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PERSPECTIVE

Perspectives on Dual Targeting Delivery Systems for Brain Tumors

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Abstract Brain tumor remains one of the most serious threats to human beings. Different from peripheral tumors, drug delivery to brain tumor is largely restricted by the blood brain barrier (BBB). To fully conquer this barrier and specifically deliver drugs to brain tumor, dual targeting delivery systems were explored, which are functionalized with two active targeting ligands: one to the BBB and the other to the brain tumor. The development of dual targeting delivery system is still in its early stage, and attentions need to be paid to issues and concerns that remain unresolved in future studies.

Keywords Dual targeting · Brain tumor · Blood brain barrier · Drug delivery systems

Introduction

Brain tumors remain one of the most serious threats to human beings. According to the National Cancer Center of China mortality caused by brain cancer is 3.77/100,000, ranking it the 8th highest in mortality of all cancers (Chen et al. 2015a; b). In the USA, it was estimated that 15,320 people would die from brain and spinal cord tumors (Mishra and Kesharwani 2016). Unlike peripheral tumors, the most important problem in the treatment of brain tumors is a barrier which restricts drugs and imaging probes from gaining access to the tumor in the brain, making it a challenging cancer for doctors and researchers.

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The Blood brain barrier (BBB) is the most important barrier to overcome in the treatment of brain tumors (Pardridge 2002). The BBB consists of brain microvascular endothelial cells, astrocytes, microglial cells and pericytes (Begley 2004; Zhang et al. 2016a, b). This barrier has a high transendothelial electrical resistance (TEER) which prohibits the diffusion and transport of therapeutics from the blood to brain. The BBB TEER is about 1500 ~ 2000 $\Omega \cdot cm^2$, while in other vascular tissues it is only about 20 $\Omega \cdot cm^2$ (Butt et al. 1990). Additionally, efflux transporters, such as p-glycoprotein, multiple drug resistance protein 4 and breast cancer cell resistance protein, are present on the BBB. They pump drugs out of the brain which further attenuates drug concentrations (Qosa et al. 2015). As a result, only about 98 % of small molecules and nearly all of free peptides, proteins and genes cannot directly penetrate through the BBB (Pardridge 2007).

To improve drug delivery to tumors of the brain, numerous strategies have been developed. Nanoparticle (NP) based drug delivery systems are an impressive non-invasive method to deliver drugs into tumors of the brain (Gao and Jiang 2015; Wong et al. 2012). Initial studies focused on improving BBB penetration by increasing its permeability or bypassing it to facilitate drug delivery to the brain (Gao et al. 2013a, b; Mishra and Kesharwani 2016; Zhang et al. 2016a, b). During the past decade, many kinds of strategies have been developed that have utilized receptor mediated transcytosis, transporter mediated transcytosis, adsorptive mediated transcytosis, intranasal administration, and temporal opening of the BBB by ultrasound, hypertonic solutions, and vasoactive agents. Although these strategies improved drug concentrations in the brain, their lack of selective distribution or targeting in the brain has emerged as an increasing concern as untargeted drug delivery in the brain may cause serious side effects. Thus a dual targeting delivery strategy was proposed to resolve this issue. In this perspective, progress on dual

targeting delivery systems have been reviewed, problems regarding current strategies and potential research directions are discussed in this article.

Is the BBB Integrated with the Brain Tumor?

The BBB is the most important barrier to overcome for delivering drugs to brain tumors. However, the BBB in the presence of a brain tumor, especially in an advanced brain tumor, may be compromised. Several studies show that NPs could deliver drugs into brain tumors simply because of the enhanced permeability and retention (EPR) effect (Gao et al. 2014b). On the other hand, the EPR effect was much weaker than in peripheral tumors. The vasculature pore size in a brain tumor is only 7–100 nm, which is much smaller than that in peripheral tumors (Zhan and Lu 2012). The upper pore size in a RG2 glioma model was as low as 12 nm (Sarin et al. 2009). Thus transportation from the blood to the brain tumor is still difficult. Even for small molecule drugs, the distribution in brain tumors was still much lower than that in peripheral tumors. For example, the lapatinib concentration in lung metastasis is 5.15 times higher than that in brain metastasis (Taskar et al. 2012). Additionally, most of brain tumors are highly invasive (Agarwal et al. 2011), after intravenous administration of erlotinib, the concentration in the brain tumor core was 1.83 μg/g tissue, while the number in the invasive part decreased to only 0.39 μg/g (Agarwal et al. 2013). Thus the BBB is still a main barrier to overcome for brain tumor treatment. For effective delivery drugs to tumors of the brain targeting delivery systems are encouraged that have the ability to penetrate through the BBB.

The Concept of Dual Targeting Delivery

As discussed above, the BBB is still a barrier for treatment of brain tumors, especially for low grade brain tumors and the infiltrating part of high grade brain tumors. Traditionally, brain targeting delivery systems were developed to overcome the restriction of the BBB (Gao et al. 2013a, b). However, these systems were not efficient enough for brain tumor treatment because of several reasons. (1) Generally, brain tumor cells are restricted in one or several regions rather than spread throughout the brain, thus further transportation in to the brain is a critical matter when the drug delivery systems penetrate the BBB and enter brain (Gao et al. 2013a, b). The poor targeting efficiency to brain tumor cells results in low drug concentration in the brain tumor cells. (2) The diffuse distribution of drugs in the normal brain, most of which are toxic, would cause serious neurotoxicity and side effects, which is hardly tolerant for patients. (3) The poor selection between brain tumor cells and normal brain cells makes it difficult to clearly delineate the margin of the brain tumor, which is essential for surgery resection. Due to the important functions of the brain, resection of the tumor is often restricted and the residual brain tumor cells frequently lead to tumor recurrence. To overcome this problem dual targeting drug delivery strategies were proposed.

Since 2009, several groups raised the concept of dual targeting delivery. Lu's group named it "dual targeting delivery" (Du et al. 2009), Jiang's group named it "cascade targeting delivery" (Gao et al. 2011) and Li's group named it "two-order targeting delivery" (Yan et al. 2012). Although the names are different, the concept was similar and they are referred to as "dual targeting delivery" in the following discussion. Basically, the dual targeting delivery strategy is established to conquer the two barriers faced in the delivery of drugs or probes to brain tumors or other brain disorders. That means the dual targeting delivery systems should function to penetration through or bypass the BBB (first barrier) and then specifically target to brain tumor cells or other diseased cells (second barrier) (Fig. 1). However, during the development of targeting delivery systems, some systems were also named with "dual targeting delivery" when they were designed to target two sites (for example, two different kinds of cells, tumor cells and neovasculature) and even target two receptors in the same cell type (for example, the folic acid receptor and transferrin receptor on tumor cells). Thus the concept of dual targeting delivery was expanded to the strategies that could target two sites.

Application of Dual Targeting Delivery for Brain Tumor

Since 2009, there have been many studies that proposed dual targeting delivery systems to image or treat brain tumors. According to the target sites, we can generally categorize them into the following applications (Table 1).

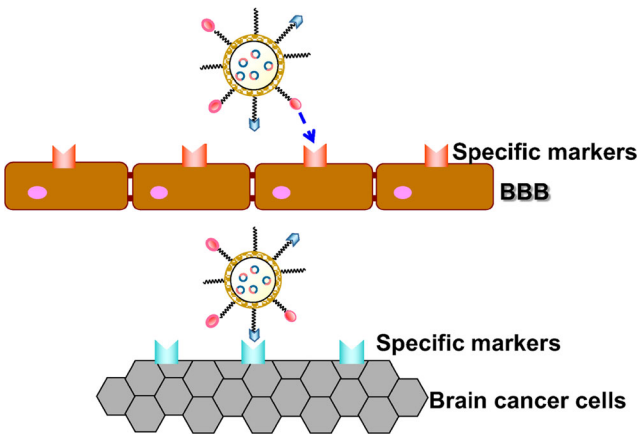

Fig. 1 Elucidation of the dual targeting delivery system for brain tumor. Normally, dual targeting delivery systems should function to penetration through or bypass the BBB (*first barrier*) and then specifically target to brain tumor cells or other diseased cells (*second barrier*)

Table 1 The different kinds of dual targeting delivery systems

Dual targeting strategy	Specific ligand	Reference
Targeting BBB and brain tumor using different ligands	TGN peptide for BBB targeting and AS1411 aptamer for brain tumor targeting	Gao et al. 2012a, b
	des-octanoyl ghrelin for BBB targeting and folate for brain tumor targeting	Chen et al. 2014
	Angiopep-2 for BBB targeting and c(RGDyK) for brain tumor targeting	Yan H, et al. 2012
Dual targeting with one ligand	Angiopep-2 targeting for LRP1	Bruun et al. 2015
		Ni et al. 2014
		Ruan et al. 2015c
		Sun et al. 2012
	Lactoferrin targeting for LRP1	Tomitaka et al. 2015
	R8-RGD for integrin	Liu et al. 2014
		Ruan et al. 2015b
	2-deoxy-D-glucose targeting for GLUT	Jiang et al. 2014
	Angiopep-2 targeting for LRP1 and activatable CPP for improving penetration	Gao et al. 2014f
	Mannose targeting for GLUT and cationic albumin targeting for BBB	Byeon et al. 2016
Target to two cell types in brain tumor	IL-13p for tumor cell targeting and RGD for neovasculature targeting	Gao et al. 2014c, d
	C(RGDyK) for endothelial cell targeting and folate for brain tumor cell targeting	Zhang et al. 2016a, b
	CK peptide that composed of a human sonic hedgehog targeting peptide and a KDR targeting peptide for brain tumor cells targeting and neovasculature targeting	Feng et al. 2015
	Epidermal growth factor and transferrin	Dixit et al. 2015

Targeting the BBB and Brain Tumor Using Different Ligands

As discussed above, the initial concept of dual targeting delivery was penetration through the BBB and then targeting to the brain tumor. To address this requirement, two ligands were often used for constructing targeting delivery systems, one for penetration of the BBB and the other for the brain tumor. TGN peptide (TGNYKALHPHNG) was selected by in vivo phage display from a 12-mer peptide library which showed high BBB targeting efficiency (Li et al. 2011). AS1411 aptamer is a brain tumor targeting ligand as the receptor of AS1411 aptamer, nucleon, is overexpressed on brain tumor cells (Bates et al. 2009), and AS1411 aptamer functionalized NPs showed impressive brain tumor targeting efficiency (Guo et al. 2011). So we functionalized these two ligands onto NPs for BBB and brain tumor dual targeting drug delivery (Gao et al. 2012a, b). In vitro, the constructed dual targeting delivery system, AsTNPs, could firstly penetrate through the BBB model and then be taken up by C6 cells (a brain tumor cell line), with a significantly higher penetration ratio and uptake intensity than the single ligand modified NPs. Furthermore, in an in vitro bEnd.3 monolayer and C6 cell coculture system (mimics the in vivo brain tumor microenvironment), the docetaxel loaded AsTNPs induced a high percentage of apoptosis C6 cells (Gao et al. 2014e). In vivo, the accumulation of AsTNPs in the brain tumor was 4.91-fold higher than that of unmodified NPs (Fig. 2). Then the intensity ratio of glioma to normal brain (T/N ratio) was introduced to evaluate glioma selective distribution. Although TGN modification could enhance glioma intensity contributed by BBB penetratiB11on, the T/N ratio of TGN modified NPs was only 1.2, which was even lower than unmodified NPs (T/N ratio was 1.6). In contrast, T/N ratio of AsTNPs was as high as 2.6, suggesting dual modification could not only improve the accumulation in the brain tumor but also the selectivity in the brain. Consequently, low dose of docetaxel loaded AsTNPs could effectively prolong the median survival time of brain tumor bearing mice from 17 days to 32 days, while at this dose, the unmodified NPs and free docetaxel showed no therapeutic effect. This study clearly demonstrated the superiority of dual targeting drug delivery systems to traditional brain targeting drug delivery systems.

Des-octanoyl ghrelin is a 28-mer peptide that mediates vector transportation through the BBB mediated by des-octanoyl ghrelin binding sites (Banks et al. 2002), while folate binds the folate receptor, which highly expressed on tumor cells (Weitman et al. 1992). Chen et al. modified the des-octanoyl ghrelin and folate onto polymersomes for doxorubicin delivery (Chen et al. 2014). In an in vitro BBB model and C6 brain tumor cell co-culture system, the dual targeting delivery system showed good C6 cell uptake after penetrating through the BBB model, which was about 2 times higher than that of folate modified polymersomes. In the brain tumor, the dual targeting delivery system achieved 3.7 fold higher doxorubicin concentration than that of unmodified polymersomes,

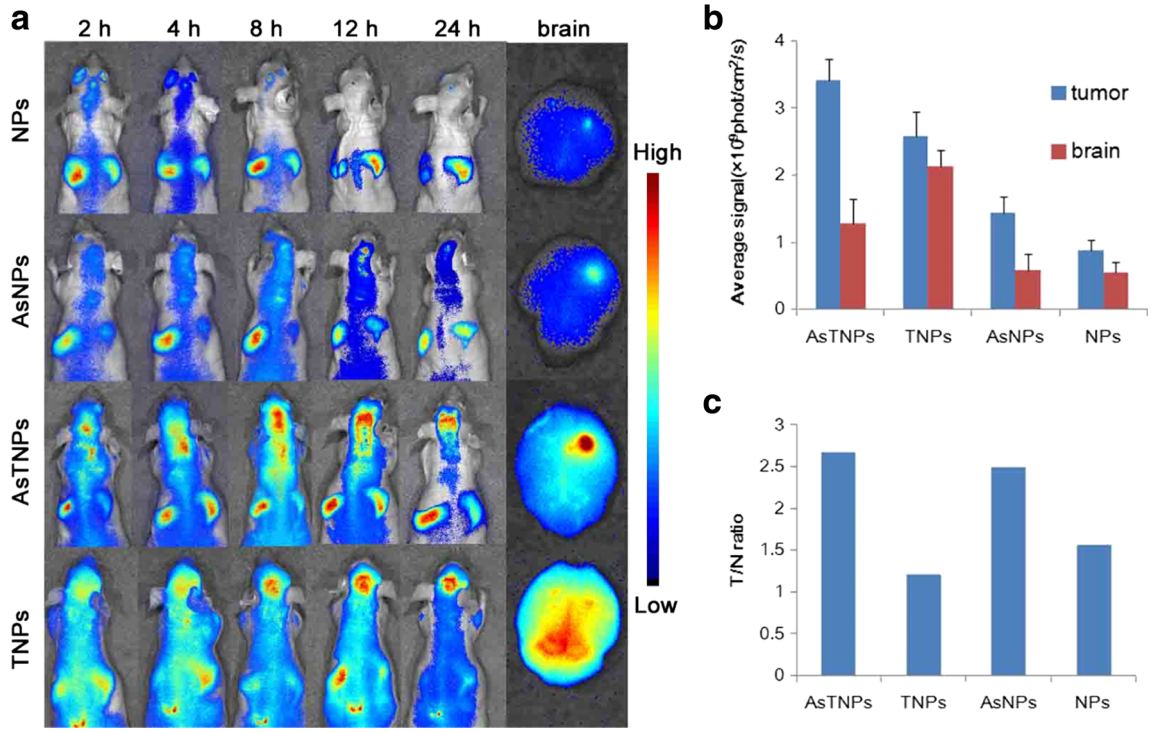

Fig. 2 **a** The in vivo imaging of DiR-loaded NPs, AS1411 conjugated NPs (AsNPs), AsTNPs and TGN modified NPs (TNP) in the brain glioma bearing nude mice at several time points with ex vivo imaging of the brain at 24 h. **b** Brain and glioma fluorescent intensity at 24 h. **c** The T/N ratio of the brains 24 h after treatment with different formulations. Reprinted from ref.(Gao et al. 2012a, b) with permission of the copyright holder, Elsevier, Amsterdam

while the concentration in normal brain of dual modified polymersomes was only 1.2 fold higher than that of unmodified polymersomes, suggesting the dual modification improve both brain tumor targeting and T/N ratio.

Dual Targeting with one Ligand

There are some ligands that could directly target both the BBB and brain tumor due to their receptors or transporters that are overexpressed on both the BBB and brain tumor cells, such as low-density lipoprotein receptor-related protein (LRP) and transferrin (Tf) receptor (TfR) (Ito et al. 2006; Li and Qian 2002; Maletinska et al. 2000). Thus, these ligands could serve as dual targeting for both BBB penetration and brain tumor targeting.

Angiopep-2 (TFFYGGSRGKRNNFKTEEY) belongs to a peptide family called angiopep, which was derived from the Kunitz domain of aprotinin (Demeule et al. 2008). Angiopep-2 has high LRP binding affinity, thus it was a candidate for a dual targeting ligand for NPs to penetrate the BBB and target the brain tumor. Our group conjugated angiopep-2 onto gold NPs (AuNPs) to construct a dual targeting delivery system (Ruan et al. 2015c). The model drug, DOX, was anchored onto the AuNPs surface through hydrazone, a pH sensitive linker, which enabled the system to release drug into the tumor and tumor cells in the acidic environment of the tumor. Due to the hydrazone between AuNPs and DOX, the release of DOX was pH sensitive. The 48 h cumulative release in pH 7.4 was 21.9 %, while in pH 5.0 it was elevated to 88.3 %. Combining the BBB and brain tumor targeting of anigopep-2 and tumor specific release of DOX, DOX loaded angiopep-2 modified AuNPs significantly prolonged the medium survival time of brain tumor bearing mice from 19 days to 55 days.

The angiopep-2 modification also elevated gene expression in brain when using dendrimer and liposomes as gene delivery carriers (Ke et al. 2009; Sun et al. 2012). Sun et al. utilized cationic liposomes as carriers for co-loading tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) genes and paclitaxel decorated with angiopep-2 for BBB and brain tumor dual targeting delivery (Sun et al. 2012). The gene expression of TRAIL loaded angiopep-2 modified cationic liposomes in U87 brain tumor cells was considerably higher than that of unmodified cationic liposomes. As a result, the co-loading of angiopep-2 modified cationic liposomes induced 81.99 % apoptosis after crossing the bEnd.3 monolayer, while unmodified cationic liposomes was only 60.51 %. The cell apoptosis induction efficiency of the co-loading system was also significantly better than that of the angiopep-2 modified cationic liposomes that were only loaded with a TRAIL gene (40.12 %) or paclitaxel (58.67 %). In vivo, the co-loading dual targeting delivery system greatly prolonged the median survival time of brain tumor bearing mice from 23 days to 69.5 days, which was higher than the temozolomide positive control group (47 days).

Recently, several studies have used angiopep-2 as a dual targeting ligand to successfully deliver imaging probes to brain tumors. Ni et al. modified angiopep-2 onto upconversion NPs that were loaded with gadolinium for magnetic resonance imaging (MRI) and fluorescence imaging (Ni et al. 2014). Results showed this dual targeting system provided better contrast for brain imaging than the commercial MRI contrast (Gd-DTPA) and fluorescence probe (five-aminolevulinic acid). Our group also used angiopep-2 modified carbonaceous dots for delivering doxorubicin to brain tumors, which showed a better delivery efficiency than unmodified carbonaceous dots (Chen et al. 2015a, b).

Tandem peptides may display higher BBB penetration and brain tumor targeting effects. RGD is an extensively evaluated targeting ligand that has high affinity with integrin $\alpha_v\beta_3$, which is overexpressed on endothelial cells and tumor cells (Barczyk et al. 2010; Schottelius et al. 2009). However, receptor mediate endocytosis and transcytosis are lead to low transmembrane efficiency and easy to saturation. Thus conjugating the specific ligand with a cell penetrating peptide (CPP) may be a useful strategy. Liu et al. conjugated c(RGDyK) with octa-arginine to form the tandem peptide R8-RGD (Liu et al. 2014). The cellular uptake of R8-RGD modified liposomes by bEnd.3 cells and C6 cells was 30-times higher than that of RGD modified liposomes. More importantly, the penetration ratio through the bEnd.3 monolayer of R8-RGD modified liposomes was higher (around 100-times) than that of RGD modified liposomes. Consequently, the R8-RGD modified liposomes showed higher location in the tumor site of brain tumor bearing mice. After loading with paclitaxel, the median survival time prolonged from 26 days to 48 days, which was significantly longer than those treated with paclitaxel loaded RGD modified liposomes (38 days). To further improve homogenous tumor distribution, our group established a size shrinkable system: gelatin NPs fabricated with AuNPs (AuNPs-GNPs) (Ruan et al. 2015a, d). The size of the system decreases from 186.5 nm to 59.30 nm under the degradation by matrix metalloproteinase-2 (MMP-2) (Ruan et al. 2015a) which is overexpressed in many kinds of tumors (Forsyth et al. 1999). The reduced size promotes the intratumor penetration of the drug delivery systems, enabling homogenous distribution in the tumor. We decorated R8-RGD onto AuNPs-GNPs for brain tumor targeting imaging and doxorubicin delivery (Ruan et al. 2015b). The R8-RGD modified AuNPs-GNPs showed higher localization in the brain tumor than the unmodified AuNPs-GNPs. The T/N ratio of R8-RGD modified AuNPs-GNPs was as high as 2.3, while the ratio of unmodified AuNPs-GNPs was only about 1.2. Additionally, doxorubicin showed homogenous and high distribution in the entire brain tumor as demonstrated by confocal fluorescent imaging of brain slices.

Besides receptors, there are some nutrient transporters overexpressed on both the BBB and brain tumor cells due to the enhanced requirement of nutrients for growth of the brain tumor. D-Glucose transporter protein (GLUT) shows a particularly high concentration in brain microvessels, which is about 100-fold higher than the transferrin receptor (Wiley et al. 2013). P-aminophenyl-α-D-mannopyranoside, a substrate of GLUT1 and GLUT3, improved brain accumulation of liposomes (Du et al. 2014). Additionally, GLUT is also overexpressed on brain tumor cells (Gorin et al. 2004). Thus, Jiang et al. utilized 2-deoxy-D-glucose, a substrate of GLUT, as dual targeting ligand for brain tumor targeting delivery (Jiang et al. 2014). The cellular uptake of 2-deoxy-D-glucose modified NPs by RG2 brain tumor cells was considerably higher than that of unmodified NPs. At the same time, the transportation ratio of modified NPs across the bEnd.3 monolayer after 24 h incubation was about 17 %, which was much higher than that of unmodified NPs (only about 6 %). The enhanced brain tumor cell uptake and bEnd.3 monolayer transportation was significantly attenuated by the addition of 2-deoxy-D-glucose, demonstrating that 2-deoxy-D-glucose was critical for targeting delivery. In vivo, the brain tumor accumulation of 2-deoxy-D-glucose modified NPs was considerably higher than that of unmodified NPs, resulting in greatly longer median survival time (38 days) of brain tumor bearing mice compared with that of control group (29 days).

Combining the dual targeting ligand with CPP may further improve the distribution in brain tumors. Our group dual functionalized NPs with angiopep-2 and activatable CPP for brain tumor targeting delivery of docetaxel (Gao et al. 2014f; Mei et al. 2014). Angiopep-2 mediated BBB penetration and brain tumor targeting. At the site of the brain tumor, CPP was activated by MMP-2 in tumor and then mediated tumor penetration, resulting in higher tumor cell internalization and deeper tumor penetration (Gao et al. 2014f).

Similarly, it is also useful to combine an active targeting ligand with cationic proteins as cationic proteins target to the brain through adsorptive mediated endocytosis (Lu et al. 2007). Byeon et al. synthesized NPs using cationic albumin and mannose modified albumin (c/m-HSA NPs) to target the BBB and brain tumor through both absorptive mediated endocytosis and GLUT mediated endocytosis (Byeon et al. 2016). The c/m-HSA NPs showed the most prominent performance in transport across the bEnd.3 monolayer and uptake in U87 brain tumor cells and spheroids. The half inhibition concentration (IC_{50}) of doxorubicin loaded c/m-HSA NPs was 2.2–15.6-fold lower than those of doxorubicin or other albumin NPs. In vivo, the accumulation of c/n-HSA-NPs in the brain tumor was higher than all other groups (Fig. 3).

Targeting two Cell Types in the Brain Tumor

Since there are many types of cells in a tumor, inhibiting the proliferation of single types of cells may not be sufficient enough for tumor treatment. For example, anti-angiogenesis treatment could inhibit tumor blood vessel growth thus preventing the tumor from accessing nutrients and inhibiting

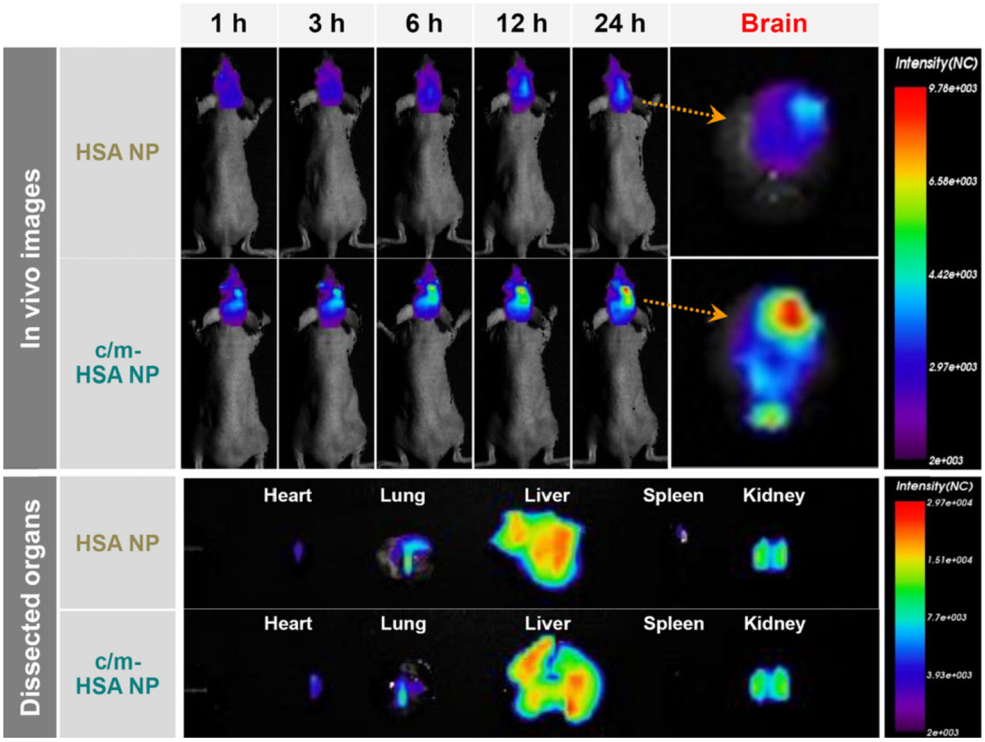

Fig. 3 Biodistribution of HSA NP and c/m-HSA NP in orthotopic glioma-bearing mice until 24 h after intravenous injection (*left, upper*) and ex vivo image of the brains excised at 24 h (right, upper). Ex vivo images of other major organs at 24 h (*lower*). Reprinted from ref. (Byeon et al. 2016) with permission of the copyright holder, Elsevier, Amsterdam

tumor growth. However, it also increased tumor invasiveness and resistance to chemotherapy (Sengupta et al. 2005). Thus combining anti-angiogenesis with anti-tumor treatment may improve treatment outcomes (Sengupta et al. 2005). Our lab modified NPs with RGD and IL-13p peptide to target both brain tumor neovessels and brain tumor cells as αvβ3 (receptor of RGD) is overexpressed on neoendothelial cells and IL13Rα2 (receptor of IL-13p) is overexpressed on brain tumor cells (Gao et al. 2014c,d; Mintz et al. 2002). In an endothelial cell and brain tumor cell co-culture model, RGD modification selectively elevated the uptake of NPs by endothelial cells, IL-13p modification selectively elevated the uptake by brain tumor cells while dual modification could enhance the uptake by both cells (Gao et al. 2014c). In vivo immunofluorescent imaging also demonstrated the specific targeting ability of RGD and IL-13p (Fig. 4). RGD and IL-13p dual modified NPs could deliver docetaxel to both neovasculature and tumor cells of a brain tumor and showed a better anti-brain tumor effect than the single ligand modified NPs. These data suggest this as a promising strategy to target more than one cell type in a tumor.

Zhang et al. used c(RGDyK) and folate for targeting endothelial cells and tumor cells respectively (Zhang et al. 2016a, b). The in vivo fluorescence imaging demonstrated the concentration of dual modification iron oxide NPs in brain was much higher than other NPs. These studies demonstrated the superiority of dual targeting to more than one cell type in brain tumors. Tandem peptides could be used for this purpose. CK peptide is composed of a human sonic hedgehog targeting peptide and a KDR targeting peptide, while human sonic hedgehog signaling pathway is aberrantly activated in tumor cells and KDR receptor overexpressed in tumor neovasculature. The CK modified NPs showed a higher affinity with U87 brain tumor cells and HUVEC cells than the unmodified NPs (Feng et al. 2015). As a result, the treatment with paclitaxel loaded CK modified NPs achieved the longest median survival time of brain tumor bearing mice.

Problems and Perspectives of Dual Targeting Delivery Systems

Although dual targeting delivery systems considerably improved the delivery efficiency of drugs or probes to brain tumors, a number of issues remain to be elucidated.

Linker Chemistry

Normally, polyethylene glycol (PEG) or other polymers are used to link the ligands onto NPs. The linker could not only decrease serum protein adsorption and reticuloendothelial system (RES) recognition but also improve the elasticity of the ligand, which is important for interaction between ligand and receptor and the further internalization (Kolate et al. 2014; Larson et al. 2012; Owens and Peppas 2006). Cruz et al. conjugated antibodies onto NPs through PEG of various length (2000 ~ 20,000) to determine the influence of PEG length on the cell targeting effect of the particles (Cruz et al.

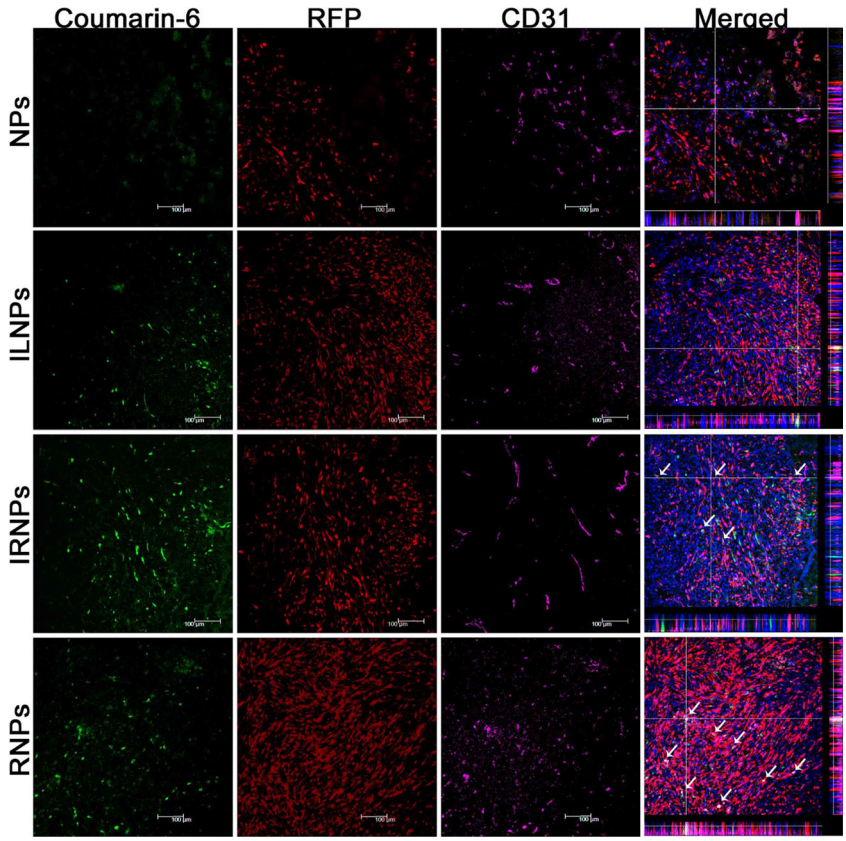

Fig. 4 Distribution of various particles in brain tumors and colocalization with microvessels (indicated by white arrows) 2 h after injection. Nuclei were stained by DAPI, particles were marked with coumarin-6, brain tumor cells were RFP-C6, and microvessels were stained by CD31antibody. The bar represents 100 μm. IRNPs: Il-13p and RGD dual modified NPs; RNPs: RGD modified NPs, ILNPs: IL-13p modified NPs. Reprinted from ref. (Gao et al. 2014c) with permission of the copyright holder, American Chemical Society

2011). The antibody conjugated NPs with PEG length of 3000 showed higher cell binding efficiency and uptake intensity compared with that of 2000, 6000, 10,000 and 20,000. Although this study was not designed for brain tumor targeting, it demonstrates the important effect of PEG linker on targeting delivery efficiency. Thus it is important to optimize the linker length when constructing a brain tumor targeting delivery system.

The length of linkers for the two ligands also should be taken into consideration because one ligand may influence the interaction of another ligand with its receptor. In some studies, linkers with the same length were used for modification of two ligands. For example, our studies used PEG3400 for the conjugation of both ligands (Gao et al. 2014d), Chen's study used PEG with molecular weight around 2000 for the modification of des-octanoyl ghrelin and folate (Chen et al. 2014). But other studies used linkers with different lengths. Li's study used tocopheryl polyethylene glycol 1000 succinate (TPGS1000) for conjugation of mannose and polyethylene glycol 2000-distearoyl phosphatidyl ethanolamine (PEG2000-DSPE) for the conjugation of dequalinium (Li et al. 2014). Although the different lengths of PEG or other linkers were used, there are few studies that carefully evaluate the best length of linkers for dual ligands modification. The dual targeting delivery systems, especially dual ligand modification for different functions, should use different linker lengths. For the BBB targeting ligand, the linker should be longer to enable the ligand to effectively interact with the BBB while the linker for the brain tumor targeting ligand should be shorter to minimize disturbing the interaction between the BBB targeting ligand and the BBB. However, if the ligand has more than one function, the proper length would be changed.

Ligand Density, Orientation and Ratio between two Ligands

The ligand density could also influence the targeting efficiency of drug delivery systems. Although higher ligand density showed higher avidity and selectivity with target cells through multivalent binding (Choi et al. 2010), increasing the ligand density could also inhibit the disassociation from cells, resulting in decreased transcytosis and reduced accumulation in brain parenchyma (Wiley et al. 2013). However, too low density of the ligand made the particles unable to engage the BBB, resulting in low brain targeting delivery. Thus a certain density is required for brain targeting delivery.

The ligand orientation would also affect the specific interaction efficiency between ligand and receptor. If the ligand binds to the surface of NPs through hydrophobic residues, the targeting efficiency may be attenuated and even lost (Lee and Ytreberg 2012). To keep the ligand in a preferred orientation, several methods were developed, including conjugation

through affinity tags into the targeting protein sequence, oriented functionalization onto NPs driven by recombinant protein linkers and using site-specific chemo-selective reaction (Mazzucchelli et al. 2013). In constructing a brain tumor targeting delivery system, site-specific reactions are widely used, such as the reaction of maleimide with thiol on the cysteine, the reaction of carboxyl with amino on the end of the ligand and the click chemistry between azide and alkyne (Avvakumova et al. 2014). However, detailed evaluation of the orientation influence has not been evaluated on brain targeting delivery.

Additionally, the ligand could also elevate the serum protein adsorption and RES recognition after intravenous injection (Gao and He 2014). The interaction with NPs with serum proteins could significantly change the in vivo behavior of NPs (Mahmoudi et al. 2015). Several studies showed the specific interaction between ligand and receptors was considerably attenuated after protein adsorption (Salvati et al. 2013; Xu et al. 2016). Thus there was a balance density to maximum the targeting effect and minimize protein adsorption and RES clearance. Some studies have optimized the density. For example, Pang et al. compared the OX26 conjugated polymersomes with the density of 0, 5, 34 or 92 (number of OX26 molecule per particle) (Pang et al. 2008). Increasing OX26 density led to shorter blood circulation time. As a result, OX26 conjugated polymersomes with the density of 34 not 92 displayed best brain targeting delivery. Thus the density should be optimized for a given ligand because there are no general rules that could tell the best ligand density. Except the ligand density, the density ratio between two ligands may also affect the targeting delivery efficiency of the dual targeting systems. However, no study has been reported to evaluate this concern.

Ligand Function in Transcytosis

For most active targeting systems, active targeting was mediated by specific interactions between ligand and receptor and further internalization. However, for BBB targeting, the dual targeting drug delivery systems should be internalized into brain endothelial cells from the blood side and then excreted to the brain side. Unfortunately, the high affinity between ligand and receptor/transporter increased the transportation of NPs from blood but inhibited the disassociation from the cell membrane and thus decreased the transcytosis efficiency. Some studies reported reducing the affinity of anti-TfR antibody with TfR led to more antibody entering the brain because the high affinity anti-TfR antibody trafficked more to lysosomes of BBB endothelial cells (Bien-Ly et al. 2014; Yu et al. 2011). To solve this issue, Clark et al. conjugated Tf with AuNPs (Tf-AuNPs) through acid cleavable linker (Clark and Davis 2015). When the Tf-AuNPs bound with the Tf receptor and were internalized into cells, the Tf was detached and released AuNPs into the brain due to the acidic condition in the endosomes. After 8 h incubation of Tf-AuNPs with bEnd.3 monolayers, the transportation ratio of Tf-AuNPs with the acidic cleavable linker was 10-fold higher than that of Tf-AuNPs with an uncleavable linker. In vivo, the acidic cleavable linker also considerably increased the brain delivery of Tf-AuNPs. This concern would also affect the efficiency of dual targeting delivery systems, which should be taken into consideration in the future studies.

The recycling of receptors may further attenuate the transcytosis efficiency. For example, apolipoprotein E (ApoE) is a ligand of LRP, thus ApoE could be used for brain targeting delivery. However, a study showed approximately 30 % of the internalized ApoE was recycled after 4 h (Farkas et al. 2004). Therefore, for effective transcytosis, ligand modified NPs should detach from the receptors after internalization or cleave between the ligand and NPs to release NPs, which was discussed above. However, for single ligand modified NPs that are designed to target both the BBB and brain tumor cells, the only applicable method is releasing the intact ligand modified NPs from the receptors, which requires careful optimization of the affinity of ligand with receptors.

Particle Size and Shape

The properties of NPs greatly influence the targeting delivery efficiency. In tumors, NPs with larger size showed poorer penetration ability. Compared to NPs 60 nm and 125 nm, the NPs with 12 nm could penetrate into the area that is 80 μm away from the blood vessels, while the NPs with 125 nm could only locate around the vessel (Popovic et al. 2010). On the other hand, the small size NPs tend to be quickly excreted from the tumor, resulting in a low retention efficiency (Kibria et al. 2013). Thus there were several studies constructed using size-changeable systems to increase both the tumor retention and penetration (Hu et al. 2015; Wong et al. 2011).

The size also affects the BBB targeting and penetrating efficiency. Shilo et al. evaluated the uptake of 20 ~ 110 nm NPs by bEnd.3 cells. 70 nm NPs were taken up by most of the cells (Shilo et al. 2015). Gao and Jiang evaluated the brain delivery efficiency of polysorbate 80 coated NPs with sizes of 70, 170, 220 and 345 nm (Gao and Jiang 2006). The NPs with a size lower than 100 nm showed the highest concentration in the brain. Thus using nanoparticles with a size smaller than 100 nm may be the most efficient for brain targeting delivery. The nanoparticle shape can also influence the interaction between nanoparticles and the membrane and the resulting internalization. Compared with spherical NPs, rod-shaped NPs showed a higher specific and lower nonspecific accumulation using in vitro microfluidic systems (Kolhar et al. 2013). In vivo, the vascular targeting NPs with a rod shape displayed a higher accumulation in the lung and brain than that of spherical NPs. Peiris et al. established chain-like NPs, which showed much higher brain tumor accumulation (Peiris et al. 2015). Compared with traditional chemotherapy, the chain-

like NPs delivered 18.6-fold greater drug to the brain tumor, resulting in considerably better anti-tumor effect. Except the size and shape, many other properties have the ability of affecting the interaction between a targeting delivery system and cells, for example, rigidity of nanoparticles, surface charge, the length, rigidity and water solubility of the ligands (Ding and Ma 2012; Huang et al. 2016; Mahmoudi et al. 2015; Valencia et al. 2011). However, researchers don't pay enough attention on these aspects when constructing dual targeting delivery systems.

Conclusion and Perspectives

In summary, several dual targeting delivery systems have been developed to achieve brain tumor targeting. In general, such systems are designed to penetrate the BBB while achieving specific delivery to brain tumor cells or other stroma cells in brain tumors. The dual targeting delivery systems are proven to deliver more drugs to brain tumors thus leading to improved anti-tumor effect in vivo. In future studies, more efforts should be dedicated to elucidate the impact of various parameters such as linker length, ligand density and ratio, particle size and shape on the delivery efficiency of dual drug targeting delivery systems.

Although the dual targeting delivery systems are still in the infant stage, clinical translation of such delivery systems appears a general trend. However, a number of issues and concerns need to be well addressed before translational studies. Safety is a major concern for clinical use, and thus, biodegradable NPs are recommended, such as liposomes, albumin based NPs and polylactic acid (PLA) based NPs (Weissig et al. 2014). The preparation procedure is another concern, because methods used in laboratory, especially for ligand modification, are not suitable for large-scale production. Conjugation ligands with materials before NPs preparation is suggested, and several candidate nanomedicines using this method are currently under clinical evaluation (van der Meel et al. 2013).

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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