNPs caused extensive and histological damages to the liver and the kidney, whereas no visible macroscopic and histological change was observed in major organs for three weeks post-injection in the case of targeted NPs (Kwon *et al.*, 2014).

2.1.3. Anti-EGFR repebodies

Anti-EGFR repebodies are another kind of protein that can be applied in EGFR active targeting with nanomedicines. Repebodies are a scaffold protein composed of leucine-rich repeated modules. In

addition to a relatively good binding affinity towards EGFRs ($K_d = 9.18$ nM), simple engineering and good specificity, these non-antibody proteins possess a smaller molecular size (30 kDa) and present better tissue penetration compared to entire antibodies (Table 2). For the production, anti-EGFR repebodies can be cloned into a pET21a vector with polyhistidine and polyanionic tags at the N-terminal and C-terminal ends. The constructed vector is then transformed into an *E. coli* host strain (Pyo *et al.*, 2018).

Due to its EGFR-binding ability, anti-EGFR repebodies were conjugated onto NPs in several studies for better targeting EGFR-positive tumors. For instance, Lee and co-workers fused an anti-EGFR repebody to apoptin, which is a tumor-selective apoptotic protein for cancer treatment. The resulting amphiphilic monomers could be self-assembled into micelles with a size around 48 nm, a high homogeneity and stability level. Consequently, the cellular uptake of the targeted NPs increased significantly according to the EGFR expression level of the cancer cells, which confirmed the targeting activity of the ligand. In addition, the cytotoxicity of the EGFR positive cancer cells (A431 and MDA-MB-468 cells) treated with the targeted NPs was remarkably enhanced compared to the negligible apoptotic activity of the free protein, or modest cytotoxicity against H1650 cells expressing a moderate level of EGFR, or low cytotoxicity against EGFR low-expressing cells (MCF-7) (Lee *et al.*, 2017).

Similar to the role of EGF protein in targeted nanomedicines with dendrimers, anti-EGFR repebodies once conjugated to highly charged NPs, can both help increase the drug delivery efficiency and attenuate the cytotoxicity linked to high charges. Using this strategy, Kim and colleagues fused genetically a polyanionic peptide to their anti-EGFR repebody that allowed its further conjugation with positively charged dendrimers. As expected, the gene delivery efficiency of the targeted dendrimers was correlated with the EGFR expression level due to the ligand targeting properties. In addition, the toxicity of the targeted dendrimers decreased with an increase in the repebody's amount on the NPs. This result indicated the perspective of using active targeting ligands to overcome the limitations of toxicity in dendrimers' application (Kim *et al.*, 2018).

Both anti-EGFR affibodies and repebodies belong to the group of non-antibody scaffolds and appear as interesting alternatives to conventional antibodies in EGFR targeting with nanomedicines. As therapeutic agents, the small size of these proteins may pose a major disadvantage in terms of short plasma half-life time (Gebauer and Skerra, 2020; Vazquez-Lombardi *et al.*, 2015), but in the case of nanomedicines functionalized with targeting moieties, the half-life time of the finalized NPs depends mainly on the properties of nanocarriers. Nevertheless, if the blood circulating time of the resulting NPs is not sufficient to have efficient therapeutic activities, one of the roots may come from these fragments. Therefore, several methodologies may be applied on targeting ligands to improve the stealthiness of the finalized NPs such as Fc fusion or polyethylene glycol (PEG) conjugation.

2.2. Anti-EGFR whole antibodies

Monoclonal antibodies (mAbs) are largely recognized as the most popular targeting ligand in the field of nanomedicines due to their safety; high tendency and specificity to target receptors (Alibakhshi *et al.*, 2017a).

They are Y-shaped proteins made of four polypeptide subunits and composed of identical heavy and light chains. Their arms are termed "fragment antigen-binding domains" (Fab) and the tail is termed "fragment crystallization domain" (Fc) with the role of activating cascades (Table 2). Each heavy chain and each light chain are composed of heavy and light variables (VH, VL), which create the site-binding region and constant (CH, CL) regions (Alibakhshi *et al.*, 2017a).

After binding to target receptors expressed at the cell surface, mAbs can cause a direct cytotoxic effect by inducing the apoptosis or block the corresponding ligands/cognate receptors. For tumor cells, as soon as mAbs interact with their receptors, the Fc domain can trigger cell destruction by the engagement of host immune effector mechanisms. This process includes two main pathways: i) the activation of cytotoxic enzymes of the complement cascade and/or ii) the triggering of cytotoxic effects of innate immune cells such as natural killer cells via the activation of Fc receptors or the phagocytosis activation by monocytes/macrophages (Marabelle and Gray, 2015).

For the EGFR-targeting, a number of human mAbs have been developed such as cetuximab; necitumumab; matuzumab; nimotuzumab and panitumumab (Li *et al.*, 2015b; Maya *et al.*, 2013). Among these mAbs, cetuximab (Cet; C225 or Erbitux®) is the most widely used in cancer treatment both as a therapeutic agent and as an active targeting ligand (Maya *et al.*, 2013). Cetuximab is the first FDA-approved EGFR-specific mAb for the treatment of EGFR-expressing metastatic colorectal cancer in combination with FOLFIRI (irinotecan, 5-fluorouracil, and leucovorin) (Li *et al.*, 2016). It is also indicated for the treatment of the locally advanced squamous cell carcinoma of the head and neck in combination with radiation therapy and for the recurrent or metastatic squamous cell carcinoma. This mAb is a chimeric human antibody (MW = 145.78 kD) from the immunoglobulin IgG1 class that specifically targets the human EGFR with a higher binding affinity ($K_d = 0.39$ nM) than EGFR natural ligands such as EGF protein or the transforming growth factor- α . Cet blocks ligand-binding and inhibits ligand-induced phosphorylation and activation of the EGFR tyrosine kinase. C225 has been shown to be effective in several antitumor processes including the G0/G1 cell-cycle arrest, the induction of apoptosis, the inhibition of DNA repair, the anti-angiogenesis, the tumor cell motility, invasion, and metastasis. However, the application of Cet remains challenged by potential cytotoxic effects through antibody-dependent cell-mediated cytotoxicity (Baselga, 2001; Specenier and Vermorken, 2013).

Numerous studies proved that the functionalization of NPs with Cet can enhance effectively drug cellular internalization into EGFR-overexpressed cancer cells. Furthermore, Cet-modified nanoparticles are able to recognize specifically EGFR-overexpressing cells and present better therapeutic and diagnostic effects with less toxicity on normal cells (Chu *et al.*, 2015; Costa *et al.*, 2017). Up to now, Cet remains the most used active targeting ligand in EGFR targeted nanomedicines. Liu *et al.* developed a multifunctional Cet-conjugated liposomal system encapsulating doxorubicin, AuNPs and SPIONs for theranostic application. Efficient Cet-mediated targeting with a higher cellular uptake of NPs in the EGFR-overexpressed skin cancer (A431 cells) was achieved for the targeted NPs compared to the free drug or the non-targeted NPs. This better cellular uptake not only increased the cytotoxicity of drug towards cancer cells but also improved the PDT activity of its metallic component. *In vivo*, the MR and optical imaging reconfirmed the specific tumor targeting of the targeted NPs with a clear tumor accumulation and its promotion on the tumor destruction. Finally, without major morphological damages to normal tissues, these targeted NPs clearly demonstrated the benefit of having Cet on the surface and presented their potency as a theranostic tool for cancer treatment (Liu *et al.*, 2019). In another study, Yoon and co-workers conjugated Cet onto their PEGylated dendrimers for better gene delivery. The authors were successful in delivering more efficiently oncolytic adenovirus into EGFR-positive tumors with the targeted dendrimers than the non-targeted dendrimers or the naked gene. In EGFR positive cancer cells (A549 cells), the targeted dendrimers with Cet showed an 8.6-fold and a 6.5-fold higher gene cellular uptake than the naked gene or the Cet-free dendrimers, respectively. Furthermore, the immunogenicity and hepatotoxicity linked to the highly charged dendrimers were also significantly attenuated thanks to the charge neutralization of Cet (Yoon *et al.*, 2016).

Besides the benefit as an active targeting ligand, conjugated Cetuximab on NPs can also present its therapeutic potency. Qian *et al* reported that the conjugation of Cet on AuNPs enhanced the cytotoxicity of Cet in non-small lung cancer (NSCLC) compared to the free Cet. Indeed, the therapeutic effect of Cet-decorated AuNPs was more pronounced in EGFR-overexpressed A459 cells compared to low EGFR-expressed H1299 cells that reflected a selective targeting of Cetuximab. Furthermore, due to the enhancement in EGFR endocytosis and the subsequent suppression of the downstream signaling pathway, the apoptotic activity of Cet-AuNPs was significantly higher than that of free Cet, as reflected by a higher reduction in tumor weight and volume with a lower toxicity level (Qian *et al.*, 2015). This discovery suggested a new strategy to use multifunctional ligands as both active targeting and therapeutic agents in nanomedicines.

In addition to cetuximab, other human mAbs such as panitumumab (Vectibix®) and nimotuzumab (h-R3) may also be used for nanoconjugation (Li *et al.*, 2015b; Yook *et al.*, 2015). Panitumumab is a fully humanized mAb belonging to the class IgG2a and clinically used to inhibit metastatic colorectal cancer in combination with chemotherapy (Lin *et al.*, 2018; Yook *et al.*, 2015). This IgG2 mAb exhibits an approximate 8-fold greater binding affinity than cetuximab (K_d around 0.05 nM) and contains no murine protein sequences that may reduce the risk of hypersensitive reactions. In clinical applications, the anti-tumor activity and toxicity of panitumumab was similar to that of cetuximab despite its higher binding affinity (Kim and Grothey, 2008). This observation may call into question the necessity of using ligands with a super-high affinity. Similar to that of Cetuximab, the conjugation of panitumumab onto NPs also presented several benefits such as selective drug delivery into EGFR positive tumors. As an example, Yook *et al* constructed a novel radiation nanomedicine for the treatment of EGFR positive breast cancer by modifying its AuNPs with panitumumab. By comparing the cell-bound radioactivity of the antibody-conjugated NPs and the bare NPs, the authors demonstrated the specific binding of the targeted NPs to EGFR positive cancer cells and this binding was found to be dependent to their EGFR density. In fact, this value was 3.1 times and 22.1 times greater in MDA-MB-468 cells than in MDA-MB-231 and MCF-7 cells with high, moderate and low EGFR density respectively. At the same dose of radioactivity, the survival rate of MDA-MB-468 cells treated with targeted NPs was clearly low compared to that of MDA-MB-231 cells or MCF-7 cells (0.001 % vs 33.8 % and 25.8 %). Compared to the non-targeted NPs, the radiotoxicity of the targeted NPs towards MDA-MB-468 cells was also significantly greater (0.001 % vs 8.4 %) (Yook *et al.*, 2015).

Nimotuzumab (h-R3) is a humanized IgG1 isotype mAb that binds to the extracellular domain of the EGFR and inhibits the EGFR-ligand binding. This mAb has been approved for the treatment of head and neck tumors, and is in clinical trials for other kinds of cancers with a high-level expression of EGFR. Compared to cetuximab, h-R3 shows a lower binding affinity towards EGFRs in human cells with a K_d around 4.53 nM. Nevertheless, one of the biggest advantages of nimotuzumab compared to Cet or panitumumab is the absence of severe adverse effects such as skin, renal, and gastrointestinal mucosa toxicities (Li *et al.*, 2015b; Ramakrishnan *et al.*, 2009). In EGFR-targeting nanomedicines, NPs functionalized with nimotuzumab exhibited satisfactory results in terms of cellular uptakes and tumor accumulation. As an example, in an attempt to better deliver gene into EGFR-overexpressed cancer cells, Li and colleagues decided to introduce h-R3 onto their dendrimers. The decoration of h-R3 on dendrimers helped enhance the gene transfection efficiency with higher nuclear accumulation in the EGFR positive HepG2 cells compared to the free-antibody dendrimers. On the other hand, the innate cytotoxicity of dendrimers due to their high charges was significantly decreased due to the neutralizing effect of h-R3 (Ramakrishnan *et al.*, 2009). All these results demonstrated that panitumumab or nimotuzumab could be used as alternative to Cetuximab in EGFR-targeted nanomedicines.

2.3. Anti-EGFR antibody fragments

Although the presence of mAbs on NPs presents undeniable benefits, antibody-functionalized NPs also encounter several restrictions including i) poor stability due to the antibody large size (MW=150 kDa) (Richards *et al.*, 2017), ii) high immunogenicity (Byrne *et al.*, 2008; Richards *et al.*, 2017), iii) rapid elimination (Ashton *et al.*, 2018; Richards *et al.*, 2017), and iv) the lack of conjugation specificity that may hinder ligand-receptor binding (Byrne *et al.*, 2008). To overcome these issues and thanks to insight knowledge in the antibody structure, researchers are able to disassemble an antibody into fragments in accordance with specific purposes such as: Fab, Fab', F(ab')₂ and Fv regions. In addition, with the help of protein engineering, a novel class of antibody fragments has been constructed and can serve as alternatives to conventional mAbs. In the case of EGFR-targeting, several antibody fragments of this novel class have been engineered and applied in nanomedicines including Fabs, scFvs, nanobodies, and bispecific antibodies (Alibakhshi *et al.*, 2017b; Richards *et al.*, 2017). All these antibody fragments preserve the specificity of an antibody by retaining at least one antigen-binding region. Therefore, once these fragments are conjugated to NPs, the active targeting is preserved or even better than the original mAbs. Numerous advantages for the functionalization of NPs with these fragments compared to the whole mAbs have been revealed such as better conjugation of targeting ligands and attenuated immunogenicity. In fact, as these fragments are devoid of an Fc effector region and 100 % humanized, the complement activation might be avoided and the risk of hypersensitive reactions are reduced (Byrne *et al.*, 2008). In addition to less immunogenicity, the smaller size of these fragments leads to higher loading capacities and a superior orientation of conjugated ligands on the NPs. This property may result in an overall enhancement in conjugation efficacy (Byrne *et al.*, 2008; Richards *et al.*, 2017).

2.3.1. Anti-EGFR Fab fragments

In the field of medicine, antigen-binding fragments (Fabs) are considered the conventional form of antibody fragments and were first introduced in 1995 (Alibakhshi *et al.*, 2017a; Nelson, 2010). Fabs can be produced by disassembling an intact mAb into two Fabs of 50 kDa using the protease papain or via artificial engineering. A Fab is composed of one VH and one VL chain linked together by disulfide bonds and contains a single antigen-binding site (Alibakhshi *et al.*, 2017a). In comparison to full antibodies, Fab fragments are less immunogenic and smaller (MW = 50 kDa). The smaller size of Fabs may allow a higher density, a better orientation of conjugated ligands on NPs, and can cause less perturbation to the NPs physicochemical properties (**Table 2**) (Houdaihed *et al.*, 2020). Once Fabs are conjugated to NPs, their presence has been proved to provide higher cellular internalization and improved the therapeutic efficacy in many studies.

As an example, Zhai *et al* conjugated an anti-EGFR Fab with a K_d at 5.6 nM onto their lipid NPs. Consequently, a higher specificity towards EGFRs was achieved with the targeted NPs compared to the bare NPs (Zhai *et al.*, 2015). In another study, Houdaihed and co-workers combined the EGFR and HER2 targeting by decorating their polymeric NPs with anti-HER2 and anti-EGFR Fab fragments to co-deliver three therapeutic agents into EGFR positive breast cancer tumors. In fact, trastuzumab and panitumumab antibodies were digested using protease papain to produce anti-HER2 and anti-EGFR Fab fragments, respectively. These fragments were then conjugated onto PEG-PLGA NPs using the covalent method. As a result, a better cellular uptake due to Fab fragments was confirmed by a clearly higher cytotoxicity of the dual-targeted NPs than the HER2 mono-targeted and the non-targeted NPs in HER2-EGFR overexpressed cells (SK-BR-3). On the contrary, no significant difference in cytotoxicity was observed in HER2-EGFR negative cells (MCF-7) treated with these three types of NPs. This study not only demonstrated the benefit of using anti-EGFR Fab fragments as active

targeting ligands but also suggested that the dual-targeting strategy may help to better deliver drug into tumors with the heterogeneity of receptor expression (Houdaihed *et al.*, 2020).

2.3.2. Single chain antibody fragments

Single chain antibody fragments or scFv are the second type of antibody fragments used as active targeting ligands in nanomedicines. It is made of a single polypeptide chain with the size of 25 kDa. In their structure, these antibody fragments (Table 2) have a monovalent structure with a good affinity for a single antigen and comprises a heavy chain and a light chain linked together via a short flexible peptide linker. The glycine and serine residues in the linker sequence make the scFv flexible and resistant to protease (Ahmad *et al.*, 2012; Alibakhshi *et al.*, 2017a). In order to generate high-affinity and high-specificity scFv fragments, new selection and display technologies such as phage display or ribosome display are available to isolate scFv with desirable properties (Alibakhshi *et al.*, 2017a; Holliger and Hudson, 2005).

As an example for EGFR-targeting with scFv in nanomedicines, Yang and colleagues used an anti-EGFR with a good binding affinity ($K_d=3.36$ nM) to functionalize their quantum dots (QD) and superparamagnetic iron oxides nanoparticles (SPIONs) for breast cancer and pancreatic tumor *in vivo* imaging. The results showed that both the targeted QD and SPIONs with anti-EGFR scFv specifically bound and better internalized into EGFR-expressing breast cancer (MDA-MB-231 and 4T1) and pancreatic cancer (MIA Paca-2) cells. As a result, these targeted NPs provided a significantly better fluorescent signal and contrast for the fluorescence and MR imaging compared to their counterparts (Yang *et al.*, 2009). In our team, NPs functionalized with anti-EGFR scFv also draw a great interest. We developed a novel nanomedicine based on the complexation between a humanized anti-EGFR scFv-SPION, siRNA and cationic polymers for a better gene delivery into triple negative breast cancer cell (MDA-MB-231). Our study pointed out that a better cellular uptake could be achieved thanks to the presence of anti-EGFR scFv and the cellular uptake can be increased with an increase in the scFv amount on NPs. As a result, this better cancer cellular uptake led to better gene silencing effects compared to the non-targeted NPs (69.4 % vs 25.3 %) (Vinh Nguyen *et al.*, 2020). All the above results have demonstrated the promising application of NPs decorated with anti-EGFR scFv in the enhancement of NPs' therapeutic efficiency.

2.3.3. Anti-EGFR nanobodies

Nanobodies (Nbs) or VHH single-domain antibodies are the next type of EGFR-antibody fragments used in nanomedicines. This kind of fragment (Table 2) is an antigen-binding fragment of camelid and shark heavy-chain antibodies (HcAbs) with dimensions in the nanometer range (Muyldermans, 2013). Compared to the traditional mAbs, nanobodies offer several advantages including i) a small size (4 nm long and 2.5 nm wide) and a low molecular weight (15 kDa) that facilitate tumor accumulation and offer a higher density of ligands on the NPs surface, ii) thermal and chemical resistance that permits its use in hard conditions, iii) high solubility and stability allowing an easier conjugation onto nanoparticles, iv) high specificity and binding affinity, and v) low immunogenic potential due to the lack of Fc components (Alibakhshi *et al.*, 2017a; Altintas *et al.*, 2013; Kooijmans *et al.*, 2016; Muyldermans, 2013; Wang *et al.*, 2017). Moreover, the production of nanobodies is easier than that of conventional mAbs, which refers to a multi-stage process with several steps including i) the immunization of camels or lama with the target antigen, ii) the cloning of the V gene repertoire from peripheral blood lymphocytes and iii) the selection of target-specific Nbs through phage display (Alibakhshi *et al.*, 2017a).

In targeting nanomedicines, the anti-EGFR nanobodies have been engineered and are getting increasing attention. Roovers *et al* succeeded to produce anti-EGFR nanobodies with good binding

affinities (K_d from 5 to 20 nM) that may be used for further functionalization in NPs. These nanobodies potentially inhibited EGF-dependent cell proliferation and their antitumor activity was found to be similar to that of Cetuximab (Roovers *et al.*, 2011). Altintas and co-workers constructed novel EGFR targeting nanobody-functionalized albumin NPs to deliver a multikinase inhibitor to EGFR overexpressing tumor cells. Compared to non-targeted NPs, nanobody-conjugated NPs exhibited a 40-fold higher binding affinity to EGFR-positive 14C squamous head and neck cancer cells. Therefore, the targeted NPs led to a successful release of drug in cancer cells and inhibited cell proliferation, whereas the non-targeted formulation showed no anti-proliferative activity on cancer cells (Altintas *et al.*, 2013). In another study, Wang *et al.* developed quantum-dot based polymeric micelles conjugated with an anti-EGFR nanobody and encapsulating aminoflavone for EGFR-overexpressing MDA-MB-468 triple-negative breast cancer theranostics. Due to the enhancement and selectivity in the cellular uptake compared to the non-targeted NPs (67-fold), the targeted NPs offered a better fluorescence signal for *in vivo* NPs biodistribution studies. Furthermore, aminoflavone encapsulated in the targeted NPs was found to better accumulate in tumor sites and exhibited a more effective tumor inhibition without observable systemic toxicity (Wang *et al.*, 2017). All above studies revealed remarkable advantages of using anti-EGFR nanobodies as active targeting ligands in nanomedicines, especially in terms of tumor penetration ability. In this aspect, the nanobody-decorated NPs are one of the most effective nanocarriers in the frame of this review.

2.3.4. Anti-EGFR bispecific antibodies

Due to the contribution of multiple factors in cancer and the blockage of several targets that might result in better efficacy, the concept of bispecific antibodies (BsAb) for dual-targeting appears as a potential strategy for better targeting with NPs (Krishnamurthy and Jimeno, 2018). A BsAb (Table 2) is made of two different antibodies or antibody derivatives (such as scFv, Fab, etc.) that allows a simultaneous recognition of two different receptors (Alibakhshi *et al.*, 2017a). BsAb was initially produced by the reduction and re-oxidation of hinge cysteines in mAb or through the fusion of two hybridoma cell lines. Recently, the development of recombinant DNA technology helps to facilitate the BsAb production with a greater variety (Krishnamurthy and Jimeno, 2018). In comparison to conventional antibodies, several advantages can be achieved with BsAb such as: i) an enhancement in tumor killing by redirecting cytotoxic T and natural killer (NK) cells, ii) the retargeting of toxins, radionuclides or cytotoxic agents, iii) the blockage of two different mediators or pathways, iv) an increase in binding specificity via interactions with two different receptors (Alibakhshi *et al.*, 2017a).

A standard example of the BsAb application in EGFR targeting NPs was performed in the study of Wu *et al.* The authors developed a bispecific antibody conjugated to SPIONs for the targeted MR imaging in both HER2 and EGFR-overexpressing tumors. The bispecific antibody was created by linking an anti-EGFR scFv to an anti-HER2 scFv via a flexible peptide linker. The binding affinity (K_d) of the resulting BsAb was determined to be 10 and 3 nM towards EGFR and HER2 protein respectively, and was comparable to that of the corresponding scFv. Compared to the non-targeted NPs, the dual-targeted SPIONs exhibited their targeting properties both for EGFR and HER2 receptors. In fact, compared to the non-targeted NPs, a better targeting activity was achieved for the dual-targeted NPs incubated with HER2+/EGFR+ cancer cells (SK-BR-3) and HER2+/EGFR++ cancer cells (A431). On the contrary, both the dual-targeted NPs and the non-targeted NPs binds poorly to HER2-/EGFR-cancer cells (Colo-205). Moreover, this specific targeting ability was clearly pronounced in SK-BR-3 cells compared to Colo-205 cells by a factor of 61.5 greater in the contrast enhancement at 24 hours post-injection. As a result, the contrast of MR imaging in HER2-EGFR positive tumors (SKBR-3 and A431 cells) was obviously enhanced 94.8 and 84.1 %, respectively (Wu *et al.*, 2016).

Besides the dual-targeting application to increase the drug delivery efficiency, bispecific antibodies can also facilitate the conjugation of active targeting ligands onto the NPs surface. This strategy relies on the use of BsAb that, one side, can recognize the target receptor on the cell surface, and on the other side, can bind to a component of NPs such as their polymer layer. Using this strategy, Cui and colleagues developed a bispecific antibody targeting polyethylene glycol (PEG) and EGFRs by connecting two corresponding scFv specificities via a glycine-serine linker (G4S). The bispecific antibody was able to bind to the PEGylated NPs and allowed the conjugation of anti-EGFR ligands onto the PEGylated NPs without any modification on anti-EGFR ligands. *In vitro*, the resulting NPs exhibited an enhancement in the targeting and internalization into triple-negative breast cancer cells overexpressing EGFRs (MDA-MB-468) compared to their PEGylated counterparts. This result confirmed the EGFR targeting activity of the anti-EGFR scFv in this bispecific antibody and demonstrated the perspective of this conjugation method. However, there was no difference in tumor accumulation for the targeted and the non-targeted NPs *in vivo*. The authors claimed that the BsAb functionalization might influence the stealthiness of the NPs in *in vivo* environment with complicated conditions (Cui *et al.*, 2019). Although there are still steps to optimize, this study highlighted the perspective of using BsAb in NPs functionalization when physical and chemical methods are not suitable.

The application of antibody fragments have shown a great interest in EGFR targeting with nanomedicines for both, therapeutic and diagnostic applications in cancer therapy. These antibody-based fragments not only help to address the issues that normally we confront with traditional mAbs but also offer unique milestones, especially in terms of tumor penetration and double or even multiple targeting. However, similar to scaffold proteins, the quick clearance is one of the biggest limitations for the use of antibody fragments in *in vivo* level and needs to be carefully considered in design steps. Besides, while antibody-based strategies have proven their potency in clinical applications for cancer treatment for long time, the use of these fragments are relatively new. Therefore, their safety must be evaluated in deep.

2.4. GE11 peptide

Recently, peptides have emerged as one of the most promising alternatives to antibodies and their derivatives have been used as active targeting ligands in nanomedicines. In comparison with conventional mAbs, several advantages can be enlisted for peptides including i) lower immunogenicity, ii) good penetration into tumors, iii) easy synthesis, and iv) better conjugation (Pi *et al.*, 2017). To achieve good targeting properties, the selection of appropriate peptides for each kind of receptors plays a key role. Nowadays, the screening of phage display libraries is normally used to select ligands for a specific receptor (Li *et al.*, 2005).

Among available peptides for EGFR-targeting, the GE11 peptide (Table 2) is the most widely exploited as active targeting agent in EGFR-targeting nanomedicines. GE11 is a small peptide of 12 amino acids (MW = 1540 g/mol) whose sequence is NH₂-Y-H-W-Y-G-Y-T-P-Q-N-V-I-CO₂H. In terms of EGFR binding affinity, the GE11 peptide bind specifically and efficiently to one EGFR region with a K_d ~22 nM (Li *et al.*, 2005). This peptide has a relatively lower affinity than EGF protein (K_d = 2 nM) or mAbs. However, it is much smaller, does not have any mitogen activity and manifests less immunogenicity. Furthermore, GE11 can be easily produced by microwave-assisted solid phase automated synthesis (Pi *et al.*, 2017).

Recent studies have revealed that GE11 is able to accelerate the cellular uptake of NPs via the EGFR-dependent endocytosis. This enhancement in internalization helps improve tumor inhibition effects and significantly reduce the NPs toxicity against normal cells (Pi *et al.*, 2017). GE11-decorated NPs

provide a new strategy for cancer treatment with higher therapeutic efficacy and fewer side effects. As an example, Milane and co-workers introduced GE11 onto their polymeric NPs to co-deliver paclitaxel and lonidamine for the treatment of multidrug-resistant in breast and ovarian tumors. The authors investigated the intracellular internalization pathway of the targeted and non-targeted NPs and showed that the targeted NPs entered the EGFR positive cancer cells (OVCAR5, SK-OV-3-TR and MDA-MB-231 cells) and through the EGFR-mediated pathway, whereas the non-targeted NPs internalized via the non-specific endocytosis. In addition, the GE11-NPs exhibited a greater cellular uptake with an increasing EGFR expression level and a superior pharmacokinetic profile than the free drug and the GE11-free NPs. Finally, in EGFR positive multidrug-resistant ovarian and breast cancer cells (hypoxic and normoxic SK-OV-3 and MDA-MB-231 cells, respectively), the targeted NPs exhibited very interesting anti-tumor activities by causing the cell death up to 90-95 %. This study showed that the targeted NPs with the GE11 peptide may be a good solution to overcome the problem of the multi-drug resistance in EGFR-overexpressing cancers (Milane *et al.*, 2011).

Thanks to its smaller size than other kinds of ligands, GE11 occupies smaller space on the NPs surface and facilitates other ligands' introduction that might help target better tumors and minimize off-target effects. Using this strategy, Talekar and colleagues designed a dual CD44/EGFR targeting NPs to deliver miRNA into lung cancer cells (SK-LU-1). In comparison with the single CD44-targeted NPs, the dual-targeted NPs showed a 20-fold increase in the expression of loaded miRNA in the target cells. *In vivo*, when the single CD44-targeted NPs exhibited a 7-fold increase in the miRNA expression than the non-targeted NPs, an increase by a factor of 12 in the gene expression was obtained for the dual-targeted NPs (Talekar *et al.*, 2016).

Another benefit of GE11 is the possibility for it to be introduced onto amphiphilic molecules that can later self-assemble into micelles with good drug loading capacity. Using this strategy, to co-deliver gemcitabine and olaparib for the treatment of pancreatic cancer with breast cancer 2-mutation, Du *et al* introduced GE11 on the hydrophilic domain of an amphiphilic molecule that self-assembled into micelles. The targeted micelles presented better tumor targeting in EGFR-overexpressed pancreatic cancer cells (Capan-1) than the non-targeted micelles. On the other hand, the extensive internalization of the targeted NPs in EGFR positive pancreatic cancer Capan-1 cells compared to other cell lines (primary human pancreatic stellate H-PSC cells and human umbilical vein endothelial HUVEC cells) with a lower EGFR level highlighted the EGFR targeting activity of the conjugated GE11. As expected, a clear enhancement of antitumor effects was obtained for the targeted NPs among all the treatment groups with the non-targeted NPs or the free drug (Du *et al.*, 2018).

Interestingly, Biscaglia and co-workers investigated the benefit of using GE11 as a targeting ligand compared to Cetuximab. In this study, the authors introduced GE11 or Cet onto their AuNPs and compared their EGFR targeting activity *in vitro*. The GE11-AuNPs on one side exhibited better targeting towards EGFR positive cancer cells (Caco-2 and SW480) compared to EGFR negative cells (SW620). Besides, despite its lower binding affinity, GE11-AuNPs achieved a better targeting activity than Cet-NPs in EGFR-overexpressed cells. To explain this phenomenon, the authors proposed that the organization of GE11 on the surface of nanosystem was more favorable for its interaction with EGFRs on the cell surface and resulted in a better active targeting activity. Although these results are limited to AuNPs and needed more studies, especially *in vivo* ones, to confirm, this study pointed out the importance of controlling the organization of conjugated ligands for an efficient targeting activity (Biscaglia *et al.*, 2017).

All previous studies indicate that GE11 is a useful targeting ligand for EGFR targeting with the help of nanomedicines. Compared to other kinds of EGFR ligands, GE11 is outstanding regarding its ease in conjugation to nanocarriers, which makes it suitable to most kinds of nanoparticles and conjugation

strategies. However, as GE11 binding affinity is relatively lower than other kinds of EGFR targeting ligands, its density on NPs plays an essential role in the targeting properties of the finalized NPs, and extensively depends on the conjugation methods. Therefore, one of the most important parameters to be carefully considered during the development of targeting nanomedicines with GE11 is the selection of appropriate conjugation strategies. To this end, the conjugation method should be selected according to each kind of NPs and GE11 density needs to be controlled after the conjugation to ensure that the resulted NPs possess an efficient targeting activity for EGFR.

2.5. Anti-EGFR aptamers

In addition to peptides, aptamers are another alternative to protein ligands. Aptamers are considered “chemical antibodies” due to their functional similarity and the possibility to recognize their target with high specificity and affinity. Furthermore, while traditional mAbs are highly immunogenic, expensive and difficult to synthesize, aptamers provide remarkable advantages over protein antibodies, both in synthesis and application including i) a smaller molecular weight (8-25 kDa), ii) better and faster tissue penetration, iii) non-immunogenicity, iv) high thermal stability, v) low cost and faster synthesis, and vi) easy modification (Chen *et al.*, 2016; Sun and Zu, 2015; Yoo *et al.*, 2019).

Aptamers belong to a class of short RNA or single-stranded DNA nucleic acids with a unique 3D structure that is designed for the specific binding to their target. Concerning the synthesis, the systematic evolution of ligands by exponential enrichment or SELEX is normally applied. This process consists of five steps including: i) mixing the target with a random oligonucleotide library, ii) separating target bound sequences, iii) amplifying the target-bound sequences to generate an enriched library, iv) mixing the target with the new library for the next round of enrichment, and v) sequencing the enriched aptamer sequences after a number of selection rounds (Sun *et al.*, 2014).

Besides promising advantages, there also remain some drawbacks of aptamers due to their innate structure that might hinder *in vivo* application. The most relevant limitation is their degradation by nucleases in biological fluids. However, the development in biochemistry can help solve this issue. Many efficient solutions have been developed to improve impressively their nuclease stability such as: i) the substitution of the 2'-OH RNA functional group with 2'-fluoro, 2'-amino or 2'-O-methoxy, ii) the substitution of the phosphodiester backbone with boranophosphate or phosphorothioate, iii) the substitution with phosphorodithioate and 2'-O-methyl in one nucleotide, iv) the locked nucleic acid technology, and v) the generation of mirror RNA sequences (Sun and Zu, 2015).

In EGFR targeting with nanomedicines, anti-EGFR aptamers have been conjugated to different kinds of NPs (Table 2). For instance, Ilkhani *et al* introduced an anti-EGFR aptamer with a high affinity ($K_d = 2.4$ nM) to citrate-coated gold NPs (AuNPs) for better detecting EGFRs in biological fluids (Ilkhani *et al.*, 2015). Lv and colleagues constructed novel gene-drug co-delivery polymeric NPs functionalized with anti-EGFR aptamers to improve the therapeutic index in drug-resistant lung cancer. In comparison with the non-targeted NPs, a better drug delivery and efficient gene transfection efficiency were achieved with the targeted NPs. Consequently, the EGFR positive non-small lung cancer cells (PC-9 and H1975) treated with the targeted NPs encountered a remarkable proliferation inhibition, a better induced apoptosis, a down regulation of target protein expression and an enhanced drug cytotoxicity compared to cells treated with the unmodified NPs (Lv *et al.*, 2018).

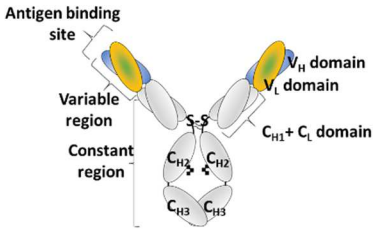

In addition to their targeting properties, these “chemical antibodies” can also exhibit their therapeutic potency once introduced onto NPs. Chen *et al* developed albumin-ciplastin NPs conjugated with anti-EGFR aptamers for better efficacy and tolerability of cisplatin in the treatment of EGFR positive cervical cancer. Both the free aptamers in solution and the conjugated aptamers on NPs were able to bind specifically to the EGFR positive cervical cancer Hela cells. Moreover, a greater tumor accumulation of cisplatin was obtained with the aptamer-functionalized NPs and resulted in a




significant reduction of the IC₅₀ of cisplatin (26 μ M for the targeted NPs vs 55 μ M for the free drug or 56 μ M for the non-targeted NPs). Better tumor accumulation also improved the tolerability of cisplatin with less systemic toxicity and nephrotoxicity. Interestingly, the conjugated aptamers also presented their therapeutic activity by blocking the EGFR activation and contributed to the antitumor activity of the final NPs (Chen *et al.*, 2016).

Kang *et al* carried out an interesting study to compare the benefit of anti-EGFR aptamers and cetuximab in EGFR-targeting activity with nanomedicines. In this study, two lipid NPs co-loading paclitaxel and quantum dots were functionalized with cetuximab or anti-EGFR aptamers. Compared to the non-targeted NPs, both the NPs functionalized with antibodies or aptamers clearly exhibited higher localization in tumor tissues and enhanced the therapeutic efficacy with stronger tumor inhibition by a factor of five. Although the binding affinity of the anti-EGFR aptamer is only a half of that of cetuximab (K_d = 0.62 nM vs 0.38 nM, respectively) and the number of ligands per NP was similar, the aptamer-NPs and the antibody-NPs demonstrated similar binding capacity to the target cells. The authors proposed that due to the smaller size compared to that of cetuximab, the conjugated aptamers might have a higher chance for multivalent interaction with EGFRs on target cells than the conjugated cetuximab and could explain the similar binding activity despite its lower binding ability. These results suggested that the interaction capacity between targeting ligands and receptors is also important to their targeting ability and needs to be considered in the design step of nanomedicines (Kang *et al.*, 2018).

A novel and efficient modality for EGFR positive cancers' treatment can be developed by the functionalization of nanomedicines with anti-EGFR aptamers. Compared to other kinds of EGFR ligands that are protein-based ones, anti-EGFR aptamers offer a unique advantage in terms of thermal stability. This property may be very helpful for the future large-scale production and long-term storage. Nevertheless, as aptamers can only target the receptors if they are in a correct conformational structure, their conformation on the surface of NPs must be considered with vigilance to select appropriate conjugation methods, and ensure their targeting properties.

598 **Table 2 EGFR-targeting ligand available for conjugation onto nanoparticles**

Targeting moiety	Structure	MW (KDa)	K _d (nM)	Advantages	Drawbacks	Ref.
EGF protein	A single polypeptide of 53 amino acids	6	1.0-2.0	Small size, natural ligand for EGFRs, low risk of immunogenicity, easy conjugation	High-cost and complicated production, susceptibility to hypersensitive reactions for the murine EGF	(Grapa <i>et al.</i> , 2019; Master and Sen Gupta, 2012; Negahdari <i>et al.</i> , 2016; Silva <i>et al.</i> , 2016)
Affibody	Scaffold protein of 58 amino acids	7	2.8	Low molecular weight and size, high stability, easy conjugation, low immunogenicity	Lower binding affinity compared to mAbs, lack of data on safety and short half-life time	(Lucky <i>et al.</i> , 2016; Y. Zhang <i>et al.</i> , 2018)
Repebody	Scaffold protein with a leucine-rich repeated module	30	9.18	Small size, better tissue penetration than mAbs	Lack of data on safety	(Pyo <i>et al.</i> , 2018)
Monoclonal antibody	 <p>Antigen binding site Variable region Constant region V_H domain V_L domain C_{H1} + C_L domain C_{H2} C_{H3}</p>	150	<p>Cetuximab : 0.39 Panitumumab : 0.05 Nimotuzumab : 4.53</p>	Highest binding affinity, longest history of utilization, safety	Big size, limited number of conjugated ligands, high-cost production, immunogenicity, stability, rapid capture by liver and spleen	(Chu <i>et al.</i> , 2015; Kim and Grothey, 2008; Master and Sen Gupta, 2012; Ramakrishnan <i>et al.</i> , 2009)
Fab fragment	<p>VH-VL via disulfide bonds</p> 	50	5.6	Small size, easy production, low immunogenicity, high stability, longer circulation time than mAbs	Lower binding affinity than mAbs	(Alibakhshi <i>et al.</i> , 2017b; Zhai <i>et al.</i> , 2015)

ScFv fragment	VH-VL linked via a peptide linker 	25	3.36	Small size, high affinity, easy production, low immunogenicity, orientated conjugation	Lower binding affinity than mAbs	(Master and Sen Gupta, 2012; Yang <i>et al.</i> , 2009)
Nanobody	Camelid and shark VH 	15	5-20	Small size, high thermal and chemical resistance, high solubility, easy conjugation, low immunogenicity	Scale-up, difficulty in synthesis	(Master and Sen Gupta, 2012; Roovers <i>et al.</i> , 2011)
Bispecific antibody	Two scFv linked via the CH ₃ domain 	50	Comparable to corresponding scFv	Increased binding specificity, blockage of two different mediators, better conjugation and tumor penetration in NPs		(Cui <i>et al.</i> , 2019; Master and Sen Gupta, 2012)
GE11 peptide	Small peptide of 12 amino acids	1.5	22	Small size, low immunogenicity, easy synthesis, easy conjugation with good orientation	Lower affinity than mAbs	(Li <i>et al.</i> , 2005; Pi <i>et al.</i> , 2017)
Aptamer	Single stranded nucleic acid with a 3D specifically designed structure	8-25	2.4	Small size, better and faster tumor penetration, non-immunogenicity, high thermal stability, low cost and faster synthesis, possible surface modification	Nuclease degradation	(Chen <i>et al.</i> , 2016; Sun and Zu, 2015)

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3. Methodologies involved in the conjugation of EGFR active targeting ligands onto NPs' surface

A wide variety of approaches have been developed to introduce targeting moieties onto nanocarriers including both physical interactions and chemical conjugations. For EGFR targeting, the physical method is normally based on physical adsorption or electrostatic interactions and chemical strategies involving carbodiimide, maleimide and click chemistry. In the following section, the advantages and drawbacks of each conjugation method will be *discussed*.

3.1. Non-covalent adsorption

The first and easiest conjugation method is to adsorb targeting ligands onto the surface of NPs via non-covalent linkages. Non-covalent adsorption may be divided into physical adsorption and ionic binding. Physical adsorption occurs via weak interactions such as hydrogen bonding, hydrophobic and Van der Waals attractive forces, while ionic binding is based on the interactions between the opposite charges of ligands and NPs (**Table 3**). Compared to other conjugation strategies, this method provides several advantages including i) avoiding complex chemical conjugation that helps preserve ligand biological activities, ii) easy and fast formulation, iii) fast release of the ligand in tumor sites and possibility to deploy the ligand antitumor activities, iv) possibility to minimize the cytotoxicity linked to highly charged NPs (Juan *et al.*, 2020; Li *et al.*, 2015a; Y. Zhang *et al.*, 2018). Nevertheless, its weak stability in biological fluids due to weak interactions established between ligands and NPs remains one of the biggest challenges for its *in vivo* applications. Among the available targeting moieties for EGFR-targeting, this conjugation method can be applied to EGF proteins (Li *et al.*, 2015a; Silva *et al.*, 2016; Zhang *et al.*, 2012); affibodies (Y. Zhang *et al.*, 2018); repebodies (Kim *et al.*, 2018) and mAbs (Li *et al.*, 2015b; Lu *et al.*, 2018; Qian *et al.*, 2015). As charged NPs are normally required in this strategy, dendrimers, gold nanoparticles (AuNPs) or cationic liposomes are the most widely used. Consequently, the surface charges of the resulting NPs are significantly changed in most cases compared to the non-targeted NPs. Thus, the change in the surface charge of the ligand-conjugated NPs can serve as an indicator to confirm a successful conjugation process.

In the ionic binding for positively charged NPs such as dendrimers, active targeting moieties need to be negatively charged. As an example, Zhang *et al* succeeded in adsorbing negatively charged EGF proteins onto highly positively charged dendrimers. The zeta potential of the finalized NPs declined significantly from $+18.3 \pm 0.3$ mV to $+3.3 \pm 0.5$ mV, whereas the size remained unchanged around 350 nm (Zhang *et al.*, 2012). In another study, Kim *et al* introduced a polyanionic peptide to an anti-EGFR repebody and then exploited the electrostatic interactions between the negative charges of this peptide with the positive charges of dendrimers to formulate a final dendrimer complex for a targeting gene delivery. The resulting dendrimer complex was also significantly neutralized in terms of charge (Kim *et al.*, 2018). Always in order to introduce targeting ligands onto dendrimers, Li and colleagues adsorbed h-R3 mAbs to PAMAM dendrimers and created final self-assembled h-R3-PAPAM-DNA complexes. In this complexation, electrostatic associations were created between the positively charged dendrimers and the negatively charged h-R3 and DNA (Qian *et al.*, 2015). In addition to the targeting properties, conjugated ligands may play also the role of toxicity-reducing agent for the highly charged dendrimers. In fact, the only possible way for non-targeted dendrimers to enter the cell is through non-specific endocytosis that may damage the lipid bilayer structure of the cell and cause cell death. On the contrary, neutralized dendrimers with conjugated ligands can enter the cells through a non-specific pathway and also through the EGFR signal pathway endocytosis, which is a normal physiological process. Therefore, the less charged NPs and the two internalization pathways may cause less damage to the cell membrane than their counterparts may. This phenomenon was confirmed in the studies of Zhang *et al* and Kim *et al* for EGF-conjugated and anti-

EGFR repebody-conjugated dendrimers, respectively. In these studies, an attenuated cytotoxicity level related to an increase in the amount of anti-EGFR ligands on the NPs surface was recorded for the targeted dendrimers (Kim *et al.*, 2018; Zhang *et al.*, 2012). However, in case dendrimers are used for gene delivery, ligand adsorption to dendrimers might increase steric hindrance and electrostatic repulsion against nucleic acids. To overcome these problems, the ratio of ligand/DNA or the orders of the complexation need to be optimized. As an example, Li *et al* showed that the antibody/DNA ratio needed to be inferior to five in order to complex completely DNA (Qian *et al.*, 2015). In another study, Zhang and co-workers found that if EGF adsorption occurs after the formation of DNA-dendrimer complexes, it did not affect the interaction of DNA-dendrimers (Zhang *et al.*, 2012). To conclude, there are two possible solutions to tackle the hindrance of ligand adsorption to the complexation between DNA and dendrimers including i) the use of an appropriate ratio of ligand/nucleic acid or ii) the post-conjugation strategy of the charged ligand.

The positively charged anti-EGFR ligands can be also introduced onto negative metallic NPs such as SPIONs, AuNPs or AgNPs via electrostatic interactions. Zhang *et al* used the electrostatic interactions between positive charges of the histidine residues in anti-EGFR affibodies and negative charges of COOH groups in quantum dots for the photoacoustic imaging of EGFR positive tumors. A remarkable increase in the zeta potential from -37 mV to +0.31 mV was recorded after the affibody conjugation that revealed the presence of affibody ligand (Y. Zhang *et al.*, 2018). For mAbs, Cetuximab possesses an isoelectric point at $pH_i=8.48$. Therefore, Cet is positively charged in $pH < pH_i$ and negatively charged in $pH > pH_i$ (Chu *et al.*, 2015). Therefore, a possible adsorption of Cet onto positively charged NPs can be achieved in $pH < pH_i$.

Besides the ionic binding, physical adsorption can also be used to conjugate anti-EGFR fragments onto NPs. However, the biggest issue of this method is that it is impossible to ensure the functionality of the adsorbed antibodies on NPs surfaces. Moreover, due to the weak linkage between the conjugated ligands and NPs, once these targeted NPs are in contact with the biological fluids, a competition between ligands and the complex mixtures of serum or plasma proteins may occur and the targeting ligands may be covered or replaced by these proteins (Tonigold *et al.*, 2018). Despite its disadvantages, this strategy can be used in cases where no other methods are suitable. Using this strategy, two studies of Lu *et al* and Qian *et al* conjugated Cetuximab onto Fe_3O_4 -AuNPs and AuNPs, respectively. In this conjugation, the pH of the reaction medium was adjusted to the isoelectric point of the antibody and a blocking treatment step with BSA (bovine serum albumin) was performed to fix the conjugated ligands on the NPs surface. As a result, the targeted NPs in these studies were able to target specifically the EGFR positive lung cancer (A549) and human glioma (U251) cells, which confirmed the functionality of the adsorbed antibodies (Lu *et al.*, 2018; Qian *et al.*, 2015).

All the previous studies demonstrate the potential application of the non-covalent adsorption strategy in the introduction of anti-EGFR ligands to NPs. However, its application remains limited due to its weak stability. To overcome the challenge in the stability of non-covalent interactions, covalent strategies have been developed.

3.2. Carbodiimide chemistry

Among available covalent strategies, carbodiimide chemistry is the most used, as synthetic schemes are generally easy to carry out. This strategy refers to the reaction between carboxyl groups and primary amine groups present in ligands and on the surface of NPs without the need of performing any further chemical modification (**Table 3**). Before the reaction, the functional carboxyl groups must be activated by the addition of cross-linking agents, where the free-length linker 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) is the most used. However, only in an acidic medium (pH

4.5) that this reaction is the most efficient, and it must be performed in buffers devoid of extraneous carboxyl groups and amine groups. In order to improve conjugation efficiency, N-hydroxysuccinimide (NHS) or N-hydroxysulfosuccinimide (sulfo-NHS) are normally added (Juan *et al.*, 2020).

In terms of EGFR-targeting, carbodiimide chemistry can be used for most ligands such as EGF proteins (Gill *et al.*, 2018; Tsai *et al.*, 2018), reprobodies (Lee *et al.*, 2017), mAbs (Huang *et al.*, 2015; Maya *et al.*, 2013; Wang and Zhou, 2015), Fab fragments (Houdaihed *et al.*, 2020), bispecific antibodies (Wu *et al.*, 2016), and aptamers (Chen *et al.*, 2016; Lv *et al.*, 2018; Xie *et al.*, 2019). Using this strategy, Gill *et al.* were able to conjugate EGF proteins in DTPA-EGF to their PLGA nanoparticles via the reaction between primary amines on EGFs and carboxyl groups on the polymeric NPs. The resulting nanoparticles allowed further complexation with a radioactive agent and the conjugation yield was determined to be around 2 µg of EGF per mg of NPs (Gill *et al.*, 2018). In another study of Lee *et al.*, an anti-EGFR reprobod body was successfully conjugated to an oncolytic protein that could later self-assemble into nanoparticles of around 48 nm via the reaction between NH₂ in reprobodies and the COOH terminus of the protein. The resulting self-assembled nanoparticles showed a high homogeneity and stability level in physical conditions (Lee *et al.*, 2017). In addition, Maya *et al.* firstly activated the carboxyl groups on O-carboxymethyl chitosan NPs and then conjugated them with the amino groups of cetuximab. The Cet conjugation efficiency in this study was found to be 42±6 % (Maya *et al.*, 2013). Using this strategy, Houdaihed and colleagues were also able to introduce an anti-EGFR Fab onto COOH-PEG-PLGA NPs for a targeted delivery of paclitaxel into breast cancer cells. The reaction occurred between the primary amine functions of Fab fragments and the carboxyl groups of the polymeric NPs (Houdaihed *et al.*, 2020). For anti-EGFR aptamer conjugation, Lv *et al.* activated the COOH groups of anti-EGFR aptamers and subsequently coupled them with the NH₂-terminated dendrimers to tackle drug resistance in non-small cell lung cancer (Lv *et al.*, 2018).

In the study of Yoon *et al.*, instead of using physical adsorption to conjugate cetuximab onto dendrimers as previously described, the authors applied the reaction between free primary amine groups on the dendrimers and carboxyl groups on the Fc region of Cet that provided a better stability level than non-covalent adsorption (Yoon *et al.*, 2016). The same observation was shown in the study of Zhang *et al.* to conjugate Cet onto dendrimers for EGFR targeting. The authors showed that the multiple amino groups of dendrimers made them outstanding in the chemical conjugation of antibodies and may enhance the yield as well as the stability of ligand conjugation in comparison with electrostatic interactions (Zhang *et al.*, 2016).

Although this strategy is easy and does not require further chemical modification, the coupling between functional groups and crosslinkers is not highly selective due to the abundance of primary amines functions or carboxyl groups in ligands or NPs themselves or in biological fluids. Moreover, the absence of control over the ligand orientation present on the NPs surface might hinder its targeting properties (Juan *et al.*, 2020).

3.3. Maleimide chemistry

Recently, the maleimide chemistry has been widely exploited to functionalize NPs with active targeting ligands. This strategy is based on the reaction between free sulfhydryl groups (-SH) on ligands and maleimide groups on NPs to form a stable thioether linkage (**Table 3**). This strategy offers several advantages compared to carbodiimide chemistry including i) much faster reactions and efficiency at a neutral pH suitable for ligands or NPs with a low stability level in acidic media and ii) better selectivity and ligand orientation on NPs due to the fact that functional groups are much less abundant in biological fluids (Juan *et al.*, 2020).

As free sulfhydryl moieties are required in this method, two strategies can serve to generate free sulfhydryl groups or the de-protonated form of thiol at physiological pH. The first way is via the reduction of native disulfide bonds of the cysteine residues in ligands with reducing reagents. To this end, dithiothreitol (DTT) and tris(carboxyethyl)phosphine (TCEP) are the most used reducing reagents (Agarwal and Bertozzi, 2015). In addition, ligands can also be thiolated by modifying the primary amine groups on lysine residues with sulfhydryl-addition reagents such as 2-iminothiolane (Traut's reagent) and N-succinimidyl S-acetylthioacetate (SATA) (Yoo *et al.*, 2019; Yu *et al.*, 2012). On the other side, to introduce maleimide groups on NPs, maleimide cross-linking reagents such as NHS/maleimide heterobifunctional linkers are normally used. A wide variety of linkers can be applied including PEGylated analogues (NHS-PEG-maleimide), succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) and sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC). Among these linkers, PEGylated analogues are preferred in nanomedicines due to the resulting improving stealthiness effect (M. Cardoso *et al.*, 2012).

Maleimide chemistry can be used for nanoconjugation with all kinds of EGFR-targeting ligands mentioned in this review. As an example, Faucon *et al* firstly introduced maleimide units onto the surface of NPs using N-(maleimidoethyl) amine via carbodiimide chemistry. Subsequently, free sulfhydryls were generated using DTT from the six cysteine residues in the EGF structure and reacted with maleimide-functionalized NPs. The resulting NPs possessed around 4.7 EGF ligands per NP (Faucon *et al.*, 2017). In another effort to conjugate anti-EGFR affibodies onto upconversion NPs for photodynamic therapy in solid oral cancers, Lucky and colleagues transformed the non-activated thiols in cysteine-residues of affibodies into activated thiols with the reducing agent DTT and subsequently conjugated them to the bifunctional maleimide-PEG-COOH linker via thioether bonds. Afterwards, the nanoparticles were coated by the silane groups to provide primary amine groups, which allowed subsequent conjugation with carboxyl groups in affibody-PEG (Lucky *et al.*, 2016). Kang and co-workers managed to conjugate cetuximab and an anti-EGFR aptamer onto lipid micellar NPs for co-loading quantum dots and paclitaxel. In this study, sulfhydryl groups were introduced into Cet or aptamers via the reaction with the Traut's reagent and then the thiolated ligands reacted with maleimide groups on lipid micelles. Interestingly, the number of conjugated ligands was the same for cetuximab and the anti-EGFR aptamer (Kang *et al.*, 2018). Our group also succeeded in developing theranostic EGFR-targeting NPs with a humanized anti-EGFR scFv. In our study, a cysteine-tag was introduced into the C-terminus of our anti-EGFR scFv and the NPs were previously coated by an NHS-PEG-Mal polymer layer. After the reduction with TCEP to generate free sulfhydryls, the activated scFvs were conjugated onto our NPs via maleimide chemistry. The number of anti-EGFR scFv was determined to be around 13 scFvs per SPION (Vinh Nguyen *et al.*, 2020).

In addition to protein ligands, maleimide chemistry was also applied for GE11 peptide and anti-EGFR aptamers. In fact, GE11 is usually grafted with four glycine residues as the spacer and a terminal cysteine (GGGGC) that allows the application of maleimide chemistry. As an example, Chariou *et al* firstly grafted a bifunctional PEG linker onto their virus-based nanoparticles (VNPs) and subsequently linked them with the cysteine-terminated GE11 for the detection and imaging of various kinds of EGFR-overexpressed cancers including skin epidermoid carcinoma (A431), colorectal cancer (HT-29) and triple-negative breast cancer (MDA-MB-231) (Chariou *et al.*, 2015). In another study, Kim and co-workers used maleimide chemistry to introduce anti-EGFR aptamers onto a liposomal system for co-delivering quantum dots and nucleic acids. Aptamers were thiolated with the DTT reagent and then reacted with the maleimide groups present on liposomes. The resulting liposomes were shown to be efficient as nanocarriers for tumor-directed gene delivery and bio-imaging (Kim *et al.*, 2017).

Furthermore, maleimide chemistry also appears as a good alternative in case carbodiimide chemistry is not appropriate due to the accessibility issue of functional groups on NPs/ligands or aggregation problems because of acidic pH. As an example, Lee and co-workers developed Cet-conjugated quantum dots for cancer imaging using the maleimide strategy instead of the carbodiimide one. The carboxylated polymer-coated quantum dots were modified into amine via reactions with diamino-PEG and subsequently thiolated by a reducing reagent DTT. On the other side, the cetuximab antibody was activated and conjugated to one side of sulfo-SMCC. Finally, free thiol groups on the activated nanoparticles were connected to maleimide groups on the other end of sulfo-SMCC. As a result, approximately 6 cetuximabs per NP were introduced onto the finalized NPs (Lee *et al.*, 2010).

Despite its robust covalent conjugation with a greater stability level than the non-covalent approaches and better selectivity than the carbodiimide strategy, maleimide chemistry also suffers from several issues. Especially, the thiolation step may change the chemical structure of the ligand and hinder its binding affinity. Furthermore, the non-selectivity of maleimide to cysteine-rich reaction media has also been reported. This lack of selectivity is due to exchange reactions with thiol-containing proteins in serums. As a result, non-homogenous conjugates and poorly defined yielding off-target cytotoxicity may occur (Juan *et al.*, 2020).

3.4. Click chemistry

To tackle the problem of non-selectivity in the ligand conjugation, site-specific conjugation strategies such as click chemistry is one of the most suitable approaches (Table 3). Click chemistry refers to the formation of a stable triazole linkage by the reaction between alkyne and azide groups. To do so, a linker is normally used to connect ligands and NPs. In most cases, the functionalization of NPs or ligands with azide or alkyne moieties goes through carbodiimide or maleimide conjugation. Therefore, NHS esters or maleimide groups need to be present on one side of the linker, while azide or alkyne groups must be located on the other side. Compared to the previous methods, this reaction can occur under mild conditions (in aqueous solvents at room temperature), does not require sophisticated purification and provide irreversible chemical bonds with the absence of cytotoxic byproducts (Juan *et al.*, 2020). Moreover, thanks to the low presence of these functional groups in biological systems or biomolecules, this strategy can be exempt from undesirable reactions with other functional groups and provides a specific conjugation at the desired location on the biomolecules. Therefore, a highly oriented ligand conjugation can be achieved and click chemistry is highly recommended for the functionalization of NPs with targeting moieties. However, the original kind of this strategy requires the presence of a Cu(I) catalyst, which is susceptible to toxicity. Recently, Weisseleder *et al.* have developed a novel copper-free click chemistry procedure to overcome the toxicity linked to Cu(I)-catalyzed reaction by exploiting the reaction between azide and alkyne groups in trans-cyclooctene (TCO).

This conjugation method is attracting an increasing interest and more and more applied in nanoconjugation approaches involving EGFR-targeting NPs. As an example, Kotagiri and co-workers constructed a copper-free chemical process to conjugate cetuximab onto their quantum dots for a rapid analysis of biological samples. On one side, amine-coated quantum dots were activated with an NHS-PEG-Azide cross-linker. On the other side, cetuximab was functionalized with NHS-PEG-dibenzocyclooctyne (NHS-PEG-DBCO). These intermediate products then engaged in a click reaction to obtain the finalized antibody-decorated quantum dots (Ab-QD) with a high yield (around 88 %). Interestingly, the authors also prepared different Ab-QD NPs as comparative NPs, using traditional strategies such as maleimide chemistry with the SMCC linker or the biotin-streptavidin strategy. The recorded number of conjugated antibodies from click chemistry was two times and five times higher than those of the maleimide and the biotin-streptavidin strategies, respectively (8.4 vs 3.9 and 1.6

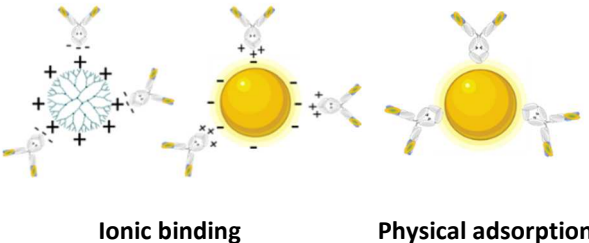
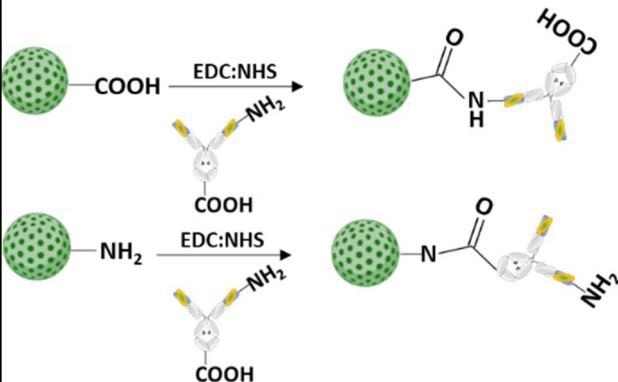
antibody per QD). Moreover, Cet-QD NPs with click chemistry showed a better binding ability to EGFR positive human pancreatic and breast cancer cells (BxPc-3 and MDA-MB-231, respectively) than Cet-NPs prepared with other strategies. These results suggested that click chemistry was more selective with a higher yield and improved the orientation of conjugated ligands on the NPs surface resulting in a better binding affinity towards EGFRs on the cell surface (Kotagiri *et al.*, 2014).

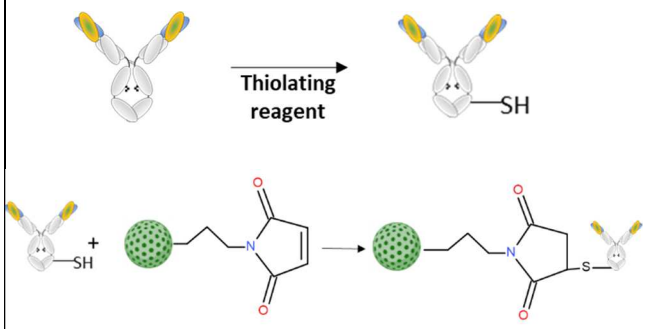
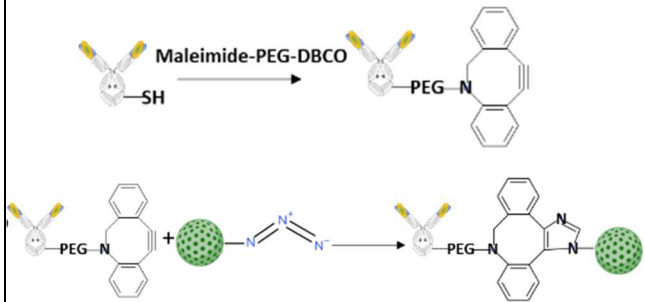
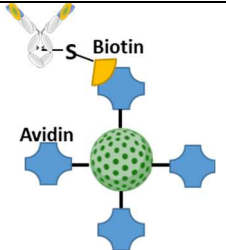
Despite its high conjugation yield and high selectivity, few studies have applied click chemistry for nanoconjugation with EGFR-targeting ligands so far. This chemical strategy is relatively new and more complicated than traditional methods. Furthermore, while the reaction between alkyne and azide groups is highly specific, the introduction methods of these functional groups onto targeting ligands or NPs normally involves classical conjugation chemistries such as carbodimide or maleimide ones. As these conventional chemistries are not entirely specific, a careful consideration on this aspect is necessary, especially in the case of targeting ligands that require highly conformational structure for targeting properties such as aptamers. In all cases, click chemistry has proved its great potential for the conjugation of targeting moieties to nanoparticles.

3.5. Non-covalent binding by adapter molecules

The study of Kotagiri and co-workers also suggested another selective conjugation strategy for EGFR targeting NPs that is based on another non-covalent approach using adapter biomolecules. This strategy can help avoid randomly oriented antibodies and enhanced the stability of linkage. The most relevant used method in this strategy exploits the strong binding affinity between biotin and a biotin-binding protein such as avidin or its analogues (Juan *et al.*, 2020). As biotin and avidin need to be present on ligands and NPs, other chemical strategies are normally applied to conjugate these moieties. Despite its potency, this conjugation strategy has not been applied so far to introduce anti-EGFR ligands onto NPs. Nevertheless, its promising application was demonstrated with similar receptors such as HER2. Warlick and colleagues managed to prepare targeted NPs for HER2 active targeting using biotin/neutravidin. In this study, an anti-HER2 antibody (trastuzumab) was biotinylated using carbodiimide chemistry and maleimide chemistry was used to functionalize albumin NPs with neutravidin. As a result, specific targeting to HER2-overexpressing cells was obtained (Wartlick *et al.*, 2004). This study demonstrated the perspective of applying the biotin-avidin strategy to functionalize NPs with anti-EGFR fragments.

859 **Table 3 Available conjugation strategies for the functionalization of nanoparticles with anti-EGFR ligands**

Conjugation strategy	Mechanism	Appropriate EGFR-targeting ligands	Appropriate NPs	Advantages	Drawbacks	Ref.
Non-covalent adsorption	 <p>Ionic binding Physical adsorption</p>	EGF proteins, affibodies, reprobodies, mAbs	Charged NPs such as dendrimers, AuNPs, AgNPs	Fast and easy formulation; avoidance of complex chemical conjugation; release of ligands in the tumor with antitumor activity; reduced toxicity due to high charges of NPs	Weak interactions and stability	(Krishnamurthy and Jimeno, 2018; Pi <i>et al.</i> , 2017; Wu <i>et al.</i> , 2016; Yoo <i>et al.</i> , 2019)
Carbodiimide chemistry		EGF proteins, reprobodies, mAbs, Fabs, bispecific antibodies, peptides, aptamers, etc.	Micelles made of polymers, amphiphilic molecules, lipids; chitosan NPs; liposomes; inorganic NPs, etc.	High stability; higher conjugation efficiency than the physical method; no chemical modification on the ligands	Low selectivity; absence of control over ligand orientation; reactions in acidic and water-free media	(Holliger and Hudson, 2005; Lv <i>et al.</i> , 2018; Roovers <i>et al.</i> , 2011; Talekar <i>et al.</i> , 2016; Tsai <i>et al.</i> , 2018; Yook <i>et al.</i> , 2015)
Maleimide		EGF proteins;	Micelles	Faster and more	Thiolation	(Chariou et

chemistry		affibodies; reprobodies; mAbs; Fabs; scFvs; nanobodies; bispecific antibodies; GE11 peptides; aptamers	made of polymers, amphiphilic molecules, lipids; chitosan NPs; liposomes; inorganic NPs, etc.	selective reactions; neutral pH media that avoid NPs aggregation	may hinder biological activities of ligands; non- selectivity in thiol- containing media	al., 2015; Faucon et al., 2017; Juan et al., 2020; Kang et al., 2018; Kim et al., 2017; Lucky et al., 2016; Sandoval et al., 2012)
Click chemistry		EGF proteins; affibodies; reprobodies; mAbs; Fabs; scFvs; nanobodies; bispecific antibodies; GE11 peptide; aptamers	Micelles made of polymers, amphiphilic molecules, lipids; chitosan NPs; liposomes; inorganic NPs, etc.	Site-specific conjugation; higher yield; control of ligand orientation; mild conditions, unsophisticated purification	Relatively new and more complicated than other methods; possible toxicity with Cu(I) catalysts	(Juan <i>et al.</i> , 2020; Kotagiri <i>et al.</i> , 2014; Yu <i>et al.</i> , 2012)
Biotin-avidin		Not applied yet for EGFR	Most of kinds of NPs	Good orientation of conjugated ligands	Non- covalent strategy and more complicated than other methods	(Juan <i>et al.</i> , 2020)

4. Main analytical methods used for detecting the presence of conjugated ligands on the surface of NPs

Following ligand conjugation, analytical methods are necessary to confirm whether the ligand is successfully introduced onto NPs or not. Currently, most studies are relying on the changes in NPs' physico-chemical properties (e.g., their size and charge) as indicators of successful conjugation. Nevertheless, to the best of our knowledge, such indicators are not sufficient, and in some cases, might even be misleading. The following part of this review is designed to provide information on the available analytical methods for the detection of conjugated anti-EGFR ligands on NPs.

4.1. Size and charge of the nanoparticles

The introduction of a ligand to NPs might lead to an increase in their size and a change in their surface charge, compared to those of the bare NPs. Such changes in physico-chemical properties may be indicators of successful conjugation.

In terms of size, the hydrodynamic diameter (D_H) is normally used and can be determined using the dynamic light scattering (DLS) method. Other techniques for size determination such as TEM images, in most cases, are not appropriate for detecting the presence of conjugated ligands. In EGFR-targeting, an increase in D_H is particularly noticeable when large-size ligands such as whole antibodies (e.g., cetuximab, whose size is around 10-15 nm) are conjugated. As an example, the polymeric NPs in the study of Wang et Zhou increased in size from 105 nm to 122 nm after antibody-conjugation (Wang and Zhou, 2015). An increase in size (from 175 nm to 185.7 nm) was also used to reveal the presence of Cet on the liposome surface in the study of Liu and colleagues (Liu *et al.*, 2019). In the study of Maya *et al.*, after Cet conjugation, the size of their chitosan NPs dramatically increased from 130 ± 25 nm to 180 ± 35 nm (Maya *et al.*, 2013). In our opinion, this large increase in size (around 50 nm) may come not only from the introduction of Cet onto the NPs, but also from other factors such as nanoparticles' aggregation. Apart from whole antibodies, other anti-EGFR targeting ligands are, in most cases, relatively small, making the DLS method not sensitive enough to distinguish the difference in size before and after ligand conjugation. For example, our team managed to conjugate an anti-EGFR scFv ligand onto SPIONs but there was no significant difference in the size of NPs after the conjugation (Vinh Nguyen *et al.*, 2020). Zhai *et al.* observed a similar phenomenon for the conjugation of an anti-EGFR Fab that was the second biggest kind of anti-EGFR fragments onto their lipid NPs. The authors found that this conjugation barely changed the size of the NPs, which remained around 230 nm (Zhai *et al.*, 2015). On the contrary, using the similar kind of ligand (anti-EGFR Fabs) for their lipid NPs, Zhou *et al.* detected an increase in size from 96.3 nm to 111.7 nm after Fab conjugation (Zhou *et al.*, 2007). These studies demonstrate that using only an increase in the size of NPs may not be sufficient to confirm the presence of conjugated ligands.

Similarly to the size of NPs, the surface charge may also be modified after ligand conjugation. To evaluate surface charges, measuring the zeta potential is the most widely used technique, as a clear change in this parameter is usually recorded in non-covalent conjugation methods. Lu *et al.* carried out a standard example by adsorbing Cetuximab on NPs. The zeta potential of their Fe_3O_4 -Au NPs was totally reversed from -23.2 ± 1.8 mV for the bare NPs to $+11.1 \pm 1.8$ mV for the Cet-conjugated NPs (Lu *et al.*, 2018). Besides, a change in the zeta potential was also observed for other kinds of EGFR-targeting ligands using electrostatic forces. For instance, in the study of Yin and co-workers, the authors constructed self-assembled complexes of the EGF protein, DNA and dendrimers. While the size was almost unchanged, the zeta potential declined significantly from $+16.2 \pm 0.2$ mV to $+2.4 \pm 0.8$ mV, in line with an increasing ratio of EGF/DNA (Zhang *et al.*, 2012). Zhang and colleagues decorated their negatively-charged quantum dots with positive anti-EGFR affibodies. As a result, the

zeta potential dramatically increased from -37 mV to +0.31 mV, which demonstrated successful conjugation (Y. Zhang *et al.*, 2018). In the case of other conjugation strategies, the zeta potential may be either unvaried or changed. For example, Sandoval *et al* conjugated the EGF protein onto their liposomal NPs using maleimide-thiol chemistry. However, no difference in the zeta potential before and after EGF conjugation was observed (Sandoval *et al.*, 2012). In our previous publication, the zeta potential of NPs also remained at the same value, despite the successful introduction of an anti-EGFR scFv ligand onto NPs using maleimide chemistry (Vinh Nguyen *et al.*, 2020).

In conclusion, an increase in the size of NPs or changes in their zeta potential after ligand conjugation might prove the presence of a ligand on the NPs' surface, especially in the case of antibody conjugation and conjugation methods using electrostatic interactions, respectively. Although many studies in the field of nanomedicines are currently using these methods, such physico-chemical property changes are insufficient, and other accurate techniques are required.

4.2. Spectroscopic analysis

Using spectroscopic analyses such as Fourier transform infrared (FT-IR) spectroscopy to analyze NPs before and after ligand conjugation may help confirm successful coupling.

FT-IR analyses rely on the detection of special functional groups that are not present on NPs before the conjugation and are only introduced onto NPs because of the presence of targeting ligands. The apparition of these functional groups may be due to i) available groups present on ligands themselves such as amine or carboxyl groups, ii) a new linkage established due to chemical conjugation such as amide or thioether linkages, iii) functional groups on other molecules that are linked beforehand to the ligand such as PEGylated ligands. FT-IR analyses have been deemed suitable for detecting successful conjugation onto inorganic NPs using biological ligands. As an example, Zhang *et al* confirmed the successful functionalization of their quantum dots with anti-EGFR affibodies by means of FT-IR analyses combined with the detection of physico-chemical property changes. In fact, the amine and the carboxyl groups from the conjugated affibodies were detected by the peaks at 1645 cm⁻¹ and 1538 cm⁻¹ for the N-H stretching and N-H bending of the amine group, respectively; and at 1715 cm⁻¹ and 1345 cm⁻¹ for the C=O and C-O stretching of the carboxyl group, respectively (Y. Zhang *et al.*, 2018). In another study, Lucky and co-workers used FT-IR analyses to confirm the successful grafting of PEGylated affibody conjugates on the surface of their TiO₂-upconversion NPs. On one hand, the characteristic peaks of PEG at 2885 and 1470-1350 cm⁻¹, corresponding to C-H stretching and C-H bending, appeared after the grafting. On the other hand, the presence of an amide linkage created after the conjugation was detected at 3500 and 1690 cm⁻¹, corresponding to N-H stretching and C=O stretching (Lucky *et al.*, 2016). Chen and colleagues analyzed the FT-IR spectra of the NPs before and after the conjugation to confirm the presence of anti-EGFR aptamers on their albumin NPs. The characteristic peaks of an amide linkage confirmed the crosslink between aptamers and albumin NPs after the conjugation (Chen *et al.*, 2016).

However, this method is not always feasible due to possible weak signals of linkages created from the conjugation. Furthermore, a suitable purification method is necessary to eliminate all non-reacted ligands.

4.3. Thermogravimetric analysis

Thermogravimetric analyses (TGA) also appear among the analytical methods that might be used to confirm the presence of EGFR-targeting ligands on the NPs' surface. As this method is based on the weight loss of samples according to an increased temperature, it might be used to evaluate the characteristic weight loss profile of the NPs before and after ligand conjugation due to the organic

content. Similarly to spectroscopic analyses, TGA are more suitable to confirm the presence of organic ligands conjugated to inorganic NPs rather than in organic NPs.

Marega and co-workers carried out a good example for the application of TGA in EGFR-targeting NPs. The authors managed to conjugate cetuximab onto their nanozeolites and used TGA to confirm this coupling. TGA results showed that in the non-targeted nanozeolites (Zeo-NH₂), the amount of the organic content and the oligoethylene-derived pyrolysis product was low, but increased significantly in the targeted-NPs (Cet-Zeo-NH₂) due to the presence of cetuximab. Moreover, the characteristic weight loss profile (minimum at 320 °C) obtained from the Cet-conjugated NPs confirmed the presence of the antibody on the surface of NPs. Interestingly, this strategy also allowed the authors to estimate the quantity of the grafted cetuximab in NPs (1.7-2.0 nmol of Cet per mg of NPs) (Marega *et al.*, 2016).

4.4. Structural and elemental analysis

Recently, precise techniques such as structural or elemental analyses with ¹H-NMR or X-ray photoelectron spectroscopy (XPS), respectively are increasingly used to verify qualitatively the presence of biological ligands on NPs.

¹H-NMR can be used to confirm the chemical structure of monomers used for further nanoformulation, especially in the case of targeted micelles and liposomes. As an example, Nan *et al* used this strategy to confirm the successful conjugation of the EGF protein onto PEGylated phospholipid (PEG-DSPE) molecules by means of the maleimide chemistry, after which their EGFR-targeting liposomes were constructed. The results obtained from ¹H-NMR spectra presented chemical structure shifts, in comparison with the initial monomer (before the conjugation). Furthermore, the structure of the ligand-functionalized monomer was confirmed with ¹H-NMR spectra (Nan, 2019).

The introduction of organic ligands onto inorganic NPs normally results in an increase in organic components in the finalized NPs and can be detected with XPS elemental analyses. In fact, once a ligand is successfully coupled to non-organic NPs, C1s and O1s peak areas in XPS analyses will increase significantly. Moreover, the presence of new elements such as N or S atoms due to ligand coupling may also reveal the presence of active targeting moieties. In the study of Yamamoto and co-workers on the conjugation of cetuximab onto gold NPs, the authors found that when the AuNPs surface was functionalized with the EGF protein, the C1s and O1s peak areas increased significantly (12.1 to 22.2 for C_{1s} and 6.14 to 13.0 for O_{1s}) (Yamamoto *et al.*, 2019). Marega and colleagues used XPS spectra and TGA to confirm the coupling of cetuximab on their nanozeolites (Zeo-NH₂ NPs). Before the conjugation, the XPS spectrum of Zeo-NH₂ NPs showed their expected elemental composition including O, Si, Al, C for the ethylene glycol and N atoms of the terminal amino groups (fewer than 1 % of N atoms). After the conjugation, the XPS spectrum clearly displayed the presence of C-centered peaks that fingerprinted both the remained ethylene glycol on Zeo-NH₂ NPs and the polypeptidic molecular fragments from the conjugated antibodies. Moreover, an enhanced content of N atoms (around 11 %) coming from the conjugated cetuximab was recorded, denoting the occurrence of bioconjugation. In addition, this analytical method may also help compare the conjugation efficiency of two different conjugation strategies. In the same study Marega *et al*, by comparing the “organic to inorganic” proportion of the targeted NPs prepared with covalent chemistry and that obtained from non-covalent adsorption of the antibody, the authors were able to compare the yield of the non-covalent and the covalent methods. In fact, the sum of the percentage of C1s and N1s divided by the sum of the percentage of Si2p and Al2p for each kind of NPs was calculated. This ratio remained at the value of 4.0 for the antibody-conjugated NPs with non-covalent

or covalent strategies, suggesting that the conjugation yield of these strategies was similar in this study (Marega *et al.*, 2016).

Despite their high precision, these techniques in most cases are unfeasible due to the small quantity of conjugated targeting moieties present on NPs. Furthermore, a good purification method is essential to preclude any interference of non-conjugated ligands. Nevertheless, these methods can provide a firm conclusion on the presence of targeting ligands on NPs and worthy of consideration.

4.5. Fluorescence spectroscopy and imaging

A more selective and illustrating method for the detection of conjugated ligands is based on fluorescence spectroscopy. There are two available strategies for such detection. The first solution is to use a secondary antibody that is previously fluorescence-labeled and that can bind to the conjugated ligand. In this strategy, the targeted NPs are firstly incubated with the fluorescence-labeled antibody to create an antibody-ligand-NPs complex. This complex can be further detected thanks to fluorescence signals on the antibody. Besides, targeting ligands can also be previously fluorescence-labeled with a fluorescent dye that, once grafted onto NPs, can be detected by means of fluorescence spectroscopy.

Lu *et al* conjugated cetuximab onto Fe₃O₄@Au NPs using physical adsorption and the presence of cetuximab was confirmed with the use of fluorescence spectroscopy. The targeted NPs were first incubated with the FITC-conjugated anti-Human IgG to form the FITC-anti IgG-Cet-NPs complex. Then, the fluorescence from FITC on this complex was detected by a confocal laser-scanning microscope. A green fluorescence signal was observed for the targeted NPs, whereas no fluorescence was detected in the case of the non-targeted NPs (Lu *et al.*, 2018).

In another effort to confirm the presence of coupled anti-EGFR aptamers on their dendrimers, Lv and co-workers introduced a fluorescence dye (FAM dye) onto the 3' terminal of their anti-EGFR aptamers with excitation and emission wavelengths at 491 nm and 519 nm, respectively. After the aptamer-conjugation, fluorescence signals of the FAM dye were revealed for all aptamer-modified NPs, but not with the bare NPs. This result indicated that aptamers were successfully introduced onto the NPs. Moreover, conjugation efficiency could also be estimated using the calibration curves of FAM-modified aptamer solutions. As a result, conjugation efficiency was found to be around 20 % in this study (Lv *et al.*, 2018). In another study of Xie and colleagues, the authors conjugated cyanine-modified aptamers onto their mesoporous silica NPs. By following the cyanine fluorescence signal, the authors were able to determine both qualitatively and quantitatively the grafted aptamers on NPs. In this study, each NP was shown to have around 344 aptamers on its surface (Xie *et al.*, 2019). However, as the non-conjugated ligands can interfere or even misinform the success of the conjugation, an appropriate strategy of purification plays an important role.

Although its promising application has been proved, the introduction of a fluorescent dye onto EGFR targeting ligands may significantly change their structure, and results in a decrease in their targeting ability towards EGFRs. As a result, while applying this strategy, a complementary step of verifying the binding affinity of ligands before and after the introduction of fluorescent dye is necessary.

4.6. SDS gel electrophoresis

Among the available methods for the detection of ligands derived from proteins, one of the most efficient methods is sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The principle of this technique is based on the non-migration or retarded migration of nanoparticles in gels that allows the separation of unattached ligands from conjugated ones. In this technique, gel

electrophoresis is performed with the targeted NPs, the non-targeted NPs as negative controls and solutions containing free ligands as positive controls. After the migration, the protein-staining reagent is added to reveal the presence of the protein. If the conjugation is successful, no protein band is observed for the targeted NPs at the location found using the free ligand solution, as it has already been co-localized with NPs. Using this strategy, Gill *et al* confirmed the conjugation of the EGF protein onto their polymeric NPs. In this study, after the revelation with the protein-staining reagent, the band corresponding to the free EGF protein (at MW = 6.4 kDa) appeared at the bottom of the gel and was used as the positive control. For the targeted NPs, that band was absent, but a strong protein band at the top of the gel co-localizing with the NPs confirmed the successful conjugation (Gill *et al.*, 2018).

In addition to the application as a qualitative method, SDS-PAGE can also be used to estimate the quantity of the grafted ligands. To this end, serial dilution of ligand solutions is conducted to construct a calibration curve for the determination of conjugated ligands. Using this method, Kao and colleagues showed that around 124 cetuximab molecules per NP were coupled onto their AuNPs for an EGFR-targeting theranostic strategy (Kao *et al.*, 2013). In another study of Zhai and co-workers, the authors used SDS-PAGE to confirm the presence of conjugated anti-EGFR Fabs on their NPs and then used integrated images analyses to estimate the efficiency of this ligand conjugation (Zhai *et al.*, 2015). However, image analyses do not seem to provide precise results. In our point of view, this method should only be used as a qualitative method or a control on the conjugation yield.

4.7. Protein dosage

For active targeting ligands with a protein origin, protein assays such as BCA and Bradford assays are widely used to estimate the quantity of the grafted ligands on the NPs surface.

The BCA (bicinchonic) protein assay is a widely used method for the colorimetric detection and quantification of total protein in a solution. This Copper-based protein assay was introduced for the first time in 1985, involving two steps including protein-copper chelation and reduced copper detection. The resulting complex in BCA is water-soluble and exhibits a strong linear absorbance at 562 nm with increasing protein concentrations. BCA is sensitive and has a broad dynamic range with capability of measuring protein concentrations from 0.5 µg/mL to 1.5 mg/mL. In addition, BCA is stable under alkaline conditions, and can be included in copper solutions to allow a one-step procedure. However, substances that reduce proteins or chelate copper also produce colors in the BCA assay, thus interfering with the accuracy of protein quantitation. This interference is also observed for certain single amino acids (such as cysteine, tyrosine and tryptophan). Therefore, the presence of these substances needs to be avoided in BCA assays (Walker, 2009).

Another protein assay method that can be used for ligand quantification is the Bradford protein assay. The Bradford method is a dye-based assay that exploits the binding ability of the Coomassie blue to proteins. In acidic environments, proteins bind to the Coomassie dye and a spectral shift from the red form of the dye (maximal absorbance at 465 nm) to its blue form (maximal absorbance at 610 nm) can be observed. Although the difference between the two forms of the dye is the greatest at 595 nm, this wavelength can be adjusted to be suitable for each solution. In terms of measuring capability, the Bradford assay is linear in a range of protein concentrations up to 2.0 mg/mL. Compared to other methods such as BCA, Bradford's is considered the fastest and the easiest method. Moreover, it is also compatible with most salts, solvents, buffers, thiols, reducing reagents and metal chelating agents.

In the case of NPs functionalization using targeting ligands, both the BCA and the Bradford methods can be used, but each method has its own advantages and drawbacks. Therefore, the choice of a

quantification method should be meticulously considered and can be based on i) the compositions of samples, ii) the stability and solubility of NPs in each method conditions, and iii) the required sensitivity of quantification. In fact, as reducing agents and amino acids are normally present in the conjugation reaction, the results from the BCA method are prone to being interfered and the Bradford method appears to be more suitable. On the contrary, the Bradford method needs to be performed in an acidic environment that is not appropriate for samples with poor stability or poor solubility in an acidic pH. Besides, the BCA method is more sensitive than the Bradford method (Kruger, 2009).

Maya *et al* carried out a standard example of the application of the BCA to quantify conjugated anti-EGFR ligands on NPs. In their study, the authors developed cetuximab-conjugated chitosan NPs to deliver paclitaxel onto EGFR-overexpressing tumors and used the BCA to confirm the presence and determine the efficiency of cetuximab conjugation. The conjugated NPs were incubated with the BCA reagent and the absorbance of the obtained solution was firstly recorded at 562 nm and subsequently compared to that of the calibration curve. The results showed that the Cet conjugation efficiency was $42 \pm 6 \%$ for this study (Maya *et al.*, 2013).

In our team, we use the Bradford assay as our daily control of ligand conjugation. In our previous publication, we used the Bradford method to estimate the amount of anti-EGFR scFv fragments that were successfully conjugated onto our SPIONs. Due to the binding ability of the Bradford's reagent to the kappa light chains present on scFv fragments, this method was suitable for scFv detection and quantification. The absorbance of the incubated samples with the reagent was measured at 630 nm and the concentration of the grafted scFv ($\mu\text{g/mL}$) was determined using a calibration curve (scFv concentrations of 0; 2.5; 5; 10; 15; 20; 25 $\mu\text{g/mL}$) with scFv-free SPIONs as the negative control. The results showed that around 13 targeting scFv fragments per NP were introduced onto our SPIONs (Vinh Nguyen *et al.*, 2020). In another study, Houdaihed and colleagues also used the Bradford method to confirm the presence and quantify the amount of the grafted anti-EGFR Fab fragments onto their polymeric NPs. A similar number of grafted ligands to our study was found in this study, with approximately 12 conjugated ligands per NP (Houdaihed *et al.*, 2020).

In conclusion, a broad range of analytical methods can be used to confirm the success of ligand bioconjugation from simple methods to sophisticated ones. In addition to "daily control" such as changes in size and charge of finalized NPs compared to initial NPs, other analytical methods are highly recommended to provide a clear and precise confirmation. The choice of appropriate methods can be made according to the kind of ligands and NPs. As an example, if ligands are protein-based, it is useful to apply SDS-PAGE and protein dosage as complementary methods to size-charge controls in order to have both qualitative and quantitative information on conjugated ligands.

1120 **Table 4 Main analytical methods used for the detection of conjugated anti-EGFR ligands on NPs surface**

Analytical method	Qualitative/ Quantitative	Appropriate cases	Advantages	Drawbacks	References
Size and charge measurements	Qualitative	Increase in size (antibody-conjugation); changes in zeta potential (conjugation using electrostatic interactions)	Easy procedure; regular control	Lack of relevance	(Liu <i>et al.</i> , 2019; Lu <i>et al.</i> , 2018; Maya <i>et al.</i> , 2013; Wang and Zhou, 2015; Zhang <i>et al.</i> , 2012; Y. Zhang <i>et al.</i> , 2018)
FT-IR		Organic ligands on inorganic NPs; chemical conjugation strategies		Limited applications to organic ligands on inorganic NPs and chemical conjugation; low sensibility if the conjugation efficiency is not sufficient, and demand for a good purification method to eliminate non-reacted ligands	(Chen <i>et al.</i> , 2016; Lucky <i>et al.</i> , 2016; Y. Zhang <i>et al.</i> , 2018)
Thermogravimetric analysis		Organic ligands on inorganic NPs		Limited applications to organic ligands on inorganic NPs	(Marega <i>et al.</i> , 2016)
Elemental analysis techniques		Ligand-conjugated monomer for NMR; organic ligands on inorganic NPs for XPS		Limited applications to organic ligands on inorganic NPs; invasive method	(Marega <i>et al.</i> , 2016; Nan, 2019; Yamamoto <i>et al.</i> , 2019)
Fluorescence spectroscopy and imaging	Qualitative or semi-quantitative	All kinds of ligands and NPs	More selective and illustrating method;	A second fluorescence-labeled antibody or a	(Lu <i>et al.</i> , 2018; Lv <i>et al.</i> , 2018; Xie <i>et</i>

			possibility for quantification	labeled-ligand is required, a good purification method to eliminate non-reacted ligands is needed	<i>al.</i> , 2019)
SDS page gel electrophoresis	Qualitative or semi-quantitative	Protein-based ligands	The most used method for detecting of protein-based ligands; possibility to be used as a semi-quantitative method	Not suitable for non-protein ligands	(Gill <i>et al.</i> , 2018; Kao <i>et al.</i> , 2013; Zhai <i>et al.</i> , 2015)
Protein dosage	Quantitative	Protein-based ligands	Sensitive method for protein-based ligand (BCA assay); easiest and fastest method (within few minutes for the Bradford assay)	BCA is interfered by reducing agents, chelators and certain amino acids. The Bradford method needs to be performed in an acidic pH	(Houdaihed <i>et al.</i> , 2020; Maya <i>et al.</i> , 2013; Vinh Nguyen <i>et al.</i> , 2020)

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5. Conclusions

Nanoparticles functionalized with EGFR-targeting ligands are becoming an interesting field of study in the quest for better treatment of various types of cancer. The overexpression of EGFRs in various types of solid tumors makes them one of the most promising oncomarkers for targeted nanotherapy. Therefore, several types of anti-EGFR ligands with high binding affinity have been developed for nano-functionalization including the EGF protein and its derivatives, whole antibodies or antibody fragments, peptides and aptamers. The functionalization of NPs with these fragments offers many advantages including i) an enhancement in cancer cellular uptake via EGFR receptor-mediated endocytosis, ii) a higher *in vivo* tumor accumulation and retention level of loaded agents resulting in better therapeutic efficacy, iii) less systemic toxicity thanks to tumor targeting properties or neutralizing effects for highly charged NPs, and iv) possibility to be used spontaneously as therapeutic agents and as active targeting ligands.

Despite these promising benefits, nanomedicines targeted with EGFR-targeted ligands should be designed to meet i) the pathophysiology of the disease due to possible mutations in the oncomarker and ii) the individual need of each patient in terms of drug content that we call “personalized nanomedicine”. Moreover, physicochemical properties, and the safety of nanomedicines should be also meticulously considered to better exploit the EPR effect (enhanced permeability and retention effect), and to address the risk of nano-toxicity.

Secondly, as there is a strict correlation between the type, the number and the conformation of the conjugated ligands and the therapeutic efficiency, these aspects need to be optimized. One of the solutions to address these issues is to use an appropriate conjugation strategy. A variety of conjugation methods including both physical interactions and chemical reactions have been developed for the introduction of EGFR-targeting ligands onto NPs. As each conjugation method presents both advantages and drawbacks, the choice of a conjugation strategy should be considered regarding i) the type of NPs and targeting ligands, ii) their stability in reactive media or biological fluids and iii) eventual toxic byproducts. Among these methods, a selective conjugation method such as click chemistry is preferable. This method may not only help introduce a higher amount of ligands onto NPs but also control the orientation of the grafted ligands on the surface of NPs, resulting in better interactions between the ligands and their targets.

After the conjugation, the presence of conjugated ligands need to be confirmed with suitable analytical methods. Currently, changes in colloidal properties of NPs after the conjugation remain the principal control of successful conjugation. However, the lack of relevance of this method needs to be underlined and more accurate methods should be more widely applied.

Taken together, nanomedicines functionalized with EGFR-targeting ligands are remarkably promising for the cancer treatment but future optimization to obtain maximal therapeutic effects with minimal side effects is still needed.

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