Hexavalent sperm-binding IgG antibody released from vaginal film for development of potent on-demand non-hormonal female contraception

Bhawana Shrestha[1], Kathleen Vincent[2], Alison Schaefer[3], Yong Zhu[4], Gracie Vargas[5], Massoud Motamedi[4], Kelsi Swope[6], Josh Morton[6], Carrie Simpson[6], Henry Pham[7], Miles B. Brennan[7], Michael H. Pauly[7], Larry Zeitlin[7,8], Barry Bratcher[6], Kevin J. Whaley[7,8], Thomas R. Moench[8,9], Samuel K. Lai[1,3,9,10*]

[1] Department of Microbiology & Immunology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA.
[2] Department of Obstetrics and Gynecology, University of Texas Medical Branch, Galveston, TX 77555, USA.
[3] UNC/NCSU Joint Department of Biomedical Engineering, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA.
[4] Department of Ophthalmology, University of Texas Medical Branch, Galveston, TX 77555, USA.
[5] Department of Neuroscience and Cell Biology, University of Texas Medical Branch, Galveston, TX 77555, USA.
[6] Kentucky BioProcessing, Inc., Owensboro, KY 42301, USA.
[7] ZabBio, Inc., San Diego, CA 92121, USA.
[8] Mapp Biopharmaceutical, Inc. San Diego, CA 92121, USA.
[9] Mucommune, LLC., Durham, NC 27709, USA.
[10] Division of Pharmacoengineering and Molecular Pharmaceutics, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA.

[*]Samuel K. Lai
University of North Carolina at Chapel Hill
Marsico 4213, 125 Mason Farm Road
Chapel Hill, NC 27599
Phone: (919) 966-3024
Email: lai@unc.edu

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Abstract
Non-hormonal products for on-demand contraception are a global health technology gap; this unmet need motivated us to pursue the use of sperm-binding monoclonal antibodies to enable effective on-demand contraception. Here, using the cGMP-compliant *Nicotiana*-expression system, we produced an ultra-potent sperm-binding IgG antibody possessing 6 Fab arms per molecule that bind a well-established contraceptive antigen target, CD52g. We term this hexavalent antibody "**F**ab-**I**gG-**F**ab" (FIF). The *Nicotiana*-produced FIF had at least 10-fold greater sperm agglutination potency and kinetics than the parent IgG, while preserving Fc-mediated trapping of individual spermatozoa in mucus. We formulated the *Nicotiana*-produced FIF into a polyvinyl alcohol-based water-soluble contraceptive film, and evaluated its potency in reducing progressively motile sperm in the sheep vagina. Two minutes after vaginal instillation of human semen, no progressively motile sperm were recovered from the vaginas of sheep receiving FIF-Film. Our work supports the potential of multivalent contraceptive antibodies to provide safe, effective, on-demand non-hormonal contraception.

Significance Statement
Nearly half of all pregnancies in the U.S. are unintended, due to millions of women avoiding available hormonal contraceptive methods as a result of real and/or perceived side-effects associated with the use of exogenous hormones. Topical vaginal delivery of anti-sperm monoclonal antibodies that could agglutinate sperm into clusters too large to penetrate mucus and prevent sperm from reaching the egg represents a potentially safe and potent mechanism for non-hormonal contraception. We report here the engineering of a vaginal film loaded with hexavalent (i.e., 6 Fab) anti-sperm IgG, made using GMP manufacturing processes, that possess significantly superior agglutination potency than the parent IgG, enabling potent on-demand non-hormonal contraception via effectively agglutinating all human sperm within minutes.

Main Text

Introduction
Despite the availability of potent and low-cost, long-acting, reversible contraceptives (LARCs), many women continue to use on-demand contraceptives due to infrequent sexual activity. In addition, many women strongly prefer non-hormonal contraceptives because of the real and/or perceived side-effects associated with existing hormonal methods(1–3). Indeed, the FDA-approved Vaginal Contraceptive Film (VCF) meets the contraceptive needs of many women as it provides a contraceptive method that is women-controlled, inexpensive, non-hormonal, discrete, and readily available over the counter. Unfortunately, VCF and most other spermicides use nonoxynol-9 (N9) as an active ingredient. N9 can damage the mucosal surfaces by disrupting the vulvar, vaginal, and cervical epithelium, and substantially increases the risks of sexually transmitted infections(4–6). We believe there is a substantial unmet need for alternatives that can offer effective on-demand contraception, and are free of exogenous hormones or detergents.

Anti-sperm antibodies (ASA) to surface antigens on sperm(7) represent a promising class of molecules that could enable safe, on-demand, non-hormonal contraception. ASAs found in the vaginal secretions of some immune infertile women could prevent fertilization by stopping sperm from reaching the egg via two distinct mechanisms(8). First, ASAs can agglutinate multiple motile sperm into clumps that stop forward progression(9, 10). This mechanism is most effective at high sperm concentrations, and is more potent with polyvalent antibodies (Abs) such as IgM. Second, ASAs can trap individual spermatozoa in mucus by forming multiple low-affinity Fc-mucin bonds between sperm-bound ASA and mucin fibers(11), resulting in individual sperm that simply shake in place, unable to assume progressive motility needed to reach the upper reproductive tract. Over time, sperm that are agglutinated or immobilized in mucus either die or are eliminated from the female reproductive tract by natural mucus clearance mechanisms.

Years ago, the discovery of the contraceptive potential of ASAs motivated the development of contraceptive vaccines. ASAs elicited by vaccination with sperm antigens offered considerable contraceptive efficacy, but this approach stalled due to unresolved variability in the intensity and duration of the vaccine responses in humans, as well as concerns that active vaccination might lead to irreversible infertility(12–14). In contrast, topical delivery of pharmacologically active doses of ASA in the vagina can overcome many of the key drawbacks of contraceptive vaccines by providing consistent amounts of antibodies needed without risks of inducing immunity to sperm, thus making possible both consistently effective contraception and rapid reversibility. In good agreement with this concept, vaginal delivery of a highly multivalent anti-sperm IgM reduced embryo formation by 95% in a highly fertile rabbit model(15).

This approach of topical passive immunocontraception has not been reported in humans, due in part to manufacturing and purification challenges with polyvalent Abs such as sIgA and IgM, and the lower agglutinating potencies of IgG. To overcome these challenges, we report here a highly multivalent IgG that possesses 6 Fabs per IgG molecule, with Fab domains interspersed by flexible glycine-serine linkers arranged in a **F**ab-**I**gG-**F**ab orientation; we term this molecule FIF **(Fig. 1A).** To determine whether FIF may be useful for on-demand contraception, we produced FIF using a cGMP-compliant *Nicotiana benthamiana* manufacturing platform, and formulated the FIF into a dissolvable vaginal film comprised of polyvinyl alcohol. We report here the *in vitro* characterization and *in vivo* potency of this vaginal FIF Film.

Results

cGMP production of FIF in *N. benthamiana*

Efficient agglutination requires ASA to bind a ubiquitous antigen that is highly expressed on the surface of human sperm. For these reasons, we chose to engineer a monoclonal antibody (mAb) targeting a unique glycoform of CD52 (hereafter referred to as CD52g) that was previously shown to be produced and secreted by epithelial cells lining the lumen of the epididymis, and present on sperm, white blood cells in semen, and the epithelium of the vas deferens and seminal vesicles(16, 17). The CD52g glycan-based antigen appears to be universally present on all human sperm while absent in most other tissues(17). Using a Fab-domain isolated from a healthy but immune infertile woman(18, 19), we designed a 6 Fab antibody construct, cloned the sequences into the magnICON® vector system, and transfected *Nicotiana benthamiana* using agrobacterial-infiltration process(20–22). This system allows for rapid and scalable production of full-length mAbs in two weeks; the same system has been used to produce various cGMP-compliant mAbs for clinical studies(23). To generate mAbs with homogeneous mammalian glycans, we used a transgenic strain, Nb7KOΔXylT/FucT of *N. benthamiana* which yields mAb with predominantly G0 N-glycans. Without optimization, the production yields of the *Nicotiana*-produced FIF (FIF-N) post-protein A chromatography were approximately 29 mg/kg of plant tissue **(Fig. S1A)**. The mAbs were further purified using ceramic hydroxyapatite chromatography prior to further biophysical characterization. SDS-PAGE analysis demonstrated the correct assembly of FIF-N at its theoretical molecular weight, ~350 kDa (**Fig. 1B**). Purified FIF contained >99% monomeric form as determined by high performance liquid size exclusion chromatography analysis **(Fig. 1C and Fig. S1B).** FIF-N demonstrated excellent stability, with no appreciable aggregation or degradation upon storage at room temperature for 3 weeks and freezing at -70°C **(Fig. S1, B and C).**

Production of FIF-N-Film

Polyvinyl alcohol (PVA) is a polymer routinely used in biomedical applications. Low molecular weight PVA is widely used in female reproductive health products suitable for intravaginal administration, with no appreciable vaginal toxicity or irritation. Similar to prior work in formulating a vaginal film releasing both an anti-HIV (VRC01) and anti-HSV (HSV8) mAb(24) evaluated in a Phase 1 trial, we prepared water-soluble PVA films comprised of PVA 8-88 (67 kDa) together with 10 mg of FIF-N, using an aqueous casting method. As a control, an IgG-N-Film with 20 mg of anti-CD52g IgG was also prepared.

Both films were fabricated to 2”x2” in dimensions, clear in visual appearance with few bubbles present, homogeneous, and resistant to tear **(Fig. 1D)**. Both films showed no significant levels of endotoxin, and no detectable bioburden (CFU/mL), indicating efficient and aseptic removal of potential contaminants **(Table S1)**.

FIF-N-Film possesses superior agglutination potency

We next assessed the sperm-agglutinating potencies of dissolved IgG-N and FIF-N films. We focused on assessing the reduction in progressive motile (PM) fraction of sperm, since it is the PM sperm fractions that reach the uterus and penetrate the zona pellucida to fertilize the egg. We first assessed the agglutination potencies of FIF-N-Film vs. IgG-N-Film using a sperm escape assay with purified sperm. The sperm escape assay uses Computer Assisted Sperm Analysis (CASA) to quantify the number of PM sperm that escapes agglutination over 5 min when mixed with specific mAbs at different mAb and sperm concentrations. We elected to first assess agglutination at a low concentration of 5 million PM sperm/mL, the minimal PM sperm concentration in semen associated with fertility, which limits sperm collision frequency and making it more challenging to achieve rapid and complete agglutination. FIF-N-Film exhibited at least 16-fold greater agglutination potency than IgG-N-Film, defined here as the minimal mAb concentration at which PM sperm are reduced by >98%. The minimum concentration of IgG-N-Film needed was ~6.25 μg/mL, whereas just 0.39 μg/mL of FIF-N-Film was sufficient **(Fig. 2A).**

To confirm efficient agglutination also occur with native semen, we further assessed the agglutination potency of the FIF-N-Film vs IgG-N-Film using whole semen. FIF-N-Film again exhibited at least 10-fold greater agglutination potency than IgG-N-Film **(Fig. 2C**). Both FIF-N-Film and IgG-N-Film required ~16-fold more mAb to achieve >98% agglutination of PM sperm in whole semen compared to in purified motile sperm, likely due to CD52g present on other components in whole semen, including non-PM sperm, seminal leukocytes, as well as on exosomes from the epithelium of the vas deferens and seminal plasma(25).

FIF-N-Film exhibits faster sperm agglutination kinetics

For effective vaginal immunocontraception based on limiting sperm motility in mucus, mAbs must agglutinate/immobilize sperm before they reach the upper reproductive tract; thus, rapid reduction of PM sperm is likely an important factor in contraceptive efficacy. Thus, we next quantified the kinetics of sperm agglutination by quantifying the number of PM motile sperm present at 30 s intervals following treatment of purified sperm (5 million PM sperm/mL) with IgG-N-Film and FIF-N-Film. IgG-N-Film reduced PM sperm by ≥90% within 90 s in 5 of 6 semen samples at 6.25 μg/mL, but failed to do so in 6 of 6 samples at 1.56 μg/mL **(Fig. 3A).** In contrast, FIF-N-Film agglutinated ≥90% of PM sperm within 30 s in all cases at both 6.25 μg/mL and 1.56 μg/mL concentrations **(Fig. 3A)**. Even at 0.39 μg/mL, FIF-N-Film still agglutinated ≥90% of PM sperm within 90 s in 5 of 6 samples. Notably, the agglutination kinetics of FIF-N-Film was markedly faster and more complete than the parent IgG at all mAb concentrations and across all time points **(Fig. 3B)**.

Similar to the sperm escape assay, we also assessed agglutination kinetics of FIF-N-Film vs. IgG-N-Film using whole semen. Again, a higher concentration of FIF-N-Film and IgG-N-Film was required to obtain comparable agglutination kinetics vs. purified sperm. Nonetheless, FIF-N-Film exhibited markedly faster and more complete sperm agglutination kinetics than IgG-N-Film at all mAb concentrations and all time points in whole semen **(Fig. 3D)**. At 25 μg/mL, FIF-N-Film agglutinated ≥90% of PM sperm within 30 s in 6 of 6 whole semen samples while IgG-N-Film agglutinated ≥90% of PM sperm in 90 s in only 2 of 6 specimens at the same concentration **(Fig. 3C)**. Lower sperm concentration (as found in semen from oligospermia, sub-fertile individuals) may limit sperm agglutination due to reduced likelihood of a sperm-sperm collision, whereas higher sperm amounts may saturate the agglutination potential. We thus further assessed whether FIF-N-Film can effectively reduce PM sperm at 1 million PM sperm/mL and 25 million

PM sperm/mL. FIF-N-Film maintained similar superior agglutination kinetics over IgG-N-Film across both lower and higher sperm concentrations **(Fig. S2)**. These results underscore the increased potency for FIF-N-Film compared to the IgG-N-Film across diverse conditions.

FIF-N and FIF-Expi293 exhibit equivalent agglutination

To confirm that the production of FIF in *N. benthamiana* and their subsequent formulation into PVA films did not reduce their agglutination activity, we further compared the sperm agglutination potencies of FIF-N, before and after film formulation, to Expi293-produced FIF. At 0.39 μg/mL, FIF-Expi293, FIF-N, and FIF-N from four dissolved FIF-N-Films all demonstrated comparable sperm agglutination potencies **(Fig. S3A)**. Similarly, FIF-Expi293, FIF-N, and FIF-N from dissolved FIF-N-Films all agglutinated all sperm within 60 s in 3 of 3 samples at 1.56 μg/mL **(Fig. S3B).** The agglutination kinetics profile of Expi293- and *Nicotiana*- produced FIF, pre- and post- film formulation, were also virtually identical **(Fig. S3C).** These results underscore that neither production of FIF in *Nicotiana* nor formulation of FIF-N into films had any significant impact on the actual agglutination potencies of FIF.

FIF-N-Film traps individual spermatozoa in vaginal mucus

Previous work has shown that IgG and IgM Abs can retard the active motility of individual spermatozoa in mucus despite continued vigorous beating action of the sperm flagellum; clinically, this is referred to as the "shaking phenomenon"(11). This muco-trapping function is similar to recent observations with Herpes Simplex Virus (HSV)(26, 27), whereby multiple HSV-bound IgGs formed polyvalent adhesive interactions between their Fc domains and mucin fibers in cervicovaginal mucus (CVM). Anti-HSV IgG-mediated effective trapping of individual viral particles in CVM, and blocked vaginal Herpes transmission in mice(26). We thus assessed whether FIF-N-Film can reduce progressive motility of fluorescently labeled spermatozoa in the relatively thin (low viscosity) CVM using multiple particle tracking. FIF-N-Film reduced progressively motile spermatozoa to the same extent as the IgG-N-Film, indicating that the addition of Fabs to both the N- and C-terminus of the IgG did not interfere with Fc-mucin crosslinking **(Fig. 4).**

FIF-N-Film rapidly eliminates PM sperm in sheep vagina

Since the unique glycoform of CD52g is only found in human and chimpanzee sperm(28), there is no practical animal model to perform mating-based contraceptive efficacy studies. Instead, we designed a sheep study that parallels the human post-coital test (PCT)(29–33), which assesses the reduction of PM sperm in the female reproductive tract (FRT) given that PM sperm are required for fertilization. Clinical PCT studies have proven to be highly predictive of contraceptive efficacy in clinical trials(30, 34–39). The sheep vagina is physiologically and anatomically very similar to the human vagina(40, 41), making it the gold standard for assessing vaginal products. We instilled either Placebo-Film (no mAb) or FIF-N-Film into the sheep vagina, allowed 4 hr for the film to dissolve, followed by brief simulated intercourse with a vaginal dilator (15 strokes), vaginal instillation of fresh whole human semen, brief simulated intercourse (5 strokes), and finally, recovery of the semen mixture from the sheep vagina 2 min post semen instillation for immediate visual assessment of sperm motility via quantifying progressively motile sperm. Despite these exceptionally stringent criteria, FIF-N-Film reduced 100% of PM sperm in all four of the animals studied over two independent studies, with no observable PM sperm **(Fig. 5**; $p<0.0001$**)**. In contrast, there were high PM sperm fractions recovered from all four sheep receiving the placebo film, with a few to several hundred PM sperm counts in the microscopy field, comparable to those from sheep treated with saline control.

Finally, we utilized colposcopy (white light magnification) to assess for signs of irritation, including erythema, vascular disruption, epithelial abrasion, or disruption. We have shown that colposcopy in sheep is predictive of toxicity in humans; use of a vaginal ring releasing TDF that caused ulceration in women was tested in the sheep model, with similar findings of ulceration and epithelial disruption in the sheep when compared to the women in the clinical study(42). We observed no sign of gross toxicity by

colposcopy during these acute studies. This is in good agreement with our earlier study using the same film formulation releasing two different antibodies, where we observed no significant colposcopic or cytokine abnormalities(43).

Discussion

The sperm agglutination potency of FIF-N-Film in sheep reported here is likely attributed in large part to the additional Fab arms of the FIF molecule. By delivering FIF directly to where it is needed i.e., the vagina, the fraction of FIF available to bind sperm is maximized, thereby enabling complete agglutination and immobilization of progressively motile sperm within just two minutes of semen exposure, in good agreement with our recent work(44). In contrast, only a tiny fraction of systemically delivered mAb will be available to bind sperm because of the large blood volume (~5 L), distribution to non-target tissues, natural catabolic degradation, and finally limited and delayed distribution into the FRT, including the vagina. As a result, markedly lower total amount of FIF is needed with vaginal delivery to achieve contraceptive levels in the FRT compared to delivering the same mAb systemically. An added advantage of vaginal delivery is that the entire dose of FIF delivered is quickly available, without any delays in reaching C_{max} in the vagina from delayed extravasation from the systemic circulation. Vaginal IgG has a half-life of ~9 hr(45); thus, even after 24 hr, there will likely be sufficient quantities of FIF from the original 10 mg film to maintain effective sperm agglutination, given *in vitro* measurements that showed highly effective sperm agglutination even at FIF concentrations as low as ~390 ng/mL.

Decades ago, the high costs of mAb production and emphasis on systemic administration critically limited the feasibility of passive immunization with ASA as a strategy for non-hormonal contraception. However, the cost of mAb production has declined over the years due to advances in CHO cell production. It reportedly costs between $95–$200/gram to produce currently marketed mAbs using a CHO expression system(46), and between $100–$200/gram using Nicotiana-expression system with production yield of ~1-2 g/kg (47–49). Such rapid decline in manufacturing costs likely make cost-effective antibody-based contraception feasible in the not-too-distant future. Since FIF exhibits considerable agglutination potencies down to 390 ng/mL, additional dose optimization may further reduce the amount of FIF needed per film, thus further decreasing costs and improving scale.

mAbs based topical contraceptives, such as the FIF-N-film reported here, are likely to be safe due to their binding specificity, particularly when targeted to epitopes present primarily on sperm. Vaginally dosed mAbs are poorly absorbed into the systemic circulation(50, 51), and the vaginal immune response is limited even when vaginally vaccinating with the aid of highly immunostimulatory adjuvants(52). Vaginal secretions naturally contain high levels of endogenous IgG (i.e., 1-2 mg/mL(53, 54), making it unlikely that vaginal delivery of FIF, which is comprised of fully human Fabs and Fc, would trigger inflammation, sensitization, or other local toxicities. Finally, PVA (67 kDa) film, which is widely used in pharmaceutical applications as well as in contraceptive products such as VCF, has been found to be safe and non-immunogenic to use. Altogether, these features make PVA film delivering mAb vaginally for immunocontraception likely to be exceptionally safe.

Typically, only ~1% of the ejaculated sperm enter the cervix, even fewer reaching the uterus, and only dozens of sperm (out of the ~200 million in the ejaculate) reach the neighborhood of the egg(55). Accordingly, poor sperm motility in mid-cycle cervical mucus and low total sperm count are considered good correlates to low conception rates. Human semen averages between 45-65 million sperm/mL(56), 15 million sperm/mL marks the lowest 5th percentile in men with proven fertility(57), and <5 million sperm/mL is often considered severe oligospermia that correlates with very low fertility[37]. These observations suggest a marked reduction of progressive sperm motility, even if incomplete (e.g., a 10-fold reduction in PM sperm fractions), may likely provide substantial contraceptive efficacy. This expectation is also consistent with the observations that even under ideal circumstances, with unprotected intercourse on the cycle day of maximum fertility, the odds of conceiving are only about ~10%(59). This indicates

that only a small (i.e., limiting) number of motile sperm would reach the egg per intercourse; thus, reducing progressive sperm motility in the vagina and cervical canal should proportionally reduce the likelihood of conceiving. These findings, together with the contraceptive success with topical ASA against rabbit sperm(15), suggest arresting progressive sperm motility in mucus using mAb (which can reduce PM sperm by >99.9%) should provide an effective form of contraception.

One potentially important mechanism of vaginal HIV transmission is cell-associated HIV transmission, whereby HIV in immune cells of HIV+ semen facilitates direct cell-to-cell spread of the virus to target cells in the female reproductive tract(60). Cell-associated HIV transmission may be more efficient than cell-free HIV transmission, since intracellular viruses are not exposed to the same host restriction factors and innate immune molecules the FRT. Since CD52g is adsorbed on the surface of immune cells originating from the male reproductive tract, it is possible that FIF can also agglutinate such immune cells and limit their access to target cells in the FRT, thereby limiting cell-associated vaginal HIV transmission. Combining contraception with the prevention of sexually transmitted infections is also an attractive public health strategy. With further reduction in manufacturing costs and greater availability of multi-metric ton manufacturing capacity for mAbs, it may be possible to create a cost-effective, on-demand multi-purpose technology product based on a cocktail of antiviral and anti-sperm mAbs that can simultaneously afford potent contraception and effective protection against STI transmission.

Polymeric vaginal films are advantageous for delivering active pharmaceutical ingredients (API) and are preferred over other delivery methods due to enhanced bio-adhesive properties, ease of use, compact size and negligible vaginal leakage(61–65). Currently, multiple vaginal films with anti-retroviral microbicides are under development and evaluation(66–69). In a recent Phase I study, a vaginal film formulated with the microbicide drug candidate, dapivirine, was found to be safe and acceptable with uniform vaginal distribution while exhibiting considerable efficacy against *ex vivo* HIV-1 challenge model(63). Similarly, vaginal films could be formulated with contraceptive mAbs and microbicides or anti-fungal agents to achieve multipurpose prevention. Finally, it may be possible to formulate vaginal films to provide sustained release in the vagina spanning days to weeks(70, 71).

There are a number of limitations to our current study. First, we did not directly demonstrate efficacy by preventing pregnancies. We are unable to do so due to the unique antigen (CD52g) that our antibody targets: prior work has shown that, besides humans, only chimpanzees possess CD52g(28), and it is not possible to conduct chimpanzee studies in the U.S. Instead, for our *in vivo* proof-of-concept study, we were forced to adopt a sheep model designed to closely mimic the human post-coital test that is routinely used to assess the efficacy of sperm-targeted contraceptives in the early phase clinical studies. Fortunately, the human cost-coital test has been shown to correlate well with eventual efficacy in preventing pregnancies. Second, the precise dose of antibodies needed to ensure highly effective sperm agglutination remains not well understood. In the current study, to ensure success, we incorporated a relatively large dose of mAb (10 mg) into the vaginal film formulation. Although we expect this dose of mAb to be commercially viable, it is possible we can achieve effective agglutination of sperm with even lower quantities of mAb by engineering more potent mAbs(72). We are also pursuing the development of other vaginal delivery formats, such as an intravaginal ring that can afford sustained release of our mAbs across the potential fertility window, which may further reduce the dose needed.

Materials and Methods

Experimental design and ethics

The objective was to assess the sperm-agglutinating and -trapping potency of PVA film formulated with *Nicotiana*-produced FIF Ab *in vitro* and *in vivo*. The *in vitro* studies using human semen and human cervicovaginal mucus samples were approved by the Institutional Review Board (IRB) of the University of North Carolina at Chapel Hill (IRB-101817). Prior to the collection of semen and mucus samples,

informed written consents were obtained from all male and female subjects. Mass student emails and printed posters were utilized to recruit subjects for the UNC-Chapel Hill studies. The sheep surrogate post-coital test using human semen samples was approved by the IRB of the University of Texas Medical Branch (UTMB). Informed written consent was obtained from the pre-screened male volunteers. Sheep studies were approved by the UTMB Institutional Animal Care and Use Committee (IACUC) and utilized 5 female Merino crossbred sheep. IgG-N-Film and FIF-N-Film were dissolved in ultra-pure water before all *in vitro* experiments.

Construction of *N. benthamiana* expression vectors

The variable light (V_L) and variable heavy (V_H) DNA sequences for anti-sperm IgG antibody were obtained from the published sequence of H6-3C4 mAb(18, 19). For the construction of expression vector encoding light chain (LC), a gene fragment consisting of V_L and C_λ DNA sequences was cloned into PVX viral backbone (Icon Genetics)(20). For the construction of an expression vector containing IgG1 heavy chain (HC), a gene fragment consisting of V_H and C_H1-C_H2-C_H3 DNA sequences was cloned into TMV viral backbone (Icon Genetics)(20). For the construction of expression vector containing FIF HC, a gene fragment consisting of V_H/C_H1-$(G_4S)_6$ Linker-V_H/C_H1-C_H2-C_H3-$(G_4S)_6$ Linker-V_H/C_H1 DNA sequences was cloned into TMV viral backbone (Icon Genetics).

Production of mAbs in Nb7KOΔXylT/FucT *N. benthamiana*

Briefly, IgG and FIF mAbs were expressed in *N. benthamiana* plants using "magnifection" procedure(23). Cloned expression vectors i.e., PVX-LC, TMV-IgG-HC, and TMV-FIF-HC were transformed into *Agrobacterium tumefaciens* strain ICF320 (Icon Genetics) and grown overnight at 28.0°C followed by 1:000 dilution in infiltration buffer [10 mM MES (pH 5.5) and 10 mM $MgSO_4$]. The combinations of diluted bacterial cultures (TMV-IgG-HC + PVX-LC and TMV-FIF-HC + PVX-LC) were used to transfect 4 wk old *N. benthamiana* plants (ΔXTFT glycosylation mutants) using vacuum infiltration. Using a custom-built vacuum chamber (Kentucky Bioprocessing), the aerial parts of entire plants were dipped upside down into the bacterial/buffer solution and a vacuum of 24'' mercury was applied for 2 min. Infiltrated plants were allowed to recover and left in the growth room for transient expression of antibodies. 7 days after infiltration, plants were harvested and homogenized in extraction buffer containing 100 mM Glycine, 40 mM Ascorbic Acid, 1 mM EDTA (pH 9.5) in a 0.5:1 buffer (L) to harvested plants (kg) ratio. The resulting green juice was clarified by filtration through four layers of cheesecloth followed by centrifugation at 10,000 g for 20 min. Next, mAbs were captured from the clarified green juice using MabSelect SuRe Protein A columns (GE Healthcare). The mAbs were eluted from Protein A columns were further purified using equilibrated Capto Q columns (GE Healthcare) and flow-through fractions, which contain mAbs, were collected. The mAb-containing fractions were finally polished with CHT chromatography with type II resin (Bio-Rad).

Biophysical characterization of mAbs

SDS-PAGE at reducing and non-reducing conditions was performed to determine the molecular weight of FIF-N. Briefly, 1 μg of mAb was denatured at 70°C for 10 min. Next, 0.3 μL of 0.5 M tris (2-carboxyethyl) phosphine (TCEP) was added as a reducing agent to the denatured protein for a reduced sample and incubated at room temperature for 5 min. After the incubation, samples were loaded, and the gel was run for 40 min at a constant voltage of 200 V. Bio-Rad Precision Protein Plus Unstained Standard was used as a protein ladder. Imperial Protein Stain (Thermo Scientific) was used to visualize the protein bands. The brightness and contrasts of the SDS-PAGE image were linearly adjusted using Image J software (Fiji).

HPLC-SEC was performed to determine the purity of IgG-N and FIF-N mAbs. The HPLC-SEC system consisted of a TSK Gel Super SW3000 column (Tosoh Biosciences) connected to Agilent 1260 HPLC system and a UV detector. The flow rate was maintained at 0.2 mL/min. The column was equilibrated with 0.1 M sodium phosphate, 0.15M NaCl buffer, pH 7.2 before loading the samples. 100 μg of each

mAbs (50uL) were injected onto the column, and data were collected and analyzed using the ChemStation chromatography data system and software (Agilent). The proportion of monomers, aggregates, and fragments present in each mAb sample were calculated using ChemStation software (Agilent).

Production of IgG-N and FIF-N films

Films were manufactured using the solvent casting method(61). Briefly, PVA 8-88 (67 kDa; 25%, w/w) was dissolved in MilliQ water. Next, IgG and FIF mAbs suspended in 10 mM Histidine + 0.005% Polysorbate 20, pH 6.5 were slowly added into the PVA solution followed by 200 mg/mL maltitol. The solution was stirred over 15 minutes to ensure uniform distribution of mAbs and to remove the entrapped air bubbles. The final uniform polymer solution was cast onto a polyester substrate attached to a glass plate using a 2''x2''x0.020'' die press. The film sheet was allowed to dry for 20 min before it was removed from the substrate, and then cut into 2"x1.8" individual unit doses using a scalpel. Placebo film was prepared using the same method as described above except without drug substances in the polymer solution. IgG-N-Film and FIF-N-Film were dissolved in ultra-pure water prior to *in vitro* experiments.

Semen collection and isolation of purified motile sperm

Healthy male subjects were asked to refrain from sexual activity for at least 24 hr prior to semen collection. Semen was collected by masturbation into sterile 50 mL sample cups and incubated for a minimum of 15 min post-ejaculation at RT to allow liquefaction. The density gradient sperm separation procedure (Irvine Scientific) was used to extract motile sperm from liquefied ejaculates. Briefly, 1.5 mL of liquified semen was carefully layered over 1.5 mL of Isolate® (90% density gradient medium, Irvine Scientific) at RT, and centrifuged at 300 g for 20 min. Following centrifugation, the upper layer containing dead cells and seminal plasma was carefully removed without disturbing the motile sperm pellet in the lower layer. The sperm pellet was then washed twice with the sperm washing medium (Irvine Scientific) by centrifugation at 300 g for 10 min. Finally, the purified motile sperm pellet was resuspended in the sperm washing medium, and an aliquot was taken for the determination of sperm count and motility using CASA. All semen samples used in the functional assays exceeded lower reference limits for sperm count (15×10^6 total sperm/mL) and total motility (40%) as indicated by WHO guidelines(57).

Sperm count and motility using CASA

The Hamilton-Thorne computer-assisted sperm analyzer, 12.3 version, was used for the sperm count and motility analysis in all experiments unless stated otherwise. For each analysis, 4.4 µL of the semen sample was inserted into MicroTool counting chamber slides (Cytonix). Then, six randomly selected microscopic fields, near the center of the slide, were imaged and analyzed for progressively motile and non-progressively motile sperm count. The complete parameters that were assessed by Hamilton-Thorne Ceros 12.3 software for motililty analysis are listed in **Table S2**. PM sperm were defined as having a minimum of 25 µm/s VAP and 80% STR(73).

Sperm escape assay

This assay was conducted using whole semen and purified motile sperm at the starting concentration of 10×10^6 PM sperm/mL. Briefly, 40 µL aliquots of purified motile sperm or whole semen were transferred to individual 0.2 mL PCR tubes. Sperm count and motility were performed again on each 40 µL aliquot using CASA. This count serves as the original (untreated) concentration of sperm for evaluating the agglutination potencies of respective Ab constructs. Following CASA, 30 µL of purified motile sperm or native semen was added to 0.2 mL PCR tubes containing 30 µL of Ab constructs, and gently mixed by pipetting. The tubes were then held fixed at 45° angles in a custom 3D printed tube holder for 5 min at RT. Following this incubation period, 4.4 µL was pipetted from the top layer of the mixture with minimal perturbation of the tube and transferred to the CASA instrument to quantify the number of PM sperm. The percentage of the PM sperm that escaped agglutination was computed by dividing the sperm count obtained after treatment with Ab constructs by the original (untreated) sperm count in each respective tub

followed by multiplication with 2 to correct for the 2-fold dilution that occurs upon Ab-treatment. Each experimental condition was evaluated in duplicates on each semen specimen, and the average from the two experiments was used in the analysis. At least 6 independent experiments were done with at least 6 unique semen samples.

Agglutination kinetics assay

FIF-N-Film vs IgG-N-Film: This assay was conducted using both whole semen and purified motile sperm at the starting concentration of 2 x 10^6 PM sperm/mL, 10 x 10^6 PM sperm/mL, and 50 x 10^6 PM sperm/mL. Briefly, 4.4 µL of purified motile sperm or whole semen was added to 4.4 µL of Ab constructs in 0.2 mL PCR tubes, and gently mixed. A timer was started immediately while 4.4 µL of the mixture was transferred to chamber slides with a depth of 20 µm (Cytonix), and video microscopy (Olympus CKX41) using a 10x objective lens focused on the center of the chamber slide was captured up to 90 s at 60 frames/s. PM sperm count was measured by CASA every 30 s up to 90 s. Each experimental condition, except for 50 x 10^6 PM sperm/mL, was evaluated in duplicates on each semen specimen, and the average from the two experiments was used in the analysis. At least 6 independent experiments were done with at least 6 unique semen samples.

CVM collection and processing

CVM was collected as previously described(26). Briefly, undiluted CVM secretions, averaging 0.5 g per sample, were obtained from women of reproductive age, ranging from 20 to 44 years old, by using a self-sampling menstrual collection device (Instead Softcup). Participants inserted the device into the vagina for at least 30 s, removed it, and placed it into a 50 mL centrifuge tube. Samples were centrifuged at 230 g for 5 min to collect the secretions. Samples were collected at various times throughout the menstrual cycle, and the cycle phase was estimated based on the last menstrual period date normalized to a 28-day cycle. Samples that were non-uniform in color or consistency were discarded. Donors stated they had not used vaginal products nor participated in unprotected intercourse within 3 days before donating. All samples had pH < 4.5.

Fluorescent labeling of purified sperm

Purified motile sperm were fluorescently labeled using Live/Dead Sperm Viability Kit (Invitrogen Molecular Probes. Briefly, SYBR 14 stock solution was diluted 50-fold in sperm washing media. Next, 5 µL of diluted SYBR 14 and PI dye were added to 1 mL of purified sperm resulting in a final SYBR 14 and PI concentration of 200 nM and 12 µM respectively. The sperm-dye solution was incubated for 10 min at 36°C followed by centrifuging at 300 g for 10 min. Next, the labeled motile sperm pellet was resuspended in the sperm washing medium, and an aliquot was taken for the determination of sperm count and motility using CASA.

Multiple particle tracking studies

Fluorescent motile sperm in human CVM was tracked as previously described(72). CVM was first diluted three-fold using sperm washing medium and titrated to pH 6.8-7.1 using NaOH. Next, 4 µL of Ab constructs or control (anti-RSV IgG1) was added to 60 µL of modified CVM and mixed well in a CultureWell™ chamber slide (Invitrogen) followed by the addition of 4 µL of 1 x 10^6 PM sperm/mL of fluorescently labeled sperm. Once mixed, sperm, Ab, and CVM were incubated for 5 min at RT. Then, translational motions of the sperm were recorded using an electron-multiplying charge-coupled-device camera (Evolve 512; Photometrics, Tucson, AZ) mounted on an inverted epifluorescence microscope (AxioObserver D1; Zeiss) equipped with an Alpha Plan-Apo 20/0.4 objective, environmental (temperature and CO2) control chamber, and light-emitting diode (LED) light source (Lumencor Light Engine DAPI/GFP/543/623/690). 15 videos (512 × 512 pixels, 16-bit image depth) were captured for each Ab condition with MetaMorph imaging software (Molecular Devices) at a temporal resolution of 66.7 ms and spatial resolution of 50 nm (nominal pixel resolution, 0.78 µm/pixel) for 10 s. Next, the acquired videos were analyzed via a neural network tracking software (74) modified with standard sperm motility

parameters (**Table S2**) to determine the percentage of PM sperm. At least 6 independent experiments were performed, each using a unique combination of CVM and semen specimens.

In vivo surrogate efficacy studies
On the test day, each sheep received a randomized unique Ab treatment and all sheep were dosed with the same semen mixture that was pooled from 3-5 donors. Briefly, placebo film or FIF-N-film (provided under blind to the animal facility) or saline were instilled into sheep's vagina and incubated for 4 hr, followed by thorough mixing using a vaginal dilator for 15 strokes. Next, 1 mL of pooled whole semen was pipetted into the sheep's vagina, followed by simulated intercourse with a vaginal dilator for 5 strokes. Two minutes after the introduction of semen, fluids from the sheep vagina were recovered and assessed for the PM sperm count in a hemocytometer (Bright-Line™ Hemacytometer) under a light microscope (Olympus IX71) using a 20x objective with Thorlabs camera. Each Ab condition was repeated three more times in the same group of sheep (n=5) with at least 7 days interval in between experiments. Treatments and quantifications were performed in a blinded fashion.

In vivo colposcopy
Sheep were anesthetized with ketamine/diazepam and isoflurane, intubated, and positioned supine on a V-tilt table. A speculum was placed in the vagina and a Leisegang OptiK Model 2 colposcope used to visualize the vagina and cervix by white light low power magnification (3.5-7.5X). An experienced colposcopist recorded findings on examination records as previously reported(75) using the WHO/CONRAD guidelines for colposcopic evaluation of vaginal products(76). Findings were noted as erythema, vascular disruption (petechiae or ecchymosis), superficial epithelial disruption (peeling, abrasion), or deep epithelial disruption (bleeding, ulceration, laceration).

Statistical analysis
All analyses were performed using GraphPad Prism 8 software. For multiple group comparisons (Fig. 2 and Fig. S3), P values were calculated using a one-way ANOVA with Dunnett's multiple comparisons tests. To compare the percent reduction of PM sperm *in vitro* by IgG-N-Film vs FIF-N-Film, using whole semen as well as purified semen at the final concentration of 1 x 10^6 PM sperm/mL, 5 x 10^6 PM sperm/mL, and 25 x 10^6 PM sperm/mL, one-tailed t-test was performed. Similarly, the comparison between control- and anti-sperm Ab-treated fluorescent PM sperm was performed using a one-tailed t-test. Lastly, to compare the percent reduction of PM sperm *in vivo* by Placebo-Film vs FIF-N-Film one-tailed t-test was performed. In all analyses, α=0.05 for statistical significance. The values for *N*, *P*, and the specific statistical test performed for each experiment are included in the appropriate figure legends. All data are presented as the mean ± standard deviation.

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Figures legends

Fig. 1. Production of FIF-N-Film. (A) Schematic diagrams of anti-sperm Fab-IgG-Fab (FIF). The additional Fab is linked to the N-terminal and C-terminal of parent IgG using flexible glycine-serine linkers to assemble FIF. **(B)** SDS-PAGE analysis of FIF-N in native (non-reducing) and reducing conditions. Non-reducing SDS-PAGE showcases the total molecular weight of the Ab and reducing SDS-PAGE displays the molecular weight of the individual heavy chain and light chain of Ab. **(C)** Demonstration of the homogeneity of FIF-N after protein A and ceramic hydroxyapatite (CHT) Chromatography using high performance liquid size exclusion chromatography (HPLC-SEC) analysis. Y-axis indicates the total percentage of Abs representing their theoretical molecular weights. **(D)** Image of water-soluble polyvinyl alcohol (PVA) film comprising of *Nicotiana*-produced FIF Ab.

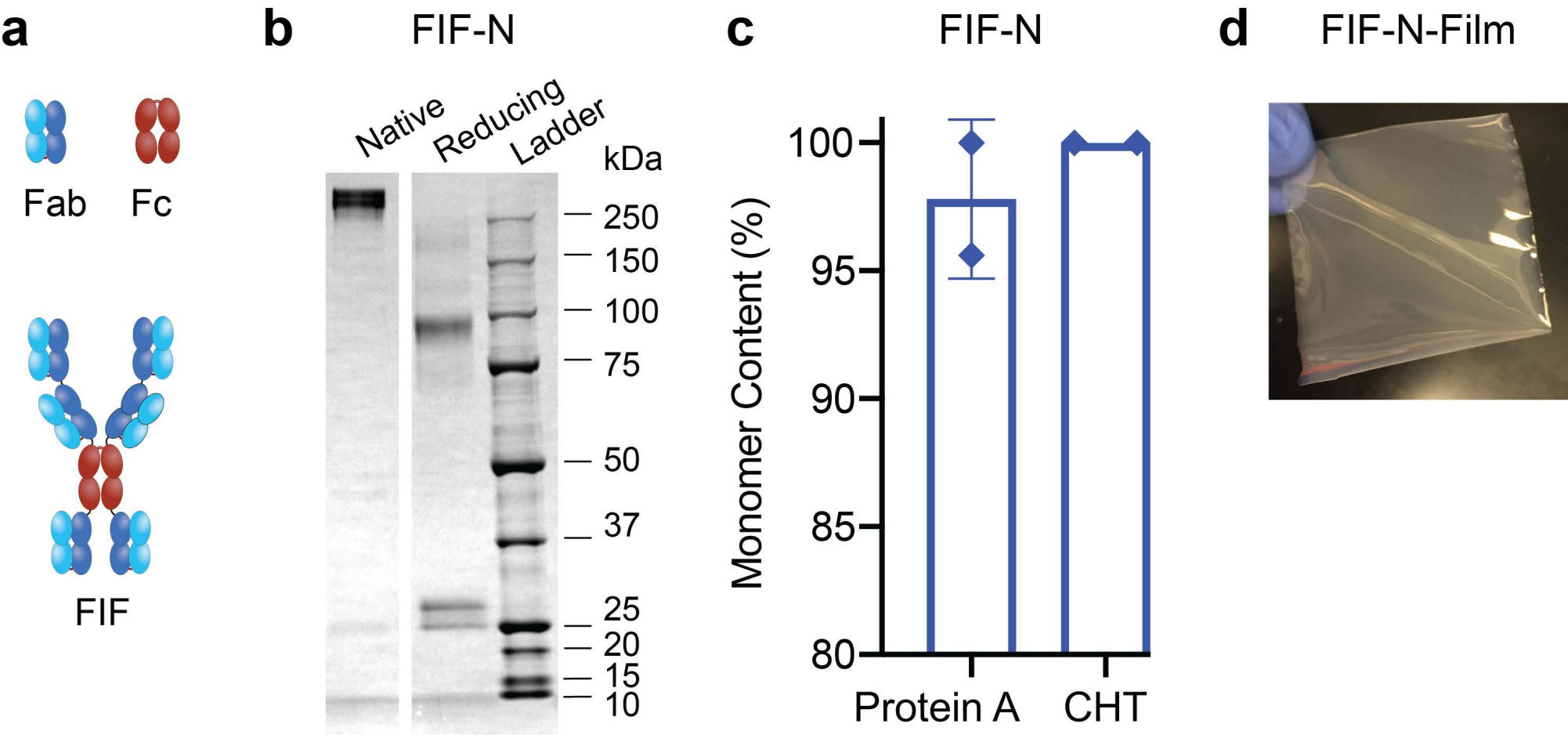
Fig. 2. FIF-N-Film possesses markedly greater agglutination potency than IgG-N-Film. (A) Sperm agglutination potency of the IgG-N-Film and FIF-N-Film determined by quantifying PM sperm that escaped agglutination after Ab-treatment compared to pre-treatment condition using CASA. Purified sperm at the final concentration of 5 x 10^6 PM sperm/mL was used. **(B)** Sperm agglutination potency of the Abs normalized to the media control. **(C)** Further assessment of sperm-agglutination potency of the IgG-N-Film and FIF-N-Film using whole semen. **(D)** Sperm-agglutination potency of the IgG-N-Film and FIF-N-Film against whole semen normalized to the sperm washing media control. Data were obtained from N=6 independent experiments using 6 unique semen specimens. Each experiment was performed in duplicates and averaged. P values were calculated using a one-way ANOVA with Dunnett's multiple comparisons test. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001. Data represent mean ± standard deviation.

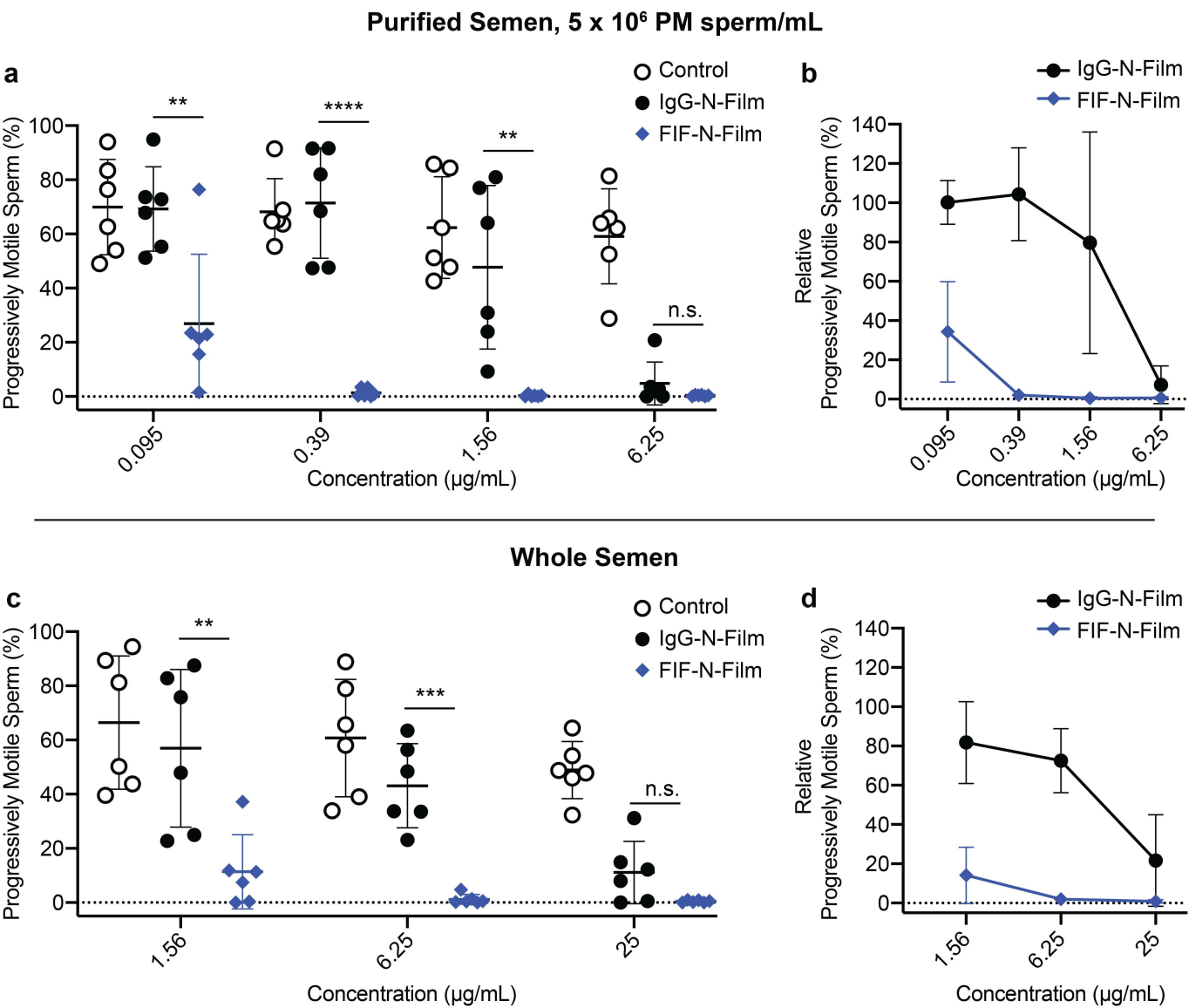
Fig. 3. FIF-N-Film exhibits markedly faster agglutination kinetics than IgG-N-Film. (A) Sperm agglutination kinetics of IgG-N-Film and FIF-N-Film measured by quantifying the time required to achieve 90% agglutination of PM sperm compared to sperm washing media control. **(B)** The rate of sperm agglutination determined by measuring the reduction in the percentage of PM sperm at three timepoints after Ab-treatment compared to sperm washing media control. Purified sperm at the final concentration of 5 x 10^6 PM sperm/mL was used. **(C)** Sperm agglutination kinetics and **(D)** The rate of sperm agglutination assessed for IgG-N-Film and FIF-N-Film using whole semen. Data were obtained from N=6 independent experiments using 6 unique semen specimens. Each experiment was performed in duplicates and averaged. P values were calculated using a one-tailed t-test. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001. Data represent mean ± standard deviation.

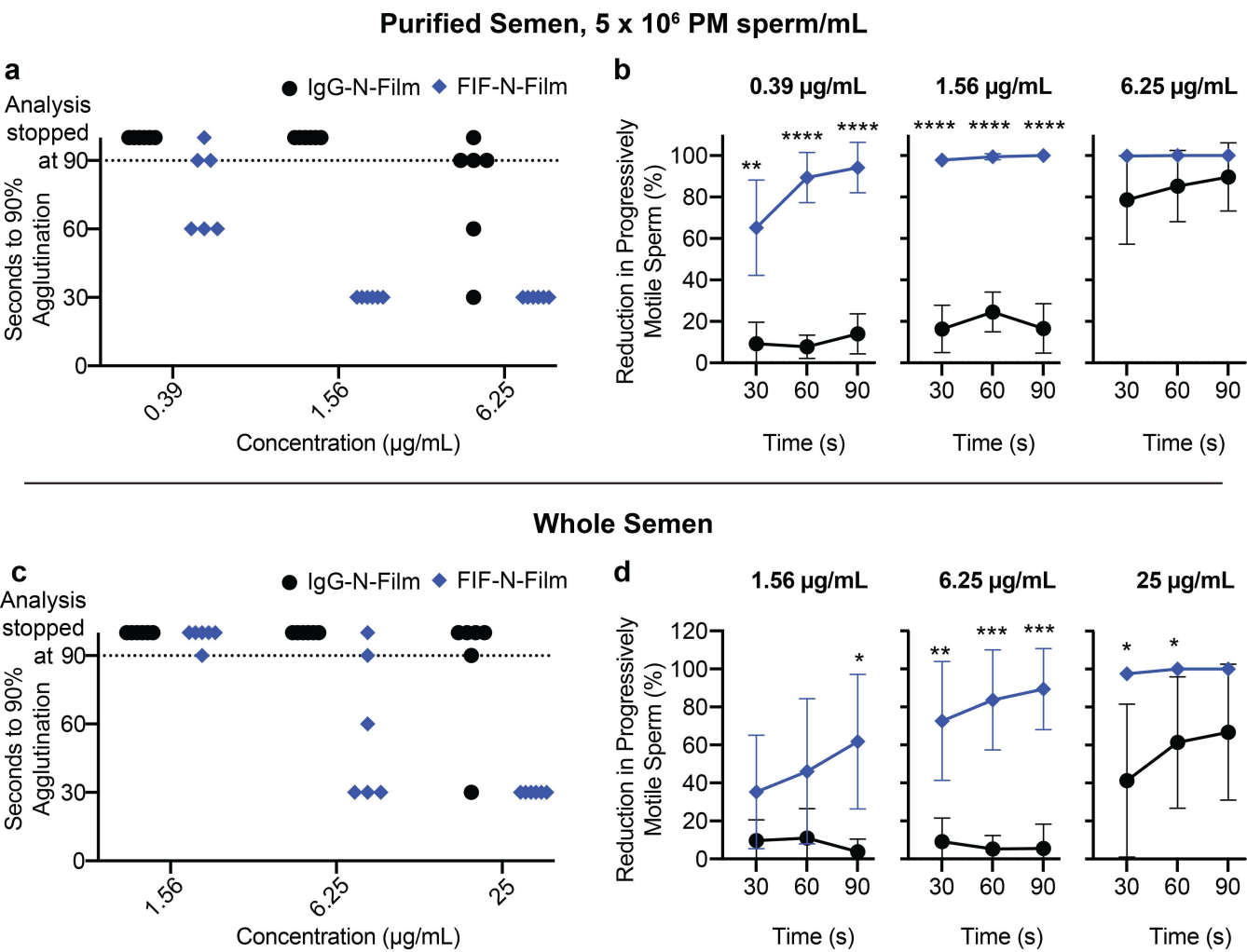
Fig. 4. FIF-N-Film maintains the trapping potency of IgG-N-Film. (A) The trapping potency of the indicated Abs (25 μg/mL) measured by quantifying fluorescently labeled PM sperm in Ab-treated CVM using neural network tracker analysis software. Purified sperm at the final concentration of 5.8 x 10^4 PM sperm/mL was used. Data were obtained from N=6 independent experiments using 6 unique combinations of semen and CVM specimens. P values were calculated using a one-tailed t-test. *P < 0.05 and **P < 0.01. Data represent mean ± standard deviation. **(B)** Representative 4 s traces of sperm within one standard error mean of average path velocity at a timescale τ of 1 s in CVM treated with control (anti-RSV IgG), IgG-N-Film, and FIF-N-Film.

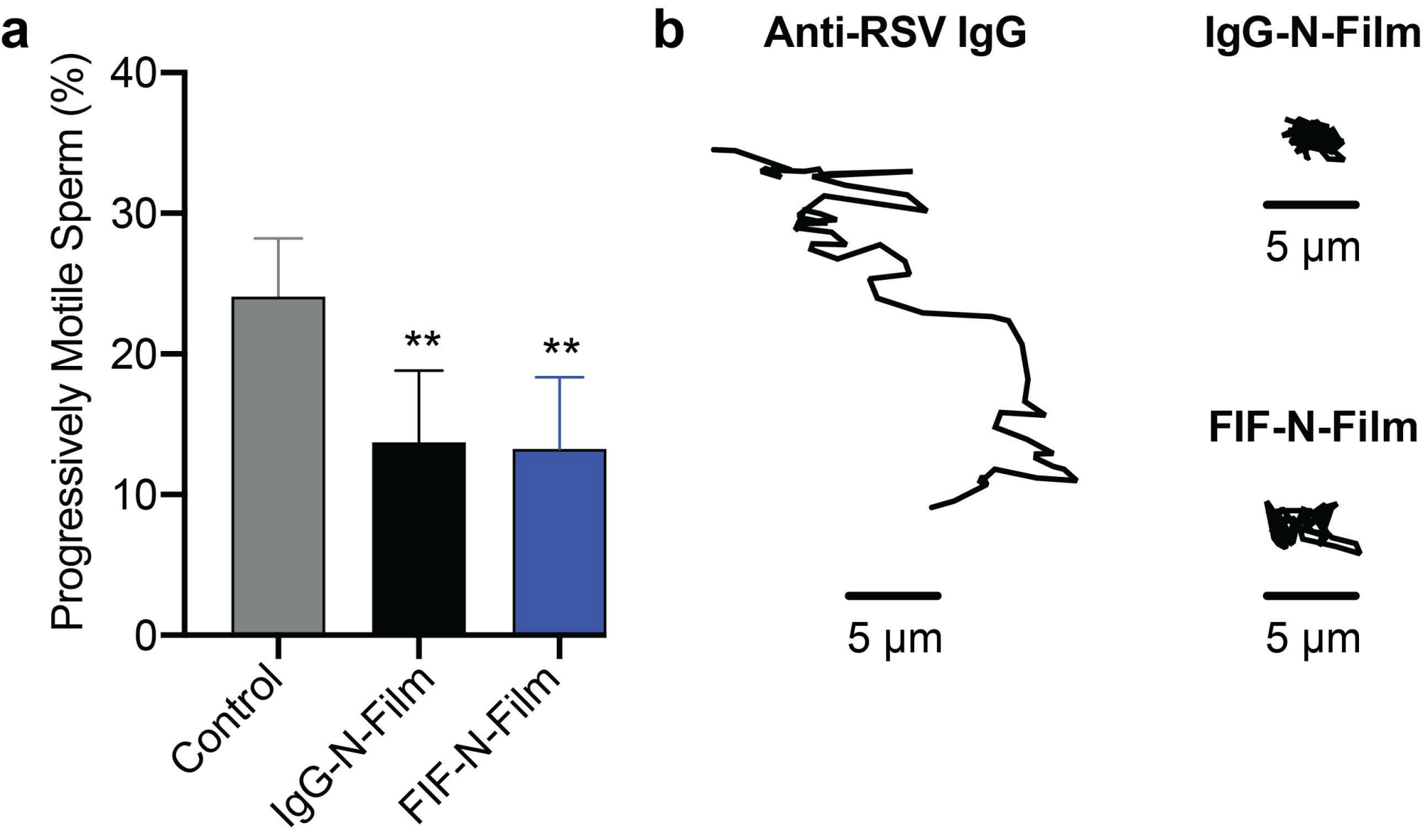
Fig. 5. FIF-N-Film exhibits complete agglutination in surrogate sheep studies.
(A) Schematic of the study design. **(B)** The potency of Placebo-Film and FIF-N-Film measured by quantifying PM sperm in sheep's vaginal fluid after Ab- or Placebo- treatment compared to saline-treatment. Treatment administration was blinded, and quantifications were performed using a neural network tracker modified with sperm motility parameters. Data were obtained from N=4 independent experiments. P values were calculated using a one-tailed t-test. *P < 0.05, **P < 0.01, ***P < 0.001 and

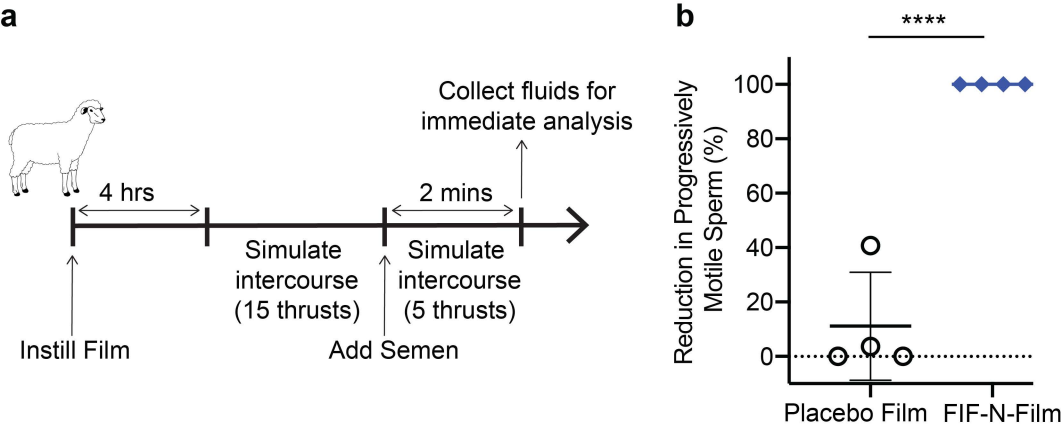
****P < 0.0001. Data represent mean ± standard deviation.


a
Fab
Fc
FIF
b
FIF-N
Native
Reducing
Ladder
kDa
250
150
100
75
50
37
25
20
15
10
c
FIF-N
Monomer Content (%)
100
95
90
85
80
Protein A
CHT
d
FIF-N-Film


Purified Semen, 5 x 10⁶ PM sperm/mL
a
Control
IgG-N-Film
FIF-N-Film
Progressively Motile Sperm (%)
**
****
**
n.s.
0.095
0.39
1.56
6.25
Concentration (µg/mL)
b
IgG-N-Film
FIF-N-Film
Relative Progressively Motile Sperm (%)
0.095
0.39
1.56
6.25
Concentration (µg/mL)
Whole Semen
c
Control
IgG-N-Film
FIF-N-Film
Progressively Motile Sperm (%)
**
***
n.s.
1.56
6.25
25
Concentration (µg/mL)
d
IgG-N-Film
FIF-N-Film
Relative Progressively Motile Sperm (%)
1.56
6.25
25
Concentration (µg/mL)


Purified Semen, 5 x 10⁶ PM sperm/mL
a
IgG-N-Film
FIF-N-Film
Analysis stopped at 90
Seconds to 90% Agglutination
0
30
60
90
0.39
1.56
6.25
Concentration (µg/mL)
b
0.39 µg/mL
1.56 µg/mL
6.25 µg/mL
Reduction in Progressively Motile Sperm (%)
0
20
40
60
80
100
**
****
****
****
****
****
30
60
90
Time (s)
Whole Semen
c
IgG-N-Film
FIF-N-Film
Analysis stopped at 90
Seconds to 90% Agglutination
0
30
60
90
1.56
6.25
25
Concentration (µg/mL)
d
1.56 µg/mL
6.25 µg/mL
25 µg/mL
Reduction in Progressively Motile Sperm (%)
0
20
40
60
80
100
120
*
**
***
***
*
*
30
60
90
Time (s)


a
Progressively Motile Sperm (%)
40
30
20
10
0
**
**
Control
IgG-N-Film
FIF-N-Film
b
Anti-RSV IgG
5 µm
IgG-N-Film
5 µm
FIF-N-Film
5 µm


a
Collect fluids for
immediate analysis
4 hrs
2 mins
Simulate
intercourse
(15 thrusts)
Simulate
intercourse
(5 thrusts)
Instill Film
Add Semen
b
****
Reduction in Progressively
Motile Sperm (%)
100
80
60
40
20
0
Placebo Film
FIF-N-Film

Supplementary Materials for

Hexavalent sperm-binding IgG antibody released from vaginal film for development of potent on-demand non-hormonal female contraception

Bhawana Shrestha, Kathleen Vincent, Alison Schaefer, Yong Zhu, Gracie Vargas, Massoud Motamedi, Kelsi Swope, Josh Morton, Carrie Simpson, Henry Pham, Miles B. Brennan, Michael H. Pauly, Larry Zeitlin, Barry Bratcher, Kevin J. Whaley, Thomas R. Moench, Samuel K. Lai*

*Corresponding author: Samuel K. Lai. Email: lai@unc.edu

This PDF file includes:

Supplementary Methods

Agglutination kinetics assay

FIF-N vs FIF-Expi293: This experiment was conducted using the starting concentration of 10 x 10^6 PM sperm/mL. Briefly, 4.4 µL of purified motile sperm was added to 4.4 µL of Ab constructs in 0.2 mL PCR tubes, and gently mixed. A timer was started immediately while 4.4 µL of the mixture was transferred to chamber slides with a depth of 20 µm (Cytonix), and video microscopy (Olympus CKX41) using a 10x objective lens focused on the center of the chamber slide was captured up to 90 s at 60 frames/s. PM sperm count was measured by CASA every 30 s up to 90 s. At least 3 independent experiments were done with at least 3 unique semen samples.

Safety assessment of mAbs

Endotoxin levels in mAbs were measured with Endosafe PTS (Charles River), which detects by measuring color intensity related to endotoxin concentration.

Bioburden was determined for IgG-N and FIF-N by counting the number of colony-forming units that formed after mAbs were incubated overnight on the bacterial agar plate at 37°C.

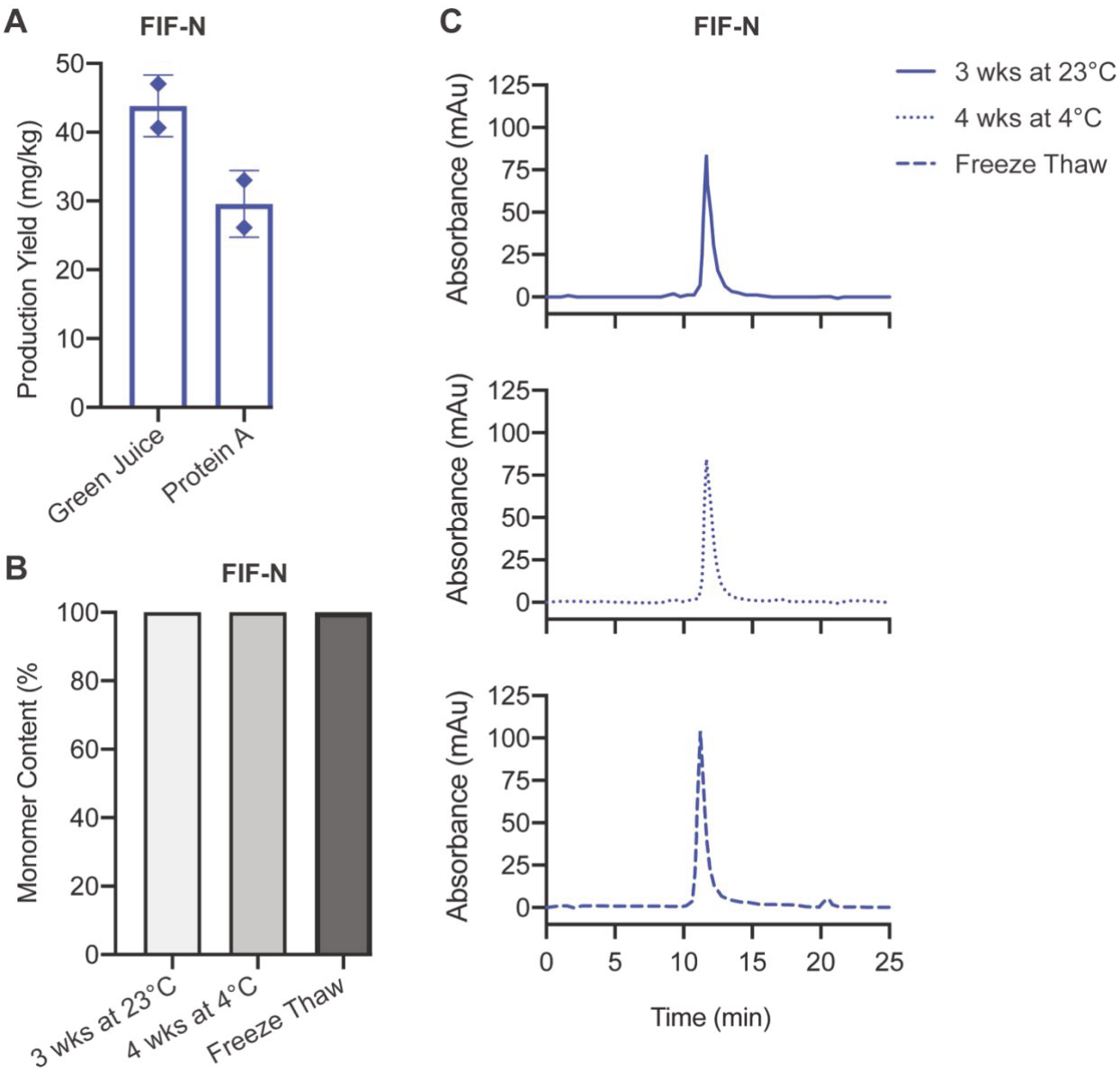


Fig. S1. Additional characterization of *Nicotiana*-produced FIF. (A) The production yield of IgG and FIF from *Nicotiana benthamiana* expression (Green Juice) followed by purification using protein A chromatography. Data were obtained from 2 independent transfections. Lines indicate arithmetic mean values and standard deviation. **(B)** Demonstration of the homogeneity of the FIF-N under different storage conditions using size exclusion chromatography (SEC) analysis. Y-axis indicates the total percentage of Abs representing their theoretical molecular weights. **(C)** SEC curves of the FIF-N stored under different conditions.

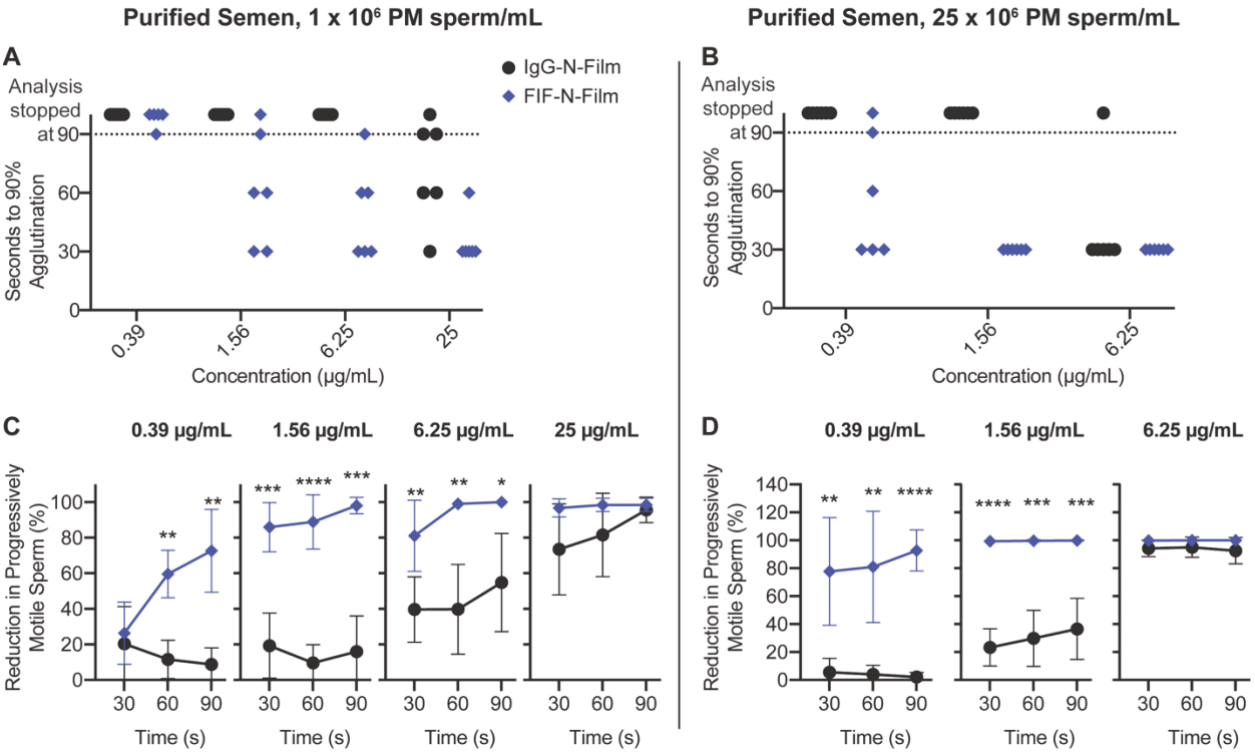


Fig. S2. FIF-N-Film demonstrates faster agglutination kinetics than IgG-N-Film at both low and high sperm concentrations. (A) Sperm agglutination kinetics of the IgG-N-Film and FIF-N-Film measured by quantifying time required to achieve 90% agglutination of PM sperm compared to media control using a final concentration of 1 x 10^6 PM sperm/mL and **(B)** 25 x 10^6 PM sperm/mL. **(C)** The rate of sperm agglutination of the IgG-N-Film and FIF-N-Film measured by measuring the reduction in the percentage of PM sperm at three different time points after Ab-treatment compared to negative control using a final concentration of 1 x 10^6 PM sperm/mL and **(D)** 25 x 10^6 PM sperm/mL. Data were obtained from N=6 independent experiments using 6 different semen donors. The experiment involving 1 x 10^6 PM sperm/mL was performed in duplicates and averaged. P values were calculated using a one-tailed t-test. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001. Data represent mean ± standard deviation.

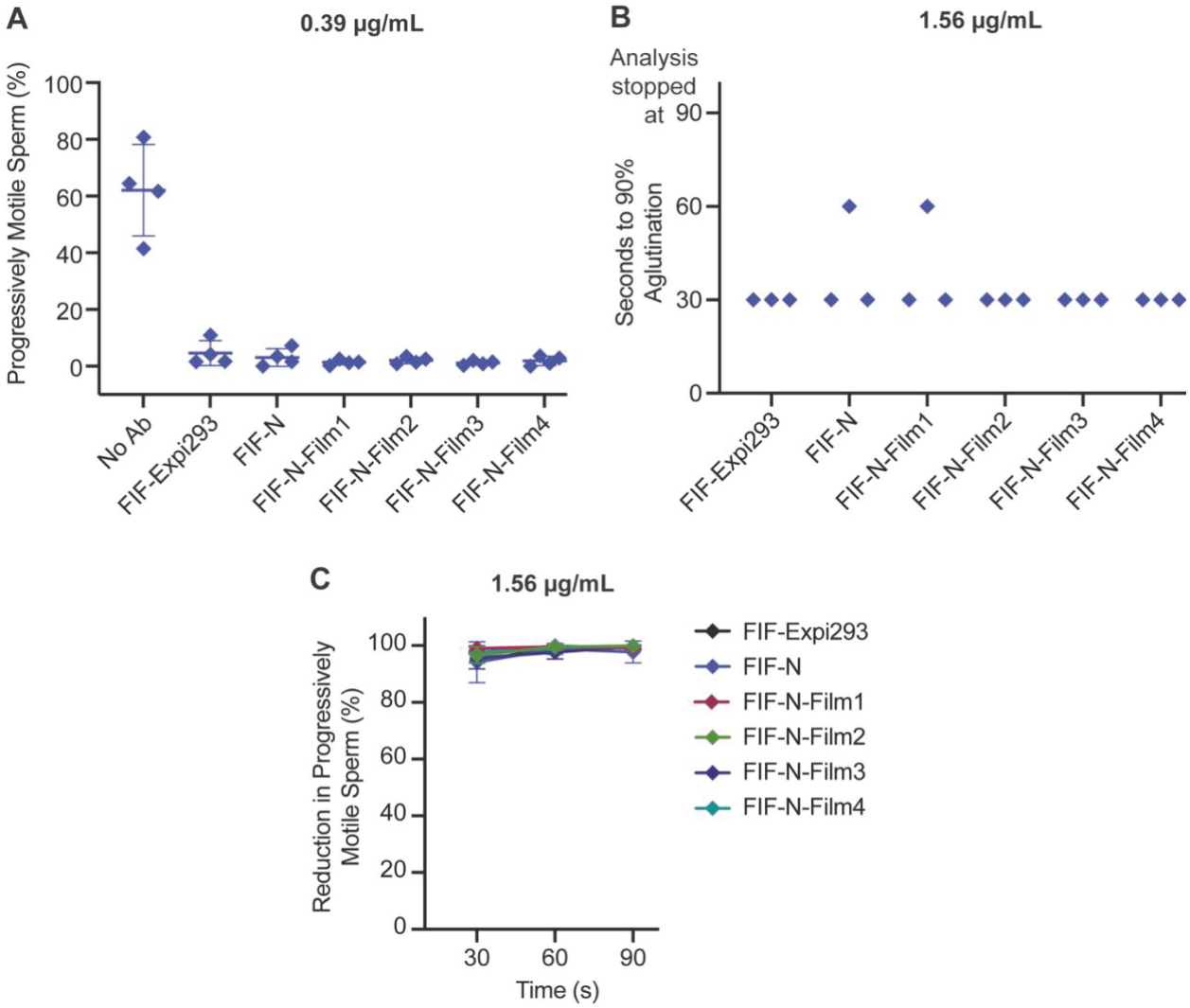


Fig. S3. *Nicotiana*-produced FIF exhibit agglutination comparable to Expi293-produced FIF. **(A)** Sperm agglutination potency of the indicated mAbs determined by quantifying PM sperm that escaped agglutination after Ab-treatment compared to pre-treatment condition using CASA. FIF-N-Film1 and FIF-N-Film2 are the film duplicates from one batch whereas FIF-N-Film3 and FIF-N-Film4 are the duplicates from a separate batch. **(B)** Sperm agglutination kinetics of the indicated mAbs measured by quantifying the time required to achieve 90% agglutination of PM sperm compared to media control. **(C)** The rate of sperm agglutination of the indicated mAbs measured by measuring the reduction in the percentage of PM sperm at three different time points after Ab-treatment compared to the negative control. Purified sperm at the final concentration of 5 x 10^6 PM sperm/mL was used for all experiments. A significant difference was not observed between the agglutination potency and kinetics of Expi293- and *Nicotiana*- produced mAbs upon one-way ANOVA with Dunnett's multiple comparisons tests. Agglutination potency data were obtained from N=4 independent experiments using 3 unique semen specimens. Agglutination kinetics data were obtained from N=3 independent experiments using 3 unique semen specimens. Data represent mean ± standard deviation.

Table S1. Safety parameter results for IgG-N-Film and FIF-N-Film.

Test Method	IgG-N-Film	FIF-N-Film
Endotoxin	Film1: <0.958 Film3: <26.1	Film1: 1.505 Film3: <0.953
Bioburden	Film1: 0 Film3: <1	Film1: 2 Film3: 0

Table S2. The sperm motility parameters of the Hamilton-Thorne Ceros 12.3.

Parameter	Value	Parameter	Value
Frames Per Sec	60	Path Velocity (VAP)	25 μm/s
No. of Frames	60	Straightness (STR)	80 %
Minimum Cell Size	3 pixels	VAP Cutoff	10 μm/s
Default Cell Size	6 pixels	VSL Cutoff	0 μm/s
Minimum Contrast	80	Slow Cells	Motile
Default Cell Intensity	20	Standard Objective	10X
Chamber Depth	20 μm	Magnification	1.87

Data S1. Raw data for figures.