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Liposomal nanoparticle armed with bivalent bispecific single-domain antibodies, novel weapon in HER2 positive cancerous cell lines targeting

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ABSTRACT

Breast cancer is the leading cause of mortality among all cancers. HER2, human epidermal growth factor receptors type 2, a receptor tyrosine kinase that induces interminable cell proliferation, is overexpressed in 20–25 percent of breast cancers. In spite of significant progress in nanomedicine in the past decade, being subjected to genetic drift that hides many paramount epitopes has rendered targeting HER2 as a big challenge. In the present study, we developed monovalent and bivalent monospecific along with bivalent bispecific VHH targeting different epitopes on HER2, and showed that bivalent bispecific VHH has the highest affinity among other tested modalities. Then we covalently coupled VHHs to the fluorescent labeled liposomal nanoparticle to produce targeted liposomes. Based on flow cytometry results, bivalent bispecific VHH targeted liposomes showed the highest fluorescent intensity, on HER2 breast cancer cells. Liposomes conjugated to bivalent monospecific VHH exhibited enhanced affinity toward HER2 positive cell lines compared to monovalent targeted liposomes, with bivalent bispecific liposomes appearing as the most robust probe.

1. Introduction

Growth made in cancer research has led to the development of a wide range of novel anticancer agents. Despite positive results in vitro, these agents have some adverse effects in vivo, such as reduced efficacy, low solubility, off-target effects and significant toxicity. To address these pharmacological limitations, several nano-delivery systems have been designed and developed for a safe, efficient and controlled delivery and release of therapeutic agents (Chang and Yeh, 2012). Nanosystems have some theoretical advantages over conventional low molecular weight agents, including large loading capacity, protection of the payload from degradation, specific delivery and controlled or sustained release (Nakamura et al., 2016). Among different types of nanocarriers, only liposomes have FDA approval. These nanoparticles are similar to natural cell membrane regarding their components and structure. Typically, 70% of eukaryotic cells are made up of phosphatidylethanolamine. Cholesterol and other phospholipids are other constituents of liposomes (Khaleghi et al., 2016). Liposomes are small, spherical, and enclosed compartments separating an aqueous medium from another by the phospholipid bilayer. Because of their innate properties, many of drugs and molecules, including anticancer and antimicrobial agents, chelating agents, peptide hormones, enzymes, proteins, vaccines, and genetic materials can be incorporated into the aqueous or lipid phases of liposomes (Chang and Yeh, 2012). By designing and changing the specific features of liposomes such as size, composition, and surface decoration selective delivery to the target site can be provided. In the case of cancer, nano-carriers can be directed to the tumor through both passive and active targeting mechanisms. The basis of nano-system delivery is accumulation of the agent within tumors through passive targeting, due to the enhanced permeability and retention (EPR) effect, followed by release of their therapeutic payloads (Wang et al., 2011). Compared to normal tissue, EPR can increase the tumor delivery of nanoparticles by less than 2-fold. The suitable size of liposomes can partly increase drug accumulation within the tumor, but the effective release and loading of a drug into target cells can be improved by the mean of active targeting strategies. One of the drawbacks of liposomes is the fast elimination of the blood and being captured by the reticuloendothelial cells, primarily in the liver. Several approaches have aimed to tackle this problem, one of which is PEGylation and immunotargeting preparation are the most common ones (Torchilin, 2005). Immunoliposomes can deliver encapsulated agents into the cells by a receptor-dependent manner, and bypass the drug resistance and off-target cytotoxicity effects. Conventional immunoglobulins and their corresponding fragments are widely used as targeting moieties for liposomes (Torchilin, 1984), which can be covalently attached to liposomes, without affecting their functional integrity (Torchilin, 2005).

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Table 1
Bivalent VHH primers.

Fragment 1	T7 promoter primer	TAATACGACTCACTATAGGG
	rev1	CGGTTTCGGGGTCTGCGGACCACCGTGCGGACCTTGACCTGCCTGACCGCTGCTAAC
Fragment 2	for2	GGTCCGCACGGTGGTCCGCAGACCCCGAAACCGGAAGTTCAGCTGGAAGAAAGC
	T7 Terminator primer	GCTAGTTATTGCTCAGCGG

Despite improvements in targeting efficacy, whole antibodies have been incapable of resolving the problems entirely (Ji et al., 2013; Rahbarizadeh et al., 2011a). Trastuzumab, a conventional monoclonal antibody broadly utilized as a targeting moiety for anti-HER2 targeting, have been associated with cytotoxicity, which is reportedly exacerbated when applied in combination with anticancer agents such as doxorubicin and anthracycline (Bubalo, 2018). Fortunately, nature has introduced antibody fragments to us as alternatives to invigorate the targeted drug delivery technology. A novel type of antibodies found in Camelidae, lack the light chains and CH1 domain but surprisingly represent a broad antigen-binding repertoire which can recognize various antigens with high specificity (Moghimi et al., 2013; Rahbarizadeh et al., 2011b). The binding domain of these heavy-chain antibodies is limited to a 15–20 kDa single variable domain called VHH (Sharifzadeh et al., 2013). With no reported immunogenic responses thus far, this targeting moiety has nano-molar affinity due to their elongated CDR3 that can recognize epitopes in protein cavities, generally inaccessible for whole antibodies (Hassanzadeh-Ghassabeh et al., 2013). Some studies show the bivalent structures of antibodies may be ideal candidates for targeting of nanocarriers, based on many valuable features including longer clearance time and more effector functions. To prepare a super functional VHH molecule, we developed a bivalent VHH construct by fusing the genes coding for two single domain antibodies. Based on previous studies, bivalent VHH shows enhanced affinity and augmented potency in targeting extracellular domain of cell surface receptors (Panetta and Greenwood, 2008). Bivalent and biparatopic VHHs are magic bullets for multifactorial diseases, such as cancer, where targeting more than one targets is demanded (Panetta and Greenwood, 2008). In the case of breast cancer, targeting and blocking multiple targets including EGFR (Cheong et al., 2015), HER2 (Nikkhoi et al., 2017) and MUC1 (Sadeqzadeh et al., 2011) can be highly advantageous. A pantamerized VHH constructed by Huet and coworkers aimed to elicit a TRAIL-associated apoptotic response proved that using multivalent VHH significantly elevates affinity and avidity, compared to the monovalent counterpart (Huet et al., 2014).

Breast cancer is the second most common cancer that occurs in women, and aberrant expression or activity of two members of the HER family, HER1, and HER2, have been connected to 20–25% of breast cancer cases (Yarden, 2001). HER2 is a signaling tyrosine kinase receptor that causes increased cell proliferation, tumor invasiveness, accelerated angiogenesis and reduced apoptosis, which ultimately translates into an aggressive disease, which is resistant to traditional systemic therapy with increased probability of recurrent disease and decreased survival (Moghimi et al., 2013). To prepare immunoliposomes against HER2 positive cells, in the present study, HER2-specific VHHs (RR2 and RR4), previously selected by panning from immune one-humped camel VHH gene library (Jamnani et al., 2012), were cloned and expressed as monomeric, dimeric-monospecific, dimeric-bispecific VHHs in *Escherichia coli*. Herein, we report the functional evaluation of a novel anti-HER2 intelligent liposomal nano-system with high potential for targeted cancer therapeutic applications.

2. Materials and methods

2.1. Cell lines and culture conditions

BT-474 and SKBR-3 human breast cancer cell lines were used as HER2-positive cells. MDA-MB-231 was used as a HER2-negative cell line. The cell lines were obtained from DSMZ (Braunschweig, Germany). The BT-474 cell lines were cultured in RPMI-1640 (Gibco/Invitrogen, Carlsbad, CA, USA). The SKBR-3 cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM) (Gibco/Invitrogen). The MDA-MB-231 growth medium consists of DMEM/F12 (Gibco/Invitrogen) supplemented with 5% donor horse serum, 100 units/mL penicillin, and 100 μg/ml streptomycin.

2.2. Constructing bivalent VHH

Two single-domain antibody fragments (VHH) were fused together using splicing by overhang extension (SOE) PCR to generate bivalent VHHs. Briefly, the genes encoding the VHHs were PCR amplified using two sets of primers. The *rev1* and *for2* primers (Table 1) contain the *hinge* sequence (GPHGGPQTPKP) at both extremities (underlined nucleotide). PCR amplification of RR2 and RR4 via two sets of primers yielded four VHH fragments containing *hinge* sequence, H-RR2, H-RR4, RR2-H and RR4-H. Equimolar amounts of all RR2, and RR4 fragments were mixed and fused to construct bivalent VHHs. Bivalent monospecific RR2 (RR2-H-RR2) and bivalent monospecific RR4 (RR4-H-RR4) are composed of two identical VHHs. Bivalent bispecific VHHs, RR2-H-RR4, and RR4-H-RR2, were prepared by fusing two different VHHs. The PCR-amplified monovalent and bivalent VHHs were then purified with Qiaquick PCR purification kit (Qiagen, Valencia, CA, USA) and double-digested overnight with *Nco*I-*Xho*I. *pET26b* plasmid (EMD Millipore, USA) containing the *pelB* sequence (MKYLLPTAAAGLLLLAAQPAMA) was linearized overnight with *Nco*I-*Xho*I enzymes and treated with alkaline phosphatase. Finally, the linearized *pET26b* expression plasmid and the monovalent and bivalent VHHs were mixed and ligated with *T4 DNA ligase*. The ligated products were used to transform *E. coli BL21 DE3* electrocompetent cells. Transformants were screened by colony PCR, using the universal T7 primers, and double digested to confirm the constructs. Finally, monovalent and bivalent VHHs constructs were sequenced to certify the lack of accidental mutations.

2.3. Expression and purification of mono- and bivalent VHH

For the purification, *E. coli* strain *BL21 DE3* harboring recombinant expression plasmid encoding VHHs were cultured in terrific broth (TB) medium containing kanamycin (50 mg/liter) until OD of 0.6 at 650 nm was attained. After induction for 18 h at 24 °C using 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), cells were harvested by centrifugation at 4000*g* for 10 min at 4 °C. Cell fractionation was carried out by resuspending the cell pellets in TES buffer (0.2 M tris-HCl, pH 8.0; 0.5 mM EDTA; 0.5 M sucrose) (10 ml per liter of original culture). The cells were then subjected to a mild osmotic shock by addition of TES, diluted 1:4 with H_2O. After incubation on ice for 30 min, the suspension was centrifuged (5000*g*, 10 min) and the supernatant was centrifuged again (48,000*g*, 15 min). Periplasmic extracts were extensively dialyzed against purification buffer (500 mM NaCl, 50 mM Tris-HCl; pH 8.0) to remove EDTA and then concentrated to a volume of approximately 2.5 ml per liter of original culture using Amicon 3000 Da. The concentrated periplasmic extract was applied on Ni-NTA resins to purify recombinant VHHs. After washing with gradient concentration of imidazole (from 50 mM to 150 mM), pure VHHs were eluted with a solution of 500 mM imidazole in purification buffer.