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molecular pharmaceutics

Anionic Amino Acid Dendrimer−Trastuzumab Conjugates for Specific Internalization in HER2-Positive Cancer Cells

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Received April 12, 2010; Revised Manuscript Received June 2, 2010; Accepted June 7, 2010

Abstract: Trastuzumab, a humanized monoclonal antibody against human epidermal growth factor receptor 2 (HER2), offers a promising strategy of anticancer drug targeting to HER2-expressing cancer cells. Conjugation of trastuzumab to dendrimers, repeatedly branched polymers with a highly functionalized surface, can enhance the drug loading capacity. However, typical dendrimers such as cationic polyamidoamine dendrimers have exhibited a nonspecific cytotoxicity. In the present study, we developed a novel biocompatible amino acid dendrimer with potentially less toxicity by surface modification of the sixth generation lysine dendrimer with glutamate (KG6E). The synthesized KG6E showed a well-controlled particle size around 5−6 nm with low polydispersibility and negative surface potentials for negligible cytotoxicity. Next, the targeting efficiency of the fluorescent-labeled KG6E−trastuzumab conjugate was evaluated in HER2-positive (SKBR3) and -negative (MCF7) human breast cancer cell lines compared to free trastuzumab and KG6E dendrimers. The KG6E−trastuzumab conjugate was specifically bound to SKBR3 cells in a dose-dependent manner with low binding affinity to MCF7 cells. Furthermore, the conjugate was significantly internalized in SKBR3 cells and then trafficked to lysosomes. These results indicate the potential of KG6E−trastuzumab conjugates as HER2-targeting carriers for therapeutic and diagnostic approaches to cancer therapy.

Keywords: Amino acid dendrimer; cytotoxicity; dendrimer−antibody conjugates; HER2; intracellular delivery; tumor targeting

Introduction

The lack of tumor specificity is one of the most important factors for the failure of cancer chemotherapy and diagnosis. Tumor-targeted delivery of chemotherapeutic drugs and imaging probes is believed to enhance therapeutic efficacy in tumors with low systemic side effects in noncancerous tissues and tumor diagnosis, respectively. Monoclonal antibodies (mAbs) against tumor antigens show a high specificity and affinity for tumor cells, which has led to the development of antibody-based cancer therapy.[1−3] Human epidermal growth factor receptor-2 (HER2/ErbB-2/neu), a tyrosine kinase transmembrane receptor which is overex-

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10.1021/mp100105c
Published on Web 06/07/2010

pressed in various cancers including breast, ovarian and lung cancer, is associated with a poor prognosis and invasive carcinomas;[4,5] therefore, it is a potential candidate for targeted antibody therapy.[6,7] Humanized anti-HER2 mAb trastuzumab (Herceptin), which has been approved by the U.S. Food and Drug Administration for clinical use, is specifically bound to HER2 receptors and mediates antiproliferation of breast cancer cells;[8−10] however, the therapeutic effects of mAb monotherapy tend to be limited.[10] Antibody-chemotherapeutic drug conjugates are reported as an alternative approach to synergize antibody-mediated cellular toxicity and chemotherapy for effective clinical translation.[11−13] For example, direct conjugation of chemotherapeutic drugs, such as geldanamycin[14,15] and maytansinoids,[16] to trastuzumab showed improved pharmacokinetics for effective antitumor activity compared with trastuzumab monotherapy. The drug payload using this conjugation method, however, is restricted due to a significant decrease in the receptor recognition,[12] immunoreactivity,[17] and water solubility of the conjugates.[18]

Nanoparticles, including liposomes, micelles, polymers and dendrimers, have been reported as effective drug carriers offering a high drug payload by physical incorporation or chemical conjugation.[19−21] Among them, dendrimers possess attractive features including (i) their nanosize range, (ii) monodispersity, (iii) rigid globular structure with high physical stability and (iv) a large number of functional groups for versatile chemical modification.[22−24] Nevertheless, conventional dendrimers, such as cationic polyamidoamine (PAMAM) and poly-L-lysine dendrimers, tend to exhibit nonspecific dose-dependent interactions and cytotoxicity due to the positive charge of the peripheral amino groups.[25−27] In this regard, Baker and colleagues have reported partially acetylated PAMAM−trastuzumab conjugates for reduced

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nonspecific interactions[28] in HER2-expresssing tumor targeting.[29,30] Indeed, acetylated modification possibly alters (i) the physicochemical properties of the dendrimers including their water solubility, (ii) structural homogeneity and (iii) the number of peripheral amino groups available for antibody−drug conjugation;[31] therefore, precise control of the degree of acetylation is necessary.

Amino acid dendrimers are stepwise synthetic macromolecules of amino acid branch units which typically are lysine, obtained by peptide or amide bond formation.[32−34] The designed structure of the amino acid dendrimers can be controlled by organization of the repeated amino acid branches at the internal or peripheral layers called "generations".[32] We and other groups have reported the influence of surface modification on the *in vivo* distribution behaviors of amino acid dendrimers in that neutral and anionic dendrimers were less recognized by the reticuloendothelial system.[33−35] The potentially anionic amino acid dendrimers are of interest because they offer an expected improvement in nonspecific interactions with retention of the monodispersity, water solubility, platform for drug conjugation and biodegradability.[36] Therefore, anti-HER2 mAb conjugated anionic amino acid dendrimers could be potential candidates for HER2-expressing tumor targeting.

As far as intracellular delivery of anticancer drugs in tumor cells is concerned, dendrimer−drug conjugates should be ideally cleaved only upon internalization in the lysosomal compartment to release free anticancer drugs from dendritic scaffolds for anticancer activity in the tumor cells.[37] Accordingly, the *in vitro* evaluation of HER2-specific internalization as well as the intracellular distribution of anionic amino acid dendrimer−trastuzumab conjugates is a prerequisite for their therapeutic application.

In this study, we synthesized anti-HER2 trastuzumab conjugated to a glutamate-modified sixth generation of lysine dendrimer (KG6E) for targeting to HER2-positive cancer cells. The surface-modified glutamate provided an equal number of carboxylic acid and amine groups on the periphery of the KG6E dendrimer. Then, we examined the relationship between the zeta-potentials and the cytotoxicity of KG6E dendrimer−trastuzumab conjugates in HER2-positive (SK-BR3) and -negative (MCF7) human breast cancer cells. The binding affinity, internalization efficiency and lysosomal trafficking were determined to evaluate the feasibility of potentially anionic KG6E dendrimers as targeted drug carriers using fluorescently labeled KG6E−trastuzumab conjugates.

Materials and Methods

Materials. 1-[Bis(dimethylamino)methylene]-1*H*-benzotriazolium 3-oxide hexafluorophosphate (HBTU) was obtained from Peptide Institute (Osaka, Japan). 1-Hydroxybenzotriazole hydrate (HOBt), Boc-Lys(Boc)-OH dicyclohexylamine and Boc-Glu(OtBu)-OH were purchased from Watanabe Chemical Industries (Hiroshima, Japan). Trifluoroacetic acid (TFA) and *N*-ethylmaleimide were provided by Nacalai Tesque (Kyoto, Japan). PAMAM dendrimer generation five (PAMAM G5) was purchased from Sigma-Aldrich (St. Louis, MO). Trastuzumab was obtained from Chugai Pharmaceutical (Tokyo, Japan). Sulfosuccinimidyl 6-(3′-[2-pyridyldithio-]propionamido)hexanoate (sulfo-LC-SPDP) and sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) were from Molecular Biosciences (Boulder, CO). AlexaFluor 488 succinimidyl ester and anti-AlexaFluor 488 Rabbit IgG were obtained from Molecular Probes (Eugene, OR).

Synthesis of Glutamate-Modified Sixth Generation Lysine Dendrimers (KG6E). KG6E was synthesized according to previous reports with a minor modification.[33,34] In brief, the first generation of lysine dendrimer (KG1) was synthesized by activation of Boc-Lys(Boc)-OH dicyclohexylamine in 1.1 molar excess of HBTU and HOBt to the number of amines in the hexamethylenediamine core molecule in DMF for 30 min. The coupling reaction was performed in pH 9−10 adjusted with triethylamine and stirred at room temperature (RT) for 3 h. The Boc-protected KG1 was purified by liquid−liquid extractions with 5%

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sodium bicarbonate, 10% citric acid and saturated sodium chloride solution, respectively. The KG1 was obtained after deprotection of Boc-protected KG1 by TFA. For the higher generations, the coupling reactions were repeated. Finally, KG6 was reacted with Boc-Glu(OtBu)-OH and deprotected with TFA to produce KG6E. This was purified by ultrafiltration using VIVASPIN-20 (MWCO 10,000), lyophilized and characterized by ^{1}H NMR and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

Synthesis of Thiol-KG6E−AlexaFluor 488 Conjugates (Thiol-KG6E−AF). For SPDP−KG6E, KG6E (40.0 mg, 1.22 μmol) was reacted with sulfo-LC-SPDP cross-linking agent (3.22 mg, 6.10 μmol) in PBS pH 7.4 at RT for 1 h. The reaction mixture was purified by ultrafiltration as above and then lyophilized to obtain SPDP−KG6E. The number of SPDP per KG6E molecule was determined by pyridine-2-thione assay based on absorbance at 343 nm following cleavage of the disulfide bond by dithiothreitol (DTT).[38]

For SPDP−KG6E−AF, SPDP−KG6E (30.0 mg, 0.90 μmol) in PBS was added to AlexaFlour 488 succinimidyl ester (3.19 mg, 4.95 μmol) in DMSO, and then incubated at RT for 3 h. The reaction mixture was purified by ultrafiltration as described previously and then lyophilized to obtain SPDP−KG6E−AF. The purified conjugate was characterized by UV−vis spectroscopy. The degree of AlexaFluor 488 labeling on dendrimer was calculated based on the absorption at 494 nm and molar extinction coefficient of 71000 M^{-1} cm^{-1} as described in the manufacturer's instruction (Invitrogen). Prior to further conjugation, the disulfide bond on SPDP−KG6E−AF dendrimer (7.70 mg, 0.220 μmol) was reduced with DTT (17.7 mg, 115 μmol) in PBS containing 5 mM EDTA (PBS-EDTA) at RT for 30 min. The reaction mixture was purified by ultrafiltration in PBS−EDTA to obtain a thiol-KG6E−AF. The obtained thiol-KG6E−AF conjugate was immediately used for the next reaction.

Synthesis of Trastuzumab−KG6E−AlexaFluor 488 Conjugates (Trast−KG6E−AF). In order to synthesize Trast−KG6E−AF, the thiol reactive maleimide group was first introduced into anti-HER2 antibody. Trast−maleimide was given after conjugation of trastuzumab (33.0 mg, 0.220 μmol) and sulfo-SMCC cross-linker (0.672 mg, 1.50 μmol) in PBS−EDTA at RT for 30 min and purification by ultrafiltration. The obtained trast−maleimide (30.0 mg, 0.200 μmol) was immediately reacted with thiol-KG6E−AF (6.88 mg, 0.200 μmol) in PBS−EDTA at RT for 1 h. The unreacted thiols were neutralized with *N*-ethylmaleimide (2.50 mg, 20.0 μmol) in PBS at RT for 1 h to minimize the dimerization via disulfide bond formation of dendrimers and antibodies. After removal of excess reagent by ultrafiltration, the final reaction mixture was purified by gel filtration on a HiPrep Sephacryl S-300 column in PBS at 0.5 mL/min. The conjugate fractions containing trastuzumab and AlexaFluor 488 determined by absorbance at 280 and 494 nm, respectively, were pooled and concentrated by ultrafiltration in PBS. The number of trastuzumab modification on dendrimer was indirectly determined using a mole ratio of AlexaFluor 488 modified on KG6E and trastuzumab because the absorption peak of KG6E was overlapped with trastuzumab spectra in the far UV region. The molar ratio of AlexaFluor 488 to trastuzumab was calculated as described in the manufacturing instruction (Invitrogen) where the molar extinction coefficient of antibody and a correction factor for absorption of AlexaFluor 488 at 280 nm were 2.03×10^5 M^{-1} cm^{-1} and 0.11, respectively.

Particle Sizes and Zeta Potentials. The particle sizes and zeta potentials of dendrimers at a concentration of 1 mg/mL in PBS were measured using a Zetasizer Nano ZS instrument (Malvern Instruments, Worcestershire, U.K.).

Cell Lines and Culture Conditions. HER2-positive (SKBR3) and -negative (MCF7) human breast cancer cells were obtained from DS Pharma Biomedical (Osaka, Japan) and European Collection of Animal Cell Cultures (ECACC) (Porton Down, U.K.), respectively. SKBR3 and MCF7 cells were maintained in RPMI1640 and 1% nonessential amino acid−Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin, respectively, at 37 °C in 5% CO_2 atmosphere.

Cytotoxicity Assay. The cytotoxicity of dendrimers and conjugates was evaluated based on a WST-8 assay using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). Cells were seeded at 5×10^3 cells/well in 96-well plates and incubated for 24 h before experiments. After 24 h incubation of dendrimers at indicated concentrations, cells were treated with 10 μL of WST-8 reagent at 37 °C for 2 h. The absorbance at 450 nm normalized with the absorbance at 630 nm was measured. Cell viability was expressed as a percentage relative to PBS treatment controls.

Cellular Uptake. Cells were seeded at 5×10^4 cells/well in 12-well plates and incubated for 48 h before uptake experiments. For binding or competitive binding studies, cells were incubated with AF-labeled conjugates at 4 °C for 1 h. For internalization studies, cells were incubated with AF-labeled conjugates at 4 °C for 1 h, then washed with culture medium and incubated at 37 °C (pulse−chase method). The extracellular AF-labeled conjugates were quenched with anti-AlexaFluor 488 Rabbit IgG (0.25 μg/mL) on ice for 30 min. At indicated times, cells were washed, and collected in PBS before flow cytometry analysis using a Becton Dickinson FACScan analyzer (Franklin Lakes, NJ). The cellular-associated fluorescence of 1×10^4 cells was measured, and the mean fluorescent intensity of gated viable cells was quantified.

Intracellular Localization of Trast−KG6E−AF Conjugates. Cells were seeded at 1×10^4 cells/well on glass-bottom 48-well plates. After a 24 h cultivation, cells were incubated with 50 nM AF-labeled conjugates at 37 °C for the indicated times. For the competitive inhibition study, cells were pretreated with 6 μM free trastuzumab at 37 °C for 30

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min. For the intracellular localization study, lysosomes were labeled with 50 μM LysoTracker Red DND-99 (Molecular Probe) for 1 h following compound treatment. After fixation with 4% paraformaldehyde−PBS for 20 min, cells were washed and mounted using a SlowFade Gold with DAPI nuclear staining (Molecular Probe) followed by imaging using a Biozero BZ-8000 fluorescent microscope (Keyence, Osaka, Japan).

Statistical Analysis. Statistical analysis was performed using an ANOVA followed by the two-tailed unpaired Student's *t* test between groups. $P < 0.05$ was considered to be indicative of statistical significance.

Results and Discussion

Synthesis and Characterization of Anionic Amino Acid KG6E Dendrimers. In order to overcome any nonspecific cytotoxicity, an anionic amino acid dendrimer was designed. Although PEGylation by polyethyleneglycol and acetylation by acetic anhydride have been used to alter the positively charged surface of macromolecules, these methods affect the homogeneity and solubility of dendrimers, respectively.[18,31] We, therefore, developed anionic amino acid dendrimers by glutamate-modification of the sixth generation of lysine dendrimers (KG6E) as shown in Figure 1a. Since the glutamate residues on the peripheral structure consist of an equal number of chemically reactive amine and carboxylic acid groups, KG6E could uniquely offer flexibility in chemical conjugation to drugs or ligands via a range of chemical bonding including ester,[30] pH-dependent hydrolytic amide,[39] thioether[17] and hydrazone,[37] and compromise positive surface potentials, respectively. KG6E was prepared by HBTU/HOBt synthesis (Scheme 1), and a high yield (75%) was obtained after purification using liquid−liquid extraction method. The purified KG6E was characterized by ^{1}H NMR (Figure 1b) and MALDI-TOF-MS (Figure 1c). The measured chemical shifts and integrals of KG6E from ^{1}H NMR analysis substantially corresponded to the predicted chemical shifts and integrals calculated from 128 groups (Figure 1a) of lysine and glutamate residues at δ4.53 and δ3.56, respectively. The found average mass of KG6E ([M + H] = 32751.88) by MALDI-TOF-MS analysis was in agreement with the theoretical average mass ([M + H] = 32793.99).

Physicochemical Properties and Cytotoxicity of KG6E Dendrimers. The physicochemical properties and cytotoxicity of KG6E were evaluated in comparison with lysine (KG6) and PAMAM G5 dendrimers which represent a typical cationic dendrimer containing the same number of amines as KG6E (Figure 1a). The mean particle sizes of these dendrimers were around 5−6 nm with a very low polydispersibility index (0.176−0.184) (Figure 2a). Moreover, KG6E was slightly larger than KG6 in accordance with an

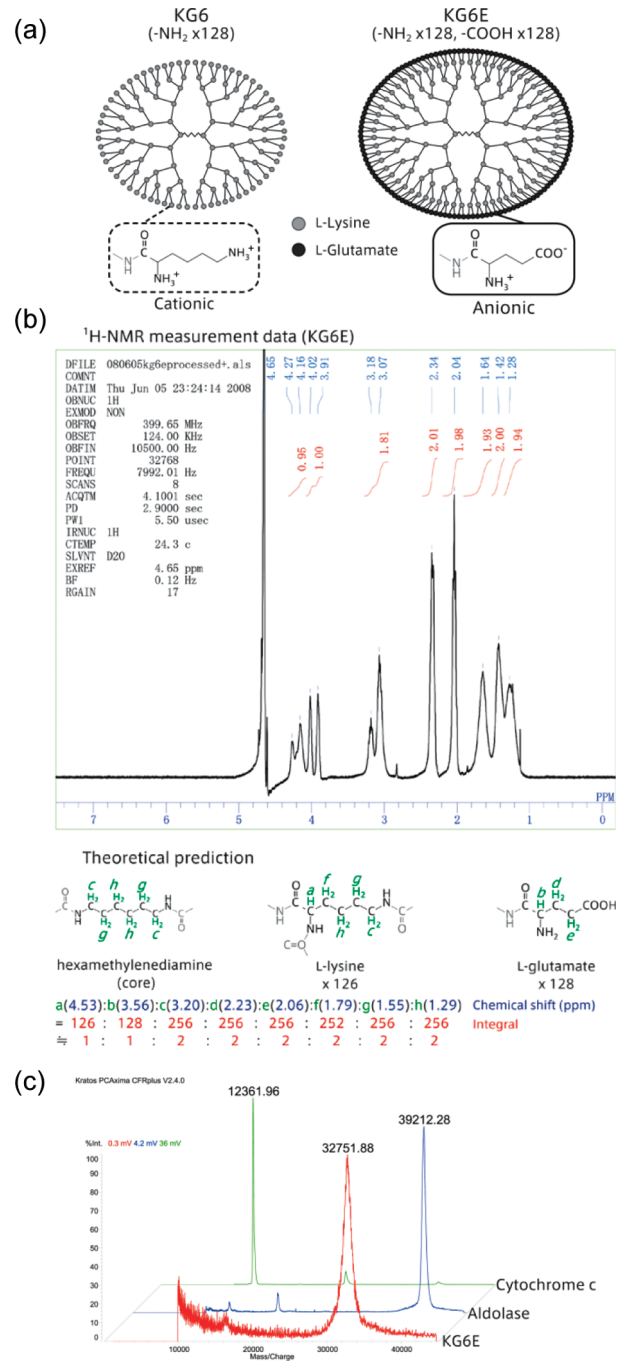

Figure 1. (a) Chemical structure of KG6 and KG6E dendrimers. (b) ^{1}H NMR analysis of KG6E. KG6E was dissolved with D_2O and measured by ^{1}H NMR. The chemical shifts (blue) and the integrals (red) are indicated at a corresponding peak in the upper panel. Predicted chemical shifts (blue) and integral ratio (red) of protons (green) are shown with the structures in the lower panel. The other protons (black) were not detected due to the proton exchange. (c) MALDI-TOF-MS spectrum of KG6E. The matrix sinapinic acid and samples were dissolved in dH_2O:acetonitrile:trifluoroacetate (50:50:0.2) solution before measurement using a Kratos PC Axima CFR plus (Shimadzu, Kyoto Japan). Aldolase ([M + H] = 39212.28) and cytochrome *c* ([M + H] = 12361.96) were used as external calibration samples. The theoretical average mass of KG6E is 32792.98 ([M + H] = 32793.99).

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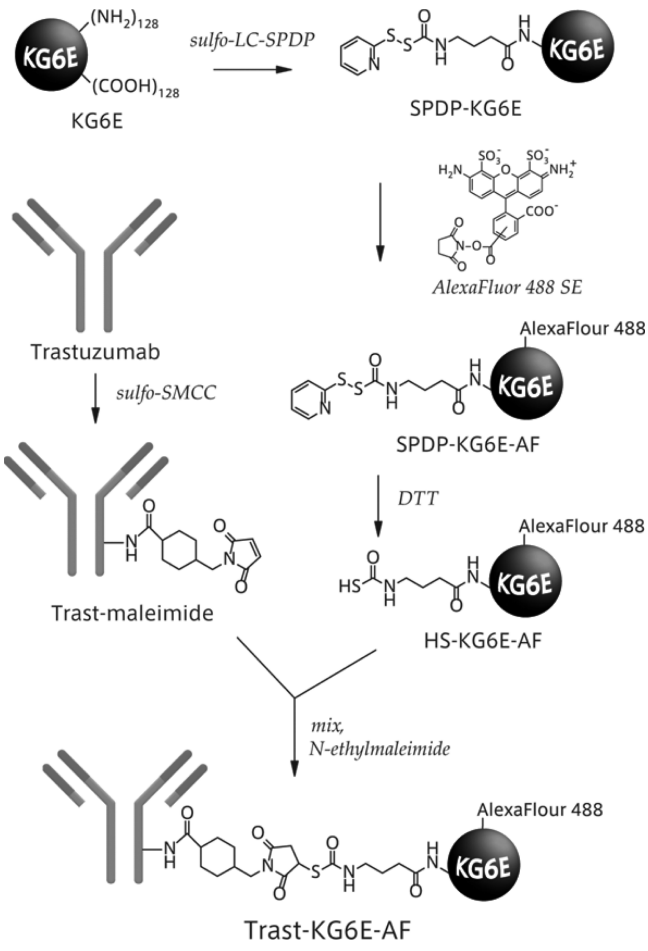
Scheme 1. Synthesis scheme for the Trastuzumab−KG6E−AlexaFluor (Trast−KG6E−AF) Conjugate



increased glutamate generation corresponding to our previous report.[33] As expected, PAMAM G5 and KG6 dendrimers showed positive surface potentials of 20.00 and 17.67 mV, respectively (Figure 2b). On the other hand, KG6E dendrimer showed a negative surface charge at −22.40 mV although there are an equal number of 128 positive amines and negative carboxylates. This observation might be explained

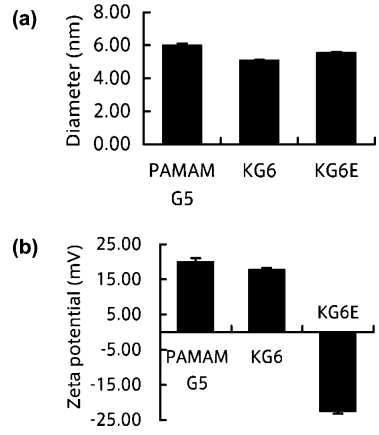


Figure 2. Particle sizes (a) and zeta potentials (b) of dendrimers in PBS, pH 7.4, were measured using a Zetasizer Nano ZS. Each value represents the mean ± SEM of three experiments.

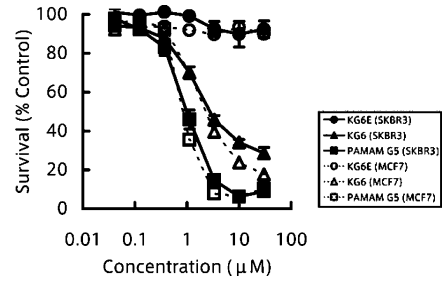


Figure 3. Cytotoxicity of PAMAM G5 (square), KG6 (triangle) and KG6E (circle) dendrimers in SKBR3 (filled) and MCF7 cells (open). Cells were incubated with dendrimers for 24 h before the determination of cell viability by WST-8 assay. Each value represents the mean ± SEM of three separate experiments.

by the more extended bond of the delta carboxylates on the dendritic surface resulting in negative surface potentials.

To evaluate the biocompatibility of KG6E dendrimers, cytotoxicity testing was performed in both breast cancer cell lines. Figure 3 shows similar cell viability profiles of each dendrimer in both cells. The cytotoxicity of PAMAM G5 and KG6 was dose-dependent with an IC_{50} around 0.93 and 1.8 μM, respectively. In contrast, KG6E itself exhibited only a very low toxicity to these cells as shown by more than 85% of cell viability at the maximal concentration (30 μM). This is in agreement with the low cellular membrane interaction of the negative net charge of KG6E dendrimers since the positive potentials induce an electrostatic attraction to anionic cell membranes.[40] These findings indicate that the KG6E dendrimer is a well-controlled and safe carrier for drug payload modification and therapeutic application.

Synthesis of Trast−KG6E−AF Conjugates. To design HER2-targeting drug carriers, anti-HER2 mAb trastuzumab was covalently conjugated to KG6E dendrimers. The chemical nature of the linkage plays a key role in the bond stability of conjugates in a physiological environment before reaching target cells[16] and antibody−receptor recognition.[17] The nonreducible thioether-linked trastuzumab[16] and anti-MUC1 mAb[41]−drug conjugates were well-tolerated in the blood circulation leading to effective drug targeting in HER2-positive breast and MUC1-positive cancer cells, respectively, for therapeutic efficacy whereas disulfide-linked conjugates were associated with premature reductive release of anticancer drugs before reaching tumor cells resulting in poor efficacy and unwanted toxicity.[16] In this regard, trastuzumab was stably bound to KG6E via thioether formation by the reaction of a heterobifunctional cross-linking agent sulfo-LC-SMCC to the thiol group (Scheme 1). The protected thiol groups were introduced into KG6E using a sulfo-LC-SPDP

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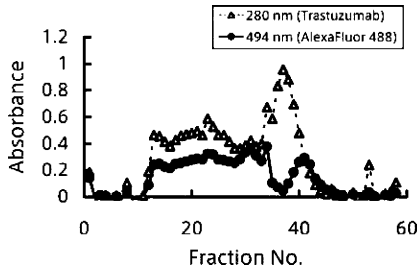

Figure 4. Gel filtration chromatography of Trast−KG6E−AF conjugates after purification using a HiPrep Sephacryl S-300 column. Samples were eluted with PBS at 0.5 mL/min and collected as 1.0 mL/fraction. Trastuzumab and AlexaFluor 488 were detected by UV absorbance at 280 (open triangle) and 494 nm (filled circle), respectively.

cross-linker to provide SPDP−KG6E in a high yield (81.7%). A molar ratio of 2.7 SPDP linkers per KG6E was calculated. In this study, AlexaFluor 488 was used as a fluorescent probe as well as model molecule for dendrimer conjugation by a pH-dependent hydrolytic amide bonding which can be applied for drug conjugation since amide-bonded conjugates are stable in extracellular media with a low drug release[42] but labile in lysosomes for drug liberation and antitumor activity.[37,39,42] The amine groups of SPDP−KG6E reacted with AlexaFluor 488 succinimidyl ester to obtain SPDP−KG6E−AF. Since KG6E showed the UV absorption peak at a wavelength of 210 nm with negligible absorption at a wavelength more than 250 nm, the number of AlexaFluor 488 in KG6E−AF conjugate was absolutely calculated from the absorbance at 494 nm without interference of KG6E dendrimers (data not shown). The number of AlexaFluor 488 molecules was calculated to be 3.0 per KG6E dendrimer. Trastuzumab was derivatized with a thiol reactive maleimide group using sulfo-LC-SMCC to obtain Trast−SMCC, and the number of maleimide molecules on trastuzumab was calculated to be 3.7. Trast-SMCC was reacted with DTT-reduced thiol-KG6E−AF at a 1:1 molar ratio. Trast−KG6E−AF conjugates were obtained after purification using gel filtration with a HiPrep Sephacryl S-300 column, and the fractions absorbing at 280 and 494 nm were collected and pooled (Figure 4). The yield of

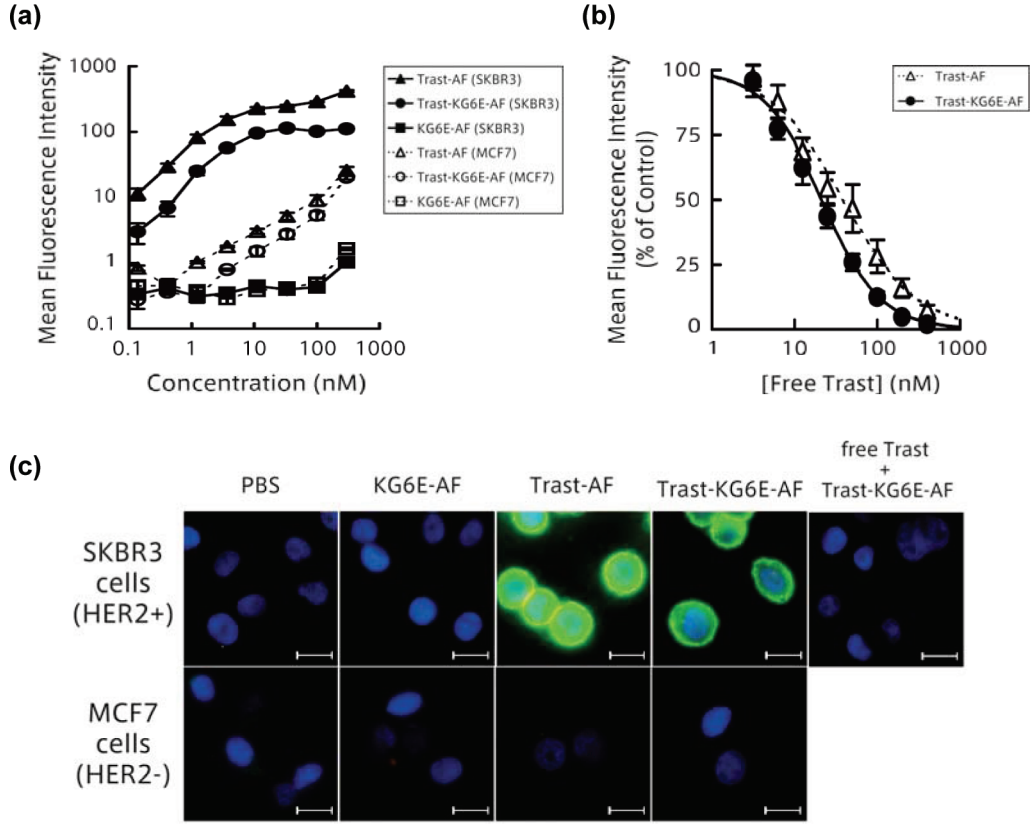

Figure 5. Binding and internalization of Trast−KG6E−AF conjugates in HER2-positive breast cancer cells. (a) Binding affinity of Trast−KG6E−AF conjugates (circle) in HER2-positive SKBR3 (filled) and -negative MCF7 cells (open) was evaluated in the 0.1−500 nM concentration range of antibody at 4 °C for 1 h compared with Trast−AF (triangle) and KG6E−AF (square) conjugates. (b) Competitive binding of 50 nM Trast−KG6E−AF conjugates (filled circle) by free trastuzumab at 3−400 nM in SKBR3 cells at 4 °C for 1 h compared with 50 nM Trast−AF conjugates (opened triangle). Mean fluorescence intensity (MFI) was measured by flow cytometry. Each value is the mean ± SEM of three or four separate experiments. (c) Specific binding of Trast−KG6E−AF conjugates observed by fluorescence microscopy. Cells were incubated with 50 nM conjugates at 37 °C for 1 h or preincubated with 6 μM free trastuzumab at 37 °C for 30 min before conjugate incubation. After fixation with 4% paraformaldehyde, cells were mounted with a SlowFade Gold and DAPI before photography. Scale bars, 20 μm.

Trast−KG6E−AF was 66%. The 3.3 molar ratio of AlexaFluor 488 to trastuzumab was calculated based on the corresponding absorbance. Regarding the 3.0 molecules of AlexaFluor 488 in KG6E−AF conjugate, one molecule of trastuzumab was estimated in Trast−KG6E−AF conjugate.

Binding Affinity of Trast−KG6E−AF Conjugates. In order to evaluate the targeting efficiency of Trast−KG6E−AF conjugates in HER2-positive breast cancer cells, the binding affinity was determined in HER2-positive SKBR3 and HER2-negative MCF7 cells by flow cytometry analysis. After 1 h incubation at 4 °C, Trast−KG6E−AF and AF-labeled trastuzumab (Trast−AF) conjugates were selectively bound to SKBR3, rather than MCF7 cells, in a dose-dependent manner (Figure 5a). As expected, AF-labeled KG6E (KG6E−AF) was weakly bound to these breast cancer cells. For a more clear indication of the HER2-mediated binding specificity of Trast−KG6E−AF conjugates, SKBR3 cells were pretreated with free trastuzumab prior to Trast−KG6E−AF conjugates in a competitive binding assay (Figure 5b). The binding affinity of Trast−KG6E−AF and Trast−AF conjugates were markedly inhibited by free trastuzumab in a dose-dependent fashion with an IC_{50} of 19.8 nM and 36.2 nM, respectively. The quantitative specific binding study of Trast−KG6E−AF conjugates was in parallel with the fluorescent microscopy study (Figure 5c). These results indicate that Trast−KG6E−AF conjugates maintain HER2-specificity for effective targeting to HER2-positive breast cancer cells via an HER2 receptor-mediated binding mechanism. This finding is in agreement with a previous report that the targeting specificity of anti-HER2 liposomes is improved by surface modification with anionic molecules.[43] Thus, it is suggested that KG6E dendrimers are potentially conjugated to antibody for tumor targeting without loss of antibody−antigen recognition.

Internalization and Intracellular Localization of Trast−KG6E−AF Conjugates. To evaluate whether Trast−KG6E−AF conjugates can be internalized into cells after binding, the internalization efficiency was studied by the pulse−chase method. Cells were pulsed with Trast−KG6E−AF or Trast−AF conjugates at 4 °C, chase-incubated at 37 °C, and then surface-quenched with anti-AlexaFluor 488 IgG before flow cytometry analysis. Under these conditions, anti-AlexaFluor 488 IgG achieved more than 80% surface-quenching to remove surface-bound conjugates since it could not cross the cell membrane (data not shown). Therefore, the fluorescence intensity obtained after surface-quenching by anti-AlexaFluor 488 IgG is indicated as the internalized

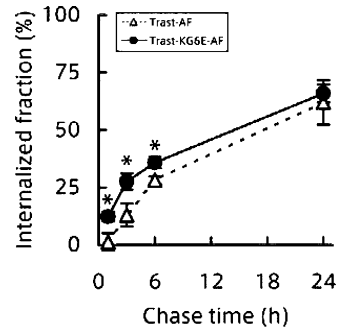

Figure 6. Internalization of Trast−KG6E−AF conjugates in HER2-positive breast cancer cells by the pulse−chase method. SKBR3 cells were incubated with 50 nM Trast−AF (open triangle) or Trast−KG6E−AF (filled circle) conjugates at 4 °C for 1 h (pulse). After washing, cells were incubated at 37 °C for the indicated time (chase). The internalized fraction was evaluated by surface quenching with 0.25 μg/mL of anti-AlexaFluor 488 IgG on ice for 30 min. The mean fluorescence intensity (MFI) was measured by flow cytometry. Each value shows the mean ± SEM of five to eight separate experiments. Statistically significant differences ($^*P < 0.05$) compared with Trast−AF conjugates at each time point.

fraction of Trast−KG6E−AF conjugates. Although Trast−KG6E−AF and Trast−AF conjugates exhibited similar internalization profiles, Trast−KG6E−AF conjugates were significantly taken up in SKBR3 cells with higher internalizing rates during the early uptake period compared with Trast−AF conjugates (Figure 6). These results are consistent with previous reports that trastuzumab−PAMAM[29] and −PLGA nanoparticle[44] conjugates were more rapidly internalized in HER2-positive breast cancer cells in comparison with free trastuzumab.[15,45] It is known that the Fc region of the antibody plays an important role in the inhibition of receptor-recycling after cellular binding and trafficking.[46] In this content, conjugation of trastuzumab with KG6E may sterically interfere with the Fc-induced inhibition of receptor-recycling; therefore, the internalizing rate of Trast−KG6E−AF conjugates was considered to be increased. These results suggest that Trast−KG6E−AF conjugates are efficiently internalized into HER2-positive breast cancer cells upon binding.

To confirm the intracellular localization of Trast−KG6E−AF conjugates, cells were incubated with lysosomal marker

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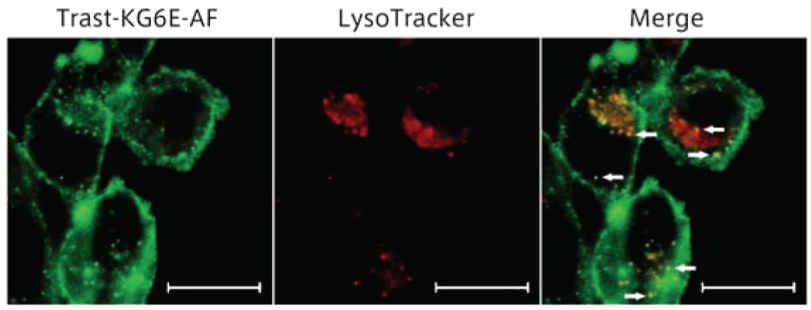

Figure 7. Lysosomal localization of Trast−KG6E−AF conjugates. SKBR3 cells were incubated with Trast−KG6E−AF conjugates at 37 °C for 2 h followed by lysosome staining using 50 μM LysoTracker in red for an additional 1 h. Cells were fixed with 4% paraformaldehyde and mounted with a SlowFade Gold before fluorescence observation. The colocalization of Trast−KG6E−AF conjugates (green) and LysoTracker (red) is indicated in yellow (white arrow). Scale bars, 20 μm.

(Red-LysoTracker) after treatment with Trast−KG6E−AF conjugates. After a 3 h incubation, Trast−KG6E−AF conjugates were internalized and partly sorted to lysosomes as determined by colocalization with LysoTracker (Figure 7). This result corresponds to previous reports that trastuzumab−PAMAM−methotrexate and trastuzumab−acid labile fluorescent probe conjugates were localized in lysosomal vesicles after 24−48 h treatment in HER2-positive cells.[30,47] Lysosomal trafficking is emphasized as an essential pathway for release of not only chemotherapeutic drugs for antitumor activity but also imaging probes for highly sensitive tumor imaging after linker cleavage by lysosomal enzymes at a low pH.[12,16,47−49] Upon cleavage of pH-sensitive linkers, chemotherapeutic drugs such as doxorubicin passively cross lysosomal membrane based on hydrophobicity and notional hydrogen bonding capacity.[50] Importantly, an insufficient cleavage due to rapid lysosomal escape of amide-bonded methotrexate−cationic PAMAM dendrimer was reported for low anticancer activity; on the other hand, conjugation to anionic PAMAM dendrimer could prolong lysosomal residence time which was responsible for sufficient drug liberation from conjugates resulting in effective treatment of methotrexate-resistant tumors.[42] Recently, Kobayashi and colleagues have reported a highly sensitive HER2-selective tumor imaging using pH-activatable fluorescent probe−trastuzumab conjugates which are highly fluorescent only after cleavage in lysosomes.[47] These findings suggest the potentially chemical conjugation with tunable pH-sensitive linkages at least as amide bonding of trastuzumab−anionic KG6E conjugate to chemotherapeutic drugs such as doxorubicin or geldanamycin, and imaging reagents including near-infrared fluorescent probes for effective chemotherapy and diagnosis. Likewise, lysosomal sorting would enable antibody−amino acid dendrimer−chemotherapeutic drug or imaging probe conjugates with pH-sensitive linkers to release drugs or imaging agents and, moreover, to decompose amino acid dendrimers into amino acids by proteolytic enzymes in the lysosomes.[36,37,51] Regarding the *in vitro* assumption of HER2 targeting with high internalization and lysosomal trafficking, trastuzumab-conjugated potentially anionic KG6E dendrimers would be an attractive carrier for HER2-positive cancer targeting of chemotherapeutic drugs and imaging agents.

Conclusion

In this study, we demonstrated that KG6E dendrimers possess an anionic potential with a uniform structure and low cytotoxicity in breast cancer cell lines. Trastuzumab-conjugated KG6E showed HER2-specific binding, high cellular internalization rates and trafficking to lysosomes. These observations indicate that trastuzumab-conjugated anionic amino acid dendrimers are promising carriers for HER2-expressing tumor-selective delivery. These findings provide useful information for the rational design of biocompatible dendrimers and development of mAb−dendrimer conjugates for targeted chemotherapy and bioimaging.

Acknowledgment. This work was supported in part by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan for Scientific

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Research, by a Grant-in-Aid from the Uehara Memorial Foundation, by a Grant-in-Aid from the Mochida Memorial Foundation for Medical and Pharmaceutical Research, and by the Japan Society for the Promotion of Sciences (JSPS) through a JSPS Research Fellowship for Young Scientists. We would like to thank Dr. Takuro Niidome, Department of Applied Chemistry, Faculty of Engineering, Kyushu University, Fukuoka, Japan for valuable advice in dendrimer synthesis and Mr. Ryo Masuda, Department of Physical and Organic Chemistry, Bioorganic Medical Chemistry, Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan for experimental support in the MALDI-TOF-MS analysis.

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