Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 309 (2003) 724–732
BBRC
www.elsevier.com/locate/ybbrc

A hierarchy of lipid constructs for the sperm plasma membrane☆

Meirav Apel-Paz,[a] T. Kyle Vanderlick,[a,*] Neelima Chandra,[b] and Gustavo F. Doncel[b]

[a] *Department of Chemical Engineering, Princeton University, Princeton, NJ 08544, USA*
[b] *CONRAD Program, Department of Obstetrics and Gynecology, Eastern Virginia Medical School, 601 Colley Avenue, Norfolk, VA 33507, USA*

Received 13 August 2003

Abstract

We have presented a series of lipid constructs as models of the sperm plasma membrane. We also isolated the plasma membrane from rabbit sperm cells and characterized the lipid composition. The behavior of these various membrane systems was evaluated using a vesicle leakage assay, in which surfactant (nonoxynol-9, N-9; or benzalkonium chloride, BZK) exposure induced membrane permeabilization. These studies shed light on the relative importance and significance of particular components present in the lipid constructs. In particular, a highly unsaturated phospholipid component characterized by an ether-linkage to position 1 of the glycerol backbone (as opposed to the more conventional ester linkage) as well as the presence of sulfogalactosyl ceramide were found to have an effect on the surfactant-induced leakage response. The presence of cholesterol had the greatest influence on membrane behavior. The construct series also demonstrated the ability of the surfactants studied to discriminate between different membrane systems. We found that N-9 displayed little sensitivity to membrane composition while BZK showed specific behavior with the various membrane systems.
© 2003 Elsevier Inc. All rights reserved.

Keywords: Benzalkonium chloride; C31G; Carboxyfluorescein; Cholesterol; Leakage; Nonoxynol-9; Phospholipids; Plasma membrane; Sperm (rabbit); Surfactants

Sperm cells are highly differentiated, polarized, and compartmentalized cells with a high membrane to cytoplasm ratio. Sperm membranes have many physical and functional features that distinguish them from most somatic cell membranes. Many of these features allow sperm cells to accommodate the range of environments experienced within the male and female reproductive tracts.

Sperm plasma membranes are organized in several specific regions. The sperm head is composed of three distinct parts; the acrosomal area or anterior head, the equatorial segment, and the post acrosomal area; and the sperm tail is composed of two regions, the midpiece and principal piece [1,2]. Studies have shown that in contrast to most cell membranes, inter-regional diffusion is very limited, preventing membrane components from mixing and randomizing, and thus assuring proper and specific functioning [2,3].

As with somatic cells, the lipid matrix of the sperm cell membrane is a heterogeneous mix of phospholipids, glycolipids, and sterols, which are distributed asymmetrically between the inner and outer leaflets of the membrane. Despite their general compositional similarity to somatic cells, the lipid matrix of the sperm plasma membrane has several distinguishing features (in spite of variations observed between different animal species [4–7]). One key example is a particularly high fraction of polyunsaturated fatty acid chains, in particular clupanodonic (22:5) and docosahexaenoic (22:6) [2,8,9].

Another unique characteristic is a significant fraction of phospholipid that is not based on the common diacyl

☆ *Abbreviations:* BZK, benzalkonium chloride; CF, 5(6)-carboxyfluorescein; Chol, cholesterol; DCIP, 2,6-dichloroindophenol sodium salt; DPG, diphosphatidylglycerol; GC, gas chromatography; N-9, nonoxynol-9; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; PS, phosphatidylserine; SDS, sodium dodecyl sulfate; SGC, 3′-sulfogalactosyl ceramide; SGG, 3′-sulfogalactosylglycerolipid; SPH, sphingomyelin; STD, sexually transmitted disease; TLC, thin layer chromatography.

* Corresponding author. Fax: 1-609-258-0211.
E-mail address: vandertk@princeton.edu (T. Kyle Vanderlick).

0006-291X/$ - see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2003.08.065

linkage between constituent fatty acids and the glycerol backbone. In particular, the sperm plasma membrane is populated with lipids in which one of the two fatty acids is linked via an ether bond (at the *sn*-1 position) to a PC or PE headgroup.

Yet another specialized lipid found in the sperm plasma membrane is 3′-sulfogalactosylglycerolipid (SGG), which constitutes 5–8% of the total lipid. Apart from sperm cells, this glycolipid, known also as seminolipid, is found only in the brains of mammals of certain species, but in much lower concentrations (0.2%) [10,11].

Cholesterol (Chol) is the major sterol component of the sperm plasma membrane. Interestingly, sperm cells continuously modulate membrane cholesterol content during their journey from the testis through the female reproductive tract. Membrane cholesterol, well known to influence molecular packing, is present in high proportions in the epididymis where the stability and resiliency of the membrane is most critical. However, it is continuously shed from the membrane on arrival of sperm to the female reproductive tract, in preparation for the fusion steps [9,12]. This example, along with those mentioned above, makes clear that the sperm plasma membrane is comprised of a finely tuned, complex, and specialized lipid mixture that contributes to many of cell's vital functions.

The significance and accessibility of the sperm plasma membrane also make it a prime target for contraceptive intervention. One of the simplest vaginal contraceptive strategies involves exposing sperm to membrane active agents, such as the surfactant nonoxynol-9 (N-9) found in various commercial spermicides [13,14]. Although the exact mechanism is not known, evidence strongly suggests that sperm are inactivated as a result of surfactant incorporation into the membrane, with associated deleterious consequences [14]. Such intercalation and disruption of lipid membrane structures are also believed to be responsible for N-9's in vitro potent virucidal activity [15].

Of course a key problem with this mode of contraception and STD prevention is the issue of cell discrimination. Ideally, a spermicidal/microbicidal agent should selectively perturb sperm and pathogen's membranes in favor of those of other cell membranes in the vicinity, such as those of vaginal epithelial cells or the beneficial lactobacilli of the vaginal microflora. Chronic problems with irritation [16], as well as the possibility that N-9 exposure may even enhance HIV transmission [17], make clear that this goal is far from being achieved. It is, however, conceivable that the unique compositional characteristics of sperm membranes could serve as a basis for the design of more selective membrane active agents.

For both fundamental biological studies, as well as for biomedical applications such as those just mentioned, it would be extremely useful to have accurate, and affordable, synthetic models to represent the lipid matrix of sperm plasma membranes. In this paper, we introduce a hierarchy of lipid constructs for the anterior head of the sperm plasma membrane. We show their similarity in composition, and in behavior, to reconstituted membranes formed from lipid extracts of rabbit sperm plasma membranes. As part of this work, we present a characterization of the lipids in this natural membrane. The behavior of the different membranes is examined using vesicle leakage assays, in which encapsulated fluorophores permeate across the membrane as a result of vesicle exposure to a membrane-active agent. The membranes are exposed to two different surfactants: (1) the non-ionic N-9, which we show interacts very indiscriminately with the membranes and (2) the cationic surfactant, benzalkonium chloride (BZK), which displays very specific interactions. The results of these experiments enable us to identify the importance of specific membrane constituents and also to comment on the targeting capability of surfactant candidates for contraception and STD prevention.

Materials and methods

Materials. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), brain sulfatide, 1-palmitoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine, C16:0–22:6 1-alkyl-2-acyl-sn-glycero-3-phosphocholine, 1, 2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine], egg-sphingomyelin, and cholesterol (Chol) were obtained from Avanti Polar Lipids (Birmingham, AL). Benzalkonium chloride (BZK), infinity cholesterol reagent, cardiolipid control-level 1, and protein assay kit were obtained from Sigma (St. Louis, MO). 5(6)-Carboxyfluorescein (CF) was obtained from Molecular Probes (Eugene, OR). Nonoxynol-9 (N-9) was a gift from Biosyn, Philadelphia, PA (as Rhone–Poulenc's Igepal CO-630 Special at a purity of 95%). All surfactants were used without further purification. All solutions were prepared using Millipore water.

Isolation of sperm plasma membranes. Semen were collected from adult White New Zealand male rabbits with the aid of an artificial vagina [18]. Spermatozoa were freed from seminal plasma by dilution in TALP medium containing 0.1% bovine serum albumin (BSA) and centrifugation at 500*g* for 10 min. After washing spermatozoa three times, the sperm pellet was resuspended in 1 mL TALP + 0.1% BSA. Sperm concentration and motility was assessed using a computer assisted semen analyzer (IVOS v.10.8, Hamilton Thorne, Beverly, MA). Samples were immediately frozen at −20 °C and stored at −120 °C until further processing.

The plasma membrane from the anterior head region of rabbit spermatozoa was isolated following the procedure described by Wolf and coworkers [19] with minor modifications. Briefly, washed sperm (in TALP + 0.1% BSA) was pooled and centrifuged using a J2HS Beckman centrifuge at 1200*g* for 20 min in Dulbecco's phosphate-buffered saline solution (calcium chloride—132.50 mg/L; magnesium chloride—100.00 mg/L; potassium chloride—200.00 mg/L; potassium phosphate, monobasic—200.00 mg/L; sodium chloride—8000.00 mg/L; and sodium phosphate, dibasic—1500.00 mg/L) as a washing step. The saline solution was removed and this step was repeated twice more. The pellet was cooled to 4 °C and maintained at this temperature for the remainder of the procedure. The pellet was re-suspended roughly 10 times in Ca^{2+} and Mg^{2+}-free Hanks' balanced solution ($2–4 \times 10^9$ sperm/mL). The suspension was then submitted to nitrogen cavitation

in a Parr Instrument cell disruption bomb packed in ice. The cells in the bomb were pressurized to 750 psi and equilibrated for 10 min. The suspension was then centrifuged at 6000*g* for 10 min. The supernatant of the last step was centrifuged at 100,000*g* for 30 min using a Beckman L7-55 ultracentrifuge. To remove any remaining large materials, the pellet was resuspended in Hanks' solution and subjected to another centrifugation step at 10,000*g* for 10 min.

Lipid extraction procedure. Lipids of the suspended membranes were extracted following the procedure described by Wolf and coworkers [19] with minor modifications. To every 1 mL of the suspended sample, 3.75 mL of a mixture of methanol–chloroform (2:1) was added. The sample was shaken for 1 h at room temperature. The mixture was centrifuged at 900*g* for 5 min. Next, 2.5 mL of 1:1 chloroform–H_2O was added per 1 mL of original suspension. The sample was then centrifuged at 10,000*g* for 10 min. The bottom phase was collected and dried in a rotary evaporator at 30 °C for 1 h. The lipids were then dissolved in 3 mL chloroform and stored at −20 °C.

Lipid analysis. Detailed analysis of the lipid extract was performed by Lipid Analysis Laboratories (Ontario, Canada). Briefly, lipids were fractionated on 0.25 mm-thick Silica Gel 60 TLC plates (Merck) and developed according to Skipski et al. [20]. The lipids were visualized using 2,7-dichlorofluorescein and illuminated with ultraviolet light. Each head group was then eluted from the plate and methylated with boron trichloride methanol and the fatty acid composition was determined by GC using internal standards on a Varian 3800 chromatograph equipped with a 60 m, 0.32 mm ID, DB-23 capillary column, 0.10 μm film thickness. Initial column and injector temperatures were 60 °C, while the detector was maintained at 250 °C, and the carrier gas was hydrogen at a flow rate of 70 cm/s. Total phospholipid content was measured on 0.25 mm-thick Silica Gel 60 TLC plates (Merck) and developed using heptane/isopropyl ether/acetic acid (60/40/3). In addition, the sample was saponified; cholesterol esters were converted to free cholesterol, sample was derivatized using TMS (trimethylsilyl) and run by GC for total cholesterol using di-hydrocholesterol (DHC) as an internal standard.

The total phospholipid content of the extract was also determined in our laboratory according to the ascorbic acid spectrophotometric method for total phosphorous assay, in a procedure made available by Avanti Polar Lipids. The measurements were performed using a Genesys 2 spectrophotometer (Thermo Spectronic Instruments).

We also determined the cholesterol content using the following enzymatic cholesterol assay (Sigma Procedure No. 401 for total cholesterol based on Roeschla et al. procedure). In particular, 20 μL of the sample was incubated with 2 mL of the Infinity cholesterol reagent for 15 min at room temperature, after which the signal at 500 nm was measured. Cardiolipid control, level 1 was used as assay control.

Biochemical assays. Three enzyme assays were used to evaluate the purity of the lipid extract product. The method of Labbe and Loir [5] was used for the determination of 5′-nucleotidase (EC 3.1.3.5; plasma-membrane marker). The amount of phosphate released was determined as described in Lipid analysis. The membrane fraction was checked for mitochondrial contamination with succinic dehydrogenase assay (EC 1.3.99.1; mitochondrial marker) according to the following protocol. A mixture of 0.004 M sodium azide (final concentration), 0.02 M sodium succinate, 5 μM 2,6-dichloroindophenol sodium salt (DCIP), and 0.1 mL membrane fraction was added to a cuvette. The volume was made up to 1 mL with phosphate-buffered saline (PBS; pH 7.4). Absorbance at 600 nm was read every 5 min for 30 min against a

Table 1
Fatty acid compositions of lipids extracted from rabbit sperm plasma membranes

Fatty Acid	SPh	PC	PS	PI	PE	DPG
C14:0	3.85 ± 0.43	0.96 ± 0.09	2.78 ± 0.38	5.65 ± 0.13	1.35 ± 0.16	2.15 ± 0.29
C14:1	0.42 ± 0.09	0.69 ± 0.22	0.84 ± 0.03	0.69 ± 0.2	0.00 ± 0.01	0.21 ± 0.09
C15:0	1.16 ± 0.19	0.34 ± 0.02	2.45 ± 0.35	3.48 ± 0.34	1.26 ± 0.07	2.67 ± 0.36
C16:0	73.56 ± 0.63	18.24 ± 1.77	18.61 ± 1.46	38.13 ± 1.76	23.93 ± 3.34	46.97 ± 3.15
C16:1	0.33 ± 0.10	0.20 ± 0.03	2.09 ± 1.20	0.00 ± 0.00	0.61 ± 0.13	0.05 ± 0.02
C18:0	9.25 ± 0.99	18.55 ± 0.87	27.72 ± 1.33	26.20 ± 1.43	9.47 ± 0.28	12.84 ± 1.36
C18:1	0.81 ± 0.30	5.79 ± 0.31	15.41 ± 0.42	6.41 ± 0.43	11.69 ± 2.97	7.30 ± 0.85
C18:2N6	0.72 ± 0.27	3.82 ± 0.60	14.78 ± 0.78	3.48 ± 0.47	10.64 ± 0.62	4.87 ± 0.76
C18:3N6	0.04 ± 0.01	0.22 ± 0.07	0.24 ± 0.13	0.00 ± 0.00	0.06 ± 0.02	1.36 ± 0.26
C18:3N3	0.14 ± 0.08	0.00 ± 0.01	0.39 ± 0.11	0.00 ± 0.00	0.59 ± 0.15	0.49 ± 0.18
C18:4N3	0.24 ± 0.03	0.00 ± 0.00	0.53 ± 0.30	0.16 ± 0.09	0.30 ± 0.17	0.22 ± 0.10
C20:0	3.86 ± 0.01	0.00 ± 0.01	0.79 ± 0.13	2.51 ± 0.44	0.55 ± 0.31	2.98 ± 1.71
C20:1	0.02 ± 0.01	2.85 ± 0.25	0.26 ± 0.09	0.24 ± 0.13	0.00 ± 0.01	0.00 ± 0.00
C20:2N6	0.16 ± 0.05	0.28 ± 0.07	0.46 ± 0.04	0.85 ± 0.31	3.90 ± 2.07	0.66 ± 0.37
C20:3N6	0.02 ± 0.01	0.57 ± 0.01	1.27 ± 0.33	0.00 ± 0.00	0.47 ± 0.14	0.29 ± 0.08
C20:4N6	0.00 ± 0.00	0.95 ± 0.09	1.87 ± 0.35	0.46 ± 0.13	1.29 ± 0.37	0.75 ± 0.06
C20:3N3	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.17 ± 0.09	0.00 ± 0.06
C20:4N3	0.00 ± 0.00	0.03 ± 0.01	0.00 ± 0.00	0.25 ± 0.14	0.00 ± 0.04	0.14 ± 0.08
C20:5N3	0.04 ± 0.02	0.25 ± 0.14	0.48 ± 0.18	0.13 ± 0.07	0.05 ± 0.03	0.00 ± 0.00
C22:0	2.70 ± 0.20	0.00 ± 0.00	0.12 ± 0.74	0.03 ± 0.01	0.98 ± 0.06	0.65 ± 0.37
C22:1	0.03 ± 0.01	0.36 ± 0.13	0.52 ± 0.30	0.49 ± 0.21	1.68 ± 0.38	0.82 ± 0.47
C22:2N6	0.04 ± 0.01	0.03 ± 0.00	0.10 ± 0.05	0.08 ± 0.02	0.21 ± 0.12	0.37 ± 0.13
C22:4N6	0.11 ± 0.06	1.05 ± 0.12	0.14 ± 0.05	0.18 ± 0.08	1.27 ± 0.22	0.12 ± 0.04
C22:5N6	0.34 ± 0.09	36.46 ± 2.19	4.34 ± 0.91	4.08 ± 1.42	22.97 ± 6.58	2.99 ± 0.81
C22:5N3	0.05 ± 0.00	0.04 ± 0.10	0.26 ± 0.05	0.75 ± 0.21	0.74 ± 0.19	0.34 ± 0.07
C22:6N3	0.25 ± 0.13	8.21 ± 1.16	1.26 ± 0.69	0.60 ± 0.17	1.32 ± 0.15	1.14 ± 0.62
C24:0	1.04 ± 0.40	0.03 ± 0.00	0.69 ± 0.16	3.96 ± 2.04	4.51 ± 2.55	2.26 ± 1.07
C24:1	0.80 ± 0.16	0.20 ± 0.04	0.33 ± 0.06	1.17 ± 0.61	0.30 ± 0.13	0.99 ± 0.04
Saturated	95.42 ± 0.70	38.12 ± 1.19	54.45 ± 2.33	79.96 ± 1.9	42.05 ± 7.28	76.51 ± 1.29
Monounsaturated	2.42 ± 0.29	10.09 ± 0.90	19.44 ± 1.19	9.00 ± 0.34	14.29 ± 2.70	9.36 ± 0.81
Polyunsaturated	2.16 ± 0.40	51.92 ± 2.52	26.11 ± 1.14	11.04 ± 1.99	43.98 ± 4.72	13.72 ± 0.50

Numbers represent mole percent (mean ± S.E.); based on three replicates.

blank containing all components except succinate. All necessary controls were carried out. Acrosomal contamination was checked by an acrosin assay (EC 3.4.21.10; acrosomal marker) following the procedure of Zahler and Doak [21].

The protein content of lipid extracts was determined using a procedure based on Peterson's modification to Lowry procedure (Sigma Procedure No. P 5656 for protein). Bovine serum albumin was used as a standard.

Surfactant and CF solutions preparation. Surfactant stock solutions (N-9 and BZK at 0.1 mM solutions) were prepared by dissolving the surfactant in 16.66 mM Hepes buffer containing 125 mM NaCl. The same buffer solution including salt was prepared in the absence of surfactant; we refer to this in particular as the 'external solution' from here on. The pH of the solutions was adjusted to pH 7.4 with 2 M NaOH. One hundred millimolar of 5,6-carboxyfluorescein (CF) solution was prepared using 16.66 mM Hepes buffer and also adjusted to pH of 7.4. Iso-osmotic conditions of the surfactant, external, and CF solutions were assured by measuring osmolarity using a Fiske Micro-Osmometer Model 210. The osmolarity was matched if needed to a value of 265 mOs by the addition of NaCl.

Vesicle preparation and characterization. Large unilamellar vesicles were prepared by using the procedure previous described in detail [22]. Briefly, a chloroform solution of lipid(s) was dried overnight in a vacuum oven. In those cases where membranes with more than one component were created, the mixture was sonicated for 5 min before drying it. The dried lipid film was redissolved in CF solution to yield a 10 mM lipid solution. In the case of the extract a factor of 686.6 mg/mmol was used for conversion of milligrams of phospholipid to millimoles. This factor is the weighted average of the individual phospholipids in the isolated plasma membrane as determined according to their relative abundance as indicated by the data provided in Table 1. A multilamellar vesicle dispersion was prepared via 5 cycles of vortex mixing followed by freeze–thawing (using liquid nitrogen). The dispersion was extruded (Lipex biomembranes) through 400 and 100 nm polycarbonate filters (Nucleopore). Vesicles were separated from unencapsulated dye by passing the solution through a Sephadex G-50 medium resin column, using the external solution as the eluant. The vesicle fraction was collected and characterized as described below.

The phospholipid content of vesicles was determined as described in Lipid analysis. The average vesicle diameter was determined to be 100 ± 8 nm using dynamic light scattering experiments (Brookhaven, BI-200Smlaser light scattering goniometer equipped with solid-state laser [$\lambda = 532.5$ nm] and an ALV-5000 correlator). The size distribution was reasonably homogeneous (polydispersity lower than 0.065). All measurements were performed at 25 °C under a scattering angle of 90°.

Leakage experiments. Membrane permeability was studied by monitoring the increase in the fluorescence signal, $I(t)$, associated with the release of the fluorophore CF from the interior of vesicles exposed to surfactant in solution. At high concentrations as encapsulated in the vesicle, CF has negligible fluorescence due to very efficient quenching. As the CF is diluted (after leaking to the exterior solution), the self-quenching efficiency decreases and the observed fluorescence increases.

Fluorescence measurements were made using a Hitachi F-4500 fluorescence spectrophotometer. The samples were excited at 490 nm and an emission maximum was read at 519 nm. Surfactant solution was added to vesicle solution in such amounts as to yield the desired final surfactant and lipid concentrations.

The extent of vesicle leakage is determined by normalizing the intensity of the fluorescence signal:

$$\%\text{Leakage} = \left(\frac{I(t) - I_0}{I_\infty - I_0}\right) \times 100, \quad (1)$$

where I_0 is the initial signal before adding the surfactant and I_∞ corresponds to the fluorescence signal at $t \to \infty$, after all the dye is released. The latter is determined by complete vesicle lysis as accomplished by addition of 100 μL of 2% (w/w) Triton X-100.

We have previously shown that the release of CF is continuous and goes to completion with first-order rate kinetics [22]. As a means of comparing the performance of membrane/surfactant system to the next, we report the percent of leakage recorded after 4 h. The lipid concentration in all experiments was the same (4.6×10^{-3} mM final lipid concentration). Each experiment was repeated at least three times. Repeatability was within ± 5%, unless otherwise stated.

Results

Isolation and analysis of rabbit sperm plasma membranes

The regional structure of the sperm plasma membrane permits isolation of specific membrane domains. Several studies [5,6,19] have shown that the nitrogen cavitation procedure used in this work is effective in removing nearly all the plasma membrane from the anterior region of the head. Similar to the findings of Parks and Hamstead [23] and Wolf [19], we found that the procedure also generates a product of high purity, not contaminated with other intracellular membrane components. By testing for succinic dehydrogenase, we found the membrane free of any mitochondrial contamination. The isolated fraction was essentially free of acrosin, a marker for the acrosomal membrane (found only in trace amounts). As it should be, the released membrane was found to be enriched in the cell surface enzyme marker 5′ nucleotidase as compared to the cavitate.

The protein contents of the cavitate and the released membrane were found to be 11.8 ± 4.3 and 0.143 ± 0.03 mg/10^9 sperm cells, respectively (standard deviations based on nine trials). If the plasma membrane is assumed to be composed of approximately 4% protein [24], then roughly 30% of the membrane proteins are recovered. Previous studies have reported similar recoveries, usually around 25% [5,24,25]. Using the phosphorous quantification assay described previously, we found that the phospholipid to protein ratio was 0.67.

Lipids were extracted from the released membrane fraction using the procedure described in Materials and methods. TLC/GC analysis of the lipid product revealed the relative proportions of the main phospholipid classes as shown in Fig. 1. The main classes found are PC, PE, SPH, PS, PI, and DPG. With due regard to the fact that the compositions of sperm plasma membranes differ across animal species and sometimes between studies of the same species, our results fall within the range of values reported in the literature. Directly comparing our results to the study of Hinkovska-Galcheva and Srivastava [7] that also used rabbit sperm cells, we find that the main phospholipid classes are in general agreement. With regard to details, we found lower contents of SPH, PS, PI, and DPG (14.2%, 6.9%, 3.5%, and 4.6%, respectively, as compared to 22%, 9%, 8%, and 9% in [7]) and higher PC and PE contents (54.6% and 15.9% compared to 41% and 7%).

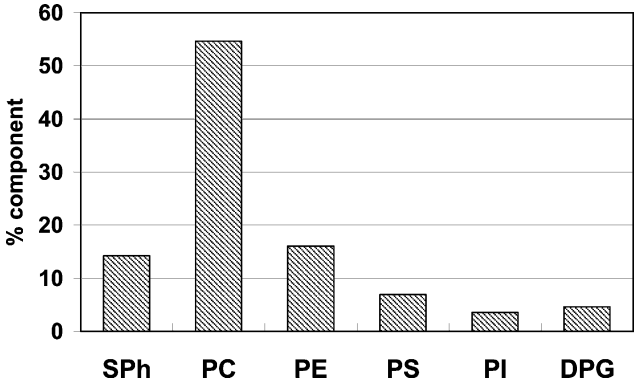

Fig. 1. Phospholipid composition of plasma membrane of the anterior head of rabbit sperm. Phospholipids were separated and identified as described in Materials and methods.

The fatty acid compositions of the main phospholipid components are summarized in Table 1. As readily seen, the fraction of the polyunsaturated fatty acids is high (38.63%), especially with regard to the PC class. The main polyunsaturated fatty acids are C22:5N6 (clupanodonic) and C22:6N3 (docosahexaenoic). The predominant saturated fatty acid is palmitic acid (C16:0) followed by stearic acid (18:0). These results are in agreement with the study of Nikolopoulou et al. [4].

Finally, we also found that cholesterol makes up 29.91% of extracted product and the cholesterol to phospholipid ratio was found to be 0.42 which is the same value found in the study of Hinkovska-Galcheva and Srivastava for rabbit sperm extract [7]. Literature values range from 0.12 to 1 depending mainly on the species from which the sperm was taken [4,5,12,25].

The hierarchy of lipid constructs for the sperm plasma membrane

Our goal was to create an artificial system for the lipid matrix of sperm plasma cell membranes, capturing essential components and features which distinguish, in a general sense, these membranes from those of somatic cells. A related goal was to better understand the role of lipids that are unique to sperm membranes (e.g., ether-linked phospholipids) as well as the role of substantial membrane components (e.g., cholesterol). A practical goal was to create such systems with commercially available, reasonably priced, ingredients.

Towards these goals, we thus put forward a hierarchy of lipid constructs, ranging in complexity from a single component system (POPC) to a multicomponent system that includes, for example, lipids with the unique ether-linkage as well as a sulfogalactolipid to represent seminolipid. The series of lipid constructs, identified as I–V, are detailed in Table 2. The models put forward were contrived on the basis of an extensive survey of the literature dealing with sperm cell membrane compositional analysis. Reasoning for the particular constructs in the hierarchal series is described below.

Lipids of the phosphatidylcholine group generally constitute the largest class of phospholipids found in most plasma membranes. Hence, our simplest membrane construct, I, is the single-component system composed of POPC. This construct was derived primarily as a reference point for comparing the properties of all other systems.

Cholesterol is well known to play a critical role in regulating the molecular packing and thus the permeability, stability, and fusogenicity of sperm cell membranes [2,26]. As stated previously, membrane cholesterol content varies along the way from the testis, through the epididymis, to the female reproductive tract, where an efflux of cholesterol occurs in preparation to the sperm–egg interaction. Thus, cholesterol was an obvious component to add to the next construct in the hierarchy, II. We chose 30% cholesterol as a representative value.

Seminolipid, SGG (3′-sulfogalactosylglycerolipid), is unique to the plasma membrane of sperm cells where it constitutes 5–8% of the matrix. Unfortunately, SGG is not commercially available and so instead we chose to

Table 2
Composition of lipid constructs in comparison to the composition of lipid extracted from rabbit sperm plasma membranes

	PC			PE	SPH	PS	Chol	SGC
	16:0–22:6 di-ester	16:0–22:6 ether-ester	16:0–18:1	16:0–16:0	egg-SPH	16:0–18:1		
I	–	–	100	–	–	–	–	–
II	–	–	70	–	–	–	30	–
III	38.9	–	13.9	16.7	20.8	2.8	–	6.9
IV	28	–	10	12	15	2	28	5
V	–	28	10	12	15	2	28	5
V†	*Construct V with only half as much 16:00-22:6 ether–ester (14%), and including 16:00-22:6 di-ester (14%)*							
V‡	*Construct V with even more 16:00–22:6 ether–ester (35%), and less of 16:00–18:1 (3%)*							
V⁻	*Construct V without SGC*							
E	38.3			11. 2	9.96	4.9	29.9	NA

Numbers represent mole percent. E stands for Extract.

include SGC, 3′-sulfogalactosyl ceramide, in the higher level constructs. SGC has the same headgroup as SGG and is found in the sperm cells of some vertebrates as well as in the myelin sheath [10]. The molecules also seemingly have similar functional abilities and SGC has been used as an analog for SGC in many studies [10,11]. Thus the higher level constructs III–V all include 5% SGC.

Our literature review revealed that the major phospholipid classes present in sperm plasma membranes are PC, PE, and sphingomyelin (SPH) which comprise 35 to 55%, 5 to 32.5%, and 10 to 23% of the total membrane lipid, respectively [4,5,7,12,27]. PS, PI, and DPG are minor components with compositions ranging from 1.5 to 17.4%, 0.4 to 8%, and 1.5 to 8% [5,7,12, 23,27]. To represent these different lipid classes, while keeping the size of the constructs as reasonable as possible, we chose to include commercially available lipids in the PC, PE, SPH, and PS families in all the higher level constructs: III–V.

The sperm plasma membrane, and in particular its PC and PE components, is rich in polyunsaturated fatty acids [9,27]. The major polyunsaturated components are clupanodonic (22:5) and docosahexaenoic (22:6). We thus chose to deliberately include PC lipids containing 22:6 in the higher level constructs III–V.

Constructs IV and V were derived to examine in particular the influence of ether-linked phospholipids, a distinctive characteristic of sperm plasma membranes. As these commercially available lipids are quite expensive, construct IV is in itself a useful model membrane system as it provides a balance of accuracy and affordability. As previously described, a high proportion of the polyunsaturated fatty acids in the sperm plasma membrane are linked at position 2 of the glycerol moiety via an acyl group whereas position 1 of these lipids is occupied with a saturated fatty acid linked via an ether bond. Construct IV contains the conventional (di ester-linked) 16:0–22:6 PC. In construct V, this particular lipid is replaced with its ether linked analog, *sn*-1 ether linked 16:0/*sn*-2 ester-linked 22:6-PC. As will be shown, the susceptibility of the membranes to surfactant attack is indeed dependent upon the presence of the ether-linked lipids. To examine this behavior in more detail, we also prepared two other model membrane systems, marked as $V^{\dagger}$ and $V^{\ddagger}$, which contain different amounts of the ether-ester linked 16:0–22:6 PC (see Table 2). In the same vein, to evaluate the specific role of sulfogalactolipid in regulating membrane behavior, we created a permutation of construct V, labeled V^{-}, that lacks SGC.

Finally, we note that construct III is equal to construct IV with cholesterol removed. Construct III thus allows us to evaluate the explicit role of cholesterol in the more sophisticated artificial systems as compared to that in much simpler systems (e.g., construct II vs. construct I).

With regard to compositional similarity, the artificial membrane systems can be compared to the lipids extracted from the rabbit sperm plasma membranes as described in Isolation and analysis of rabbit sperm plasma membranes. As shown in Table 2, the basic compositional profile of the higher level constructs (III–V) reasonably mimics that of our lipid extracts (see also Discussion). This is particularly encouraging, since we devised our lipid constructs well before the analysis of the rabbit sperm lipid extracts was achieved.

Surfactant-induced vesicle leakage experiments

We use surfactant-based vesicle leakage experiments as a platform for comparing the behavior of the lipid constructs in reference to the behavior of vesicles reconstituted from rabbit sperm plasma membrane lipid extracts. These studies also allow us to compare the performance of the prevalent spermicidal agent, N-9, to that of another surfactant, BZK, which was also used in spermicidal formulations.

We have previously studied in great detail the process of surfactant-induced vesicle leakage [22]. For the purpose at hand, we report simply the extent of leakage (fraction of encapsulated probe released from the vesicle interior) observed after four hours of vesicle incubation with surfactant solutions at various concentrations (see Leakage experiments). The leakage response curve reflects, generally speaking, the resistance of membranes to surfactant attack as the concentration of surfactant is increased. Experiments were performed in a salt solution to better approximate physiological conditions.

Fig. 2 shows the leakage response curve for the hierarchal lipid constructs as well as the rabbit lipid extract as induced by exposure to N-9. As expected, all membranes became more permeable with increasing surfactant concentration. Two basic patterns are remarkable. The leakage behaviors of constructs II, IV, V,

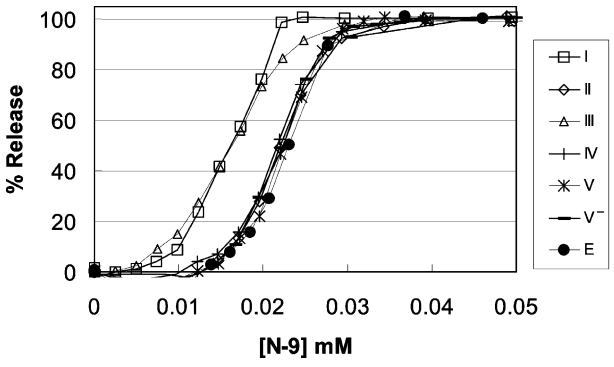

Fig. 2. Leakage response of vesicles made of the different constructs (lipid concentration 4.6×10^{-3} mM) in the presence of N-9. Extent of leakage after 4 h is plotted versus overall surfactant concentration. Data are based on a minimum of three experiments.

and the extract are very similar. The other two constructs (I and III), notably those that are void of cholesterol, also behave very similarly. In comparison to the systems containing cholesterol, these membranes are far less resistant toward surfactant attack (i.e., they leak the same fraction of contents at lower surfactant concentrations).

The leakage response curves for membranes exposed to BZK are shown in Fig. 3. For purposes of clarity we show the curves for constructs I and III and the curves for II, IV, and V in two adjacent graphs; the response of vesicles composed of the lipid extract is shown in both graphs. Similar to the case with N-9, we again find that lack of cholesterol causes a marked increase in surfactant-induced membrane perturbation. In addition, the relatively sophisticated *sans*-cholesterol construct (III) behaves nearly identically to the simple POPC system (I). Unlike in the experiment with N-9, however, the remaining constructs each exhibit different behaviors. Although not at all similar to the extract, the low-level construct (II; POPC + Chol) appears to be the most resistant of all membranes, requiring the highest BZK concentrations to obtain a given level of release. The behavior of construct IV is closer to that of the extract; however, the curves do not generally match up except at concentrations above 0.02 mM. Clearly the extract displays a very distinctive and complex response curve, with a characteristic "bend," that is mimicked only by the highest level construct, V, which contains ether-linked lipid. It follows quite closely the behavior of the extract except between BZK concentrations of 0.01 and 0.024 mM, where its behavior deviates, i.e., the "bend" occurs at higher levels of leakage.

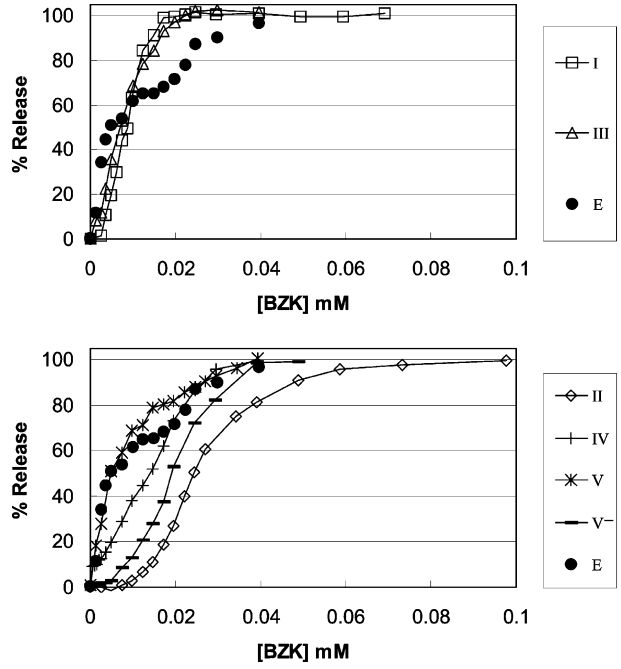

Fig. 3. Leakage response of vesicles made of the different constructs (lipid concentration 4.6×10^{-3} mM) in the presence of BZK. Extent of leakage after 4 h is plotted versus overall surfactant concentration. Data are based on a minimum of three experiments.

Since the presence of the ether-linked lipid (1-ether 2-ester linked 16:0–22:6 PC) does indeed impact the membrane response to surfactant attack (compare Constructs IV and V), we briefly attempted to vary the concentration of this specialized lipid to see if the leakage response could approximate even more closely the behavior of the extract (see Table 2, constructs $V^{\dagger}$, $V^{\ddagger}$). One permutation, $V^{\dagger}$, involved creating a construct with half as much ether-linked lipid. In this case the leakage response fell between that of construct IV and construct V, as might be expected. Another permutation, $V^{\ddagger}$, involved using a higher percentage of the ether-linked lipid at the expense of other PC components. In this case, the leakage response curve rose more steeply than that of construct V (data not shown), creating even further deviations from the behavior of the extract.

Finally, the specific influence of the sulfogalactolipid was explicitly investigated. If sulfogalactolipid is removed from the highest level construct, to form construct V^{-}, the membrane becomes more susceptible to attack by the charged surfactant BZK (see Fig. 3). Meanwhile, the perturbation induced by N-9 is not affected by the absence of this specific lipid.

Discussion

We have presented a series of lipid constructs as models for the lipid matrix of sperm plasma membranes. As a frame of reference for comparing the composition and behavior of these constructs, we also extracted lipids from the anterior head of rabbit spermatozoa and reported on this as well. The behavior of these various membrane systems was evaluated using a vesicle leakage assay in which surfactant exposure induces membrane permeabilization. These studies not only shed light on the relative importance and significance of particular components present in the lipid constructs, but they also reveal the ability of the surfactants studied to discriminate between different membrane systems.

In direct comparison to the rabbit sperm lipid extracts, we find that the two highest lipid constructs, IV and V, are quite realistic in terms of key composition classes. First, the overall relative fraction of PC is identical. The other major lipid classes compare as follows: the PE contents are similar; the PS content of the lipid extract is higher than that in the constructs; and the SPH content of the extract is lower (see Table 2 for details). The SGG content of the extract was not determined, but the fraction of sulfogalactolipid included in the constructs reflects amounts routinely reported in

the literature [10,11]. Finally, the cholesterol content of the constructs was found to be similar to that of the extract.

The fatty acid composition of the extract and the highest-level lipid constructs can also be compared. Not surprisingly, the extract includes many fatty acid components; 51.11% of these, however, are fully saturated lipids, mostly C16:0 and C18:0. Notably, the predominant saturated lipid in the constructs is C16:0; however, the total fraction of saturated lipids is higher (70.52%). The predominant monounsaturated fatty acid in the extract is C18:1; the same holds true in the hierarchal constructs. In this case, the relative fraction of monounsaturated PC lipids in the extract and the constructs is similar: 10.26% and 9.49%, respectively. The main polyunsaturated components in the extract are C22:5 and C22:6. The polyunsaturated "representative" in the constructs is a lipid composed of the latter fatty acid (C22:6). The total fraction of polyunsaturated components in the constructs is lower than that in the extract (20.08% vs. 38.62%).

As the goal of this work was to create a generic model for the sperm plasma membrane and not one necessarily specific to a particular animal species, the general similarities between the artificial constructs and actual sperm membrane lipids are a mark of success.

Direct comparisons of membrane behavior are revealed in the surfactant-induced vesicle leakage experiments. For both surfactants studied, N-9 and BZK, the top level construct (V) best mimics the behavior of vesicles formed of rabbit sperm lipid extract. Moreover, though not identical, the behavior is astonishingly similar given the comparative simplicity of the lipid construct. Thus, while the hierarchal series can readily be expanded upon, the current artificial system (V) serves as a useful model for the plasma membrane of the anterior head of the sperm cell.

The value of creating a hierarchal series of constructs also enabled us to establish the significance of certain key membrane components. For example, in the absence of cholesterol, none of the associated constructs display behavior remotely close to that of the extract. Moreover, even the sophisticated cholesterol-free construct (III) behaves in manner similar to the most basic system (i.e., just like pure POPC, construct I). We note that the amount of cholesterol in a membrane distinctly regulates the response of membranes to surfactant attack [28].

The unusual ether linkage of fatty acid to the glycerol backbone of the phospholipid also plays a role in controlling the interaction of sperm membranes with surfactant. The leakage behavior of the top-level constructs (IV and V)—which differ only by the absence/presence of a phospholipid bearing this ether linkage—does not always overlap. In particular, when the constructs were perturbed by the surfactant BZK only construct V exhibited a behavior akin to the uniquely shaped leakage response curve of the extract. Increasing amounts of ether–ester 16:0–22:6 PC in constructs IV (0%), $V^{\dagger}$ (14%), V (28%), and $V^{\ddagger}$ (35%) render the respective membranes more susceptible to attack by the cationic surfactant BZK. Interestingly similar experiments with the anionic surfactant SDS (data not shown) show the same trend. A possible explanation for this behavior originates from solvation effects. The ether oxygen is more basic than the carbonyl oxygen of the ester and is thus better able to hydrogen bond. An increase in water solvation would aid the incorporation of charged surfactants into the membrane. From the biological perspective, the existence of ether-linked lipids in sperm membranes is not completely understood, but their presence probably promotes chemical stability [2] since the ether linkage is less prone to degradation. Our studies show that this evolutionary advantage comes with a cost of susceptability to modern synthetic agents.

Our studies also show that the presence of sulfogalactolipid in the membrane has an impact on its susceptibility to attack by the charged surfactant, BZK; in this case elimination of this negatively charged lipid (construct V^{-}) decreased the extent of leakage induced by the positively charged BZK. We suspect that electrostatic interactions are at the root of this effect. Favorable interactions promote the binding of oppositely charged surfactant (e.g., BZK) to membranes containing sulfogalactolipid, increasing membrane permeabilization. Indeed, in separate experiments using the anionic SDS as the perturbant (data not shown), the opposite effect was observed—namely, that elimination of sulfogalactolipid increased membrane permeabilization.

Finally, our construct system allows us to compare directly the performance of different membrane active agents with current or potential application in contraception and prevention of sexually transmitted diseases. We discovered, for example, that the prominent surfactant in commercial spermicides, N-9, displays little sensitivity to membrane composition much beyond the fraction of cholesterol. This inability of N-9 to distinguish between different membranes is quite significant and likely the cause of its failure as a promising microbicidal candidate [29–31]. Meanwhile, we found that the surfactant BZK interacts very discriminately with the various membrane systems. This result shows that the unique composition of sperm membranes may indeed allow some "targeting" of surfactant-based perturbants to these systems.

In summary, we extracted the lipids from the plasma membranes of rabbit sperm cells. These lipids were reconstructed as bilayers in the form of vesicles and used to examine membrane perturbation as induced by surfactants. We also proposed a hierarchal series of lipid constructs for the sperm plasma membrane, designed to accurately represent both the composition and behavior

of the natural system while also allowing systematic studies of key membrane components. We note that our hierarchal constructs can be readily and systematically expanded. As well, other simple yet meaningful assays may be implemented (e.g., fusion assays) to test and guide the further development of these artificial systems. In the current form, however, the construct series has already proven especially valuable in demonstrating the specificity of interactions (or lack thereof in the case of N-9) between surfactants and membranes. This information is key to the design of specific, non-irritating of membrane active agents for spermicidal and microbicidal applications.

Acknowledgments

Support for this subproject [GMP-01-25] was provided by the Global Microbicide Project (GMP) of the CONRAD Program, Eastern Virginia Medical School. The views expressed by the authors do not necessarily reflect the views of CONRAD or GMP.

References

[1] W.V. Holt, Membrane heterogeneity in the mammalian spermatozoon, Int. Rev. Cytol. 87 (1984) 159–194.
[2] S. Ladha, Lipid heterogeneity and membrane fluidity in a highly polarized cell, the mammalian spermatozoon, J. Membr. Biol. 165 (1998) 1–10.
[3] R.A. Cardullo, D.E. Wolf, in: R.A. Bloodgood (Ed.), Ciliary and Flagellar Membranes, Plenum Press, New York, 1990, pp. 305–336.
[4] M. Nikolopoulou, D.A. Soucek, J.C. Vary, Changes in the lipid content of boar sperm plasma membranes during epididymal maturation, Biochim. Biophys. Acta 815 (1985) 486–498.
[5] C. Labbe, M. Loir, Plasma membrane of trout spermatozoa: I. Isolation and partial characterization, Fish Physiology and Biochemistry 9 (1991) 325–338.
[6] S.R. Mack, J. Everingham, L.J.D. Zaneveld, Isolation and partial characterization of the plasma-membrane from human-spermatozoa, J. Exp. Zool. 240 (1986) 127–136.
[7] V. Hinkovska-Galcheva, P.N. Srivastava, Phospholipids of rabbit and bull sperm membranes—structural order parameter and steady-state fluorescence anisotropy of membranes and membrane leaflets, Mol. Reprod. Dev. 35 (1993) 209–217.
[8] F.M. Flesch, B.M. Gadella, Dynamics of the mammalian sperm plasma membrane in the process of fertilization, Biochim. Biophys. Acta-Rev. Biomembr. 1469 (2000) 197–235.
[9] J.P. Nolan, R.H. Hammerstedt, Regulation of membrane stability and the acrosome reaction in mammalian sperm, FASEB J. 11 (1997) 670–682.
[10] M. Attar, M. Kates, M.B. Khalil, D. Carrier, P.T.T. Wong, N. Tanphaichitr, A Fourier-transform infrared study of the interaction between germ-cell specific sulfogalactosylglycerolipid and dimyristoylglycerophosphocholine, Chem. Phys. Lipids 106 (2000) 101–114.
[11] S. Tupper, P.T.T. Wong, M. Kates, N. Tanphaichitr, Interaction of divalent-cations with germ-cell specific sulfogalactosylglycerolipid and the effects on lipid chain dynamics, Biochemistry 33 (1994) 13250–13258.
[12] P. Martínez, A. Morros, Membrane lipid dynamics during human sperm capacitation, Front. Biosci. 1 (1996) d103–d117.
[13] P.S. Savle, G.F. Doncel, S.D. Bryant, M.P. Hubieki, R.G. Robinette, R.D. Gandour, Acylcarnitine analogues as topical, microbicidal spermicides, Bioorg. Med. Chem. Lett. 9 (1999) 2545–2548.
[14] O.J. D'Cruz, F.M. Uckun, Gel-microemulsions as vaginal spermicides and intravaginal drug delivery vehicles, Contraception 64 (2001) 113–123.
[15] F.C. Krebs, S.R. Miller, D. Malamud, M.K. Howett, B. Wigdahl, Inactivation of human immunodeficiency virus type 1 by nonoxynol-9, c31g, or an alkyl sulfate, sodium dodecyl sulfate, Antiviral Res. 43 (1999) 157–173.
[16] M.K. Stafford, H. Ward, A. Flanagan, I.J. Rosenstein, D. Taylor-Robinson, J.R. Smith, J. Weber, V.S. Kitchen, J. Acq. Imm. Def. Synd. Hum. Retrovirol. 17 (1998) 327–331.
[17] J. Stephenson, Widely used spermicide may increase, not decrease, risk of hiv transmission, JAMA—J. Am. Med. Assoc. 284 (2000) 949.
[18] P.E. Castle, K.J. Whaley, T.E. Hoen, T.R. Moench, R.A. Cone, Contraceptive effect of sperm-agglutinating monoclonal antibodies in rabbits, Biol. Reprod. 56 (1997) 153–159.
[19] D.E. Wolf, A.C. Lipscomb, V.M. Maynard, Causes of nondiffusing lipid in the plasma-membrane of mammalian spermatozoa, Biochemistry 27 (1988) 860–865.
[20] V.P. Skipski, M. Barclay, R.F. Peterson, Quantitative analysis of phospholipids by thin-layer chromatography, Biochem. J. 90 (1964) 374–378.
[21] W.L. Zahler, G.A. Doak, Isolation of the outer acrosomal membrane from bull sperm, Biochim. Biophys. Acta 406 (1975) 479–488.
[22] M. Apel-Paz, G.F. Doncel, T.K. Vanderlick, Membrane perturbation by surfactant candidtaes for STD prevention, Langmuir 19 (2003) 591–597.
[23] J.E. Parks, R.H. Hammerstedt, Developmental-changes occurring in the lipids of ram epididymal spermatozoa plasma-membrane, Biol. Reprod. 32 (1985) 653–668.
[24] G. Gillis, R. Peterson, L. Russell, L. Hook, M. Freund, Isolation and characterization of membrane vesicles from human and boar spermatozoa: Methods using nitrogen cavitation and ionophore induced vesiculation, Preparative Biochem. 8 (1978) 363–378.
[25] J.E. Parks, J.W. Arion, R.H. Foote, Lipids of plasma-membrane and outer acrosomal membrane from bovine spermatozoa, Biol. Reprod. 37 (1987) 1249–1258.
[26] P.L. Yeagle, Cholesterol and the cell-membrane, Biochim. Biophys. Acta 822 (1985) 267–287.
[27] D.P. Selivonchick, P.C. Schmid, V. Natarajan, H.H.O. Schmid, Structure and metabolism of phospholipids in bovine epididymal spermatozoa, Biochim. Biophys. Acta 618 (1980) 242–254.
[28] M. Apel-Paz, T.K. Vanderlick, G.F. Doncel, Impact of membrane cholesterol content of the resistance of vesicles to surfactant attack, in preparation.
[29] J. Kreiss, E. Ngugi, K. Holmes, J. Ndinya-Achola, P. Waiyaki, P.L. Roberts, I. Ruminjo, R. Sajabi, J. Kimata, T.R. Fleming, J. Am. Med. Assoc. 268 (1992) 477–482.
[30] R.E. Roddy, L. Zekeng, K.A. Ryan, U. Tamoufe, S.S. Weir, E.L. Wong, A controlled trial of nonoxynol 9 film to reduce male-to-female transmission of sexually transmitted diseases, N. Engl. J. Med. 339 (1998) 504–510.
[31] L. Van Damme, G. Ramjee, M. Alary, B. Vuylsteke, V. Chandeying, H. Rees, P. Sirivongrangson, L. Mukenge-Tshibaka, V. Ettiegne-Traore, Effectiveness of col-1492, a nonoxynol-9 vaginal gel, on hiv-1 transmission in female sex workers: A randomised controlled trial, Lancet 360 (2002) 971–977.