Published in final edited form as:

Sci Transl Med. 2012 June 13; 4(138): . doi:10.1126/scitranslmed.3003453.

Mucus-Penetrating Nanoparticles for Vaginal Drug Delivery Protect Against Herpes Simplex Virus

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Abstract

Incomplete coverage and short duration of action limit the effectiveness of vaginally administered drugs, including microbicides for preventing sexually transmitted infections. We investigated vaginal distribution, retention, and safety of nanoparticles with surfaces modified to enhance transport through mucus. We show that mucus-penetrating particles (MPPs) provide uniform distribution over the vaginal epithelium, whereas conventional nanoparticles (CPs) that are mucoadhesive are aggregated by mouse vaginal mucus, leading to poor distribution. Moreover, when delivered hypotonically, MPPs were transported advectively (versus diffusively) through mucus deep into vaginal folds (rugae) within minutes. By penetrating into the deepest mucus layers, more MPPs were retained in the vaginal tract after 6 h compared to CPs. After 24 h, when delivered in a conventional vaginal gel, patches of a model drug remained on the vaginal epithelium, whereas the epithelium was coated with drug delivered by MPP. We then developed

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Supplementary Material

Materials and methods

Movie S1. Nanoparticle motions without flow.

Movie S2. Nanoparticle motions with induced flow.

Author contributions: L.M.E. and Y.Y.W. conducted imaging experiments; L.M.E. and B.C.T. conducted animal experiments; L.M.E. and T.A.T. conducted particle formulation and characterization experiments; T.H. conducted HSV-2 infection experiments. L.M.E., R.C., and J.H. directed all studies. L.M.E. wrote the manuscript with input and editing contributions from all authors.

Competing interests: The MPP technology is being developed by Kala Pharmaceuticals, of which J.H. is a co-founder, consultant, and director. J.H. and R.C. own company stock, which is subject to certain restrictions under University policy. The terms of this arrangement are being managed by the Johns Hopkins University in accordance with its conflict of interest policies.

MPPs composed of acyclovir monophosphate (ACVp). When administered prior to vaginal herpes simplex virus 2 (HSV-2) challenge, ACVp-MPPs protected 53% of mice, compared to only 16% protected by soluble drug. Overall, MPPs improved vaginal drug distribution and retention, provided more effective protection against vaginal viral challenge than soluble drug, and were non-toxic when administered daily for one week.

Introduction

Improved methods for sustained and more uniform drug delivery to the vagina may provide more effective prevention and treatment of conditions that impact women's health, such as cervical cancer, bacterial vaginosis, and sexually transmitted infections. For example, women are disproportionately infected with HIV, partly owing to a lack of female-controlled prevention methods (1, 2). An easily administered, discreet, and effective method for protecting women against vaginal HIV transmission could prevent millions of infections worldwide. However, vaginal folds, or "rugae", that accommodate expansion during intercourse and child birth, are typically collapsed by intra-abdominal pressure, making the surfaces of these folds less accessible to drugs and drug carriers (3). Poor distribution into the vaginal folds, even after simulated intercourse, has been cited as a critical factor for failure to protect susceptible vaginal surfaces from infection (4). Distribution over the entire susceptible target surface has been proven important for preventing and treating infections (5-9). Additionally, to increase user acceptability, drug delivered to the vagina should be retained in the vaginal tract at effective concentrations over extended periods of time. Achieving sustained local drug concentrations is challenging because the vaginal epithelium is highly permeable to small molecules (10) and also because soluble drug dosage forms (gels, creams) can be expelled by intra-abdominal pressure and ambulation (7, 9). Lastly, drug delivery methods must be safe and non-toxic to the vaginal epithelium. Improvements in the distribution, retention, and safety profile of vaginal dosage forms may lead to a substantial increase in efficacy and decrease in the side effects caused by largely ineffective systemic treatments for cervicovaginal infections and diseases (11, 12).

Nanoparticles have received considerable attention owing to their ability to provide sustained local drug delivery to the vagina (1, 13). However, the mucus layer coating the vaginal epithelium presents a barrier to achieving uniform distribution and prolonged retention in the vaginal tract. Mucus efficiently traps most particulates, including conventional polymeric nanoparticles (CPs), through both adhesive and steric interactions (14). The efficiency with which mucus traps foreign pathogens and particulates implies that CPs would become trapped immediately upon contact with the luminal mucus layer, preventing penetration into and, thus protection of, the rugae. Particles and pathogens trapped in the superficial luminal mucus layer would be expected to be rapidly cleared from the tissue, limiting the retention time of mucoadhesive materials, such as CP (14).

By mimicking viruses that have evolved to penetrate the mucus barrier to establish infection, we recently engineered mucus-penetrating particles (MPPs) for mucosal drug delivery by coating CPs with an exceptionally high density of low molecular weight poly(ethylene glycol) (PEG) (15-17). MPPs diffuse through human cervicovaginal mucus (CVM) at speeds comparable to their theoretical diffusion through water (16, 17). In this work, we sought to test the hypothesis that MPPs would provide enhanced distribution and increased retention in vivo in the vagina by penetrating into the deepest mucus layers, including the more slowly cleared mucus in the rugae, thereby releasing drug in the optimal location for efficient tissue uptake (Fig. 1A). In addition to the common progestin-induced diestrus phase (DP) mouse model, we introduce an estradiol-induced estrus phase (IE) mouse model in which the mouse CVM (mCVM) more closely mimics human CVM (hCVM) and

therefore provides a more human-like model for developing and translating MPPs for human use.

Results

Transport of nanoparticles on mouse vaginal tissue *ex vivo*

Carboxylic acid-coated, fluorescent polystyrene nanoparticles (PS-COOH) were made into MPPs by covalently attaching a dense coating of low molecular weight PEG, as previously reported (15, 16). Additionally, biodegradable MPPs (BD-MPP) were formulated with a poly(lactic-co-glycolic acid) (PLGA) core and a physically adsorbed PEG coating, as previously reported (18), because biodegradable particles can be loaded with drugs and are suitable for dosing to humans. PS-COOH and PLGA nanoparticles have a highly negative surface charge, which is nearly neutralized when densely coated with PEG. Nanoparticles were determined to be well-coated by measuring the zeta potential (ζ -potential) (Table 1). A ζ -potential more neutral than -10 mV was previously found to be necessary for mucus-penetrating properties in hCVM (15).

To ensure that MPPs were mucus-penetrating in native estrus phase mCVM, the particles were administered intravaginally to mice in the estrus phase. The entire vagina was then excised and opened to visualize the motions of hundreds of individual particles with a multiple particle tracking (MPT) method (19) developed in our lab. Particle trajectories for MPPs were indicative of rapid diffusion through watery pores in the mCVM, whereas motions of uncoated PS-COOH nanoparticles (CPs) were smaller than the particle diameter (~ 100 nm) (Fig. 1B). The ensemble averaged mean squared displacement ($\langle \text{MSD} \rangle$) of MPPs in mCVM was found to be comparable to that reported for MPPs in hCVM (20) (Fig. 1C), corresponding to ensemble averaged effective diffusivity ($\langle D_{\text{eff}} \rangle$) only ~ 8 -fold slower than the theoretical diffusion of 110 nm particles in water ($\sim 4 \mu\text{m}^2/\text{s}$). Based on the measured D_{eff} for individual particles, we estimated with Fick's Second Law of Diffusion that about half of the MPP would diffuse through a 100 μm -thick layer of mCVM in about 4 h, whereas even after 24 h there would be no appreciable penetration by CPs (Fig. 1D). D_{eff} values for CPs at a time scale of 1 s corresponded to MSD values less than the particle diameter (Fig 1E, dotted line), likely revealing thermal fluctuation of particles stuck to mucin fibers and not particle diffusion. Overall, the transport behavior of both MPPs and CPs in estrus phase mCVM was very similar to their transport behavior in hCVM.

Synchronizing a large number of mice in the estrus phase for retention studies required hormonal treatment. Particle transport behavior was tested in IE mice to confirm that estradiol treatment, which has been used routinely for inducing estrus-like behavior in many animal models (21, 22), did not alter MPP and CP transport behavior prior to distribution and retention studies (Fig. 1F). Additionally, BD-MPP transport behavior was indistinguishable from MPP in IE mucus (Fig. 1G).

Distribution of nanoparticles in the vagina

We next investigated in the estrus phase mouse and IE mouse whether the ability to rapidly penetrate mucus would lead to more rapid and uniform vaginal distribution of MPPs compared to CPs. We applied MPPs and CPs in hypotonic media to mimic the way osmotically driven water flux (advective transport) rapidly transports nutrients from the intestinal lumen to the brush border epithelial surface. Ten minutes after particle administration, the entire vagina was dissected out and stained for cell nuclei. CPs aggregated in the luminal mucus and did not penetrate into the vaginal rugae (Fig. 2A). In contrast, MPPs—both nonbiodegradable and biodegradable—formed a continuous particle layer that coated the entire vaginal epithelium, including all the surfaces of the rugae. MPPs

penetrated more than ~100 μm of mucus via advection within 10 min compared to the ~4 hours it would take them to diffuse that distance through mucus (Fig. 1E). This behavior was also consistent for BD-CPs and BD-MPPs, and CPs and MPPs administered to IE mice (Fig. 2A). Videos illustrating the movement of MPPs through hCVM past muco-adhesive CPs can be found in video S1 (no flow, diffusion) and video S2 (with flow, advection). To further characterize the effects of the mucus barrier, we found that removing vaginal mucus by lavage (23, 24) prior to particle administration markedly improved CP distribution, indicating that their mucoadhesive character prevents uniform distribution in the vagina (Fig. 2B).

To quantify the difference in distribution of MPPs and CPs, fluorescent images were obtained of freshly excised, opened, and flattened mouse vaginal tissue. The adhesion of CPs to luminal vaginal mucus layers created “stripes” of mucus with particles alternating with dark “stripes” of mucus without particles, the latter corresponding to the rugae that were opened when the vaginal tissue was flattened (Fig. 3A). In contrast, transport of MPPs toward the epithelium and into the rugae created a continuous particle coating on the flattened vaginal surface (Fig. 3A). Quantification of the fluorescence on the vaginal and ectocervical tissue indicated that 88% of the flattened vaginal surface and 87% of the ectocervical surface was densely coated with MPPs, whereas only 30% of the vaginal surface and 36% of the ectocervical surface was coated with CPs. Upon further inspection at higher magnification of darker areas of the vaginal and ectocervical surfaces, a continuous, less-concentrated coating of MPPs was seen (Fig. 3A, insets), implying that there was nearly complete coverage of the vaginal and ectocervical epithelium. For CPs, a less-concentrated coating was not found at higher magnification (Fig. 3A, insets). Similar trends were found with BD-MPPs, with 85% vaginal coverage and 86% ectocervical coverage, as well as BD-CPs, with 31% vaginal coverage and 27% ectocervical coverage (Fig. 3A).

We then sought to determine whether the improved distribution of BD-MPP could improve the delivery of small molecules as compared to a gel dosage form. Lipophilic molecules are likely to enter the first epithelial surface they contact, failing to contact cells in the rugae. Conversely, hydrophilic molecules can diffuse rapidly through the vaginal epithelium and be carried away by blood and lymph circulation leading to brief periods of coverage. We loaded BD-MPP with a fluorescent, water-soluble small molecule, fluorescein isothiocyanate (FITC), as a model drug (FITC/MPP). To mimic conventional vaginal delivery, soluble FITC (FITC/gel) was administered in the universal vaginal placebo gel hydroxyethylcellulose (HEC). Twenty-four hours after administration to estrus phase mice, the vaginal tissues were excised and flattened to expose the vaginal folds. Patches of FITC coated 42% of the vaginal surface when administered as FITC/gel, whereas FITC/MPP provided a well-retained FITC coating of 87% of the vaginal surface (Fig. 3B), even 24 h after particle administration.

Retention of nanoparticles in the vagina

We next sought to determine vaginal retention of MPPs compared to mucoadhesive CPs using our IE model. Fluorescent MPPs and CPs were administered intravaginally to IE mice. At specified time points, the entire reproductive tract (vagina and uterine horns) was excised and analyzed quantitatively with fluorescence imaging (Fig. 4A). After an initial decrease in particle fluorescence that was similar for MPP and CP (likely owing to initial “squeeze out” preceding mucus penetration), the remaining amount of MPPs stayed constant at roughly 60% (Fig. 4B). In contrast, the amount of CPs steadily decreased with time to 10% (6 h). Importantly, although CPs were distributed along the length of the vagina, this longitudinal coverage did not indicate CP penetrated mucus to reach the epithelium, nor surfaces inside the vaginal folds, as shown in Figure 2A.

Nanoparticle toxicity in the progestin-treated diestrus mouse vagina

The immune system is highly active at mucosal surfaces (25), especially those with surfaces covered with living cells, such as columnar epithelia in the endocervix in humans. Inflammatory effects of nanoparticles were investigated using mice pre-treated with Depo-Provera, a long-acting progestin treatment that synchronizes mice in the diestrus phase, during which the vaginal epithelium thins and becomes covered with living cells (this is important for experiments lasting 24 h or longer). In contrast, in estrus, the mouse vagina thickens from 4 to 7 cell layers to about 12 cell layers, and the epithelial surface is protected with many layers of dead and dying cells (26). Additionally, the progestin-induced diestrus phase (DP) mouse vaginal epithelium has an increased immune cell population, leading to enhanced acute inflammatory responses, whereas the estrus phase is characterized by an absence of immune cells (27).

Standard hematoxylin and eosin (H&E) staining was used to investigate potential toxic effects of intravaginally administered nanoparticles. Nonoxynol-9 (N9), a nonionic detergent known to cause vaginal toxicity (28), was used as a positive control, and PBS (saline) was used as a negative control. The same (BD-)MPPs and (BD-)CPs that were used for distribution and retention studies were tested for toxicity. As expected, N9 caused acute inflammation at 24 h that was not seen following PBS treatment (Fig. 5A). CP, like N9, caused pronounced neutrophil infiltration into the lumen, but MPPs did not cause this inflammatory effect (Fig. 5A).

Cytokine release with repeated vaginal application

Recent studies indicate that in response to certain vaginal products, the vaginal epithelium can secrete immune mediators that may enhance susceptibility to sexually transmitted infections (29, 30). Thus, it is important that a vaginal product not induce such an immune response, particularly after repeated dosing. Because our ultimate goal was to test MPPs for protection against HSV-2, we compared an MPP formulation containing acyclovir monophosphate (ACVp) to N9, HEC placebo gel, PBS, and a gel vehicle (TFV vehicle) used in recent tenofovir clinical trials. Nanoparticle and control formulations were administered vaginally to Depo-Provera-treated mice daily for seven days. Vaginal lavages were collected on day 8 from each mouse and assessed for cytokines that have been found to be elevated in response to epithelial irritation: interleukin 1 (IL-1), interleukin 1 (IL-1), tumor necrosis factor (TNF-), and interleukin 6 (IL-6). It was found that both IL-1 and IL-1 levels were elevated in response to both the TFV vehicle and N9 solution (Fig. 5B). This was not surprising in the case of N9 treatment, considering IL-1 and IL-1 are secreted by the vaginal epithelium in response to injury (29). In contrast, the cytokine levels associated with ACVp-MPP were equivalent to the levels associated with HEC placebo gel (Fig. 5B), which has been used in clinical trials without any associated increase in susceptibility to infection (31, 32). There was no detectable elevation of either IL-6 or TNF- associated with any vaginal treatment as compared to untreated controls.

Vaginal protection against HSV-2 in the progestin-treated diestrus mouse

We finally investigated whether the improved distribution, retention, and toxicity profile of MPPs would lead to improved protection against vaginal HSV-2 challenge in mice. Depo-Provera treatment markedly increases the vaginal susceptibility of mice to infections, and candidate microbicides have provided only partial protection in the mouse model used here, even when administered immediately before the infectious inoculum (4, 33). Moreover, several vaginal product excipients actually increase susceptibility to infection in this model (34, 35). We chose to test ACVp for blocking vaginal transmission of HSV-2 infections, because acyclovir provides viral suppression in animals with repeated dosing multiple times per day (36). However, a single vaginal pretreatment with 50 mg/mL (5%) ACVp in guinea

pigs resulted in 70% of animals infected compared to controls (37). Therefore, ACVp provided a test case to determine whether MPP could significantly improve protection by a water-soluble and quickly metabolized drug by prolonging therapeutically relevant drug concentrations after a single application. Additionally, the mechanism of action of nucleotide analogs, such as ACVp, is prevention of intracellular viral replication, such that successful protection implies efficient uptake and retention in susceptible target cell populations in the vaginal and cervical mucosa.

We formulated ACVp nanoparticles with the same muco-inert coating used for all other studies. The size and ζ -potential of ACVp-MPP were similar to polystyrene (PS)-based MPP (Table 1). Mice were administered soluble ACVp or ACVp-MPP intravaginally 30 min prior to HSV-2 challenge. Soluble drug administered at the same concentration as the ACVp-MPP (1 mg/mL) was ineffective at protecting mice from viral infection (84.0% infected compared to 88.0% of controls), whereas only 46.7% of mice in the ACVp-MPP group were infected (Table 2). Groups of mice given soluble drug at 10-times the concentration in ACVp-MPPs were still infected at a rate of 62.0% (drug in PBS) or 69.3% (drug in water). Comparing soluble drug to ACVp-MPP in the same vehicle (pure water), soluble drug was significantly less protective, even at 10-fold higher concentration than ACVp-MPP (Table 2).

Discussion

The female reproductive tract is susceptible to a wide range of sexually transmitted infections (1). Biological vulnerability, a lack of female-controlled prevention methods, and inability to negotiate condom use all contribute to male-to-female transmission worldwide (1, 2). An easily administered, discreet, and effective method for protecting women against vaginal HIV, HSV-2 and other virus transmission could prevent millions of infections worldwide. After 11 unsuccessful microbicide trials, CAPRISA 004 was the first to demonstrate partial protection against HIV with a vaginally administered microbicide (tenofovir) in a gel formulation (31). An important difference between previous generation microbicides, such as N9, and the current generation of microbicides is the site of action. Many current generation microbicides, such as nucleotide analogs tenofovir and acyclovir monophosphate, work intracellularly to inhibit viral replication, whereas previous generations directly inactivated pathogens in the vaginal lumen. However, some previous generation microbicides caused toxicity to the vaginal epithelium that increased susceptibility to infection (38).

For vaginal drug delivery to be maximally effective, topically delivered drugs must be distributed uniformly, maintained at sufficiently high concentration, and remain in close proximity to the folded vaginal epithelium (rugae) and cervical mucosa. Several techniques have been used to observe distribution of gels and drugs following vaginal administration, such as MRI (6), gamma-scintigraphy (6, 9), colposcopy (8), and fiber optics (6). These techniques are adequate to observe gross distribution along the vaginal tract, but do not reveal entry into vaginal folds. Our work demonstrates that, although a topical treatment may be well-distributed longitudinally along the vaginal tract, much of the folded epithelium can be left untreated and unprotected. Such untreated surfaces could have contributed to recent failures of several candidate microbicides against HIV in clinical trials (39). Additionally, when a fluid or gel is administered to the vagina, it directly contacts the rapidly shed outer luminal mucus layer. Mucoadhesive particles, such as CP, are trapped in this superficial mucus layer and thereby excluded from the rugae. In contrast, we demonstrated that MPPs are capable of penetrating deep into the mouse rugae and, when delivered hypotonically, provided complete coverage of the epithelium within only 10 minutes.

Diffusion of particles is not rapid enough to result in such a uniform epithelial coating within minutes. Diffusion over ~100 μm would take on the order of hours. However, the vaginal epithelium has a great capacity for fluid absorption induced by osmotic gradients. Absorption of water through the mucus barrier assists MPPs in rapidly reaching the entire epithelial surface by advection, where the drug payload can then be released for optimal tissue uptake. In contrast, water absorption was not beneficial for CP, because they became adhesively trapped and immobilized in the luminal mucus (video S2).

Inadequate retention of therapeutically active compounds in the vaginal tract is another limiting factor for vaginal protection. For example, many vaginal spermicides provide protection for no more than 1 h (40). Other vaginal products are not well-retained even after 6 h (7-9), necessitating repeated administration for adequate protection. Similarly, over 90% of CPs were shed from the vagina within 6 h because they did not penetrate deep into the mucus layers. In contrast, MPPs provided enhanced delivery of an encapsulated model drug (FITC) for at least 24 h, as compared to soluble drug in a gel formulation. Thus, MPPs may provide a means for achieving potent, once-daily, topical vaginal administration for treatments such as microbicides against sexually transmitted diseases.

In prior attempts to develop mucosal drug delivery systems for the vaginal tract, a variety of “pretreatments” have been used that diminish the mucus barrier. Administering fluids (24, 41, 42), swabs (24, 43), or degradative enzymes (44) prior to administration of mucoadhesive delivery vehicles was likely essential to the drug or gene delivery achieved in these studies. Here, we found that a lavage plus swab pretreatment markedly improved distribution of CPs in the vagina, allowing the particles to coat the epithelium similarly to MPP (Fig. 2B). Barrier-removing pretreatments may be impractical for human use, and especially inappropriate for microbicides intended to prevent sexually transmitted diseases. Healthy CVM itself is a somewhat effective barrier to viral infections (45). We show that effective epithelial coverage can be achieved by using MPPs, without the need to degrade or remove the mucus barrier.

PEG coatings have been widely used in developing polymeric drug carriers that are not easily recognized by the immune system (17). We demonstrated that dense PEG coatings produce MPPs that rapidly penetrate mucus without causing inflammation in the mouse vaginal tract. In contrast, administration of uncoated CPs resulted in an acute inflammatory response similar to administration of N9. Additionally, cytokine levels associated with daily administration of MPPs were indistinguishable from HEC placebo gel. Elevated levels of IL-1 and IL-1, which are associated with epithelial injury, occurred after daily dosing with both N9 and TFV vehicle gel. The tenofovir-containing version of this gel was shown to have complete protection against HIV in a tissue explant model, and complete protection occurred in spite of visible epithelial shedding (46). Previous work suggests that glycerol in the TFV gel may be responsible for the observed toxicity in mice (35).

Mice are useful animal models for developing vaginal products, but there are key differences in vaginal physiology between mice and humans. First, the estrous cycle occurs over a 4 to 5 day period in contrast to the 28-day human menstrual cycle. Throughout the four stages of the mouse estrous cycle, substantial growth is followed by sloughing of the epithelium, whereas there is relatively little change in the human vaginal epithelium throughout the menstrual cycle (47, 48). The late proestrus and early estrus phases of the mouse estrous cycle are the most similar to that of the human vaginal epithelium (48, 49). In these stages, there is significant bacterial colonization, including a peak in the presence of lactobacilli (50). Additionally, the estradiol influence causes active secretion of mucus (50, 51), which we found in mice is both penetrable by MPPs and cleared in a matter of hours, similar to humans. Thus, we believe the IE mouse model is a valuable model in addition to

the commonly used DP model for investigating vaginal delivery methods. Estradiol can be used to synchronize mice in the estrus phase, but does not “arrest” them in estrus; they continue to cycle, whereas DP treatment can arrest mice in a diestrus-like phase for days to weeks (52).

We have not yet investigated certain conditions that might affect MPP-mediated protection; for example, the potential effects of semen on MPP movement through mucus, or the impact of the MPP formulation on vaginal pH and bacterial flora were not tested. Mice and nonhuman primates have essentially neutral vaginal pH, because their vaginal flora is not lactobacilli-dominated. Therefore, it would be appropriate to investigate potential alterations in vaginal pH and bacterial flora in humans prior to any large-scale clinical trials. After MPPs were deemed suitable for repeated vaginal application in humans, partner studies could be done that would investigate the effect of coitus and the presence of semen on vaginal tissue drug concentrations. Nevertheless, we have shown that vaginally administered MPPs loaded with acyclovir monophosphate were more effective at protecting mice against vaginal HSV-2 infection than soluble drug, even at 10-fold higher soluble drug concentration. These results motivate further development of MPPs for safe and effective vaginal drug delivery, for prevention and treatment of sexually transmitted infections, contraception, and treatment of other cervicovaginal disorders.

Materials and methods

Mouse vaginal epithelium model

To study the distribution and retention of nanoparticles at the vaginal mucosal surface and the effects of repeated dosing, 6-8 week old CF-1 mice (Harlan) were used. Mice were housed in a reversed light cycle facility (12 h light/12 h dark). For naturally cycling estrus, mice were selected for external estrus appearance and confirmed upon dissection (53). For hormonally induced estrus (IE), mice were acclimated for 3 weeks and injected subcutaneously with 100 µg of 17- estradiol benzoate (Sigma) two days prior to the experiments. It has been demonstrated in numerous studies that treatment with estradiol induces an “estrus-like” state with analogous epithelial characteristics and vaginal cell populations (22, 51). For vaginal toxicity and cytokine release, mice were injected subcutaneously with 2.5 mg of Depo-Provera (medroxyprogesterone acetate, 150 mg/mL) (Pharmacia & Upjohn Company) 7 days prior to the experiments.

Water was used as the hypotonic medium for all particle solutions. For ex vivo tracking, 5 µL of particles were administered intravaginally. After approximately 10 min, the vagina was removed and carefully sliced open to lay flat. The whole tissue was placed in a custom-made well constructed such that a cover slip could be placed on top to contact the mucus without deforming the tissue. The well was a rectangle approximately 1 mm × 0.5 mm cut out of three layers of electrical tape adhered to a standard glass slide. Cover slips were sealed around the edges with superglue and imaged immediately to prevent drying.

Mice were anesthetized prior to experimental procedures, including sacrifice by cervical dislocation. For all studies, mice were prevented from self-grooming by a collar of mildly adhesive tape around the abdomen, and from inter-grooming by housing in individual cages. All experimental protocols were approved by the Johns Hopkins Animal Care and Use Committee.

Nanoparticle preparation and characterization

For conventional mucoadhesive particles (CPs), we used fluorescent, carboxyl (COOH)-modified polystyrene (PS) nanoparticles sized 100 nm (Molecular Probes). These particles feature a negatively charged surface at neutral pH (Fig. 1B). To produce mucus-penetrating

particles (MPPs), CPs were covalently modified with 5-kDa amine-modified PEG (Creative PEGworks) via standard 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide coupling reaction. Particle size and ζ -potential were determined by dynamic light scattering and laser Doppler anemometry, respectively, using a Zetasizer Nano ZS90 (Malvern Instruments). Size measurements were performed at 25°C at a scattering angle of 90°. Samples were diluted in 10 mM NaCl solution (pH 7) and measurements performed according to instrument instructions. A near-neutral ζ -potential, measured by laser Doppler anemometry, was used to confirm PEG conjugation.

For biodegradable particles, PLGA 2A (50:50 Lakeshore Biomaterials), Lutrol F127 (BASF), and poly(vinyl alcohol) (PVA, 25 kDa, Polysciences) were used. Alexa Fluor 555 was chemically conjugated to PLGA, which was used to produce nanoparticles by nanoprecipitation as described previously (18). Briefly, 10 mg/mL of labeled PLGA was dissolved in acetone or THF (with or without 2 mg FITC), and added dropwise to 40 mL of aqueous surfactant solution. After stirring for 2 h, particles were filtered through a 5- μ m syringe filter and collected by centrifugation (Sorvall RC-6+, ThermoScientific) and washed. Particle size and ζ -potential were determined as described.

ACVp-MPPs were prepared by dissolving ACVp in ultrapure water containing Lutrol F127. Zinc acetate was added at a molar ratio of 5:1 ACVp:Zn, to chelate the ACVp and render it water insoluble, and then immediately flash frozen and lyophilized. Particle characterization was conducted after reconstitution. The powder was reconstituted with ultrapure water prior to administration, with a final concentration of 1 mg/mL ACVp and 0.8 mg/mL Lutrol. Soluble ACVp was titrated with NaOH as needed to reach pH to 6-7. Particle size and ζ -potential were determined as described.

Multiple particle tracking (MPT)

The trajectories of the fluorescent particles in ex vivo vaginal tissue samples were recorded using a silicon-intensified target camera (VE-1000, Dage-MTI) mounted on an inverted epifluorescence microscope equipped with 100 $\times$ oil-immersion objective (numerical aperture 1.3). Movies were captured with Metamorph software (Universal Imaging Corp.) at a temporal resolution of 66.7 ms for 20 s. Trajectories of $n > 130$ particles were analyzed for each experiment, and three independent experiments were performed using tissue from different mice. The coordinates of particle centroids were transformed into time-averaged mean squared displacements ($\langle \text{MSD} \rangle$), calculated as:

$$\langle \Delta r^2(\tau) \rangle = [x(t+\tau) - x(t)]^2 + [y(t+\tau) - y(t)]^2$$

where τ is time scale (or time lag), x and y are the corresponding particle coordinates at time t , and r^2 is the MSD. This equation was used to calculate particle MSDs and effective diffusivities (D_{eff}), as previously demonstrated (16, 17). The calculated D_{eff} values were used for modeling of particle penetration through a mucus slab, as described previously (17).

Distribution of nanoparticles in the mouse vagina

Five μ L of either CPs or MPPs were administered intravaginally. The entire vagina was then removed and frozen in Tissue-Tek O.C.T. Compound (Sakura Finetek U.S.A., Inc.). Transverse sections were obtained at various points along the length of the tissue (between the introitus and the cervix) using a Microm HM 500 M Cryostat (Microm International). The thickness of the sections was set to 6 μ m to achieve single cell layer thickness. The sections were then stained with ProLong Gold (Invitrogen) antifade reagent with DAPI to visualize cell nuclei and retain particle fluorescence. Fluorescent images of the sections were

obtained with an inverted fluorescent microscope. To quantify nanoparticle distribution, 5 μ L of either CPs or MPPs were administered intravaginally. Within 10 minutes, vaginal tissues, including a “blank” tissue with no particles administered, were sliced open longitudinally and clamped between two glass slides sealed shut with super glue. This procedure completely flattens the tissue, exposing the folds. The “blank” tissue was used to assess background tissue fluorescence levels to ensure that all images taken were well above background levels. Six fluorescent images at low magnification and at least one image at high magnification were taken for each tissue. The images were thresholded to draw boundaries around the fluorescent signal, and then the area covered quantified using ImageJ software. An average coverage was determined for each mouse, and then these values were averaged over a group of $n = 3$ mice.

The cervix from each mouse was cut from the uterine horns and mounted using the same custom-made wells used for ex vivo particle tracking. The wells were sealed with a cover slip, and the background fluorescence levels determined using the blank tissue. One fluorescent image, constituting nearly the entire ectocervical surface, was taken at low magnification above tissue background levels. These images were thresholded in the same manner to determine the area covered with particles. At least one higher-magnification image was taken for each tissue to show individual particles.

Effects of mucus removal on mucoadhesive nanoparticle

For distribution with mucus removal, prior to particle administration, mice were given 2 $\times$ vaginal lavage with 50 μ L of PBS followed by a single swab with a cotton-tipped applicator. Subsequently, 5 μ L of CPs were administered intravaginally. The entire vagina was then removed and frozen in Tissue-Tek O.C.T. Compound (Sakura Finetek U.S.A., Inc.). Transverse sections were obtained at various points along the length of the tissue (between the introitus and the cervix) using a Microm HM 500 M Cryostat (Microm International). The thickness of the sections was set to 6 μ m to achieve single cell layer thickness. The sections were then stained with ProLong Gold (Invitrogen) antifade reagent with DAPI to visualize cell nuclei and retain particle fluorescence. Fluorescent images of the sections were obtained with an inverted fluorescent microscope.

Distribution and retention of drug molecules in the mouse vagina

FITC dye (Sigma-Aldrich) was mixed at 1 mg/mL in HEC gel kindly provided by T. Moench (Reprotect). Biodegradable MPP were prepared as described, loaded with FITC dye and suspended in 1% Lutrol F127. To evaluate distribution, 10 μ L of either gel or particle solution was administered intravaginally. After 24 h, the vaginal tissue was removed and cut open to lie flat. The tissue was then mounted between two microscope slides and squeezed to flatten the rugae. A “blank” tissue was included to determine background autofluorescence from the vaginal tissue, to ensure that the exposure setting used was indicative of FITC presence. Fluorescent images of the dye distribution on the flattened tissue surface were obtained using a Nikon E600 inverted microscope equipped with a 2 $\times$ objective. These images were thresholded in the same manner using ImageJ to determine the coverage area.

Retention of nanoparticles in the mouse vagina

To evaluate nanoparticle retention, 5 μ L of red fluorescent CPs or MPPs were administered intravaginally. Whole cervicovaginal tracts were obtained at 0, 2, 4, and 6 h and placed in a standard tissue culture dish. For each condition and time point, $n > 7$ mice were used. Fluorescence images of the tissues were obtained using the Xenogen IVIS Spectrum imaging device (Caliper Life Sciences). Quantification of fluorescent counts per unit area was calculated using the Xenogen Living Image 2.5 software.

Acute toxicity of nanoparticles

Five μL of particles or control solutions were administered intravaginally to the DP mouse model. After 24 h, whole cervicovaginal tracts were obtained and fixed in 4% paraformaldehyde solution for 24 h. Tissues were placed in 70% ethanol and taken to the Johns Hopkins Reference Histology Laboratory for paraffin embedding and standard H&E staining.

HSV-2 challenge in the mouse vagina

Female 6-8 week old CF-1 mice were subcutaneously injected with medroxyprogesterone acetate, and one week later received 20 μL of test agent or PBS intravaginally with a firepolished positive displacement capillary pipette (Wiretrol, Drummond Scientific). Thirty minutes later, mice were challenged with 10 μL of inoculum containing HSV-2 strain G (ATCC #VR-734, 2.8×10^7 TCID₅₀ per mL). HSV-2 was diluted 10-fold with Bartel's medium to deliver 10 ID₅₀, a dose that typically infects ~85% of control mice. Mice were assessed for infection three days later after inoculation by culturing a PBS vaginal lavage on human foreskin fibroblasts (Diagnostic Hybrids, MRHF Lot #440318W), as described previously (34). In this model, input (challenge) virus is no longer detectable in lavage fluid if it is collected more than 12 hours after the challenge.

Vaginal cytokine release with repeated administration

Twenty μL of each test agent was administered intravaginally to the DP mouse model once-a-day for seven days. HEC gel and N9 were provided by T. Moench (Reprotect), and TFV vehicle gel was kindly provided by C. Dezzutti (University of Pittsburgh). On the eighth day, each mouse was lavaged twice with 50 μL of PBS. Each lavage sample was diluted with an additional 200 μL of PBS and centrifuged to remove the mucus plug. Supernatant (200 μL) was removed and split into 50 μL for each of the four (IL-1 β , IL-1 α , TNF- α , and IL-6) Quantikine ELISA kits (R&D Systems). ELISAs were conducted per the manufacturer's instructions.

Statistical analysis

All data are presented as a mean with standard error of the mean (SEM) indicated. Statistical significance was determined by a two-tailed, Student's t-test ($\alpha = 0.05$) assuming unequal variance. In the case of HSV-2 challenge, statistical significance was determined using Fisher's exact test, two-tailed distribution.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank H. Patel (ELISA analysis), K. Maisel (particle tracking), the animal husbandry staff at Johns Hopkins, and the Wilmer Microscopy and Imaging Core Facility (MICE).

Funding: This work was supported by the NIH (grants R33AI079740, R01CA140746, R21AI094519, R21EB008515) (J.H., R.C.), and National Science Foundation (L.M.E. and Y-Y.W.) and Howard Hughes Medical Institute (L.M.E) graduate research fellowships.

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Figure 1. Characterization of CP and MPP

(A) Graphical depiction of vaginal drug delivery from gel, CP, and MPP formulations. (B) Representative trajectories for particles exhibiting effective diffusivities within one SEM of the ensemble average at a time scale of 1 s. (C) Ensemble-averaged geometric mean square displacements ($\langle \text{MSD} \rangle$) as a function of time scale. Data for particles on *ex vivo* estrus phase mouse vaginal tissue (mCVM) compared to the same particles in *ex vivo* human CVM (hCVM) (20) and the theoretical diffusion rate of 110 nm particles in water. (D) Percentage of particles capable of penetrating a 100 μm -thick layer of mCVM over time, based on Fick's Second Law of Diffusion simulation of particles undergoing random diffusion. (E) Distributions of the logarithms of individual particle effective diffusivities (D_{eff}) at a time scale of 1 s. Data represent the average of three independent experiments, with $n = 150$ particles. Diffusivity values to the left of the dotted line indicate particles with MSD values less than the particle diameter. (F, G) $\langle \text{MSD} \rangle$ as a function of time scale,

comparing MPP to CP (F) as well as MPP to BD-MPP (G) in estrus and IE mice. The theoretical diffusion rate of 110 nm particles in water is included. Data (C, D, F, G) are means $\pm$ SEM (three independent experiments, n = 130 particles).

Figure 2. Particle distribution in the mouse vagina

(A) Distribution of red fluorescent non-biodegradable and biodegradable CPs and MPPs in transverse cryosections of estrus phase and IE mouse vaginal tissue. (B) Distribution of red fluorescent non-biodegradable and biodegradable CPs in transverse cryosections of mouse vaginal tissue with either an intact mucus layer (no treatment) or mucus removed by lavage and swabbing (mucus removed). Images are representative of $n = 3$ mice.

Figure 3. Quantification of nanoparticle and drug coverage in vagina and ectocervical tissue (A) Distribution of red fluorescent non-biodegradable and biodegradable CPs and MPPs on flattened estrus phase mouse vaginal and ectocervical tissue. Insets are images of dark areas at higher magnification. (B) Distribution and retention of a model drug, FITC, in the estrus mouse vagina delivered in gel form or encapsulated in biodegradable MPPs. Fluorescent images of flattened mouse vaginal tissue after 24 h. All images are representative of the averages calculated for $n = 3$ mice and were quantified as percent coverage $\pm$ SEM. $*P < 0.05$ compared to CP or FITC/gel, Student's t test.

Figure 4. Retention of non-biodegradable MPPs and CPs in the IE mouse cervicovaginal tract (A) Overlay of particle fluorescence intensity and photographic images for CPs and MPPs in whole cervicovaginal tract tissue. Images chosen are representative of the average at each time point. (B) Fraction of particles remaining over time based on quantification of particle fluorescence in (A). Data are means $\pm$ SEM ($n = 7$). * $P < 0.05$ compared to CP, Student's t test.

Figure 5. Acute toxicity and cytokine concentrations with daily administration

(A) Hematoxylin and eosin (H&E)-stained cross-sections of mouse DP vaginal tissue excised 24 h after intravaginal administration of 5% N9, PBS, CPs, MPPs, BD-CPs, and BD-MPPs. Scale bar applies to all images. Arrowheads point to clusters of neutrophils (stained purple). Images are representative of $n = 5$ mice. (B) Cytokine concentrations in DP mouse cervicovaginal lavage after daily vaginal treatments for 7 days. Data are means $\pm$ SEM. * $P < 0.05$ compared to ACVp-MPP, Student's t test.

Table 1**Particle characterization**

Data are mean $\pm$ SEM ($n = 3$). PDI = polydispersity index

Particle Type	Size (nm)	-potential (mV)	PDI
PSPEG (MPP)	112 $\pm$ 3	-4.2 $\pm$ 0.6	0.03
PS (CP)	87 $\pm$ 4	-39.0 $\pm$ 2.4	0.10
PLGA/F127 (BD-MPP)	152 $\pm$ 6	-4.2 $\pm$ 0.3	0.06
PLGA/PVA (BD-CP)	161 $\pm$ 15	-6.7 $\pm$ 0.1	0.06
ACVp-MPP	65 $\pm$ 10	-6.3 $\pm$ 1.0	0.37

Table 2
HSV-2 vaginal challenge results

Infections in DP mice resulting from vaginal challenge with HSV-2 30 minutes after drug administration. Statistical significance was determined using Fisher's exact test, two-tailed distribution. N/A, not applicable.

Group	Drug form (concentration)	Vehicle	Number of mice	Number infected	Infected mice (%)	P-value (vs. ACVp-MPP)
1	Placebo	PBS	100	88	88.0	<0.001
2	Soluble ACVp (1 mg/mL)	PBS	25	21	84.0	0.002
3	Soluble ACVp (10 mg/mL)	PBS	100	62	62.0	0.10
4	Soluble ACVp (10 mg/mL)	Water	75	52	69.3	0.02
5	ACVp-MPP (1 mg/mL)	Water	45	21	46.7	N/A