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Improvement of Targeted Gene Delivery to Human Cancer Cells by a Novel Trifunctional Crosslinker

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Abstract: A facile method for the construction of an immunoconjugate which displays targeting ligands, such as antibody fragments, with a high density is reported. For this purpose, we synthesized a novel trifunctional crosslinking reagent. By the use of this reagent, ligands targeting the specific cell can be displayed on the surface of the drug carrier with a high density. In this study, we display HER2 (human epidermal growth-factor receptor-2) binding ligands on branched polyethylenimine (PEI), which can form polyplexes with plasmid DNA. Kinetic analysis of the binding to the extracellular domain of HER2 show the PEI displaying a high density of ligands binds to the target more strongly compared to the PEI displaying ligands at a low density. The increased density of HER2 ligands displayed on the gene carrier contributes to the improved transfection efficiency. This approach can be applied to other drug delivery systems, including liposome, micelle, and so on.

Keywords: antibodies · cell recognition · conjugation · drug delivery · immunoconjugate

Introduction

Targeted delivery is a critical issue in gene therapy to reduce the amounts of drugs for the effective treatments and, accordingly, reduce side effects. In recent years, various gene delivery systems have been developed aiming for targeted-delivery, including the use of viral and non-viral vectors.[1] In case of non-viral vectors, a variety of cell-targeting ligands, such as small molecules, peptides, proteins, and antibodies, promote them into a wide range of applications in the field of gene therapy. Natural ligands such as saccharide, folate, and transferrin are inexpensive and they are easy to prepare and handle. However, these natural ligands also bind to some non-target cells, and compete for binding with native molecules in bodily fluids. Antibodies are favorable for targeted delivery because of their high affinity and specificity against their target antigen. However, the application of antibody-mediated therapeutics has several problems, in particular, the high cost of preparation of intact antibodies. To construct alternatives to antibodies, antibody fragments such as $F(ab')_2$ and Fab, which can be prepared easily in bacteria, have been developed. Moreover, with recent advances in engineering antibodies, many novel binding proteins that bind their target antigens with high specificity and affinity can now be easily generated.[2]

An antibody with two antigen-binding sites, which interact with its antigen bivalently, achieves high affinity against its antigen. In contrast, most of these antibody fragments and binding proteins are monovalent and, consequently, their affinities are relatively weak compared to an intact antibody. Therefore, dimeric and more complicated forms have been generated by genetic and chemical methods to improve their binding affinity. For example, by genetic methods, diabodies and minibodies have been developed and achieved comparable antigen binding affinity to the parental IgG.[3]

Dimeric or multimeric forms have also been generated by chemical cross-linking methods. Dimeric and trimeric antibody fragments have been developed by using bis-maleimide and tri-maleimide derivative cross-linkers, respectively.[2] For application of the multimeric fragments or ligands for ligand-targeted therapeutics, it is desirable for these multimers to modify functional molecules, such as radioisotopes, cytotoxins, and nanocarriers. The chemical cross-linking method also enables site-specific modification with these functional molecules, not only generation of dimeric or mul-

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timeric forms. King et al. designed their cross-linkers containing a 12-*N*-4-macrocycle to allow stable radiolabelling of the fragments with ^{90}Y.[4] Wilbur et al. conjugated $(Fab')_2$ with diagnostic and therapeutic agents by using the trifunctional equilibrium transfer alkylation cross-linking reagent.[5] This cross-linking reagent also permitted the site-specific PEGylation of native disulfide bonds in therapeutic proteins without destroying their tertiary structure or abolishing their biological activity.[6]

In this study, we synthesize a novel trifunctional crosslinking reagent which facilitates the dimerization of ligands such as antibody fragments and also enables modifications of these ligands with another functional molecule such as a drug, chemical probe, and nanocarrier.

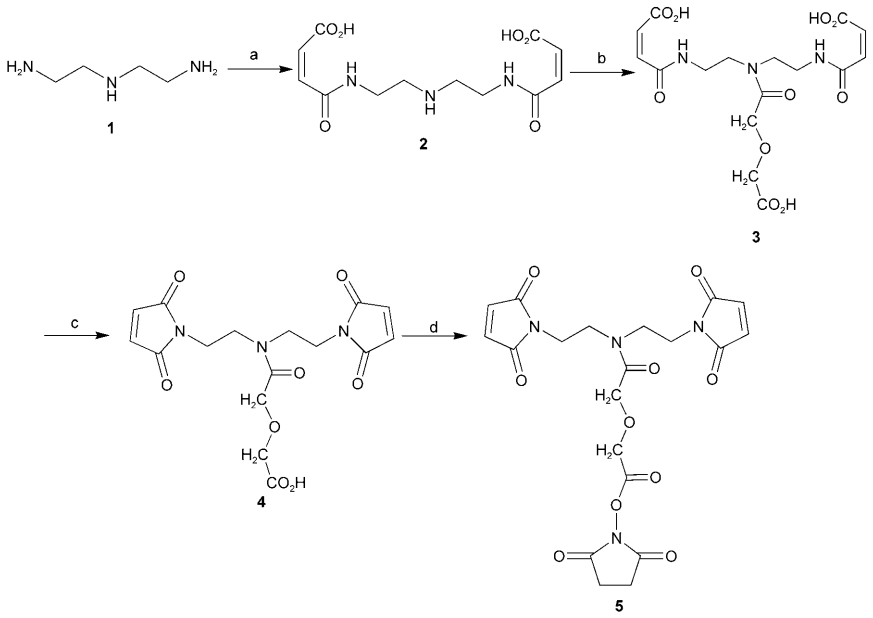
Results and Discussion

We synthesized a novel trifunctional crosslinker (Scheme 1). This reagent possesses three functional groups consisting of two maleimide residues, which react with the thiol groups, and an N-hydroxysuccinimidyl ester, which reacts with the amino groups. This crosslinker could facilitate dimerization of ligands which have thiol groups and further modification at the hinge of dimerized ligands by an activated ester. It is worth noting that only recrystallization is needed for the purification of this reagent, accordingly, this scheme is suitable for large-scale synthesis (see Experimental Section).

We attempted to dimerize HER2 (human epidermal growth factor receptor-2)-binding ligands[7] and modify the dimer with a fluorescent probe (Figure 1B). SDS-PAGE analysis clearly showed that the fluorescent probe (Dansyl moiety) was covalently conjugated with the dimerized ligands (Figure 1C). This result indicated the feasibility of the approach in which by using our trifunctional crosslinker, dimerized antibody fragments or ligands can be modified with functional molecules, such as cytotoxins, prodrugs, nucleic acids, nanocarriers, and so on.

Furthermore, by using this trifunctional crosslinker we conjugated Her2ligand with branched polyethylenimine as a gene carrier for targeted delivery (Figure 2). The cationic polymer polyethylenimine (PEI) has been successfully used as non-viral gene carriers both in vitro and in vivo.[8] We compared the efficiency of the gene delivery by two kinds of PEI–Her2ligand conjugates which were synthesized by using a commercially available bifunctional crosslinker (EMCS) and our trifunctional crosslinker (bMNHS).

First, purified Her2ligand, PEI–Her2ligand (EMCS), and PEI–Her2ligand (bMNHS) were analyzed for HER2–ECD



Scheme 1. Reagents and conditions: a) Maleicanhydride, DMF 95%; b) Diglycolicanhydride, DMF; c) NaOAc., aceticanhydride, 50% from 2; d) DCC, *N*-hydroxysuccinimide, methoxyethylether, 83%. DMF = *N*,*N*-dimethylformamide, DCC = Dicyclohexylcarbodiimide

Abstract in Japanese:

新規のクロスリンカーを合成し、抗体フラグメント等のリガンドを高密度で提示できる新しいイミューノコンジュゲートの構築法を開発した。このクロスリンカーを用いてポリエチレンイミン上にHER2リガンドを高密度で提示したところ、低密度で提示したものよりもターゲットに対して高い親和性を持つことがわかった。また遺伝子導入効率においてもリガンドを高密度で提示すると向上することが確認された。

binding by real-time biospecific interaction analysis using a BIAcore biosensor instrument. These molecules were injected over the NTA sensor chip flow-cell surface containing the immobilized target protein His-HER2-ECD and the control flow-cell.

Her2ligand, PEI–Her2ligand (EMCS), and PEI–Her2ligand (bMNHS) were all shown to bind to HER2–ECD. Upon evaluation of the binding curves of these molecules, the dissociation equilibrium constant (K_D) was determined to be 53 nM for Her2ligand, 4.7 nM for PEI–Her2ligand (EMCS), and 0.97 nM for PEI–Her2ligand (bMNHS) (Table 1). As expected, by using the trifunctional crosslinker

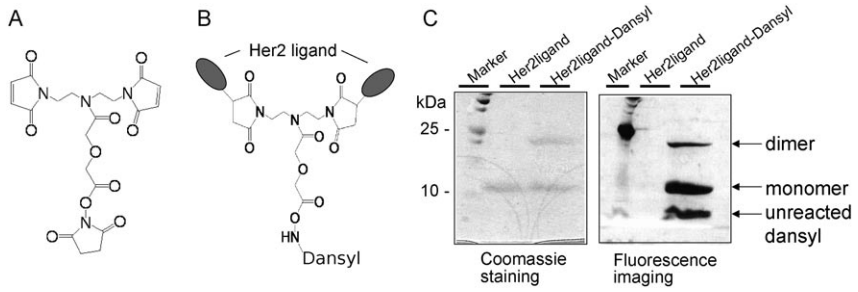


Figure 1. a) Structure of the novel trifunctional crosslinker (bMNHS). b) The modification of dimerized Her2-ligands with Dansyl probe by using the trifunctional crosslinker. c) SDS-PAGE analysis of the dimerized ligands conjugated with fluorescent probe.

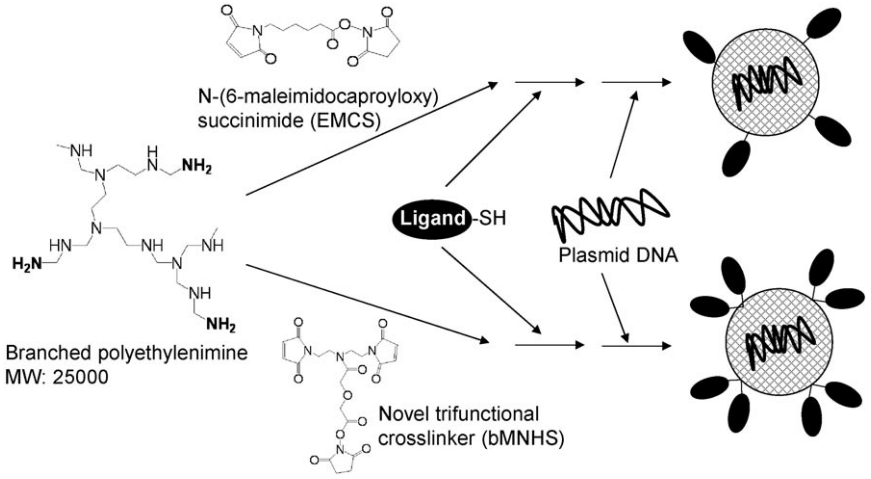


Figure 2. Schematic presentation of the synthesis of polyethylneimine–Her2ligand conjugates by using the bifunctional crosslinker (EMCS) or our trifunctional crosslinker (bMNHS). PEI–Her2ligand conjugates could form polyplexes with plasmid DNA.

Table 1. Kinetic analysis of the binding of Her2ligand, PEI–Her2ligand (EMCS) and PEI–Her2ligand (bMNHS) to HER2-ECD by biosensor binding studies.

Ligand	KD(nM)	K_a [$M^{-1}s^{-1}$]	K_d [s^{-1}]
Her2ligand	53	6.4×10^{3}	1.1×10^{-5}
PEI–Her2ligand(EMCS)	4.7	4.8×10^{3}	9.3×10^{-6}
PEI–Her2ligand(bMNHS)	0.97	3.2×10^{4}	2.2×10^{-5}

for conjugation of PEI with Her2ligand, the affinity for HER2–ECD was improved.

The PEI–Her2ligand (EMCS or bMNHS)/DNA polyplexes were evaluated for in vitro transfection efficiency in HER2 positive SKBR3 cells. Before transfection, we checked the interaction and complex formation between plasmid DNA and positively charged PEI–Her2ligand conjugates by gel retardation assays (data not shown). On the basis of the cell viability assay by Alamar Blue assay (Trek Diagnostic Systems, Inc.), we prepared the polyplexes at an N/P (nitrogen of polyethylenimine/phosphate of DNA) ratio of 7.5 because polyplexes have increased toxicities above this point. Figure 3 shows about a fivefold higher luciferase expression with PEI–Her2ligand (bMNHS) compared to with the PEI–Her2ligand (EMCS). To confirm HER2 specificity of these polyplexes, HER2 positive SKBR3 cells were preincubated with free Her2ligand, which effectively depressed subsequent transfection with both PEI–Her2ligand (EMCS) and PEI–Her2ligand (bMNHS). These results strongly imply, in this targeting system, the increased density of Her2ligand displayed on the gene carrier contributed to the improved targeting efficiency, resulting in an improved transfection efficiency.

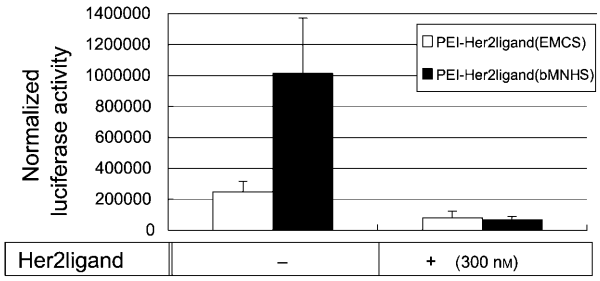


Figure 3. Delivery and expression of a luciferase gene using PEI–Her2ligand (EMCS or bMNHS)/Plasmid DNA polyplexes in HER2 positive SKBR3 cells. Values were normalized against the protein content of the sample.

Conclusions

Multivalent interactions have been extensively investigated to promote targeting to specific cells. In the case of intact antibodies, they can recognize the specific antigen with two binding sites. Our novel crosslinker facilitates dimerization of ligands and modification with another functional molecule including probe and drug. Our system exploits another strategy to construct an immunoconjugate.

In this study, we also demonstrated the enhancement of gene delivery by a densely ligand-displayed polyethylenimine. Analysis by real-time biospecific interaction showed that densely ligand-displayed polyethylenimine bound to the targeting antigen more strongly. Accordingly, it was consid-

ered that the improved affinity to the cell gave rise to the transfection efficiency.

On the other hand, especially in the case of solid tumor-targeted delivery, it became clear that the improved affinity did not always contribute to ligand-targeted delivery. When the binding affinity is too high, there are several reports that penetration of the targeting molecules into solid tumors decreases because of the "binding-site barrier", whereby the molecule binds strongly to the first targets encountered but fails to diffuse further into the tumor.[9,10] Adam et al. showed high affinity restricted the localization and tumor penetration of single-chain Fv antibody molecules (scFv) when compared to scFvs with a different affinity to the same antigen, indicating the existence of a threshold affinity for the improved delivery.[10] Recently, Zhou et al. showed the importance of the density of ligands on the nanoparticle for efficient uptake.[11] They suggest ultrahigh affinity of ligand may be unnecessary for targeted delivery if low affinity ligands are displayed at high densities.

For further developments of gene delivery in terms of specificity and efficiency, controlling the affinity and density of ligands and the choice of a proper set of ligands are demanded. By the use of our reagent, several types of ligands, which can target different antigens at the same time, can be displayed at high density, enabling the control of the affinity of the gene carrier. Research into this is currently being undertaken by our group.

Experimental Section

Synthesis

(2Z,2Z′)-4,4′-(2,2′-azanediylbis(ethane-2,1-diyl)bis(azanediyl))bis(4-oxobut-2-enoic acid) (2).

Maleicunhydride (10 g) in dimethylformamide (DMF) (30 mL) and diethylenetriamine **1** (5.4 mL) in DMF (30 mL) were added slowly to DMF (30 mL) at room temperature and further stirred for 3 h. The resulting white precipitate was filtered and washed with DMF and chloroform to afford **2** (14.2 g, 95%). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 3.06$ (t, 4H, $J = 5.6$ Hz), 3.43 (t, 4H, $J = 5.6$ Hz), 6.07 (d, 2H, $J = 12.4$ Hz), 6.31 ppm (d, 2H, $J = 12.4$ Hz); ^{13}C NMR: $\delta = 36.45$, 47.50, 131.51, 133.66, 167.59, 168.30 ppm.

2-(2-bis(2-(2,5-dioxo-2,5-dihidro-1H-pyrrol-1-yl)ethyl)amino)-2-oxo-ethoxy)acetic acid (3).

Diglycolicanhydride (7.17 g) in DMF (25 mL) was added into solution of **2** (14.2 g) in DMF (475 mL) and stirred at room temperature for 2 h. The reaction mixture was concentrated in vacuum to afford **3**.

(2Z,2Z′)-4,4′-(2,2′-(2-(carboxymethoxy)acetylazanediyl)bis(ethane-2,1-diyl)bis(azanediyl))bis(4-oxobut-2-enoic acid) (4).

Compound **3** was dissolved in acetic anhydride. Sodium acetate (2.1 g) was added to the solution and stirred at 90°C for 3 h. The reaction mixture was filtered. Water (30 mL) was added to the filtrate and stirred for 3 h. The solution was evaporated and the residue was dissolved in chloroform and washed with brine. The organic layer was dried over anhydrous sodium sulfate and concentrated in vacuum. The residue was recrystallized in methanol to yield **4** (50%). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 3.36$ (t, 2H, $J = 6.8$ Hz), 3. 63 (m, 2H), 3.70 (t, 2H, $J = 6.8$ Hz), 3. 75 (m, 2H), 4.15 (s, 2H), 4.31 (s, 2H). 6.70 (s, 2H), 6.78 ppm (s, 2H).); ^{13}C NMR: $\delta =$ 35.34, 35.64, 44.47, 44.81, 70.64, 71.45, 134.58, 134.67, 170.44, 172.67, 175.85 ppm; Anal. Calcd for $C_{16}H_{18}N_4O_6$: C 50.65, H 4.48, N 11.08; found: C 50.58, H 4.43, N 10.87.

2,5-dioxopyrrolidin-1-yl-2-(2-(bis(2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)amino)-2-oxoethoxy)acetate (5).

Dicyclohexylcarbodiimide (33 mg) and *N*-hydroxysuccinimide ware added to **4** (100 mg) in methoxyethylether (1.3 mL) and stirred at 0°C for 2 h. The reaction mixture was brought to room temperature and further stirred for 3 h. A few drops of acetic acid was added to the reaction mixture at 0°C and stirred for 1 h. Resulting white precipitate was removed by filtration and the filtrate was concentrated in vacuum. The residue was recrystallized in 2-propanol to yield **5** (83%). ^{1}H NMR (400 MHz, $CDCl_3$) $\delta = 2.83$ (s, 4H), 3.40 (t, 2H, $J = 7.2$ Hz), 3. 57 (m, 2H), 3.67 (t, 2H, $J = 7.2$ Hz), 3. 71 (m, 2H), 4.24 (s, 2H), 4.53(s, 2H), 6.68 (s, 2H), 6.71 ppm (s, 2H); ^{13}C NMR $\delta = 25.80$, 35.23, 35.66, 44.02, 44.71, 67.89, 69.25, 134.54, 134.59, 165.76, 168.95, 169.01, 170.52, 170.59 ppm.

Plasmid Construction and Expression of Her2ligand Peptides

A plasmid vector, which expressed Her2ligand with a cysteine residue at the N-terminus, was constructed as follows.

A 1 μmol amount of each synthetic DNA primer was mixed, then PCRs were carried out with *Ex Taq* DNA polymerase (Takara, Shiga, Japan) to construct a Her2ligand coding region. The DNA primers were 5′- CAT GCC ATG GTG GAT AAC AAA TTC AAC AAA GAA CTG CGC CAG GCG TAC TGG GAA ATC CAG GCG CTG CCG AAC CTG AAC TG -3′, 5′- CCG AAC CTG AAC TGG ACC CAG AGC CGC GCG TTC ATC CGC AGC CTG TAC GAT GAT CCG AGC CAG AGC GCG AAC CTG CTG -3′, and 5′- CCG GAA TTC GCT GCC ACC GCT GCC ACC TTT CGG CGC CTG CGC ATC GTT CAG TTT TTT CGC TTC CGC CAG CAG GTT CGC GC -3′. To introduce the cysteine residue at N-terminus and restriction enzyme sites, a 1 μmol amount of each synthetic DNA primer was mixed with the above PCR product and PCRs performed similarly. The DNA primers were 5′- CAT GCC ATG GGC *TGC* GTT GAT AAC AAA TTC AAC AAA GAA CTG -3′ and 5′- CGC GGA TCC TTA TTT CGG CGC CTG CGC ATC G -3′. Purified PCR product was digested with *Nco*I and *Eco*RI restriction enzymes and cloned into pTWIN2 vector (New England Biolabs) to obtain the pTWIN2Her2ligand vector.

The *E. coli* strain ER2566 (New England Biolabs) transformed with pTWINHer2ligand vector was cultured in LB medium containing ampicillin at 25°C until the turbidity at 600 nm reached 0.4. Induction was performed by adding isopropyl-β-D-thiogalactopyranoside (IPTG, final 1 mM) and the bacteria were cultured for an additional 4 h at 25°C, then, harvested by centrifugation at 4000 g for 15 min. The bacterial pellet was resuspended in 20 mM HEPES buffer (pH 8.5) containing 500 mM NaCl, 1 mM EDTA and 0.5% TritonX-100 and sonicated on ice. The lysate was centrifuged at 12000 g for 5 min and the Her2ligand protein in the supernatant was then purified using chitin beads as described in manufacture's protocol. Purified Her2ligand was dialyzed into PBS.

Modification of Dimerized Her2ligand with Dansyl

1 mg bMNHS freshly dissolved in dimethylformamide was added to 1 mg Dansyl-cadaverine and incubated at room temperature with rotating. After 12 h, 1 mM activated Dansyl was prepared in 0.1 M phosphate buffer (pH 7.0). Ligands were reduced by incubation with 2 mM dithiothreitol and 2 mM EDTA in 0.1 M phosphate buffer (pH 7.0) for 30 min at room temperature without agitation. DTT and EDTA were eliminated by gel chromatography (PD-10 column, GE Healthcare). The concentration of thiol group was measured by the Ellman test.

1 mM activated Dansyl was added to reduced ligands at a molar ratio of 1 and incubated with rotating at room temperature for 12 h. Dansyl-labelled Her2ligands were fractionated on sodium dodecyl sulfate (SDS) polyacrylamide (18%) gels. The fluorescence of the Dansyl moiety was detected by using a UV transilluminater and the ligands were detected by following Coomasie Brilliant Blue staining.

Generation of Polyethylenimine–Her2ligand(EMCS or bMNHS)

In order to generate a gene delivery carrier, branched 25 kDa polyethylenimine (PEI, Aldrich, St. Louis, USA) was dissolved in PBS to a final concentration of 2 mg mL^{-1}. To 1 mg branched PEI, the crosslinker [N-

(6-Maleimidocaproyloxy)succinimide (EMCS, Dojindo, Japan) or trifuncational crosslinker (bMNHS)] freshly dissolved in dimethylformamide was added (final 4 mM) and incubated at room temperature with gentle shaking. After 4 h, unconjugated crosslinkers were removed by ultrafiltration with a 10 kDa cut-off membrane (VIVASPIN, Sartorius, UK). 100 μg Her2ligand was reduced by incubation with 5 mM Tris(2-carboxyethyl)phosphine (TCEP) in PBS for 30 min at room temperature without agitation. The concentration of Her2ligand was measured using Protein Assay reagent (Bio-Rad). In order to generate Her2ligand–PEI conjugate, the EMCS-activated PEI or bMNHS-activated PEI dissolved in PBS was added to the reduced Her2ligand and incubated overnight at room temperature with gentle shaking. The sample complex was purified by ultrafiltaration on a 30 kDa cut-off membrane (Microcon YM-30, Millipore). The sample was washed twice with 20 mM MOPS buffer (pH 7.3) containing 1.5 M NaCl. The PEI–Her2ligand conjugate was dissolved in 0.1 M MOPS buffer (pH 7.3) containing 150 mM NaCl. The concentration of PEI was determined by copper complexation.

Cell Viability Assay

Cells (2×10^4 cells per well) were cultured in 96-well plates. PEI conjugates were mixed with 125 ng plasmid DNA at N/P ratios of 0, 1, 2, 5, 7.5, 10, 12, and 15 in PBS and incubated for 20 min at room temperature. Polyplexes were then added to the cell culture medium and incubated at 37 °C. After 24 h, Alamar Blue assay was performed as described in the manufacture's protocol. The viability of untreated cells was defined as 100 %.

Gene Transfer by PEI–Her2ligand Conjugates

Cells (2×10^4 cells per well) were cultured in 96-well plates. PEI–Her2ligand with plasmid DNA was generated as follows. Plasmid DNA encodes Luciferase. DNA (125 ng) was added to 25 μL of DMEM. Subsequently, PEI–Her2ligand (EMCS) or PEI–Her2ligand (bMNHS) was added to the DNA solution to yield an N/P ratio of 7.5. The mixture was mixed by pipetting and incubated at room temperature for 30 min. Culture medium of the adherent cells were replaced with 100 μL of fresh culture medium and the above transfection mixture was added. Cells were incubated for 48 h at 37 °C.

Binding Analysis of PEI–Her2ligand Conjugates with HER2-ECD using SPR (Surface Plasmon Resonance)

The binding analyses of PEI–Her2ligand conjugates with HER2-ECD (extracellular domain of human epidermal growth factor receptor-2) were conducted using the SPR method on a Biacore 2000, with a NTA sensor chip (Biacore, Uppsala, Sweden). Initially, the sensor chip was coated with 200 nM His-HER2-ECD (R&D systems), dissolved in 10 mM HEPES buffer (pH 7.4) containing 150 mM NaCl, 50 μM EDTA and 0.005 % Surfactant P20, by injecting 20 μL of the solution at a flow rate of 2 $\mu L\,min^{-1}$.

For the kinetic analysis, each of Her2ligand and PEI–Her2ligand conjugates were injected at final concentrations ranging from 25 to 500 nM.

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