Mechanism of cholesterol transfer from the Niemann-Pick Type C2 protein to model membranes supports a role in lysosomal cholesterol transport[+]

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Running title: NPC2 protein functions as a cholesterol transfer protein

SUMMARY

Cells acquire cholesterol either by *de novo* synthesis in the endoplasmic reticulum or by internalization of cholesterol-containing lipoproteins, particularly low density lipoprotein (LDL), via receptor-mediated endocytosis. The inherited disorder Niemann-Pick type C (NPC), in which abnormal LDL-cholesterol trafficking from the endo-lysosomal compartment leads to substantial cholesterol and glycolipid accumulation in lysosomes, is caused by defects in either of two genes that encode for proteins designated as NPC1 and NPC2. NPC2 is a small intralysosomal protein that has been characterized biochemically as a cholesterol binding protein. We determined the rate and mechanism by which NPC2 delivers cholesterol to model phospholipid membranes. A fluorescence dequenching assay was used to monitor the kinetics of cholesterol transfer from the protein to membranes. The endogenous tryptophan fluorescence of the NPC2 was quenched upon binding of cholesterol, and the subsequent addition of acceptor vesicles resulted in dequenching of the tryptophan signal, enabling the monitoring of cholesterol transfer to membranes. The rates of cholesterol transfer were evaluated as a function of acceptor vesicle concentration, acceptor vesicle phospholipid headgroup composition, and aqueous phase properties. The results suggest that NPC2 rapidly transports cholesterol to phospholipid vesicles via a collisional mechanism which involves a direct interaction with the acceptor membrane. Transfer of cholesterol to membranes is faster in an acidic environment, and is greatly enhanced by the presence of the unique lysosomal/late endosomal phospholipid lyso-bis phosphatidic acid, LBPA (also known as bis-monoacylglycerol phosphate, BMP), Finally, we found that the rate of transfer of cholesterol from vesicles to NPC2 was dramatically increased by the presence of LBPA in the donor vesicles. These results support a role for the NPC2 protein in the egress of LDL derived cholesterol out of the endosomal/ lysosomal compartment.

INTRODUCTION

Niemann –Pick type C (NPC) [1] disease is a rare autosomal-recessive disorder affecting diverse ethnic groups. NPC is a complex lipid storage disease characterized by the accumulation of unesterified cholesterol and glycolipids in the endosomal/lysosomal system (1-4). Normally, uptake of exogenous cholesterol by the cell involves endocytosis of cholesteryl ester-rich low density lipoproteins (LDL) via the receptor-mediated endocytic pathway, followed by fusion of the endosomal and lysosomal compartments, where the cholesteryl ester core of LDL is hydrolyzed to unesterifed cholesterol and free fatty acids. Cholesterol must leave this compartment to induce the homeostatic responses which regulate the cellular cholesterol pool, including the regulation of LDL receptor expression and *de novo* cholesterol synthesis (5,6). At present, the pathways for trafficking of lysosomal cholesterol remain unknown. Genetic studies of NPC patients have identified two complementation groups (7, 8). The major disease locus, NPC1, maps to chromosome 18q11 and is responsible for 95% of NPC cases (9). The minor disease locus, NPC2, maps to chromosome 14q24.3 and is responsible for 5% of cases (10). The two complementation groups have thus far shown essentially indistinguishable cellular and biochemical characteristics, suggesting that the two gene products, NPC1 and NPC2, may function in a coordinated manner (11, 12). Although there is abundant indirect evidence that suggests a role for the NPC proteins in late endosomal/lysosomal transport of cholesterol, their precise functions at the cell and molecular levels have not yet been determined.

The 131 aa NPC2 was previously characterized as HE1, a cholesterol binding protein present in mammalian epididymal fluid (13, 14). Purified NPC2 binds cholesterol with micromolar affinity and a 1:1 stoichiometery (13, 15). Recently Friedland et al (16) examined the ligand binding properties of NPC2 using the naturally occurring fluorescent sterol dehydroergosterol, and the dissociation constants obtained were approximately 0.2μM and 0.7μM at pH 5.0 and 7.0 respectively, consistent with the hypothesis that NPC2 binds cholesterol in the acidic endo-lysosomal compartment.

The tertiary structure of the unliganded form of bovine NPC2 (bNPC2) was characterized by Stock and colleagues, revealing unique properties of the cholesterol binding site (16). Interestingly, the all β-strand structure of apo bNPC2 lacks a large cavity, tunnel or surface pocket that would accommodate a cholesterol molecule, in contrast to other sterol binding proteins such as elicitin (17) and sterol esterase (18) which have a large hydrophobic cavity that is present in the absence of a ligand. In the apo NPC2 structure, several features nevertheless implicate the hydrophobic interior of the protein as the binding site for cholesterol. The hydrophobic core of the protein is not densely packed and contains three small cavities, the combined volume of which is 158 $Å^3$. While this is too small to accommodate a 741 $Å^3$ cholesterol molecule, the overall shape of the cavities was shown to match well with a space filling model of a cholesterol molecule (16). At present, therefore, it is hypothesized that for cholesterol to bind NPC2, two β-sheets would move apart forming a single interior tunnel that is sufficiently large to accommodate the NPC2 ligand. Preliminary analysis of an NPC2-cholesteryl sulfate complex supports this hypothesis (A. Stock, personal communication).

The functional implications of cholesterol binding by NPC2 are not yet understood. Since endo/lysosomes of

individuals with NPC disease accumulate free cholesterol, a logical supposition is that normal cholesterol binding to NPC2 is necessary for the transfer of cholesterol out of this subcellular compartment. In the present studies we have examined the rate and mechanism of cholesterol transfer from purified human NPC2 to model membranes using the endogenous tryptophan fluorescence of NPC2. A fluorescence dequenching assay was used to monitor the kinetics of cholesterol transfer from protein to membrane. By evaluating the rates of cholesterol transfer as a function of acceptor vesicle phospholipid concentration and composition, we were able to distinguish between an aqueous diffusional transfer mechanism, and one in which cholesterol transfer occurs during NPC2-membrane interactions. The results suggest that NPC2 delivers cholesterol to acceptor phospholipid membranes by directly interacting with the membranes, with ligand transfer occurring during this interaction. The observed rates of this collisional transfer process are very rapid, suggesting that NPC2 is functioning as a lysosomal cholesterol transport protein.

EXPERIMENTAL PROCEDURES

Purification of recombinant human NPC2 protein. A Chinese hamster ovary cell line (HE1-800#7) transfected with a human HE1 expression vector (10) which secretes large amounts of the NPC2 protein into the media was used. Cells were passaged in medium containing D/F12 and 10% FBS , and the conditioning medium consisted of D/F12, 50μM $ZnSO_4$, 1% glucose and 25mM HEPES. When cells were confluent the conditioned media was concentrated by centrifuging at 4500 rpm for 20 minutes using a YM-10 membrane. This concentrated conditioned medium containing the recombinant human NPC2 protein was further centrifuged at 4500 rpm for 30 minutes at 4° C to pellet out any remaining cell debris. The supernatant was then loaded onto a sequential DE52 (Whatman)/ SP-Sephadex C-25 (Amersham Pharmacia) column and eluted with 100mM NaCl 20mM Tris buffer at pH 7.0. The NPC2 containing fractions were identified first by O.D. at 280nm, and confirmed using SDS/PAGE with visualization using SYPRO Ruby protein stain. The pooled fractions were concentrated in an Amicon ultrafiltration cell with a YM-10 membrane, and at the same time a buffer exchange was carried out using 25mM ammonium acetate, pH 4.5. Finally, the different glycoforms of human NPC2 were separated on a Mono-S 10/100 GL column (Amersham Biosciences) using a gradient of 25-1000mM ammonium acetate buffer of pH 4.5. The different glycosylated forms of the hNPC2 protein were identified using SDS/PAGE and silver staining. The four peaks eluting first were diglycosylated forms of the NPC2 protein, and the last two were the apo and holoforms of the monoglycosylated NPC2 (H.L. Liou and P. Lobel, manuscript in preparation).

Membrane vesicle preparation. Phospholipids were obtained from Avanti Polar Lipids, Inc. Small unilamellar vesicles (SUV) were prepared by sonication and phospholipid concentration was determined by quantification of inorganic phosphate, as described previously (19). All vesicle concentrations used are indicating the concentration of phospholipid. Vesicles were maintained at temperatures above the phase transition temperatures of all constituent lipids. The standard vesicles were prepared to contain 100 mol % egg phosphatidylcholine (EPC). For some experiments, as indicated in the text, 25 mol% of other lipids such as neutral (bovine brain) sphingomyelin (SM), or anionic phospholipids (brain phosphatidylserine (PS), soy phosphatidylinositol (PI), or lyso

bis phosphatidic acid (LBPA) (also known as bis-monooleoylglycerol phosphate, BMP) were substituted for EPC in the vesicles. Vesicles were prepared in 20mM sodium citrate, 150mM NaCl pH 5.0 buffer.

Cholesterol binding and transfer assays. Endogenous tryptophan fluorescence of the NPC2 protein was used to monitor cholesterol binding and unbinding to/from NPC2. The basic principle is that upon cholesterol binding to NPC2, the tryptophan signal is quenched; upon subsequent addition of acceptor phospholipid vesicles, the transfer of cholesterol from the NPC2 protein to membranes may be monitored directly by the increase in tryptophan fluorescence over time. Human NPC2 has two tryptophan residues at positions 109 and 122. Stoichiometric amounts of cholesterol dissolved in DMSO and hNPC2 were incubated for 30 minutes at 25^{o} C to achieve quenching of the endogenous tryptophan fluorescence of NPC2. The final concentration of DMSO in the incubation mixture was 1% (v/v).

For cholesterol transfer assays, membranes of defined composition in varying concentrations were mixed with the NPC2/cholesterol donor complex using a Stopped-flow Spectrofluorimeter SX-18MV (Applied Photophysics Ltd., UK), and the increase in tryptophan signal over time was used to obtain the transfer rates. The excitation wavelength was 280nm, and emission was monitored using a 299nm cutoff filter. Transfer was monitored at 25^{o} C, and controls to ensure that photobleaching was eliminated were performed before each experiment. In a typical experiment, the donor NPC2/cholesterol concentration employed was 5μM NPC2/ 5μM cholesterol. Both monoglycosylated and diglycosylated NPC2 were used as indicated in the text. The acceptor membranes ranged in concentration from 125μM to 1000μM. Except where indicated, experiments were performed using 20mM sodium citrate, 150mM NaCl buffer of pH 5.0 to mimic the acidic lysosomal milieau. To observe the effect of pH on the transfer rates of cholesterol from monoglycosylated NPC2 to membranes, the buffer pH was varied from 4.0-7.0. To observe the effect of ionic strength on the transfer rates, NaCl concentration was varied from 150 mM to 2M.

Data were analyzed using software provided with the stopped flow instrument, and the cholesterol transfer rates were obtained by exponential fitting of the curves. All curves were well fit by a single exponential function. For each experimental condition at least 5 replicates were done, and the average ± SE for 3 or more separate experiments are reported. It is worth noting that this transfer assay uses native ligand and native protein with no exogenous probes to acquire kinetic information, thus the rates obtained are likely to be physiologically relevant.

Interaction of NPC2 with membranes containing brominated phosphatidylcholine (BrPC). 0.5 mM SUVs prepared to contain 75 mol% of either 6,7 BrPC or 11,12 BrPC and 25 mol% cardiolipin were incubated for 30 minutes at room temperature with 5 μM NPC2 at pH 5.0. Tryptophan spectra for NPC2 in the absence and presence of the BrPC vesicles were obtained at 25^{o}C.

RESULTS

Cholesterol binding and transfer from NPC2 to membranes. As shown in Figure 1, approximately 20% of the NPC2 tryptophan signal was quenched following stoichiometric addition of cholesterol. 1% DMSO alone did not quench the tryptophan fluorescence of NPC2. Figure 2 shows a representative dequenching scan for the

transfer of cholesterol from NPC2 to EPC membranes at pH 5.0.

Effect of vesicle concentration and charge on cholesterol transfer from monoglycosylated NPC2 to membranes. Monoglycosylated NPC2 was used for the majority of the present experiments. As shown below, results obtained using the diglycosylated form of NPC2 were qualitatively identical although the cholesterol transfer rates were consistently slower from the diglycosylated NPC2.

Transfer of a hydrophobic ligand from a binding protein to an acceptor membrane can be envisioned to occur by one of two basic mechanisms. One possibility is aqueous diffusion, in which the rate-limiting step is the release of the ligand from the protein. Another is a collisional mechanism, in which effective interaction between the protein and the membrane is rate-limiting for ligand transfer. To distinguish between these mechanisms, cholesterol transfer from NPC2 to model membranes was examined as a function of increasing membrane concentration and as a function of the lipid composition of the acceptor vesicles, in particular the phospholipid headgroup charge. In studies involving increasing membrane concentration, no change in transfer is expected for a diffusional mechanism because the rate of ligand dissociation from the protein is independent of the acceptor. For a collisional transfer mechanism, in contrast, the rate of ligand movement will increase with an increase in the number of protein-membrane collisions and, hence, as the acceptor membrane concentration increases (19, 20).

Figure 3 shows the results obtained when a constant concentration of NPC2/cholesterol was mixed with increasing concentrations of zwitterionic EPC SUVs. A proportional increase in transfer rate with EPC SUV concentration was clearly observed, suggesting a collisional mechanism of transfer. When increasing concentrations of vesicles containing sphingomyelin were examined, a similar increase in transfer rate was observed (Figure 3), however the rates of cholesterol transfer were approximately 4-fold lower than those for transfer to EPC membranes. To examine the effect of membrane surface charge, 25 mol% of various anionic phospholipids were incorporated into the acceptor vesicles. The rate of cholesterol transfer relative to zwitterionic vesicles was markedly increased (Figure 4A), further demonstrating the effect of specific membrane composition on ligand transfer from NPC2, and, thereby, supporting the hypothesis of a collisional mechanism of cholesterol transfer. Interestingly, the highest rates of cholesterol transfer were observed with membranes containing 25 mol% LBPA , a lysosome and late endosome-specific phospholipid. Transfer rates from NPC2 to LBPA-containing vesicles were approximately 30-fold greater than transfer to EPC vesicles, suggesting physiologically relevant specificity in the NPC2-membrane interaction (Figure 4B).

Transfer of cholesterol from diglycosylated NPC2 to membranes. Two glycosylation forms of NPC2 are present in the lysosome, one with a single oligosaccharide chain attached at Asn 58, and another with two oligosaccharides attached at Asn 58 and Asn 135 (21). A comparison of cholesterol transfer properties of the two glycoforms showed that, as for monoglycosylated NPC2, cholesterol transfer rates from diglycosylated NPC2 to EPC membranes increased with increasing concentrations of vesicles (Figure 4C). Further, the rate of cholesterol transfer was markedly increased by the presence of anionic phospholipids in the acceptor membranes (Figures 4B, 4C).

Thus, the mechanism of cholesterol transfer, via protein-membrane interaction, appears to be the same for both glycoforms. However, the cholesterol transfer rates from the diglycosylated NPC2 to zwitterionic membranes were considerably slower than those from the monoglycosylated NPC2. Transfer was 4-fold and 3-fold slower to the EPC and sphingomyelin membranes, respectively. Transfer to anionic membranes was also slower, 3-fold to PS- and PI-containing vesicles, and 2.5-fold to LBPA-containing vesicles (Figure 4B).

Effect of ionic strength on cholesterol transfer from monoglycosylated NPC2 to membranes. To further examine the mechanism of cholesterol transfer from NPC2 to phospholipid membranes, the effect of cholesterol solubility on transfer kinetics was assessed by varying the NaCl concentration of the buffer between 150 mM and 2M. Increasing [NaCl] up to 1M caused a large increase in the cholesterol transfer rate, with further increases seen up to 2M NaCl, as shown in Figure 5. Since the solubility of hydrophobic compounds in aqueous media decreases as a logarithmic function of ionic strength (22), an aqueous diffusion-based transfer mechanism would have been expected to show a *decrease* in cholesterol transfer rate with increasing NaCl concentrations. Although it is also possible that protein or membrane conformational changes as a function of [NaCl] could have effects on cholesterol transfer rates from NPC2, we found that the tryptophan fluorescence intensity and spectrum of NPC2 were unchanged at NaCl concentrations of 150mM, 1M, and 2M, and that addition of cholesterol or dehydroergosterol to NPC2 resulted in virtually identical levels of tryptophan quenching irrespective of the NaCl concentration of the buffer (data not shown). These results suggest that no major conformational changes are occurring, and that the sterol binding properties of NPC2 are not substantially modified by ionic strength.

Effect of pH on cholesterol transfer from monoglycosylated NPC2 to membranes. Since the NPC2 protein is a resident lysosomal protein, we examined the rate of cholesterol transfer as a function of pH. As seen in Figure 6, the more acidic the environment the greater the cholesterol transfer rates. Compared to the rate of transfer at pH 4.0, transfer of cholesterol from NPC2 to EPC vesicles decreased by 1.5 fold at pH 5.0, 2.5 fold at pH 6.0 and 3 fold at pH 7.0. Similar effects of pH on cholesterol transfer rates from NPC2 were observed irrespective of the composition of the acceptor vesicles (Figure 6).

NPC2 interactions with membranes. The kinetic data suggested a protein-membrane interaction mechanism for cholesterol transfer from NPC2 to membranes. Based on this hypothesis, we examined NPC2 binding to membranes directly, using vesicles containing acyl chain-brominated phospholipids. Since Br is an effective quencher of aromatic amino acid fluorescence, such an approach has been used to detect the interaction of proteins with membranes (23). Figure 7 shows that NPC2 tryptophan fluorescence is quenched upon incubation of protein with vesicles containing Br-PC. Greater quenching is observed for the vesicles containing 6,7-BrPC than for those containing 11,12-BrPC.

Cholesterol transfer from membranes to NPC2. It has long been appreciated that the spontaneous desorption rate of cholesterol from membranes is exceedingly slow (24,25). To determine whether NPC2 could serve as an acceptor of cholesterol from membranes, and whether it could accelerate the rate of cholesterol transfer from membranes, we again made use of the quenching of NPC2

tryptophan by cholesterol binding to monitor the movement of cholesterol *from* membranes *to* NPC2. 10 mol% cholesterol was incorporated into SUV composed otherwise of 100% EPC, or with substitution of 25% EPC by LBPA. The results in Figure 8 show clearly that NPC2 can serve as an acceptor of membrane-bound cholesterol, that the rate of cholesterol transfer from membranes to NPC2 is markedly faster than reported off-rates of cholesterol from membranes, and that the presence of LBPA in the donor membranes increases the rate of cholesterol transfer to NPC2 by approximately 30-fold.

DISCUSSION

Niemann-Pick C disease is characterized by the accumulation of LDL-derived cholesterol in the endosome-lysosome system. Identification of the two gene products responsible for NPC disease suggests that a derangement in cholesterol trafficking may be central to disease progression. NPC1 contains a sterol sensing domain motif (26) that has been found to be required for binding of a photoactivatable cholesterol analogue (257), and NPC2 has been shown to bind radiolabeled cholesterol and the fluorescent sterol dehydroergosterol at low micromolar affinity (13-16). The precise roles of the NPC gene products in lysosomal cholesterol transport are not yet clear. In the present study we sought to determine the mechanism of cholesterol transfer from NPC2 to model phospholipid membranes as a step toward understanding its physiological function at the molecular level.

The intrinsic tryptophan fluorescence of the human NPC2 protein was used to monitor the binding and transfer of cholesterol to acceptor membranes. Human NPC2 contains 2 Trp residues, at positions 109 and 122. These are identical with the corresponding residues in bovine NPC2, and both are seen in the X-ray crystallographic structure to be in close approximation and facing the interior of the protein (16). The fact that cholesterol quenches hNPC2 tryptophan suggests that it binds at an interior site, although conformational changes caused by ligand association in another region could also alter the Trp quantum yield. However, mutagenesis studies by Ko et al. also support the presence of a cholesterol binding site in the hydrophobic core of the NPC2 protein (15). Three point mutants, F66A, V96F and Y100A, which are located in the hydrophobic core region of NPC2 in close proximity to each other, showed a reduced ability to bind cholesterol and were unable to restore normal levels of cholesterol in npc2 fibroblasts. This strongly suggests that cholesterol binding occurs in an interior hydrophobic site and is essential for the function of NPC2 protein. Preliminary analysis of an NPC2-cholesteryl sulfate complex indicates that the ligand is bound in an interior tunnel (A. Stock, personal communication).

To determine whether cholesterol transfer from NPC2 to membranes occurs by a protein-membrane interaction-mediated mechanism, the theoretical number of collisions between protein and membrane was varied by increasing the concentration of acceptor membrane phospholipid. If transfer involved diffusion through an aqueous medium, the rate of cholesterol transfer from NPC2 would remain constant, since desorption from the protein would be the limiting step. But in transfer by collision, the rate would increase in proportion to the frequency of collision (19, 20, 28). The present results show that the rate of cholesterol transfer from NPC2 increased in proportion to the acceptor vesicle concentration and, hence, the frequency of NPC2-membrane interactions, suggesting a collisional mechanism of transfer (Figure 9).

The composition of the acceptor membranes was found to modulate the observed rates of cholesterol transfer over a very large range, almost 500-fold. The lowest transfer rates, in the range of 0.03 sec^{-1}, were found for membranes containing sphingomyelin. Specific lipid-lipid interactions between cholesterol and sphingomyelin in membranes are well appreciated (29), thus it might be expected that the presence of SM in acceptor membranes could accelerate cholesterol transfer from NPC2. It is likely, however, that effects of membrane sphingomyelin are not related to direct interaction with the NPC2-bound cholesterol, since the sterol is buried inside the protein. Rather, the SM results suggest that specific NPC2-membrane "collisional complexes" are formed based on surface properties of the protein and particular membrane structure/composition motifs. Since the presence of SM is known to increase the lipid order of fluid phase membranes (30), it is possible that decreased membrane fluidity may somewhat diminish effective NPC2-membrane interactions.

The dramatic enhancement of cholesterol transfer rates upon incorporation of anionic phospholipids into the acceptor vesicles suggests that positive charges on the protein's surface may be interacting with the negatively charged phospholipids. As noted above, the point mutagenesis studies of Ko et al. (15) clearly showed that abrogation of cholesterol binding rendered the protein unable to rescue the cholesterol accumulation phenotype in npc2 cells. Interestingly, however, several mutants that maintained normal cholesterol binding were nevertheless also unable to rescue the phenotype. Two of these were mutations in the positively charged residues K32 and K75, both of which face the exterior of the protein. We hypothesize that these residues may be involved in NPC2-membrane and/or NPC2-protein electrostatic interactions, and that these unexplained results are in fact due to derangements in cholesterol transport properties.

In a diffusion-based mechanism, the aqueous solubility of the lipid undergoing transfer would be expected to inversely modulate its rate of transfer. Such a relationship has been found for the transfer of several types of lipids from phospholipid vesicles, including cholesterol, phospholipids themselves, and fatty acids, as well as for the transfer of fatty acids from liver fatty acid binding protein (19, 20, 24, 31). In all these cases, increasing ionic strength resulted in a logarithmic decrease in lipid transfer rate. The fact that an opposite relationship was observed in the present studies, with increasing ionic strength resulting in an increase in cholesterol transfer rate from NPC2, further suggests that cholesterol transfer to membranes is not occurring via the aqueous phase. An increase in lipid transfer rate as a function of medium ionic strength was also found for fatty acid transfer from heart fatty acid binding protein to membranes, which is thought to occur via collisional interactions (32). We have shown previously that high salt concentrations increase the lipid order of fluid phase phosphatidylcholine membranes (19), thus if the mechanism of cholesterol transfer involves collisional interaction of NPC2 with the acceptor membranes, then changes in the physical state of the bilayer might be expected to influence the transfer rate. It is also possible that surface charge shielding, which would occur at high ionic strength, could modulate the electrostatic interactions that are likely to be involved in NPC2-membrane interactions, thereby altering the cholesterol transfer rate. Taken together, the effects of membrane phospholipid charge and the effects of ionic strength suggest that electrostatic interactions are likely to contribute to the

formation of the putative NPC2-membrane collisional complexes.

We have interpreted the effects of membrane phospholipid concentration and composition as likely arising from NPC2-membrane interactions. It is also possible, however, that the cholesterol association step, the on-rate to acceptor membranes, could show similar increases with membrane concentration, since the association step would also be expected to increase with membrane concentration. It is likely, however, that the results are not due to the acceptor lipid bilayers being rate-limiting for cholesterol transfer. It is generally thought that on-rates of hydrophobic monomers from an aqueous phase onto a membrane are extremely rapid. For example, we previously estimated the on-rate for a fluorescent fatty acid analogue association onto membranes at approximately 10^6 $s^{-1}M^{-1}$ (19), much faster than any of the rates observed in the present studies. More importantly, the work of Lange et al. (33) showed that the bimolecular on-rate of cholesterol from buffer to liposomes was approximately 3 x 10^6 $s^{-1}M^{-1}$. Using these estimates and the acceptor membrane phospholipid concentrations used in our NPC2 experiments yields rates of at minimum 100 s^{-1}. The fastest cholesterol transfer rates obtained in the present studies (from NPC2 to membranes containing LBPA), were in the range of 10 s^{-1}, much slower than would be expected if membrane association were the rate limiting step in cholesterol transfer from NPC2 to membranes. Another consideration is that any influence of on-rate on observed transfer rates is minimized under conditions of excess acceptors over donors (34). Although these experiments do not use identical donor and acceptor species, the lipid concentrations of the acceptor vesicles, which can accommodate well more than equimolar cholesterol while maintaining their bilayer structure, indicate that the condition of excess acceptor > donor is likely to have been met in these experiments. Further, as discussed below, the fact that the observed transfer rates for cholesterol are markedly faster than its desorption rate into the aqueous phase strongly implies that an aqueous diffusion-based mechanism cannot be involved in the mechanism of transfer of cholesterol from NPC2 to membranes.

Native protein and unmodified ligand were used for all present experiments, thus the transfer rates obtained are likely to be physiologically meaningful. The observed rates of cholesterol transfer from NPC2 to membranes varied from 0.02 sec^{-1} to 10 sec^{-1}. These rates of cholesterol transfer *to membranes* are one to more than 3 orders of magnitude more rapid than the rate of cholesterol dissociation from NPC2 into the aqueous phase, which was found to be 0.003 sec^{-1}, (15). This suggests that the membrane interaction mechanism of NPC2 would enable a very rapid rate of cholesterol transport within the endo/lysosomal compartment, thereby supporting fast and efficient egress. Indeed, Ko et al. found that a single molecule of NPC2 was capable of mobilizing ~3000 cholesterol molecules in alleviating the cholesterol accumulation of npc2 fibroblasts (15).

The physiological significance of NPC2 as a cholesterol transfer protein is further supported by its pH dependence and by effects of specific membrane lipid composition. The rate of cholesterol transfer from NPC2 to membranes was most rapid at pH 4, declining to 25% or lower at pH 7 depending on the acceptor membrane composition. As a resident lysosomal protein, NPC2 thus functions optimally in an acidic environment. It is possible that at lower pH, the positive surface charge on NPC2 is increased, thereby maximizing electrostatic interactions that are likely to contribute to, if not solely determine, the protein-membrane collisional mechanism of

cholesterol transfer. We also found that incorporation into acceptor vesicles of a phospholipid believed to be unique to endosomes, lysosomes and multivesicular late endosomes, LBPA (35), dramatically increased the rate of cholesterol transfer from NPC2. This increase was not solely related to lipid negative charge since all the anionic lipids examined would be singly charged at experimental pH, yet the effect was most dramatic for LBPA. Kobayashi et al. have shown that LBPA is present in high concentrations in the internal membrane network of endo/lysosomes. Moreover, treatment of BHK cells with an anti-LBPA antibody resulted in an NPC-like phenotype, with cholesterol accumulation in late endosomes (35). We hypothesize that LDL-derived cholesterol is transferred to the LBPA rich internal lysosomal membranes, and that NPC2 protein is centrally involved in cholesterol deposition and/or removal from these sites. Indeed, we show here that membrane-bound cholesterol can be rapidly transferred to NPC2 relative to the spontaneous desorption rate of cholesterol from a membrane, and that membranes containing LBPA produce a 30-fold increase in the rate of cholesterol transfer compared to membranes containing solely EPC.

The kinetic data indicate that cholesterol transfer from NPC2 to membranes occurs during protein-membrane 'collisions.' Quenching of NPC2 tryptophan upon incubation with BrPC-containing membranes provides direct support for such NPC2-membrane interactions. Placement of the bromine atoms at different positions along the phospholipid acyl chains can be used to provide an indication of where membrane perturbations may be occurring. However, since the hNPC2 tryptophans are not facing the exterior of the protein, the observed quenching could potentially be reflecting a conformational change in the protein upon membrane interaction.

Recently, Chen et al. reported that a small fraction, about 3%, of endo-lysosomal NPC2 remained Triton-insoluble following 16 h incubation with the detergent. It was suggested that the insoluble fraction represented insoluble membrane microdomains (36). Thus, although NPC2 is clearly not an integral membrane protein, we hypothesize that its association with membranes is likely to be part of its normal mechanism of action in regulating cholesterol trafficking in the endosomal/lysosomal compartment.

NPC2 contains three potential N-glycosylation sites, however it has been shown that only two of these are normally glycosylated. Monoglycosylated NPC2 contains a single oligosaccharide chain at Asn 58, and diglycosylated NPC2 contains oligosaccharide chains at Asn 58 and Asn 135 (21, Liou H.L. and Lobel P., manuscript in preparation). We found that cholesterol transfer from diglycosylated NPC2 was considerably slower than transfer from the monoglycosylated NPC2, irrespective of acceptor membrane composition. Since diglycosylated NPC2 would likely have a greater negative charge than the monoglycosylated protein, charge repulsion between the diglycosylated protein and anionic acidic groups on membrane phospholipids may in part account for the slower cholesterol transfer rates.

The limiting membranes of lysosomes and late endosomes contain highly glycosylated membrane proteins, including NPC1, which are thought to form a luminal carbohydrate coat that protects the proteins and the membrane in which they are embedded from degradation by lysosomal enzymes (37, 38). The functional significance of glycosylation of intra-organellar resident proteins, such as NPC2, is likely related to normal protein folding and trafficking from their site of synthesis in the endoplasmic reticulum (37). It is not

known whether the two glycoforms of NPC2 have unique functional characteristics. Chikh et al. have suggested than only the Asn 58 glycosylation is required for proper targeting of NPC2 to lysosomes (21). In NPC1 deficient mice, Chen et al. (36) reported that NPC2 glycosylation appeared to be altered, and instead of the two glycoforms typically observed in wild type mouse endo/lysosomes a broad band of somewhat higher molecular weight appeared on Western blot analysis. The altered glycosylation pattern was suggested to be specific for NPC2, since two other endosomal/lysosomal proteins showed no changes in either expression or protein species (36). It would be of interest to isolate the NPC2 protein from NPC1 deficient mice and determine whether cholesterol transfer rates were slower due to the apparent increase in protein glycosylation. Such an effect could perhaps contribute to altered cholesterol transport rates in NPC1 disease. Nevertheless, it is worth noting that Naureckiene et al. did not observe an alteration in the NPC2 glycosylation pattern in NPC1 deficient fibroblasts (10).

In summary, the present studies assessed the rates of cholesterol transfer from human NPC2 protein to model phospholipid membranes, and determined the mechanism by which cholesterol transfer occurs (Figure 9). Taken together the results suggest that NPC2 protein delivers cholesterol to membranes via direct protein-membrane "collisions," as evidenced by transport kinetic parameters as well as the direct demonstration of NPC2-membrane interaction. Cholesterol transfer from NPC2 to membranes is markedly faster than its dissociation into the aqueous phase. Moreover, cholesterol transfer is most rapid at acidic pH, and the presence of anionic phospholipids in the membranes, in particular the endosome-lysosome specific lipid LBPA, dramatically increases the rate of cholesterol transfer. Finally, NPC2 can serve as an acceptor of membrane-bound cholesterol, and the rate of cholesterol transfer from membranes to NPC2 is markedly faster than off-rates of cholesterol from membranes (24, 25). The rate of cholesterol movement from membranes to NPC2 is itself dramatically enhanced by the presence of LBPA. Thus, the results indicate that a cholesterol transport function for NPC2 is likely to be of physiologic importance, and that specific NPC2 mutations or the complete absence of its expression underlie the cholesterol accumulation phenotype of NPC2 disease.

In our working model for normal endosomal/lysosomal cholesterol transport, lyso-bis phosphatidic acid, enriched in intralysosomal membranes, may serve as a localizing site for the interaction of NPC2 with the LDL-derived, acid hydrolase-liberated free cholesterol. The unesterified cholesterol is bound by NPC2 protein within the lumen of the endo-lysosome. NPC2-bound cholesterol is then delivered to the limiting organellar membrane via direct interaction of the protein with the membrane, perhaps followed by lateral diffusion to membrane-bound proteins, or by direct interaction with membrane proteins, potentially NPC1. The mechanism of the final efflux step, by which cholesterol exits the lysosomal compartment, is not known. The present model predicts that NPC2 will not only effect the transfer of cholesterol *to* specific membranes and accelerate the transfer of cholesterol *from* membranes, but will also dramatically increase the rate of cholesterol movement *between* membranes.

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FOOTNOTES

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[1]Abbreviations used are: BMP/LBPA, bis-monoacylglycerol phosphage/lyso-bis phosphatidic acid; BrPC, brominated phosphatidylcholine; LDL, low density lipoprotein; NPC, Niemann-Pick type C; SUV, small unilamellar vesicles; EPC, egg phosphatidylcholine; PS, brain phosphatidylserine; PI, soy phosphatidylinositol, SM, brain sphingomyelin.

FIGURE LEGENDS

Figure 1. Binding of cholesterol to hNPC2 protein. 5μM NPC2 and 5μM cholesterol were incubated for 30 minutes at room temperature and pH 5.0, as described under Experimental Procedures. Final DMSO concentration was 1.0% (v/v).

Figure 2. Cholesterol transfer from NPC2 to membranes: Representative dequenching scan. Transfer of cholesterol from NPC2 to EPC membranes (5μM NPC2, 5μM cholesterol, 1000μM EPC vesicles) was monitored at 25°C and pH 5.0 using a stopped-flow fluorescence spectrometer, as described under Experimental Procedures. The transfer rate obtained for this data set is 0.22 sec^{-1}.

Figure 3. Effect of acceptor membrane concentration on cholesterol transfer from monoglycosylated NPC2 to zwitterionic membranes. Transfer of 5μM cholesterol from 5μM NPC2 to 100%EPC SUVs (▲) or 25% SM/ 75% EPC SUVs (●) was monitored at pH 5. Average transfer rates from five separate experiments ± SE are shown. The inset shows the data for SM-containing vesicles using an expanded scale.

Figure 4: Effect of acceptor membrane composition on cholesterol transfer from monoglycosylated and diglycosylated NPC2. **(A)** Transfer of 5μM cholesterol from 5μM monoglycosylated NPC2 to 100% EPC SUVs (▼), 25 %PI/ 75% EPC SUVs (■), 25 %PS/ 75% EPC SUVs (●), and 25 %LBPA/ 75% EPC SUVs (▲). Average transfer rates from five separate experiments ± SE are shown. **(B)** Comparison of cholesterol transfer from monoglycosylated and diglycosylated NPC2 to zwitterionic and anionic membranes. Transfer of 5 μM cholesterol from 5 μM monoglycosylated NPC2 (■) and diglycosylated NPC2 (□) to 500 μM SUV containing 100 mol% EPC or 25 mol% SM, PI, PS or LBPA. **(C)** Effect of acceptor membrane concentration on cholesterol transfer from diglycosylated NPC2. Transfer of 5 μM cholesterol from 5 μM diglycosylated NPC2 to 100% EPC SUVs (▼), 25 %PI/ 75% EPC SUVs (■), 25 %PS/ 75% EPC SUVs (●), and 25 %LBPA/ 75% EPC SUVs (▲). Average transfer rates from five separate experiments ± SE are shown.

Figure 5. Effect of ionic strength on cholesterol transfer rates to EPC vesicles. Transfer of 2 μM cholesterol from 2 μM monoglycosylated NPC2 to 500 μM SUVs composed of 100mol % EPC at different NaCl concentrations.

Figure 6. Effect of pH on transfer of cholesterol from monoglycosylated NPC2 to membranes. Transfer of 5 μM cholesterol from 5 μM monoglycosylated NPC2 to 500 μM SUVs containing 100mol % EPC, 25 mol % PI, PS and LBPA at different pH values. Average transfer rates from 3-4 separate experiments ± SE are shown.

Figure 7. Quenching of NPC2 Trp fluorescence by SUV containing BrPC.
5 μM NCP2 was incubated with 500 μM SUVs containing 25% cardiolipin and 75 % 6,7 BrPC or 11,12 BrPC at 25^{o}C and pH 5.0. The excitation wavelength was 280 nm; excitation and emission slits were set at 1.0 and 4.0 respectively.

Figure 8: Transfer of cholesterol from membranes to NPC2. Transfer of cholesterol from 100 μM 10% cholesterol/90% EPC SUVs or 10% cholesterol/25% LBPA/65% EPC SUVs to 5 μM NPC2 was monitored at 25^{o}C and pH 5.0. The transfer rates are 0.053 and 1.59 sec^{-1}, respectively.

Figure 9: Potential mechanisms of cholesterol transfer from the NPC2 protein (10) to membranes. (A) Collisional mechanism in which effective interactions between NPC2 and membrane are rate-limiting for ligand transfer. **(B)** Aqueous diffusion, in which the rate-limiting step is the dissociation of the ligand from the protein. As discussed in the text, it is likely that cholesterol transfer from NPC2 to membranes occurs by a collisional mechanism.

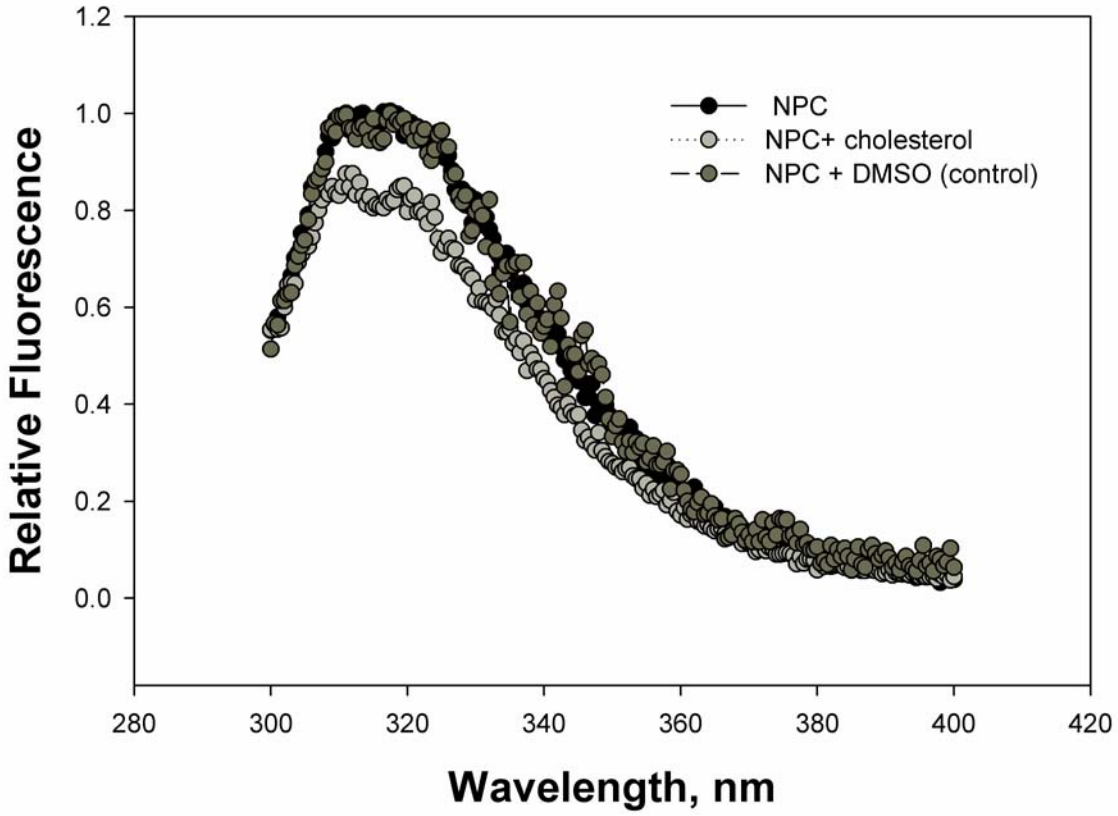

1.2
1.0
0.8
0.6
0.4
0.2
0.0
Relative Fluorescence
280
300
320
340
360
380
400
420
Wavelength, nm
NPC
NPC+ cholesterol
NPC + DMSO (control)

Figure 1

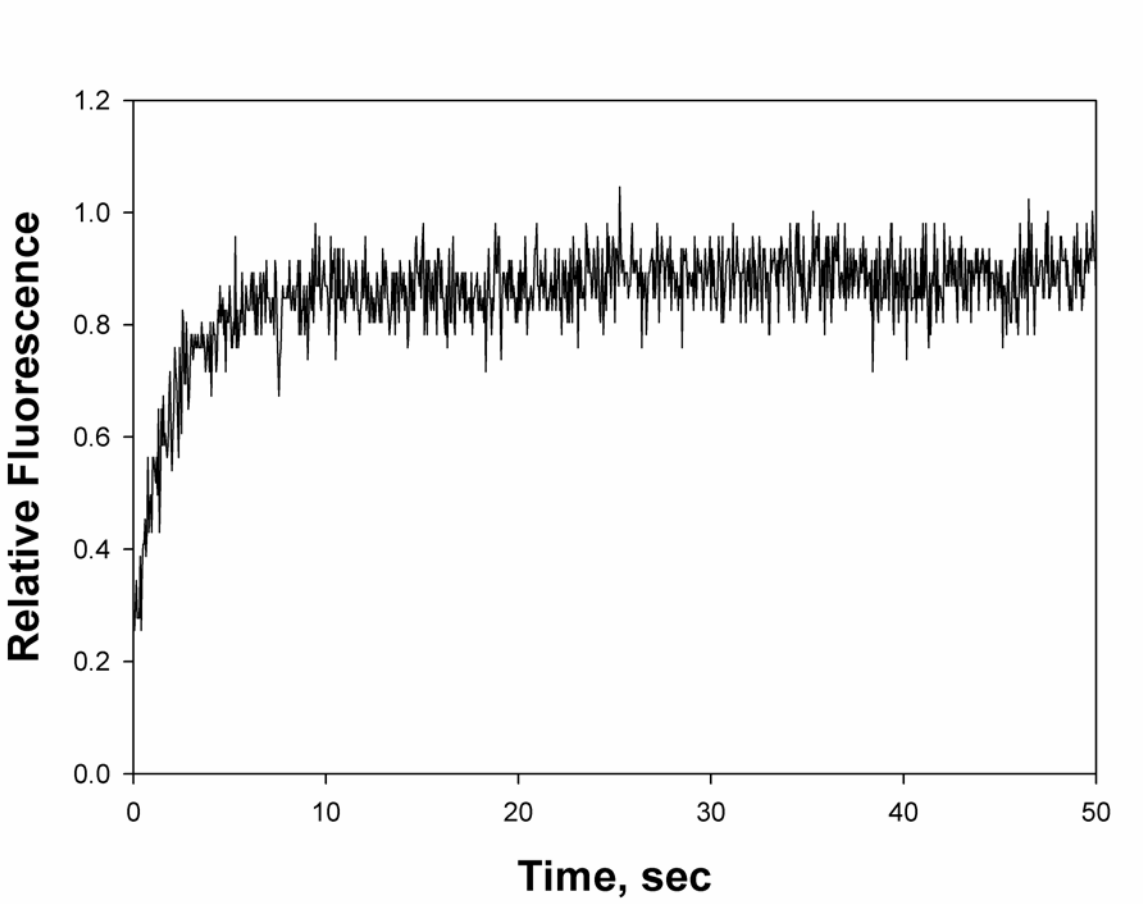

Relative Fluorescence
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
Time, sec

Figure 2

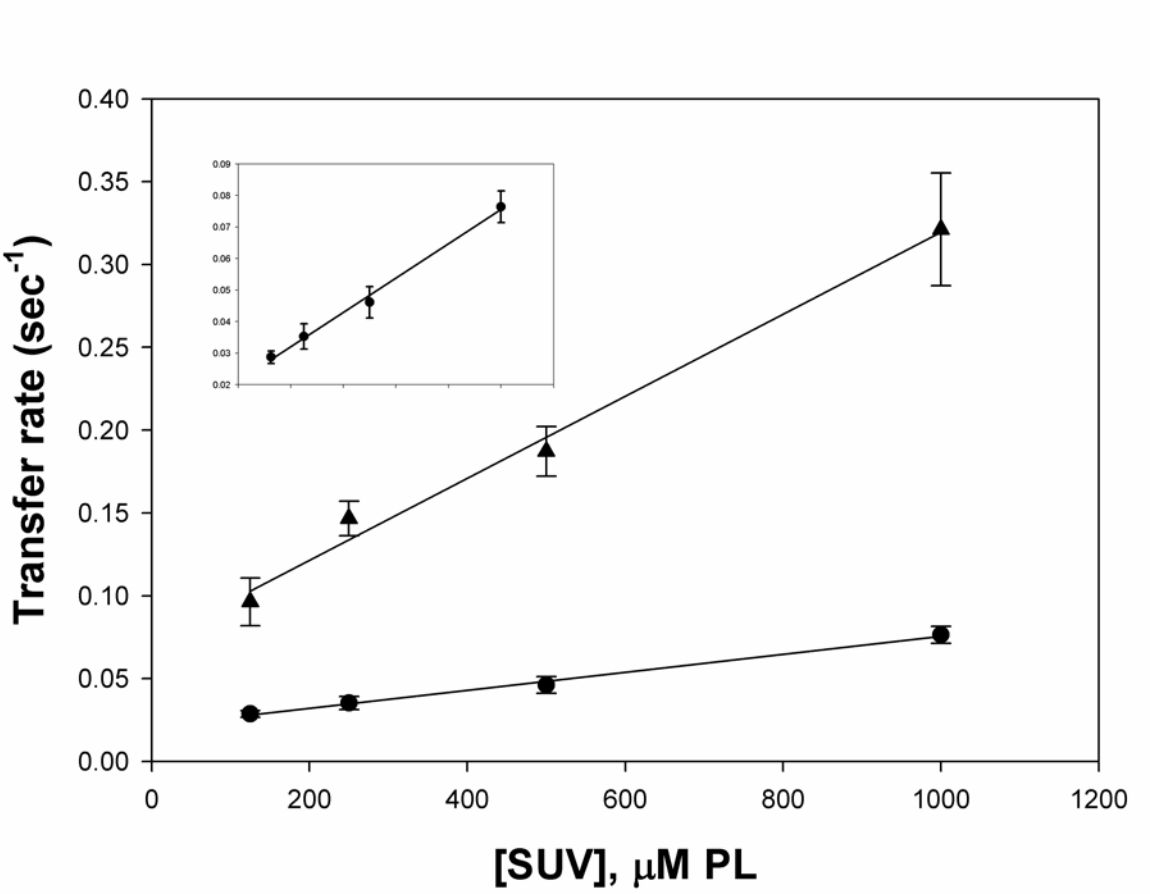

Transfer rate (sec-1)
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
200
400
600
800
1000
1200
[SUV], μM PL

Figure 3

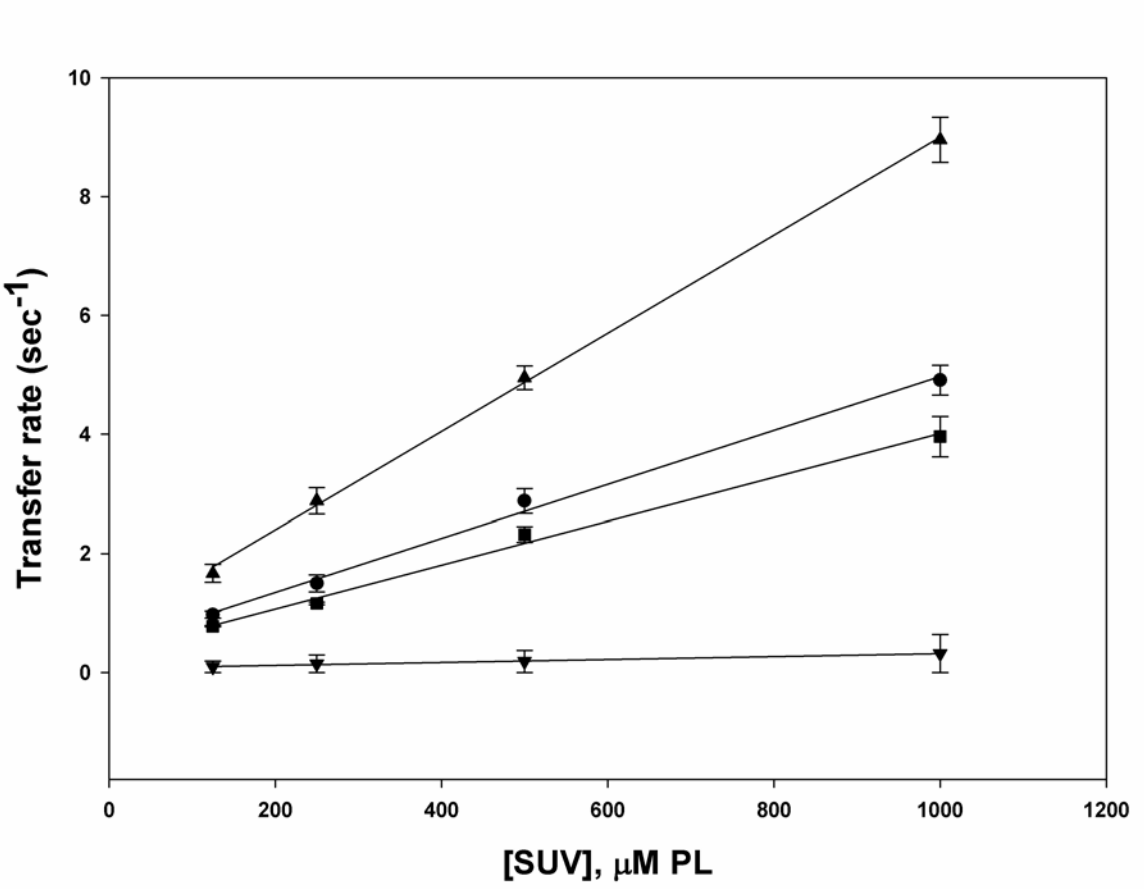
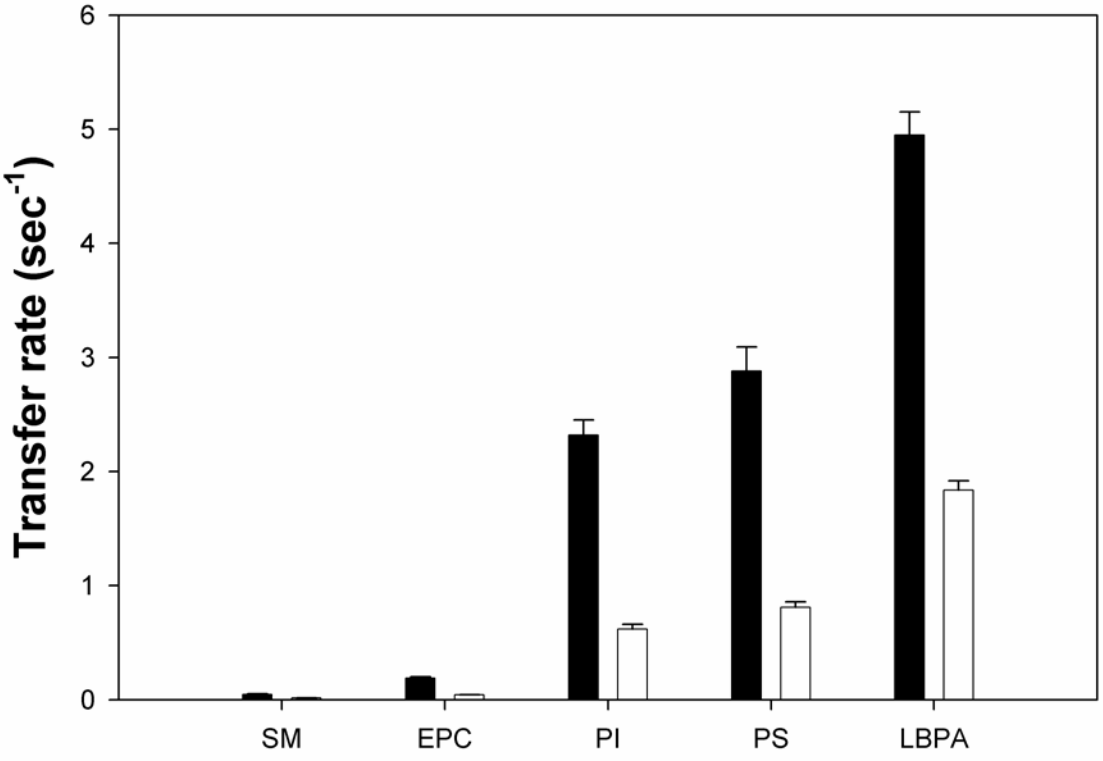

Transfer rate (sec-1)
10
8
6
4
2
0
0
200
400
600
800
1000
1200
[SUV], μM PL

Figure 4A



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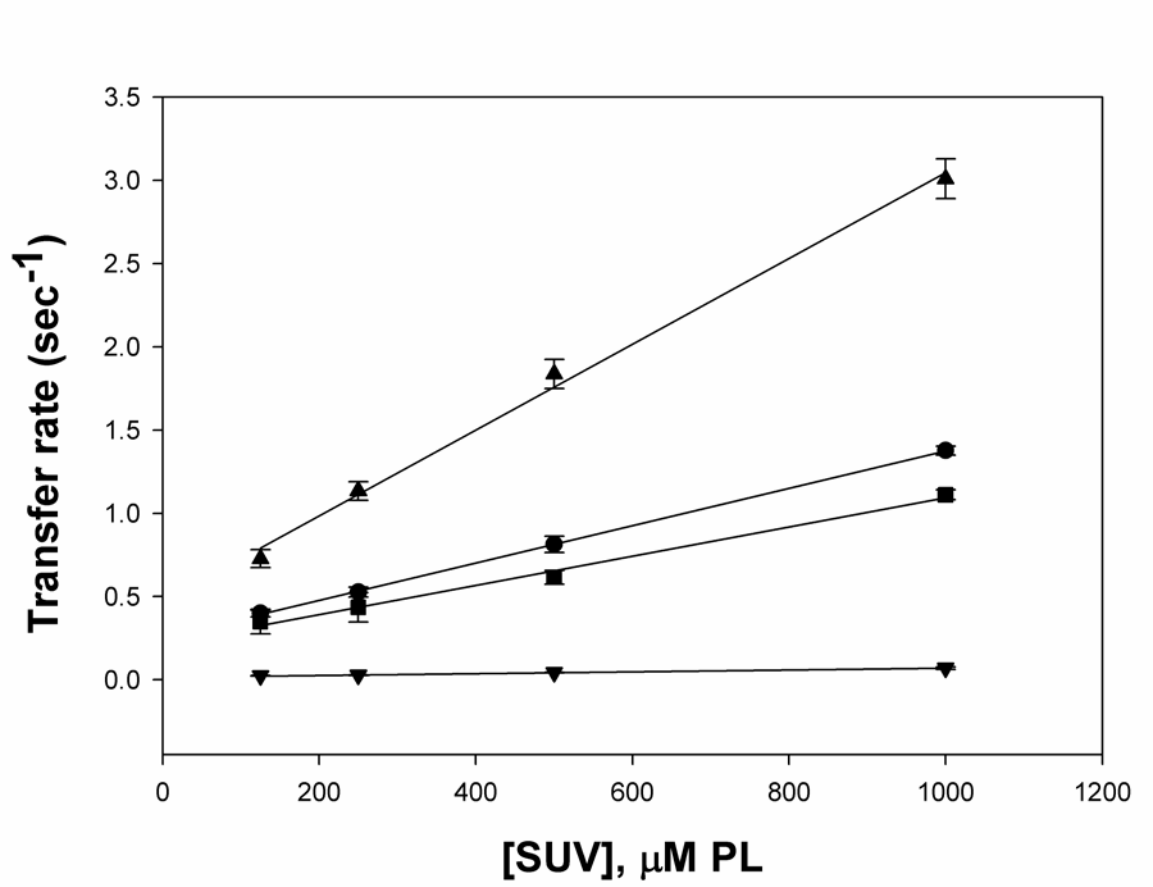
Transfer rate (sec-1)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
200
400
600
800
1000
1200
[SUV], μM PL

Figure 4C

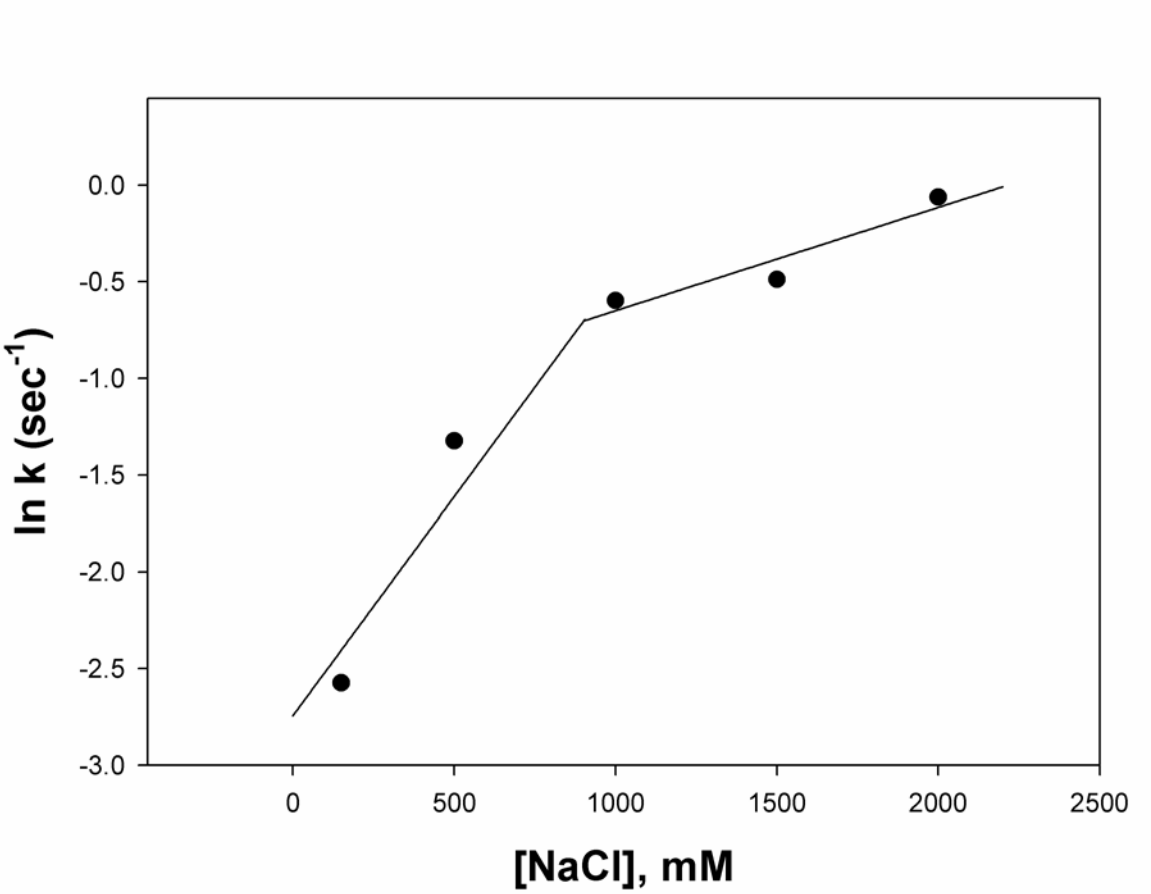

Figure 5

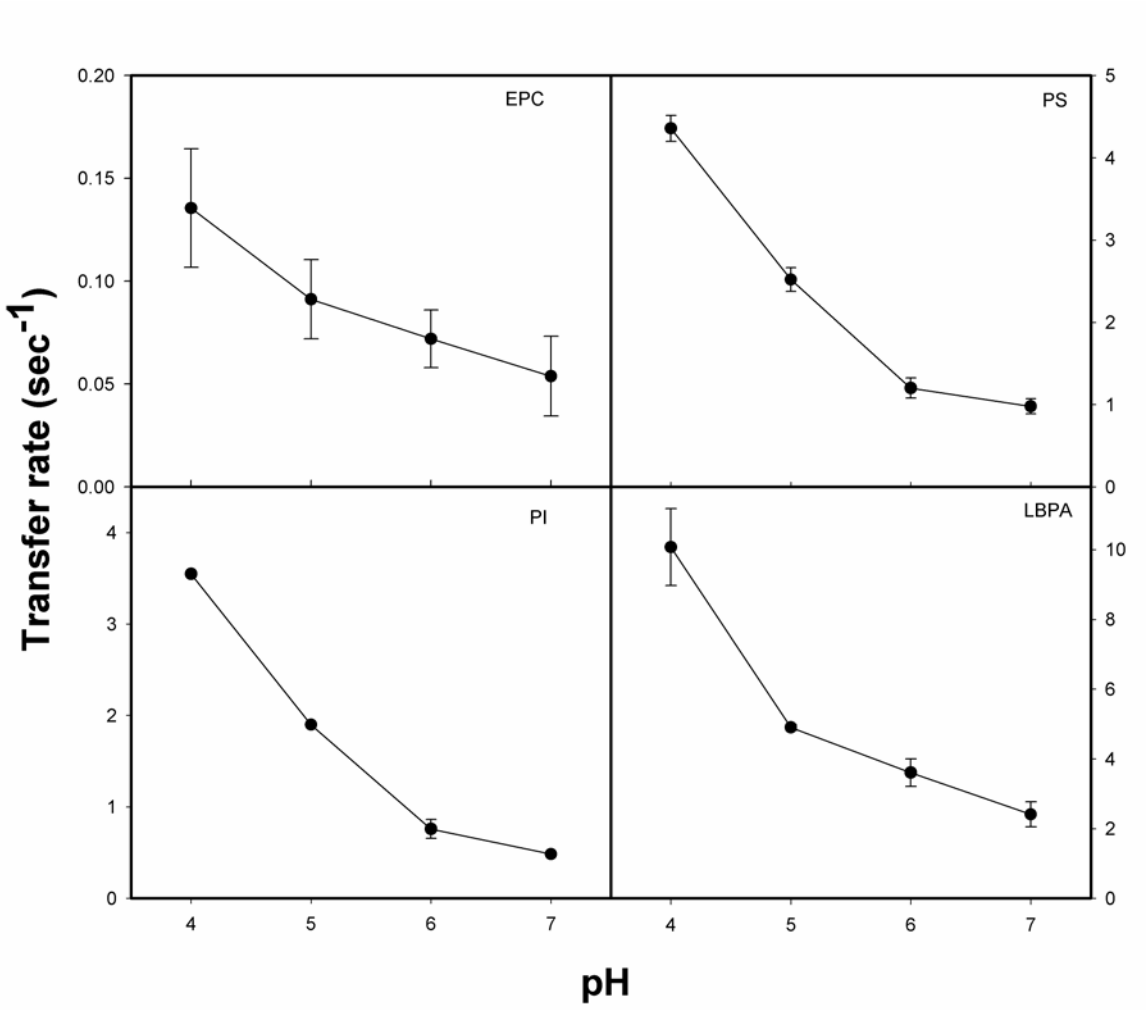
EPC
PS
PI
LBPA
Transfer rate (sec-1)
pH
0.20
0.15
0.10
0.05
0.00
5
4
3
2
1
0
10
8
6
4
2
4
5
6
7

Figure 6

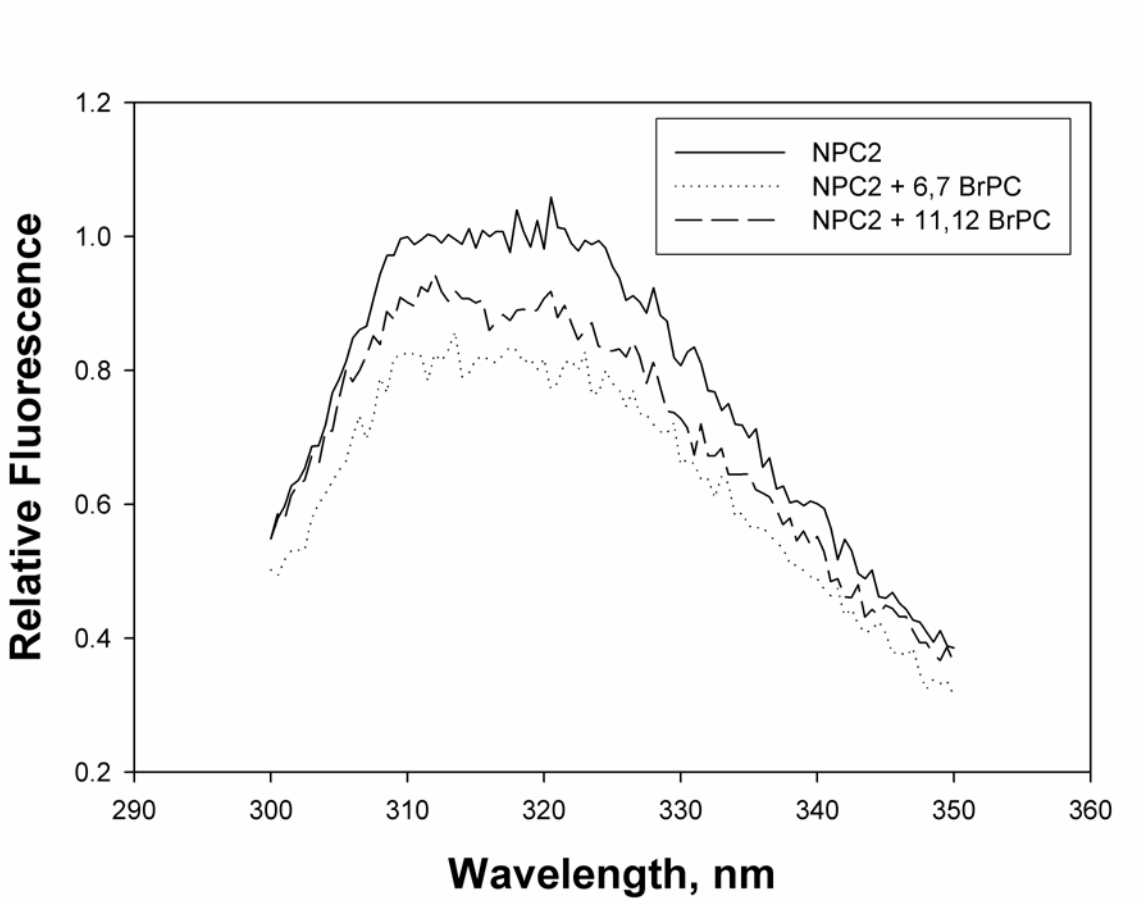
NPC2
NPC2 + 6,7 BrPC
NPC2 + 11,12 BrPC
Relative Fluorescence
Wavelength, nm
1.2
1.0
0.8
0.6
0.4
0.2
290
300
310
320
330
340
350
360

Figure 7

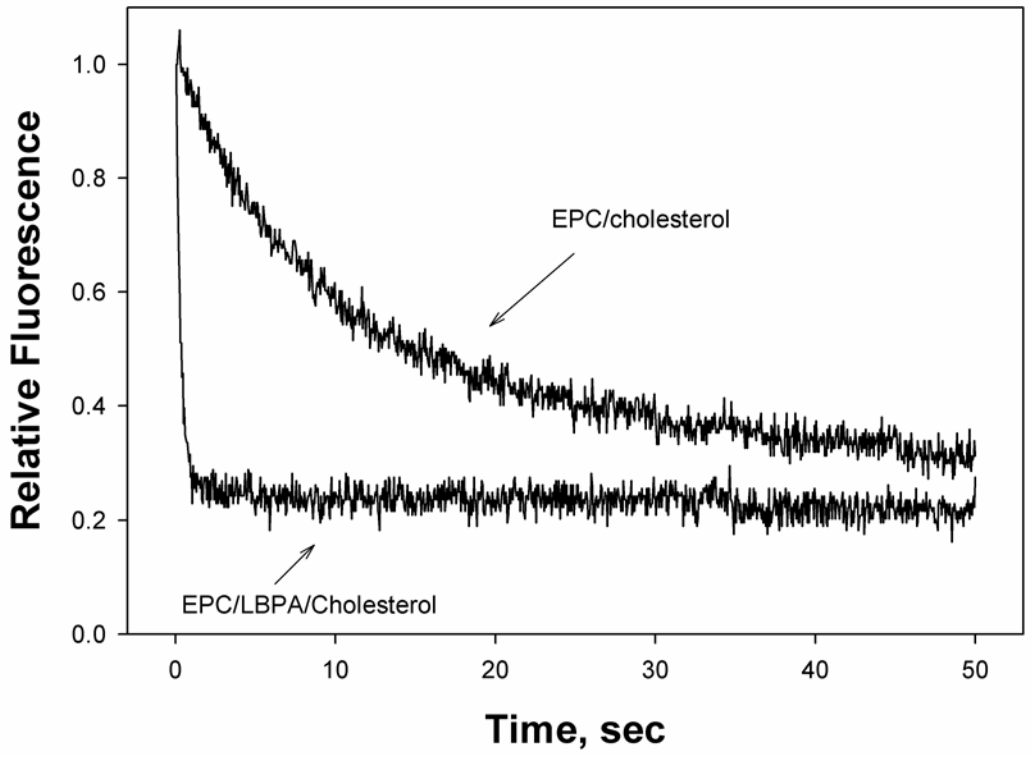
Relative Fluorescence
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
Time, sec
EPC/cholesterol
EPC/LBPA/Cholesterol

Figure 8

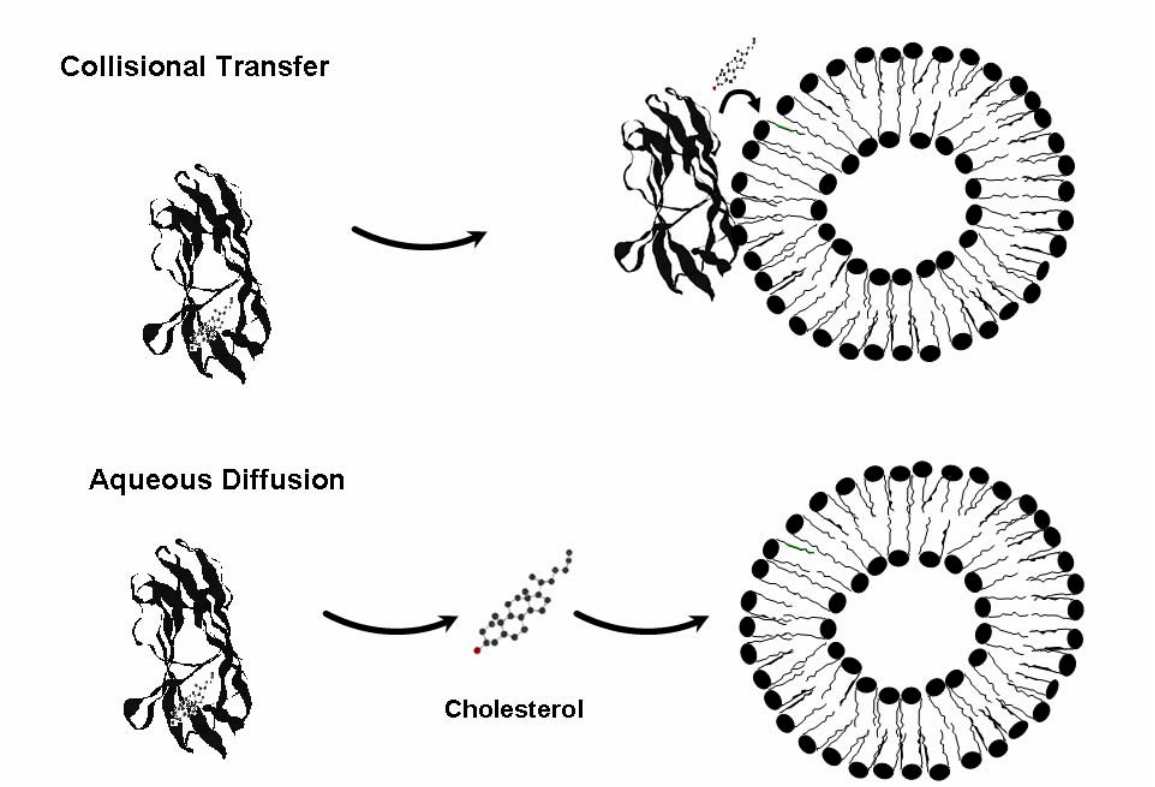

Figure 9