Behavior of a photoactivatable analog of cholesterol, 6-photocholesterol, in model membranes

Evan A. Mintzer[a], Barry-Lee Waarts[b], Jan Wilschut[b], Robert Bittman[a,*]

[a] *Department of Chemistry and Biochemistry, Queens College of the City University of New York, Flushing, NY 11367, USA*
[b] *Department of Medical Microbiology, Molecular Virology Section, University of Groningen, Ant. Deusinglaan 1, 9713 AV Groningen, The Netherlands*

Received 12 November 2001; accepted 6 December 2001

First published online 14 December 2001

Edited by Hans-Dieter Klenk

Abstract 6-Photocholesterol, a new photoactivatable analog of cholesterol in which a diazirine functionality replaces the 5,6-double bond in the steroid nucleus, was used recently to identify cholesterol-binding proteins in neuroendocrine cells [Thiele, C., Hannah, M.J., Farenholz, F. and Huttner, W.B. (2000) Nat. Cell Biol. 2, 42–49], to track the distribution and transport of cholesterol in *Caenorhabditis elegans* [Matyash, V., Geier, C., Henske, A., Mukherjee, S., Hirsh, D., Thiele, C., Grant, B., Maxfield, F.R. and Kurzchalia, T.V. (2001) Mol. Biol. Cell 12, 1725–1736], and to probe lipid–protein interactions in oligodendrocytes [Simons, M., Kramer, E.M., Thiele, C., Stoffel, W. and Trotter, J. (2000) J. Cell Biol. 151, 143–154]. To determine whether 6-photocholesterol is a faithful mimetic of cholesterol we analyzed the ability of this probe, under conditions in which it is not photoactivated to a carbene, to substitute for cholesterol in two unrelated assays: (1) to condense 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine monomolecular films and (2) to mediate the fusion of two alphaviruses (Semliki Forest and Sindbis) with liposomes. The results suggest that this analog is a suitable photoprobe of cholesterol. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Photoaffinity labeling; Sterol; Virus fusion; Membrane fusion

1. Introduction

Cholesterol is a major component of cellular membranes and serum lipoproteins. Its interactions with other membrane constituents, including glycerophospholipids [1] and sphingomyelin [2,3], have been investigated intensively, and these interactions are essential for many cellular processes [1]. Cholesterol-rich domains in membranes, or rafts, are believed to act as protein-localizing platforms; these domains have been implicated in protein and lipid sorting and trafficking [4,5]. Cholesterol is also involved in the fusion of alphaviruses, specifically Semliki Forest virus (SFV) and Sindbis virus (SIN), with their host cell target membranes [6–12].

The membrane properties of cholesterol are directly related to its structure. The rigid ring system of the sterol nucleus affords structural properties to the membrane [13,14], and the presence of the 3β-hydroxyl group is required for the condensing effect of cholesterol on phospholipid monomolecular films [15], as well as for the membrane fusion process of SFV and SIN [7,9]. In addition, subtle alterations in the structure of the isooctyl side chain perturb the interactions of cholesterol with other membrane lipids [1,16].

Photoactivatable analogs of lipids have been frequently employed to label proteins targeted by the parent lipids [17,18]. However, the introduction of a photophore into the molecule of interest may interfere with the native behavior of the compound. A photoactivatable cholesterol analog, 6-photocholesterol, in which a diazirine functionality (a carbene precursor) has been introduced into the 6 position and the C5,C6-double bond is absent, has been shown recently to bind to cholesterol-binding proteins in brain synaptic vesicles [19]. This probe was also utilized to identify a cholesterol-binding protein in *Caenorhabditis elegans* [20] and to study cholesterol–proteolipid protein interactions in oligodendrocytes [21]. In the present study, we found that 6-photocholesterol is fully capable of mimicking the behavior of cholesterol with respect to mediating the fusion of SFV and SIN fusion with target liposomes and condensing 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) films at all surface pressures we examined. Our results provide support for the use of 6-photocholesterol as a suitable probe for the identification of cholesterol-binding proteins in membranes.

2. Materials and methods

2.1. Monolayer experiments and isotherm analysis

Cholesterol and POPC were obtained from Avanti Polar Lipids (Alabaster, AL, USA) and used without further purification. 6-Photocholesterol was synthesized from 6-keto-5α-cholestan-3β-ol as previously described [19]. Lipid stock solutions were prepared by dissolving the pre-weighed solid in a known volume of spreading solvent (HPLC-grade hexane/2-propanol, 3/2). The solutions were stored at −20°C and warmed to room temperature prior to each experiment. Solutions containing 6-photocholesterol were kept in the dark, and all experiments involving this probe were carried out under subdued light. Monolayer experiments were performed on a subphase of pure water (distilled and passed through a Milli-Q (Millipore Corp., Bedford, MA, USA) purification system to a resistivity of 18 MΩ/cm) at ambient temperature (21 ± 1°C). Plots of surface pressure vs. molecular area of monolayers were constructed using a computer-controlled Kibron μ Trough S surface balance (Helsinki, Finland). Binary films were prepared by mixing appropriate volumes from stock solutions, and all mixtures were produced immediately prior to use. Lipids were spread in small aliquots (< 10 μl) with a Hamilton digital syringe, which was rinsed with spreading solvent repeatedly before and

*Corresponding author. Fax: (1)-718-997 3349.
E-mail address: robert_bittman@qc.edu (R. Bittman).

Abbreviations: SFV, Semliki Forest virus; SIN, Sindbis virus; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine

0014-5793/02/$22.00 © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.
PII: S0014-5793(01)03271-9

after use, and compressions were initiated after a 5-min delay to allow evaporation of the spreading solvent. The compression rate did not exceed 4 Å^2/molecule/min.

Data were collected with Filmware, a proprietary program from Kibron, and the resulting isotherms were analyzed using Filmfit software (Creative Tension, Austin, MN, USA). Ideal additivity of binary monolayer systems is described by:

$$A_\pi = X_1(A_1)_\pi + (1-X_1)(A_2)_\pi \quad (1)$$

where X_1 and $(1-X_1)$ are the mol fractions of components 1 and 2, respectively, and $(A_1)_\pi$ and $(A_2)_\pi$ are the molecular areas of pure components 1 and 2 at identical surface pressures, π [22]. Negative deviations from the linear relationship described by Eq. 1 are considered to indicate condensation, implying molecular packing among the components in the binary system [23].

2.2. Viral fusion assay

Fusion of SFV or SIN with liposomes was measured using a lipid-mixing assay based on pyrene excimer fluorescence as described before [9–12]. Briefly, pyrene-labeled viruses were generated by infection of BHK-21 cells, which were cultured beforehand in the presence of 16-(1-pyrenyl)hexadecanoic acid (Molecular Probes, Eugene, OR, USA). Virus collected from the cell supernatant was purified by sucrose density gradient centrifugation. Liposomes (large unilamellar vesicles, diameter 200 nm) were prepared in 5 mM HEPES, 150 mM NaCl, 0.1 mM EDTA, pH 7.4 (HNE), by a freeze/thaw extrusion procedure [24]. Liposomes consisted of phospholipids (from Avanti Polar Lipids) and high-grade cholesterol (from Solvay Pharmaceuticals, Weesp, The Netherlands) or 6-photocholesterol (see above). The phospholipids used were phosphatidylcholine (PC) from egg yolk, phosphatidylethanolamine (PE) prepared by transphosphatidylation of egg PC, and sphingomyelin (SPM) from bovine brain. Hydrated lipid mixtures, subjected to five cycles of freezing and thawing, were extruded 21 times through a Unipore polycarbonate filter with a pore size of 0.2 μm (Nucleopore, Pleasanton, CA, USA) in a LiposoFast mini-extruder (Avestin, Ottawa, Canada).

Pyrene-labeled SFV or SIN (0.5 μM viral phospholipid) and liposomes (200 μM phospholipid) were mixed in 0.665 ml of HNE in a magnetically stirred and thermostatted (37°C) quartz cuvette in an AB2 fluorometer (SLM/Aminco, Urbana, IL, USA). Fusion was triggered by the addition of 35 μl of 0.1 M MES, 0.2 M acetic acid, pretitrated with NaOH to achieve the final desired pH, and the decrease of pyrene excimer fluorescence intensity (excitation and emission at 340 and 480 nm, respectively) was monitored continuously. The fusion scale was calibrated such that 0% fusion corresponded to the initial excimer fluorescence intensity and 100% fusion to complete dilution of the probe [9–12].

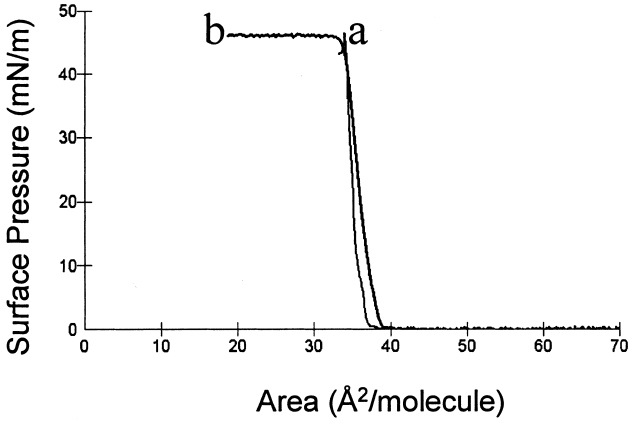

Fig. 1. Compression isotherms of pure sterols cholesterol (curve a) and 6-photocholesterol (curve b). Compressions were performed on a subphase of pure water (see Section 2.1) and are representative of at least three runs. Experiments with 6-photocholesterol were performed under subdued light.

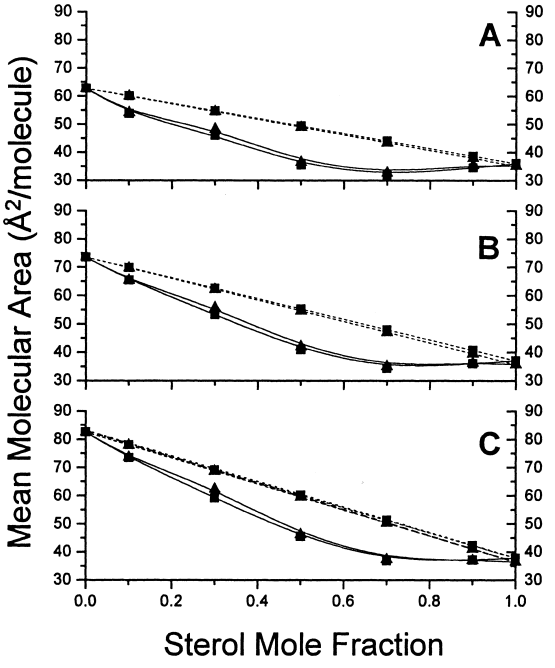

Fig. 2. Mean molecular area vs. composition at three surface pressures. A: 5 mN/m; B: 10 mN/m; C: 20 mN/m. Dashed lines represent ideal additivity as derived from Eq. 1. ▲, cholesterol; ■, 6-photocholesterol.

3. Results

3.1. Pure sterol films

The force-area isotherms for monolayers formed from pure cholesterol and 6-photocholesterol were compared (Fig. 1, curves a and b, respectively). Introduction of the diazirine functionality at the 6 position resulted in an increase of the mean molecular area of the sterol at low surface pressure by approximately 1.6 Å^2. The steep rise in the cholesterol isotherm into a condensed film, suggestive of an orientation normal to the plane of the interface [25,26], was also present in the 6-photocholesterol isotherm, although slightly attenuated. This is consistent with the molecule displaying a barely discernible tilt relative to the surface of the subphase. As the film was compressed, the interaction between the diazirine group and water (presumably the cause of this slight tilt) was abruptly eliminated, and the surface pressure increased without significant change in area. At this point, the rigid sterol nucleus prevents any further significant change in molecular area as the lateral pressure is increased. The close agreement of the collapse pressures of the two compounds (46.1 ± 0.2 mN/m; Fig. 1) indicates that monolayers of either sterol can withstand similar lateral pressures.

3.2. Mixed sterol–POPC films

It is well known that cholesterol increases the packing density of phospholipid-containing monolayers [23,27]. To further compare the interfacial behavior of 6-photocholesterol with that of cholesterol, mixed films of POPC and sterol were compressed at the air–water interface. Stable monolayers were formed from POPC and varying mol fractions of cholesterol or 6-photocholesterol at all compositions (isotherms not shown). The liquid-expanded phase of POPC persisted in mixtures with both sterols, but the curves became increasingly

steep as the mol% of POPC decreased. At 70 mol% sterol, they resembled a condensed film, consistent with strict ordering of the hydrocarbon chains at this composition. The observed increase in collapse pressure with higher sterol mol fraction indicates intimate miscibility of the two components in the monolayer (data not shown) [28].

To compare quantitatively the condensation effects we plotted the molecular areas as a function of composition at 5, 10 and 20 mN/m (Fig. 2A–C). Dashed lines represent ideal additivity according to Eq. 1. Significant reductions in molecular area were observed in each mixture and at all surface areas examined. The condensation effect induced in POPC by 6-photocholesterol or cholesterol was virtually indistinguishable at most compositions, and was greatest at high surface pressure. The effect increased with increasing mol% sterol, but displayed a smaller effect at 90 mol%.

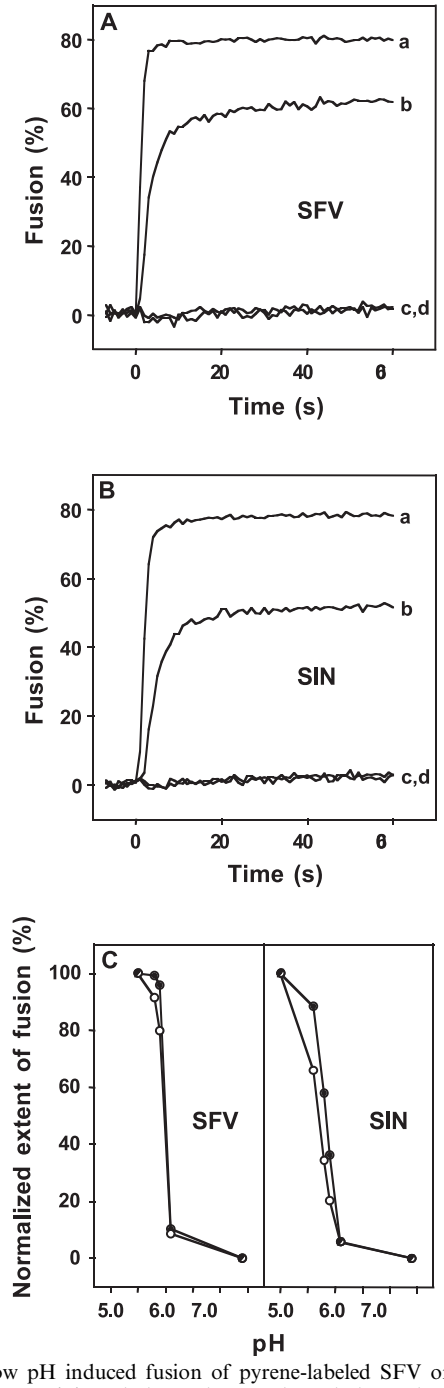

Fig. 3. Low pH induced fusion of pyrene-labeled SFV or SIN with liposomes containing cholesterol or 6-photocholesterol. Fusion was measured online as a decrease of pyrene excimer fluorescence intensity, as described in Section 2. A and B present fusion curves for SFV (at pH 5.5) and SIN (at pH 5.0), respectively; curves a, liposomes containing 6-photocholesterol at acid pH; curves b, liposomes containing cholesterol at acid pH; curves c, liposomes containing 6-photocholesterol at pH 7.4; curves d, liposomes without sterol at acid pH. C presents the detailed pH dependence of SFV and SIN fusion with liposomes containing 6-photocholesterol (solid circles) or cholesterol (open circles); the relative extents of fusion at 60 s post-acidification are presented normalized to the values at pH 5.5 (for SFV) or pH 5.0 (for SIN).

3.3. Fusion of SFV and SIN with liposomes

In order to evaluate the ability of 6-photocholesterol to support alphavirus fusion, we compared liposomes consisting of PC/PE/SPM/cholesterol or PC/PE/SPM/6-photocholesterol (molar ratios 1:1:1:1.5) as target membranes for fusion of SFV or SIN. These alphaviruses enter cells by a process of receptor-mediated endocytosis, followed by low pH induced fusion of the viral envelope with the endosomal membrane, with concomitant release of the viral genome into the cell cytosol [29,30]. It is well established that fusion of SFV or SIN requires the presence of cholesterol in the target membrane, both in model membranes and in cells [6–12].

Fusion was initiated by acidification of a mixture of pyrene-labeled virus and an excess of liposomes in the cuvette of the fluorometer. In this system, fusion is monitored by a continuous decrease of the pyrene excimer fluorescence intensity arising from dilution of pyrene-labeled lipids from the viral membrane into the target liposomes. At low pH, both SFV (Fig. 3A) and SIN (Fig. 3B) fused rapidly and extensively with the liposomes containing 6-photocholesterol (curves a). With both SFV and SIN there was no fusion at neutral pH (curves c). Fusion at low pH was dependent on the presence of 6-photocholesterol in the bilayer, since neither SFV nor SIN underwent fusion with liposomes prepared from PC/PE/SPM (curves d). Interestingly, 6-photocholesterol appeared to be even more potent than cholesterol in supporting fusion with SFV or SIN; fusion with liposomes containing 6-photocholesterol (curves a) was faster and more extensive than with those containing cholesterol (curves b). In control experiments, it became evident that 6-photocholesterol has the capacity to reversibly quench both excimer and monomer pyrene fluorescence (results not shown). This explains the apparent faster kinetics and higher extents of virus fusion with photocholesterol-containing liposomes, fusion being monitored as a decrease of pyrene excimer fluorescence intensity. In the case of photocholesterol-containing liposomes, this decrease results from the dilution of the pyrene probe from the viral into the liposomal membrane and from the quenching of pyrene fluorescence. Significantly, however, the pH threshold for fusion of both SFV and SIN with liposomes was identical, irrespective of whether cholesterol or 6-photocholesterol was included in the target liposomes, as indicated by the normalized extents of fusion shown in Fig. 3C.

4. Discussion

Recent in vivo experiments showed that 6-photocholesterol is incorporated into lipid rafts in much the same manner as cholesterol, and that the analog binds to apolipoproteins A1

and B and caveolin/VIP21, which are both known to bind to cholesterol [19,31]. This photoactivatable probe of cholesterol was also employed recently to show that ABCA1, an ATP-binding cassette transport protein, did not bind directly to cholesterol in living cells [32]. We sought here to analyze the extent to which this photoprobe mimics the behavior of cholesterol in model membranes by using two different assays: (a) the condensing effect in PC-containing monolayers and (b) the role of cholesterol in supporting the fusion of alphaviruses with target membranes.

The monolayer approach is well suited for investigation of the interfacial properties of biologically relevant lipids (e.g. [33,34]). The condensing effect of cholesterol results from the reduction of the extent of trans-gauche isomerization of the fatty acyl chains of phospholipids in the presence of the sterol [35]. In the presence of cholesterol, which is anchored by the 3β-hydroxyl group to the aqueous interface, the phospholipid's fatty acyl chains wrap around and are stabilized by the rigid sterol nucleus, resulting in acyl chain ordering. These attractive van der Waals interactions represent a measure of the affinity between the two lipids [14,36,37]. The monolayer results (Fig. 2) demonstrate that the structural features that impart these properties to cholesterol are not disturbed by the absence of the C5,C6-double bond and inclusion of the diazirine functionality at the 6 position.

It is well established that target membrane cholesterol is critically involved in the low pH induced membrane fusion process of SFV or SIN during the infectious entry of these viruses into cells [6–12]. In addition, studies in model systems have revealed that the presence of sphingolipids in the target membrane is required [11,12]. At low pH, conformational changes in the E1/E2 heterodimeric viral envelope glycoprotein mediate tight binding of the virus to target membrane cholesterol [11]. Sphingolipids appear to catalyze the fusion reaction per se, presumably through direct interaction with the E1 component of the viral spike, thus promoting the formation of an active fusion machine. Here, we demonstrate that 6-photocholesterol fully maintains the ability of cholesterol to support fusion of both SFV and SIN with liposomes. The characteristics of the fusion processes in terms of the detailed pH dependence are very similar to those with cholesterol-containing liposomes (Fig. 3). This underlines the above conclusion that 6-photocholesterol is a good mimic of cholesterol.

Acknowledgements: This work was supported by the US National Institutes of Health (Grant HL 16660) and by The Netherlands Organization for Scientific Research (NWO) under the auspices of the Foundation for Chemical Research (CW). We thank Dr. Kochurani Jacob for the synthesis of 6-photocholesterol.

References

[1] Bittman, R. (1997) in: Subcellular Biochemistry Vol. 28 (Bittman, R., Ed.), pp. 145–171, Plenum, New York.
[2] Slotte, P. (1997) in: Subcellular Biochemistry Vol. 28 (Bittman, R., Ed.), pp. 277–293, Plenum, New York.
[3] Ohvo-Rekilä, H., Ramstedt, B., Leppimäki, P. and Slotte, J.P. (2002) Prog. Lipid Res. 41, 66–97.
[4] Simons, K. and Ikonen, E. (1997) Nature 387, 569–572.
[5] Simons, K. and Ikonen, E. (2000) Science 290, 1721–1726.
[6] White, J. and Helenius, A. (1980) Proc. Natl. Acad. Sci. USA 77, 3273–3277.
[7] Kielian, M.C. and Helenius, A. (1984) J. Virol. 52, 281–283.
[8] Phalen, T. and Kielian, M. (1991) J. Cell Biol. 112, 615–623.
[9] Wahlberg, J.M., Bron, R., Wilschut, J. and Garoff, H. (1992) J. Virol. 66, 7309–7318.
[10] Bron, R., Wahlberg, J.M., Garoff, H. and Wilschut, J. (1993) EMBO J. 12, 693–701.
[11] Nieva, J.L., Bron, R., Corver, J. and Wilschut, J. (1994) EMBO J. 13, 2797–2804.
[12] Smit, J.M., Bittman, R. and Wilschut, J. (1999) J. Virol. 73, 8476–8484.
[13] Butler, K.W., Smith, I.C.P. and Schneider, H. (1970) Biochim. Biophys. Acta 219, 514–517.
[14] De Kruijff, B., De Greef, W.J., Van Eyk, R.V.W., Demel, R.A. and Van Deenen, L.L.M. (1973) Biochim. Biophys. Acta 298, 479–499.
[15] Demel, R.A., Bruckdorfer, K.R. and Van Deenen, L.L.M. (1972) Biochim. Biophys. Acta 255, 311–320.
[16] Slotte, J.P., Jungner, M., Vilchèze, C. and Bittman, R. (1994) Biochim. Biophys. Acta 1190, 435–443.
[17] Bayley, H. (1983) Photogenerated Reagents in Biochemistry and Molecular Biology, Elsevier, Amsterdam.
[18] Brunner, J. (1989) Methods Enzymol. 172, 628–687.
[19] Thiele, C., Hannah, M.J., Fahrenholz, F. and Huttner, W.B. (2000) Nat. Cell Biol. 2, 42–49.
[20] Matyash, V., Geier, C., Henske, A., Mukherjee, S., Hirsh, D., Thiele, C., Grant, B., Maxfield, F.R. and Kurzchalia, T.V. (2001) Mol. Biol. Cell 12, 1725–1736.
[21] Simons, M., Kramer, E.M., Thiele, C., Stoffel, W. and Trotter, J. (2000) J. Cell Biol. 151, 143–154.
[22] Chapman, D., Owens, N.F., Phillips, M.C. and Walker, D.A. (1969) Biochim. Biophys. Acta 183, 458–465.
[23] Cadenhead, D.A. and Müller-Landau, F. (1980) J. Colloid Interface Sci. 78, 269–270.
[24] Hope, M.J., Bally, M.B., Webb, G. and Cullis, P.R. (1985) Biochim. Biophys. Acta 812, 55–65.
[25] Theunissen, J.J.H., Jackson, R.L., Kempen, H.J.M. and Demel, R.A. (1986) Biochim. Biophys. Acta 860, 66–74.
[26] Kauffman, J.M., Westerman, P.W. and Carey, M.C. (2000) J. Lipid Res. 41, 991–1003.
[27] Ghosh, D., Lyman, R.L. and Tinoco, J. (1971) Chem. Phys. Lipids 7, 173–184.
[28] Ali, S., Brockman, H.L. and Brown, R.E. (1991) Biochemistry 30, 11198–11205.
[29] Helenius, A., Kartenbeck, J., Simons, K. and Fries, E. (1980) J. Cell Biol. 84, 404–420.
[30] Marsh, M., Bolzau, E. and Helenius, A. (1983) Cell 32, 931–940.
[31] Murata, M., Peranen, J., Schreiner, R., Wieland, F., Kurzchalia, T.V. and Simons, K. (1995) Proc. Natl. Acad. Sci. USA 92, 10339–10343.
[32] Wang, N., Silver, D.L., Thiele, C. and Tall, A.R. (2001) J. Biol. Chem. 276, 23742–23747.
[33] Phillips, M.C., Ladbrooke, B.D. and Chapman, D. (1970) Biochim. Biophys. Acta 196, 35–44.
[34] Phillips, M.C. (1972) Prog. Surface Membr. Sci. 5, 139–221.
[35] Morrow, M.R., Singh, D., Lu, D. and Grant, C.W.M. (1995) Biophys. J. 68, 179–186.
[36] Lund-Katz, S., Laboda, H.M., McLean, L.R. and Phillips, M.C. (1988) Biochemistry 27, 3416–3423.
[37] Slotte, J.P., Ostman, A.L., Kumar, E.R. and Bittman, R. (1993) Biochemistry 32, 7886–7892.