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Note

Ligand peptide-grafted PEGylated liposomes using HER2 targeted peptide-lipid derivatives for targeted delivery in breast cancer cells: the effect of serine-glycine repeated peptides as a spacer

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Abstract

Ligand peptide-grafted PEGylated liposomes have been widely studied for targeted drug delivery systems. Because ligand peptides are commonly grafted using PEG as a spacer on the surface of PEGylated liposomes, the interaction between ligand peptides and their corresponding receptors can be interrupted by steric hindrance of the PEG layer. Therefore, we aimed to develop ligand peptide-lipid derivatives to enhance the targeting efficiency of ligand peptide-grafted PEGylated liposomes, and designed a new ligand peptide-lipid derivatives having serine-glycine repeats $(SG)_n$ as a spacer based on the peptide length calculated by PyMol (v0.99). We selected KCCYSL (KCC) as the ligand peptide for binding to human epidermal growth factor receptor-2 (HER2). We synthesized new KCC-$(SG)_n$-lipid derivatives (n = 3, 5, 7) and evaluated their cellular association in breast cancer cells. KCC-$(SG)_n$/PEGylated liposomes dramatically increased cellular association on HER2-positive breast cancer cells. The results suggest that KCC can be grafted on the surface of KCC-$(SG)_n$/PEGylated liposomes prepared from KCC-$(SG)_n$-lipid derivatives (n = 3, 5, 7). In summary, we succeeded in developing KCC-$(SG)_n$-lipid derivatives for the preparation of ligand peptide-grafted PEGylated liposomes.

Keywords liposomes; targeting; PEG; drug delivery; HER2

Ligand peptide-grafted PEGylated liposomes has been widely studied for selective delivery of anticancer agents to tumor cells (Noble et al., 2014). PEGylation of liposomes enhances stability, blood circulation time, and tumor accumulation via the enhanced permeability and retention effect (Klibanov et al., 1990). Additionally, the use of a longer PEG spacer such as PEG_{2000}, PEG_{3400}, and PEG_{5000}, which forms a non-linear conformation, in ligand peptide-grafted PEGylated liposomes might reduce the accessibility of peptides to their corresponding receptors due to steric hindrance (Lehtinen et al., 2012). In contrast, a shorter PEG has been reported to enhance the targeting efficiency of ligand peptide-grafted PEGylated liposomes (Stefanick et al., 2013). However, as PEG chain becomes shorter, its hydrophobicity increases (Sadzuka et al., 2003); consequently, the addition of a short oligo-lysine chain to the ligand peptide was needed to increase their aqueous solubility in some peptides (Stefanick et al., 2015).

Taking these observations into consideration, we envisaged that ligand peptide–lipid derivatives having a linear spacer with a minimum length should be able to cross the PEG layer of PEGylated liposomes to enhance targeting efficiency via recognition by ligand peptides. Therefore, we designed new peptide-lipid derivatives with different

spacer lengths of the serine-glycine (SG) repeat peptide and a non-α-helix structure (Craig et al., 2008), to graft the ligand peptide on the surface of the PEGylated liposomes. The KCCYSL (KCC) was selected as the ligand peptide (Karasseva et al., 2002) as it specifically binds to human epidermal growth factor receptor-2 (HER2) on breast cancer cells. First, we synthesized new KCC-lipid derivatives having different length SG spacers and prepared KCC-grafted PEGylated liposomes using KCC-$(SG)_n$-lipids (KCC-$(SG)_n$/PEGylated liposomes). Then, we evaluated their physicochemical properties. Finally, we investigated the cellular association properties of KCC-$(SG)_n$/PEGylated liposomes with HER2-positive SK-BR-3 and HER2-negative MDA-MB-231 breast cancer cells. Results were compared with KCC-grafted PEGylated liposomes using KCC-PEG_{2000}-lipid employing a conventional spacer to graft the ligand peptide (KCC-PEG_{2000}/PEGylated liposomes).

1,2-Distearoyl-*sn*-glycero-3-phosphocholine (DSPC) was purchased from Avanti Polar Lipids (Alabaster, AL, USA).

1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[maleimide (polyethylene glycol)-2000] (maleimide-PEG_{2000}-DSPE) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy (polyethylene glycol)-2000] ($mPEG_{2000}$-DSPE) were purchased from NOF (Tokyo, Japan).

Cholesterol was purchased from Nacalai Tesque (Kyoto, Japan). All other chemicals were reagent grade products obtained commercially.

KCC-PEG_{2000}-DSPE was synthesized by slight modification of a method previously described (Terada et al., 2007). CKCCYSL (disulfide bridge: Cys3-Cys4) and maleimide-PEG_{2000}-DSPE were dissolved in 10 mM HEPES buffer and then mixed (5:1, molar ratio) and stirred at 4°C for 96 h. After dialysis (MW cut off 6000-8000 Da), disulfide bonds were reduced by tris-(2-carboxyethyl) phosphine (20 eq.) for 2 h, and the products were dialyzed and lyophilized. The synthesized product was identified by matrix-assisted laser desorption-ionization time of flight mass spectrometry (MALDI-TOF-MS) (Figure S1A).

KCC-$(SG)_n$-lipids were synthesized using Fmoc solid-phase peptide synthesis. The Fmoc protection was removed by treatment with 20% piperidine for 20 min. The coupling of Fmoc-amino acids was performed in the presence of HBTU/HOBt/DIEA for 30 min. Unreacted amino groups were acetylated throughout the synthesis. The KSS linker was added at the back of the SG spacer to enhance the solubility of the peptide-lipid derivatives. After the coupling of Fmoc-Lys(Fmoc)-OH and deprotection of the Fmoc group, palmitic acids (10 eq.) were coupled to the amino group of the Lys residue in the presence of HBTU/HOBt/DIEA for 3 h for four times. Lipids were

cleaved from the resin and deprotected with TFA/TIS/H_2O/EDT (94/1/2.5/2.5) for 3 h. Cleavage products were dissolved in water and dialyzed (MW cut off 6000-8000 Da) for 4 days to remove the acetylated peptides. Disulfide bonds were reduced as described above. The product was dialyzed and lyophilized. The compounds were analyzed by reversed-phased high-performance liquid chromatography (RP-HPLC) and MALDI-TOF-MS (Figure S1B-D, S2).

PEGylated liposomes and KCC-PEG_{2000}/PEGylated liposomes were prepared by slight modification of a method previously described (Terada et al., 2007). KCC-$(SG)_n$/PEGylated liposomes were prepared by slight modification of a method previously described (Garg et al., 2009). Dried lipid films were hydrated with HEPES buffer (pH 7.4) at 65°C for 10 min. The liposomes were sonicated for 15 min in a bath sonicator and for 3 min in a probe sonicator. A total of 0.5 mol% rhodamine-DOPE was incorporated to label the liposomes. The particle size and zeta-potential of the liposomes were determined using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK).

SK-BR-3 and MDA-MB-231 cells were purchased from the ATCC and ECACC, respectively. The cells were and cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (AusGene X, Brisbane, Australia), 100 U/mL

penicillin, 100 µg/mL streptomycin (Wako, Osaka, Japan). The cells were maintained under an atmosphere of 5% CO_2 at 37 °C.

For cellular association experiments, cells were seeded in 24-well plates (5.0×10^4 cells/cm^2). After 24 h, cells were incubated in serum-free RPMI-1640 containing 100 µM liposomes. After 3 h, cells were collected and resuspended in 50 µL PBS. Then cells were analyzed using a Tali® Image-Based Cytometer (Invitrogen, Carlsbad, CA, USA). For confocal laser scanning microscopy (CLSM) studies, cells were seeded in a glass-bottom dish (2.2×10^4 cells/cm^2). After 24 h, cells were incubated in serum-free RPMI-1640 containing 100 µM liposomes. After fixation, nuclei were stained with DAPI. Confocal images were obtained using a CLSM (LSM 710, Carl Zeiss, Oberkochen, Germany).

Two-group comparisons were performed by unpaired t-test. Analysis of variance was used to test the statistical significance of differences between groups. Multiple comparisons between all groups were performed with the Tukey-Kramer test.

It has been reported that the maximum PEG layer thickness is approximately 5 nm (Ceh et al., 1997; Garbuzenko et al., 2005). Peptide-lipid derivatives having different SG spacer lengths were designed based on the peptide length calculated by PyMol (v0.99) (Figure 1). The calculated length between KCC and the lipid of the

KCC-$(SG)_3$-lipid, KCC-$(SG)_5$-lipid, and KCC-$(SG)_7$-lipid was 3.5, 5.0 and 6.4 nm, respectively. Therefore, the use of $(SG)_5$ or $(SG)_7$ as a spacer is expected to provide enough length to graft KCC on the surface of PEGylated liposomes. All final products were analyzed by MALDI-TOF-MS and RP-HPLC, and they showed the expected molecular weight and were over 88% purity. The particle sizes of the KCC-$(SG)_n$/PEGylated liposomes were the same as those of the KCC-PEG_{2000}/PEGylated liposomes (Table 1). Their size, from 100 to 200 nm, is expected to achieve longer circulation and higher tumor accumulation (Ishida et al., 1999).

The cellular association of KCC-PEG_{2000}/PEGylated liposomes or KCC-$(SG)_n$/PEGylated liposomes with SK-BR-3 cells was evaluated. As shown in Figure 2A, use of KCC-PEG_{2000}/PEGylated liposomes resulted in a slight increase of the fluorescence intensity, even when the liposomes were modified with 6% KCC-PEG_{2000}-DSPE. However, a remarkable enhancement of cellular association was observed for all KCC-$(SG)_n$/PEGylated liposomes. The cellular association of KCC-$(SG)_{5,7}$/PEGylated liposomes showed very similar results in HER2-positive SK-BR-3 cells, while the cellular association of KCC-$(SG)_3$/PEGylated liposomes at a concentration of 25 μM was significantly decreased compare to that of KCC-$(SG)_5$,

$_7$/PEGylated liposomes (Figure 2B, 2C). In contrast, KCC-$(SG)_n$/PEGylated liposomes did not enhance cellular association in HER2-negative MDA-MB-231 (Figure 2D), suggesting that KCC-$(SG)_n$/PEGylated liposomes might interact with SK-BR-3 cells via binding to HER2. The cellular association of KCC-$(SG)_n$/PEGylated liposomes prepared from KCC-$(SG)_3$-lipid, KCC-$(SG)_5$-lipid and KCC-$(SG)_7$-lipid derivatives correspond with the results from the simulation of spacer length calculated by PyMol. These results suggest that the KCC-$(SG)_n$/PEGylated liposomes can bind to breast cancer cells over-expressing HER2.

To confirm the data from the cellular association experiments, the association of KCC-$(SG)_n$/PEGylated liposomes and KCC-PEG_{2000}/PEGylated liposomes in SK-BR-3 and KCC-$(SG)_n$/PEGylated liposomes in MDA-MB-231 cells was evaluated by CLSM. Minimal fluorescence was observed in SK-BR-3 cells treated with KCC-PEG_{2000}/PEGylated liposomes and MDA-MB-231 cells treated with KCC-$(SG)_n$/PEGylated liposomes (Figure 3A). However, the highest fluorescence was observed in SK-BR-3 cells treated with KCC-$(SG)_n$/PEGylated liposomes (Figure 3B). These results are in accordance with the results of the cellular association experiments.

In conclusion, we designed and synthesized ligand peptide-lipid derivatives, KCC-$(SG)_n$-lipids, for preparation of HER2 over-expressed breast cancer cells directed

to KCC-grafted PEGylated liposomes. The cellular association study revealed that KCC-$(SG)_{5,7}$/PEGylated liposomes could be efficiently targeted to HER2 over-expressing breast cancer cells. We succeeded in developing new KCC-$(SG)_n$-lipid derivatives for the preparation of ligand-grafted PEGylated liposomes. These information is valuable for the development of targeted ligand-grafted liposomes.

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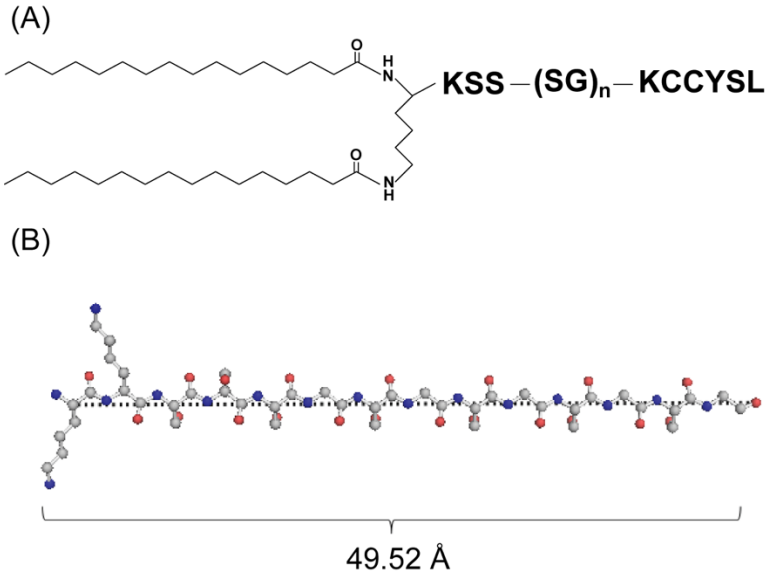

Figure 1. Chemical structures of KCC-$(SG)_n$-lipid (A) and calculated peptide length between KCC and lipid ($KKSS(SG)_5$) (B). Length was calculated by PyMol (1 Å=0.1 nm).

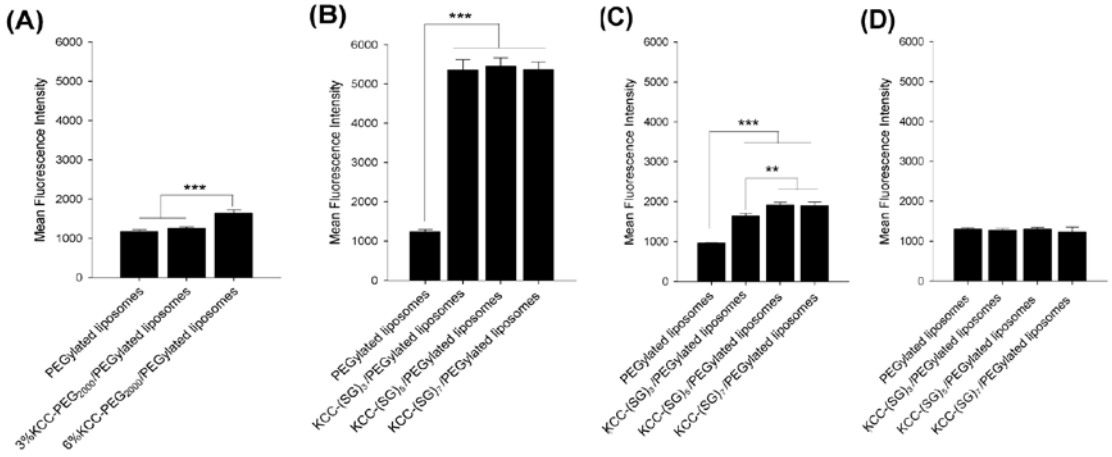

Figure 2. Cellular association of KCC-PEG_{2000}/PEGylated liposomes in SK-BR-3 cells (A), KCC-$(SG)_n$/PEGylated liposomes at a concentration 100 µM in SK-BR3 cells (B), KCC-$(SG)_n$/PEGylated liposomes at a concentration 25 µM in SK-BR3 cells (C), and KCC-$(SG)_n$/PEGylated liposomes in MDA-MB-231 cells (D). Cells were incubated with liposomes for 3 h, then analyzed using Tali® Image-Based Cytometer. Data are the mean ± SD for triplicate experiments (**$P < 0.01$, ***$P < 0.001$).

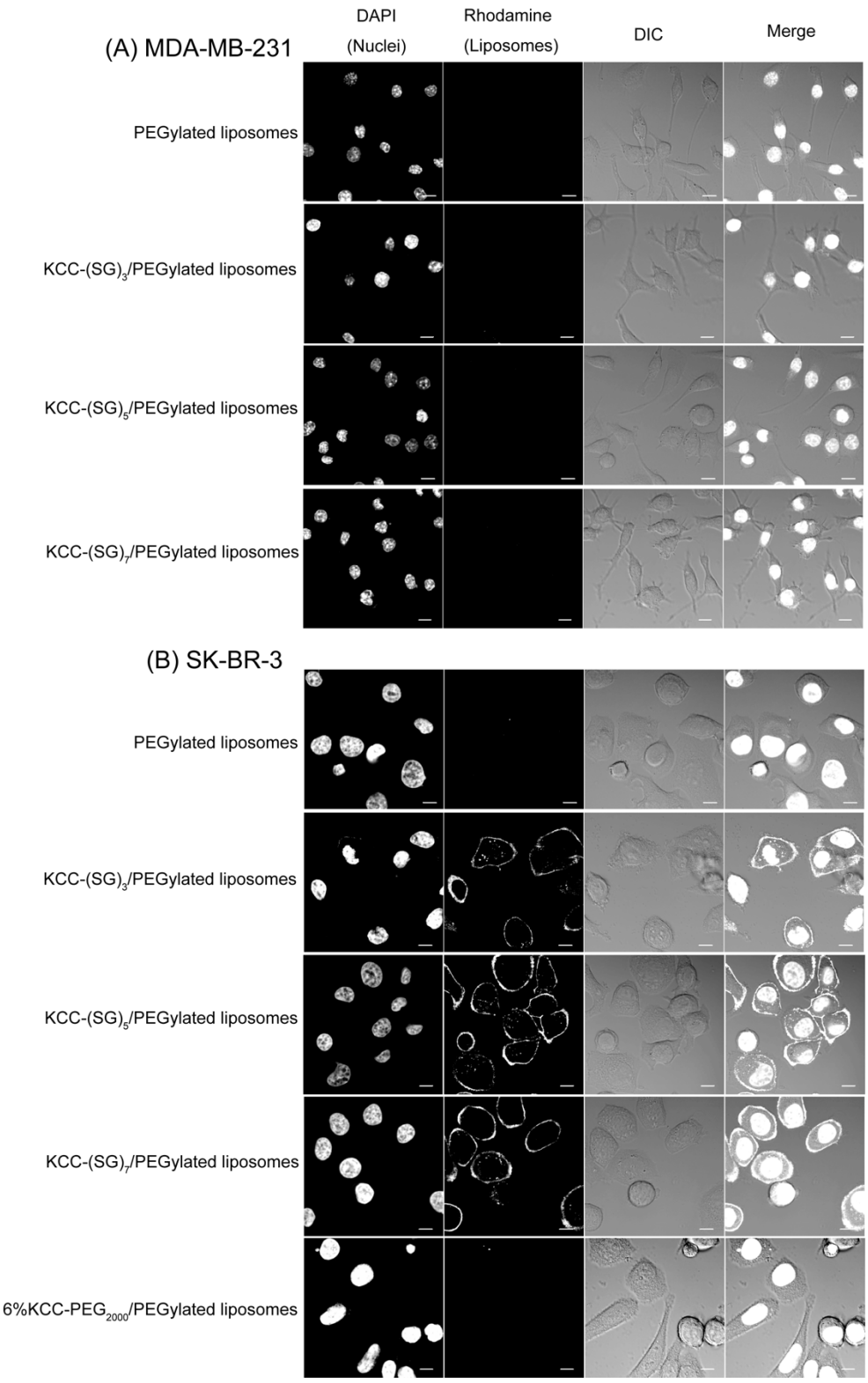

Figure 3. CLSM images of MDA-MB-231 (A) and SK-BR-3 cells (B). Cells were incubated with PEGylated liposomes, KCC-PEG_{2000}/PEGylated liposomes or KCC-$(SG)_n$/PEGylated liposomes for 3 h. Scale bars, 10 μm.

Table 1. The lipid composition, mean particle sizes and zeta-potentials of liposomes.

Lipid composition (molar ratio)	Size (nm)	Zeta-potential (mV)
PEGylated liposomes	121.9 ± 8.8	−17.6 ± 1.4
(DSPC/Cholesterol/mPEG$_{2000}$-DSPE=55/35/10)		
3%KCC-PEG$_{2000}$/PEGylated liposomes	139.4 ± 18.6	−16.4 ± 1.8
(DSPC/Cholesterol/mPEG$_{2000}$-DSPE/KCC-PEG$_{2000}$-DSPE=55/35/7/3)		
6%KCC-PEG$_{2000}$/PEGylated liposomes	155.9 ± 26.8	−17.0 ± 2.0
(DSPC/Cholesterol/mPEG$_{2000}$-DSPE/KCC-PEG$_{2000}$-DSPE=55/35/4/6)		
KCC-(SG)$_3$/PEGylated liposomes	130.8 ± 3.4	−15.3 ± 2.2
(DSPC/Cholesterol/mPEG$_{2000}$-DSPE/ KCC-(SG)$_3$-lipid=52/35/10/3)		
KCC-(SG)$_5$/PEGylated liposomes	135.5 ± 10.5	−15.0 ± 2.7
(DSPC/Cholesterol/mPEG$_{2000}$-DSPE/ KCC-(SG)$_5$-lipid=52/35/10/3)		
KCC-(SG)$_7$/PEGylated liposomes	126.5 ± 5.3	−14.9 ± 2.5
(DSPC/Cholesterol/mPEG$_{2000}$-DSPE/ KCC-(SG)$_7$-lipid=52/35/10/3)		

Results are expressed as the mean ± S.D. of three experiments.