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Intravaginal gene silencing using biodegradable polymer nanoparticles densely loaded with small-interfering RNA

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Abstract

Vaginal instillation of small-interfering RNA (siRNA) using liposomes has led to silencing of endogenous genes in the genital tract and protected against challenge from infectious disease. Although siRNA lipoplexes are easily formulated, several of the most effective transfection agents available commercially may be toxic to the mucosal epithelia and none are able to provide controlled or sustained release. Here, we demonstrate an alternate approach, using nanoparticles composed entirely of FDA-approved materials. To render these materials effective for gene silencing we developed novel approaches to load them with high amounts of siRNA. A single dose of siRNA-loaded nanoparticles to the mouse female reproductive tract caused efficient and sustained gene silencing. Knockdown of gene expression was observed proximal (in the vaginal lumen) and distal (in the uterine horns) to the site of topical delivery. In addition, nanoparticles penetrated deep into the epithelial tissue. This is the first report demonstrating that biodegradable polymer nanoparticles are effective delivery vehicles for siRNA in the vaginal mucosa.

The female reproductive mucosa is a major port of entry for many viral and bacterial pathogens that can cause infectious, inflammatory, and neoplastic diseases. Agents that can be applied topically to prevent transmission of these pathogens are widely recognized as the best defense against sexually transmitted infections (STIs) including HIV/AIDS^{1, 2}. Intravaginal delivery of small molecules³, peptides⁴, proteins^{5, 6}, and DNA⁷ are promising agents for microbicide development. Agents that can activate the RNA interference (RNAi) pathway by delivering small-interfering RNA (siRNA) or short-hairpin RNA (shRNA) targeting specific viral and bacterial pathogens may also be promising microbicides^{8, 9}. Systemic delivery of a combination of siRNAs targeting host and viral factors was shown to

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AUTHOR CONTRIBUTIONS K.A.W. and W.M.S. designed the experiments. K.A.W. prepared and characterized the siRNA polymer nanoparticles. K.A.W. and M.J.W. performed collected the *in vitro* cell culture experiments, and also collected and analyzed the data. K.A.W. and J.K.S.S performed the *in vivo* animal experiments and harvested the reproductive tissue. K.A.W. performed the tissue sectioning, and collected and analyzed the microscopy images. K.A.W. performed the immunohistochemical staining for macrophages. C.J.B. performed histological examination of sectioned tissues. Y.C. prepared fluorescent nanoparticles. Y.C. performed, collected, and analyzed the data for fluorescent polymer nanoparticle delivery to the mouse female reproductive tract.

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be effective in suppressing HIV-1 infection in humanized mice,¹⁰ but systemic delivery is not ideal for prevention of local infection.

Agents and delivery systems must be effective, safe, and nonirritating to the delicate vaginal mucosa to be useful as a topical microbicide¹¹. Microbicides that induce inflammation or cause epithelial disruption may render this tissue more permissive to infection. By far, the most widely used systems for delivering siRNA are liposomal carriers. Local and systemic delivery of siRNA lipoplexes in different disease models has produced promising results that may translate to treatments for cancers, viral diseases, and genetic disorders^{10, 12-15}. For example, lipid-mediated delivery of siRNA targeted against essential viral proteins was proven to be effective against HSV-2 challenge in mice after intravaginal application⁸. However, there are serious concerns with using lipoplexes especially for local delivery to the vaginal mucosa to protect against infectious diseases such as HIV-1. For example, some cationic lipids are known to be cytotoxic and unstable at high ionic conditions¹⁶⁻¹⁹. Lipoplexes have also been documented to cause dose-dependent toxicity and to elicit immune activation^{20, 21}. In addition, designing lipid-based delivery systems for targeting cells involved in HIV-1 infection (macrophages, dendritic cells, and T-lymphocytes) is complex and may be too costly for microbicide development²².

We developed a surprisingly simple alternative delivery strategy for siRNA based on biodegradable and biocompatible polymers. Here we show that PLGA nanoparticles can be densely loaded with siRNA and, when applied topically to the vaginal mucosa, lead to efficient and sustained gene silencing. Over one-thousand molecules of siRNA per particle were entrapped by polymer nanoparticles of <200 nm diameter. A single topical application of these siRNA nanoparticles produced effective and sustained gene silencing throughout the female reproductive tract for at least 14 d. Our nanoparticles produce significant levels of gene knockdown and are less irritating and inflammatory compared to delivery of siRNA lipoplexes. We hypothesize that these materials—which are well known to improve agent stability—will also enhance internalization^{23, 24}, enable cell-specific targeting²⁵, and prolong the gene silencing effect by providing sustained release of siRNA^{26, 27}.

A mimic was used to develop methods for formulating and characterizing siRNA loaded PLGA nanoparticles. The mimic was formed by annealing two DNA oligonucleotides to form a double-stranded product (dsDNA) that preserves the molecular weight and charge density of siRNA but uses deoxynucleotides for increased stability. We used this mimic to test the hypothesis that loading and encapsulation efficiency of small duplex nucleic acids could be improved by pre-complexing with low molecular weight, natural polyamines. We tested endogenous polyamines known to interact and stabilize nucleic acids *in vivo*^{28, 29}. Gel-shift assays were used to determine the optimal ratio to combine the mimic and polyamines, and then various dsDNA nanoparticles were formulated using a double-emulsion solvent evaporation technique³⁰. We observed that initial loading could enhance actual loading but, even at the highest initial loading tested (200 nmoles), encapsulation of naked mimic resulted in only ~6 pmoles siRNA per milligram of PLGA (<5% encapsulation efficiency). We obtained the most dramatic increase in loading and encapsulation efficiency by combining a high initial loading and co-encapsulating the naked mimic with either

spermidine or putrescine (see Supplementary Information Fig. S1). In fact, complexes prepared with spermidine improved loading of the mimic by >40-fold.

Our experience with the mimic led us to select spermidine (Spe) as a counterion for encapsulating various siRNA sequences. All nanoparticles exhibited a similar size, morphology, and surface charge that were independent of the initial loading, siRNA target sequence, or polyamine used in the formulation (see Supplementary Information Table S1 and Fig. S2). PLGA nanoparticles encapsulating an siRNA targeted against the gene encoding for mitogen activated protein kinase (MAPK1) were formulated at a molar ratio of the polyamine nitrogen to nucleic acid phosphate of 8:1 (N:P ratio)³¹. We obtained an encapsulation efficiency of 40% and a loading of 50 pmoles/mg PLGA. These values agree with the results obtained with the mimic and equate to several hundred molecules of siRNA per nanoparticle. Lower N:P ratios produced loadings of several micrograms siRNA per milligram of polymer, or >1,000 molecules of siRNA per nanoparticle.

After preparation of the siRNA nanoparticles, we extracted siERK2 and showed that it was as efficient as a positive control in silencing MAPK1 expression in cultured cells (see Supplementary Information Fig. S3). This result demonstrates that the formulation process does not alter the physicochemical or functional properties of the siRNA. Furthermore, the extracted siERK2 required a transfection agent to reduce MAPK1 expression, suggesting that siERK2/Spe alone is inefficiently transported intracellularly and therefore unable to enter the RNAi pathway and activate gene silencing.

We evaluated the cytotoxicity of our siRNA polymer nanoparticles in cell culture prior to testing their bioactivity. We did not observe a decrease in cell viability of HepG2 hepatocytes or HeLa cervical carcinoma cells to any components in our polymer nanoparticles (Fig. 1a). Free spermidine, spermidine-loaded PLGA nanoparticles, dsDNA/spermidine-, and siRNA/spermidine-loaded nanoparticles were evaluated for cytotoxicity at concentrations as high as 10 mg/mL. We observed that neither the PLGA nanoparticles singularly or in combination with spermidine, the mimic, or siRNA were cytotoxic in the cell types and over the range of concentrations that were evaluated.

To test the bioactivity of our siRNA nanoparticles, we administered siERK2 nanoparticles (0.5 mg/mL) to cultured cells and measured target mRNA levels relative to a housekeeping control gene (GAPDH). We observed targeted knockdown of *erk2* mRNA in NIH/3T3 fibroblasts and HepG2 hepatocytes (see Supplementary Information Fig. S4). However, knockdown of MAPK1 expression in HeLa cells was observed only after doubling the nanoparticle concentration in suspension (see Supplementary Information Fig. S5). This suggests that the silencing efficiency of our siRNA nanoparticles is both dose and cell dependent. A dose-response curve showed that our siRNA polymer nanoparticles produced equal or better gene silencing compared to siRNA delivered using a commercial transfection agent, Lipofectamine™ RNAiMax (LRM, Invitrogen) (Fig. 1b).

PLGA nanoparticles loaded with siMAPK1 were subsequently evaluated for their ability to sustain gene silencing in cell culture. *In vitro* controlled release profiles show that our particles produced a modest burst release after the first day followed by sustained linear

release of siRNA (see Supplementary Information Fig. S6). Even after 30 d, less than 50% of the encapsulated siERK2 had been released. These sustained release profiles translated to prolonged gene silencing in HepG2 cell cultures, where we observed that siERK2 nanoparticles produced significant and sustained gene silencing (>14 d) compared to the commercial LRM agent (< 7 d) (see Supplementary Information Fig. S7).

We evaluated the biodistribution and tissue retention of our polymer nanoparticles using PLGA particles loaded with a coumarin-6 fluorescent dye. The high loading and fluorescence quantum yield associated with using coumarin-6 particles enabled us to sensitively track the biodistribution of our nanoparticles *in vivo* compared to using PLGA nanoparticles loaded with a fluorescently-labeled siRNA (data not shown). The coumarin-6 nanoparticles (C6NP) were applied topically to the cervicovaginal tract and fluorescent imaging techniques were used to monitor their distribution in the local tissue. One day after administration, intense fluorescence was detected in the vaginal canal, where fluorescence was 30-40 times greater than the signal measured in PBS treated controls (Fig. 2a). Using multiphoton microscopy, we were able to detect fluorescent nanoparticles distributed distally in the uterine horns (Fig. 2b). Tissue cross-sections showed C6NP well below the surface of the tissue, with certain regions showing particles as deep as 75 μm below the luminal wall in the vaginal tract (Fig. 2c) and uterine horns (Fig. 2b). Multiphoton microscopy was also used to measure the residence time and tissue distribution of nanoparticles applied after only a single topical application. Indeed, we detected fluorescent nanoparticles throughout the reproductive tract and as deep as 120 μm below the luminal surface after 3, 5, and 7 d (Fig. 2d). Tissue sections taken from control animals instilled with PBS showed no fluorescence signal in the channels used to detect C6NP. Confocal microscopy confirmed that nanoparticles were internalized by cells and distributed intracellularly throughout the cytoplasm and around the nucleus (see Supplementary Information Fig. S8).

In vitro release measurements of our higher loaded siRNA nanoparticles were collected in acidic conditions to mimic the pH found in the cervicovaginal tract. After 24 h, the release of siRNA from polymer nanoparticles incubated at pH 5.0 begins to deviate from the release profile observed at pH 7.4, and showed a more rapid and linear release of the encapsulated siRNA (Fig. 3a). By two weeks, polymer nanoparticles maintained in acidic conditions released almost twice as much siRNA. SEM micrographs showed that acidic conditions led to more porous nanoparticles, whereas nanoparticles maintained at physiological pH had a 30% greater effective volume as determined by dynamic light scattering (Fig. 3a, inset). We also observed that siRNA released at pH 5.0 was chemically intact and functional (Fig. 3b). In these experiments, siRNA released at acidic pH was collected after 24 h, concentrated, and then analyzed by gel electrophoresis and for bioactivity. The released siRNA had the same electrophoretic mobility as a standard (Fig. 3b, inset). In addition, when the released siRNA (designed to target luciferase) was transfected into a HEK-293T cell line stably expressing luciferase, we observed a similar reduction in gene expression relative to a positive control.

Based on the promising results from the *in vivo* biodistribution experiments and the functional integrity of our siRNA nanoparticles in low pH conditions, we predicted that

vaginal instillation of siRNA polymer particles would lead to sustained knockdown of gene expression *in vivo*. In a study modeled after the work of Palliser et al. (2006), we tested the efficiency of our siRNA nanoparticles versus the commercial LRM transfection agent to cause gene silencing in the female reproductive tract. An siRNA targeted against EGFP was formulated into biodegradable nanoparticles and lipoplexes that were then delivered by vaginal instillation into transgenic GFP mice. The siRNA sequence targeting GFP chosen for our *in vivo* studies has been used in several studies as a control sequence and, in these widely cited publications as well as recently by Robins et al. (2008), was shown to be non-immunostimulatory^{8, 32-35}.

We tested two different nanoparticle formulations where the siEGFP was pre-complexed with either spermidine or protamine. Lipoplexes were prepared with LipofectamineTM RNAiMax (LRM) according to published procedures³⁶. A single dose of 500 pmoles siEGFP was administered per animal and GFP expression was monitored over 2 weeks (total length of experiment). In all cases, delivery of siEGFP led to a reduction in gene expression throughout the regions of the reproductive tract that we examined: vaginal tract, cervix, and uterine horns. The most significant reduction in tissue fluorescence was observed in the vaginal tract at 10 d post-administration ($p = 0.001$) (Fig. 4). Silencing of EGFP expression appeared throughout the vaginal epithelium and submucosa (see Supplementary Information Fig. S7). Both PLGA nanoparticle formulations reduced GFP expression in this region by 50-60%, which was similar to silencing produced from LRM delivery of siEGFP. Nanoparticles also produced a significant reduction in GFP expression near the cervix ($p = 0.01$) and in the uterine horns ($p = 0.05$) after 10 d. These results were consistent with our IVIS and multiphoton imaging data that showed fluorescent PLGA nanoparticles concentrated throughout the reproductive tract and penetrated deep within the vaginal tissue (Fig. 2).

To determine the onset and duration of gene silencing, we examined the time course of gene knockdown produced from lipoplex and nanoparticle delivery of siRNA (Fig. 5). Reduced EGFP expression was seen earlier throughout all regions of the reproductive tract when using lipoplexes, except in the vagina where significant gene silencing was also observed at 7 d with the nanoparticles (Fig. 5c). In general, for all treatment groups, reduction of EGFP expression was greatest in the vaginal tract followed by the cervix and then by the uterine horns. However, a similar reduction in GFP expression was seen in all areas of the reproductive tract at the 14 d time point for groups treated with siEGFP nanoparticles.

Histological examination of haematoxylin-eosin (HE) stained sections of vaginal tissues from mice treated with PBS (Fig. 6a-c) or PLGA nanoparticles (Fig. 6d-f) were within normal limits and histologically similar. In contrast, mice treated with siRNA formulated into lipoplexes using LRM had thickened (hyperplastic) vaginal epithelium with frequent foci of luminal polymorphonuclear neutrophils (Fig. 6g-h) and distinctive areas of pus. These histological features are suggestive of symptoms associated with active vaginitis. Immunohistological staining for CD45 in the vaginal tissue revealed increased positive cells within the epithelium of mice treated with lipoplexes (Figs. 6i) compared with the other treatment groups (Figs. 6c and 6f). However, we did not observe a significant difference in IL-1 β or TNF- α levels detected by ELISA (data not shown).

RNA interference mediated by small-interfering RNA is a promising approach for prevention and treatment of human disease, and is moving rapidly from basic science to clinical application^{37, 38}. But delivery of siRNA is one of the most challenging problems in modern medicine. Despite vigorous effort, there are no generalizable, simple approaches for siRNA delivery that are both effective and safe. In fact, it is widely recognized that different disease targets may require different strategies for siRNA delivery. Here we use nanotechnology to create a new approach for *in vivo* siRNA delivery to mucosal surfaces. To convert an FDA-approved material into an siRNA delivery system, we solved two problems simultaneously: 1) make the particles small to allow penetration of tissue barriers and entry into cells, and 2) stably incorporate large quantities of siRNA into these small particles. To our knowledge, this manuscript contains the first report of successful *in vivo* delivery of siRNA from PLGA polymer nanoparticles to the vaginal mucosal surface.

We chose to build a delivery system from PLGA because it is already FDA-approved for a variety of clinical applications and has been used safely in humans for several decades³⁹⁻⁴¹. PLGA encapsulation of siRNA has been described previously by others, but the ability of these systems to activate RNAi-mediated gene silencing was not demonstrated rigorously^{27, 42, 43}. Recently, Murata et al. showed inhibition of tumor growth after intratumoral injection of PLGA microspheres encapsulating siRNA targeted against vascular endothelial growth factor (VEGF) ²⁶. However, these microspheres were too large to be endocytosed (35-45 μm)⁴⁴ and required that the anti-VEGF siRNA was first released extracellularly as a polyplex with either polyarginine or PEI before being internalized by cells. These microparticles may have limited applications because of the known toxicity of these polycations and the large size of the particles. Nanoparticles (100-300 nm) of PLGA are particularly attractive carriers because—as we demonstrate here—small particles can penetrate deep into tissue and are easily internalized by cells⁴⁴. Despite their small size, our nanoparticles were loaded with a high density of siRNA by using spermidine as a co-encapsulant. This low-molecular weight natural polyamine is present intracellularly at millimolar concentration and known not to be cytotoxic⁴⁵.

It will be surprising to many experts that this simple PLGA-based nanoparticle formulation leads to significant knockdown; a recent and comprehensive review of siRNA delivery vehicles does not even mention the potential of PLGA in this regard³⁸. Yet, we show that a single *in vivo* topical application of our PLGA polymer nanoparticles leads to widespread distribution and retention in the vaginal tissue for at least one week. We are unaware of any other published study showing *in vivo* biodistribution, penetration, and retention of topically applied nanoparticles for drug delivery to the vaginal mucosa. We also show that vaginal administration of siRNA nanoparticles leads to knockdown of gene expression throughout the reproductive tract that can be sustained for at least 14 d. These results suggest that nanoparticles encapsulating siRNA are internalized by the cells of the reproductive tract, and that siRNA released from the nanoparticles enters the RNAi pathway in the cell cytosol to cause gene silencing. Our data suggest that siRNA polymer nanoparticles were equal to or better than lipoplexes in silencing gene expression after a single topical dose. Controlled release data suggests that the nanoparticle systems may have delivered a siEGFP dose of as little as 200 pmoles after 10 d compared to 500 pmoles with LRM delivery (Fig. 3). Many

applications using siRNA have administered 1-2.5 mg/kg for effective gene silencing *in vivo*; other studies of vaginal delivery of siRNA have shown effectiveness using doses as small as ~0.3 mg/kg. Here we show that siRNA nanoparticles may efficiently cause gene silencing at a dose of 0.1 mg/kg. The efficient silencing produced by the siRNA nanoparticles was surprising due to the lower dosing. Since only 30-40% of the available dose was actually delivered using siRNA polymer nanoparticles, these systems may have greater potential than lipoplexes for enhanced and more sustained gene silencing. Furthermore, the lower toxicity and reduced potential for inducing an inflammatory response as seen with our siRNA nanoparticles may be important in applications that require high or frequent dosing such as with prophylactic microbicides against HIV-1.

Although cervical mucus is widely regarded as a significant barrier for the transport of polymeric nanoparticles, recent *in vitro* studies have indicated that size, surface chemistry, and concentration can significantly influence the mucosal transport of particles^{46, 47}. Indeed, we provide evidence to suggest that PLGA nanoparticles produce surprisingly efficient and safe delivery of siRNA throughout this tissue compartment. Our results expand the systems used currently for drug delivery in the vagina, and it is reasonable to speculate that this approach can be extended to other vulnerable mucosal surfaces such as those in the eye, lung, and digestive tract.

Small-interfering RNAs were synthesized using 2'-O-ACE-RNA phosphoramidites (A4 grade, Dharmacon Research). siRNA sequences directed against *erk2* and *EGFP* were as previously described⁴⁸. The sense and anti-sense strands of siRNAs used in these studies are listed in Supplementary Table 1. Preparation of siRNA nanoparticles and characterization of their size, siRNA loading, encapsulation efficiency, and controlled-release properties are described completely in Supplementary Information. Cell lines and culturing methods used in these studies, as well as all *in vitro* gene silencing experiments are also described in Supplementary Information.

All animal procedures were approved by the IACUC at Yale University. Female mice were subcutaneously injected with 2 mg Depo-Provera (medroxyprogesterone acetate, Pfizer). Intravaginal instillation of nanoparticles or lipoplexes was performed 4-5 d later after washing the reproductive tracts three times with 50 μ L of PBS and swabbing the vaginal canal once with a calcium alginate tip (Fisher). Biodistribution studies were performed with fluorescent PLGA nanoparticles using female ICR mice (5-6 weeks, Taconic Farms). Fluorescent nanoparticles encapsulating coumarin-6 dye (Acros Inc, 0.25-25 μ g coumarin-6/mg PLGA) were delivered intravaginally in a total volume of 15-20 μ L (1-10 mg nanoparticles). Animals were under anesthesia during the instillation and for 15-30 min afterwards. Individual mice (n=1) were sacrificed on 1, 3, 5, and 7 d upon which time the entire reproductive tract was removed and visualized using the IVIS 200[®] imaging system (Xenogen). Images were acquired using the Living Image 3.0 software with a GFP excitation/emission filter set, 15 s exposure time, f/4, and medium binning. A photograph of the same field of view was also obtained and combined with the pseudo-colored fluorescent image to form the final composite picture. Multiphoton microscopy images were obtained with a TriM Scope II microscope from the Yale University Facility for In Vivo Imaging Microscopy. Excised reproductive tracts were stained with Hoescht dye (5 min), mounted on

a glass slide with CitiFluor mounting media (EMS). Images (400×400×75 µm) were obtained using a 2-photon microscope with 860 nm excitation using the Inspector 3.40 software. Hoescht dye (blue) and coumarin-6 (green) was visualized by their respective optical filters. Composite Z-stacks were constructed using Volocity software (Improvision).

Intravaginal delivery of siRNA PLGA nanoparticles and lipoplexes were performed in transgenic EGFP mice. Female FVB.Cg-Tg(GFPU)5Nagy mice (6-8 weeks old) were obtained from Jackson Laboratories. Animal procedures were as described above for the biodistribution studies. Lipoplexes were prepared by combining 500 pmoles siEGFP with Lipofectamine™ RNAiMax (Invitrogen) according to the manufacturer's protocol and then administered intravaginally in a volume of 20 µL. Nanoparticles (5 mg, 500 pmoles) were instilled in 20 µL PBS. Animals were sacrificed by carbon dioxide asphyxiation at specified times, the reproductive tract were excised, and then flash frozen.

For fluorescence microscopy, dissected tissue was placed in optimal cutting temperature compound (TissueTek) and snap-frozen in liquid nitrogen. Microscopy images were collected on 9 µm tissue sections using an Olympus IX71 inverted fluorescent microscope. Tissue GFP fluorescence was analyzed by four independent individuals blinded to the treatment study and image fluorescence was quantified by image analysis software (ImageJ). Quantification was performed to determine the average integrated fluorescence intensity per unit area over 12 tissue sections. Data were collected and expressed as mean ± s.d. for each group. An unpaired t-test was used to discriminate the significant differences between two groups (two-tailed, P<0.05). For immunohistochemical staining, slides were fixed in acetone and haematoxylin-eosin (HE) staining was performed by Yale Research Histology. Immunohistochemical staining for CD45 was performed with the DAB Substrate Kit (BD Pharmingen) according to the manufacturer's recommendations. Examination and pathological scoring of HE and anti-CD45 stained sections of reproductive tracts were performed by an individual blinded to experimental manipulations. Digital light microscopic images were recorded using a Leica DM550B microscope (Leica Microsystems) with an AxoCam MRC Camera and AxioVision 4.4 imaging software (Carl Zeiss Microimaging, Inc.).

Supplementary Material

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Figure 1. In vitro cytotoxicity and bioactivity of siRNA nanoparticles

Polymer PLGA nanoparticles encapsulating siRNA were evaluated for cytotoxicity using cultured (a) HepG2 hepatocytes and HeLa cervical carcinoma cells. The toxicity of free spermidine (green diamonds), spermidine-loaded PLGA nanoparticles (black squares), dsDNA/spermidine- (red circles), and siRNA/spermidine-loaded (purple stars) nanoparticles were evaluated over a range of concentrations from 0-10 mg/mL. CellTiter Blue® fluorescence (Promega) was used to measure cell viability compared to untreated cells (blue line/triangles). Neither the PLGA nanoparticles singularly or in combination with spermidine, the mimic (dsDNA), or siRNA showed cytotoxicity in the cell types and over this range of concentrations. (b) Dose-response curves comparing the bioactivity of siRNA delivered with a transfection agent (red circles) or using PLGA nanoparticles (open squares). An siRNA targeted against the luciferase gene (siLUC) was delivered to cultured HEK-293T cells stably expressing luciferase. Luciferase activity was measured with the Bright-Glo™ (Promega) reagent and separate treated wells were used to measure cell viability using CellTiter Blue®. Luciferase activity normalized to viable cell number and plotted against the amount of delivered siLUC. PLGA nanoparticles show equal or better activity compared to a commercial transfection agent (Lipofectamine RNAiMax).

Figure 2. Fluorescent PLGA nanoparticles appear throughout the reproductive tract and penetrate deep within the vaginal tissue after topical administration

PLGA nanoparticles loaded with a fluorescent dye (coumarin-6) were vaginally instilled into the reproductive tract of female ICR mice at a dose of 750 mg per animal. The entire reproductive tract was imaged 24 h post-administration. (a) An in vivo imaging system (IVIS) was used to obtain fluorescent images of the whole tissue. Multiphoton microscopy was used to obtain deep tissue images of the vaginal tract (b) and uterine horns (c) 24 h post-treatment. Box highlights areas where nanoparticles were detected. Image dimensions are 400 mm μ 400 mm μ 75 mm. (d) Multiphoton microscopy images of the female reproductive tract in C56Bl/6 mice at 3, 5, and 7 d after a single topical treatment of fluorescent PLGA nanoparticles. Control animals were instilled with PBS and showed no fluorescence signal in the green channel used to detect nanoparticle fluorescence. Inset shows magnified areas enclosed in white box. Image dimensions are 400 mm μ 400 mm, with depths of 50-120 mm. Green, red, and blue arrows indicated x-, y-, and z-coordinates, respectively. For (b-d), Hoescht dye (blue) and coumarin-6 nanoparticles (green) were detected by excitation at 860 nm and visualized by their respective optical filters.

Figure 3. Release of PLGA nanoparticles densely-loaded with siRNA is sustained for several weeks

A high initial loading of siRNA (200 nmoles) was combined with spermidine and formulated into PGLA nanoparticles. (a) Under physiological pH in PBS ($I = 0.2$ M, $pH = 7.4$), cumulative release of siRNA/Spe is not significant after the initial burst release. In vitro release studies performed in acidic conditions with the same ionic strength as PBS (50 mM citrate buffer, $I = 0.2$ M, $pH = 5.0$), show linear and sustained siRNA release after 48 h. Representative SEM micrographs (inset) show that acidic conditions cause nanoparticles to exhibit pores or dimples on their surface (red arrow). (b) The siRNA released at pH 5.0 from our polymer nanoparticles is chemically intact and functional. After 24 h of controlled release at pH 5.0, siRNA was collected from the buffer, concentrated, and then analyzed by gel electrophoresis and for bioactivity in a HEK293T cell line stably expressing luciferase (293T-Luc). Released siRNA had a similar electrophoretic mobility to the stock material used as a standard (inset). The siRNA (designed to target luciferase) was also able to transfect and knockdown gene expression comparable to a positive control. Values represent the mean $\pm$ s.d.

Figure 4. Intravaginal delivery of siRNA using biodegradable nanoparticles causes gene silencing throughout the reproductive tract of transgenic GFP mice

A siRNA targeted against EGFP was delivered using lipoplexes (filled bar) or PLGA nanoparticles with the siEGPF pre-complexed with spermidine (diagonal bar) or protamine (hatched bar). Quantification of EGFP fluorescence per area of tissue was performed in the three different regions of the reproductive tract. Reduction in EGFP expression was seen in the vagina, cervix, and uterine horns in GFP transgenic mice 10 days after topical administration. Open bars are the cumulative negative controls (see Materials and Methods). Bars represent the mean $\pm$ s.d.

Figure 5. A single topical administration of PLGA nanoparticles loaded with siRNA causes sustained gene silencing throughout the reproductive tract

Vaginal instillation of siRNA using a commercial transfection agent or PLGA nanoparticles leads to EGFP silencing in the (a) uterine horns, (b) cervix, and (c) vaginal tract. An siRNA targeted against EGFP was formulated in LipofectamineTM RNAiMax (circles), or pre-complexed with spermidine (triangles) or protamine (diamonds) and then encapsulated into PLGA nanoparticles. Knockdown of EGFP expression was assessed by image analysis of tissue fluorescence compared to delivery of an siRNA mimic (squares). Values represent the mean $\pm$ s.d. for $n = 3$ per treatment group except for the negative controls where $n = 18$. Statistical significance was determined by a two-sample t-test with $p < 0.05$ (*). Asterisk above the group indicates all members were statistically different compared to the control, whereas the asterisk is placed adjacent to a group when it is the only member that showed significance.

Figure 6. Lipid delivery of siRNA may be inflammatory and cause epithelial disruption in the vaginal tissue

Histopathology of vaginal tissue in mice instilled with PBS (a-c), siRNA polymer nanoparticles (d-e), and lipoplexes (g-i). Histologic analysis of tissue sections from the vagina and uteri in mice treated with PBS (a, b (inset of a)) or siRNA nanoparticles (d, e (inset of d)) showed minimal to no inflammation by haematoxylineosin staining, exhibited equivalent vaginal epithelial thickness (b, e arrows), and showed no significant pathologic findings. In contrast, lipid-treated mice had marked vaginal epithelial hyperplasia (g, *) and frequent scattered neutrophils within the lumen (h (inset of g, arrowheads)) indicative of a mild to moderate vaginitis. Comparison of sides with anti- CD45 IHC revealed numerous CD45 positive cells within the epithelium of lipid-treated mice (i) in comparison to the nanoparticle-(f) or PBS (c) treated mice. L = vaginal lumen, E = epithelium. HE staining (a-b, d-e, g-h). DAB staining with methyl green counterstain (c, f, i). Scale bars a-b, d-e, g-h = 500 μ m. Scale bar c, f, i = 100 μ m.