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# Spermatozoa as a transport system of large unilamellar lipid vesicles into the oocyte



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Nienke Geerts, PhD joined the Vanderlick group at Yale University in 2010 after receiving her PhD in biophysics in 2009 at the University of Amsterdam, The Netherlands with a special focus on DNA-driven assembly of micronsized colloids. As a postdoc at Yale she became interested in the concept of delivery and the idea of making vesicles motile. This initial thought resulted into a collaboration with G Huszar, MD. Her current interests also include DNA assembly of lipid nanodiscs and exploring other new methods of drug delivery.

Abstract In addition to their role as man-made membranes, vesicles continue to be investigated as carriers for drug delivery. While most research focuses on their injectable properties, here a new delivery strategy is proposed. It is shown that spermatozoa can transport vesicles of variable composition. For human spermatozoa, the vesicles started to show binding after 20 mol% of the nonbinding vesicle backbone lipids were substituted with positive, negative, cerebroside or ganglioside lipids. Vesicle binding is a dynamic process with constant 'on' and 'off' binding. The physiological and motility attributes of the spermatozoa are not affected by the attached vesicles. Sperm swimming characteristics changed only marginally. Also, the activation status of the acrosomal membrane, tested with the fluorescent probe *Pisum sativum* agglutinin, was not affected by vesicle binding. Moreover, the hyaluronic acid-binding test showed that viable, fully developed spermatozoa will attach and remain bound to hyaluronic acid-coated slides regardless of vesicle binding. Therefore a new 'hybrid' delivery system was created with human spermatozoa, and tested with a mouse IVF system. Large unilamellar vesicles physisorbed to mouse spermatozoa can not only penetrate the mouse oocytes in these proof-of-principle experiments, but also deliver the cargo placed within the vesicles. ©

KEYWORDS: lipid vesicles, mouse, spermatozoa, transport, delivery, hybrid

# Introduction

Two significant barriers must be overcome for applications of man-made nanostructures in the human body: the

immune response and the delivery scheme (Immordino et al., 2006; Langer, 1990; Tianshun and Rodney, 2001). Lipid vesicles can be designed to mimic human cells closely enough to manage the immune response (Maurer et al.,

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1999), thus making these versatile membrane sacs attractive cargo carriers for drug delivery applications. Still, these simple nanostructures lack self-propulsion and thus a transport mechanism is necessary for each delivery application. For instance, most research on vesicle drug delivery (Jung et al., 2010; Moghimia and Szebenib, 2003) utilizes the blood stream for transport. Likewise, less prevalent means of delivery – e.g. oral (Chen et al., 1996), aerosols (Vyas et al., 2004) or topical applications (Cevc, 2004) – all rely on external forces to transport vesicles to their intended destinations.

Vesicle locomotion requires some type of machinery to provide sufficient energy to overcome friction, drag and inertia. Motile objects on a similar scale in nature make use of one or more lash-like appendages, called flagella (Haimo and Rosenbaum, 1981). One option to obtain self-propulsion in vesicles is to reconstruct such known swimming machinery. Unfortunately, flagella consist of an arrangement of at least 50 different proteins (Bardy et al., 2003), thus in-vitro restoration in a vesicle membrane is an extremely difficult prospect.

In lieu of flagella reconstruction, a more viable approach is simply to attach vesicles to motile microscopic swimmers. Indeed, the microbial world provides an incredibly large and diverse population of candidates, but toxicity obviously limits human-based applications. In the eukaryotic domain, there is an obvious candidate with potential applications in human reproduction, including fertility, contraception and the prevention of sexual transmitted diseases: the sperm cell, which uses its flagellum to propel itself through the female reproductive tract. In humans, sperm cells consist of a head of size  $5-3 \,\mu\text{m}$  and a tail approximately  $50-80 \,\mu\text{m}$  long (Katz et al., 1986). Although vesicles can be fabricated across different length scales, the dimensions of spermatozoa make the use of large unilamellar vesicles (LUV; referred to as vesicles), in this case 200 nm in diameter, a natural choice, allowing multiple vesicles to bind to each sperm cell (Figure 1A).

Interactions between spermatozoa and lipid vesicles have been studied previously, albeit not in the context of movement or transport. Since the discovery of egg yolk as a protectant for cryopreservation of spermatozoa (Phillips and Lardy, 1940), there has been a desire to use lipid vesicles for this purpose (Pillet et al., 2012; Ropke et al., 2011; Wilhelm et al., 1996). Another field where spermatozoa and vesicles have crossed paths is the field of transfection. Here, complexes of cationic vesicles and DNA are used to introduce foreign genetic material into sperm heads (Bachiller et al., 1991; Gershon et al., 1993). The cationic vesicles increase the uptake of DNA by fusing with the sperm membrane. Later, the concept of vesicle fusion with sperm membranes was further explored with regards to lipid composition (Arts et al., 1993) and used for the loading of sperm cells with membrane-impermeable agents (Garrett et al., 1999).

In contrast, the motivation of this work was to explore the use the spermatozoa as vehicles to transport vesicles and their encapsulated cargo. Success in doing so would allow new general delivery possibilities, including applications specifically related to human or animal reproduction.



**Figure 1** Vesicle binding to human sperm cells. (A) Cartoon of a sperm cell carrying several lipid vesicles. The vesicle membrane consists of two lipids: neutral POPC and one of the four binding lipids tested (positively charged DOTAP, negatively charged DOPG, cerebroside or ganglioside). The enlargement clarifies that the ultimate goal was not the transport of the lipid vesicle, but the cargo within the vesicle; not to scale. (B) Confocal image of spermatozoon—vesicle binding. All vesicle types bound to spermatozoa, this example used 50 mol% DOPG; bar = 10  $\mu$ m. (C) Resonance energy transfer showing that vesicles adhered, rather than fused, to the sperm cells. At time point 1 (arrow 1), either spermatozoa (Ce-, sperm-experiment) or vesicle buffer (CE-, sperm-control) were added to all samples. At time point 2 (arrow 2), Triton X-100 (Ce-, sperm-experiment) was added, to eliminate all quenching of NBD, or vesicle buffer (CE-, sperm-control) was added once more. CE = cerebroside veiscles.

This paper addresses the first set of fundamental issues: (i) how should lipid vesicles be adapted, to be able to bind (adhere) to sperm cells? (ii) can spermatozoa operate naturally when loaded with the extra vesicular cargo? and (iii) are spermatozoon-vesicle hybrids capable of transporting the vesicles and delivering the encapsulated cargo during fertilization?

## Materials and methods

#### **Reagents and sperm specimens**

The lipids 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC; neutral/uncharged), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP; positively charged), 1,2-dioleoylsn-glycero-3-phospho-(1'-rac-glycerol) (DOPG; negatively charged), cerebrosides (total; porcine brain), gangliosides (ovine brain) and the fluorescent lipids lissamine rhodamine B 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (Rh-DOPE), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(cap biotinyl) (biotin-lipid) were all purchased from Avanti Polar Lipids (Alabaster, AL, USA). The fluorescent lipid N-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (NBD-PE) was purchased from Invitrogen Molecular Probes (Grand Island, NY, USA). Green fluorescent neutravidin was purchased from Invitrogen (Grand Island, NY, USA).

The studied specimens originated from the leftover portion of normozoospermic semen specimens within 1 h of ejaculation. Sperm concentration and motility were determined using a computer-assisted semen analysis system (Hamilton-Thorn, Beverly, MA, USA).

#### Vesicle preparation

Large unilaminar vesicles (~200 nm) were prepared by the use of the extrusion method (Hope et al., 1985). A lipid mixture (1 mmol/l), containing all lipids to make up the vesicle membrane in chloroform, was placed into a glass vial and evaporated under vacuum (>4 h) to remove all the solvent and leave a lipid film at the bottom of the vial. Vesicle buffer (2 ml; 125 mmol/l NaCl, 10 mmol/l HEPES, pH 7.2) was added to the vial. The sample was vortexed, frozen and then thawed. This cycle was repeated five times before conversion to a uniform size by repeated extrusion (10 times) of the 2-ml sample through a pair of trach-etch membranes (Whatman Nucleopore, pore size 0.2  $\mu$ m).

The vesicle types that were studied were created from two lipids to be able to control the percentage of potential binding enhancing lipids: POPC and either a positive lipid (type 1; DOTAP), a negative lipid (type 2; DOPG), a cerebroside or a ganglioside (type 3 and 4; hyaluronic acid (HA) chemical structure mimics). The four lipid types were added in concentrations ranging from 20–50 mol% (a percentage of total moles of lipids present) of POPC lipid replacement.

The biotinylated vesicles were prepared as described above, but consisted of three lipids: 10 mol% of biotin—lipid, 40 mol% of POPC and 50 mol% of cerebrosides. For visualization, 0.5 mol% of fluorescent lipid (Rh-DOPE) was added (the fluorescent lipid NBD-PE was used as a second colour). Lipid concentrations were measured using a standard phosphate assay (Chen et al., 1956). Final vesicle concentrations were determined at  $\sim 1 \times 10^{10}$  vesicles/ml.

#### Binding of vesicles to human spermatozoa

Spermatozoa were mixed with a vesicle suspension in a 1:1 (v/v) ratio for 30 min. Semen was either added as raw semen or, if the sperm concentrations were high (>100 × 10<sup>6</sup> spermatozoa/ml), the semen was diluted to  $60 \times 10^6$  spermatozoa/ml in either human tubal fluid (HTF; Irvine Scientific, Santa Ana, CA, USA) or in vesicle buffer. Vesicle binding was determined by confocal microscopy. It was found that 20 µl of vesicle suspension was sufficient to saturate 200 µl of semen specimen ( $60 \times 10^6$  spermatozoa/ml).

#### NBD-rhodamine resonance energy transfer assay

To distinguish between vesicle fusion and adhesion to sperm cells, a resonance energy transfer assay (Struck et al., 1981) was performed. Microscopy alone was incapable of resolving this distinction, because membrane fusion between the vesicles and the spermatozoa would result in similar fluorescent images (Arts et al., 1993; Gadella et al., 1999). Briefly, vesicles of all studied lipid compositions were dually labelled with 0.8 mol% of each of the the fluorescent phospholipid analogues NBD-PE (green) and Rh-DOPE (red). Photons emitted by the excited NBD-PE will be transferred to Rh-DPPE, thus NBD fluorescence will be quenched. Upon fusion the probes will be diluted, and hence an increase in NBD fluorescence will occur. Upon adhesion NBD fluorescence will remain as is.

NBD fluorescence was measured with a SynergyMx spectrofluorometer (BioTek Instruments USA) at excitation and emission wavelengths of 465 nm and 530 nm, respectively. Vesicle suspension (150  $\mu$ l) was added to 96 wells at 37°C. NBD fluorescence was monitored for several minutes to establish baseline emissions. Then either 15 × 10<sup>6</sup> spermatozoa (in 50  $\mu$ l) or 50  $\mu$ l of vesicle buffer were added. The fluorescence scale was calibrated by setting the initial fluorescence of the nonfused (labelled) vesicles to 1. Quenching was eliminated by disruption of the vesicles in Triton X-100 (2%, v/v, infinite dilution). The experiment was repeated three times.

#### **Ethical approval**

For the study, semen aliquots left over from samples submitted for semen analysis were used. Each sample was deidentified prior to research utilization. All experimental applications of semen have been approved by the Yale School of Medicine Human Investigation Committee (1311013005; 2009). All animal procedures were performed in accordance with an approved protocol from the Institutional Animal Care and Use Committee of Yale University.

# Creatine kinase immunostaining and aniline blue staining of vesicle-loaded spermatozoa

For these experiments, low normozoospermic samples were preferred to achieve an adequate representation of spermatozoa with normal and arrested development. Arrested development was shown by cytoplasmic retention, as detected by higher concentrations of surplus creatine kinase (CK; visualized by green fluorescent CK immunocytostaining), which has been shown to be associated with failure of plasma membrane remodelling (Huszar and Vigue, 1993; Huszar et al., 1997, 2007). Incomplete chromatin development was detected by aniline blue staining. Aniline blue, being a marker of immature chromatin, shows diminished nuclear development (Aoki et al., 2005; Sati et al., 2008).

For CK staining, spermatozoa were washed in phosphate buffer supplemented with sucrose (PB-sucrose; 20 mmol/l sodium phosphate buffer, pH 7.0 containing 5% sucrose). Sperm cells were resuspended to  $20-30 \times 10^6$  spermatozoa/ml and 40 µl vesicles were added. After 30 min at room temperature, 15 µl of the sample was placed on a microscope slide and fixed for 20 min with 3.7% formalin in PB-sucrose solution. The slides were blocked with a protein-block solution (Abcam, Cambridge, MA, USA) for 5 min. After a short wash, the first CK antibody is applied for 2 h (1:500 dilution; Abcam), followed by repeated washing and the second antibody (FITC-conjugated; Abcam) for 1.5 h (1:250 dilution). Samples were analysed by confocal microscopy. The proportion of spermatozoa with three CK staining patterns were evaluated in more than 1000 sperm cells from semen of seven men.

For aniline blue staining, spermatozoa were washed in 20 mmol/l phosphate buffer containing 0.9% NaCl (pH 7.2). Sperm cells were resuspended at  $20-30 \times 10^6$  spermatozoa/ml and 40 µl vesicles were added. After 30 min at room temperature, 15 µl of the sample was placed on a microscope slide and allowed to air dry. The sperm smear was then stained for 5 min with 5% aniline blue (Sigma–Aldrich, St Louis, MO, USA) acidified to pH 3.5 with acetic acid. Subsequently, bright-field and a fluorescent images were taken. The proportion of spermatozoa with three aniline blue staining patterns were evaluated in more than 600 sperm cells from semen of seven men.

# Acrosomal status of vesicle-loaded and control spermatozoa

Spermatozoa were separated from the seminal fluid by centrifugation (600g for 20 min) over 45% Isolate gradient solution (Irvine Scientific, Santa Ana, CA, USA). The pellet was washed in 20 mmol/l phosphate buffer containing 0.9% NaCl (pH 7.2), resuspended to  $20 \times 10^6$  spermatozoa/ml and divided into two aliquots. One aliquot was left as is; to the other, 20 µl of vesicles were added. After 30 min at room temperature,  $15 \,\mu$ l of each aliquot were applied to a microsope slide, which was allowed to air dry, fixed with 95% ethanol and dried at 4°C. A drop of *Pisum sativum* lectin solution (100 mg/ml in PBS, FITC conjugate labelled, FITC-PSA; Sigma-Aldrich) known to bind to the acrosomal matrix (Cross et al., 1986; Mendoza et al., 1992) was placed over the spermatozoa-containing areas, and the samples were placed in the dark for 15 min at room temperature. After three washes, the slides were analysed by fluorescent microscopy. More than 1500 sperm cells from 15 independent samples were evaluated.

## HA binding of vesicle-loaded spermatozoa

Vesicle-loaded sperm samples and nonloaded controls were diluted to  $20\times10^6$  spermatozoa/ml in HTF. 7  $\mu l$  was

incubated layered onto a HA-coated glass slide (Biocoat, Horsham, PA, USA) for 10 min at room temperature. To determine the percentage of motile spermatozoa that bound to HA, numbers of bound and unbound motile spermatozoa and immotile spermatozoa were determined in  $2 \times 100$  spermatozoa in two different microscopic fields. The percentage of total bound motile spermatozoa was determined (mean of five independent samples).

#### Binding of vesicles to mouse spermatozoa

Spermatozoa from mature male mice (C57BL/6J; Jackson Labs, Bar Harbor, ME, USA) were isolated by dissection of the cauda epididymis and vas deferens followed by release of the spermatozoa into modified HTF (Sigma–Aldrich) after cutting off the tissue with fine scissors. Vesicles (20  $\mu$ l of 50 mol% cerebroside) were added to a portion (~400  $\mu$ l) of the sperm suspension (~1 × 10<sup>6</sup> spermatozoa/ml) and incubated for 30 min at 37°C with 5% CO<sub>2</sub> in the gas phase. Vesicle binding to spermatozoa was determined by confocal microscopy.

DNA for the green fluorescent protein (GFP)-mRNA was obtained from a CS2P eGFP X/P plasmid and transcribed with a mMessage mMachine SP6 kit (Life Technologies). GFP-mRNA loaded vesicles were obtained similar to vesicles, except for two adjustments: (i) buffers were made RNAse free; and (ii) RNAse was added after vesicle preparation to degrade all mRNA outside the vesicles.

#### IVF experiments with mouse spermatozoa

Oocytes were isolated from hormonally primed females (5 IU pregnant mare serum gonadotrophin (Sigma-Aldrich) followed by 5 IU human chorionic gonadoptropin (HCG; Sigma-Aldrich) 48 h later, both delivered by intraperitoneal injection). Thirteen hours after HCG injection, females were killed with CO2 and the oviducts removed. Cumulusoocyte complexes were released from the oviducts with a pair of fine watchmaker forceps. Oocytes were placed in 250 µl drops of pre-equilibrated modified HTF medium with 4 mg/ml bovine serum albumin (Pentax Fraction V; BSA) under silicone oil (50 cS viscosity; Sigma) and approximately 20 µl of vesicle-loaded sperm suspension was added to each drop of oocytes. Gametes were coincubated for 6 h at 37°C in a 5% CO<sub>2</sub> incubator. After 6 h, the oocytes were washed with four drops of potassium simplex optimised medium (KSOM; Ho et al., 1995) with 4 mg/ml BSA and cultured overnight in 5%  $CO_2$  in air at 37°C. Fertilization was judged by the number of 2-cell-stage embryos per total number of embryos after 24 h of in-vitro culture. If needed, the zona pellucidae were removed by incubating in Acid Tyrode's solution (pH 2.5; Sigma) for 3-4 min, and washed through four drops of KSOM immediately before visualization. For confocal imaging, oocytes were immersed in  $\sim$ 120  $\mu$ l of PBS (Sigma-Aldrich) in a Lab-Tek chamber (Nunc).

Biotinylated vesicles were used to determine whether the transported vesicles were located on the inside or the outside of the oocyte membranes. Oocytes were fertilized with spermatozoon-vesicle hybrids (carrying biotinylated vesicles) as described above. Following the wash in KSOM, the oocytes were placed inside a Lab-Tek microscopy chamber and incubated for a short time (10 min) with green fluorescent neutravidin in the surrounding medium (10 ul: 1 mg/ml PBS). After this short incubation. the oocytes were visualized directly by confocal imaging. Patches outside the membrane are accessible to biotin-neutravidin binding and can easily be distinguished from vesicle membrane fully transported into the oocyte by microscopy. As neutravidin cannot by itself pass through the membrane, binding to biotin vesicles inside the oocyte is not possible, therefore the green fluorescent neutravidin remains in solution in its diluted form (and thus remains invisible). In contrast, green fluorescent neutravidin will bind to patches outside the membrane, thereby increasing its local concentration. This will lead to a bright green fluorescent spot colocated with the membrane patch (red fluorescent). As controls, two concentrations of vesicle suspensions (no spermatozoa;  $1 \times 10^4$  or  $10^{10}$  vesicles/ml) were tested for their capability to penetrate the zona pellucidae of the oocytes as well as spermatozoa without vesicles.

#### Laser scanning confocal microscopy

Confocal images were obtained using a Leica TCS SP5 confocal system, with a Leica  $\times 63/1.3$  NA Plan Apo DIC glycerine immersion lens (Leica Microsytems, Germany). The sperm cells were imaged by bright-field microscopy, while the position of the vesicles was determined by exciting the Rh-DPPE probe with a HeNe laser at 543 nm. FITC-PSA, GFP-mRNA and the FITC-conjugated second CK antibody were excited with the 488 nm line of an argon laser.

#### Bright-field and fluorescence microscopy

Sperm cells were visualized using a Nikon Eclipse TS100 microscope ( $\times$ 60 oil immersion objective) equipped with fluorescein isothiocyanate and Texas red filters and a monochrome Spot camera running Spot 4.0.1 software (Diagnostic Instruments, Sterling Heights, Michigan, USA). Images were pseudocoloured and compiled using the same Spot software.

## Results

# Adapting lipid vesicles to obtain spermatozoonvesicle binding

The key variable underlying spermatozoon-vesicle binding was the lipid composition of the man-made vesicle membrane. Vesicles consisting purely of noncharged lipids (POPC) were unable to bind to sperm cells (data not shown). Therefore, the vesicle membrane was enriched with one of four selected lipid types: DOTAP, DOPG, cerebrosides or gangliosides. All vesicle types studied exhibited strong binding to spermatozoa as soon as 20-50 mol% POPC was replaced (Figure 1B). Notably, the binding was enhanced at the tops of the heads and the midpieces of the spermatozoa. In each case, equal volumes of the vesicle suspensions were mixed with sperm suspensions ( $\sim 60 \times 10^6$  cells/ml) at room temperature and incubated for 30 min. Although positively charged vesicles (type 1) bound to spermatozoa, they also caused large-scale aggregation (with spermatozoa and vesicles falling out of suspension) and so this type of vesicle was not included in any of the following studies.

**Figure 1**C clearly shows that the vesicles adhered to, and did not fuse with, the sperm membrane. While performing a resonance energy transfer experiment, an increase in emission is only observed after addition of Triton X-100, a detergent that disassembles membranes.

Moreover, the binding of vesicles to spermatozoa was confirmed to be a reversible dynamic process. To show this, spermatozoa were incubated with red-fluorescent-labelled cerebroside vesicles for 1 h and then transferred to a bath of green-fluorescent-labelled cerebroside vesicles. The resultant spermatozoa displayed both fluorescent colours (Figure 2) confirming that vesicles were exchanged by an 'on' and 'off' binding process.

Three vesicle types (DOPG, cerebrosides and gangliosides) were also compared for their loading time, amount of vesicles bound and the possibility to reverse vesicle binding. In short, when given enough time, all vesicle types bound equally well. However, the cerebroside vesicles were the fastest binders (Supplementary Figure 1, available online only), showing high fluorescence, indicative of high vesicle binding, after just 30 min. Also, all vesicle types bound could be removed by a change in pH of  $\pm 1.0$  (relative to neutral), followed by a subsequent wash (data not shown).

# Spermatozoon—vesicle hybrid kinematic and functional attributes

To serve as a transport vesicle carrier, the spermatozoon should maintain motility after vesicle binding. To test the effects of vesicle binding on sperm kinematic attributes, computer-assisted semen analysis was used to assess percentage sperm motility, sperm velocity, cross-beat frequency and linearity. As shown in Figure 3, all three spermatozoon—vesicle hybrids (DOPG, cerebrosides, gangliosides) exhibited only minimal differences to the control sperm sample.

As differences in binding kinetics were the only distinguishing characteristic between the three vesicle systems, the fastest binding cerebroside vesicles were selected as the system of choice in all further experiments. The binding of vesicle-loaded versus control spermatozoa to HA-coated slides was compared and no measurable differences in the percentage of sperm binding to HA were found (data not shown). Thus, vesicle binding to spermatozoa did not affect HA binding.

To test whether vesicle binding affected acrosomal integrity, both vesicle-loaded and control spermatozoa were stained with a green-fluorescent lectin, FITC-PSA. Complete loss of the acrosomal gap resulted in a complete lack of fluorescence (Figure 4C), while intact acrosomes appeared bright (Figure 4A). A patchy pattern of fluorescence was expected for spermatozoa undergoing the capacitation process (Figure 4B). Both the control spermatozoa population and spermatozoon—vesicle complexes showed near equal occurrences of acrosomal loss (Figure 4D). Thus, vesicle binding did not affect the acrosomal membrane.

This study examined whether vesicles bound all spermatozoa equally or preferentially, based on the state of sperm development. To test this, spermatozoa were first allowed to bind to vesicles (monitored by red fluorescence) and then stained with either a cytoplasmic (CK) or a nuclear (aniline blue) marker specific to arrested sperm development



**Figure 2** Binding of vesicles to sperm cells is a dynamic process. Spermatozoa are incubated for 1 h with red fluorescent 50 mol% cerebroside vesicles, followed by 1 h with green fluorescent 50 mol% cerebroside vesicles. (A) Spermatozoa appear red, indicative of vesicle binding. (B) The same spermatozoa appear green fluorescent as well, indicating that red vesicles can be replaced by green vesicles (dynamic 'on' and 'off' binding). (C) Overlay of A and B. Bars =  $10 \,\mu m$ .



**Figure 3** Swimming characteristics of spermatozoa versus vesicle-loaded spermatozoa. The four main criteria (percentage of motile spermatozoa, track speed, beat frequency of the sperm tail and linearity of the swimming trajectory) show only marginal changes. Values are means, with error bars representing standard errors of the mean from six independent samples. LUV = large unilamellar vesicles.

(Sati et al., 2008). Using fluorescent CK immunocytochemistry, the sperm population could be divided into three categories (Supplementary Figure 2): normally developed (nonfluorescent), fully arrested (green fluorescent) and intermediate state. For each spermatozoon, this work determined the developmental state and whether vesicles were bound or not. Interestingly, as shown in Figure 5, vesicle binding did depend on the state of sperm development. Close to 100% of normally developed spermatozoa had vesicles bound to them, whereas >30% of arrested spermatozoa did not exhibit vesicle binding. In a second experiment, spermatozoa were classified by assigning the population into three developmental categories, according to their aniline blue-staining pattern (Supplementary Figure 3). This constituted corroborating evidence that vesicle binding depended on the state of sperm development.

# Transport of vesicles and delivery of cargo during fertilization

To study the transport of vesicles and the delivery of their encapsulated cargo, a mouse model system was used.

Similar to the binding experiments with human spermatozoa, mouse spermatozoa were mixed with cerebroside vesicles. After 30 min, the result was checked by confocal microscopy. Mouse spermatozoa displayed areas of red fluorescence, which were indicative of vesicle binding (Figure 6A).

To determine whether spermatozoon-vesicle complexes may indeed deliver vesicles or their cargo into an oocyte, a mouse IVF system was used. First, as a control, it was examined whether vesicles were capable of penetrating the oocyte without the presence of spermatozoa. Figure 6B clearly shows that without spermatozoa available to carry the vesicles through the zona pellucida, delivery was not possible. Even at a significantly higher vesicle concentration  $(1 \times 10^{10} \text{ vesicles/ml})$ , the vesicles remain either in solution or adhere to the zona pellucida, but fail to penetrate (Figure 6C).

Next, differences in oocyte fluorescence and fertilization after incubation with spermatozoa or vesicle-loaded spermatozoa for over 6 h were compared. The presence of red fluorescence was indicative of successful sperm penetration



**Figure 4** FITC-PSA staining of spermatozoa alone versus spermatozoon—vesicle complexes. (A–C) Acrosomal membrane status determined by lectin fluorescence in an intact membrane (A), an intermediate case (B), and a dissipated membrane (C). (D) Vesicle binding does not affect the acrosome, as the proportions of light, patchy and dark spermatozoa are similar to unbound spermatozoa. Values are means, with error bars representing standard errors of the mean from 15 independent samples. LUV = large unilamellar vesicles. Bars =  $5 \mu m$ .



**Figure 5** Spermatozoon–vesicle binding according to sperm developmental state. After 30-min incubation with vesicles, creatine kinase (CK) immunostaining or aniline blue (AB) staining was performed to categorize the spermatozoa into three developmental groups. Values are means, with error bars representing standard errors of the mean from seven independent samples.

and vesicle transport. For this first proof-of-principle experiment, vesicles without additional cargo were used (Figure 6F). Over the nine experiments performed, only marginal differences in the number of oocytes fertilized were found (fertility: control 70%; vesicle-loaded spermatozoa 74%). However, a measurable difference in fluorescence within the oocytes was seen (Figure 6D and E): Figure 6D shows an image of an oocyte fertilized with a control spermatozoon (no vesicles around), and the level of auto-fluorescence of such a cell was negligible; in contrast, as shown in Figure 6E, an oocyte fertilized with a vesicle-loaded spermatozoon resulted in a consequential display of bright fluorescent spots. As the fluorescence sometimes occurred close to the membrane, it was not clear if all spots were within the oocyte's inner membrane or trapped in between the zona pellucida and the inner membrane. Location of the vesicles was determined in experiments which included: (i) removal of the zona pellucida before imaging, but after fertilization; and (ii) addition of 10 mol% biotinylated lipid to the vesicles studied. Confocal analysis showed that, of the oocytes fertilized with vesicle-loaded spermatozoa, ~60% (31 oocytes, two independent samples) had their fluorescent patches inside the cell (data not shown). Spermatozoon—vesicle fertilized oocytes developed normally compared with controls up to the blastocyst stage (development was never continued after this stage).

The final step, to show the utility of spermatozoonvesicle complexes for transport into oocytes, was to load the vesicles with actual cargo (Figure 7). To show that not only the vesicles were transported, but also that the encapsulated cargo reached the oocytes, a GFP-mRNA construct was used. If transported, the mRNA gets translated into GFP, a fluorophore readily detected by confocal microscopy. The level of green fluorescence was measured in both fertilized and nonfertilized oocytes and compared with the level of fluorescence in control experiments (spermatozoon-vesicle hybrids with empty vesicles; Figure 7). Data was collected for >150 oocytes from nine independent experiments. To compare fluorescent levels, the value was normalized to the mean values of fertilized control oocytes. A measurable increase in green fluorescence was detected for oocytes fertilized with GFP-mRNA-containing spermatozoon-vesicle hybrids.

## Discussion

The present work studied the possibility of creating a hybrid transport system consisting of multiple vesicles bound to sperm cells (Figure 1A). To obtain a functional spermatozoon-vesicle hybrid, a few conditions have to be met. First, and most importantly, vesicles need to bind to the



**Figure 6** Vesicle binding to mouse spermatozoa and consequent IVF experiments. (A) Confocal image of vesicle binding to mouse spermatozoa; insert: binding pattern of vesicles on one spermatozoon. (B) Cerebroside vesicles  $(1^4/ml)$ , visualized by red fluorescence, added to oocytes remain outside the zona pellucidae. (C) Cerebroside vesicles, at a high concentration  $(1^{10}/ml)$ , also appear on the outside of the oocyte. (D) An oocyte fertilized with control spermatozoa (no vesicles) shows no fluorescence. (E) An oocyte fertilized with spermatozoa loaded with cerebroside vesicles shows bright fluorescent spots on and within the oocyte. All micrographs shown are z-projections of an overlay of a bright-field image and red laser data. (F) Cartoon of spermatozoa used in (E).



**Figure 7** Oocyte green fluorescence measured after fertilization with control spermatozoa (empty vesicles bound) and spermatozoa loaded with vesicles containing GFP-mRNA. Values are means, with error bars representing standard errors of the mean from nine independent samples.

spermatozoa. Second, the extra vesicle load should not interfere with the sperm cell's basic functions (e.g. the kinematic and functional sperm attributes in zona pellucida and oocyte interactions should remain unaffected). Third, spermatozoon—vesicle hybrids should be capable of delivery of both the vesicles and the encapsulated cargo during fertilization.

The key variable underlying spermatozoon—vesicle binding is the lipid composition of the man-made vesicle membrane. The most commonly used lipid vesicle is made with only one neutral lipid: POPC. Vesicles consisting purely of this noncharged lipid were unable to bind to sperm cells. Thus, to obtain spermatozoon—vesicle binding, the vesicle membrane had to be adapted. There are two routes that can be followed in exploring possible lipid mixtures that will result in spermatozoon—vesicle binding: replacing some of the POPC lipids with either charged lipids or specific uncharged lipids to obtain an adhering vesicle membrane.

Charged lipids are natural candidates, as sperm membranes consist of random negatively and positively charged patches (Cooper and Bedford, 1976; Yanagimachi et al., 2005). Although charged vesicles are likely to bind sperm cells, usage of such vesicles may lead to problems as charged vesicles may exhibit nonspecific binding to surfaces other than the sperm membrane. A more challenging route would be creating vesicles containing uncharged lipids exclusively. In the search for suitable uncharged lipids, inspiration was found from HA, which is known to strongly attract sperm binding (Huszar et al., 2003, 2007). Two lipids that closely resemble the chemical structure of HA were used to create strong sperm binding: cerebrosides and gangliosides.

To obtain vesicle sperm binding, three suitable vesicle types: negative (DOPG), ganglioside and cerebroside were found. Having a larger array of available vesicles will increase the chance that a suitable match can be found for various applications. In this work, only vesicles with a membrane composed of two main lipid components were selected. Judicious choices of vesicle membrane lipid compositions lead to strong physisorption of vesicles to the outer sperm membrane and hybrid transport systems consisting of spermatozoa and vesicles were created without the need to incorporate sophisticated attachment methodologies. Obviously in the future, additional lipids could be supplemented to create more complex vesicles. Also, while only noncovalent binding between vesicles and sperm cells have been discussed, it is of note that binding may be achievable with special ligands (e.g. biotin-streptavidin; Noppl-Simson and Needham, 1996), DNA linkers (Beales and Vanderlick, 2007) or even designated antibodies (Lewis et al., 1980).

As spermatozoa have a limited motility time span, the duration of the binding procedure is an important condition, with quick loading times desired for most potential applications. The three vesicle types tested all loaded quickly and efficiently, resulting in sperm-mediated motile hybrid complexes. As it was aspired to develop a spermatozoon–vesicle hybrid system that allows sperm cells to carry a large load of vesicles, the amount of vesicles bound is also of importance. The steady rise in fluorescent intensity over time for spermatozoa incubated with a vesicle suspension indicated that sperm cells were loaded with increasing number of vesicles (Supplementary Figure 1).

The binding of vesicles to spermatozoa is not irreversible; indeed, it was confirmed that membrane fusion does not occur. Instead, a dynamic process ensued, with a binding preference for the head and midpiece areas of the spermatozoon. Given the noncovalent connection between vesicles and spermatozoa, it was examined if pH changes could serve as a trigger for dissociation. Three vesicle types were tested: negative (DOPG), ganglioside and cerebroside. A small change in pH did indeed cause complete vesicle dissociation for all three vesicle types. Simple electrostatic changes to the vesicle membrane cannot explain this behaviour, as the pH change was too small to alter the charge of either the zwitterionic POPC backbone or the other lipid types present. Although there is no theoretical explanation for this behaviour, the result might nevertheless have great practical utility in applications that require controlled shedding of vesicles from spermatozoa.

In the spermatozoon-vesicle hybrids, spermatozoa conserved unaffected motility attributes, the ability to bind HA and acrosomal integrity. These findings suggest that the spermatozoon-vesicle complex could be optimal carriers of vesicles, and its encapsulated cargo, into the oocyte. First, the spermatozoa were not affected by the extra loading. Secondly, HA binding by human spermatozoa indicated cellular maturity, viability and an unreacted acrosomal status. Moreover, HA-coated slides are often used as surrogates for zona pellucida binding, indicating that HA-binding spermatozoa should be capable of fertilization (Huszar et al., 2007). Thirdly, the acrosome and the acrosome reaction is an important marker of human sperm function, as a sperm cell can no longer penetrate the zona pellucida once this membrane is dissipated. The results described above are based on populations of spermatozoa examined collectively. Vesicle-binding properties could also be selectively examined in individual spermatozoa, in line with the idea that human semen is comprised of a heterogeneous cell population with different degrees of development, varying in functional integrity and fertilizing potential (Huszar et al., 2003, 2007). It was shown that vesicles tended to have a preference to binding to those spermatozoa that had completed cytoplasmic extrusion and plasma membrane remodelling (i.e. fully developed sperm cells in the pool of fertilizing spermatozoa).

Utilizing spermatozoa to transport cargo into the oocyte is a perfect example of natural noninvasive drug administration. This is not the first study that has investigated the sperm cell for transport. The pioneer work of Brackett et al. (1971) resulted in the first report of spermatozoa carrying cargo into the oocyte during the process of fertilization. Here, the researchers made use of the capacity of sperm cells to spontaneously take up exogenous DNA. After a silence of 20 years, the important implications of this finding were rediscovered. Lavitrano et al. (1989) suggested using this finding as a new method to obtain transgenic mice. While the interaction between foreign DNA and the spermatozoa of a variety of species are undisputed (Atkinson et al., 1991; Castro et al., 1990. Horan et al., 1991), the actual gene transfer initially received negative reports (Al-Shawi et al., 1990; Brinster et al., 1989). Nevertheless, others have also reported successful sperm gene transfer experiments (Arezzo, 1989; Shemesh et al., 2000; Wang et al., 2003) and now this technology is considered reproducible.

The major improvement of the system described here, compared with the pioneer work described above, is that the transport is now no longer limited to DNA. This used to be a constraint, as sperm cells are not known to take up a wide variety of therapeutic molecules. In contrast, the spermatozoon—vesicle hybrids of this study can carry and transport virtually any hydrophilic molecule added to the interior of the vesicle, and even hydrophobic moieties in the phosphate bilayer surrounding it. Moreover, vesicles containing different cargo could be bound to spermatozoa simultaneously.

The transport of vesicles and their cargo was proven with two functional tests. First the presence of fluorescence, originating from the lipids of the vesicle membrane, was used to determine that transport of the vesicles into the oocyte was feasible. Second, the vesicles were filled with a GFP-mRNA construct (cargo) to confirm that transport and delivery of the cargo within the vesicles was accomplished. In future experiments, this work will be extended upon by examining the transport of nucleic acids, antibodies and/or proteins, the main candidates for vesicle cargo.

In summary, this work created a new hybrid delivery system in which large unilamellar vesicles were physisorbed to spermatozoa in physiological conditions, with no significant alterations in sperm motility and functional attributes. This system was tested for its ability to transport vesicles during IVF and deliver cargo within the oocyte after fertilization. With the mouse model system, it was confirmed that the hybrids were indeed capable of both transport as well as intraoocyte delivery of the encapsulated cargo. The positive results indicate the viability of the spermatozoon–vesicle hybrids. With transport cargo options limitless, future experiments should confirm the extent of the usability of this exciting finding.

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# Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10. 1016/j.rbmo.2013.11.009.

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