Synthesis and Characterization of Chelator-Lipids for Reversible Immobilization of Engineered Proteins at Self-Assembled Lipid Interfaces[§,⊥]

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Abstract: In molecular biology and protein engineering the immobilized metal ion affinity chromatography (IMAC) using a NTA-chelator is a very powerful technique in identification and isolation of oligo-histidine-tagged fusion proteins. This concept was transferred to the properties of self-assembling systems with the aim of reversible immobilization, orientation of biomolecules, and functionalization of lipid interfaces. Here we describe the synthesis and the chemical and physical characterization of such metal affinity lipids. The NTA-chelator was coupled either to a phospholipid, DPPE, or to a synthetic lipid, DODA. Metal complex formation was investigated by means of TLC and FTIR techniques. Using film balance techniques the generation of metal sensitive lipid films is demonstrated. In the presence of Ni^{2+} drastic changes of the area-pressure isotherms were observed. Furthermore, the specific ligand binding of imidazole as a model compound for oligo-histidine-tagged fusion proteins to these functionalized metal-lipid films was investigated.

Introduction

Immobilized metal ion affinity chromatography (IMAC) was introduced by Porath et al.[1] as a new tool for the purification of proteins and peptides. Since that time IMAC has been used in the isolation of proteins,[2] peptides,[3] and nucleic acids.[4] In IMAC the metal ion, e.g. Ni^{2+}, Zn^{2+}, or Cu^{2+}, is complexed and immobilized by a chelator, which is covalently linked to a solid matrix. The metal complex contains free coordination sites, which can be occupied by additional electron donor groups. Of special interest are histidine residues on protein surfaces.[5] Combining this principle with protein engineering leads to an elegant strategy for identification and rapid one-step purification of gene products expressed as fusion proteins with an oligo-histidine tag. The oligo-histidine tag should serve as a high affinity binding sequence for the purification of any fusion protein via metal chelating adsorbents. One important and well characterized chelator for the oligohistidine tag is the *N*-nitrilotriacetic acid (NTA) derivate *N*-(1-carboxy-5-aminopentyl)iminodiacetic acid.[6]

Functionalized lipids play an increasing role in biotechnology, for example, in the biofunctionalization of membranes,[7] for the liposome targeting with antibodies,[8] for drug delivery systems,[9] for immobilizing receptors on lipid surfaces,[10] and in biosensor applications.[11] The advantage of functionalized lipid systems, apart from being bioadaptable, lies in their two-dimensional fluidity, their inherent potential of self-organization and pattern formation.

To link lipid technology to the concept of fusion proteins, we have synthesized lipid molecules, which contain NTA as a metal chelator head-group able to bind electron donor groups such as imidazole or histidine. The synthesis was carried out with two different amphipatic molecules, a phospholipid (DPPE), and a dioctadecylamine (DODA), summarized schematically in Figures 1 and 2. Because of the chemical instability of phospholipids the head-group and the lipophilic part have been processed separately; only in the last step of the synthesis have they been condensed via the chemistry of active esters (Figure 1). Since DODA is highly stable chemically, an alternative reaction pathway could be followed. Starting with DODA the molecule was build up step by step as illustrated in Figure 2.

In this report the synthesis of the two NTA-lipids is described. The products are characterized chemically and physically. Specific ligand binding of imidazole as a model compound

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⊥ Abbreviations: abs, absolute; AcOH, acetic acid; Boc, *tert*-butyloxycarbonyl; CH_2Cl_2, methylene chloride; $CHCl_3$, chloroform; DCC, dicyclohexylcarbodiimide; DMAP, 4-(dimethylamino)pyridine; DMF, dimethylformamide; DMPG, 1,2-dimyristoyl-sn-glycero-3-phospho-[1-*rac*-glycerol]; DPPE, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine; DODA, dioctadecylamine; DODA-Suc, *N*-succinyldioctadecylamine; DODA-Suc-NHS, *N*-[(hydroxysuccinimidyl)succinyl]dioctadecylamine; DODA-Suc-Lys-Boc, N^{α}-[*tert*-butyloxycarbonyl]-N^{ϵ}-[(dioctadecylamino)succinyl]-L-lysine; DODA-Suc-Lys, N^{ϵ}-[(dioctadecylamino)succinyl]-L-lysine; NTA-DODA, N^{α},N^{α}-bis[carboxymethyl]-N^{ϵ}-[(dioctadecylamino)succinyl]-L-lysine; EDTA, ethylenediaminetetraacetic acid; FAB, fast atomic bombardment; FTIR, Fourier transformed infrared spectroscopy; IMAC, immobilized metal ion affinity chromatography; MS, mass spectroscopy; NEt_3, triethylamine; NHS, *N*-hydroxysuccinimide; NMR, nuclear magnetic resonance spectroscopy; NTA, N^{α},N^{α}-bis[carboxymethyl]-L-lysine; NTA-DPPE, N^{α},N^{α}-bis[carboxymethyl]-N^{ϵ}-[(1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamino)succinyl]-L-lysine; Suc-, succinyl-; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; Z, benzyloxycarbonyl.

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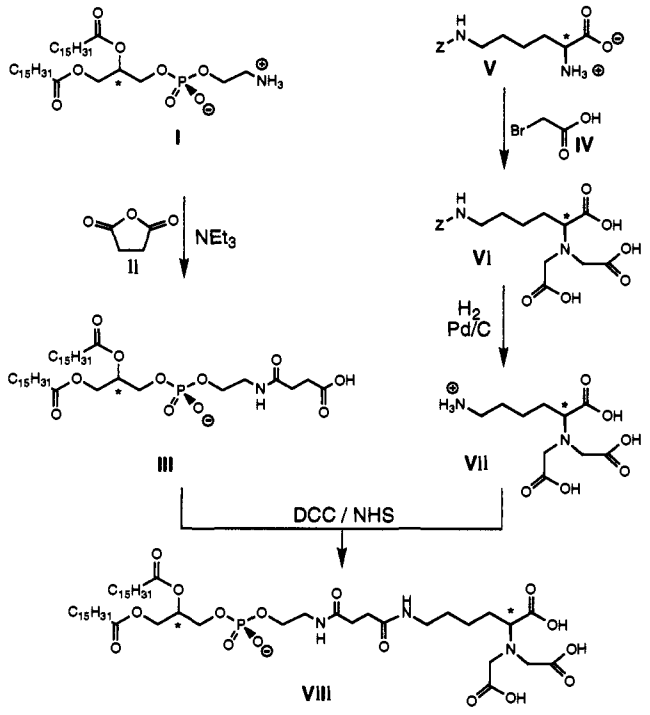

Figure 1. Synthesis of the NTA–lipid including phospholipids.

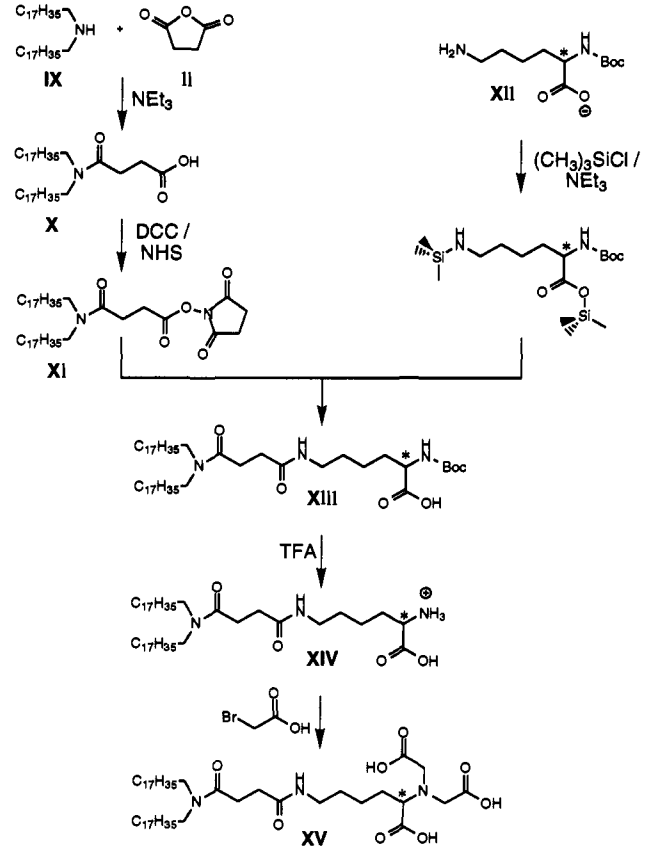

Figure 2. Synthesis of the NTA–lipid including synthetic lipids.

of oligo-histidine-tagged fusion proteins has been confirmed with a lipid monolayer system at the air/water interface.

Materials and Methods

Materials. The following chemicals were used: DPPE (Avanti Polar Lipids, Birmingham, AL), DODA, N^{α}-Boc-lysine, and trimethylchlorosilane (Fluka, Neu-Ulm, Germany), N^{ϵ}-Z-L-lysine, bromoacetic acid, succinic anhydride, bromocresol green spray reagent, molybdenum blue spray reagent, fluorescamine spray reagent, and Pd/C (5%) (Aldrich Chemie, Steinheim, Germany). Thin-layer chromatography (TLC) plates (Kieselgel 60 F_{254}), Lichroprep Diol (40–63 μm, 60-230 mesh), and silica gel (40–63 μm, 230–400 mesh) were ordered by Merck (Darmstadt, Germany). All other chemicals and solvents were purchased from Fluka (Neu-Ulm, Germany) and were reagent p.a. grade. Solvents were used without further purification unless otherwise stated.

Methods. Reactions were monitored by TLC (precoated plates 0.25 mm, silica gel 60 F_{254}) and visualized by UV (Z-group), fluorescamine (amino group), molybdenum blue (phosphate group), bromocresol green (acids and bases), or iodine. Solvent ratios are given in volume/volume. If necessary, products were purified by silica gel column chromatography (Merck Lichroprep Diol or silica gel). ^{1}H-NMR (500 MHz) spectra were recorded on a Bruker AM 500, and ^{13}C-NMR (100 MHz) and ^{31}P-NMR (161 MHz) were recorded on a Bruker AM 400. Chemical shifts (δ) are given in ppm relative to the solvent for ^{1}H and ^{13}C and relative to 85% phosphoric acid as an external standard for ^{31}P-NMR.

Mass spectroscopy (Finnigan, MAT, Forster City, CA) was performed in the negative or positive ion mode.

Fourier transformed infrared spectra were measured with a Nicolet 60 SXR (wavenumber resolution of 2 cm^{-1}) using a liquid helium cooled ACT detector. The samples were placed on a ATR crystal (ZnSe), dried under nitrogen, and subsequently measured (average of 1000 scans for each spectrum). Data were analyzed using the software provided by Nicolet.

Film balance measurements were performed with the following setup: The film balance consists of a trough (Teflon) of 450 × 900 mm^2 area and a movable barrier. The surface tension is measured with a Wilhelmy system. For temperature control (accuracy ± 0.5 °C), thermostated water was circulated through copper tubes attached to the bottom plate of the trough. For fast heat transfer, the floor of the trough was a thin Teflon film (0.1 mm) attached to the thermostated ground plate. Temperature was measured by a Pt 100. The entire film balance was covered by a glass hood. Pressure/area diagrams of lipid monolayers were obtained by isothermal compression and expansion. Barrier speed and film pressure were measured and controlled by an IBM-compatible PC-AT.

Results

Synthesis of NTA-Lipids. Synthesis of *N*-Succinyl-[1,2-dipalmitoyl)-sn-glycero-3-phosphoethanolamine] (Suc-DPPE) III. DPPE **I** (250 mg, 0.361 mmol) and 150 μL of Et_3N were dissolved in 20 mL of $CHCl_3$/MeOH (9:1). A solution of 50 mg (0.5 mmol) of succinic anhydride **II** dissolved in 20 mL of CH_2Cl_2 was added, and the solution was stirred at room temperature. Total conversion of compound **I** was achieved after 4 h as monitored by TLC in $CHCl_3$/MeOH/H_2O (65:25:4) developed with fluorescamine. The reaction mixture was acidified by adding 40 mL of $CHCl_3$ and 40 mL of 0.02 M citrate/0.02 M phosphate buffer (pH 5.5) and stirred for 30 min. The aqueous phase was extracted three times with 60 mL of $CHCl_3$ and discarded. The combined organic phases were dried over anhydrous sodium sulfate, and solvent was removed in vacuo: yield 265 mg **III** (0.343 mmol) 95%; TLC R_f (**III**) 0.63 in $CHCl_3$/MeOH/H_2O (65:25:4), R_f(**I**) 0.78 in $CHCl_3$/MeOH/H_2O (65:25:4); FTIR 3354 cm^{-1} (OH), 2922 cm^{-1} (CH_2), 2858 cm^{-1} (CH_2), 1726 cm^{-1} (C=O), 1666 cm^{-1} (Amid I), 1549 cm^{-1} (Amid II); ^{1}H NMR (500 MHz, CD_2Cl_2) δ 0.95 (t, 6H, $CH_3(CH_2)_{14}$), δ = 1.1–1.4 (m, 48H, $CH_3(CH_2)_{12}CH_2$), δ = 1.6 (m, 4H, $CH_3(CH_2)_{12}CH_2CH_2$-CO), δ = 2.3 (t, 4H, $CH_3(CH_2)_{12}CH_2CH_2CO$), δ = 2.5 (m, 4H, $NHCOCH_2CH_2COO^-$), δ 3.4 (dt, 2H, $PCOCH_2CH_2NH$), δ = 3.8 (m, 2H, $POCH_2CH_2NH$), δ = 4.15 (m, 2H, CH_2CHCH_2-OP), δ = 4.3 (dd, 2H, CH_2CHCH_2OP), δ = 5.2 (m, 1H, CH_2CHCH_2), δ 7.4 (t, 1H, NHCO); ^{13}C-NMR (100 MHz, CD_2-Cl_2) δ 8.7 (CH_3), δ = 14.17, 22.98, 25.20, 29.44, 29.65, 29.84, 30.00 ($CH_3(CH_2)_{13}CH_2CO$), δ 31.56 ($NHCOCH_2CH_2COO^-$), δ = 32.24 ($NHCOCH_2CH_2COO^-$), δ = 34.32 ($CH_3(CH_2)_{13}CH_2$-COOCH), δ = 34.52 ($CH_3(CH_2)_{13}CH_2COOCH_2$), δ = 46.02 ($POCH_2CH_2NH$), δ = 62.69 ($POCH_2CH_2NH$), δ = 64.0 (CH_2-$CHCH_2OP$), δ = 64.60 (CH_2CHCH_2OP), δ = 70.53 (CH_2-$CHCH_2OP$), δ = 172.62 (NHCO), δ = 173.21 (CH_2COOCH_2),

δ = 173.54 (CH_2COOCH), δ = 175.90 (COO^-); ^{31}P-NMR (161 MHz, CD_2Cl_2) δ = 0.42 ppm (s); MS ($C_{41}H_{78}O_{11}NP$, FAB negative) M – H^+ = 790 g/mol.

Synthesis of (N^α,N^α-Bis[carboxymethyl]-N^ϵ-[benzyloxycarbonyl]-L-lysine (N^ϵ-Z-NTA) VI. Bromoacetic acid **IV** (4.17 g, 30.2 mmol) was dissolved in 15 mL of 1.5 N NaOH and cooled to 0 °C. N^ϵ-Benzoyloxycarbonyl-L-lysine **V** (2 g, 15.1 mmol) in 25 mL of 1.5 N NaOH was added dropwise to this solution. After 2 h cooling was stopped, and the solution was stirred over night at room temperature. After heating at 50 °C for 2 h and cooling, 45 mL of 1 M HCl was added dropwise. The precipitate was filtered off and dried in vacuo over $CaCl_2$: yield 3.4 g of **VI** (8.5 mmol) 56%; TLC R_f (**VI**) 0.15 ($CHCl_3$/MeOH 1:1); ^{1}H-NMR (500 MHz, DMSO) δ = 1.2–1.8 (m, 8H, $CHCH_2CH_2CH_2CH_2NH_2$), δ = 3.3 (t, 1H, CH_2CHN), δ = 3.5 (s, 4H, NCH_2COO), δ = 5.1 (s, 2H, phenyl-CH_2O), δ = 7.1 (m, 5H, phenyl-H); ^{13}C-NMR (100 MHz, DMSO) δ = 174.02 ($CHCOOH$), δ = 173.35 (CH_2COOH), δ = 156.08 ($OCONH$), δ = 137.31 (phenyl-C1), δ = 128.34 (phenyl-C-2,3), δ = 127.70 (phenyl-C-4), δ = 65.10 (NCH_2COOH), δ = 64.44 (CH), δ = 59.59; (phenyl-CH_2O), δ = 53.56 ($OCONHCH_2CH_2$), δ = 47.30 ($OCONHCH_2CH_2$), δ = 29.13 ($CH_2CH(COO)NH_2$), δ = 22.99 ($OCONHCH_2CH_2CH_2CH$).

Synthesis of N^α,N^α-Bis[carboxymethyl]-L-lysine (NTA) VII. N^ϵ-Z-NTA **VI** (3 g, 7.5 mmol) was dissolved in 50 mL of MeOH/H_2O (20:1) and, after the addition of a spatula tip of 5% Pd/C, hydrogenated at room temperature and normal pressure. Total hydrogenation of compound **VI** was achieved after 90 min as monitored by TLC in CH_3CN/H_2O (4:1) developed with UV, I_2, and fluorescamine. The catalyst was filtered off, and solvent was removed in vacuo. The resulting precipitate was redissolved in 5 mL of H_2O and 200 mL of EtOH was added. The product crystallizes at 0 °C within 2 days. The crystals were filtered off and dried in vacuo: yield 1.7 g of **VII** (5.7 mmol) 76%; TLC R_f (**VII**) = 0.2 in CH_3CN/H_2O (4:1). ^{1}H-NMR (500 MHz, D_2O): δ = 1.4–2.0 (m, 8H, $CHCH_2CH_2CH_2CH_2NH_2$), δ = 3.0 (t, 1H, $NCHCH_2$), δ = 3.8 (s, 3H, NCH_2COO). ^{13}C-NMR (100 MHz, D_2O): δ = 174.98 ($CHCOOH$), δ = 173.06 (CH_2COOH), δ = 70.63 (NCH_2COO^-), δ = 58.11 (CH), δ = 41.85 ($^+H_3NCH_2$), δ = 31.56 ($CHCH_2$), δ = 29.20 ($CHCH_2CH_2$), δ = 25.89 ($CHCH_2CH_2CH_2CH_2NH_3^+$).

Synthesis of N^α,N^α-Bis[carboxymethyl]-N^ϵ-[(1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamino)succinyl]-L-lysine (NTA-DPPE) VIII. NHS (42 mg, 0.365 mmol) in 100 μL DMF was added under nitrogen to a solution of 283 mg (0.365 mmol) Suc-DPPE **III** and 82.8 mg (0.401 mmol) DCC in 10 mL $CHCl_3$. The mixture was stirred under nitrogen at room temperature over night. NTA **VII** (200 mg, 0.742 mmol) and 845 μL 4-ethyl-morpholine in 20 mL MeOH were added subsequently. The solution was then stirred at room temperature under nitrogen over night. Solvent was removed in vacuo, the precipitate suspended in 60 mL $CHCl_3/H_2O$ (1:1) and stirred for 20 min. The aqueous phase was extracted three times with 60 mL $CHCl_3$ and discarded. Combined organic phases were dried over anhydrous sodium sulfate and solvent was removed in vacuo. NTA-DPPE **VIII** was purified by silica gel column chromatography. The precipitate was redissolved in 3 mL $CHCl_3$/MeOH/4-ethyl-morpholine (27:3:1) and applied to a silica gel column (Lichroprep DIOL, 2×20 cm). Unreacted lipids were eluted with 120 mL $CHCl_3$/MeOH/4-ethyl-morpholine (27:3:1) as monitored by TLC. The product was eluted with $CHCl_3$/MeOH (1:1) and solvent was removed in vacuo. Yield: 70 mg **VIII** (0.066 mmol) 18%; TLC: R_f (**VIII**) = 0.25 in $CHCl_3$/MeOH/H_2O (65:25:4). FTIR: 3295 cm^{-1} (OH), 2920 cm^{-1} (CH_2), 2851 cm^{-1} (CH_2), 1740 cm^{-1} (C=O), 1655 cm^{-1} (COO^-), 1649 cm^{-1} (Amid I), 1551 cm^{-1} (Amid II), 1467 cm^{-1} (COO^- stretching vibration), 1220 cm^{-1} (P=O). ^{1}H-NMR (500 MHz, CDCl3/MeOD 1:1): δ = 0.95 (t, 6H, $CH_2(CH_2)_{14}$), δ = 1.1–1.4 (m, 48H, $CH_3(CH_2)_{12}CH_2$), δ 1.4–1.9 (m, 12H, $CH_3(CH_2)_{12}CH_2$-CH_2,$CH(C_2)_4CH$), δ = 2.3 (m, 4H, $CH_2(CH_2)_{13}CH_2COO$), δ = 2.5 (t, 4H, $COCH_2CH_2CO$), δ = 3.0 (m, 1H, $NCH(COOH)$-CH_2), δ = 3.6–3.9 (m, 8H, NCH_2COO^-, $POCH_2CH_2NH$, $POCH_2CH_2NH$), δ = 4.15 (m, 2H, CH_2CHCH_2OP), δ = 4.3 (dd, 2H, CH_2CHCH_2OP), δ = 5.2 (m, 1H, CH_2CHCH_2), δ = 7.2 (t, 1H, $NHCO$) ^{31}P-NMR (161 MHz, $CDCl_3$/MeOH 1:1): δ = 0.5 (s). MS ($C_{51}H_{91}O_{16}N_3P$, FAB negative): M-H^+ = 1033.8 g/mol.

Synthesis of N-Succinyl-dioctadecylamine (DODA-Suc) X. DODA **IX** (992 mg, 1.9 mmol) and 380 mg (3.8 mmol) succinic anhydrid **II** were dissolved in 40 mL $CHCl_3$ and 2.4 mL Et_3N were added. The solution was stirred under nitrogen at room temperature over night. Total conversion of compound **IX** was monitored by TLC in $CHCl_3$/MeOH/NEt_3 (40:8:1), developed with I_2 and bromocresol green. The organic solution was extracted two times with 40 mL 1N HCl and three times with 40 mL H_2O. After drying over anhydrous sodium sulfate and removing of the solvent in vacuo, the product **X** was recrystallized from acetone. Yield: 1.08 g **X** (1.74 mmol) 92%; TLC: R_f (**X**) = 0.8 in $CHCl_3$/MeOH (9:1). R_f (**IX**) = 0.7 in $CHCl_3$/MeOH (9:1). FTIR: 3300 cm^{-1} (OH), 2910 cm^{-1} (CH_2), 2849 cm^{-1} (CH_2), 1733 cm^{-1} (C=O), 1646 cm^{-1} (Amid I), 1533 cm^{-1} (Amid II), 1466 cm^{-1} (COOH). ^{1}H-NMR (500 MHz, $CDCl_3$): δ = 0.85 (t, 6H, $CH_3(CH_2)_{17}$), δ = 1.2–1.35 (m, 60H, $CH_3(CH_2)_{15}CH_2$), δ = 1.45–1.6 (m, 4H, $(CH_2)_{15}CH_2CH_2$), δ = 2.7 (m, 4H, $NHCOCH_2CH_2COO^-$), δ = 3.3 (dt, 4H, $(CH_2)_{16}CH_2N$). ^{13}C-NMR (100 MHz, $CDCl_3$): δ = 1.69 ($CH_3(CH_2)_{17}$), δ 14.79 ($CH_3CH_2(CH_2)_{16}$), δ = 23.38 ($CH_3CH_2CH_2(CH_2)_{15}$), δ = 27.60, 27.71, 28.35, 28.73, 29.51, 30.06, 30.37 ($CH_3(CH_2)_{13}CH_2CH_2N$), δ = 32.62 ($NCOCH_2CH_2COO^-$), δ = 47.36 ($CH_3(CH_2)_{13}CH_2CH_2N$), δ = 48.99 ($CH_3(CH_2)_{16}CH_2N$), δ = 172.71 (NCO), δ = 176.03 (COO). MS ($C_{40}H_{79}O_3N$, FAB positive) M + H^+ = 622.1 g/mol.

Synthesis of N-[(Hydroxysuccinimidyl)succinyl]dioctadecylamine (DODA-Suc-NHS) XI. NHS (230 mg, 2 mmol) and 11 mg of DMAP in 3 mL of acetone were added to a solution of 1.04 g (1.67 mmol) of DODA-Suc **X** in 15 mL of absolute CH_2Cl_2 at room temperature. After cooling to 0 °C 412 mg (2 mmol) of DCC in 4 mL of CH_2Cl_2 were added. The solution was stirred under nitrogen for 1 h and then over night at room temperature. The precipitated dicyclohexyl urea was filtered off, and solvent was removed in vacuo. The product **XI** was recrystallized from acetone: yield 1.14 g of **XI** (1.59 mmol) 95%; MS ($C_{44}H_{82}O_5N_2$, FAB positive) M + H^+ = 719.6 g/mol.

Synthesis of N^α-[*tert*-Butyloxycarbonyl]-N^ϵ-[(dioctadecylamino)succinyl]-L-lysine (DODA-Suc-Lys-Boc) XIII. Trimethylchlorosilane (340 μL, 5.2 mmol) was added under nitrogen to a suspension of 640 mg (2.6 mmol) of N^α-Boc-lysine **XII** in 5 mL of absolute CH_2Cl_2. The suspension was heated for 1 h to 60 °C. After cooling to room temperature, 380 μL (2.6 mmol) of Et_3N were added, and the suspension was heated again to 60 °C, until the solution remained clear after cooling to room temperature. 967 mg (1.35 mmol) of DODA-Suc-NHS **XI** in 10 mL of absolute CH_2Cl_2 were added at room temperature and the solution was stirred for 24 h and then refluxed for 2 h.

The reaction mixture was acidified by adding 50 mL of CH_2Cl_2/0.1 N AcOH (2:3) and stirred for 30 min. The organic phase was extracted twice with 30 mL of 0.1 N AcOH amd twice with 30 mL of H_2O. After drying over anhydrous sodium sulfate and removal of the solvent in vacuo, the product **XIII** was recrystallized from acetone: yield 1.06 mg of **XIII** (1.25 mmol) 93%; TLC R_f (**XIII**) 0.6 in $CHCl_3$/MeOH (9:1); ^{1}H-NMR (500 MHz, $CDCl_3$) δ = 0.85 (t, 6H, $CH_3(CH_2)_{17}$), δ = 1.15–1.25 (m, 60H, $CH_3(CH_2)_{15}CH_2$), δ = 1.35 (s, 9H, $(CH_3)_3$), δ = 1.40–1.55 (m, 4H, $CH_3(CH_2)_{15}CH_2CH_2N$), δ = 1.65–2.0 (m, 4H, $NHCH_2(CH_2)_2CH_2CH$), δ = 2.5 (m, 2H, $CONHCH_2$-

$CH_2(CH_2)_2CH_2CH$), δ = 2.7 (m, 4H, $COCH_2CH_2CO$), δ = 3.3 (m, 6H, $(CH_2)_{16}CH_2N$, $CONHCH_2(CH_2)_3CH$), δ = 4.2 (m, 1H, $CH_2CH(COO)NH$); ^{13}C-NMR (100 MHz, $CDCl_3$) δ = 1.67 ($CH_3(CH_2)_{17}$), δ = 14.77 ($CH_3CH_2(CH_2)_{16}$), δ = 22.81 ($CONH(CH_2)_2CH_2CH_2CH$)), δ = 23.35, 25.50, 26.20, 27.61, 27.71, 28.38, 29.02, 29.32, 29.49, 29.54, 30.02, 30.09, 30.37, ($CH_3(CH_2)_{15}CH_2CH_2N$), δ = 29.02 (($CH_3)_3C$), δ = 32.47 ($CONHCH_2CH_2(CH_2)_2CH$), δ = 32.59 ($NCOCH_2CH_2$-CONH), δ = 34.36 ($CONH(CH_2)_3CH_2CH$), δ = 39.60 ($NHCH_2(CH_3)_3CH$), δ = 47.30 ($CH_3(CH_2)_{15}CH_2CH_2N$), δ = 49.01 ($CH_3(CH_2)_{16}CH_2N$), δ = 54.02 (CH), δ = 80.39 ($C(CH_3)_3$), δ = 156.35 ($NHC(O)O$-$C(CH_3)_3$), δ = 172.71 ($NHCO$), δ = 173.62 (NCO), δ = 175.43 (COO^-); MS ($C_{51}H_{99}O_6N_3$, FAB positive) M + H^+ = 850.4 g/mol.

Synthesis of N^{ϵ}-[(Dioctadecylamino)succinyl]-L-lysine (DODA-Suc-Lys) XIV. DODA-Suc-Lys-Boc **XIII** (1.05 g, 1.24 mmol) was dissolved in 20 mL of $CHCl_3$/TFA (95:5) and stirred under nitrogen at room temperature. Total cleavage of the protecting group was achieved after 3 h as demonstrated by TLC in $CHCl_3$/MeOH/NEt_3 (40:8:1), developed with I_2 and bromocresol green. The reaction mixture was extracted twice with 15 mL of 1 N NaOH and twice with 15 mL of H_2O and dried over anhydrous sodium sulfate, and solvent was removed in vacuo. The product was recrystallized from acetone: yield 882 mg of **XIV** (1.17 mmol) 95%; TLC R_f (**XIV**) 0.3 in $CHCl_3$/MeOH/NEt_3 (40:8:1); FTIR 3320 cm^{-1} (OH), 3160 cm^{-1} (NH_3^+), 2910 cm^{-1} (CH_2), 2850 cm^{-1} (CH_2), 1679 cm^{-1} (C=O), 1626 cm^{-1} (Amid I), 1535 cm^{-1} (Amid II), 1467 cm^{-1} (COOH); ^{1}H-NMR (500 MHz, $CDCl_3$) δ = 0.85 (t, 6H, $CH_3(CH_2)_{17}$), δ = 1.15=1.25 (m, 60H, $CH_3(CH_2)_{15}CH_2$), δ = 1.40–1.55 (m, 4H, $(CH_2)_{15}CH_2CH_2$), δ = 1.65–2.0 (m, 4H, $NHCH_2(CH_2)_2CH_2CH$), δ = 2.4 (m, 2H, $CONH(CH_2)_3CH_2CH$), δ = 2.6 (m, 4H, $COCH_2CH_2CO$), δ = 3.3 (m, 6H, $(CH_2)_{16}CH_2N$, $CH_2CONHCH_2(CH_2)_3$), δ = 3.9 (m, 1H, $CH_2CH(COO)NH$), δ = 7.2 (m, NH_3^+); ^{13}C-NMR (100 MHz, $CDCl_3$) δ = 1.71 ($CH_3(CH_2)_{17}$), δ = 14.78 (CH_3CH_2-$(CH_2)_{16}$), δ 22.18 ($NH(CH_2)_2CH_2CH_2CH$), δ = 23.39, 25.47, 26.13, 27.63, 27.71, 28.33, 28.78, 29.22, 29.45, 30.06, 30.44 ($CH_3(CH_2)_{15}CH_2CH_2N$), δ = 31.83 ($NHCH_2CH_2(CH_2)_2CH$), δ = 32.63 ($NCOCH_2CH_2CONH$), δ = 34.19 ($NH(CH_2)_3CH_2$-CH), δ = 39.11 ($NHCH_2(CH_2)_2CH_2CH$), δ = 47.23 ($CH_3(CH_2)_{15}CH_2CH_2N$), δ = 49.04 ($CH_3(CH_2)_{16}CH_2N$), δ = 54.51 ($NH(CH_2)_4CH$), δ = 172.85 ($NHCO$), δ = 174.23 (NCO), δ = 174.97 (COO^-); MS ($C_{46}H_{92}O_4N_3$, FAB positive) M + H^+ = 750.5 g/mol.

Synthesis of N^{α},N^{α}-Bis[carboxymethyl]-N^{ϵ}-[(dioctadecylamino)succinyl]-L-lysine (NTA-DODA) XV. DODA-Suc-Lys **XIV** (846 mg, 1.13 mmol) and 490 μL of NEt_3 in 20 mL of $CHCl_3$ were added dropwise to an ice-cooled solution of 314 mg (2.26 mmol) of bromoacetic acid **IV** and 370 μL of NEt_3 in 10 mL of $CHCl_3$. After cooling for 1 h the solution was stirred at room temperature over night and then heated to 50 °C for 2 h. The solution was extracted twice with 30 mL of 1 N NaOH and twice with 30 mL of H_2O. After drying over anhydrous sodium sulfate, solvent was removed in vacuo. The product was purified by silica gel column chromatography with $CHCl_3$/MeOH/H_2O (65:25:4) as eluant. After removing the solvent in vacuo the product was recrystallized from acetone. Yield 292 mg of **XV** (0.34 mmol) 30%; TLC R_f (**XV**) 0.2 in $CHCl_3$/MeOH/H_2O (65:25:4); FTIR 3292 cm^{-1} (OH), 2922 cm^{-1} (CH_2), 2852 cm^{-1} (CH_2), 1671 cm^{-1} (C=O), 1635 cm^{-1} (Amid I), 1554 cm^{-1} (Amid II), 1466 cm^{-1} (COO^- stretching vibration); ^{1}H-NMR (500 MHz, $CDCl_3$/MeOD + TFA) δ = 0.85 (t, 6H, $CH_3(CH_2)_{17}$), δ = 1.15–1.25 (m, 60H, $CH_3(CH_2)_{15}CH_2$), δ = 1.40–1.55 (m, 4H, $(CH_2)_{15}CH_2$-CH_2), δ = 1.65–2.0 (m, 4H, $NHCH_2(CH_2)_2CH_2CH$), δ = 2.4 (m, 4H, $COCH_2CH_2CO$), δ = 2.6 (t, 2H, $CONH(CH_2)_3CH_2$-CH), δ = 3.1–3.3 (m, 7H, $(CH_2)_{16}CH_2NCO$, $NHCH_2(CH_2)_3$-CH, $CH_2CH(COOH)N$), δ = 3.7–3.9 (m, 4H, $CH_2CH(COO)$-NCH_2COOH).

Table 1. R_f Values of NTA–Lipids in Different Solvent Systems With and Without Ni^{2+} [a]

solvent system	NTA–DPPE with/without Ni^{2+}	NTA–DODA with/without Ni^{2+}
$CHCl_3$/MeOH/H_2O (65:25:4)	0.30/0.25	0.25/0.20
$CHCl_3$/MeOH/H_2O/NH_3 (65:25:2:2)	0.16/0.10	0.47/0.40
$CHCl_3$/MeOH/H_2O/AcOH (65:25:2:2)	0/0	0.21/0.21

[a] Spots were visualized with I_2 and molybdenum blue in the case of NTA–DPPE, respectively, bromocresol green in the case of NTA–DODA. Each value is the average of three experiments.

Table 2. Interpretation of the FTIR Signals of NTA–DPPE With and Without Ni^{2+}

group	wavenumber for NTA–DPPE (cm^{-1})	wavenumber for Ni^{2+}–NTA–DPPE (cm^{-1})
C=O	1740	1740
COO^-	1655	1657[a]
amid I	1649	1649[a]
amid II	1551	1551[a]
COO^- (stretching vibration)	1467	1462

[a] In this region no changes in the position of the signals occurred, but the intensity decreased and the system became more ordered.

Characterization of NTA-Functionalized-Lipids

For chemical and physical characterization of the products, the following experiments were performed: thin-layer chromatography, FTIR spectroscopy, mass spectroscopy, and film balance experiments. All the experiments, except film balance measurements, were carried out with the same solutions. For complexing Ni^{2+} ions with the NTA-lipids, 2 mM NTA-lipid solution in $CHCl_3$/MeOH (1:1) and a 2 mM $NiCl_2$, 100 mM Tris/HCl pH 8.5 solution were mixed and stirred for 30 min. The solutions were dried in vacuo and redissolved in $CHCl_3$/MeOH (1:1) to obtain a stock solution of 2 mM. For the experiments in the absence of Ni^{2+} a 2 mM solution of the NTA-lipids in $CHCl_3$/MeOH (1:1) was used.

Thin-Layer Chromatography. Five microliters of each solution was used. With this small amount of lipid an overloading of the TLC plates could be excluded. A small, but significant shift, of the R_f values of NTA-DPPE in two of three solvent systems, was detected (Table 1). In neutral or basic solvent systems, the R_f values were shifted in the presence of Ni^{2+} toward higher values. In the acidic system no migration of either compound was detected. Ni^{2+} showed no influence on the R_f value of the Suc-DPPE in all of the solvent systems used (data not shown).

The results obtained for NTA-DODA are similar to those for NTA-DPPE. In neutral and in basic solvent systems, a shift of about 10% was detected in the presence of Ni^{2+}. NTA-DODA also migrated in the acidic system, and no shift was determined. As a control, the also negatively charged DODA-Suc did not show any shift in the presence of Ni^{2+} for all solvent systems.

FTIR Experiments. Three hundred microliters of solution were used for each measurement (see Materials and Methods). The intensities and signal positions of the spectra with and without Ni^{2+} were compared (Table 2). In both spectra the baseline has not been refined. In the presence of Ni^{2+}, a significant shift toward smaller wavenumbers for the stretching vibration of the carboxy anion was detected (5 cm^{-1}). In addition, the carbonyl vibration frequency of the carboxy group was increased by 2 cm^{-1} and a more ordered system was found for the intensity of these carbonyl oxygens. For the vibrations of the alkyl ester, and phosphate groups and for both amide vibrations, no shift was found.

Film Balance Experiments. The experiments described above, dealt with complex formation in solution. The amphipatic nature

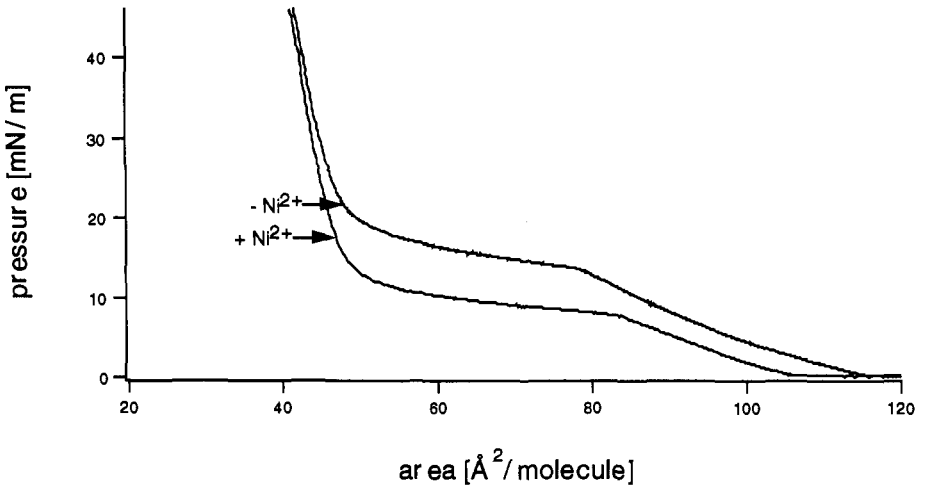

Figure 3. Influence of Ni^{2+} on a NTA–DODA monolayer: subphase, 10 mM HEPES pH 8.0, 140 mM NaCl. Compression in the absence of Ni^{2+} (upper curve). Compression of a Ni^{2+}–NTA–DODA monolayer (molar ratio Ni^{2+}:NTA–DODA 1:1) (lower curve). Compression rate 1.8 $Å^2$/molecule*min, 22 °C, for all compressions.

of these molecules is taken into account in film balance experiments, where molecules are organized in two dimensions in a monolayer. Both NTA-lipids, NTA-DPPE and NTA-DODA, could be spread at the air/water interface. They show stable behavior during several cycles of compression and expansion up to a lateral pressure of at least 30 mN/m in the case of NTA-DPPE, and 40 mN/m in the case of NTA-DODA. The isotherms are strongly dependent on the temperature, ion-strength, and pH-value of the subphase. The area/molecule of the NTA-lipids was estimated using a molecular weight of 863 g/mol for NTA-DODA and 1031 g/mol for NTA-DPPE, respectively. Countercations were not taken into account.

In the first set of experiments, the influence of Ni^{2+} ions on the NTA-lipid monolayers was investigated. Figure 3 illustrates the influence of Ni^{2+} on a NTA-DODA monolayer. Either NTA-DODA or complexed Ni^{2+}-NTA-DODA was spread at the air/water interface. When Ni^{2+} ions were spread together with the NTA-lipid (44 nmol Ni^{2+}, equivalent to 190 nM $NiCl_2$ in the subphase) the pressure of the main-phase transition was lowered by 7 mN/m. The magnitude of this effect may indicate an electrostatic interaction or structural changes in the monolayer. No effect on the specific area corresponding to the space requirement in the highly condensed state was detected. Since this drastic effect at extremely low Ni^{2+} concentration occurs at high NaCl concentration (140 mM), a specific, high affinity interaction is assumed rather than an electrostatic interaction. In the case of NTA-DPPE, changes in the isotherms were also observed in the presence of Ni^{2+}. In contrast to NTA-DODA, additional electrostatic interactions of the metal ion with the phosphate group could not be excluded in experiments with NTA-DPPE (data not shown).

In a second set of experiments, lipids with a Ni^{2+}-NTA head group were spread for investigating specific interactions with ligands and complex formation. Figure 4a/b illustrate the effect of imidazole in the subphase on the NTA-DODA monolayer in the presence and absence of Ni^{2+}. The specific interaction of a Ni^{2+}-NTA-DODA monolayer with imidazole is illustrated in Figure 4a. Two effects were observed: The pressure of the main-phase transition was shifted toward higher values (shift of 4 mN/m), and the specific area was increased (shift of 1.8 $Å^2$/molecule). In the absence of Ni^{2+}, as shown in Figure 4b, no effect of imidazole was detected. In a further control experiment with imidazole and a Ni^{2+}-DMPG monolayer prepared in the same manner, again no effect could be observed (data not shown). Thus, under these conditions electrostatic interaction with the highly negatively charged lipid surface can be excluded. The observed effects of Ni^{2+} and Ni^{2+}/imidazole on a NTA-DODA monolayer can be explained by (i) specific Ni^{2+}–NTA complex formation and (ii) the specific ligand binding at the lipid interface.

Discussion

Suc-DPPE **III** (Figure 1) and DODA-Suc **X** (Figure 2) could both be synthesized quantitatively and without further purification by reacting the lipid with succinic anhydride using the method of Kung and Reedeman.[12] The total conversion of the free amino group was detected by TLC and HPLC (data not shown).

In the case of NTA-DPPE, NTA was synthesized according to the method of Hochuli[6,13] (Figure 1), but the protecting group could not be cleaved in basic solution as mentioned by the author.[6] In contrast, the cleavage was quantitative in MeOH/AcOH(95:5). Purity of the isolated product was determined by TLC, FTIR, and NMR. In the last step of the synthesis, the addition of the NTA-derivative with the NHS-activated Suc-DPPE **III** (Figure 1), problems concerning solubility had arisen. Both educts were soluble only in $CHCl_3$/MeOH, while in DMSO, DMF, or mixtures with $CHCl_3$, the phospholipid was insoluble. In MeOH the yield decreased because of the side reaction of the solvent with the activated lipid. In place of standard silica gel a specifically modified silica material (silica diol) was used to purify the product by a gradient from apolar to polar solvent systems. NTA-DPPE could be isolated quantitatively and pure as determined by TLC, NMR, and MS.

Because of the low solubility of the educts and the low yield in the last step of the synthesis of NTA-DPPE, NTA-DODA was synthesized by an alternative method (Figure 2). For solubilization of Nα-Boc-lysine **XII** in organic solvents, the method of silanization of Krichelbaum[14] was used. Both the coupling of the nonisolated N,O-bis-silanized intermediate with DODA-Suc-NHS **XI** and the cleavage of the protecting group from **XIII** with TFA were quantitative as determined by TLC and NMR. In the last step bromoacetic acid **IV** was applied in a way similar to the method used for the synthesis of the NTA. Using silica gel column chromatography the product could be isolated with an overall yield of 30%. Purity and identity were determined by TLC and NMR.

Complex formation of the NTA-lipids was demonstrated by thin-layer chromatography. The differences in the R_f values of about 10% corresponding to the basic and neutral solvent systems are small but significant and in the same range for both solvent systems and both NTA-lipids. The complexing of Ni^{2+} reduces

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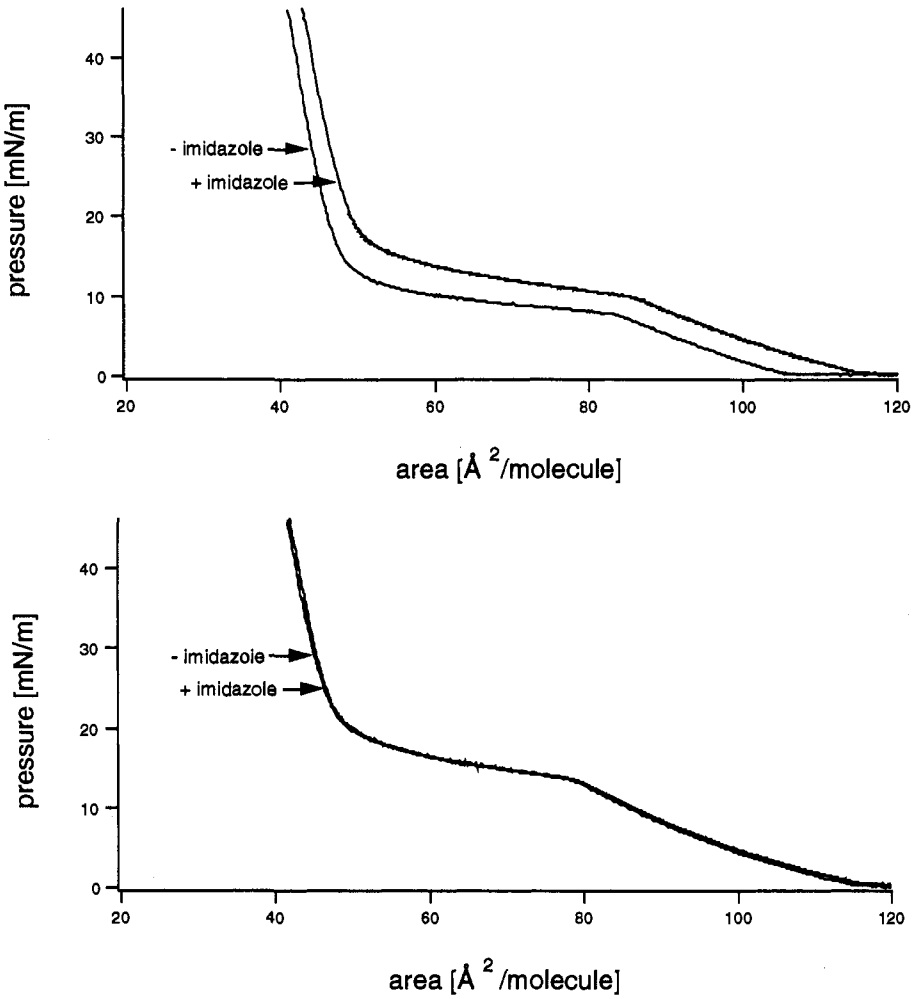

Figure 4. Influence of imidazole on a Ni^{2+}-NTA-DODA monolayer (a) and on a NTA–DODA monolayer (b); subphase, 10 mM HEPES, pH 8.0, 140 mM NaCl. Compression in the presence of 3 mM imidazole in the subphase (upper curve). Compression in the absence of imidazole in the subphase (lower curve). Compression rate: 1.8 $Å^2$/molecule*min, 22 °C, for all compressions. Molar ratio Ni^{2+}:NTA–DODA, 1:1.

the net charge and polarity. Therefore, the molecules should become more mobile. In the acidic solvent system no change in the R_f value occurred, which supports the explanation of complex formation. This solvent system protonates the carbon acids in the head group of the lipid and hinders complex formation. The possibility of electrostatic interactions causing the shifts can be excluded, because the also negatively charged DPPE-Suc, respectively DODA-Suc, shows no effect.

Further support for complex formation of the NTA-lipids was obtained by FTIR spectroscopy. Complex formation should split the binding order of 1.5 for the free carboxy group to 1 respectively 2. This point was confirmed for the stretching vibration of the carboxy groups (decrease of about 5 cm^{-1}). In addition to the small increase (2 cm^{-1}) for the carbonyl vibration of the carboxy group, we detected a more ordered system. This is in agreement with complex formation. Changes in the microstructure of this complex region require further investigation. These two shifts and the more ordered system were the only changes detected in the presence of Ni^{2+}. The intensity as well as the position of the signals for the alkyl ester, the phosphate, and the amide moieties were not altered. While we found no change in the vibration of the negatively charged phosphate group, an electrostatic interaction is negligible and the changing for the carboxyl head-groups indicates complex formation in the presence of Ni^{2+}.

In all experiments, complex formation was demonstrated in solution. The combination of results from TLC and FTIR indicated drastic changes in the properties of the lipid head-group. Because of the FTIR and TLC experiments a possible electrostatic interaction between Ni^{2+} and the negatively charged head-group of these newly synthesized NTA-lipids can be excluded. Thus, the lipid part of the NTA-lipid system does not influence the behavior of the NTA-head-group since the complex formation and the properties are comparable to NTA alone. To generate a highly metal sensitive and ligand binding surface, which can be structured in two dimensions, film balance technique was used as a very sensitive method in determining effects of the lipid head-group. As expected, a lowering of the main-phase transition pressure for the NTA-DODA system was observed in the presence of Ni^{2+} (Figure 3). The concentration of Ni^{2+} ions does not influence the specific area. The lowering of the main-phase transition clearly indicates an interaction between the lipid and the divalent cation. As determined by Lösche et al.,[15] divalent cations, in concentrations up to 10^{-3} M, reduce the pressure of the main-phase transition by up to 2 mN/m, neglecting any structural changes in the monolayer. Additionally, in our experiments different concentrations of NaCl up to 140 mM had little or no effect on the pressure of the phase-transition (data not shown). Therefore, it can be assumed that high ion-strength suppresses any electrostatic interaction between the divalent Ni^{2+} (190 nM) and the highly charged NTA-head-group. In combination with the observed reduction of the main-phase transition of about 7 mN/m caused by 44 nmol Ni^{2+}, specific complex formation was demonstrated at the air/water interface.

Oligo-histidine-tagged fusion proteins are known to interact specifically to Ni^{2+}-NTA complexes via coordinative binding of

(15) Lösche, M.; Helm, C.; Matte, H. D.; Möhwald, H. *Thin Solid Films* **1985**, *133*, 51–64.

the imidazole ring. Thus, imidazole is an ideal model compound for testing ligand binding, avoiding any additional steric or electrostatic effects of the protein. In the case of a binding, the size of the lipid head region is augmented causing an increase of the pressure of the main-phase transition. The value of the specific area is mainly determined by the tail of the lipid, and only a small effect of higher areas should occur. As shown in Figure 4a, both effects were observed in the presence of Ni^{2+}, while no effect was detected in its absence (Figure 4b). The pressure of the main-phase transition is significantly raised by 4 mN/m caused by a larger space requirement of the complex with the additional bound imidazole molecules. This is in line with the increase of the specific area. But, this should not be over-interpreted since the shift is near the range of experimental error. To exclude the possibility that the observed effects of imidazole on a Ni^{2+}-NTA-DODA monolayer are not due to a complexation of Ni^{2+} only by imidazole, the behavior of a negatively charged DMPG-monolayer on imidazole was also determined in the absence and presence of Ni^{2+}. No effects were detectable (data not shown).

The NTA-lipid system and the observed effects of Ni^{2+} and imidazole are summarized in Figure 5. Due to the high affinity of the NTA-complex Ni^{2+} ions are complexed at the lipid interface. An octahedral complex is formed causing drastic changes in the electrical and structural properties of the lipid head group as demonstrated by film balance measurements. Additionally, two coordination sites are free, which can be occupied by electron donors such as imidazole alone or attached to the protein surface. In such a way, oligo-histidine-tagged fusion proteins could be immobilized, enriched, oriented, and structured at the lipid interface. Reversibility is achieved by the use of EDTA, which possesses a higher binding constant for the Ni^{2+} than NTA. Therefore, the immobilization of any bound ligand can be disrupted under mild conditions. After deimmobilization, the system can be reused for further experiments, simply by rearrangement of the Ni^{2+}–NTA complex.

In spite of the specificity and high binding constant, the well-characterized biotin-streptavidin system lacks its reversibility.[16] The immobilization of biotinylated molecules via the streptavidin binding or vice versa is nearly irreversible and can only be disrupted by harsh conditions. The NTA–DODA system offers more applications than just lipid monolayer experiments. The combination of modern methods of molecular biology and protein engineering with the properties of membrane and thin film technology leads to a concept of reversible immobilization, orientation, functionalization of lipid surfaces including lateral organization, and pattern formation. In addition, these chelating lipids might be useful for two-dimensional crystallization of histidine-tagged fusion proteins at lipid interfaces.

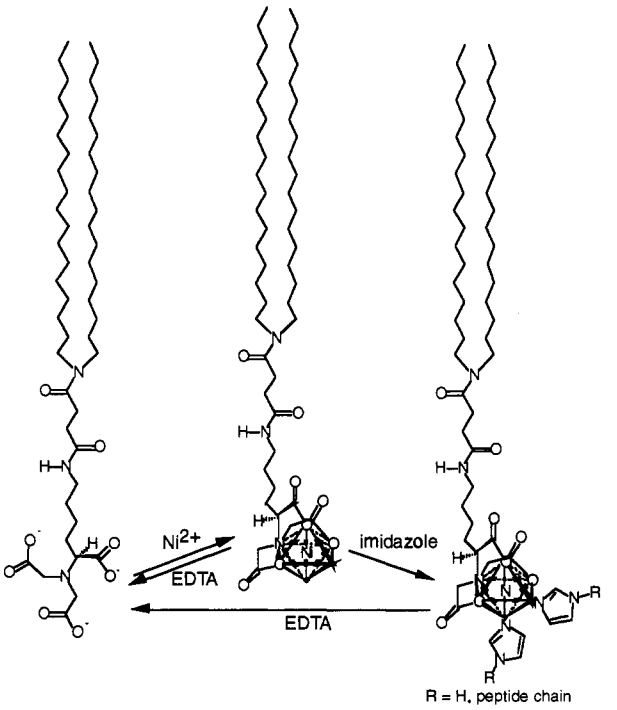

Figure 5. Schematic illustration of complex formation, imidazole binding to Ni^{2+}–NTA–DODA at the lipid surface. Reversibility was achieved by the addition of EDTA.

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