A surface-modified functional liposome capable of binding to cell membranes

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A liposome including the lipopeptide RGD-C4A2, whose surface is modified by a GRGDS-repeating peptide ligand, is found to bind to NIH3T3 cells *via* the interaction between the peptide ligand and the membrane receptor.

Liposomes have been extensively examined as potential carriers of drugs,[1] proteins[2] and DNA[3–5] for drug delivery systems (DDS), in spite of their nonspecificity of delivery due to entrapment by reticuloendothelial systems.[6] We present here a new functional liposome (RGD liposome) whose surface is modified with a high density of peptide chains. Lipopeptide **RGD-C4A2** composed of peptide and lipid parts was synthesized in advance by a method similar to that previously reported,[7,8]† and was then incorporated into the liposome bilayer membrane when the liposome was prepared using the freeze-thaw method.[9]‡ **RGD-C4A2** has a five-times repeated GRGDS sequence, which is the same as the cell adhesion sequence of fibronectin,[10] and therefore is thought to bind to the cell membrane receptor.

The peptide ligand extruded from the RGD liposome into the aqueous phase was expected to bind specifically to an anti-peptide antibody. We therefore confirmed using immunoelectron microscopy[11,12]§ that the peptide ligand was actually placed in the outer aqueous phase. Experimentally,gold colloidal particles were clearly observed on the surface of the liposome when **RGD-C4A2** was located in the liposome membrane, while they were rarely seen in the control liposome.

We applied flow cytometry to evaluate the binding between RGD liposomes and the NIH3T3 cell line (Fig. 1).¶ NIH3T3 was established from mouse fibroblasts, had normal cell-like properties, and was thought to have fibronectin receptors and bind to the present modified liposome. It is worth noting that RGD liposome/NIH3T3 [Fig. 1(*c*)] gave greater fluorescence intensity than either the NIH3T3 alone [Fig. 1(*a*)] or the control liposome/NIH3T3 [Fig. 1(*b*)]. This indicates that the RGD liposome can bind to the cells to a greater degree than the control liposome. We undertook an inhibition experiment to confirm that the peptide chain of **RGD-C4A2** participates in the binding to NIH3T3. The fluorescence intensity was actually reduced by 34.8% in the presence of the free peptide, H-Trp-(Gly-Arg-Gly-Asp-Ser)$_5$-NH$_2$ [Fig. 1(*d*)]. This strongly suggests that the binding between the RGD liposome and the NIH3T3 cell takes place *via* the interaction between the peptide chain of **RGD-C4A2** and the membrane receptor of NIH3H3.

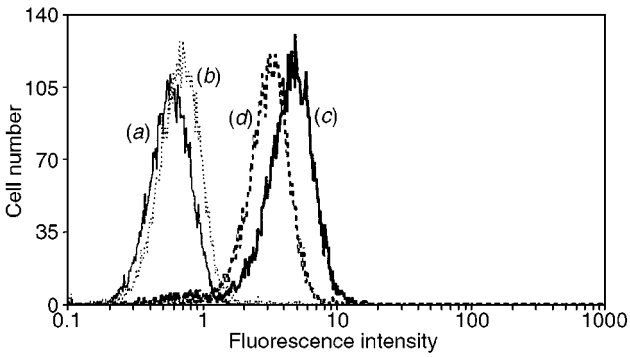
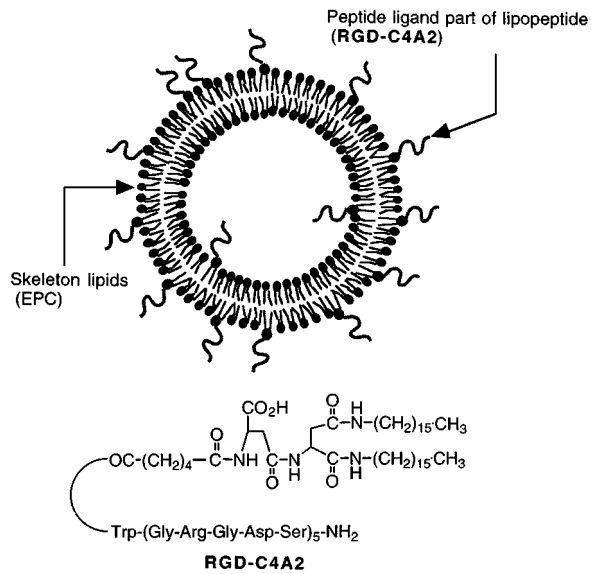


Fig. 1 Flow cytometry of (*a*) NIH3T3 only, (*b*) NIH3T3 + control liposome, (*c*) NIH3T3 + RGD-liposome and (*d*) NIH3T3 + RGD liposome + H-Trp-(Gly-Arg-Gly-Asp-Ser)$_5$-NH$_2$.



Viruses have been used as vectors in the field of gene therapy.[13] In spite of their prominent function as drug carriers, problems endangering vital functions have not yet been completely solved and thus their clinical use is still limited. Compared to viruses, however, liposomes are superior in safety and reproducibility if only the problem of specificity can be solved. Many trials have been made to give liposome the binding specificity to cells. For example, the liposome surface has been equipped with a glycolipid,[14] transferrin[15] or an antibody[16,17] as a binding anchor to cells. Among various ligand candidates, peptides seem the most appropriate for the present purpose, since information about amino acid sequences and corresponding receptors is easily available and the synthetic methods are also well established.[18] In ordinary methods such as the 'succinimide method',[16] proteins or peptides are reacted with amino groups of specific lipid molecules already incorporated into lipid bilayer membranes. However, it is very difficult to modify the surface of liposomes at high density by this ordinary method, because the reactivity is reduced owing to the steric repulsion between the liposome membrane and the protein or peptide ligand fragments. In contrast to the succinimide method, our new technique was highly efficient in incorporating the lipopeptide in the liposome membrane, *viz.* more than 90% of the added **RGD-C4A2** was introduced into the liposome.

In conclusion, we have succeeded in preparing a liposome whose surface is modified by peptide chains at high density. This liposome has been shown to bind to the membrane receptor of NIH3H3 cells using the peptide ligand on its surface. This method may open a way to wide application of liposomes for clinical use.

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Notes and references

† *Selected data* for **RGD-C4A2**: $\delta_H(D_2O)$ 7.34–7.60 (m, 2H, Trp), 7.19 (s, 1H, Trp), 6.98–7.14 (m, 2H, Trp), 4.68–4.82 (m, 6H, CH), 4.28–4.50 (m, 10H, CH), 3.75–4.12 (m, 30H, Ser-CH_2, Gly-CH_2), 3.21 (m, 10H, Arg-$CH_2CH_2CH_2N$), 2.74–2.94 (m, 10H, Asp-CH_2), 2.04–2.34 (m, 4H, $COCH_2$), 1.54–1.98 (m, 20H, Arg-$CH_2CH_2CH_2N$), 1.36–1.54 (m, 4H, $NHCH_2CH_2$), 1.27 (s br, 52H, $2(CH_2)_{13}CH_3$), 0.88 (t, 6H, $2CH_2CH_3$, *J* 6.6); MALDI-TOFMS: calc. 3369.8 (MH^+), found: 3370. Retention time in HPLC (YMC-Pack ODS-AM, 4.6 × 250 mm, eluant: 0.08% aq. TFA–MeCN from 100 : 0 to 50 : 50 over 30 min, flow rate: 0.8 ml min^{-1}): 17.4 min.

‡ After **RGD-C4A2** was dissolved in 10 μl of 20% AcOH, 100 μl of egg phosphatidylcholine (EPC)–chloroform solution (5 μmol ml^{-1}) was added. These two separated phases became homogeneous after addition of a few drops of MeOH. The solvent was evaporated under reduced pressure to form a thin lipid film on the wall of a test tube. This film was left *in vacuo* for at least 12 h to thoroughly remove the solvent. Hank's buffer solution (pH 7.2) (1 ml) was added to the thin lipid film, which was vortexed for 5 min at room temperature and subjected to repeated (7 times) freezing (−196 °C) and thawing (70 °C). The solution was then extruded 5 times through a polycarbonate membrane (pore size: 0.1 μm).

§ An antibody against H-Cys-Trp-(Gly-Arg-Gly-Asp-Ser)$_5$-NH_2 [one cysteine residue was added to H-Trp-(Gly-Arg-Gly-Asp-Ser)$_5$-NH_2] was prepared by an ordinary method from a female New Zealand white rabbit using KLH (Keyhole Limpet, Hemocyanin)-conjugated Cys-Trp-(Gly-Arg-Gly-Asp-Ser)$_5$-NH_2. After labeling the modified liposome by the prepared primary antibodies and a secondary antibody (goat anti-rabbit IgG), the samples were negatively stained with uranyl acetate, stabilized by perpendicular carbon evaporation, and observed under a transmission electron microscope.

¶ To the lipopeptide (0.1 μmol) dissolved in 20% AcOH (10 μl) was added a chloroform solution containing EPC (1.6 μmol), cholesterol (Ch) (0.4 μmol) and *N*-(fluorescein-5-thiocarbamoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (FITC-PE) (0.02 μmol). A few drops of MeOH were added to make the solution homogeneous and the modified liposome was prepared in a similar manner as described above. After the liposome and NIH3T3 cells had been incubated at 37 °C for 30 min, the cells were washed by centrifugation (1000 rpm, 3 min) at 37 °C, twice with Dulbecco's modified Eagle's medium (DMEM) and once with CMF-PBS. The cells were then suspended in 1.0 ml of CMF-PBS containing BSA (0.1%) and sodium azide (0.1%), and the fluorescence emitted from the cells was measured on the flow cytometer.

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