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Internalization of a Peptide into Multilamellar Vesicles Assisted by the Formation of an α-Oxo Oxime Bond

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Abstract: As part of a drug-delivery project, we designed and synthesised a novel hydroxylamine cholesterol-based anchor to ensure the chemoselective ligation of recognition patterns onto multilamellar vesicles by oxime ligation. The entry of a glyoxylyl peptide into the vesicles was unexpectedly assisted by the formation of the α-oxo oxime bond. We studied extensively the kinetic and thermodynamic aspects of this phenomenon. Briefly, for a glyoxylyl peptide, the speed and ability to enter the vesicle were dependent upon 1) the presence of a hydroxylamine anchor of the type Chol-E_3ONH_2, 2) the amount of peptide engaged in the ligation and 3) the flip-flop motion permitted by the different formulations, in which the presence of cholesterol seems to play an important role.

Keywords: cholesterol · drug delivery · lipids · membranes · peptides

Introduction

In drug-delivery strategies, the active targeting of synthetic lipidic vesicles, such as liposomes, is permitted by a molecular recognition pattern (MRP) anchored at their surface. This MRP can be either a small peptide, an organic molecule like folic acid, an antibody or a glycomimetic. The covalent anchorage of such MRPs is achieved by the chemoselective ligation with amphiphilic compounds (named "anchors") that are incorporated into the lipid bilayers. These anchors expose reactive functions on the surface of the vesicles, which are able to link the MRP that is solubilised in the outer aqueous compartment. In this context, chemical bonds are classically chosen from amide bonds,[1] thioether bonds[2] or disulfide bridges,[3] whereas non-covalent links are usually obtained by the avidin/biotin couple[4] or the Ni^{2+}/histidine complex.[5]

We have developed the α-oxo hydrazone bond in chemical ligation projects together with the chemistry of hydrazino acetyl peptides[6] to obtain various bioconjugates: a lipopeptide vaccine cocktail[7] and some peptido[8,9] and glycomimetic liposomes[10] were successfully produced. In parallel work, some authors have developed the oxime ligation in related subjects. Peptide–oligonucleotide conjugates[11] or template-assembled cyclopeptides as multimeric systems for integrin targeting[12] have recently been prepared by means of the α-oxo oxime ligation. The syntheses of branched oxime-linked peptides were also reported in the field of immunotherapy,[13] or in the rational construction of well-defined immunogens.[14] As part of a drug-delivery project based on multilamellar vesicles called spherulites™, and with the experience of α-oxo hydrazone chemistry in our hands, we studied the glyoxyl oxime chemistry for the purpose of anchoring MRPs.

Spherulites™ are multilamellar vesicles with no aqueous core, obtained by shearing a lipidic lamellar phase (see Supporting Information). Easy to produce, both in the laboratory and on an industrial scale, these vesicles were first designed and studied by Roux and co-workers,[15] and further developed industrially.[16] Spherulites™ consist of alternating lipidic bilayers and aqueous layers and, therefore, display an onion-like structure. Phosphatidylcholine (PC), cholesterol and various surfactants constitute their lipidic bilayers, and vesicle size is related to their formulation and the shearing's strength and duration. Theoretically, spherulites™ can encapsulate either hydrophilic or hydrophobic active compounds[17] in the aqueous phase or lipidic bilayers, respectively. This indicates their potential in drug encapsulation, and also, in drug targeting. We envisaged the use of spheru-

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lites™ in this field, and chose particularly the targeting of tumor cells as a way to validate these vesicles.

Spherulites™ were designed as neutral species to avoid any nonspecific cell adhesion and interaction with proteins and other electrolytes present in blood. To take advantage of the versatility of ligation methods, and to immobilise either peptides or antibodies on the vesicles, we designed and synthesised the new anchor "CholE_3ONH_2". Ready to be incorporated into lipidic bilayers, it was designed to react with glyoxylyl MRPs to form an α-oxo oxime conjugate. The accessibility and reactivity of its hydroxylamine head was extensively studied with an α-oxo-aldehyde peptide as a model MRP. The kinetic and thermodynamic aspects of this ligation are reported here. In addition, an unexpected result was that the glyoxylyl oxime bond formation was a serendipitous means of allowing the peptidic MRP to enter the vesicles and to react with the anchor localised in the internal membrane of the spherulites™.

Experimental Section

Materials: NMR spectra were recorded by using a 300 MHz Bruker spectrometer calibrated with δ^1H ($CHCl_3$) = 7.26 ppm and δ^{13}C ($CDCl_3$) = 77 ppm. Attributions of typical signals of cholesterol protons or carbon atoms were performed following the conventional numerotation of steroid compounds. Mass spectra were recorded by using a Perspective Biosystems Voyager-DE STR, Biospectrometry Workstation MALDI-TOF spectrometer, and measurements were acquired after deposition on a dihydroxybenzoic acid (DHB) matrix.

Chemicals: All 9-fluorenylmethyloxycarbonyl (Fmoc)-protected amino acids (L-Arg(Pmc) (Pmc = 2,2,5,7,8-pentamethylchroman-6-sulfonyl) L-Ser(*t*Bu), L-Thr(*t*Bu), L-Trp(*t*Bu), L-Tyr(*t*Bu)), coupling agents (1-hydroxybenzotriazole (HOBt), 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) and the resin (Rink® Amide) were purchased from Novabiochem. Chemicals were from Sigma-Aldrich. Solvents were from Acros. Membranes of spherulites™ were made of soy lecithin (Phospholipon 90, Natterman), and either Tween 80 (Sigma), Simulsol 2599 (Seppic) or Solutol HS 15 (a gift from BASF), and cholesterol (Chol) (Sigma).

Synthesis

Compound 2: In a round-bottomed flask, 15 g (100 mmol, 1 equiv) of the diol **1** were mixed with 3.02 mL (33 mmol, 0.33 equiv) of 3,4-dihydropyran and two drops of 35% HCl (*w*/*w* in water) overnight. The reaction medium was extracted with saturated $NaHCO_3$ (20 mL) and CH_2Cl_2 (30 mL). The aqueous layer was extracted twice with CH_2Cl_2. Combined organic layers were dried over $MgSO_4$ and concentrated under vacuum. The crude product was purified on silica gel (SiO_2 500 g, ethyl acetate/methanol 9:1) and 5.688 g of **2** were isolated. Yield 74%. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.49–1.79 (m, 6H), 3.45–3.50 (m, 1H), 3.57–3.62 (m, 2H), 3.66 (s, 8H), 3.69 (t, J = 7.42 Hz, 2H), 3.82–3.88 (m, 1H), 4.61 ppm (t, J = 3.49 Hz, 1H); ^{13}C NMR: δ = 19.4, 25.3, 30.4, 61.6, 62.2, 66.4, 70.3, 70.4, 70.5, 72.5, 98.9 ppm; MALDI-TOF MS (DHB): *m*/*z* calcd for [M+Na^+]: 257.14; found: 257.2.

Compound 3: An amount of the alcohol **2** (2 g, 8.53 mmol, 1 equiv) was mixed with 2.44 g (12.8 mmol, 1.5 equiv) of tosyl chloride and 1.04 mL of pyridine (12.8 mmol, 1.5 equiv) in dry CH_2Cl_2 under nitrogen overnight. The reaction medium was washed with 10% HCl (*w*/*w* in water) and distilled water. The organic layer was dried over $MgSO_4$ and concentrated under vacuum. The crude product was purified on silica gel (SiO_2 400 g, petroleum ether/ethyl acetate 7:3) and 1.968 g of **3** was isolated. Yield 59%. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.49–1.84 (m, 6H), 2.43 (s, 3H), 3.47 (m, 1H), 3.60 (s, 6H), 3.64 (m, 2H), 3.68 (m, 2H), 3.84 (m, 1H), 4.14 (t, J = 4.85 Hz, 2H), 4.60 (t, J = 3.47 Hz, 1H), 7.32 (d, J = 8.34 Hz, 2H), 7.78 ppm (d, J = 8.04 Hz, 2H); ^{13}C NMR: δ = 19.4, 21.5, 25.3, 30.4, 62.1, 66.5, 68.5, 69.2,70.4, 70.6, 98.8, 127.8, 129.7, 132.9, 144.7 ppm.

Compound 5: An amount of cholesterol (2.35 g, 6.1 mmol, 1.2 equiv) was dissolved in dry DMF/dioxane (1:1 mixture, 40 mL) under nitrogen, and 0.728 g (18 mmol, 3.6 equiv) of NaH was added. After 1 h, 1.968 g (5.1 mmol, 1 equiv) of compound **3** was added to the medium and allowed to react for 80 h. Distilled water (20 mL) was added to the reaction medium, which was extracted with CH_2Cl_2. The aqueous layer was extracted once with CH_2Cl_2 and the combined organic layers were dried over $MgSO_4$, concentrated under vacuum and purified on silica gel (SiO_2 200 g, CH_2Cl_2/acetone 9:1) to give 3.284 g of the product **4** mixed with unreacted cholesterol. All of this mixture was dissolved in absolute ethanol (30 mL) and 250 mg of 10-camphorsulfonic acid. After 8 h, ethanol was evaporated under vacuum and the crude product was purified on silica gel (SiO_2 300 g, CH_2Cl_2/acetone 9:1) to give 1.636 g of **5**. Yield 62%. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.66 (s, 3H; H^{18}), 0.87 (d, J = 3 Hz, $H^{26,27}$), 0.91 (d, H^{21}), 0.99 (s, H^{19}), 2.18–2.46 (m, 2H; H^4), 2.55 (brs), 3.18 (m, 1H; H^3), 3.59–3.72 (m, 12H), 5.33 ppm (m, 1H; H^6).

CholE_3ONH_2: Under a nitrogen atmosphere, 1.636 g (3.1 mmol, 1 equiv) of **6** was dissolved with 0.771 g (4.7 mmol, 1.5 equiv) of *N*-hydroxyphthalimide and 1.240 g (4.7 mmol, 1.5 equiv) of triphenylphosphine (PPh_3) in THF freshly distilled over sodium in the presence of benzophonone (40 mL). 735 μL of diethylazodicarboxylate (DEAD) were then added drop by drop. After 90 min the medium was extracted with diethyl ether and distilled water. The organic layers were dried over $MgSO_4$ and concentrated under vacuum. The crude product was purified on silica gel (SiO_2 400 g, CH_2Cl_2/acetone 9:1) to give 2 g of **6**, which was then dissolved in distilled THF (20 mL)and 6 mL of aqueous hydrazine (25% *w*/*w* in water). After 2 h, the solvent was evaporated and the crude was extracted with CH_2Cl_2. The aqueous layer was extracted twice with CH_2Cl_2, and the combined organic layers were dried over $MgSO_4$ and concentrated under vacuum. The product was purified on silica gel (SiO_2 300 g, CH_2Cl_2/methanol 95:5) to give 1.335 g of CholE_3ONH_2. Yield 80%. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.65 (s, 3H; H^{18}), 0.85 (d, J = 3 Hz, $H^{26,27}$), 0.90 (d, H^{21}), 0.97 (s, H^{19}), 2.20–2.45 (m, 2H; H^4), 3.17 (m, 1H; H^3), 3.61–6.68 (m, 10H), 3.81 (t, J = 4.55 Hz, 2H), 5.31 (m, 1H; H^6), 5.50 ppm (brs); ^{13}C NMR: δ = 11.8 (C^{18}), 18.7 (C^{21}), 19.3 (C^{19}), 22.8 ($C^{26,27}$), 39.0 (C^4), 67.2, 69.5, 70.5, 70.8, 74.7, 79.4 (C^3), 121.5 (C^6), 140.9 ppm (C^5). IR (film): $\tilde{\nu}$ = 3313, 2934, 1592, 1467, 1366, 1111, 949, 839, 800 cm^{-1}; HRMS (FAB$^+$): *m*/*z* calcd for $C_{33}H_{60}O_4N$ [M+H^+]: 534.4522; found: 534.4534 (±2.4 ppm); MALDI-TOF MS (DHB): *m*/*z* calcd [M+Na^+]: 556.43; found: 556.5.

H-CO-CO-ATWLPPR-NH_2: H-SATWLPPR-NH_2 was synthesised according to a Fmoc/*t*Butyl solid-phase strategy by using a Pioneer synthesiser (Applied Biosystems) with an in situ activation of Fmoc amino acid (9 equiv) in the presence of TBTU, HOBt and diisopropylethylamine (DIEA). After RP-HPLC purification, a periodic oxidation gave H-CO-CO-ATWLPPR-NH_2 with 33% overall yield.

RP-HPLC analyses of H-CO-CO-ATWLPPR-NH_2 were performed by using a Shimadzu SCL-6A (3 mL min^{-1}) and a WATERS 2695 (1 mL min^{-1}) for preparative and analytical separations, respectively. RP-HPLC eluents: TFA 0.05% in H_2O; TFA 0.05% in CH_3CN/H_2O 80:20 (*v*/*v*), respectively. Column C18 Nucleosil® at 50 °C.

Spherulites™ were prepared by dissolving lipids in dry CH_2Cl_2. The solvent was then evaporated under vacuum and the mixture of dried lipids was hydrated with distilled water in the following proportions: 60% *w*/*w* for **S1**, **S2**, **S3** and 35% for **S4** and **S5**. After homogenisation, the mixtures were sheared and dispersed in distilled water (8% *w*/*w*).

Reaction of peptide H-CO-CO-ATWLPPR-NH_2 onto spherulites™: Spherulites™, 8% (*w*/*w*), were diluted twice with solutions of different concentrations of H-CO-CO-ATWLPPR-NH_2. The reaction was followed by capillary electrophoresis by using a fused silica capillary (75 μm diameter, 27 cm length) in 50 mM acetate buffer pH 5.5, electrical field: E = 259 V cm^{-1}, on a Beckman Coulter PAGE-MDQ electrophoresis apparatus. Before capillary electrophoresis, samples were diluted five- and tenfold with distilled water to give peptide concentrations of 0.9 and 1.9 equivalents, respectively ([H-CO-CO-ATWLPPR-NH_2] = 3.0 and

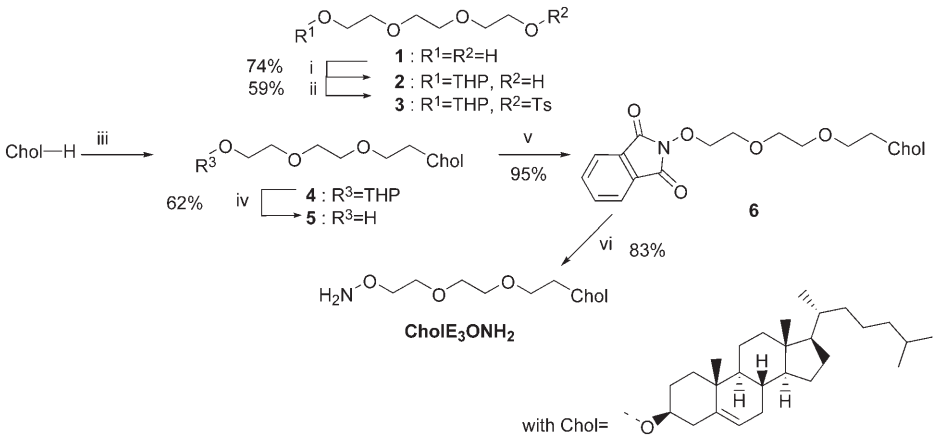
6.1 mM), to maintain the linearity of the peak areas. Mass spectroscopy of spherulites™ was performed after filtration over commercial NAP 5 columns (Amersham Pharmacia Biotech) (stationary phase Sephadex G-25). The suspension was then dissolved in THF before deposition on DHB and subjection to mass analysis.

Fluorometry: A Quantamaster C60 fluorometer was used. The samples were placed in a quartz vessel (10×2 mm). Emission spectra were recorded at between 310 and 500 nm, with excitation wavelength at 295 nm, as shown in Figure 4. Fluorescence intensity was recorded at 488 nm over time, with excitation at 366 nm (Figure 5).

Dynamic light scattering: Vesicle size was measured by using a Dyna-ProMS800. Spherulite™ suspensions were diluted by 1/1000 or 1/2000 (*m*/*m*) in distilled water and filtrated over 0.22 μm. Mean results are given for 100 acquisitions.

Results

Design and synthesis of the new anchor: $CholE_3ONH_2$ was designed to comprise three parts: 1) the reactive head hydroxylamine, 2) a short spacer triethyleneglycol to modulate the distance between the MRP and the vesicle and 3) a cholesterol moiety as the lipophilic group to be incorporated into the lipidic membranes. The synthesis of this new anchor was performed in three main steps (Scheme 1). Firstly, the commercial triethylene glycol **1** was asymmetrised to get synthon **3**. Next, **3** was coupled to the cholesterol and finally, the hydroxylamine head was introduced onto the alcohol end of **5**.



Scheme 1. Synthetic route to the anchor $CholE_3ONH_2$. i) 0.3 equiv DHP, two drops HCl, 18 h, 74%; ii) 1.5 equiv tosyl chloride, 1.5 equiv pyridine, CH_2Cl_2, 24 h, 59%; iii) a: 3 equiv NaH, 1 h, DMF/dioxane 1:1; b: 0.8 equiv **3**, 48 h; iv) 0.2 equiv 10-camphorsulfonic acid, absolute ethanol, 8 h, 62% (steps iii + iv); v) 2 equiv PPh_3, 2 equiv DEAD, 2 equiv *N*-hydroxyphthalimide, anhydrous THF, 3 h, 95%; vi) 10 equiv aqueous hydrazine, absolute ethanol, 2 h, 83%. Ts= tosyl, THP= tetrahydropyranyl, Chol= cholesterol.

In detail, as shown in Scheme 1, the asymmetrisation of the triethylene glycol **1** occurred by the protection of one hydroxyl end by 2,3-dihydropyrane (DHP) in the presence of a catalytic amount of hydrochloric acid to form a tetrahydropyranyl (THP) protecting group.[18] Next, **3** was isolated after reaction of **2** on tosyl chloride in the presence of pyridine.[19] Once obtained, the tosylate was allowed to react on the alcoholate of cholesterol, which was formed by the reaction of cholesterol with sodium hydride.[20] After purification on a silica column, compound **4** was isolated together with an amount of unreacted cholesterol, which was more easily removed after deprotection of the THP. The hydroxylamine function was introduced by coupling the *N*-hydroxyphthalimide by means of a "Mitsunobu" reaction.[21] Finally, the phthalimide group was removed by hydrazinolysis to give the anchor $CholE_3ONH_2$ with 21% overall yield, and 49% yield starting from cholesterol.

Reaction of a glyoxyl peptide onto spherulites™ functionalised with the $CholE_3ONH_2$ anchor: The anchor $CholE_3ONH_2$ was incorporated into the membrane of spherulites™ by replacing the cholesterol of the initial formulation mol by mol (Table 1). Because the pK_a of an alkyl hydroxylamine is approximately 4.5, we assumed that, when functionalised with the anchor $CholE_3ONH_2$, spherulites™ would be free of electrical charges at physiological pH 7.4. The size distribution of each kind of spherulite™ was measured by using the dynamic light scattering technique. The mean diameter was found to be close to 250 nm, with a polydispersity of 50%. No significant difference in size was observed between **S1** and **S2**, containing cholesterol or $CholE_3ONH_2$, respectively.

Table 1. Massic compositions of spherulites™[a].

	PC	Tween 80	Chol	$CholE_3ONH_2$	Water	Mean diameter [nm] (polydispersity)
S1	23.2	13.6	3.2	0	60	236 (50%)
S2	22.5	13.2	0	4.3	60	262 (51%)

[a] Values are given as percentages.

We used capillary electrophoresis to monitor the concentration of H-CO-CO-ATWLPPR-NH_2 (a peptidic recognition pattern of the receptor VEGF-R2 encountered on angiogenic cells[22]) in the presence of spherulites™ **S1** or **S2**. As shown in Figure 1, for spherulites™ prepared without $CholE_3ONH_2$ (**S1**), no significant decrease in the concentration of peptide was observed over a period of two days. In addition, after two days of contact, no fluorescence of the tryptophan residue was observed following gel filtration (Figure 2a). We concluded that there was no significant nonspecific adsorption of peptide H-CO-CO-ATWLPPR-NH_2 onto the outer membrane of spherulites™.

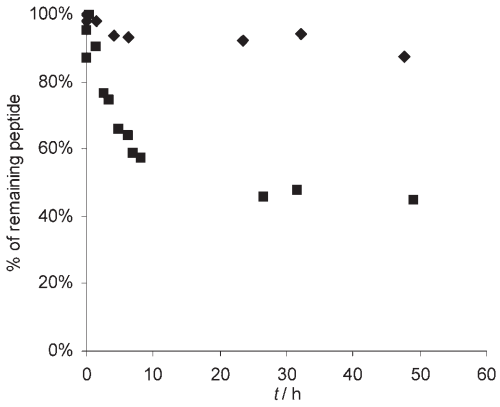


Figure 1. Percentage of peptide H-CO-CO-ATWLPPR-NH_2 remaining in the suspension as a function of time, measured by capillary electrophoresis. ◆: spherulites **S1**, ■: spherulites **S2**. Initial [peptide] = 3.17 mM. Spherulites™ were dispersed in water at 4%.

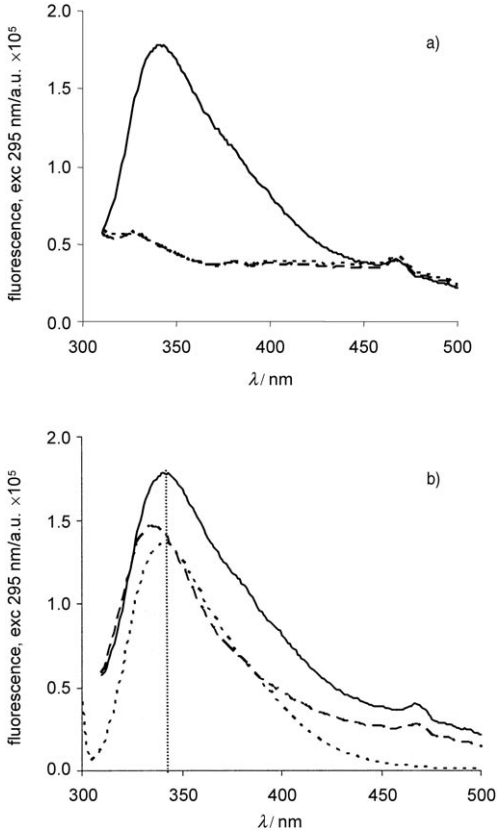


Figure 2. Intensity of fluorescence of the tryptophan residue of peptide H-CO-CO-ATWLPPR-NH_2 in response to excitation at 295 nm as a function of emission wavelength. a) Suspensions of **S1**: (——) dispersed in a solution of peptide of 608 μM, (– – –) dispersed in a solution of peptide then submitted to gel filtration and (•••••) dispersed in water. b) Suspensions of **S2**: (——) dispersed in a solution of peptide, (– – –) dispersed in a solution of peptide then submitted to gel filtration and (•••••) a solution of H-CO-CO-ATWLPPR-NH_2 of 60.8 μM in water.

On the other hand, for spherulites™ prepared with Chol-E_3ONH_2 (**S2**), the concentration of the glyoxylyl peptide decreased over time (t; Figure 1). This decrease could be well fitted by applying the monoexponential function defined in Equation (1), in which A is the percentage of peptide that reacted with the anchor, B is the percentage of unreacted peptide ($A+B=100$), and τ is the characteristic time of the reaction.

$$\%\ \text{peptide in solution} = A\, e^{-t/\tau} + B \qquad (1)$$

The fluorescence emission spectrum obtained after gel filtration of spherulites **S2** reveals that, firstly, when excited at the tryptophan excitation wavelength, the vesicles emit fluorescence, and secondly, the maximum of emission was 5 nm lower than that of the free peptide (Figure 2b). Thus, we concluded that 1) the peptide was immobilised onto the vesicles and 2) the environment of the peptide was less polar than in aqueous solution, in other words, the peptide was close to a lipidic membrane.

To confirm that the detection of peptide fluorescence correlated to the expected α-oxo oxime bond formation, a MALDI-TOF mass spectrum was recorded. To permit this analysis, the spherulites™ were disrupted by THF. The MALDI-TOF mass spectrum showed a peak at $m/z=1411.3$ and the ligation adduct $[M+H^+]$ was calculated to be 1410.9 (see Supporting Information). We could, therefore, conclude that the α-oxo oxime ligation reaction occurred between the glyoxylyl peptide and the spherulites™ functionalised with the new anchor CholE_3ONH_2.

To quantify this reaction, we measured the kinetics of the ligation of different proportions of peptide H-CO-CO-ATWLPPR-NH_2 onto spherulites **S2**. The change in percentage of peptide in solution was fitted by Equation (1), and all the parameters of the kinetics are reported in Table 2. Characteristic times of the reaction were between 15 and 20 h, if less than 0.2 equivalents of peptide, with respect to total anchor, was introduced into the suspension **S2**. By increasing the ratio of peptide:anchor, the characteristic time decreased, falling to less than half an hour for 1.9 equivalents of peptide. Moreover, from the parameter A of Equation (1), we could estimate the quantity of peptide consumed by the ligation. By plotting the number of equivalents consumed in the reaction as a function of the number

Table 2. Adjusted parameters of Equation (1) describing the kinetics of the ligation reaction. Spherulites™ were suspended at 4% *w*/*w*, and [CholE_3ONH_2] = 3.2 mM.

Equivalents of peptide	A	B	τ [h]	R^2
0.04	93.1 (±3.0)	6.8 (±3.3)	17.4 (±1.8)	0.9948
0.09	87.0 (±3.1)	12.9 (±3.6)	20.3 (±1.9)	0.9957
0.09	85.5 (±3.2)	14.6 (±3.4)	18.3 (±2.4)	0.9867
0.15	87.1 (±2.4)	12.9 (±2.3)	13.4 (±1.3)	0.9922
0.19	90.5 (±3.8)	9.5 (±4.1)	22.6 (±2.7)	0.9874
0.23	75.2 (±6.5)	24.8 (±6.1)	12.1 (±3.2)	0.9497
0.38	86.3 (±3.8)	13.7 (±2.6)	11.2 (±1.6)	0.9759
0.95	53.0 (±3.9)	47.0 (±2.7)	5.7 (±0.95)	0.9530
0.95	68.6 (±5.0)	31.4 (±3.4)	10.2 (±2.4)	0.9413
1.9	29.9 (±4.1)	70.1 (±0.99)	0.43 (±0.12)	0.9192

of equivalents of peptide engaged by the ligation (Figure 3), we saw that only 60% of the anchors were accessible to the peptide. Indeed, the number of equivalents of consumed

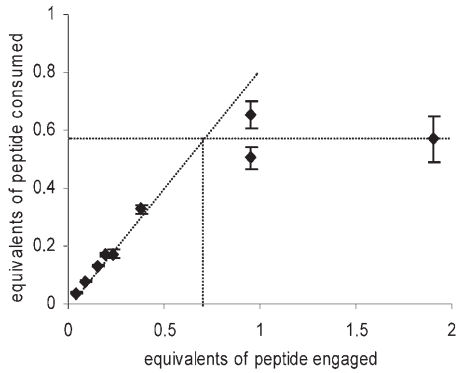


Figure 3. Number of equivalents of peptide consumed by the ligation as a function of the number of equivalents of peptide H-CO-CO-ATWLPPR-NH_2 engaged in the reaction.

peptide increased to 0.6 before 0.6 equivalent of peptide was engaged. Subsequently, the number of equivalents of consumed peptide remained constant at 0.6. A plot of the number of equivalents of peptide consumed against the fluorescence emission of tryptophan in spherulites™ reacting with different proportions of peptide (Figure 4) reveals a

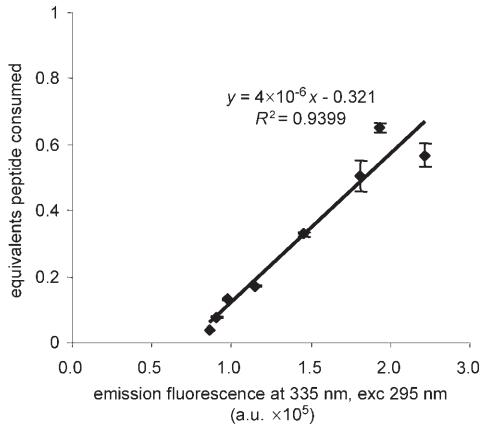


Figure 4. Linear correlation between the number of equivalents of peptide consumed by the ligation measured by capillary electrophoresis and the fluorescence of the spherulites™.

good linear correlation between these two parameters. Thus, we can reasonably suppose that all of the peptide consumed for the ligation remains in the spherulites™.

Effect of membrane composition on the kinetics of the reaction: The anchor $CholE_3ONH_2$ was incorporated into spherulites™ with various membrane compositions (Table 3). The main differences were in the surfactant used (Tween 80 for **S3**, Simulsol 2599 for **S4** and Solutol HS 15 for **S5**), and the presence (**S3** and **S5**) or absence (**S4**) of cholesterol.

Table 3. Massic compositions of spherulite™ membranes[a] that were used to study the accessibility of the internal anchor $CholE_3ONH_2$.

	PC	Cosurfactant	Chol	$CholE_3ONH_2$	Water
S3	22.7	Tween 80: 13.4	1.2	2.7	60
S4	41.9	Sim[b]: 18.9	0	4.2	35
S5	41.1	Sol[c]: 13	6.6	4.4	35
S6	48	Sol[c]: 14.3	11.4	0	35

[a] Values are given as percentages. [b] Simulsol 2599. [c] Solutol HS 15.

The kinetics of the ligation of peptide H-CO-CO-ATWLPPR-NH_2 were monitored by conducting capillary electrophoresis, and were subsequently fitted by Equation (1) (Table 4). For suspensions of spherulites **S3** and **S5** with the same concentrations of peptide, the characteristic times τ were close to 20 h. In contrast, for spherulites **S4**, the rate of reaction was ten times quicker, with a τ of approximately 2 h.

Table 4. Kinetic parameters for the ligation of peptide H-CO-CO-ATWLPPR-NH_2 (3.17 mM) onto spherulites **S3**, **S4** and **S5** (4% *w*/*w* in peptidic solution).

	% Consumed peptide	τ [h]	% Exposed anchor	$\frac{n\ consumed\ peptide}{n\ exposed\ anchor}$
S3	91 (±5)	24.9 (±3.2)	15	9.6 (±0.5)
S4	87 (±11)	2.2 (±0.7)	6	14.6 (±1.6)
S5	63 (±20)	20.7 (±10.3)	6	10.5 (±2.1)

Notably, in contrast to **S3** and **S5**, the suspension of **S4** has no cholesterol in its membrane. We can, therefore, suppose that the presence of cholesterol slows down the kinetics of the reaction. Moreover, if we consider the ratio of "consumed peptide/anchor exposed on the surface of spherulites™" (mol/mol), we see that, in each case, about ten times more anchor is engaged in the ligation than is exposed on the surface. Thus, not only the anchor exposed on the surface is accessible to the glyoxylyl peptide in solution, but also the anchor trapped within the vesicles.

Evidence for the internalisation of the glyoxylyl peptide: We confirmed that only the spherulites™ functionalised with the anchor $CholE_3ONH_2$ were capable of internalizing the glyoxylyl peptide. This was demonstrated by encapsulating 3-methyl-2-benzothiazolinone hydrazone (MBTH), a nonfluorescent, hydrazine-like molecule that becomes fluorescent only after it has reacted with a glyoxylyl compound,[23] in spherulites **S5** (containing anchor) and **S6** (without anchor). **S5** and **S6** were incubated with an aqueous solution of the studied glyoxylyl peptide and the fluorescence intensity of the suspensions was recorded over time (excitation at 366 nm, emission at 488 nm). As shown in Figure 5, fluorescence of the ligation adduct between MBTH and the glyoxylyl peptide was detected and increased only in the case of spherulites™ containing $CholE_3ONH_2$ in their mem-

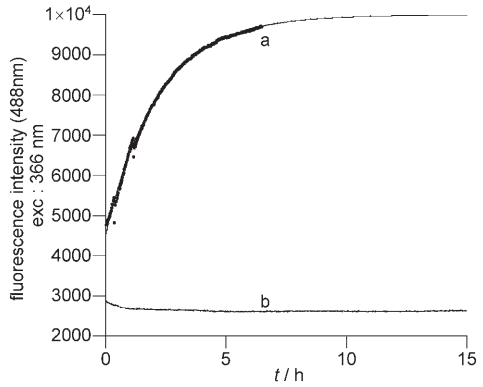


Figure 5. Evolution of fluorescence intensity at 488 nm (excitation at 366 nm) of suspensions of spherulites **S5** containing the anchor $CholE_3ONH_2$ (curve a), or spherulites **S6** without the anchor (curve b), at 4% (*w*/*w*) in a 3.17 mM solution of H-CO-CO-ATWLPPR. The internal aqueous layers of spherulites™ contained 10 mM of MBTH.

branes (**S5**). The fluorescence intensity reached a plateau after 10 h of reaction. In contrast, the level of fluorescence of spherulites **S6** remained low and constant for at least 15 h, confirming that there was no leakage of MBTH, and no entrance of the glyoxylyl peptide into spherulites™ that did not have the anchor.

Discussion

The fact that the anchor is also localised within the internal membranes of spherulites™ does not hinder its reactivity toward a peptide dissolved in the outer solution. In contrast, membrane composition can dramatically affect the kinetics of ligation onto and within the spherulites™. Moreover, Figure 4 shows that all the peptide consumed for the ligation remains incorporated within the spherulites™. As the proportion of the consumed anchor is greater than the proportion of the anchor exposed on the surface, we can conclude that the functionalisation of spherulites™ by the α-oxo oxime ligation also involves some internal anchors. Furthermore, a glyoxylyl peptide can react with a hydrazine-like molecule encapsulated within spherulites™ that contain the hydroxylamine anchor $CholE_3ONH_2$.

Spherulites™ do not encapsulate unligated peptides. Indeed, we proved by fluorescence spectroscopy after gel filtration that no detectable peptide remains within spherulites™ if it is not ligated with $CholE_3ONH_2$. We propose that the first step is the rapid reaction of the outer anchor with the solubilised glyoxylyl peptide.

As the lipid composition changed, the physical properties of the membranes were modified, in particular the lipid mobility in the flip-flop motion. The kinetics of the ligation differ as the formulation of the membrane is changed, although the concentrations of reactants are kept constant. Therefore, the chemical reaction on the surface of spherulites™ is not the limiting step. Thus, we suppose that the reaction involving the internal anchors confers the kinetic features to the system.

We propose the following explanation, represented in Figure 6: In the first step, the glyoxylyl peptide reacts with hydroxylamine anchors on the external membrane surface of the spherulites™. In the second step, by means of a flip-flop motion, ligation adducts can switch from the outer to the internal membrane surface. In the same manner, anchors firstly localised on the internal surface of the first bilayer can exchange and move to the outer surface. Thus, ligation adducts and anchors that have not yet reacted are

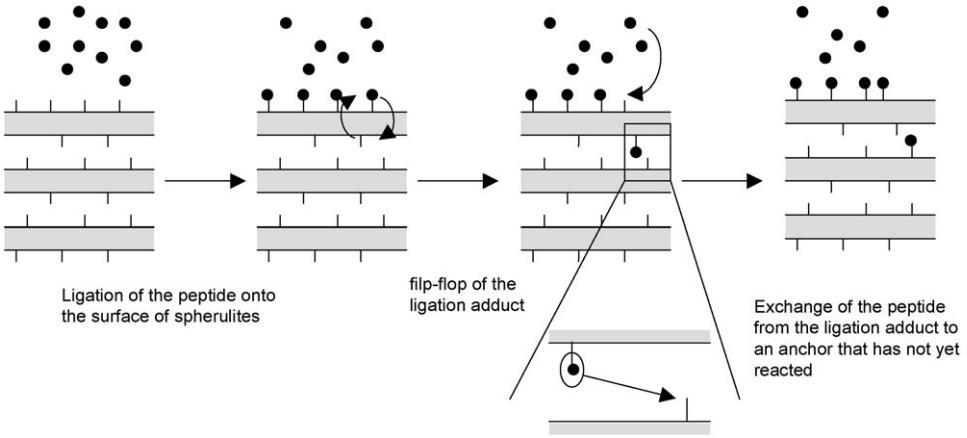


Figure 6. Hypothetical mechanism of ligation between a peptide and anchors contained both on and within the spherulites™.

"squeezed" into a limited space. As the formation of an oxime is in equilibrium, the bond between the peptide and the anchor can be hydrolysed, and the peptide can then react with anchors from deeper membrane layers. This hypothesis was confirmed by the ability of the glyoxylyl peptide to react with MBTH encapsulated in spherulites™, provided the anchor $CholE_3ONH_2$ was present in their membranes (Figure 5). In this case, after flip-flop exchanges and hydrolysis of the lipopeptide, the glyoxylyl peptide can react with MBTH present in the aqueous layers of spherulites™. By this mechanism, it is reasonable to think that 1) the oxime bond can be hydrolysed in the inside of the vesicles, thus releasing free glyoxylyl peptide ready to react again and that 2) the flip-flop motions are the limiting step and confer the functionalisation kinetics to the spherulite™ suspensions. We saw previously that the presence of cholesterol in membranes decreases significantly the kinetics of the functionalisation of the spherulites™. These results converge and are consistent with the rigidification of the mem-

brane generally observed by cholesterol's contribution to membrane formulations. Indeed, in this case, the cholesterol seems to reduce the motion of the lipids in the bilayers and, in particular, the flip-flop velocity. These effects seem to decrease the kinetics of peptide internalisation.

Conclusion

We observed that, due to the reversibility of the α-oxo oxime bond, a glyoxylyl peptide can enter multilamellar vesicles by passing through lipidic bilayers. We calculated the kinetic and thermodynamic aspects of this entry and analysed the reasons why this phenomenon occurs. The reaction was dependent upon the presence of a hydroxylamine cholesterol-like anchor. The more peptide submitted for ligation, the more entered the vesicles, until a limit of 60% was reached. We can say that the presence of the hydroxylamine anchor in internal layers of spherulites™ acted as a "driving force" for the encapsulation of a glyoxylyl peptide by these multilayer vesicles. Finally, the components of the membrane played a key role in internalisation, especially cholesterol, whose presence seemed to be correlated to membrane rigidity and the flip-flop motions. These results suggest that multilamellar onion-like vesicles can be regarded not only as cargo vesicles, but also as nanoreactors. Two reagents displaying opposite physico-chemical properties and partitioned in two different compartments, even those present in the deep membranes of the vesicle, were able to react due to their confinement. In this field, a reduction reaction has already been reported.[24]

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