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Active targeting strategy in nanomedicines using anti-EGFR ligands - a promising approach for cancer therapy and diagnosis

Phuoc Vinh Nguyen, Katel Hervé-Aubert, Igor Chourpa, Emilie Allard-Vannier*

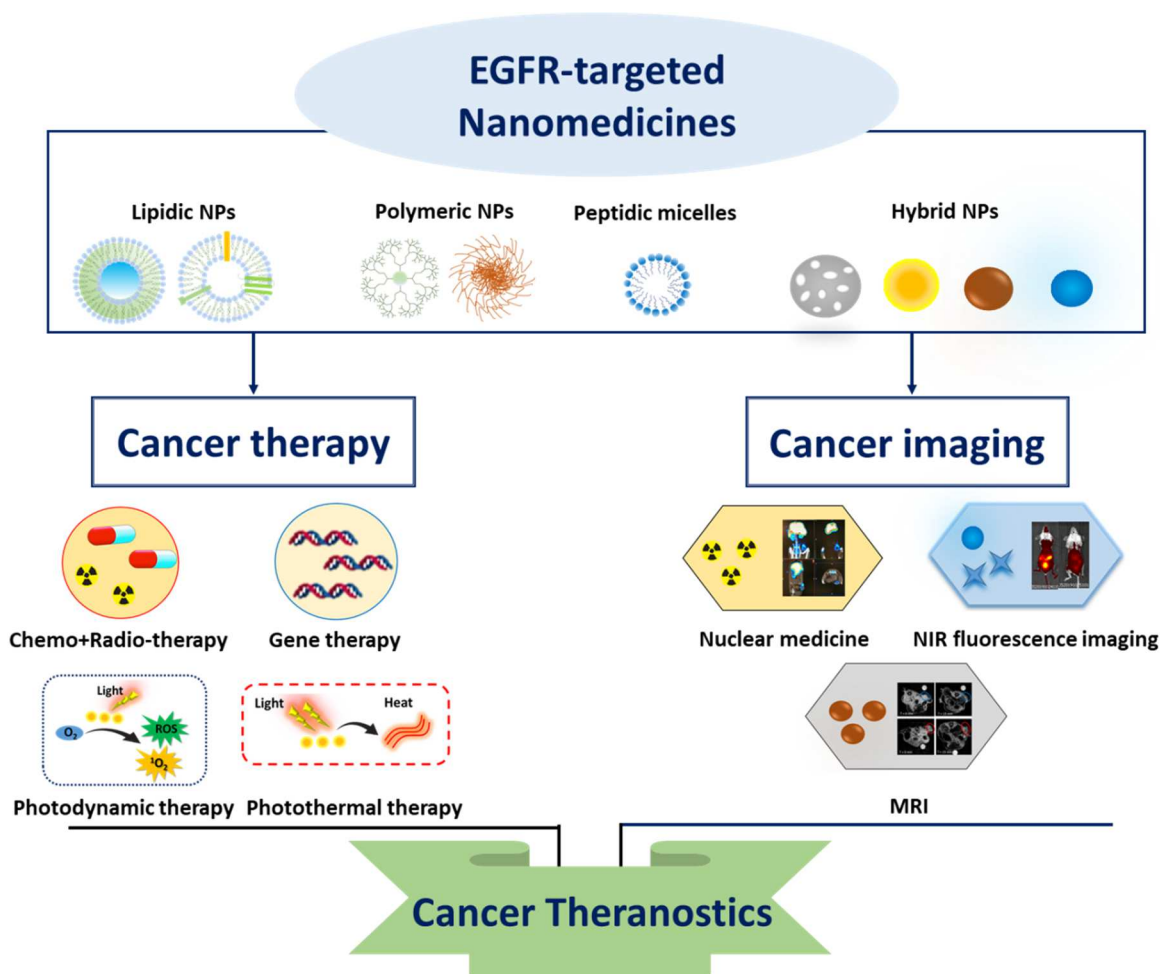
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Abstract

As active targeting using nanomedicines establishes itself as a strategy of choice in cancer therapy, several target receptors or ligands overexpressed in cancer cells have been identified and exploited. Among them, the epidermal growth factor receptor (EGFR) has emerged as one of the most promising oncomarkers for active targeting nanomedicines due to its overexpression and its active involvement in a wide range of cancer types. Henceforth, many novel EGFR-targeted nanomedicines for cancer therapy have been developed, giving encouraging results both *in vitro* and *in vivo*. This review focuses on different applications of such medicines in oncotherapy. On an important note, the contribution of EGFR-targeting ligands to final therapy efficacy along with current challenges and possible solutions or alternatives are emphasized.

Keywords: EGFR; nanomedicine; active targeting; cancer therapy, imaging, theranostics

Graphical abstract



19
20

21 1. Introduction

22 Cancer is one of the most prevalent, predominant and fatal diseases in the world with an increasing
23 number of new cases each year. In the United States, it is estimated that around 1,898,160 new
24 cases will be detected and 608,570 Americans will die from cancer in 2021 (Siegel *et al.*, 2021). With
25 the advent and the development of new modalities used in diagnosis and/or early treatment, the
26 cancer death rate has continuously fallen since 1991, reaching a total decline percentage of 31% in
27 2018. Despite this positive sign, the overall survival rate has yet to be improved, especially in the era
28 of new public health threats posed by the novel coronavirus, also known as SARS-Cov-2, during which
29 many studies have reached a consensus on the increasing vulnerability of cancer patients to COVID-
30 19 complications (Moujaess *et al.*, 2020).

31 Currently, several types of cancer treatment are available including surgery, radiotherapy, and
32 chemotherapy that can be applied either alone or in combination with each other (Huang *et al.*,
33 2017). Chemotherapy remains the standard modality for cancer therapy, but confronts several issues
34 such as quick clearance, low stability, low drug tolerance due to non-specific biodistribution, and the
35 emergence of drug resistance. One of the most promising strategies to overcome these problems is
36 to use nanoscale drug delivery systems (Pawar and Prabhu, 2019a). These nanosystems are
37 developed not only to improve drug delivery but also to cover a broad spectrum of cancer therapy
38 including gene delivery, phototherapy, diagnosis, and theranostic purposes. Compared to
39 conventional adjuvant agents, nanoparticles (NPs) offer remarkable benefits such as: i) improving the
40 delivery of highly hydrophobic drug, ii) facilitating the passage of drugs across biological barriers, iii)
41 protecting and improving the delivery of therapeutic molecules to their action sites, iv) combining
42 imaging and therapy, and v) specifically reducing drug toxicity (Hirsjarvi *et al.*, 2011).

43 Tumor targeting with NPs can be achieved by two strategies namely passive or active targeting.
44 Passive targeting relies on the Enhanced Permeation and Retention (EPR) effect or abnormalities of
45 the tumor microenvironment to better deliver therapeutic agents (Attia *et al.*, 2019; Byrne *et al.*,
46 2008; Hirsjarvi *et al.*, 2011). Although passive targeting can enhance drug tumor accumulation, it
47 cannot specifically distinguish between tumors and healthy tissues and may lead to intolerant side
48 effects. Therefore, recent studies are focusing on a new strategy termed “active targeting” to
49 maximize the therapeutic efficiency and minimize toxicity on normal tissues. Active targeting refers
50 to the functionalization of NPs’ surface with biological ligands that can bind specifically to the target
51 molecules or receptors present on cancer cells (Yoo *et al.*, 2019). Among various kinds of target
52 molecules or receptors, Epidermal Growth Factor Receptors (EGFRs) appear among the most
53 interesting targets for active targeting with nanomedicines due to their remarkable overexpression in
54 a broad spectrum of cancer types and their involvement in diverse cancer cell processes (Nguyen *et al.*,
55 2021). EGFR is a 170 kDa transmembrane glycoprotein in the tyrosine kinase family that plays a
56 key role in the control of cell proliferation in normal tissues. In cancer, the EGFR-overexpression is a
57 common phenomenon in various kinds of human cancers such as lung, head and neck, colon,
58 pancreas, breast, ovary, and bladder and kidney cancers. Its overexpression has been proven highly
59 related to multiple cancer-implicated signaling pathways and significantly contributes to cancer
60 angiogenesis as well as cancer resistance. Considering its active involvement in cancer, many
61 researchers are currently working on this field and achieved important advances in the development
62 of active targeted nanomedicines with anti-EGFR ligands. As information on its structure, signaling
63 pathways and available EGFR-targeting ligands has been precisely described in our previous review
64 (Nguyen *et al.*, 2021), this paper will specifically focus on the application of such nanomedicines in
65 cancer therapy and diagnosis.

66

67 2. Structure of EGFR-targeted nanomedicines

68 Various kinds of NPs, EGFR-targeting moieties and conjugation methods have been developed for
69 cancer therapy (Nguyen *et al.*, 2021). Diverse nanomaterials are available and can be classified into
70 two main groups including organic and inorganic NPs. **Table 1** provides information on the structures,
71 advantages, and drawbacks of different kinds of EGFR-targeted NPs and different strategies to
72 conjugate anti-EGFR ligands onto these NPs.

73 In general, these NPs can be used for various kinds of applications. However, according to a specific
74 objective, some kinds of NPs can be preferred over others. For example, in EGFR-targeted NPs, to
75 deliver a hydrophobic drug, organic NPs such as liposomes, solid lipid NPs, polymeric NPs or micelles
76 are more frequently used than inorganic NPs. In contrast, for phototherapy or imaging agents
77 delivery, inorganic NPs such as gold nanoparticles (AuNPs), superparamagnetic NPs (SPIONs) or
78 quantum dots (QDs) are usually employed. In EGFR-targeted NPs, these inorganic NPs are normally
79 attached to organic components such as polymer layers or EGFR active targeting ligands in order to
80 improve their stability and functionality, and therefore, belong to the family of hybrid NPs (Yao *et al.*,
81 2020). For gene therapy with EGFR-targeted NPs, dendrimers are employed in many studies.

82 Since their discovery in 1961, *liposomes* have been widely used as drug delivery systems in cancer
83 treatment. These NPs comprise a bilayer lipid membrane made of nontoxic phospholipids and
84 cholesterol surrounding an aqueous core. Thanks to their small size ranging from 50 nm to 100 nm,
85 their hydrophilic and hydrophobic structural properties, liposomes can be used to deliver both
86 hydrophilic and hydrophobic therapeutic agents and are considered one of the most widely used
87 nanosystems in drug delivery (Aghebati-Maleki *et al.*, 2020; Akbarzadeh Khiavi *et al.*, 2020).
88 Doxorubicin encapsulated in liposomes (Doxil[®]) is the first approved nanoformulation in cancer
89 treatment (Barenholz, 2012). In EGFR-targeted NPs, their application is highly regarded in drug and
90 gene delivery. As an example, Wang *et al* conjugated anti-EGFR scFv fragments onto their liposomes
91 to improve the therapeutic index of chemotherapy (doxorubicin and vinorelbine) in head and neck
92 cancer (HNC). On the one hand, with a size inferior to 150 nm (100 nm for non-targeted liposomes
93 and 120 nm for targeted ones), both targeted and non-targeted liposomes demonstrated an efficient
94 EPR effect, as reflected by better inhibition on tumor growth compared to free drugs. On the other
95 hand, the advantage of EGFR-active targeting was clearly revealed by a significant reduction in the
96 IC₅₀ values of various cell lines of HNC (FaDu and Ca9-22 cells), and a prolonged median survival time
97 of animals treated with targeted NPs compared to non-targeted counterparts or free drugs (94 days
98 versus 60 days and 56 days, respectively) (Wang *et al.*, 2020).

99 *Polymeric NPs* are the most common kind of NPs used for drug delivery, with Abraxane[®] (approved in
100 2005) being the first polymeric nanodrug in the market. The choice of polymers is made according to
101 the type of application and the encapsulated agents. However, these polymers, either natural or
102 synthesized, have to be biocompatible, nontoxic and have a suitable half-life time (Aghebati-Maleki
103 *et al.*, 2020). Among different kinds of polymers used in nanomedicines, poly(lactic-co-glycolic acid)
104 or PLGA and poly(lactic acid) or PLA are the most successful nanomaterials due to their capability of
105 being hydrolyzed inside the body into metabolite monomers with minimal toxicity (Aftab *et al.*, 2018).
106 Not surprisingly, many EGFR-targeted polymeric NPs have been developed and cover a large
107 spectrum of cancer therapy from therapeutic to diagnostic applications. This aspect will be further
108 illustrated in each section of this review.

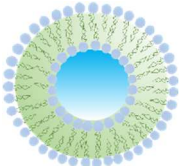
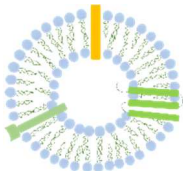
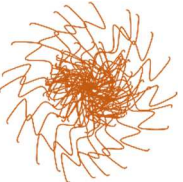
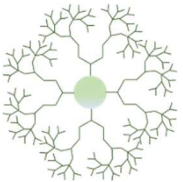
109 *Micelles* are colloidal carriers made of amphiphilic molecules that can self-assemble into nanosized
110 drug delivery systems. Most micelles are made of polymers (polymeric micelles) but amphiphilic
111 molecules such as amphiphilic peptides can also be used. As regards EGFR-targeting application with

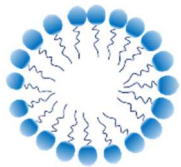




112 non-polymeric micelles, anti-EGFR peptides such as GE11 peptides have been extensively applied as a
113 component to construct micelles while preserving their EGFR-targeting properties. In this strategy,
114 the GE11 serves as the hydrophilic head and a hydrophobic tail is introduced, as shown in a study of
115 Liang *et al* (Liang *et al.*, 2016). In another study, Du *et al* have demonstrated the potency of self-
116 assembly amphiphilic peptide NPs with GE11 in the delivery of gemcitabine and olaparib to treat
117 pancreatic cancer with breast cancer 2 mutation. An extra glycine and two glutamic-acid containing
118 carboxyl groups were added into GE11 to increase its hydrophilicity. Furthermore, to have sufficient
119 hydrophobicity, a C18 tail was added into the monomer. The targeted micelles were stable, exhibited
120 high encapsulation efficiency for both drugs (up to 98%), had their half-life prolonged (>3h vs 1h for
121 the free drugs), and provided better tumor suppression compared to non-targeted ones with
122 minimal side effects (Du *et al.*, 2018).

123 *Dendrimers* are highly branched polymers that comprise three main components: a central core,
124 repetitive branching units, and terminal groups. There are three types of dendrimers. The most
125 widely used in research included polyamidoamine (PAMAM), poly(propylene imine) (PPI), and poly-L-
126 lysine (PLL) dendrimers. Compared to conventional linear and branched polymeric nanocarriers,
127 dendrimers present several advantages such as: i) higher control in terms of physico-chemical
128 properties with good solubility, ii) good ability to pass through cell barriers, and iii) high possibility of
129 surface functionalization. Therefore, dendrimers are getting increasing attention, and their great
130 potency as a drug or gene delivery system has been confirmed (Abedi-Gaballu *et al.*, 2018;
131 Palmerston Mendes *et al.*, 2017). In EGFR-targeted nanomedicines, the application of dendrimers in
132 gene delivery is very promising. To be precise, dendrimers are able to protect nucleic acids from
133 nuclease degradation by forming stable dendrimer-nucleic acid complexes via electrostatic forces.
134 Furthermore, the possibility of modifying their surface with different kinds of anti-EGFR ligands can
135 contribute effectively to enhancing the efficacy of the finalized nanocarriers (Abedi-Gaballu *et al.*,
136 2018). Therefore, the application of EGFR-targeted dendrimers in gene therapy are focused in this
137 paper.

138 In addition to the previous “organic” NPs, inorganic nanomaterials have also been extensively applied
139 in EGFR-targeted nanomedicines. These NPs include gold NPs (AuNPs), mesoporous silica NPs
140 (MSNPs), superparamagnetic iron oxides NPs (SPIONs) and quantum dots (QDs). Compared to
141 organic NPs, inorganic NPs can offer unique advantages including high stability, easy synthesis, easy
142 modification and inertness. Their application can cover a broad spectrum in cancer therapy, e.g. the
143 delivery of radiotherapy, phototherapy or imaging agents thanks to their unique intrinsic properties.
144 For example, AuNPs are of great interest owing to their unique properties in size and shape-
145 dependent optical and electronic characteristics. MSNPs are extensively used in the medical field due
146 to several advantageous properties such as their large surface area, well-defined morphology, and
147 biocompatibility. SPIONs can be served as excellent contrast agents in magnetic resonance imaging
148 (MRI). QDs are outstanding in biomedical imaging for cancer compared to traditional organic dyes
149 due to their unique optical and electronic characteristics. In addition to their unique intrinsic
150 properties, these NPs can also be served as promising drug nanocarriers due to the possibility of
151 easily modifying their surface (Bayda *et al.*, 2018). In EGFR-targeted nanomedicines, these inorganic
152 NPs can be easily functionalized with various kinds of anti-EGFR ligands via different conjugation
153 methods and have been exploited in many studies (Nguyen *et al.*, 2021). The accurate applications of
154 such nanomedicines will be discussed in the following sections.

Table 1. Different kinds of NPs with their advantages and challenges used in EGFR targeting nanomedicines

| NPs | Illustration | Structure | Anti-EGFR conjugation | Advantages | Limitations | Ref. |
|-------------------------------------|---|---|--|--|--|---|
| Liposomes |  | Lipid bilayer with a central aqueous cavity | Via chemical conjugation or electrostatic interaction for cationic liposomes | Biocompatibility, high drug loading capacity, possibility to load large molecules, surface functionalization | Difficulty to sterilize, low stability | (Alavi <i>et al.</i> , 2017; Dong <i>et al.</i> , 2018; Riaz <i>et al.</i> , 2018; Wang <i>et al.</i> , 2020) |
| Extracellular vesicles (EVs) |  | Cell-derived nanosized vesicles made of cellular membrane surrounding an aqueous core | Via electrostatic interaction or chemical conjugation | Enhanced drug cellular uptake, safety, low risk of immunogenicity, increased half-life time of drugs | Problems in heterogeneity of isolated vesicle populations, negative impacts of isolation methods on vesicle composition and properties, difficulty in scale-up | (de Jong <i>et al.</i> , 2019; Pi <i>et al.</i> , 2018; Surman <i>et al.</i> , 2019) |
| Polymeric NPs |  | NPs made of polymers such as polysaccharides, poly (lactic-co-glycolic acid) PLGA, amino acids, proteins (albumin), poly(methyl methacrylate), poly(ethylenimines), polyesters, poly(methylene malonates), etc. | Attached to the monomer of micelles via chemical conjugation | Biocompatibility and biodegradability, long half-life time, high drug stability and drug payload | Sophisticated synthesis that leads to difficulty in purification and large-scale manufacture | (Cheng <i>et al.</i> , 2021; Huang <i>et al.</i> , 2015; Milane <i>et al.</i> , 2011a; Parveen and Sahoo, 2008) |
| Dendrimers |  | Artificial macromolecular nanocarriers with well-defined topological structures | Mainly via electrostatic interaction but also via chemical conjugation | High payload capacity, high monodispersity, easy-to-control characteristics | Toxicity due to high positive charges, high-cost production | (Cheng <i>et al.</i> , 2011; Chis <i>et al.</i> , 2020; Kim <i>et al.</i> , 2018; Zhang <i>et al.</i> , 2012) |

| | | | | | | |
|-------------------------------|---|--|--|--|---|--|
| Non-polymeric micelles |  | Colloidal carriers made of amphiphilic molecules such as peptides | Attached to the monomer of micelles via chemical conjugation | Biodegradability, suitability for highly hydrophobic drugs and enhanced EPR effects, easy synthesis and low cost | Low thermal stability, fast drug release, suitability for lipophilic drugs only | (Branco and Schneider, 2009; Du <i>et al.</i> , 2018; Liang <i>et al.</i> , 2016) |
| Gold NPs |  | NPs made of gold | Via chemical conjugation or physical adsorption | Biocompatibility, easy surface modification, possibility for phototherapy, controlled drug release | Toxicity due to long-time retention in the body | (Amina and Guo, 2020; Chen, 2016; Silva <i>et al.</i> , 2016; Sztandera <i>et al.</i> , 2019) |
| Mesoporous silica NPs |  | NPs made of silica with well-defined porosity and morphology | Via chemical conjugation | Biocompatibility, easy surface modification, large surface area, well-defined morphology | Short circulation time, low stability | (Attia <i>et al.</i> , 2019; Pasqua <i>et al.</i> , 2016; Reda <i>et al.</i> , 2019) |
| Magnetic NPs |  | NPs made of superparamagnetic iron oxides | Via chemical conjugation | Biocompatibility, easy surface modification, easy synthesis, possibility to exploit magnetic properties | No internal loading capacity | (Chu <i>et al.</i> , 2015; Revia and Zhang, 2016; Vinh Nguyen <i>et al.</i> , 2020; Xiao and Du, 2020) |
| Quantum dots |  | Semiconductor nanocrystals with cores made of inorganic elements (e.g. Cd and Se) enclosed within a metallic shell (e.g. ZnS, Ag ₂ S) | Via chemical conjugation | Innate optical properties, easy surface modification | No internal loading capacity, potential toxicity | (Lee <i>et al.</i> , 2010; Richards <i>et al.</i> , 2017; Zhang <i>et al.</i> , 2018) |

1 **3. Applications of EGFR-targeted nanomedicines in the context of cancer therapy**

2 **3.1. Targeted delivery of chemotherapeutics or therapeutic radionuclides**

3 As EGFR expression is strictly related to a variety of cancer processes, anti-EGFR moieties on
4 nanomedicines can serve, at the same time, as active targeting ligands and therapeutic agents for
5 cancers with a high EGFR expression level. The introduction of anti-EGFR moieties onto
6 nanomedicines can offer several advantages as follows.

7 **3.1.1. Higher drug accumulation in cancer cells and tumors overexpressing EGFR**

8 At the cellular level, EGFR-targeted NPs can improve drug cellular uptake via specific EGFR-mediated
9 endocytosis. While non-targeted NPs internalize into cancer cells via non-specific pathways (diffusion
10 or membrane fusion), the targeted NPs can enter directly into cancer cells via both non-specific and
11 specific pathways. As regards the specific pathway, the targeted NPs firstly bind to cancer cells via
12 the EGFR ligand-receptor complex, and subsequently activate the corresponding endocytosis, which
13 involves endosomal or lysosomal uptake of these NPs. Due to this specific pathway, a better and
14 faster cellular internalization process can be achieved with EGFR-targeted NMs (Clemons *et al.*, 2018;
15 Singh *et al.*, 2016). Following the endocytosis, NPs are usually disassembled and release the
16 entrapped drugs, directly delivering them into the site of cell activation, which is not observed in the
17 case of non-targeted NPs (Clemons *et al.*, 2018). On another aspect, the functionalization with EGFR-
18 targeting ligands can help distinguish tumor cells from healthy ones, this may be useful to reduce the
19 side effects related to encapsulated drugs (Du *et al.*, 2018). This advantage is based on the relatively
20 low expression of EGFR in normal cells ($4.0-10.0 \times 10^4$ EGFRs/cell) compared to the high EGFR
21 expression level in cancer cells (up to over 10^6 EGFR/cell) (Akbarzadeh Khiavi *et al.*, 2020). Finally, as
22 EGFR-overexpression is a common phenomenon in a large spectrum of cancers, the possibility to
23 develop a nanosystem that can cover several kinds or cancers or highly heterogeneous cancers can
24 be achieved (Reda *et al.*, 2019).

25 *In vivo*, EGFR-targeting provides substantial benefits in enhancing anti-tumor activities of NPs and
26 reducing severe side effects compared to non-targeted NPs or free drugs. Many studies have
27 developed EGFR-targeted NMs for various kinds of cancers, which are resumed in Table 2. Herein, a
28 standard example was carried out by Sandoval *et al.* The authors developed gemcitabine-loaded
29 liposomes functionalized with EGF proteins for breast cancer treatment. While the cellular uptake of
30 non-targeted nanoparticles exhibited no difference among different breast cancer cell lines, EGFR-
31 targeted nanoparticles exhibited a better cellular internalization capacity according to an increasing
32 level of EGFR density in cancer cells (MDA-MB-468 > MDA-MB-231 > MCF-7, respectively). As a result,
33 a 2-fold higher tumor accumulation level in MDA-MB-468 tumors of gemcitabine was obtained and a
34 significant slower tumor growth rate of MDA-MB-468 tumors treated with EGFR-targeted NPs was
35 achieved compared to that treated with non-targeted ones (Sandoval *et al.*, 2012).

36 As the advantages of EGFR-targeted NMs compared to conventional NMs have clearly been observed
37 both *in vitro* and *in vivo* for a wide range of cancers, it is reasonable to state that EGFRs are among
38 the most appealing oncomarkers for the development of targeted nanotherapies and worthy of
39 further clinical trials.

40 **3.1.2. Overcoming multidrug resistance**

41 In addition to severe secondary effects, multidrug resistance (MDR) is another challenge imposed to
42 chemotherapy application in cancer treatment. In MDR-related processes, drugs are pumped out of
43 cancer cells before they can exert any therapeutic function. It has been reported that EGFR-

44 overexpression plays an important role in the survival of MDR-associated phenotypes (hypoxic MDR
45 tumors). To be more precise, EGFR-overexpression upregulates the hypoxia inducible factor (HIF-1 α),
46 which activates several genes related to MDR such as *mdr1* and *mrp1*. These genes subsequently
47 cause the overexpression of two essential MDR-related proteins including P-glycoprotein (P-gp) and
48 multidrug resistant protein 1 (MRP-1). The upregulation of these transmembrane drug efflux pumps
49 induces the MDR phenomenon by pumping out anticancer drugs, resulting in little or no therapeutic
50 action (Huang *et al.*, 2015). Therefore, EGFR-targeted NPs may help to overcome this problem by
51 significantly increasing drug accumulation inside MDR tumors with EGFR overexpression. On the
52 other side, the therapeutic activities of conjugated EGFR ligands on the NPs may also be helpful to
53 inhibit the production of factors such as HIF-1 α linked to MDR and provide a potential solution to
54 overcome MDR.

55 As an example, Milane *et al* used GE11-conjugated polymeric NPs (PCL, PLGA-PEG NPs) for paclitaxel
56 and lonidamine co-delivery in MDR human breast and ovarian tumor cells. The authors showed that
57 hypoxia promoted the MDR characteristics in EGFR-positive ovarian cancer (SK-OV-3 cells) and breast
58 cancer cells (MDA-MB-231 cells), as reflected by a clear increase in the IC₅₀ of paclitaxel compared to
59 those obtained from corresponding wild types. As expected, the expression of EGFRs in the hypoxic
60 SK-OV-3 and MDA-MB-231 cells was found to be significantly upregulated compared to normoxic
61 ones. As a result, a higher endocytosis level of the targeted NPs in normoxic cells (SK-OV-3 and MDA-
62 MB-231 cells) was recorded even after only 30 minutes of treatment. This phenomenon reconfirmed
63 the interest of using EGFR as a target in case of MDR. In addition, the targeted NPs loaded with the
64 two drugs demonstrated a superior pharmacokinetic profile in comparison to free drugs or non-
65 targeted NPs, resulting in 5-10% cell viability for normoxic and hypoxic cell lines, respectively. All
66 these results proved that relying on EGFR-targeted NPs is a promising strategy to actively target and
67 overcome MDR (Milane *et al.*, 2011a, 2011b). Using a similar strategy, Huang *et al* coated their
68 curcumin-loaded chitosan NPs with cetuximab and used them to treat normoxic and multidrug
69 resistant hypoxic lung cancer cells overexpressing EGFR (A549 cells). *In vitro*, free drugs were
70 ineffective against hypoxic A549 cells and lightly effective against normoxic A549 cells with an IC₅₀ of
71 40 $\mu\text{g}/\text{mL}$, whereas cetuximab-coated NPs achieved high cytotoxicity in both hypoxic and normoxic
72 A549 cells with IC₅₀ of 10 $\mu\text{g}/\text{mL}$ and 5 $\mu\text{g}/\text{mL}$, respectively. In addition, the authors showed that the
73 improvement in cytotoxicity of the targeted NPs was clearly noticeable in the case of hypoxic A549
74 cells, highlighting the efficacy of EGFR targeting in MDR hypoxic tumors (Huang *et al.*, 2015).

75 In a word, targeted nanomedicines with anti-EGFR ligands can be used as a promising strategy to
76 improve the therapeutic index of conventional chemotherapy in hypoxic MDR tumors. To better
77 exploit EGFRs, their involvement in other kinds of MDR needs to be better clarified. Besides, the
78 therapeutic activity of anti-EGFR ligands on nanomedicines in MDR cancers is another interesting
79 aspect to investigate.

80 **3.1.3. Change in the downstream signaling pathway**

81 Anti-EGFR ligands on NPs can bind to EGFRs present on cancer cells, block the binding of EGFRs on
82 cells with other EGFR ligands, suppress or disrupt the downstream signaling pathway, and lead to a
83 better therapeutic activity. As a proof of concept, a double advantage of anti-EGFR ligands on NPs
84 was highlighted in the study of Reda *et al*. On the one hand, the authors demonstrated that targeted
85 mesoporous silica NPs (MSNPs) better internalized into EGFR positive cancer cells (A549 and H460
86 cells) by a factor of eight compared to the low-expressed EGFR normal lung cell line (NL20 cells). On
87 the other hand, the grafted cetuximab on NPs could also provide a therapeutic effect by binding to
88 EGFRs on cancer cells and changing the signaling pathway. In fact, following X-ray irradiation,
89 activated EGFRs on cancer cells are normally phosphorylated and translocated to the nucleus, where

90 they play a role in mediating DNA repair. This process can reduce the efficiency of radiotherapy.
91 Therefore, if it is blocked, cancer cells will be more sensitive to radiotherapy, improving treatment
92 efficiency. On this point, it is interesting to note that conjugated cetuximab NPs were more effective
93 than free mAbs in reducing cell surface EGFR receptors despite their lower concentration. This
94 phenomenon was probably due to the higher density of grafted antibodies on NPs and resulted in
95 higher interaction mAbs-EGFRs. As a result, by analyzing the level of DNA damage, the authors found
96 that cetuximab grafted on NPs could prevent cancer cells from repairing DNA damage induced by
97 irradiation (Reda *et al.*, 2019).

98 In another study, Chen *et al* developed EGFR-targeted albumin-cisplatin NPs functionalized with anti-
99 EGFR aptamers to improve the efficacy and tolerability of cisplatin in EGFR-positive cervical cancer.
100 The results indicated that both free aptamers in solution and aptamers conjugated with
101 functionalized NPs could specifically bind to EGFR-positive cancer cells (Hela). Thanks to this specific
102 binding, targeted NPs achieved higher tumor inhibition effects with the highest cytotoxicity level (IC_{50}
103 = 26 μ M) that is twice better than that of free cisplatin (IC_{50} = 55 μ M) and that of non-targeted NPs
104 (IC_{50} = 56 μ M). Moreover, cisplatin encapsulated with targeted NPs accumulated significantly better
105 than that with non-targeted NPs and free cisplatin in tumors (1.4 and 1.75 times, respectively), and
106 accumulated less in healthy organs (1.1 and 3.9 times in liver, respectively). For example, *ex vivo* data
107 of drug accumulation in liver showed that free cisplatin accumulated more in liver by a factor of 3.5
108 and 3.9 than cisplatin conjugated with non-targeted and targeted NPs, respectively. This selective
109 tumor accumulation not only helped to enhance drug therapeutic index but also reduced systemic
110 toxicity. As cisplatin application in clinical trials is usually limited due to its high toxicity, aptamer-
111 functionalized NPs are a promising strategy to address this problem. In addition to active targeting
112 properties, the authors also insisted that EGFR-aptamers conjugated on these NPs played a vital role
113 in the antitumor effects of the finalized NPs. The conjugated anti-EGFR aptamers effectively blocked
114 EGF, induced EGFR phosphorylation, changed the signaling downstream pathway, and contributed
115 significantly to the final antitumor activities of the targeted NPs (Chen *et al.*, 2016).

116 Although the therapeutic activity of anti-EGFR ligands on nanomedicines has been proven, only
117 several kinds of ligands such as aptamers or mAbs can be applied. Moreover, their activity is highly
118 dependent on their quantity and their conformation on the NPs. Therefore, appropriate conjugation
119 strategies need to be considered in the design step (Nguyen *et al.*, 2021). Moreover, as the yield of
120 ligand conjugation is relatively low, the quantity of ligands present on NPs is far from that required to
121 have any therapeutic activity. In addition, the introduction of so many ligands onto NPs may have a
122 negative impact on colloidal properties of the finalized NPs. As a result, an equilibrium between cost-
123 benefit and efficacy-stability is a relevant issue to take into consideration.

Table 2. Applications of different kinds of EGFR-targeted nanomedicines in delivering chemotherapy or radiotherapy agents for various kinds of cancers

| Cancer | NPs | EGFR-ligands | API | IV dose (mg/kg) | Advantage of EGFR-targeted NPs compared to non-targeted NPs | Ref. |
|---------------|-----------------------|--------------|------------------------------------|-----------------|--|----------------------------------|
| Breast (TNBC) | Polymeric NPs | Peptides | Docetaxel | – | - Reduction in IC ₅₀ in MDA-MB-231 cells (5.1 ng/mL vs 30.1 ng/mL) for non-targeted NPs | (Clemons <i>et al.</i> , 2018) |
| | Polymeric NPs | EGF proteins | Paclitaxel | 2 | - At 48 h, cell viability at 10.6% vs 18.2% of non-targeted NPs - A 93-fold higher drug tumor accumulation | (Venugopal <i>et al.</i> , 2018) |
| | AuNPs | Panitumab | Radionuclides (¹⁷⁷ Lu) | – | - Higher cellular uptake into cancer cells - Cellular uptake corresponding to EGFR-expression level - Survival rate of MDA-MB-468 cells was 0.001% vs 8.4% of non-targeted NPs | (Yook <i>et al.</i> , 2015) |
| | Polymeric NPs | Peptides | Aminoflavone | 7 | - Better cellular uptake in EGFR+ cells (MDA-MB-468 cells) - No difference in cellular uptake in EGFR-cells (BT474 cells) - Stronger cytotoxicity in MDA-MB-468 + no cytotoxicity on BT474 cells - Greater tumor suppression than bare NPs or free drugs - No tissue damage. | (Brinkman <i>et al.</i> , 2016) |
| Lung (NSCLC) | AuNPs | EGF proteins | Radionuclides (¹¹¹ In) | – | - 10-fold higher cellular uptake in MDA-MB-468 than MCF-7 cells - Surviving fraction of 17.1% vs 89.8% for MDA-MB-468 and MCF-7 cells | (Song <i>et al.</i> , 2016) |
| | Polymeric NPs | Cetuximab | Gemcitabine | – | - Two-fold higher cellular internalization - Enhancement of cytotoxicity (40%) | (Wang and Zhou, 2015) |
| | Mesoporous silica NPs | Cetuximab | PLK1-siRNA | – | - Eight-fold higher cellular uptake in EGFR+ cells (A549 and H460 cells) than EGFR- ones (NL20 cells) | (Reda <i>et al.</i> , 2019) |
| | Lipid NPs | EGF proteins | Cisplatin and doxorubicin | 5 and 2.5 | - Higher tumor accumulation of drugs after 24 h than that of non-targeted or free drugs - Tumor inhibition ratio up to 74.5% with a smaller tumor volume | (Nan, 2019) |

| | | | | | | |
|---------------|---------------|---------------|-----------------------------|----------------|--|---------------------------------|
| | Chitosan NPs | Cetuximab | Paclitaxel | – | - Reduction in IC ₅₀ for A549 at 25 µg/mL vs 100 µg/mL of bare NPs - Higher cellular uptake in A549 and A431 cells than MIAPaCa-2 cells | (Maya <i>et al.</i> , 2013) |
| Pancreas | Gelatin NPs | Peptides | Gemcitabine | 5 | - Faster <i>in vitro</i> cellular internalization than non-targeted NPs, - 68% reduction in tumor growth vs 50.3% of non-targeted NPs or 14.5% of free drug | (Singh <i>et al.</i> , 2016) |
| | Micelles | Peptides | Gemcitabine and Olaparib | 5 and 50 | - Better and extensive cellular uptake capan-1 cells - Selective cellular uptake in capan-1 and not in HUVEC and H-PSC cells-- - Better tumor growth inhibition than non-targeted NPs or free drugs - No side effects for targeted NPs but severe side effects for free drugs | (Du <i>et al.</i> , 2018) |
| Colon | Micelles | Peptides | Evodiamine | 0.005 | - Reduction in IC ₅₀ of Lovo cells to 9.73 µg/mL from 19.51 for bare NPs - Greater <i>in vivo</i> anti-tumor activity than non-targeted or free drugs | (Li <i>et al.</i> , 2019) |
| Head and neck | Albumin NPs | Nanobodies | Multikinase inhibitor | – | - 40-fold higher binding affinity to 14C cells - Good inhibitory activity of cancer cells vs no activity of non-targeted NPs | (Altintas <i>et al.</i> , 2013) |
| | Liposomes | scFv | Doxorubicin and vinorelbine | 1 and 2 | - Reduction in IC ₅₀ of FaDu and Ca9-22 cells by a factor of 0.33 and 0.0028 for doxorubicin and 0.21 and 0.14 for vinorelbine, respectively - Prolonged survival time by a factor of 1.28 (72 days vs 56 days) | (Wang <i>et al.</i> , 2020) |
| Liver | Polymeric NPs | Fab fragments | Adriamycin | 1.8 | - Selective cellular uptake in EGFR-positive cells (SMMC-7721 and HepG2) but not in EGFR-negative ones (Huh7) - Higher cytotoxicity in SMMC-7721 cells - Better <i>in vivo</i> tumor accumulation and higher antitumor activity than non-targeted NPs or free drugs (p < 0.001) | (Gao <i>et al.</i> , 2014) |
| Stomach | Selenium NPs | Peptides | Oridonin | 2.5, 5 and 7.5 | - Cellular uptake according to the EGFR-expression level of cells (KYSE-150 > FLS > THP-1 cells) - Reduction of drug cytotoxicity in normal cells - <i>In vivo</i> tumor suppression activity (60%) without any body weight loss | (Pi <i>et al.</i> , 2017) |
| | Polymeric NPs | EGF proteins | Radionuclide (Ru1) | – | - Cellular uptake dependent to EGFR-expression (OE21 > OE33 = FLO-1 > HFF-1 cells) - Higher cytotoxicity of targeted NPs in OE21 cells than free drugs or non-targeted NPs | (Gill <i>et al.</i> , 2018) |

1 **3.2. Gene delivery**

2 Recently, gene therapy increasingly arouses interest in cancer therapy. Thanks to their potency and
3 their ability to silence specific oncogenes in a selective manner, interfering nucleic acids (iNAs) have
4 been widely applied to ensure targeted molecular intervention and achieve a higher level of specific
5 actions than conventional cytotoxic chemotherapy. Nevertheless, the clinical application of naked
6 iNAs is limited due to their high charge, hydrophilicity, big size, and nuclease degradation. To
7 overcome these problems, a suitable delivery system is needed to deliver these potent agents into
8 target sites. One promising approach is to nanovectorize these iNAs. Among different available
9 nanosystems, EGFR-targeted nanomedicines have demonstrated their potency in cancer treatment.

10 **3.2.1. Targeted intracellular iNAs delivery**

11 Similar to drug delivery with nanomedicines, EGFR-targeted nanomedicines have been shown to be
12 effective in protecting siRNA and are better delivered into cancer cells. Among various kinds of NPs,
13 the choice of targeting NPs with anti-EGFR ligands for gene delivery is quite limited and only certain
14 kinds of NPs have been applied including cationic polymers or liposomes, cationic polymers NPs,
15 dendriplexes and extracellular vesicles.

16 The association of iNAs with cationic polymers is normally achieved via electrostatic forces between
17 the negative charges of iNAs and the cationic charges on NPs. Several cationic polymers can be
18 applied for this purpose such as chitosan, poly-L-arginine, polyethylenimine (PEI), poly-lysine (Ben
19 Djemaa *et al.*, 2019). In a previous study, our team developed for the first time a novel EGFR-
20 targeted nanomedicine based on the complexation between EGFR-scFv fragment-decorated SPIONs,
21 siRNA and cationic polymers (chitosan and poly-L-arginine) for better gene delivery into triple
22 negative cancer cells (MDA-MB-231 cells). The results showed that by increasing the number of EGFR
23 fragments on the NPs surface, a better cellular uptake level by a factor of two can be obtained.
24 Moreover, the conjugation of EGFR scFv fragments helped increase gene silencing effects from 25.3%
25 to 69.4% in TNBC (triple negative breast cancer) cell lines (MDA-MB231 cells) (Vinh Nguyen *et al.*,
26 2020).

27 Recently, extracellular vesicles are considered emerging therapeutic carriers, especially in gene
28 delivery due to their role in intercellular communication and their ability to enter cells through
29 multiple routes that result in a long half-life time (**Table 1**). However, the lack of specificity remains
30 one of the biggest challenges for their application. To address this problem, Pi *et al* incorporated
31 EGFR-specific 2' F-RNA aptamers to extracellular vesicles to enhance targeting gene delivery into
32 EGFR-positive breast cancer cells. This targeted nanosystem exhibited successful targeted delivery of
33 surviving siRNA to breast tumor cells and inhibited the expression of the target gene at both protein
34 and mRNA levels. Moreover, targeted NPs were able to accumulate more in tumors than non-
35 targeted NPs. Consequently, with a dose of 0.5 mg of siRNA per kg of mice body weight (six doses
36 weekly), the targeted NPs significantly suppressed *in vivo* tumor growth, as monitored by the tumor
37 volume with and without NPs (tests versus controls) (Pi *et al.*, 2018).

38 **3.2.2. Reduced toxicity of iNAs-loaded dendrimers**

39 Compared to conventional cationic polymers, dendrimers are interesting materials and are
40 considered a revolution in gene delivery with nanocarriers (Dufes *et al.*, 2005). However, their
41 application is still limited due to their cytotoxicity and hemolytic properties (**Table 1**). Numerous
42 studies have revealed that this toxicity is highly related to the strong cationic charges of these
43 nanocarriers. In fact, the highly positive charge interacts strongly with the negatively charged cell
44 membranes and results in cell destabilization, cytoplasmic proteins leakage and eventual lysis

45 (Palmerston Mendes *et al.*, 2017). Hence, recent studies with dendrimers are focusing on strategies
46 to reduce or mask their charge, and surface modification with EGFR-targeting ligands may help solve
47 this problem.

48 The first example is a novel EGFR-targeted complex of EGF proteins, plasmid DNAs and PAMAM
49 dendrimers developed by Zhang *et al.* The authors employed electrostatic absorption to conjugate
50 negatively charged EGF proteins and DNAs onto their cationic dendrimers. This strategy was shown
51 to be effective in reducing the positive charge of the finalized dendrimers (from $+16.2 \pm 0.2$ mV to
52 $+2.4 \pm 0.8$ mV, respectively), which may attenuate the dendrimers toxicity. Using Renilla luciferase
53 DNAs as models, the targeted NPs exhibited higher gene transfection efficiency both *in vitro* in MCF-
54 7/EGFR and MDA-MB-231 cells and *in vivo* in MDA-MB-231 tumors, as reflected by a higher *in vivo*
55 luciferase expression level by a factor of around two. Furthermore, when non-targeted dendriplexes
56 exhibited remarkable toxicity, the cellular toxicity of the targeted dendrimers was significantly
57 decreased according to an increasing amount of EGF proteins on their surface. Firstly, this result may
58 be explained by the decrease in the charge of the targeted dendrimers. Secondly, while the only
59 possible way for non-targeted NPs to enter the cell is the non-specific pathway that may damage the
60 lipid bilayer structure and cause cell death, targeted NPs can internalize through both non-specific
61 and EGFR-specific endocytosis, which is a normal physiological process and causes less damage to the
62 cell (Zhang *et al.*, 2012).

63 In another study of Kim and colleagues, a polyanionic peptide was genetically fused to the EGFR
64 rebody that subsequently enabled the complexation of DNA, dendrimers and rebody through
65 electrostatic interactions. In this study, the authors compared the gene delivery efficiency of the
66 resulting targeted dendrimer into cell lines with different levels of EGFR expression including MDA-
67 MB-231 (high expression), HeLa (moderate expression) and HepG2 (low expression). The gene
68 delivery efficiency of the targeted NPs in cells was correlated with the EGFR expression level,
69 revealing their selective binding activity. Furthermore, a reduced cytotoxicity level related to an
70 increasing amount of EGFR rebody was observed for the targeted dendriplexes. It is likely that
71 the EGFR rebody may play a dual role as a targeting moiety and a cytotoxicity-reducing agent for
72 gene delivery with dendrimers (Kim *et al.*, 2018).

73 For gene delivery with viral vectors, EGFR-targeted NPs have been proven beneficial to improving the
74 specificity and therapeutic efficacy of gene therapy. As an example, Yoon *et al.* showed that by
75 conjugating cetuximabs onto their PEGylated dendrimers and complexing the latter with an oncolytic
76 adenovirus (Ad), the finalized formulation was efficient to enhance the delivery of this oncolytic Ad
77 into EGFR-positive tumor cells with attenuated toxicity compared to non-targeted dendrimers or
78 naked oncolytic adenovirus. Adenovirus (Ad) is a mediated cancer gene therapy that contains a linear
79 double-stranded 34-36 kb DNA genome within an icosahedral capsid, and is considered a promising
80 alternative to conventional therapy for cancer (Uusi-Kerttula *et al.*, 2015). However, its application in
81 clinical trials is limited due to its highly immunogenicity and its weakness in cellular internalization.
82 To solve this problem, the authors complexed their oncolytic Ad with cetuximab-conjugated
83 dendrimers to better deliver Ad into the tumor sites. Among three cell lines with different levels of
84 EGFR expression including EGFR-overexpressed lung cancer (A549), EGFR low-expressed breast
85 cancer (MCF-7) and normal fibroblast (HDF) cells, the EGFR-targeted complex showed a selective
86 binding capacity with the highest cellular uptake into A549 cells. Moreover, in these cancer cells, the
87 finalized formulation exhibited a cellular uptake level that was 8.6 and 6.5 times higher than those of
88 the naked Ad and the non-targeted dendrimers, respectively. The immunogenicity and hepatotoxicity
89 of the EGFR-targeted formulation were also reduced in comparison to the naked Ad (Yoon *et al.*,
90 2016).

91 **3.2.3. Combination of gene therapy and chemotherapy**

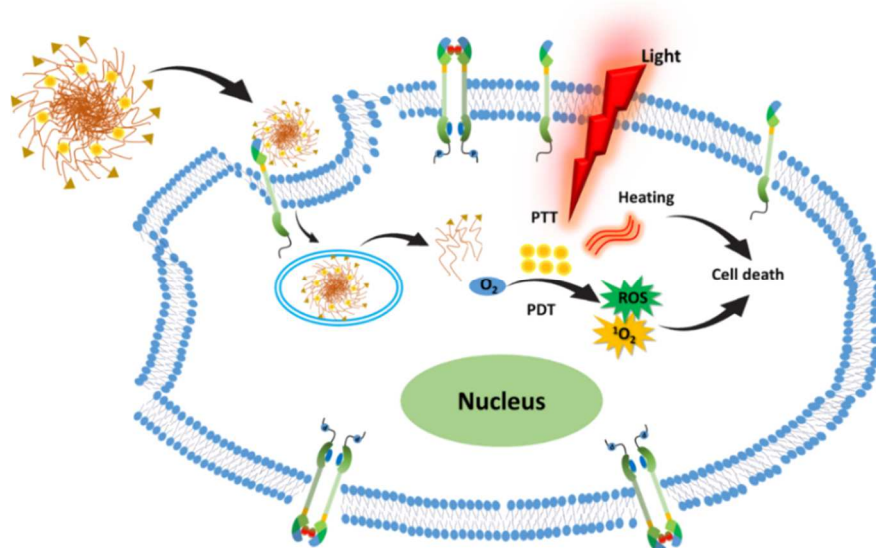
92 Drug resistance is currently a challenge to cancer treatment with chemotherapy. To address this
93 issue, higher doses of adjuvant agents are normally used, but result in more severe side effects. The
94 combination between gene therapy mediated by EGFR-targeted NMs and chemotherapy may allow
95 the use of lower but more efficient doses of anticancer drugs and effectively address this issue. To
96 this end, EGFR-targeted NMs can be used to deliver interfering nucleic acids (iNAs) in combination
97 with free drugs or to co-deliver iNAs along with drugs. As an example, Nascimento and colleagues
98 developed GE11 peptide-conjugated chitosan NPs as a versatile delivery system for silencing the
99 essential mitotic checkpoint gene Mad2 that induces cell death. The GE11-decorated NPs showed
100 efficient and targeted delivery of Mad2-siRNA in the EGFR positive A549 cell lines, resulting in a
101 better gene silencing effect than non-targeted NPs. These results were also confirmed in tumor-
102 bearing mice, as the targeted NPs were shown to have higher targeting efficiency and accumulate
103 better in tumor cells. Importantly, in cisplatin-resistant tumors, monotherapy using EGFR-targeted
104 NPs loading siRNA presented an efficient inhibitory activity on tumor growth better than that
105 involving non-targeted ones, as reflected by a delayed tumor growth rate of 51.2% vs 43%,
106 respectively, in comparison to non-treated groups. In combination with cisplatin (DDP), either EGFR-
107 targeted NPs + DDP or non-targeted NPs + DDP demonstrated a greater antitumor efficiency level
108 than that of cisplatin alone (70.6% and 58.9% in comparison to that of DDP alone, respectively).
109 Besides, the co-therapy also helped to reduce dramatically the effective dosage of cisplatin with
110 negligible toxicity in comparison with free cisplatin (Nascimento *et al.*, 2017, 2016). In another effort
111 to combine drug and gene delivery, Steinborn *et al* co-delivered methotrexate (MTX) and siRNA using
112 cationic liposomes functionalized with GE11 peptides. As expected, with the help of EGFR-targeting
113 properties from GE11 peptides, in EGFR-positive and drug-resistant cancer cell lines (KB cells), an
114 enhanced cellular internalization level and improved antitumor activities were observed for GE11-
115 modified NPs compared to free drugs or non-targeted NPs (Steinborn *et al.*, 2018). Liang *et al* used
116 GE11-binding amphiphilic peptides to construct their ultra-stable self-assembling peptide
117 nanovesicles. Anti-luciferase siRNA transfection efficiency was twice and five times higher in EGFR-
118 positive SMMC-7721 and Eca-109 cells, respectively, when GE11-conjugated nanovesicles were used
119 instead of GE11-free NPs. In contrast, no observable difference between these nanovesicles was
120 recorded for EGFR-negative cells (SGC-7901 cells). These results demonstrated that GE11 conjugation
121 helped selectively deliver siRNA into cells with high EGFR expression. Furthermore, this system can
122 be used to co-deliver doxorubicin and siRNA to EGFR-overexpressed cancer cells. Indeed, the rate of
123 tumor growth in nude mice treated with drugs/genes co-delivered with targeted NPs dramatically
124 decreased compared with that of mice treated with non-targeted NPs, whereas the free drugs or
125 genes exhibited a negligible antitumor activity (Liang *et al.*, 2016).

126 All aforementioned studies have demonstrated the great potential of NPs functionalized with EGFR
127 ligands in gene delivery. However, their application remains limited to a few kinds of NPs and a few
128 types of nucleic acids. Hence, there remains so much room for improvement as regards the
129 development of gene delivery with EGFR-targeted nanomedicines. Moreover, as the mechanism of
130 gene therapy with nanomedicines is not completely clarified, the intracellular trafficking of such NPs
131 also needs to be studied in depth to provide a new and efficient method for cancer treatment.

132 **3.3. Phototherapy**

133 In addition to traditional therapies, phototherapy has recently drawn increasing attention in cancer
134 treatment due to its remarkable benefits, especially in terms of safety compared to other methods.
135 Phototherapy application can be classified into two main branches including photodynamic and

136 photothermal therapies (**Figure 1**). EGFR-targeted nanomedicines have been applied to
137 phototherapy and demonstrated excellent results in cancer treatment.



138

139 **Figure 1. Photodynamic therapy (PDT) and photothermal therapy (PTT) with EGFR-targeted**
140 **nanomedicines in cancer treatment**

141 3.3.1. Photodynamic therapy with EGFR-targeted nanoparticles

142 Photodynamic therapy (PDT) employs light sensitive molecules, which once exposed to a specific
143 wavelength, will produce cytotoxic Reactive Oxygen Species (ROS) (Lim *et al.*, 2013). PDT is a
144 minimally invasive therapy approved for the treatment of carcinoma because photosensitizers (PS)
145 are only cytotoxic when they are activated in tumor regions. This property is its key advantage
146 compared to conventional chemotherapy. PDT efficacy has been clinically tested in various kinds of
147 cancer and was found to have limited side effects and a low risk of resistance (Master *et al.*, 2012;
148 Pawar and Prabhu, 2019a). However, the poor solubility and low tumor specificity of PS entail many
149 issues in PDT application such as risks of phototoxicity, photosensitivity and an undesirable
150 pharmacokinetic profile (Master *et al.*, 2012; Pawar and Prabhu, 2019a). One of the most promising
151 ways to overcome these challenges is targeting nanoparticles with biological ligands such as anti-
152 EGFR moieties. On the one hand, the presence of anti-EGFR ligands on NPs can help PS to better
153 internalize into cancer cells, which ensures a sufficient quantity of PS for efficient activities and
154 resolves the problem of low photosensitivity. On the other hand, the selective uptake only in cancer
155 cells and not in healthy cells can reduce the risk of phototoxicity for EGFR-targeted NPs. As an
156 example, Tsai *et al* constructed EGFR-targeted chitosan nanoparticles to deliver curcumin, a
157 photosensitizer, into gastric cancer cells. In this study, the chitosan NPs were decorated with EGF
158 proteins to target EGFR-overexpressed gastric cancer cell lines (MKN45). The cytotoxicity caused by
159 PDT with the targeted NPs was approximately four times higher than that of non-targeted NPs, with
160 IC₅₀ values at 3.4 μM and 11.9 μM, respectively. In contrast, similar IC₅₀ values (13.1 μM and 12.8 μM,
161 respectively) were observed for these NPs in EGFR-negative cells (GES), suggesting a selective uptake
162 of these targeted NPs for EGFR-positive cells. More interestingly, the authors revealed that EGFR-
163 targeted NPs exhibited a better PDT effect than folate-targeted NPs in MKN45 cells by a factor of two.
164 This could be due to different expression levels of EGFR and folate receptors in MKN45 and
165 confirmed the importance of choosing the appropriate targeting ligands to ensure treatment
166 efficiency (Tsai *et al.*, 2018). In another study, Chu *et al* developed multifunctional micelles with GE11
167 peptides to target EGFR and better deliver the photosensitizer chlorin e6 (Ce6) into colorectal cancer

168 cells. Thanks to GE11 specificity, Ce6 uptake from Ce6/GE11-micelles in EGFR-positive cancer cells
169 (HCT-116 cells) was 1.7 times higher than that from non-targeted micelles, whereas no significant
170 difference in Ce6 uptake was observed for targeted and non-targeted NPs in EGFR-negative cancer
171 cells (SW620 cells). Consequently, Ce6 released from targeted NPs exhibited a better cancer cell
172 elimination activity than its counterpart (Chu *et al.*, 2018). Liu *et al* also developed EGFR-targeted
173 polymeric micelles with anti-EGFR nanobodies as selective photodynamic therapy in head and neck
174 cancer. The targeted NPs presented not only their selectivity as their binding affinity with EGFR-
175 overexpressing A431 cells was higher than that with low-EGFR expressing HeLa cells, but also higher
176 photocytotoxicity (four times) on A431 cells compared to non-targeted micelles (Liu *et al.*, 2020).

177 For non-targeted NPs, the non-specific pathway is the only way for them to enter cancer cells. This
178 process depends on time-resolved cell membrane-mediated processes. *In vivo*, accumulated NPs via
179 the passive pathway (EPR phenomenon) can re-enter the blood stream via the diffusion mechanism
180 over time, resulting in decreased drug concentrations at tumor tissues. The active targeting that
181 highly upregulates internalization processes via specific receptor-mediated pathways can either
182 prevent the re-diffusion of drug *in vivo* or promote rapid intracellular uptake. For instance, Master
183 and colleagues utilized GE11-decorated polymeric NPs (PEG-PCL NPs) to encapsulate a PDT agent (Pc
184 4) for a better delivery, rapid intracellular uptake and enhanced PDT activity in EGFR-positive cancer
185 cells (A431). In comparison with non-targeted ones, the targeted NPs presented not only a higher but
186 also a faster intracellular uptake of drugs into cancer cells. As a result, the targeted NPs enhanced the
187 PDT-induced cytotoxicity in cancer cells within a shorter time after PDT application (10 minutes vs 5
188 hours) (Master *et al.*, 2012). This result is promising especially for further application *in vivo* and in
189 clinical trials. Currently, the interval for various current clinical formulations of PS such as Cremophor
190 is unpredictable and varies between 1-4 days. This long waiting time can hinder the treatment
191 process and reduce its efficacy especially in the cases of certain aggressive types of tumors that
192 require repeated locoregional therapy after primary treatment such as head-and-neck cancer. At this
193 point, targeted nanomedicines with anti-EGFR ligands offer an additional advantage.

194 Despite its potency and safety, PDT clinical applications are limited to the treatment of superficial
195 cancers due to the low tissue penetration ability of visible light. To address this issue, the application
196 of deep tissue penetrating near infrared (NIR) excitable upconversion NPs loaded with suitable PDT
197 agent has shown great potential in improving the penetration depth of conventional PDT. The
198 upconversion NPs are able to convert low-energy NIR light to high-energy visible or ultra-violet (UV)
199 light. The up-converted light excites subsequently the PS attached on the surface of the NPs and
200 exhibits the PDT activity. In addition, the treatment efficacy of these NPs in PDT therapy can be
201 promoted if they are decorated with targeting ligands such as anti-EGFR moieties. Lucky *et al*
202 employed this strategy for better PDT application against a non-superficial cancer (head and neck
203 cancer) by functionalizing their upconversion NPs with EGFR targeting ligands. The authors chose
204 EGFR-affibodies as targeting agents due to their small size and low immunogenicity. As a result, the
205 affibody-decorated NPs were internalized much more rapidly and efficiently (3.8 times) than the
206 unmodified NPs in EGFR-overexpressed cancer cells (A431 and H596 cells), whereas no significant
207 difference between the targeted and the non-targeted NPs was observed in EGFR-negative cells
208 (MCF-7 and HepG2 cells). In *in vivo* model, the targeted NPs exhibited higher PDT toxicity in EGFR-
209 positive cells than in EGFR-negative cells (35% vs 0%), a significant delay in tumor growth, an
210 enhanced survival rate, and long-term safety (up to 120 days) (Lucky *et al.*, 2016).

211 All these studies demonstrated that targeting nanomedicines with EGFR-targeting ligands could
212 provide efficient strategies to promote PDT application in EGFR-overexpressing cancers. Compared to
213 conventional PDT, this strategy offers outstanding advantages such as enhancement in drug cellular

214 uptake in cancer cells for a sufficient drug dose, and faster internalization resulting in a shorter drug-
215 light interval. Further studies on this application in other EGFR-positive cancers, especially non-
216 superficial ones, may offer promising methods for better treatment of these so-called “incurable”
217 diseases.

218 **3.3.2. Photothermal therapy with EGFR-targeted nanoparticles**

219 In addition to PDT, photothermal therapy (PTT) is the second type of phototherapy, which is based
220 on the use of biocompatible and special materials (PTT agents) to convert light energy into heat
221 energy, resulting in cancer cells destruction. Non-invasive and localized treatments are PTT
222 advantages compared to traditional chemotherapy. Due to minimal absorption by the human body,
223 near infrared (NIR) light (700-900 nm) is normally employed in PTT. As one of the key parameters in
224 PTT is the choice of the PTT agent, such agents need to have a good photostability region,
225 photothermal efficiency in NIR and a desirable safety profile. A variety of PTT agents are currently
226 available including carbon-nanotubes, quantum dots, graphene oxides, and metal NPs (Kwon *et al.*,
227 2014; Pawar and Prabhu, 2019b).

228 One of the most important challenges that hinders PTT clinical applications is its lack of specificity.
229 This phenomenon may cause severe side effects and reduce drug tumor accumulation, leading to
230 insufficient PTT efficiency, especially in the case of AuNPs. In fact, PTT using AuNPs is normally
231 limited due to its toxicity that originates from the long retention time of AuNPs in the body (**Table 1**).
232 AuNPs with a size superior to 40 nm can cause severe tissue damages in the liver, spleen, and kidney
233 after 28 days post intravenous injection. On the one side, the active targeting of AuNPs can attenuate
234 this phenomenon by selective delivery into tumor tissues and their reduced accumulation in healthy
235 ones. On the other side, the amount of AuNPs required to achieve efficient PTT therapeutic effects
236 can be reduced with the help of these EGFR-targeted nanomedicines. Several studies have proven
237 that this strategy may be the right solution to overcome these issues. As an example, Kwon *et al*
238 developed proteinicle/gold core/shell nanoparticles for targeted and nanotoxicity-free cancer
239 therapy. The core of NPs is made of the hepatitis B virus capsid decorated with EGFR-affibodies for
240 active targeting and dotted with many small gold NPs for PTT activity. The anti-EGFR affibodies
241 helped the NPs to be effectively delivered into EGFR-expressing breast cancer cells (MDA-MB-468
242 cells) and exhibited increased tumor cell necrosis and remarkable tumor size reduction after NIR
243 irradiation compared to AuNPs. In addition, while non-targeted AuNPs caused extensive and
244 histological damages to the liver and kidney of healthy mice, no visible macroscopic and histological
245 change was recorded in major organs in healthy mice for 3 weeks post-injection in the case of
246 targeted NPs (Kwon *et al.*, 2014). In another effort to make use of targeted PTT in glioma, Lu *et al*
247 functionalized their Fe₃O₄@Au NPs with cetuximab. In EGFR-overexpressed (U251 tumor) animal
248 cancer models, targeted NPs showed higher tumor inhibitory effects than non-targeted NPs. In
249 addition, targeted NPs did not affect the viability of cancer cells when hyperthermia was not applied.
250 As soon as hyperthermia was applied, enlarging zones of necrosis following the diffusion of targeted
251 NPs were observed, resulting in tumor growth suppression (Lu *et al.*, 2018). In another effort to
252 enhance the effectiveness of PTT in superficial bladder cancer, Chen *et al* functionalized their AuNPs
253 with anti-EGFR scFv. The targeted NPs exhibited sufficient thermal energy to kill urothelial
254 carcinomas (MBT-2 cells) both *in vitro* and *in vivo*. Under the same conditions for MBT-2 cells, the
255 NPs conjugated with EGFR-scFv were able to damage cancer cells at a 3-fold lower energy level than
256 that required for non-targeted NPs. As a result, the lower energy required by the NPs helped to
257 reduce possible toxicity related to PTT on healthy tissues *in vivo* (Chen, 2016).

258 Interestingly, a combination of PTT, chemotherapy and EGFR targeting can be achieved within a
259 single nanocarrier. He *et al* developed PLGA/polydopamine core-shell NPs for cancer photothermal

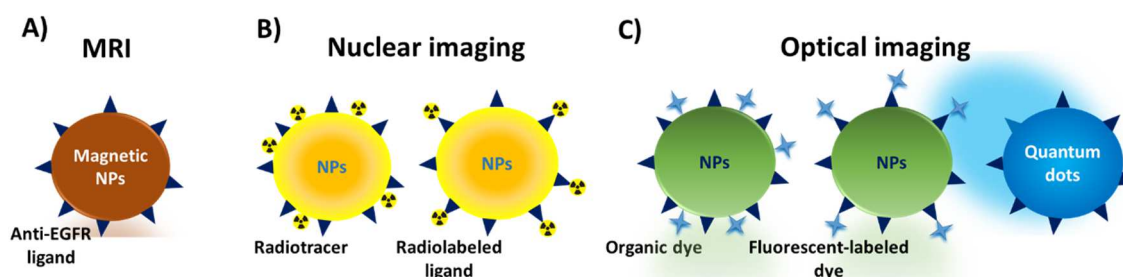
260 therapy and chemotherapy delivery. Compared to other conventional NPs such as AuNPs, the first
261 advantage of this nanosystem is its biodegradability. In addition, with the help of cetuximab as the
262 active targeting ligand, the targeted NPs could effectively internalize into head and neck cancer cells,
263 convert NIR light to heat, and trigger drug release from the PLGA core. As expected, in combination
264 with doxorubicin, targeted NPs presented a better tumor suppression rate without drug-associated
265 side effects than that of non-targeted NPs. On the other hand, the EGFR targeted NPs could quickly
266 elevate the temperature of tissues to 55 °C within 2 min and maintain this temperature during the
267 whole course of NIR irradiation (He *et al.*, 2017).

268 Although the potential of targeting nanomedicines with anti-EGFR ligands in PTT has clearly been
269 confirmed for a broad range of cancers, several critical parameters need to be carefully considered in
270 the design steps. The first problem is the photochemistry from UV wavelengths that may be
271 detrimental to the stability and recognition properties of the conjugated biomolecules. Thus,
272 protecting strategies are required to preserve the biological functions of these ligands. As an example,
273 Silva and colleagues protected the functionality of EGF proteins conjugated on their AuNPs from UV
274 wavelengths using oleic acid (OA) and hyaluronic acid (HA). The results showed that the presence of
275 polymeric-coated AuNPs helped reduce or even avoid the formation of photoproducts when EGF was
276 exposed to UV light, protecting the structure and function of EGF proteins. In addition to EGF
277 protection capacity, the HA present on NPs surface was shown to effectively provide other
278 internalization pathways than EGFR-mediated endocytosis due to its binding ability to the CD44
279 receptor (Silva *et al.*, 2016). Besides, the high temperature emitted by PTT can also have a negative
280 impact on the biological functions of anti-EGFR ligands. Especially, if NPs are not accumulated
281 enough in tumor tissues and photo-irradiation is applied, such a high temperature can deactivate the
282 targeting properties of NPs in blood stream and lead to both insufficient therapeutic activity and off-
283 site effects. Consequently, the interval time between NPs administration and photo-irradiation needs
284 to be evaluated with more accuracy.

285 4. EGFR-nanomedicines for diagnostic and theranostic applications

286 4.1. Diagnostic applications

287 In addition to perspective applications in the delivery of therapeutic agents, diagnostic tools
288 developed with EGFR-targeted NPs also provide a myriad of benefits. Thanks to its targeting delivery
289 and high loading capacity of different molecules, this strategy can enable efficient accumulation of
290 imaging agents in tumors, increase the signal-to-noise ratio and/or the intrinsic imaging functionality
291 of NPs as in the case of SPIONs, AuNPs or Quantum dots (QDs). Many studies have indicated that
292 EGFR-targeted NPs are useful not only for early detection of related cancers but also for therapy
293 response monitoring during treatment, contributing actively to the final treatment efficacy, allowing
294 better drug monitoring. Therefore, a large variety of EGFR-targeted nanomedicines have been
295 developed for Magnetic Resonance Imaging (MRI), nuclear imaging, and optical imaging used in
296 cancer (**Fig. 2**).



297

4.1.1. Targeted MRI agents

Due to its noninvasive and nondestructive nature and multidimensional tomographic capabilities, Magnetic Resonance Imaging (MRI) has emerged as one of the most powerful imaging modalities in the diagnosis and clinical staging of cancer. Due to its noninvasive and nondestructive nature and multidimensional tomographic capabilities, Magnetic Resonance Imaging (MRI) has emerged as one of the most powerful imaging modalities in the diagnosis and clinical staging of cancer. However, one of the biggest limitations of MRI is its low contrast that results in low sensitivity. To enhance the contrast in MRI, several kinds of NPs can be applied. In EGFR-targeted NPs for MRI, thanks to their innate magnetic properties, their biocompatibility and facile synthesis, superparamagnetic iron oxides NPs (SPIONs) have become the most widely used for MRI (Leary and Key, 2014) (**Table 1**). Nevertheless, for early tumor detection, MRI application with SPIONs is limited because of its low-signal sensitivity and its specificity. To address this issue, the use of EGFR-targeted NPs as contrast agents presents potential properties for improving the specificity and sensitivity of related-cancers' imaging. As an example, Tseng *et al* developed cetuximab-conjugated SPIONs for MRI in EGFR-overexpressing tumor cells. Around 31 antibody molecules were conjugated to each SPION, which helped the latter to specifically bind to EGFR-positive tumor cells (A431 cells) and enhanced the contrast on MRI in comparison to free-antibody SPIONs. In addition, antibody-dependent cell-mediated cytotoxicity of cetuximab was observed for antibody-conjugated SPIONs, resulting in a significant increase in apoptosis. These results showed a promising strategy for early detection with MRI and the treatment of EGFR-overexpressing tumor cells (Chu *et al.*, 2015). In another study, to identify patients who could benefit from EGFR-targeted therapies, Chen and colleagues also constructed cetuximab-conjugated SPIONs for their targeted MRI. While Tseng *et al* used dextran to cover their SPIONs, whose net charge remained and influenced their non-targeted internalization, Chen *et al* covered their SPIONs with a lipid coating to mask SPION charges and allow further antibody conjugation via maleimide chemistry. However, only 1.5 cetuximab molecules were conjugated per NP, much lower than the number reported in the study of Tseng *et al* (31 molecules). Despite a lower number of EGFR-targeting ligands, the targeting efficiency of the targeted SPIONs was confirmed and the targeted NPs were able to distinguish EGFR-positive cancer cells (U-251 cells) from U-87MG cells with low EGFR expression (Chen *et al.*, 2017). These results have clarified the advantage of the presence of EGFR-targeting ligands even in a small quantity on the active targeting of the finalized NPs. However, the over-presence of such ligands might also have a negative impact on the colloidal properties of the finalized NPs. Therefore, in order to have optimal efficacy, this aspect requires further investigations.

In addition to single imaging and targeting, dual imaging and double targeting are feasible with EGFR-targeted NPs. Wu *et al* carried out an interesting study in which a bispecific antibody fragment able to target both EGFRs and HER2s was developed. This fragment was constructed by connecting EGFR-scFv (from Erbitux) and HER2-scFv (from Herceptin) via a (Gly₄Ser)₃ linker. The obtained fragments were subsequently conjugated onto SPIONs, which were labeled beforehand with the NIR fluorescence dye. *In vitro* results with high HER2 and moderate EGFR expression cancer cells (SK-BR-3) showed that the dual-targeted SPIONs internalized better than the non-targeted or single-HER2 targeted SPIONs. Furthermore, the cellular uptake of the dual-targeted NPs into both HER2+/EGFR-MCF-7 cells and HER2-/EGFR+ MDA-MB-231 cells was higher compared to that of the non-targeted NPs. These results indicated that the dual-targeted NPs possess a high binding activity towards cancer cells with high HER2/EGFR expression. Remarkably, in *in vivo* MRI, the contrast enhancement of the dual-targeted NPs with SK-BR-3 cells (high HER2 and moderate EGFR expression) was 61.5

344 times higher than in HER2-/EGFR- cancer cells (Colo-205 cells) at 24 hours after IV injection at a dose
345 of 10 mg/kg (Wu *et al.*, 2016).

346 **4.1.2. Targeted nuclear imaging agents**

347 Nuclear medicine is a branch of medicine that uses radiation to provide functional and anatomical
348 data from a specific organ. The application of nuclear medicine in cancer treatment is considered the
349 most powerful technology available for early cancer detection. Several nuclear imaging techniques
350 are available for cancer imaging including Positron Emission Tomography (PET) and Single Photo
351 Emission Computed Tomography (SPECT). Currently, the application of nanotechnology in nuclear
352 medicine is drawing increasing attention and presents remarkable advantages such as its ability to
353 deliver a large quantity of imaging agents and to achieve better sensitivity imaging, along with the
354 possibility to perform multimodality imaging by delivering different kinds of imaging agents (Welch *et al.*,
355 2009). In addition to the above advantages, NPs with active targeting ligands such as EGFR can
356 enhance the selective delivery of imaging agents and result in a better diagnosis tool. Two strategies
357 can be applied to deliver nuclear imaging agents into cancer tumors including i) radiolabeling of the
358 NPs' surface and ii) radiolabeling of the active targeting ligand, and its subsequent conjugation onto
359 NPs (**Fig. 2B**).

360 Currently, the direct conjugation of imaging agents onto EGFR-targeting ligands is widely used for
361 EGFR-targeting nuclear imaging. Among the available ligands, cetuximab radiolabeled with ^{99m}Tc ,
362 ^{111}In for SPECT imaging or ^{64}Cu , ^{89}Zr , ^{86}Y for PET has been largely utilized (Eiblmaier *et al.*, 2008;
363 Menke-van der Houven van Oordt *et al.*, 2015; Nayak *et al.*, 2010). However, antibody-based imaging
364 agents confront several limitations due to their slow penetration rate into the tumor, leading to a
365 low tumor-to-blood ratio, low contrast and therefore low-sensitivity imaging. Other targeting ligands
366 such as affibodies, reprobodies or GE11 peptides may be alternatives to antibodies. As an example,
367 Burley *et al* developed a constructed ^{89}Z -EGFR affibody for targeted PET imaging in head and neck
368 cancer. PET imaging data in this study showed a clear differentiation in tumor accumulation between
369 tumors with varying EGFR expression levels, demonstrating the benefit of using anti-EGFR affibodies
370 (Burley *et al.*, 2019). In another study, Pyo and colleagues used EGFR- reprobodies labeled with ^{64}Cu
371 for PET imaging in EGFR-expressing tumors. The ^{64}Cu -reprobodies exhibited a specific uptake in EGFR-
372 positive cancer cells (H1650 cells), which was over 4 times higher than that in EGFR-negative cells
373 (SW620 cells). Moreover, these targeted complexes demonstrated a rapid uptake in EGFR-positive
374 tumors within 1h and with high tumor-to-background ratios, and therefore, were deemed potential
375 as novel EGFR imaging agents for PET (Pyo *et al.*, 2018). In another study of Paiva *et al*, the authors
376 introduced ^{64}Cu -NOTA (2,2',2''-(1,4,7-triazacyclonane-1,4,7-triyl)triacetic acid) onto the tyrosine
377 residues of GE11 peptides using azo coupling. These labeled-GE11 peptides were then added onto
378 polymeric NPs for EGFR targeting. *In vivo* analysis of ^{64}Cu -labeled polymeric NPs with PET imaging
379 demonstrated a higher accumulation level in HCT116 colon tumors for GE11-functionalized NPs than
380 non-targeted NPs. Moreover, increased tumor-to-muscle ratios for both targeted and non-targeted
381 NPs over 48h were also observed in comparison to the case of free radionuclides (Paiva *et al.*, 2020).

382 These studies have not only presented the interest of using EGFR-targeting for nuclear imaging in
383 related cancer diagnosis but also inspired scientists in the field of nanotechnology to functionalize
384 their NPs with anti-EGFR ligands for EGFR-targeting diagnosis with nuclear medicine, and more
385 importantly, for the development of multifunctional or "theranostic" NPs. As an example, Gill *et al*
386 conjugated DTPA-modified EGF proteins to their polymeric NPs for a bimodal activity including
387 nuclear imaging with $^{111}\text{Indium}$ and radiotherapy with a radiosensitizer $\text{Ru}(\text{phen})_2(\text{tpphz})^{2+}$ (Ru1). The
388 targeted NPs accumulated preferentially by EGFR-positive esophageal cancer cells (OE21 cells) and
389 exerted efficient radiotoxicity. Furthermore, this radiotoxicity was enhanced by the combinational

390 activity between ¹¹¹Indium and Ru1. Besides, substantially attenuated radiotoxicity of targeted NPs
391 for esophageal cancer cells (OE33 cells) and normal human fibroblast (HFF-1 cells) with a normal
392 EGFR level in comparison to OE21 cells confirmed the advantage of the EGFR-targeting strategy (Gill
393 *et al.*, 2018).

394 **4.1.3. Targeted optical imaging agents**

395 Optical imaging agents delivery with EGFR-targeted NPs can be achieved via three main strategies
396 including i) direct conjugation of imaging agents such as organic fluorescent dyes onto the targeted
397 NPs surface, ii) utilization of fluorescent-labeled targeting ligands, or iii) utilization of nanomaterials
398 with optical intrinsic properties (**Fig. 2C**).

399 For the first strategy, these EGFR-targeted NPs can help to distinguish EGFR-positive tumors from
400 other kinds of cancers or normal tissues and enhance the signal of loaded imaging agents. As an
401 example, in the study of Ryu *et al.*, the authors conjugated a near-infrared (NIR) fluorophore (Cy5.5)
402 and a quencher (BHQ-3) onto EGF proteins. The quencher in these nanoprobe helped to reduce
403 background signals and boost strong fluorescence intensity. The EGFR-targeted and fluorescent
404 nanoprobe exhibited a stronger *in vivo* fluorescence signal in EGFR-overexpressing tumors (MDA-
405 MB-468 cells) than that in EGFR-negative tumors (MDA-MB-436 cells). Furthermore, in comparison
406 with Cetuximab-Cy5.5, the EGF-decorated nanoprobe showed a much faster tumor accumulation
407 signal that was explained by the better tissue permeability of low-molecular weight EGF proteins
408 (Ryu *et al.*, 2013). In another study of Chariou and colleagues, virus-based NPs conjugated with GE11
409 peptides were used to carry imaging agent-Fluor 647. In different cancer cells with EGFR-
410 overexpression including A431, HT-29 and MDA-MB-231, the presence of GE11 helped to increase
411 the cellular uptake of these NPs in an EGFR-expression level-dependent way. In contrast, no
412 difference in cell uptake between targeted and non-targeted NPs was observed in EGFR-negative
413 ductal breast carcinoma (BT-474 cells) (Chariou *et al.*, 2015).

414 Despite their effectiveness, conventional labeling methods using organic fluorophores or fluorescent
415 proteins confront several restrictions such as photobleaching (photochemical degradation of the dye)
416 or low quantum yields. Therefore, NPs made of innately fluorescent materials such as quantum dots
417 (QDs) are preferred. Moreover, the possibility of surface modification allows the introduction of
418 targeting ligands such as anti-EGFR ones onto QD (**Table 1**). A good example for this phenomenon is
419 illustrated in the study of Lee *et al.* The authors conjugated cetuximab onto QDs and compared it
420 with Cy5-conjugated cetuximab. As expected, QDs-cetuximab clearly exhibited higher photostability
421 than that of Cy5-cetuximab, whose fluorescent signal decreased about 50% after 30 min. Besides,
422 cetuximab conjugated on QDs presented a high EGFR targeting property, as reflected by an increase
423 by a factor of two in fluorescence intensity in EGFR-positive A549 cells compared to EGFR-negative
424 CT26.WT cells (Lee *et al.*, 2010). Although QDs are widely used as imaging agents, they suffer from
425 the presence of cytotoxicity linked to heavy metals, photoblinking (physically on/off state of QDs)
426 and a short blood circulation time (Faucon *et al.*, 2017). Therefore, the functionalization of QDs with
427 anti-EGFR ligands is a feasible solution to overcome these problems. As an example, Zhang *et al.*
428 conjugated anti-EGFR affibodies onto Ag₂S quantum dots for photoacoustic imaging in EGFR-
429 overexpressed tumors (A431 tumors). *In vitro*, the presence of EGFR-affibodies was shown to
430 increase the cellular uptake of the obtained NPs by a factor of two in A431 cells. In A431 tumor-
431 bearing mice, after IV injection (10 mg/kg) of QDs, the targeted QDs not only presented a much
432 higher fluorescence signal than the non-targeted NPs in tumor regions but also exhibited a longer
433 half-life compared to bare QDs (3.21±0.12 h vs 11.8±1.4 min), and around 30% of the targeted NPs
434 remained in blood after 8 h of injection. This prolonged blood circulation time of QDs due to the
435 presence of anti-EGFR affibodies may be of great interest when it comes to monitoring the

436 effectiveness of cancer treatment. Moreover, the targeted QDs were shown to be accumulated
437 significantly in tumors compared to those in healthy tissues such as the kidney and lung,
438 demonstrating their biocompatibility and suitability for *in vivo* applications (Zhang *et al.*, 2018).

439 All previous studies have demonstrated the promising application of EGFR-targeted NPs in cancer
440 diagnosis that involves various kinds of diagnostic methods (Table 3). These strategies represent a
441 potential approach for the early detection of tumors, which resulted in an effective treatment plan.
442 However, as the success of these strategies is strictly dependent on the choice of targeting ligands,
443 the role of EGFR in the early stages of tumor growth needs to be clearly understood.

Table 3. Different diagnostic applications of EGFR-targeted nanomedicines in cancer

| Imaging | Type of NPs | Ligands | Type of cancer | Advantage of EGFR-targeted NPs compared to non-targeted NPs | Ref. |
|--------------|-------------------------|-----------------------|------------------------|--|--------------------------------|
| NIR | QDs | Cetuximab | Skin | - Higher photo-stability than Cy5-cetuximab - Two-fold higher fluorescence intensity in EGFR-positive cancer cells than EGFR-negative cells | (Lee <i>et al.</i> , 2010) |
| | QDs | Affibodies | Skin | - Two-fold higher cellular uptake - Higher <i>in vivo</i> fluorescence signal and longer half-life | (Zhang <i>et al.</i> , 2018) |
| | Fluorescent organic NPs | EGF proteins | Breast | - Strong asymmetric clustering of targeted NPs at the membrane of MDA-MB-468 cells | (Faucon <i>et al.</i> , 2017) |
| | Virus-based NPs | GE11 peptides | Skin, colon and breast | - Better cellular uptake into highly EGFR-expressed cells - Similar cellular uptake in low EGFR-expressed cells | (Chariou <i>et al.</i> , 2015) |
| Nuclear | Polymeric NPs | EGF proteins | Oesophagus | - Increased ¹¹¹ In cellular uptake in cancer cells - Reduced radiotoxicity of targeted NPs for normal cells | (Gill <i>et al.</i> , 2018) |
| MR | SPIONs | Cetuximab | Skin | - Specific binding to A431 cells - Enhancement in MRI contrast - Better apoptotic activity of mAbs in cancer cells | (Chu <i>et al.</i> , 2015) |
| | | Cetuximab | Glioblastoma | - Possibility to distinguish EGFR-positive cancer cells from EGFR-negative ones | (Chen <i>et al.</i> , 2017) |
| MR + optical | | Bispecific antibodies | Breast | - Higher cellular uptake in EGFR-positive cells than EGFR-negative cells - 61.5-fold better MRI contrast | (Wu <i>et al.</i> , 2016) |

1 4.2. Theranostic tools

2 The term “theranostics” was first introduced in 2002 and refers to co-delivering therapeutic agent
3 platforms (single use or combination of drugs/genes/drug + gene/ + photothermal probes/drugs +
4 photosensitizers) and imaging probes (ultrasound/magnetic contrast/fluorescent agents). These
5 multifunctional systems can deliver therapeutic agents, detect cancer cells, track the intracellular
6 localization of drugs, and monitor the efficacy of treatment. Targeting NPs with anti-EGFR ligands are
7 among the most widely used theranostic tools for cancer treatment.

8 EGFR-targeted nanoparticles for theranostic purposes have been developed and presented promising
9 results. These targeting NPs can spontaneously deliver drugs + optical imaging agents, PTT agents +
10 nuclear imaging, drugs + MRI agents, etc. As an example, Wang *et al* designed quantum dots (QDs)-
11 based polymeric micelles conjugated with anti-EGFR nanobodies and loaded with aminoflavone for
12 EGFR-overexpressing breast cancer theranostics. On the one hand, the NIR fluorescence of QDs
13 allowed *in vivo* tracking of these NPs' biodistribution. On the other hand, EGFR-nanobodies on QDs
14 helped to improve the cellular uptake (67 times higher) and the cytotoxicity of aminoflavone in EGFR-
15 positive breast cancer cells (MDA-MB-468 cells) in comparison with non-targeted NPs. Moreover, a
16 better tumor accumulation resulting in better tumor inhibition without systemic *in vivo* toxicity was
17 obtained with the targeted NPs (Wang *et al.*, 2017). In another study of Kao and colleagues, novel
18 theranostic NPs for targeted radionuclide therapy and radioimmunotherapy in lung cancer was
19 achieved with ¹³¹I-labeled immuno-cetuximab-decorated AuNPs. With the help of around 124
20 cetuximab molecules per NP, the targeted NPs demonstrated an enhancement in endocytosis and
21 cytotoxicity in high EGFR-expressed lung cancer cells (A549 cells). Furthermore, the microSPECT/CT
22 images also confirmed a better tumor accumulation for the targeted NPs compared to the non-
23 targeted ones (Kao *et al.*, 2013). Kim *et al* formulated anti-EGFR aptamers-conjugated lipid NPs for
24 siRNA and quantum dots co-delivery into triple negative breast cancer tumors. The finalized
25 aptamers-conjugated NPs were able to effectively and specifically bind to MDA-MB-231 cells,
26 whereas none of these results was observed in EGFR-negative MDA-MB-453 control cells. Moreover,
27 *in vivo* fluorescence imaging of tumor-bearing mice treated with the targeted NPs showed a higher
28 tumor accumulation and a better gene delivery for functionalized NPs than that of non-targeted NPs
29 (Kim *et al.*, 2017).

30 Besides the dual-modality, the combination of more than two therapies and diagnosis methods can
31 be achieved with EGFR-targeted nanomedicines. As an example, Liu *et al* developed multifunctional
32 cetuximab-conjugated liposomes, encapsulating doxorubicine, AuNPs and SPIONs for an efficient
33 combination of chemotherapy, photothermal therapy and MR imaging in EGFR-positive tumors. As a
34 result, efficient cetuximab-mediated targeting with a higher drug uptake in A431 cells was observed
35 for the antibody-decorated NPs compared to the non-targeted NPs. Moreover, after laser irradiation,
36 the targeted NPs exerted higher cytotoxicity towards cancer cells than doxorubicin alone, revealing a
37 synergistic effect between PTT and chemotherapy. *In vivo*, the targeted NPs promoted tumor
38 destruction by laser activation without major morphological damages to normal tissues. The
39 combination of MR and optical imaging also showed that the targeted NPs selectively targeted A431
40 tumor cells (Liu *et al.*, 2019). In another study, Li *et al* combined photodynamic therapy,
41 immunotherapy, optical and MR imaging in their novel EGFR-targeted liposomal nanohybrid
42 cerasomes. The theranostic NPs consisted of porphyrin-containing liposomes decorated with
43 cetuximab, conjugated with the NIR dye for optical detection, DOTA-Gd as MRI contrast, and
44 programmed death ligand-1 (PD-L1) as an adjuvant agent. The dual-modality imaging with the
45 targeted liposomes provided better and selective images of EGFR-positive tumors than the non-

46 targeted NPs. In addition, the antibody-decorated NPs were more effective against tumor growth
47 compared to non-targeted NPs after laser irradiation and PD-L1 immunotherapy (Li *et al.*, 2018).

48 These studies suggested a perspective approach to combine several therapies and imaging strategies
49 in just one nanosystem. Nevertheless, the introduction of so many components onto NPs may lead to
50 negative impacts on therapeutic or targeting functions of these components. Therefore, a careful
51 consideration is required to have an appropriate design of theranostic NPs.

52 **5. Conclusions**

53 It is becoming more and more evident that EGFR is a potential target for the development of
54 targeted nanomedicines in a wide range of cancers. Although such anticancer nanomedicines are still
55 in early stages of development, their potential to overcome the current issues related to adjuvant
56 treatment strategies and their innovative applications in diagnosis or even theranostics have been
57 confirmed. Especially, their perspective results in certain drug-resistant cancers such as hypoxic ones
58 provide a promising strategy of treatment and are worthy of further investigations. According to
59 different clinical needs, various kinds of nanomaterials (liposomes, dendrimers, polymeric, micelles
60 and inorganic NPs) and anti-EGFR ligands (EGF proteins, antibodies, antibodies fragments, peptides,
61 aptamers) for EGFR-targeted nanomedicines have been successfully designed and demonstrated
62 promising results both *in vitro* and *in vivo*. Nevertheless, an EGFR-targeted nanomedicine should be
63 appropriately designed to tackle several issues as follows.

64 i) the type of cancer in which such targeted nanomedicines can offer additional advantages over non-
65 targeted NPs. To be precise, EGFR-overexpression has been reported in a wide range of cancers but
66 this level of EGFR-expression may not be sufficient to bring additional benefits from active targeting
67 over the passive one. In addition, in case EGFRs are abundant in a kind of cancer, the therapeutic
68 activities of conjugated anti-EGFR ligands on NPs seem to be interesting and may improve treatment
69 efficacy. Therefore, this parameter requires deeper understanding of EGFR's involvement in each
70 kind of cancer.

71 ii) the choice of nanomaterials, EGFR-targeting moieties, their binding affinity, density, and
72 conformation on NPs are highly critical and directly affect the effectiveness of the obtained NPs.
73 With the help of advances in molecular engineering and nanotechnology, numerous types of
74 nanomaterials and targeting ligands are available, which may lead to a dilemma in the design of
75 nanomedicines. Therefore, the selection of these components should be based on the type of cancer
76 and the needs of patients in terms of the loaded drug, and possibility for cancer detection or
77 monitoring.

78 iii) even though EGFR-targeted nanomedicines have proven their improvements in terms of toxicity
79 and side effects compared to conventional NPs, they might remain in the body for a long period of
80 time. Therefore, further investigation on their long-term safety is needed before clinical trials are
81 carried out.

82 iv) the large-scale production of these nanomedicines might be costly and time-consuming. As a
83 result, these promising strategies remain in the laboratory scale. Studies on the transitional phase
84 between laboratories and the industry are needed to ensure the quality and efficacy of these
85 nanomedicines.

86 Taken altogether, targeted nanomedicines with anti-EGFR ligands are considered useful, potential
87 and multifunctional tools to improve both the diagnostic and the treatment of cancer. It is highly

88 expected that such promising nanomedicines will become commercialized and available for use in
89 clinical settings in the near future.

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