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A carbohydrate-antioxidant hybrid polymer reduces oxidative damage in spermatozoa and enhances fertility

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Gamete-gamete interactions are critically modulated by carbohydrate-protein interactions that rely on the carbohydrate-selective recognition of polyvalent carbohydrate structures[1,2]. A galactose-binding protein has been identified in mammalian spermatozoa[3] that has similarity to the well-characterized hepatic asialoglycoprotein receptor[4]. With the aim of exploiting the ability of this class of proteins to bind and internalize macromolecules displaying galactose, we designed hybrid carbohydrate-antioxidant polymers to deliver antioxidant vitamin E (α-tocopherol) to porcine spermatozoa. Treatment of sperm cells with one hybrid polymer in particular produced large increases in intracellular sperm levels of α-tocopherol and greatly reduced endogenous fatty acid degradation under oxidative stress. The polymer-treated spermatozoa had enhanced physiological properties and longer half-lives, which resulted in enhanced fertilization rates. Our results indicate that hybrid polymer delivery systems can prolong the functional viability of mammalian spermatozoa and improve fertility rates, and that our functionally guided optimization strategy can be applied to the discovery of active glycoconjugate ligands.

The critical role of long-chain, polyunsaturated fatty acids and lipids in animal cells is well known[5]. Among these, α-tocopherol as a defense against oxidative damage is primary. However, controlled delivery of hydrophobic metabolites such as α-tocopherol is difficult[6], especially where predominantly aqueous environments restrict solubility and transport.

Spermatozoa represent a challenging cell type that, for a critical period of their function (for example, during artificial insemination), may be uniquely vulnerable to oxidation[7]. Pig spermatozoa represent a stringent test because of their combination of aqueous environment, large volume and high lipid levels. The latter are rich (60%) in two primary targets of oxidation, docosapentaenoic (22:5n-6) and docosahexaenoic (22:6n-3) acids (**Supplementary Methods** online; ref. 8). In the field, this risk is increased by high seminal volume that precludes additional protection such as freezing.

Highly specific carbohydrate-binding proteins are found on spermatozoa[9–11]. The identification[3] of a galactose (Gal)-binding protein (GBP) in mammalian spermatozoa with similarity to the well-characterized hepatic asialoglycoprotein receptor (ASGPR) suggested endocytotic potential. ASGPR allows hepatocytes to internalize Gal-bearing macromolecules through receptor-mediated endocytosis (RME)[4]. This has been successfully exploited for liver-targeted delivery of Gal-bearing macromolecules[12], including elegant polymeric systems[13]. However, cell-type generality is largely unexplored[12]. We hoped to characterize and exploit the GBP by the discovery of appropriate carbohydrate glycoconjugate ligands.

The identification of appropriate ligands for carbohydrate-binding proteins is an intriguing challenge[14–16]. Limited access to complex sugars and oligosaccharides has resulted in simple monosaccharides typically being cited as molecular determinants, for example, Gal-binding protein. Yet, given the higher complexity of glycosylated interfaces (cell surfaces decorated with multiple glycolipids or glycoproteins), monovalent ligands are unlikely to be optimal. The 'cluster glycoside effect'[17] indicates that greater binding can be achieved with multivalent carbohydrate ligands. Some impressive binding increases have been observed for carbohydrate polymers[14,15,18–20]. However, binding may not correlate with enhanced endocytosis[21]. We show here that optimization guided by relevant intracellular parameters and phenotypic markers allows direct identification of appropriate glycoconjugates.

We constructed a representative array of terpolymers containing three key structural components (**Supplementary Methods** online and **Fig. 1**): antioxidant α-tocopherol, linked to polymer by an enzyme-labile[9] aryl ester linkage (component **A**); sperm-targeting group Gal, as a ligand of GBP (component **B**); and a solubility-enhancing group, hydroxyethyl or dimethylaminoethyl (DMAE; component **C**). Such multicomponent polymers are an embodiment of a prior prescient concept[22]. Screening of polymers varying in permutation and composition (**Supplementary Table 1**) for optimal aqueous solubility and functional efficacy toward spermatozoa identified two for further evaluation, with compositions of α-tocopherol/Gal/DMAE components (w/w) of 20:20:60 (**1**) and 30:20:50 (**2**), respectively.

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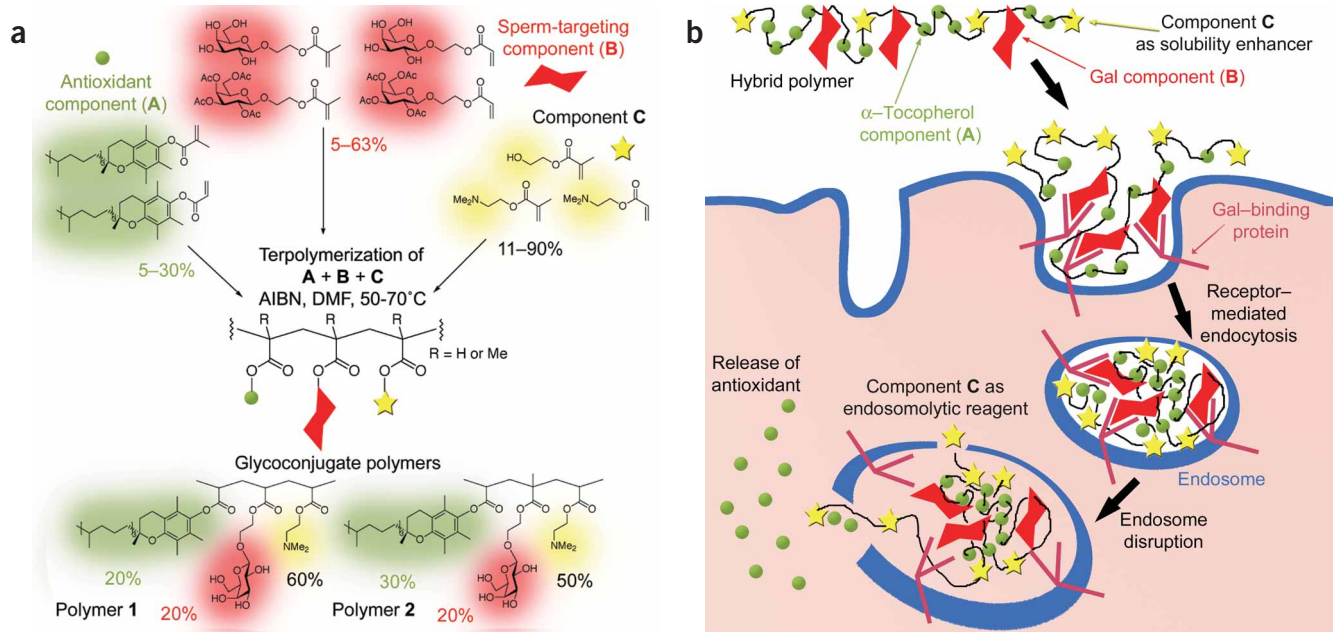

Figure 1 Design of glycoconjugate polymer ligands for endocytosis. (**a**) The design and construction of an array of hybrid carbohydrate-antioxidant polymer systems containing three components: antioxidant (**A**), sperm-homing device (**B**) and solubility enhancer and putative endosomolytic agent (**C**). Screening for function and efficacy identified polymers **1** and **2**. (**b**) The successful synergistic requirement for these three components **A**, **B**, and **C** in polymers **1** and **2**. the high levels of α-tocopherol delivery and apparent polymer internalization suggest a putative mechanism of action involving receptor-mediated endocytosis and subsequent release of key antioxidant α-tocopherol from the polymer within endosomal compartments.

Precise construction of **1** and **2** necessitated development of new carbohydrate-monomer protection strategies[23] and high monomer purity. Scavenger α-tocopherol was rigorously excluded from the radical polymerization conditions used. Through such terpolymerization strategies, simple variation of monomer feedstock allowed ready fine-tuning of composition and properties in the polymer array, greatly aiding rapid identification of suitable glycoconjugate ligands. The fact that only the use of each of the components Gal, α-tocopherol and DMAE gave polymers capable of enhanced α-tocopherol delivery in aqueous media highlights their synergism. Consistent with the role of GBP in the internalizing mechanism, the use of protected carbohydrate ligand 2,3,4,6-tetra-*O*-acetyl galactose ($GalAc_4$), in which critical hydroxyl GBP recognition groups are inaccessible, resulted in a polymer with negligible α-tocopherol delivery. Finally, to allow visualization of uptake, a fluorescently labeled polymer (**3**) was prepared by replacing the α-tocopherol component with the benzothioxanthene fluorophore, hostasol yellow, and revealed striking intracellular localization (**Fig. 2**). Comparison with control fluorescent polymer containing no carbohydrate revealed that localization is dependent on the presence of the Gal (**Fig. 2b**).

Taken together, these component properties suggest a possible mechanism (**Fig. 1b**) and the ready discovery of a glycoconjugate ligand that exploits it. Indeed, prior examples of putative RME have been noted in human sperm[24]. Nonetheless, it cannot be discounted that the higher delivery levels of α-tocopherol to sperm cells may also result from enhanced surface binding[25] to GBP or other Gal-binding surface proteins[11] followed by release of α-tocopherol before membrane penetration. Regardless, treatment with **1** and **2** resulted in dramatically higher α-tocopherol delivery to sperm than no treatment or incubation with α-tocopherol alone[8].

We evaluated **1** and **2** (**Supplementary Methods** online) for α-tocopherol delivery and subsequent intracellular action (**Fig. 3**). The ability of **1** and **2** to deliver and release α-tocopherol within sperm cells was markedly dependent on the polymer dose (**Fig. 3a**) and exposure time (**Fig. 3b**). **1** and especially **2** supplied α-tocopherol to spermatozoa over a period of time commensurate with and beyond normal commercial boar semen storage (5 d). Indeed, treatment with **2** for 8 d even at a low dose of 0.02 mg ml^{-1} showed the very high levels (>830% higher than control) to which cellular α-tocopherol could be raised. There was a significant difference in the level of uptake of α-tocopherol after exposure to **2** (30% w/w α-tocopherol) as compared with exposure to **1** (20% w/w). The effect was dose dependent, with higher polymer doses resulting in even higher levels of α-tocopherol uptake (>20-fold higher for 0.4 mg ml^{-1} of **1**, **Fig. 3a**). Dose-response analysis showed the optimal doses of **2** to be 0.01–0.02 mg ml^{-1}.

We assessed the benefit of these strongly enhanced levels of α-tocopherol by examining the ability of sperm to withstand oxidative stress (**Fig. 3c**; ref. 26). **2** afforded excellent protection against cellular oxidation and accumulation of oxidized metabolites: incubation for 2 h at 37 °C at 0.1 mg ml^{-1} significantly decreased evolution of malondialdehyde (MDA), a specific indicator of fatty acid oxidation. We observed even greater protection after 3 or 8 d under standard commercial storage conditions at 18 °C, with **2** reducing oxidation to <10% of control under these extremes.

Protection from oxidation had significant beneficial effects on three major practical parameters of spermatozoa: viability, overall motility and relative motility (**Fig. 3d**, **Supplementary Methods** online and **Supplementary Videos 1** and **2**). Thus, over an 8-d storage period (under standard commercial conditions), viabilities of treated and untreated sperm deviated significantly after 5 d: untreated sperm viability declined rapidly, whereas sperm treated with **2** retained >85% of initial viability, which lasted even after 8 d. Standard commercial practice precludes the use of samples beyond this time period, because of associated neomycin-resistant growth. Nonlinear

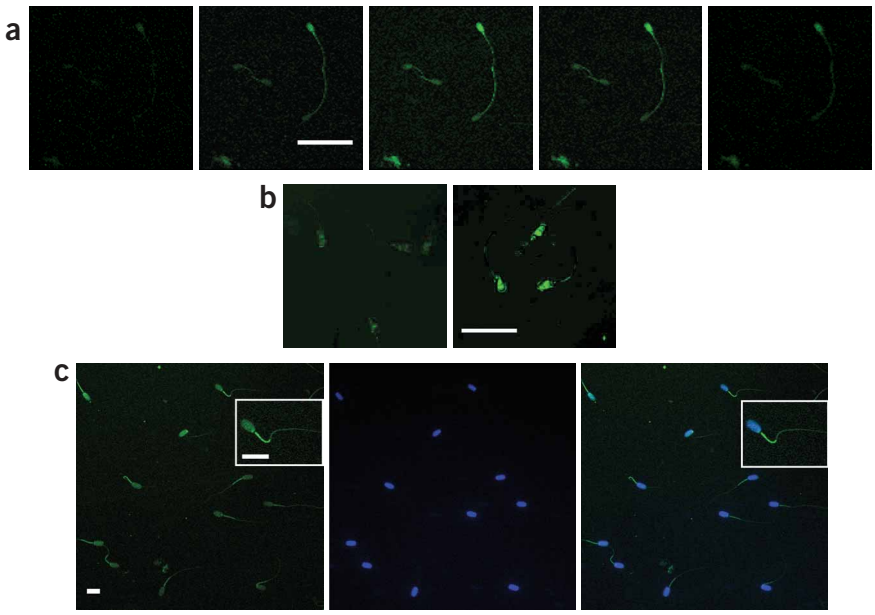

Figure 2 Fluorescence microscopy of spermatozoa. (**a**) Laser scanning confocal microscopy of the uptake of fluorescent polymer **3** into spermatozoa treated at 0.02 mg ml^{-1}. Each frame shows successive cross-sectional depth slices starting from above the spermatozoa and passing through (0.8 μm *Z* slice) and then below. Increasing fluorescence intensity at internal cross sections shows localization of polymer throughout spermatozoa. White bar shows 50 μm. (**b**) Comparative uptake into spermatozoa of Gal-targeted fluorescent polymer **3** (right) as compared with a control fluorescent polymer that does not contain Gal (left) (both at 0.02 mg ml^{-1}). White bar shows 50 μm. (**c**) Two-color comparative localization of fluorescent polymer **3** (left) and the dsDNA minor groove–binder fluorescent small-molecule probe 4′,6-diamidino-2-phenylindole (DAPI only, middle and overlay right) showing (i) broader localization for **3** as compared to DAPI and (ii) higher concentrations of **3** in the mid-piece and rear of the acrosomal cap than in the tail or head (insets show enlargement). White bars show 25 μm.

regression ($R^2 > 0.975$) revealed that the rate of decline at day 8 after treatment with **2** was only 37% that of untreated (4.1% versus 11.2% per day), and extrapolation suggests that **2** extends predicted half-life by ~3 d to 12.2 d. We also observed parallel enhancements of overall motility (**Supplementary Methods** online). Although after 8 d the overall percentage of motility is low even in the treated (+**2**) sample (9.8 ± 0.6%), this is some five-fold higher than that of d control (1.8 ± 0.5%). Furthermore, treated samples contained spermatozoa that remarkably retained individual motilities as high as their initial levels; the control did not (**Supplementary Methods** online and **Supplementary Videos 1** and **2**). No significant correlation was observed between treatments and other underlying parameters, such as sperm cell performance parameters.

Finally, *in vitro* fertilization (IVF) trials showed higher motilities, reduced agglutination and a 20% average fertilization rate ($n = 3$, 25–80 oocytes) in spermatozoa samples treated with **2**, as compared with only a 12.5% fertilization rate for untreated samples, representing a 60% increase in fertility. Chromatin structure assay, based on flow cytometry of the metachromic dye acridine orange[27], showed that DNA integrity was unaltered by treatment, and no differences were observed in embryo quality throughout development. In treated (+**2**) and untreated groups, cells were tightly packed together at compaction, and blastocysts demonstrated a clear blastocoel with a distinct trophectoderm. Moreover, in subsequent field trials ($n = 5$) with Large White × Landrace sows, the maintenance of embryo quality was further supported by undiminished

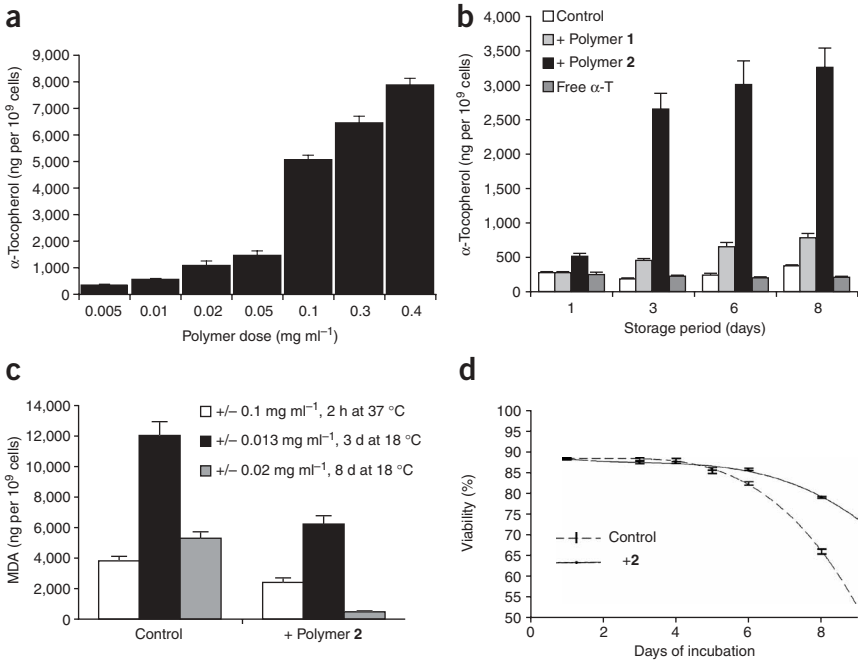

Figure 3 Enhancement of sperm parameters upon treatment. (**a**) Cell-associated α-tocopherol levels in spermatozoa following *in vitro* incubation with differing concentrations of the hybrid carbohydrate-antioxidant polymer **1** for 8 d at 18 °C in commercial standard BTS diluent. (**b**) Cell-associated α-tocopherol levels in spermatozoa during commercial storage at 18 °C in BTS diluent over an 8-d period in the presence of 0.02 mg ml^{-1} of hybrid carbohydrate-antioxidant polymers **1**,**2**. (**c**) Cell-associated MDA levels in spermatozoa as a specific marker of lipid peroxidation, as determined by TBARS analysis[26], following incubation in BTS with polymer **2** for the times (2 h, 3 d, 8 d) and at the doses (0.01–0.1 mg ml^{-1}) shown. (**d**) Percentage of viable spermatozoa over an 8-d storage period at 18 °C in BTS diluent +/– polymer **2** (0.013 mg ml^{-1}). Trend lines fitted with third-order polynomial nonlinear regression analysis ($R^2 > 0.975$) were extrapolated to spermatozoa half-lives with and without protection by **2** of 12.2 and 9.3 d, respectively. All assays were conducted on 3–5 replicates and statistical analyses applied.

average fruition level (average litter 13.5 for control versus 13.8 for treated).

In conclusion, radical terpolymerization with modulation of monomer feedstock created and fine-tuned complex glycoconjugate ligands that, in this case, allowed the discovery of multicomponent, hybrid carbohydrate-antioxidant polymers. These act as targeted spermatozoa cellular delivery systems by attachment of α-tocopherol through a covalent but labile linker and by virtue of Gal 'glycotargeting'[12] components; a third amine component ensured solubility and may enhance endosomal disruption to aid intracellular trafficking of α-tocopherol. The details of uptake mechanism are currently being elucidated. Nonetheless, this glycoconjugate prodrug polymer system is unique in its ability to transfer the essential, protective lipid metabolite α-tocopherol (enhanced up to 20-fold) to pig spermatozoa; α-tocopherol alone results in no significant uptake[8]. Reductions in intracellular α-tocopherol of as little as 32% cause significant oxidative damage to sperm DNA[28], and even slight changes in viability can influence fertility dramatically[29]. Pig semen is a particularly challenging model (highly unsaturated, oxidation-sensitive lipid; aqueous seminal fluid; routine ambient storage). Our results therefore would seem to have clear positive implications and practical applications for sperm function and artificial insemination not only in pigs but in other mammals. Moreover, the only modest enhancement in binding to other Gal-binding proteins[30] shown by the polymers identified here strongly indicates that in the design of carbohydrate ligands, *in vitro* parameters such as binding may not necessarily correlate with *in vivo* efficacy; functionally guided screens allow direct access to relevant glycoconjugate ligands.

METHODS

Polymer synthesis. All syntheses were carried out with standard laboratory glassware (with the exception of the array polymerization). Syntheses were carried out under inert atmospheric conditions (N_2) with dry solvents (dichloromethane was distilled from calcium hydride; methanol and DMF were purchased from the Aldrich Chemical Company).

Free-radical terpolymerizations were conducted in 20 permutations containing one from each of the following three acrylate (A) or methacrylate (MA) monomer component classes: (**A**) antioxidant monomers (5–30% w/w): α-tocopheryl acrylate (α-tocopheryl-A); α-tocopheryl methacrylate (α-tocopheryl-MA); (**B**) sperm-targeting monomers (5–63% w/w): (2-*O*-methacryloyl)ethyl β-D-galactoside (Gal-MA); (2-*O*-methacryloyl)ethyl 2,3,4,6-*O*-tetraacetyl-β-D-galactoside ($GalAc_4$-MA); (2-*O*-acryloyl)ethyl β-D-galactoside (Gal-A); (2-*O*-acryloyl)ethyl 2,3,4,6-*O*-tetraacetyl-β-D-galactoside ($GalAc_4$-A); and (**C**) solubility-enhancing monomers (11–90%): 2-hydroxyethyl methacrylate (HEMA); 2-(dimethylamino)ethyl methacrylate (DMAEMA); 2-(dimethylamino)ethyl acrylate (DMAEA). For all chosen compositions see **Supplementary Table 1**. Representative protocol example: A solution of α-tocopheryl-A (component **A**; 150 mg, 0.309 mmol), Gal-A (component **B**; 150 mg, 0.539 mmol), DMAEA (component **C**; 450 mg, 3.14 mmol) and AIBN (polymerization initiator; 11.3 mg, 1.5 wt% with respect to monomers) in anhydrous DMF (2.2 ml) was degassed and flushed with nitrogen and then heated at 50 °C under an atmosphere of nitrogen with continuous stirring for 24 h. Subsequently the solvents were removed under vacuum and the polymer product was purified by precipitation from acetone into petroleum ether, collected by filtration and dried *in vacuo*. The polymer was then purified by dialysis (with a dispodialyzer (SpectraPor), 1,000 MWCO) and was lyophilized to yield polymer **1** (647 mg, 86%). Polymers were characterized by optical rotation, IR, NMR, GPC, combustion analysis and AFM.

Semen evaluation. All ejaculates were obtained from standard commercial breeding boars housed according to UK/EU standards for artificial insemination centers and approved by the Department for Environment, Food and Rural Affairs. The boars were between 9 and 14 months of age and of high reproductive capacities. Diets fed were normal for breeding boars. Semen samples were obtained and processed post ejaculation and before *in vitro* experimentation according to standard commercial procedures. Spermatozoa quality parameters, including concentration, motility (digital microscopy motion analysis), viability (fluorometric ethidium bromide exclusion) and chemical determinations (lipids and fatty acids, cellular metabolites, oxidation products, and others), were quantified by established analytical and commercial techniques as appropriate. Specifically, cell-associated α-tocopherol levels were determined by HPLC analysis following homogenization subsequent to incubation +/− polymer in commercial standard Beltsville Thawing Solution (BTS). Oxidative stability of semen (7 ml, concentration: 4–5 × 10^7 cells per ml) incubated in BTS +/− polymer was conducted on washed sperm pellets and induced by $FeSO_4$ (0.8 mM, 37 °C, 1 h). MDA levels were determined spectrophotometrically with thiobarbituric acid reactive substance analysis (TBARS)[26]. Extrapolations of viability versus time to determine half-life used third-order polynomial nonlinear regression analysis ($R^2 = 0.974$ for polymer-treated, 0.998 for control). All assays were conducted on 3–5 replicates and statistical analyses applied.

Supporting materials. Further details of syntheses of monomers, multicomponent polymers, semen evaluation and IVF are provided in **Supplementary Table 1**, **Supplementary Scheme 1** (monomer synthesis), **Supplementary Scheme 2** (polymer synthesis), **Supplementary Methods** (polymer and monomer synthesis; semen evaluation and IVF) and **Supplementary Videos 1** and **2** (.mov files of treated and untreated sperm at day 3).

Note: Supplementary information is available on the Nature Chemical Biology website.

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COMPETING INTERESTS STATEMENT

A.M., P.P. and R.C.N. are/have been employees of JSR Healthbred who may have longer-term goals in exploiting this technology. An initial patent application has now lapsed.

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1. Millette, C.F. Distribution and mobility of lectin binding sites on mammalian spermatozoa. *Clin. Exp. Immunoreprod.* **4**, 51–71 (1977).
2. Benoff, S. Carbohydrates and fertilization: an overview. *Mol. Hum. Reprod.* **3**, 599–637 (1997).
3. Abdullah, M., Widgren, E.E. & O'Rand, M.G. A mammalian sperm lectin related to rat hepatocyte lectin. *Mol. Cell. Biochem.* **103**, 155–161 (1991).
4. Ashwell, G. & Morell, A.G. The role of surface carbohydrates in the hepatic recognition and transport of circulating glycoproteins. *Adv. Enzymol.* **41**, 99–128 (1974).
5. British Nutrition Foundation, ed. *Unsaturated Fatty Acids. Nutritional and Physiological Siginificance. The Report of the British Nutrition Foundation's Task Force.* (Chapman and Hall, London, 1992).
6. Cohn, W. *et al.* Tocopherol transport and absorption. *Proc. Nutr. Soc.* **51**, 179–188 (1992).
7. Aitken, R.J. & Baker, M.A. Reactive oxygen species generation by human spermatazoa: a continuing enigma. *Int. J. Androl.* **25**, 191–194 (2002).
8. Cerolini, S., Maldjian, A., Surai, P. & Noble, R.C. Viability, susceptibility to peroxidation and fatty acid composition of boar semen during liquid storage. *Anim. Reprod. Sci.* **58**, 99–111 (2000).
9. Tulsiani, D.R. & Abou-Haila, A. Mammalian sperm molecules that are potentially important in interaction with female genital tract and egg vestments. *Zygote* **9**, 51–69 (2001).
10. Topfer-Petersen, E. Carbohydrate-based interactions on the route of a spermatozoon to fertilization. *Hum. Reprod. Update* **5**, 314–329 (1999).
11. Nixon, B. *et al.* Galactosyltransferase function during mammalian fertilization. *Cells Tissues Organs* **168**, 46–57 (2001).
12. Davis, B.G. & Robinson, M.A. Drug delivery systems based on sugar-macromolecule conjugates. *Curr. Opin. Drug Discov. Devel.* **5**, 279–288 (2002).
13. Duncan, R., Kopecek, J., Rejmanova, P. & Lloyd, J.B. Targeting of *N*-(2-hydroxypropyl)methacrylamide copolymers to liver by incorporation of galactose residues. *Biochim. Biophys. Acta* **755**, 518–521 (1983).
14. Mammen, M., Chio, S.-K. & Whitesides, G.M. Polyvalent interactions in biological systems: implications for design and use of multivalent ligands and inhibitors. *Angew. Chem. Int. Edn. Engl.* **37**, 2755–2794 (1998).

15. Ambrosi, M., Cameron, N.R. & Davis, B.G. Lectins: tools for the molecular understanding of the glycocode. *Org. Biomol. Chem.* **3**, 1593–1608 (2005).
16. Gabius, H.-J., Siebert, H.-C., Andre, S., Jimenez-Barbero, J. & Ruediger, H. Chemical biology of the sugar code. *ChemBioChem* **5**, 740–764 (2004).
17. Lee, Y.C. *et al.* Binding of synthetic oligosaccharides to the hepatic Gal GalNac lectin - dependence on fine-structural features. *J. Biol. Chem.* **258**, 199–202 (1983).
18. Choi, S.-K., Mammen, M. & Whitesides, G.M. Generation and *in situ* evaluation of libraries of poly(acrylic acid) presenting sialosides as side chains as polyvalent inhibitors of influenza-mediated hemagglutination. *J. Am. Chem. Soc.* **119**, 4103–4111 (1997).
19. Arranz-Plaza, E., Tracy, A.S., Siriwardena, A., Pierce, J.M. & Boons, G-J. High-avidity, low-affinity multivalent interactions and the block to polyspermy in *Xenopus laevis*. *J. Am. Chem. Soc.* **124**, 13035–13046 (2002).
20. Ladmiral, V., Melia, E. & Haddleton, D.M. Synthetic glycopolymers: an overview. *Eur. Polym. J.* **40**, 431–449 (2004).
21. Rihova, B. Receptor-mediated targeted drug or toxin delivery. *Adv. Drug Deliv. Rev.* **29**, 273–289 (1998).
22. Ringsdorf, H. Structure and properties of pharmacologically active polymers. *J. Polym. Sci. Polym. Symp.* **51**, 135–153 (1975).
23. Ambrosi, M. *et al.* Influence of preparation procedure on polymer composition: synthesis and characterisation of polymethacrylates bearing β-D-glucopyranoside and β-D-galactopyranoside residues. *J. Chem. Soc. Perkin Trans.* **I**, 45–52 (2002).
24. Hoyes, K., Nettleton, J., Lawson, R. & Morris, I. Transferrin-dependent uptake and dosimetry of Auger-emitting diagnostic radionuclides in human spermatozoa. *J. Nucl. Med.* **39**, 895–899 (1998).
25. Morales, C.R. Role of sialic acid in the endocytosis of prosaposin by the conciliated cells of the rat efferent ducts. *Mol. Reprod. Dev.* **51**, 156–166 (1998).
26. Ohkawa, H., Nobuko, O. & Yagi, K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.* **95**, 351–358 (1979).
27. Evenson, D., Larson, K. & Jost, L. Sperm chromatin structure assay: its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques. *J. Androl.* **23**, 25–43 (2002).
28. Fraga, C.G., Motchnik, P.A., Wyrobek, A.J., Rempel, D.M. & Ames, B.N. Smoking and low antioxidant levels increase oxidative damage to sperm DNA. *Mutat. Res.* **351**, 199–203 (1996).
29. Perez-Pe, R. *et al.* Prediction of fertility by centrifugal countercurrent distribution (CCCD) analysis: correlation between viability and heterogeneity of ram semen and field fertility. *Reproduction* **123**, 869–875 (2002).
30. Ambrosi, M., Cameron, N.R., Davis, B.G. & Stolnik, S. Investigation of the interaction between peanut agglutinin and synthetic glycopolymeric multivalent ligands. *Org. Biomol. Chem.* **3**, 1476–1480 (2005).