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Injectable PAMAM Dendrimer–PEG Hydrogels for the Treatment of Genital Infections: Formulation and *in Vitro* and *in Vivo* Evaluation

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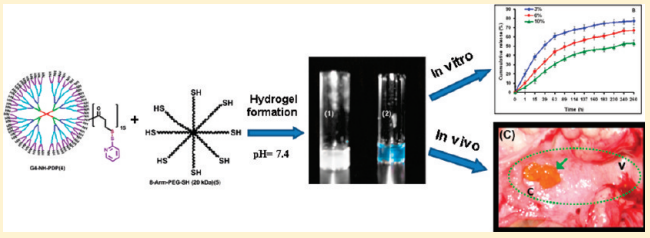
Supporting Information

ABSTRACT: Local intravaginal drug therapy is preferred for treatment of ascending genital infections during pregnancy. In the present study, an *in situ* forming biodegradable hydrogel for sustained release of amoxicillin in the cervicovaginal region is described. A generation 4 poly(amidoamine) [G4-$(NH_2)_{64}$] dendrimer with peripheral thiopyridyl terminations is cross-linked with 8-arm polyethylene glycol (PEG) bearing thiol terminations. The hydrogels were formulated and tested *in vivo* in a pregnant guinea pig model for volume, retention times, biodegradation, tolerability and transport across fetal membrane. The physicochemical characterization of the hydrogels was carried out using differential calorimetry, SEM, and confocal imaging. The hydrogels offer antibacterial activity arising from sustained release of amoxicillin from gels. The *in vivo* studies in guinea pig showed that 100–200 μL of gel sufficiently covered the cervicovaginal region with a residence time of at least 72 h and gel was primarily retained in the maternal tissues without crossing the fetal membranes into the fetus. The dendrimer gels were stable up to 72 h, and the *in vivo* biodegradation of gel occurred after 72 h; this correlated well with the *in vitro* degradation pattern. The pH of the vagina was not altered upon application of the gel, and none of the animals aborted up to 72 h after application of gel. The histological evaluation of the cervical tissues showed absence of edema in the epithelial cell layer, no sloughing of the epithelial or superficial mucous layer, and absence of necrosis and infiltration of inflammatory cells in the submucosal layers, confirming that tissues were tolerant to the gel. The immunohistofluorescence images showed the localization of the gel components on the superficial mucified epithelial layer. The cross-linking density and swelling of hydrogels was impacted by the polymer content, and the 10% hydrogels exhibited the highest cross-link density. The *in vitro* drug release studies carried out using Franz diffusion cells showed that amoxicillin release from 6 and 10% gels was sustained for 240 h as compared to 3% gels. As the polymer concentration increased to 10%, the release pattern from gels approached diffusion controlled mechanism with diffusional exponent $n = 0.49$. In conclusion, the biodegradable *in situ* forming hydrogels of the present study offer a therapeutic option to provide sustained localized delivery of amoxicillin intracervically to the pregnant woman for the treatment of ascending genital infections.



KEYWORDS: injectable hydrogels, biodegradable hydrogel, sustained release of amoxicillin, pregnant guinea pig model, pregnancy, treatment of genital infections, formulation, *in vitro* and *in vivo* evaluation

1. INTRODUCTION

Hydrogels have found varied applications such as molecularly engineered scaffolds for controlled drug release, in cellular delivery, in tissue engineering and as wound dressings due to the highly hydrated and three-dimensional properties which are similar to those of the native extracellular matrix (ECM).[1] Hydrogels have attracted a great deal of attention as a matrix for the controlled delivery of biologically active substances.[2] The suitability of hydrogels for pharmaceutical applications is mainly determined by their mechanical properties, drug loading and controlled drug release capability.[3] *In situ* forming gels have been investigated for a variety of applications such as oral, nasal, ocular, injectable, vaginal and rectal.[4−7] Thermosensitive gels are commonly investigated for the vaginal delivery of therapeutic agents as they gel in response to the body temperature. Thermosensitive

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vaginal gels for delivery of cotrimazole were formulated using Pluronic F127.[6] Polycarbophil hydrogels were investigated for intravaginal delivery of granulocyte-macrophage colony-stimulating factor (GM-CSF) for treatment of human papillomavirus (HPV)-associated genital (pre)neoplastic lesions.[8]

Over the years, the intravaginal route of drug administration has emerged as an effective means for local delivery of antibacterials, antifungals, antiprotozoals and antiviral agents.[9] The use of topical microbicides is common in pregnant women to treat yeast and bacterial infections. Bacterial vaginosis (BV) is found in 15−20% of pregnant women and is an ascending genital tract infection of chorioamnion and amniotic fluid.[10,11] Intrauterine infection during pregnancy is often responsible for disease causing spontaneous preterm birth, and infection which is associated with the microorganisms ascending from the vagina and cervix is known to affect the fetal membranes and the cervical mucosa and endometrium.[11] Local drug delivery to cervical tissues is preferred. To treat BV in pregnant women, antibiotics are administered intravaginally, and the intravaginal route is preferred to attain high local drug concentration in the vagina, which cannot be achieved by oral administrations.[12] One major problem associated with intravaginal and intrauterine drug delivery is limited contact time of administered dosage form with the mucosa due to the physiological conditions imposed by the protective mechanisms of the cervix and vagina.[6,8] This reduces the therapeutic efficacy and necessitates frequent dosing.[6,13,14] Gels are better tolerated than other conventional dosage forms.[15,16]

Dendrimer based intravaginal gels are extensively investigated as topical microbicides.[17] Polylysine dendrimer SPL7013 exhibited antimicrobial activity against herpes simplex virus,[18] and its formulation development into a prototype acidified Carbopol gel for intravaginal delivery was evaluated in animal models.[19−21] Human clinical trials (phases I and II) were conducted to determine the retention, duration of activity, safety and tolerability of a gel containing SPL7013 applied intravaginally to young nonpregnant women,[22] and the gel was found to be safe and well tolerated.[17,19] Further trials are being conducted to test its efficacy against genital herpes and HIV. Apart from SPL7013, the amine terminated PAMAM dendrimers are found to exhibit antibacterial activity toward Gram negative bacteria.[23−25] PAMAM dendrimer with hydroxyl terminations was found to effectively inhibit intrauterine *Escherichia coli* (*E. coli*) infections in guinea pigs.[25] Dendrimers are also used as carriers for antimicrobial agents (e.g., triazine antibiotics).[26] Quinolone drugs encapsulated in PAMAM dendrimers are highly active when used as topical microbicidal agents.[27] Silver complexes and nanocomposites with PAMAM dendrimer result in increased antibacterial activity toward *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *E. coli*.[28] Further, dendrimers are also extensively evaluated in several gel formulations.[29−31] Many polymers are currently evaluated as topical microbicides or as a component of the topical microbicide formulations to be applied on the vagina or rectal mucosa.[9,32]

In the present study, we have investigated the *in situ* forming biodegradable hydrogels obtained by cross-linking of thiopyridyl functionalized PAMAM dendrimer $[(NH_2)_{49}$-G4-$(NH\text{-}PDP)_{15}]$ with 8-arm polyethylene glycol (20 kDa) for sustained intravaginal delivery of amoxicillin to treat ascending genital infections during pregnancy. Multiple thiopyridyl surface functionalities of the dendrimer and the star-PEG are utilized to create a biodegradable gel with disulfide linkages. This offers the potential for the dendrimer and drug to be released, as the hydrogel degrades. Further, the dendrimers offer the potential to target selectively inflammatory cells.[33] The hydrogels were investigated for biodegradation, retention, tolerability and volume of distribution by intravaginal application in the pregnant guinea pig model. In the past hydrogels containing dendritic materials obtained by photo-cross-linking, radiation, thermal gelation, ion interactions and freeze thaw cycles of polymers have been described.[31,34−38] The hydrogels of the present study are formed *in situ* by chemical cross-linking resulting from simple mixing of the $[(NH_2)_{49}$-G4-$(NH\text{-}PDP)_{15}]$ dendrimer and the PEG polymer solutions in buffer through the formation of disulfide bonds, and these hydrogels possess the properties of both the PEG hydrogels and the dendrimer.

The cervical infections in pregnant women caused by pathogens such as *Streptococcus* group B, *E. coli* and *Gardnerella vaginalis* are responsible for premature rupture of the fetal membranes, chorioamnionitis and prematurity.[39] PAMAM dendrimer with amine terminations exhibits antibacterial activity against *E. coli*, *P. aeruginosa* and *S. aureus*[23,24] by formation of nanoscale holes in lipid bilayers of the bacterial cell membrane causing cell lysis.[25] The partially PEGylated amine terminated PAMAM dendrimers exhibit antibacterial activity against *E. coli* bacteria and *P. aeruginosa*.[23,24] The PEGylation of dendrimers reduces their cytotoxicity[17,40] and yet retains the antibacterial activity.[23,24] *In situ* forming hydrogels from dendrimers with multifunctional groups was recently reported by our group.[41] In the present manuscript, the amine terminated PAMAM dendrimers are partially modified to thiopyridyl terminations which are chemically linked to the 8-arm-PEG by disulfide bridges to form the hydrogel while few of the amine groups are retained unmodified. The covalent linking of PEG to dendrimer during gel formation was expected to overcome the cytotoxicity. Further, the amoxicillin loaded hydrogels of this study are expected to exhibit dual acting mechanism toward the bacteria, arising from sustained release of the antibiotic and antibacterial activity exhibited by the amine terminated dendrimer released from the degrading gel. The present manuscript discusses the influence of formulation additives on the gel retention times, role of humectant to avoid gel dehydration and enhancement in gel adhesive properties.

2. MATERIALS AND METHODS

2.1. Materials. Amine terminated, ethylenediamine-core poly(amidoamine) dendrimer $[G4\text{-}(NH_2)_{64}]$ (diagnostic grade generation-4 with $-NH_2$ groups) was purchased from Dendritech, and 8-arm-PEG-SH (20 kDa) (**5**) was purchased from NOF America Corporation, USA. Other reagents were obtained from assorted vendors in the highest quality available. Of these, amoxicillin, *N*-succinimidyl 3-(2-thiopyridyl)propionate (SPDP), polyvinylpyrrolidone (PVP 30 kDa), PEG 600, glycerol, glutathione (GSH), dimethyl sulfoxide (DMSO), fluorescein isothiocyanate (FITC), dimethylformamide (DMF), ethanol, phosphate buffer saline (PBS, pH, 7.4), and HPLC-grade solvents were obtained from Sigma-Aldrich.

2.2. Preparation of $[(NH_2)_{49}$-G4-$(NH\text{-}PDP)_{15}]$ (4). $[G4\text{-}(NH_2)_{64}]$ (**2**) dendrimer (500 mg, 0.0351 mmol) was dissolved in PBS buffer pH 7.4 (20 mL), and a solution of SPDP (**3**) (351 mg, 1.126 mmol) in ethanol (10 mL) was added to it under stirring to provide sufficient modification while preventing loss of product due to the insolubility of highly modified dendrimer. The reaction mixture was stirred at room temperature for 2 h. After completion of the reaction, solvent was removed under reduced pressure to get a solid compound. The

Table 1. Hydrogel Compositions and Stoichiometric Ratio between Thiopyridyl and Thiol Groups

% hydrogel	hydrogel vol (μL)	wt of polymer and ratio (1:1)	stoichiometric ratio of thiopyridyl to thiol groups	gelation time (s)	polymer content (%)
3	200	$[(NH_2)_{49}$-G4-$(NH\text{-}PDP)_{15}]$ (3 mg) + PEG-SH(3 mg) (1:1)	2:1	30	3
6	200	$[(NH_2)_{49}$-G4-$(NH\text{-}PDP)_{15}]$ (3 mg) + PEG-SH(3 mg) (1:1)	2:1	20	6
10	200	$[(NH_2)_{49}$-G4-$(NH\text{-}PDP)_{15}]$ (3 mg) + PEG-SH(3 mg) (1:1)	2:1	<10	10

crude product obtained from the reaction mixture was purified by dialysis using Spectra/Por dialysis membranes (MW cutoff 1000 Da) against water (pH = 5 on addition of 1% HCl) changing the solvent every 8 h for 24 h to remove byproduct and the excess of reactants. After dialysis, the solvent was removed using lyophilization to get pure compounds with yield of 78% (458 mg, 0.0274 mmol). Solid was reconstituted in desired amount of PBS (pH 7.4) and used for hydrogel formulation.

2.3. Preparation of $[(NH_2)_{47}$-G4-$(NH\text{-}FITC)_2(NH\text{-}PDP)_{15}]$. FITC (0.082 g, 0.210 mmol) was added to a solution of $[G4\text{-}(NH_2)_{64}]$ dendrimer (1 g, 0.070 mmol) in DMSO (20 mL) under stirring, and the reaction was continued in the dark for 18 h. To remove unreacted FITC, the reaction mixture was dialyzed (membrane molecular weight cutoff 1000 Da) in DMSO for 24 h changing DMSO every 8 h. After dialysis the DMSO was lyophilized to get pure $[(NH_2)_{62}$-G4-$(NH\text{-}FITC)_2]$ conjugate as a dark orange color solid in 81% yield (0.854 g, 0.0569 mmol). The $[(NH_2)_{62}$-G4-$(NH\text{-}FITC)_2]$ conjugate was dissolved into methanol and precipitated in acetone. The absence of free FITC in the conjugate was verified by TLC using chloroform and methanol (ratio 1:1) as mobile phase. After purification of the $[(NH_2)_{62}$-G4-$(NH\text{-}FITC)_2]$ conjugate the number of FITC molecules in $[(NH_2)_{62}$-G4-$(NH\text{-}FITC)_2]$ was calculated on the basis of ^{1}H NMR and MALDI-TOF (data not shown); the above-described procedure was used to prepare $[(NH_2)_{47}$-G4-$(NH\text{-}FITC)_2(NH\text{-}PDP)_{15}]$ for hydrogel formulation for *in vivo* applications.

2.4. Hydrogel Formation. Hydrogels were prepared by cross-linking of the branched thiol terminated PEG polymer (8-arm-PEG-SH, 20 kDa) with $[(NH_2)_{49}$-G4-$(NH\text{-}PDP)_{15}]$ (or $[(NH_2)_{47}$-G4-$(NH\text{-}FITC)_2(NH\text{-}PDP)_{15}]$). Hydrogels containing 10, 6 and 3% w/v of polymers were prepared by mixing equal volumes (1:1 v/v, 100 μL each) of the 10, 6 and 3%w/v polymer solutions of $[(NH_2)_{49}$-G4-$(NH\text{-}PDP)_{15}]$ and 8-arm-PEG-SH in PBS (pH = 7.4) as shown in Table 1. The ratio of PDP to thiol functionalities in these hydrogels was 2:1. The hydrogels resulted after 10−30 s of mixing the two polymer solutions. The gelation time was determined by the vial tilting method.[41] When the sample showed no flow, it was considered as a gel. These hydrogels were further investigated to determine the degree of swelling, drug loading efficiency, *in vitro* release studies, and *in vivo* applications.

2.5. Morphology of the Hydrogel. Scanning electron microscopy (SEM) analyses were performed to investigate the morphology of hydrogel. The 10, 6 and 3% w/v hydrogels were prepared for electron microscopy at room temperature, followed by dehydration using lyophilization. It was observed that the hydrogel volume was reduced by ~75% during the dehydration process. The samples were critical point dried, sputter-coated with 9 nm of gold/palladium, and imaged using SEM (HITACHI S-2400 scanning electron microscope) at 20 kV. The cross sections of the hydrogels were observed using confocal microscopy, to determine the cross-link density. The gels were formed by cross-linking of $[(NH_2)_{47}$-G4-$(NH\text{-}FITC)_2(NH\text{-}PDP)_{15}]$ with 8-arm-PEG-SH in PBS (pH = 7.4). The 10, 6 and 3% w/v gels were embedded in OCT media (Tissue-Tek) and frozen at −80 °C until they were sectioned. Gel sections (20 μm thick) were cut using a cryostat (Leica Microsystems; Nuchloss, Germany). Images of the sectioned gels were captured on a Leica TCS SP5 laser scanning confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany).

2.6. Equilibrium Swelling of Hydrogels. The 10, 6 and 3% w/v hydrogel disks were obtained by cross-linking of $[(NH_2)_{49}$-G4-$(NH\text{-}PDP)_{15}]$ and PEG (1:1 v/v, 100 μL each) in a cylindrical glass vial (12 × 35 mm). These hydrogel disks were weighed and subsequently immersed in 5 mL of phosphate buffered saline (PBS, pH 7.4) solution at 37 °C in 30 mL scintillation vials. The swollen hydrogels were removed from PBS and weighed at various time intervals until a swelling equilibrium had been reached. All experiments were carried out in triplicate, and the results are expressed as means ± standard deviations.

The degree of swelling was calculated from the formula previously reported,[42] where W_s is the weight of the swollen hydrogel at time t and W_0 (wet) is the initial weight.

$$\%\text{swelling} = \frac{(W_s - W_0)}{W_0} \times 100$$

2.7. Formulation of Hydrogel. The prototype vaginal gels were made using excipients: glycerin (5%, v/v), PVP (4%, w/w) and PEG 600 (5%, v/v), which were included in hydrogel formulation to improve the emollient, adhesion, retention and spreadability properties of hydrogels. These excipients were dissolved in the PBS buffer at the concentrations mentioned above, and this solution was used as a vehicle to dissolve separately the $[(NH_2)_{49}$-G4-$(NH\text{-}PDP)_{15}]$ (or $[(NH_2)_{47}$-G4-$(NH\text{-}FITC)_2(NH\text{-}PDP)_{15}]$) and 8-arm-PEG-SH. The hydrogel formulation was obtained by mixing the solution of $[(NH_2)_{49}$-G4-$(NH\text{-}PDP)_{15}]$ and 8-arm-PEG-SH in the solvent vehicle at the ratio 1:1 v/v. The gelling time was recorded for the different compositions of vehicle and polymers. The optimal concentration of additives was determined by measuring the cross-linking time and retention time of hydrogel formulation on targeted area.

2.8. Reverse Phase HPLC Characterization. *In vitro* drug release and characterization of conjugates was carried out using a Waters HPLC instrument equipped with one pump, an auto sampler and dual UV, RI, and fluorescence detector interfaced to Millennium software. The mobile phase used was acetonitrile (both 0.14% TFA by weight), and the water phase had a pH of 2.25. Mobile phases were freshly prepared, filtered and degassed prior to use. A Supelco Discovery BIO Wide Pore C5 HPLC

column (5 μm particle size, 25 cm × 4.6 mm length × i.d.) equipped with C5 Supelguard cartridge (5 μm particle size, 2 cm × 4.0 mm length × i.d.) was used for characterization of the conjugates as well as *in vitro* drug release studies. Gradient elution was used for analysis water−acetonitrile (100:0) to water−acetonitrile (60:40) in 25 min followed by returning to initial conditions for 5 min. The flow rate was 1 mL/min. Calibration curves were prepared for amoxicillin, based on UV absorbance peak area at 229 nm. These calibration curves were used for estimation of *in vitro* drug release across cellulose membrane using Franz diffusion cells.

2.9. Differential Scanning Calorimetry (DSC) Analysis of Hydrogels. The neat, modified polymers and hydrogels were subjected to thermal analysis using TA Instruments DSC Q2000 V24.4 Build 116 Module DSC Standard Cell RC. The experiments were conducted in crimped sealed aluminum pans, and the weight of each sample was in the range of 1−2 mg. All the samples were analyzed using the heat−cool−heat cycles. The samples were equilibrated at −50 °C for 2 min and were heated to 150 °C at a heating rate of 5 °C/min under nitrogen flow. The samples were quench cooled to −50 °C and equilibrated for 2 min and again heated to 150 °C at a heating rate of 5 °C/min.

2.10. Degradation of Hydrogels. *In vitro* degradation of hydrogel was performed in glutathione (GSH) solutions at pH 4 and simulated vaginal fluid up to 72 h. The simulated vaginal fluid (SVF) was prepared as described previously[43] with the addition of GSH. Briefly, the SVF was prepared by dissolving 350 mg of NaCl, 140 mg of KOH, 22 mg of Ca(OH)2, 18 mg of bovine serum albumin, 200 mg of lactic acid, 100 mg of acetic acid, 16 mg of glycerol, 40 mg of urea, 500 mg of glucose, and 20 mg of GSH in distilled water, and the pH was adjusted to 4 ± 0.02 using 0.1 M HCl. Hydrogel disks obtained by cross-linking of $[(NH_2)_{49}$-G4-$(NH\text{-}PDP)_{15}]$ and 8-arm-PEG-SH (1:1 v/v, 100 μL each) were immersed into the 5 mL GSH solution at pH 4 and simulated vaginal fluid at pH 4 in 30 mL scintillation vials in triplicate and observed for degradation. The GSH solutions in which hydrogels were immersed were injected into the HPLC system using an Ultrahydrogel size exclusion column and analyzed for the generation of different molecular weight components.

2.11. Drug Loading into the Hydrogels. Antibiotic (amoxicillin) was physically entrapped into the hydrogels. The drug (0.5 mg) was added to the PEG solution (100 μL) in vehicle, and the solution of $[(NH_2)_{49}$-G4-$(NH\text{-}PDP)_{15}]$ (100 μL) in vehicle was added to this PEG solution to form the 3%, 6%, 10% dendrimer−PEG hydrogel (200 μL) (Table 1).

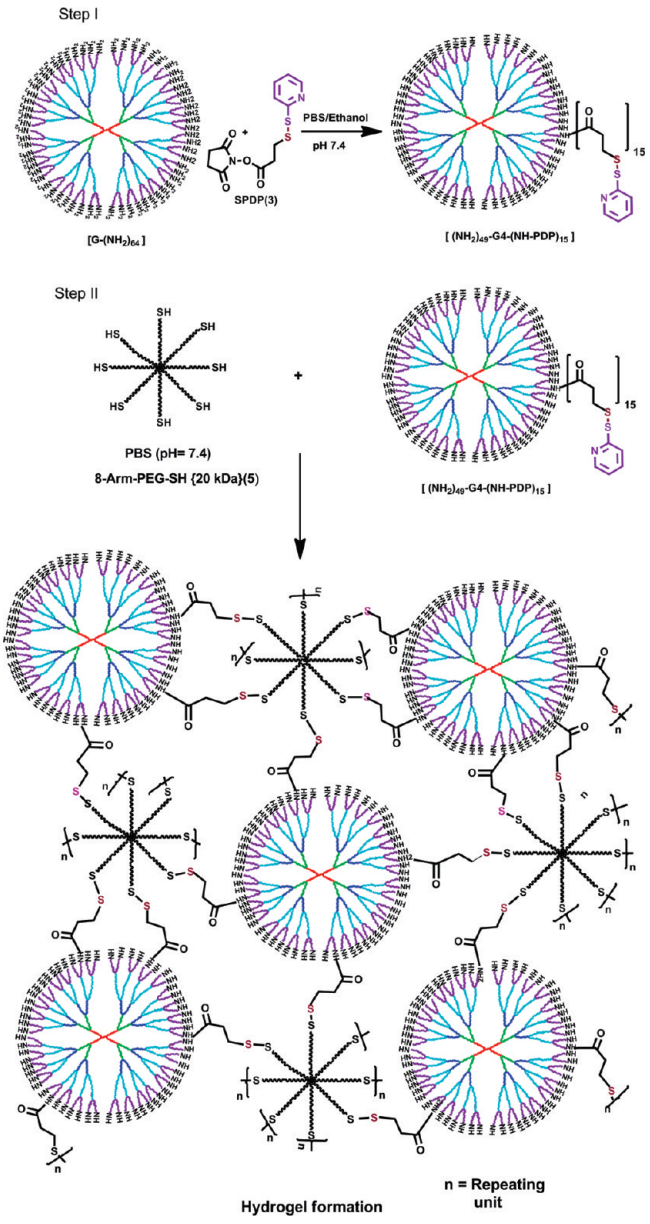
2.12. Drug Loading Efficiency. The amount of amoxicillin entrapped in the dendrimer−PEG hydrogels (10%, 6% and 3%) was determined by breaking the gel into small pieces and transferring into 1 mL eppendorf tubes filled with PBS (pH 7.4) and sonicated for 10 min. The hydrogel pieces were washed three times to extract drug. The washings were collected and filtered with 0.2 μm Millipore filter and quantified by reverse phase (RP) HPLC analysis, monitoring elution at a wavelength of 229 nm. Water:acetonitrile was used as mobile phase at a flow rate of 1 mL/min. The difference between the amount of drug taken initially and the drug content in the washings is the amount of drug entrapped.

2.13. *In Vitro* Drug Release Using Franz Diffusion Cell. For the *in vitro* drug release study, jacketed Franz diffusion cells with flat ground joints were used. The use of Franz diffusion cells to test *in vitro* drug release from topical or mucosal formulations is an accepted method.[44−46] The membrane was clamped between the donor and receiver chambers of the Franz diffusion cell apparatus with a diameter of 5 mm, a diffusional area of 0.64 cm^2 and a receptor chamber volume of 5 mL. Nitrocellulose acetate membranes (Millipore, America) with an average pore size of 0.45 μm were used. The receptor chambers filled with PBS (pH = 7.4) were maintained at 37 °C in order to ensure the body temperature. Drug (amoxicillin) is well soluble in the chosen receptor medium. Each cell contained a magnetic bar and was stirred (600 rpm) during the experiment, and the cells were equilibrated for 1 h before the samples were mounted. 200 μL samples were taken at predetermined time points and replaced with equal amounts of fresh receptor medium to maintain sink conditions. The samples were kept frozen at 4 °C prior to analysis. Quantification of drug release was carried out by reverse phase high performance liquid chromatography (RP-HPLC). All samples were run in triplicate for statistical analysis.

2.14. Evaluation of Hydrogel in Pregnant Guinea Pig Model. All the animal experimental procedures were approved by the institutional animal care and use committee of Wayne State University. Briefly, pregnant Dunkin-Hartley strain guinea pigs (n = 15) (Charles River) at 55 days of gestation (third trimester) were anesthetized by inhalation of 5.0% isoflurane in 100% oxygen at a flow rate of 2 L/min in an approved rodent anesthesia chamber. The surgical level of anesthesia was maintained with 1.5 and 2.0% isoflurane in 100% oxygen at a flow rate of 1−2 L/min via a nose cone. An endoscope was used to visualize the cervix. FITC labeled dendrimer−PEG hydrogel (100−500 μL) was injected into the cervix using an iv catheter (BD Angiocath, Infusion Therapy Systems Inc., Sandy, UT, 16GA, 5.25 in., 1.7 × 133 mm). The pH of the vagina was intermittently tested by wiping the vaginal fluid using cotton swabs. After single vaginal application, the vaginal cavity was observed for any signs of possible irritation of the vaginal mucosa (edema or redness of tissue). The observations were scored and recorded as follows: no erythema, slight erythema (light pink) and moderate to severe erythema (dark pink or light red). After 5, 12, 24 and 72 h intervention, guinea pigs were euthanized with pentobarbital sodium (120 mg/kg) and midline laparotomy was performed to expose the cervicovaginal region for further evaluation. The retention times, biodegradation and tolerability were studied *in vivo* using the guinea pigs. The vaginal and cervical tissues were used for histopathological evaluation.

2.15. Immunofluorescence Histochemistry. An immunofluorescence study was performed to investigate biodistribution of the FITC-dendrimer−PEG hydrogel in the cervicovaginal tissues of guinea pig after 24 and 72 h of treatment. Double immunofluorescent staining was performed on 20 μm thick, paraffin sections of tissues placed on silanized slides. The mucified epithelial cells were identified based on the positive staining for cytokeratin. The immunofluororescent staining was performed using Ventana Discovery autostainer for controlled and optimized reaction environment using the automation-optimized reagents from Ventana Medical Systems Inc. Briefly, paraffin wax sections were loaded onto the Ventana Discovery platform and the following steps were completed automatically: these included dewaxing by EZ prep buffer (Ventana Medical Inc.) and pretreatment in Tris/EDTA pH 8.0 antigen retrieval solution (Ventana mCC1) or protease solution for 1 h (Ventana protease 2). Endogenous peroxidase was inactivated using an enhanced inhibitor provided in the staining kit, and nonspecific antibody binding was blocked by treatment with blocking

Scheme 1. Schematic Representation of the Hydrogel Formation[a]



[a] The thiol-terminated 8-arm-PEG (20 kDa) formed gel at pH 7.4 by reacting with the thiopyridyl terminations of the $[(NH_2)_{49}$-G4-(NH-PDP$)_{15}]$ resulting in disulfide linkages.

solution for 10 min. The blocking solution was removed, and the sections were washed three times with PBS/Tween solution incubated with primary antibodies for 1 h using the liquid coverslip (Ventana Medical Inc.). The primary antibody used was monoclonal mouse anti-human cytokeratin (1:200, M7018, Dako Carpinteria, CA, USA). The sections were again washed three times with PBS/Tween solution incubated with secondary antibodies, Alexa Fluor594 goat anti-mouse IgG (1: 500, A11005, Invitrogen) for 1 h using the antibody diluent from Ventana. The sections were washed with PBS/Tween, counterstained and mounted with DAPI ProLong Gold antifade and coverslipped. Images were captured from Leica TCS SP5 laser scanning confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany). All study specimens were analyzed by a pathologist blinded to the clinical information.

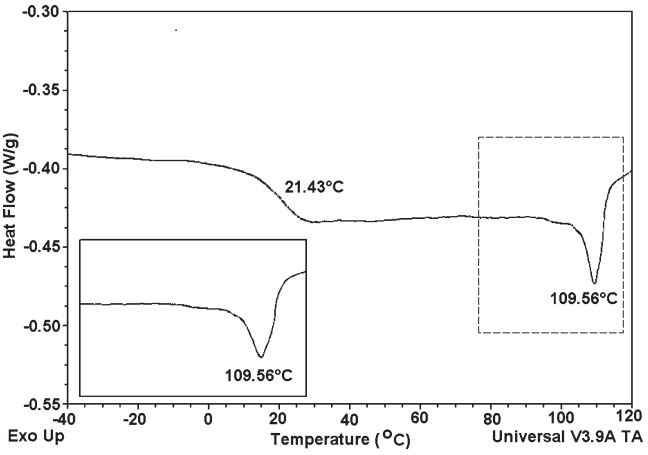


Figure 1. The DSC thermogram of $[(NH_2)_{49}$-G4-(NH-PDP$)_{15}]$ shows the T_g at 21.4 °C and an endotherm at 109.6 °C. The increase in T_g to 21.4 °C from −28 °C indicates modification of amine terminated PAMAM dendrimer with PDP groups.

3. RESULTS AND DISCUSSION

3.1. Synthesis and Characterization of $[(NH_2)_{49}$-G4-(NH-PDP$)_{15}]$. To incorporate the thiol reactive terminal groups on the amine terminated PAMAM dendrimers to form hydrogel with 8-arm-PEG-SH, the heterobifunctional cross-linker linker SPDP was appended to the dendrimer to yield thiopyridyl functionalities (Scheme 1). The *N*-succinimidyl activated ester of SPDP was coupled to the terminal amine groups to form amide-linked 2-pyridyldithiopropanoyl (PDP) groups ($[(NH_2)_{49}$-G4-(NH-PDP$)_{15}]$ (Scheme 1). The NMR analysis of $[(NH_2)_{49}$-G4-(NH-PDP$)_{15}]$ (Figure S1 in the Supporting Information) showed the presence of protons corresponding to the aromatic ring of thiopyridyl groups and the dendrimer. The protons for the aromatic ring of thiopyridyl appear at 7.20−2.26 (m, 1H, Ar), 7.74−7.82 (br d, 1H, Ar), and 8.15−8.22 (m, 2H, Ar) ppm while the other protons appeared at 2.42−2.50 (m, 4H, $-CH_2-CH_2-$ from PDP), 2.67−2.72 (m, 2H, $-CH_2-$ from interior dendrimer), 2.86−2.92 (m, 1H, $-CH_2-$ from interior dendrimers), 3.03−3.12 (m, 1H, $-CH_2-$ from interior dendrimers), 8.38−8.45 (br d, 1H, NH, from interior amide protons), and 8.52−8.59 (br d, 1H, NH, from interior amide protons) ppm as seen from the ^{1}H NMR of $[(NH_2)_{49}$-G4-(NH-PDP$)_{15}]$. The number of PDP in $[(NH_2)_{49}$-G4-(NH-PDP$)_{15}]$ was calculated on the basis of ^{1}H NMR analysis of $[(NH_2)_{49}$-G4-(NH-PDP$)_{15}]$. The integral ratio of the amide protons of dendrimer in $[(NH_2)_{49}$-G4-(NH-PDP$)_{15}]$ at 8.38−8.45, and 8.52−8.59 to the aromatic protons of thiopyridyl in $[(NH_2)_{49}$-G4-(NH-PDP$)_{15}]$ at 7.20−2.26, 7.74−7.82, and 8.15−8.22 suggests that $[(NH_2)_{49}$-G4-(NH-PDP$)_{15}]$ conjugate contains an average of 15 PDP moieties. These results are consistent with the MALDI-TOF (Figure S2 in the Supporting Information) analysis which shows molecular mass increase from 13.75 kDa to 16.70 kDa upon conjugation of PDP groups to the dendrimer (see Supporting Information). The $[$G4-$(NH_2)_{64}]$ dendrimer showed a T_g at −28 °C, and this result is in accordance with that reported previously.[44] $[(NH_2)_{49}$-G4-(NH-PDP$)_{15}]$ showed T_g at 21.4 °C, and in addition it also exhibited an endotherm at

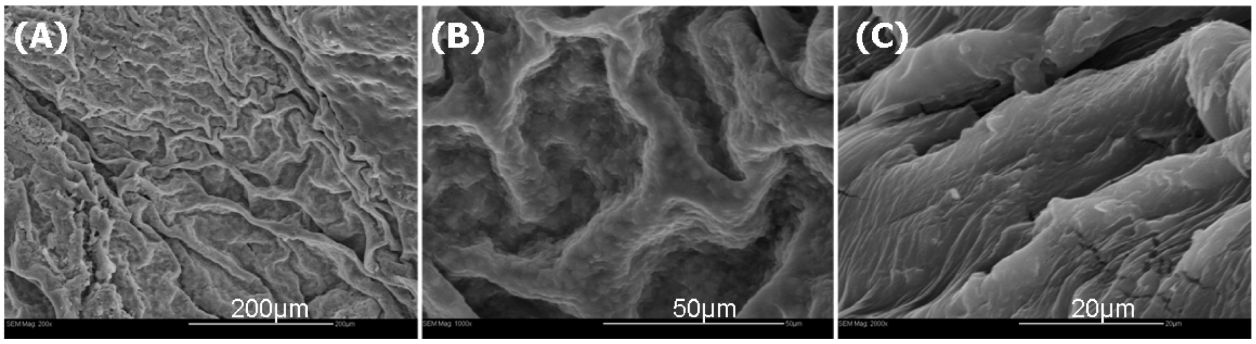


Figure 2. The SEM images of dendrimer $[(NH_2)_{49}$-G4-$(NH\text{-}PDP)_{15}]$ cross-linked with 8-PEG-SH gel (**A**) 200 μm (200x) (**B**) 50 μm (1000x) (**C**) 20 μm (2000x). These gels were dehydrated by lyophilization.

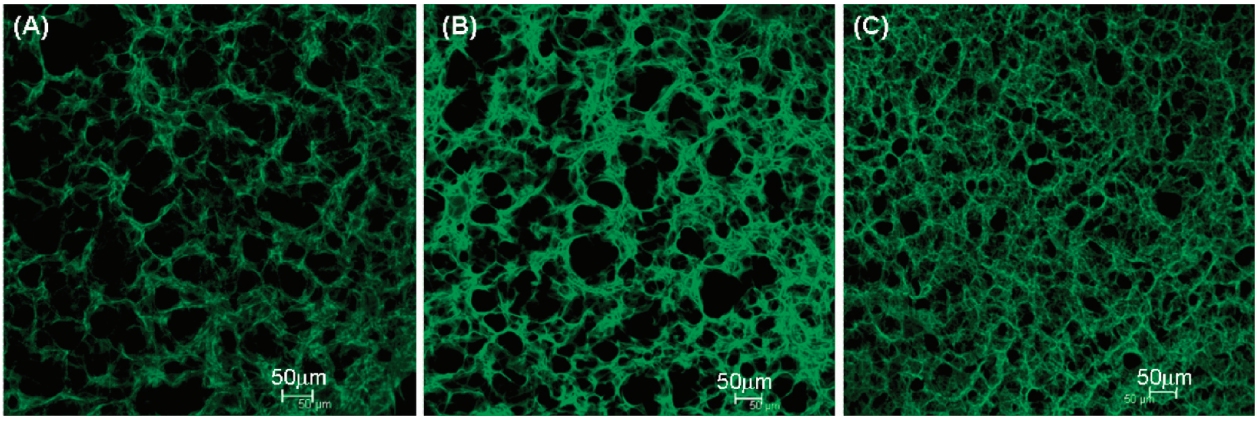


Figure 3. Hydrogel labeled with FITC to demonstrate the pore structure of the gel. By introducing the different concentration of polymers (dendrimer $[(NH_2)_{49}$-G4-$(NH\text{-}PDP)_{15}]$ and 8-PEG-SH) in the hydrogels, cross-linking density gradually increased by increasing the concentration of polymer. 3% hydrogel (A), 6% hydrogel (B), and 10% hydrogel (C) show the cross-linking network changes with increasing polymer concentration; scale bar represents 50 μm.

109.6 °C (Figure 1). The difference in the T_g between neat $[G4\text{-}(NH_2)_{64}]$ and $[(NH_2)_{49}$-G4-$(NH\text{-}PDP)_{15}]$ is attributed to the PDP groups appended on the dendrimer. The appearance of endotherm in $[(NH_2)_{49}$-G4-$(NH\text{-}PDP)_{15}]$ which was absent in neat $[G4\text{-}(NH_2)_{64}]$ dendrimer further confirms the addition of PDP groups to the dendrimer. These observations were consistent with those reported in the previous results.[47,48] This $[(NH_2)_{49}$-G4-$(NH\text{-}PDP)_{15}]$ cross-linker was used to form dendrimer−PEG hydrogel with $[(NH_2)_{49}$-G4-$(NH\text{-}PDP)_{15}]$ and 8-arm-PEG-SH. The partial modification of PAMAM G4-$(NH_2)_{64}$ dendrimer with amine terminations into $[(NH_2)_{49}$-G4-$(NH\text{-}PDP)_{15}]$ was carried out to enable the linking of PEG chains to the dendrimer by formation of disulfide bond.

3.2. Hydrogel Formation. *In situ* forming hydrogels with disulfide cross-links were investigated for intravaginal delivery. Hydrogels incorporating 3, 6 and 10% w/v of the polymer compositions were formed by mixing the solutions of $[(NH_2)_{49}$-G4-$(NH\text{-}PDP)_{15}]$ and 8-arm-PEG-SH, resulting in covalent disulfide cross-links arising from the interaction of thiol groups of the PEG with the thiopyridyl functionalities on the dendrimer surface $[(NH_2)_{49}$-G4-$(NH\text{-}PDP)_{15}]$ (Table 1). The hydrogel results from intermolecular cross-linking as shown in Scheme 1. For the formation of hydrogels, the cross-linking agent $[(NH_2)_{49}$-G4-$(NH\text{-}PDP)_{15}]$ was used in an excess of molar ratio (in terms of the functional groups) relative to PEG-SH (Table 1). The hydrogel formation was confirmed by the inverted tube method. The hydrogels were formed in 10−30 s of mixing the cross-linker and PEG solution in the upright tubes, and gel once formed was no longer pourable as seen from the inverted tubes in Figure S3 (Supporting Information). The increase in polymer concentration impacts the rate of gel formation which was higher for higher polymer concentrations (Table 1). The rapid formation of hydrogels with the increased concentration of polymers might be due to formation of rapid and intense cross-linking networks, reducing the time for gelation. For example 10% hydrogel forms in less than 10 s, while the 3% hydrogel takes nearly 30 s. Hydrogels appeared to be transparent, with uniform surface. The hydrogels were designed to facilitate the linking of PEG chains on the partially modified $[(NH_2)_{49}$-G4-$(NH\text{-}PDP)_{15}]$ dendrimer. The linking of the PEG chains by disulfide bond formation further serves to overcome the cytotoxicity of the amine terminated dendrimer. PEGylation of dendrimers to overcome cytotoxicity is well documented,[17,40] and our *in vivo* studies discussed in subsequent sections show that the gels were tolerant without any toxic effect.

3.3. Morphology of the Hydrogel. Scanning electron microscopy (SEM) experiments were performed to study the surface morphology of dendrimer−PEG hydrogel (Figure 2). SEM micrographs of critical point dried gels show a uniform dense structure with striations. The SEM experiments were performed on a dehydrated sample that exhibited significant reduction in volume from the hydrated state. It is likely that the water hydrated dendrimer−PEG hydrogel adopts a dense structure with regular cross-linking network throughout the gel. The cross

section of the hydrogels was investigated by cross-linking the $[(NH_2)_{47}$-G4-$(NH\text{-}FITC)_2(NH\text{-}PDP)_{15}]$ and 8-arm-PEG-SH. The cross section observed under the confocal microscopy shows an isotropic hydrogel that exhibits a classic uniform morphology with pores as seen in Figure 3. A characteristic change in morphology based on changes in polymer content in the hydrogel was observed (Figure 3). The 3% hydrogel does not form a dense cross-linked network as seen for the 6 and 10% gels. By introducing the different concentration PEG and dendrimer in the hydrogels, the porosity of the network changed; pore size is gradually decreased by increasing the concentration of polymer. These results suggest that dehydration of gels for SEM leads to artifact in the highly water-saturated gels, and their morphology can be better viewed by cryosectioning the gels with the presence of fluorescein isothiocynate (FITC).

3.4. Effect of Formulation Additives. The $[(NH_2)_{49}$-G4-$(NH\text{-}PDP)_{15}]$ and 8-arm-PEG-SH cross-linked hydrogels were formulated with glycerin (5%, v/v), PVP (4%, w/v) and PEG 600 (5%, v/v). The effect of formulation additives on the gelation time was investigated as shown in Table S1 in the Supporting Information. The concentration of PVP was found to affect the gelation time, and at higher concentration (8% w/v) the gels took longer than 50 s to form, hence its concentration less than 8%w/v was considered in formulation. The concentration of PEG 600 and glycerin did not significantly affect the gelation time. The vaginal musoca is moist, and at any given time the volume of the vaginal fluid is less than 1 mL and there is a possibility of fluid being reabsorbed.[49] The hydrogels of the present study will be placed in an environment with relatively low water content. The formulation additives were incorporated in the hydrogel to prevent it from becoming brittle and dehydrated. Glycerin and PEG 600 were incorporated in the hydrogels as these act as humectant and help maintain the gels in plasticized supple form.[50−53] The humectant properties of glycerin and PEG 600 are well-known. PVP was incorporated in the gel to provide mucoadhesive properties and to increase the viscosity of the gel forming solutions to prevent their leaking outside the cavity during instillation and formation of cross-linked hydrogels.[54] Use of PVP in vaginal gels for enhancing the mucoadhesive properties is well-known.[6,55−58] The optimal concentration of the additives to prevent brittleness and increase retention time on vaginal mucosa for up to prolonged periods of time was found to be glycerin (5%, v/v), PVP (4%, w/w) and PEG 600 (5%, v/v).

3.5. Thermal Analysis. The thermal behavior of the dendrimer−PEG hydrogel components and the hydrogel was investigated by DSC analysis. The DSC thermograms of dendrimer−PEG hydrogels, $[(NH_2)_{49}$-G4-$(NH\text{-}PDP)_{15}]$, 8-arm-PEG-SH, are shown in Figure 4A. 8-Arm-PEG-SH exhibits an endotherm at 51.7 °C (Figure 4e). The T_g of $[G4\text{-}(NH_2)_{64}]$ dendrimer was −28 °C, and $[(NH_2)_{49}$-G4-$(NH\text{-}PDP)_{15}]$ showed the presence of an endothermic peak at 109.3 °C with a T_g at 21.4 °C (Figure 4a). The DSC profiles show that after dendrimer was converted to its PDP derivative, the T_g shifted, indicating an altered polymer microstructure. When comparing the profiles of $[(NH_2)_{49}$-G4-$(NH\text{-}PDP)_{15}]$ and 8-arm-PEG-SH, the cross-linking of the two polymers clearly produced a new material having a microstructure different from either of its two components. In the case of hydrogels the T_g was found to be higher than that observed for the $[(NH_2)_{49}$-G4-$(NH\text{-}PDP)_{15}]$, e.g. the 3% hydrogel exhibited a T_g of 34.7 °C and the 10% and 6% hydrogels exhibited a T_g at 35.3 °C. The 3, 6 and 10% hydrogels exhibited the endotherms at 39.2, 45.9, and 46.8 °C, respectively, which was lower than that observed for the 8-arm-PEG-SH (51.7 °C). The intermolecular cross-linking of the polymer chains results in reduced mobility (resulting in increased T_g), and these polymer chains cannot reorient to form a highly ordered crystalline structure (lowered melting point). The addition of glycerin, PVP and PEG 600 slightly lowered the endotherms of 3%, 6% and 10% hydrogels when compared to hydrogels without additives (Figure 4B). The hydrogels with PEG 600 showed an endotherm corresponding to it between 15.6 and 14.3 °C in addition to the endotherm (37.9 to 38.9 °C) corresponding to 8-arm-PEG-SH (Figure 4B). The structural characteristics of both PEG hydrogel and dendrimer are seen in the dendrimer−PEG hydrogels.

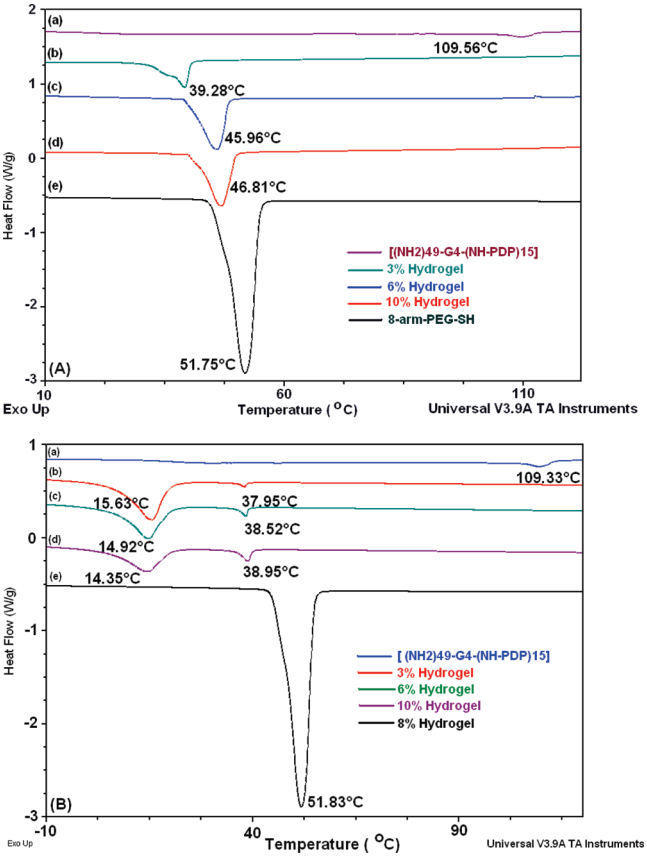


Figure 4. The DSC thermograms for the 3, 6 and 10% dendrimer−PEG hydrogels. (A) Hydrogels without formulation additives (absence of glycerin, PVP and PEG600). The 8-arm-PEG-SH (e) shows an endotherm at 51.7 °C, which is lowered on cross-linking with $[(NH_2)_{49}$-G4-$(NH\text{-}PDP)_{15}]$ as seen in curves (b), (c) and (d) for 3, 6 and 10% hydrogels, respectively. (B) Hydrogels with formulation additives (glycerin, PVP and PEG 600). In addition to the endotherms corresponding to 8-arm-PEG-SH (37.9 to 38.9 °C) in hydrogels, an endotherm for PEG 600 is seen between 15.6 and 14.3 °C.

3.6. Degradation of Hydrogels. The hydrogels investigated in the current study are biodegradable in nature, and their degradation was evaluated in simulated vaginal fluid and buffer as they are designed for intravaginal and intracervical application. The disulfide cross-links in the hydrogels are introduced to aid its slow degradation and easy self-washout from the body orifice. The female reproductive tract secretions are rich in glutathione and glutathione transferase, and the GSH levels range between 28 and 284 mg in human cervical secretions.[59,60] The disulfide

linkage or cross-links in the gel are cleavable in the presence of GSH.[61−63] Thiol−disulfide exchange is a chemical reaction in which a thiolate group S^- attacks on one of the sulfur atoms of a disulfide bond −S−S−. Under basic and mild acidic conditions, GSH is known to act as a thiolate moiety, and it gets oxidized while cleaving the existing disulfide bonds. These reactions are favored at higher basic pH, and the vaginal pH is low (3.8−4.5). It is therefore expected that *in vivo* the disulfide bonds in gel would undergo a slow degradation at vaginal pH, in the presence of GSH. The *in vitro* experiment showed that hydrogels were stable up to 3 days upon exposure to GSH solution at pH 4.0, and in simulated vaginal fluid, and did not show any signs of degradation as seen (Figure S4 in the Supporting Information). After 72 h the gels started to degrade and erode in both the solutions; this was reflected in ∼0.24% w/w reduction in the weight of the gels, and a further reduction in weight by ∼0.44% w/w over time (Figure S5 in the Supporting Information). This is consistent with the *in vivo* degradation pattern which is discussed in the subsequent sections. The chromatograms for the GSH solutions containing hydrogels did not show generation of any peaks until 50 h (data not shown). After 65 h the presence of a few small peaks could be seen, which is attributed to the breakdown of the gel into the smaller polymer components. The slow degradation of hydrogel is expected over time and would release the polymer components. The amine terminated dendrimers by themselves exhibit antibacterial activity by alteration of the bacterial cell wall.[25] The $[(NH_2)_{49}$-G4-$(NH\text{-}PDP)_{15}]$ dendrimers in the present study have partially unmodified amine groups, and these dendrimers are therefore expected to act as antibacterial agents. The antibacterial activity of partially PEGylated amine terminated dendrimers is well documented.[24] Hence the hydrogels of the present study are expected to exhibit dual antibacterial mechanism attributed to the slow release of the amoxicillin followed by release of partially amine terminated G4 PAMAM dendrimer.

3.7. Degree of Swelling. The degree of swelling in hydrogels was measured gravimetrically by calculating the equilibrium swelling obtained by comparing the ratios of the weights of the dry and water-swollen hydrogels over the time course. The degree of swelling in hydrogels influences the pore size, which affects the mechanical strength of the hydrogels and the drug release properties. The 3% hydrogel showed higher swelling when compared to that of 6% and 10% gel (Figure S6 in the Supporting Information). The equilibrium swelling state was reached for the 3, 6 and 10% hydrogels within 10, 8, and 6 h respectively. The observed pattern is attributed to the increased cross-linking density in hydrogels containing higher polymer concentration. It was observed from the confocal microscopy that the cross-link density in 3% hydrogel was lower as compared to the 6 and 10%, and the swelling results further support these results.

3.8. Drug Loading Efficiency. Amoxicillin was physically entrapped into the *in situ* forming gels. Amoxicillin was dissolved in the 8-arm-PEG solution and mixed with the $[(NH_2)_{49}$-G4-$(NH\text{-}PDP)_{15}]$ solution to form the gel. The theoretical amounts of drug used for entrapment were 0.50 mg in 200 μL of hydrogel formulation (3, 6 and 10%). The drug extracts from the hydrogel were quantified by reverse phase (RP) HPLC analysis monitoring elution with absorbance of 229 nm using water:acetonitrile as the mobile phase. The amount of drug entrapped in the 3, 6 and 10% w/v hydrogels was 52, 45 and 41% respectively. The 3% gels showed relatively higher drug loading efficiency over the 6 and 10% gels, and this difference could be attributed to higher cross-linking density in gels with higher polymer concentration and reduced pore size as seen in Figure 3.

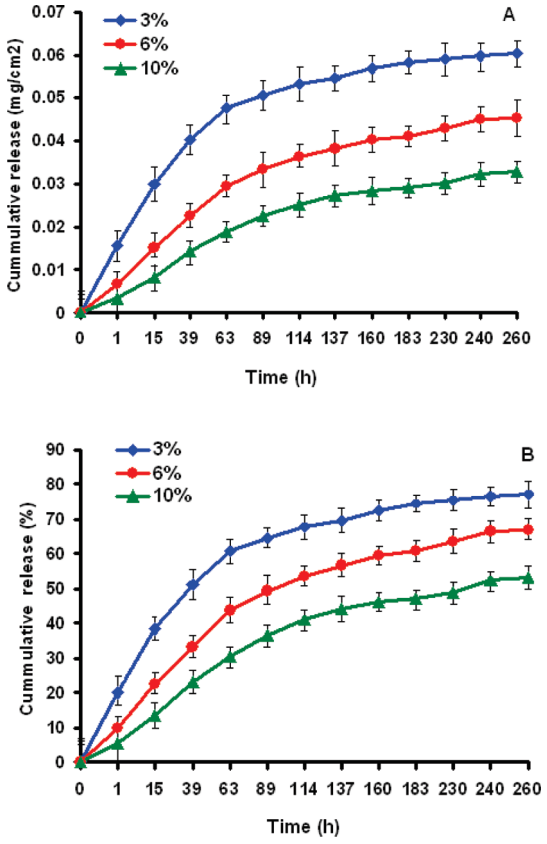


Figure 5. (A; bottom) Cumulative amount of amoxicillin released with respect to time (h) across per cm^2 area for 3, 6 and 10% hydrogels and (B; top) cumulative amount of amoxicillin released with respect to time. The release mechanism was found to be non-Fickian for 3 and 6% hydrogels while for 10% hydrogels it approached Fickian diffusion. All the experiments were carried out in triplicate, the data in the plots represents the mean of these and the error bars represent the standard deviations (±).

3.9. *In vitro* Drug Release. The *in vitro* drug release profiles from three different hydrogel formulations were studied using Franz diffusion cells. The plot of cumulative amount of drug released (mg/cm^2) as a function of time (h) from the three different types of hydrogels is shown in Figure 5A. The drug release plot shows that amoxicillin release was sustained for 260 h with a total release of 72%, 63%, 51% from 3%, 6% and 10% hydrogels respectively. A relatively slower drug release was observed from 10% hydrogel when compared to 3% hydrogel. These results are consistent with the lower swelling of the 10% hydrogel, which is attributed to the high cross-link density in polymer network obtained for higher polymer concentrations, which lowers the pore size. The plot of percent drug released versus time was used to determine the release mechanism (Figure 5B). The data (first 60% of the amount release) was fitted to explain the release mechanism and pattern using the Peppas equation as follows:

$$\frac{M_t}{M_\infty} = kt^n \qquad \text{(I)}$$

where M_t/M_∞ is the fraction of drug released, k is a kinetic (proportionality) constant dependent on the system, t is the time

Table 2. Determination of Flux, Diffusional Exponent (n) and Permeability Coefficient for Hydrogels

% w/v hydrogels	flux (J) (mg cm^{-2} s^{-1}) × 10^{-7}	diffusion exponent (n)	permeability coeff (P) (cm h^{-1}) × 10^{-6}
3	4.72	0.20	1.81
6	4.16	0.25	1.85
10	3.88	0.49	1.89

period for release, and n is the diffusion exponent indicative of the release mechanism for matrices of various shapes and swelling patterns. In the case of Fickian release, the exponent n has a limiting value of 0.50, 0.45, and 0.43 from slabs, cylinders, and spheres, respectively.[64] The values of n and k are inversely related, and a higher value of k suggests a burst release of drug from matrix. The values of diffusional exponent are shown in Table 2. At higher polymer concentration (10%) the drug release mechanism seems to approach the Fickian diffusion with n = 0.49, while the lower polymer concentrations exhibit non-Fickian release mechanism. One of the important functions of the gel is to provide extended release of the drug, which was evaluated using the Franz diffusion cell. This study shows that the drug is released by diffusion mechanism from gels at higher concentration. This study also confirms that there is no dose dumping from the gel, and it exhibits extended release to exhibit local therapeutic efficacy.[44−46]

Permeation parameters were obtained from the cumulative amounts of drug permeated (mg cm^{-2}) as a function of time (hours). The steady state flux (J) representing the absorption rate per unit area was determined from the slope of the linear portion of the plots. In all experiments the same number of data points were taken to calculate the steady state flux. The permeability constant (P) was calculated according to Fick's first law of diffusion, based on the steady state flux and the applied drug concentration (C_i) on the donor side. The permeability coefficients were deduced by dividing the flux by the initial drug load (C_i) as shown in equation

$$J = \frac{dQ}{dt\ A} \qquad \text{(II)}$$

$$P = \frac{J}{C_i} \qquad \text{(III)}$$

The flux, diffusional exponent and permeability coefficient are shown in Table 2. The flux and permeability were found to decrease with the increase in the polymer concentration. This is due to the increased cross-link density and lower swelling of the hydrogels at higher polymer content. The observed result is consistent with the lower drug release rate at higher polymer concentrations. This indicates that the dendrimer−PEG hydrogel forms a tight network as compared to the PEG cross-linked hydrogels. The dendrimer−PEG hydrogels of the present study are therefore expected to sustain the drug release efficiently.

3.10. *In Vivo* Testing of Hydrogel Formulations in Guinea Pig Model. Vaginal distribution, retention, biodegradation and tolerability of gels are important parameters to achieve sustained residence in the body cavity. The intravaginal gels of the present investigation are developed to treat the ascending genital infections during pregnancy. Since the gel is *in situ* forming, permeation and transport of the gel (PEG and [$(NH_2)_{49}$-G4-(NH-PDP)$_{15}$] polymers) across the fetal membranes into the fetus was investigated. The 10% w/v gels were used for *in vivo* testing as these were found to sustain the drug release for longer times as compared to 3 and 6% gels. The volume of the gel for intravaginal application was determined by injecting the samples 100 to 500 μL. The ideal volume for application was found to be 200 μL, and any volume above that resulted in leaking of the gel material outside the vagina. Similar volumes for intravaginal gels in guinea pigs were reported.[20] The gels without formulation additives exhibited short residence times and were leaked out as brittle particles after 24 h. The gels with formulation additives (glycerin, PVP and PEG 600) were retained in the cervicovaginal region at least up to 72 h, the end point used in this protocol. The incorporation of PVP in the gels provides the mucoadhesive effect. Figure 6 shows the presence of gel after 5, 12, 24, and 72 h of application. The visual examination revealed that 200 μL gel volume was sufficient to cover the cervicovaginal region. The gel could be seen in the cervicovaginal region in the early hours after application (5 and 12 h), and the gel was retained in this region even at later time points (24 to 72 h). The gel was found to slowly degrade with change in morphology, and the eroded material was seen on the fetal membranes of the pups positioned very close to the cervix (Figure 7A). The gel was not seen on any other pup (fetus) positioned away from the cervix. It is interesting to note that the gels with disulfide bonds exhibited a slow degradation *in vivo* at vaginal pH. This observation is similar to the *in vitro* degradation study in simulated vaginal fluid with GSH at pH 4. The gel components remained on the surface of the fetal membranes without transport across the membranes (Figures 7A and 7B). The [$(NH_2)_{49}$-G4-(NH-PDP)$_{15}$] dendrimer released due to degradation of the gel is not seen across the fetal membrane. The pups did not show traces of gel components on fur after removal of the fetal membranes (Figure 7C), indicating that the gel does not cross the fetal membranes and can be used for the selective local treatment of the pregnant mother without transfer to the fetus. Our previous *ex vivo* studies in human fetal membranes showed that the transport of FITC labeled G4 PAMAM dendrimers is restricted across the membrane.[65] The results of the present *in vivo* study combined with the previous *ex vivo* study substantiate that the hydrogels formed using the [$(NH_2)_{49}$-G4-(NH-PDP)$_{15}$] and 8-arm-PEG- SH do not cross the fetal membranes and could be used for the selective local treatment of pregnant woman without transfer to the fetus. The pH of the vagina was tested after 5 h, 12 h and 24 h after application of the gel using the swabs, and no change in pH was observed after application of the gel. The gels are formed rapidly *in situ*, and they absorb the buffer in which they were formed without affecting the pH of the vagina. None of the animals showed any discomfort after application of gel; none of the animals aborted in 72 h. The visual examination of the vaginal tissues showed no signs of edema and irritation, and the gels were well tolerated by the animals.

The histological evaluation of the uterus and cervicovaginal epithelial layer shows that the cell layer was not disrupted and no morphological changes were observed in the cells after 24 and 72 h treatment with hydrogels (Figure 8). The epithelial layer of the control tissue and the 24 and 72 h tissue with hydrogel treatment appear comparable. There are no signs of sloughing of the epithelial cells into the lumen, inflammation or edema of the epithelium. The submucosal tissues after hydrogel treatment (24 and 72 h) did not show any signs of necrosis or massive infiltration of the inflammatory cells. The cervical tissues show presence of the superficial mucous cell layer, and after treatment with hydrogels the tissues do not show any signs of sloughing of the superficial mucous layer.

No signs of atropy of the epithelial cell layer or the superficial mucous layer were observed after the hydrogel treatment for 72 h. The animals treated with hydrogels did not show any signs

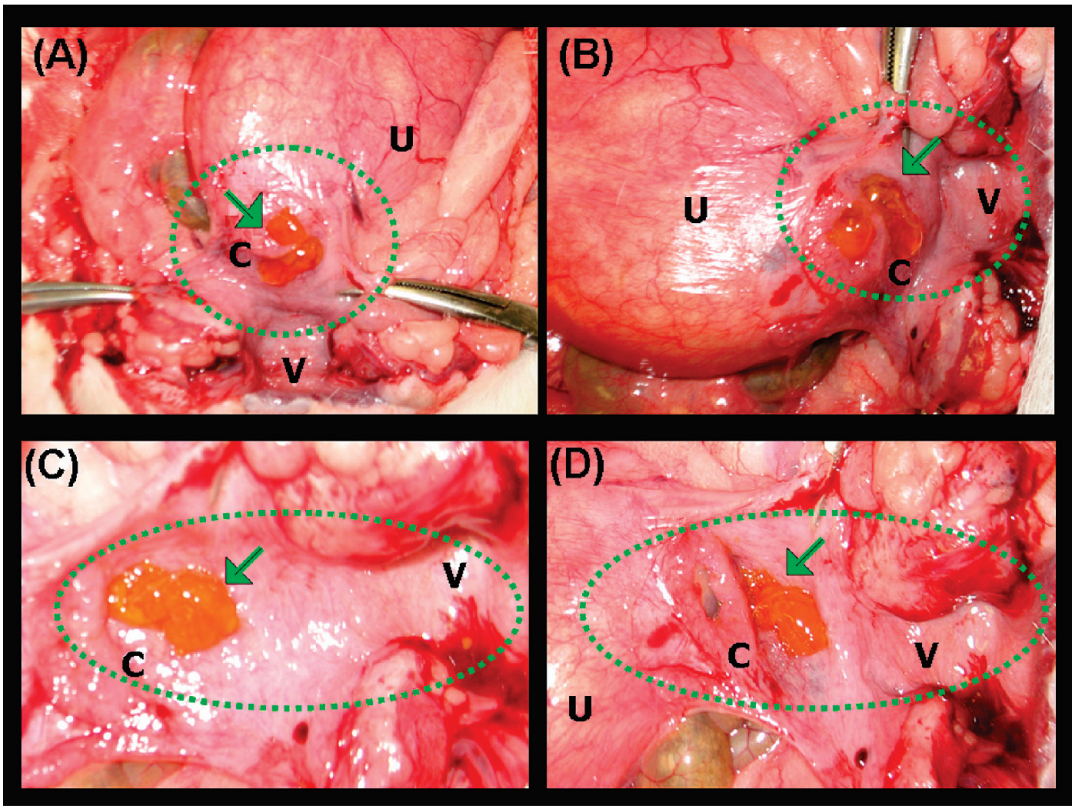


Figure 6. Intravaginal and intracervical application of *in situ* forming dendrimer−PEG hydrogels in the pregnant guinea pigs. The green arrows mark the presence of hydrogel on the tissue. (A) Day 1: hydrogel after 5 h of application. (B) Day 1: hydrogel after 12 h of application. (C) Day 2 after hydrogel application. (D) Day 3 after hydrogel application. C = cervix, V = vaginal cavity, U = uterus with pups. The hydrogel is retained in the cervix and vaginal cavity for 2 days, and on day 3 it is seen largely in the vaginal cavity of pregnant guinea pigs.

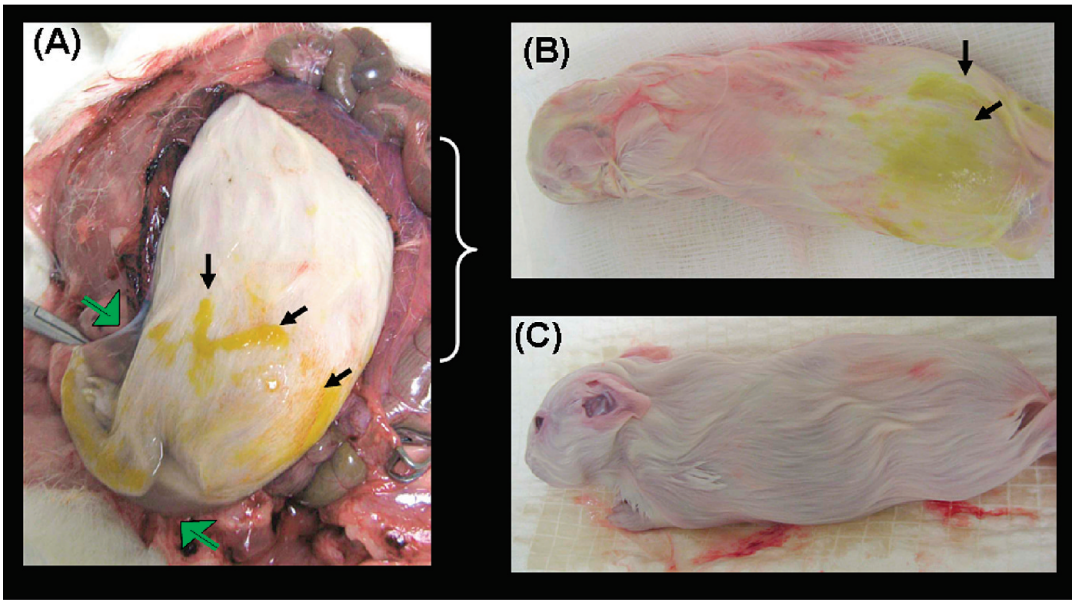


Figure 7. The dendrimer−PEG hydrogels after intravaginal and intracervical application in pregnant guinea pigs do not cross the fetal membrane and enter into the gestational (sac) cavity. (A) Day 3: hydrogel seen on the fetal membrane of the pup positioned close to the cervix. The green arrows mark the presence of fetal membrane on the pup, and the black arrows show the presence of gel outside of the fetal membrane. (B) The pup covered in fetal membrane with hydrogel on top of the fetal membrane. (C) The pup after removal of the fetal membrane showing no signs of hydrogel on the fur or inside the fetal membrane.

of thickening of the mucous cell layer when compared to the control animal (Figure 8). These results suggested that the animals were tolerant to the gels and no untoward reaction was exhibited. The residence of the gel on the mucified epithelial

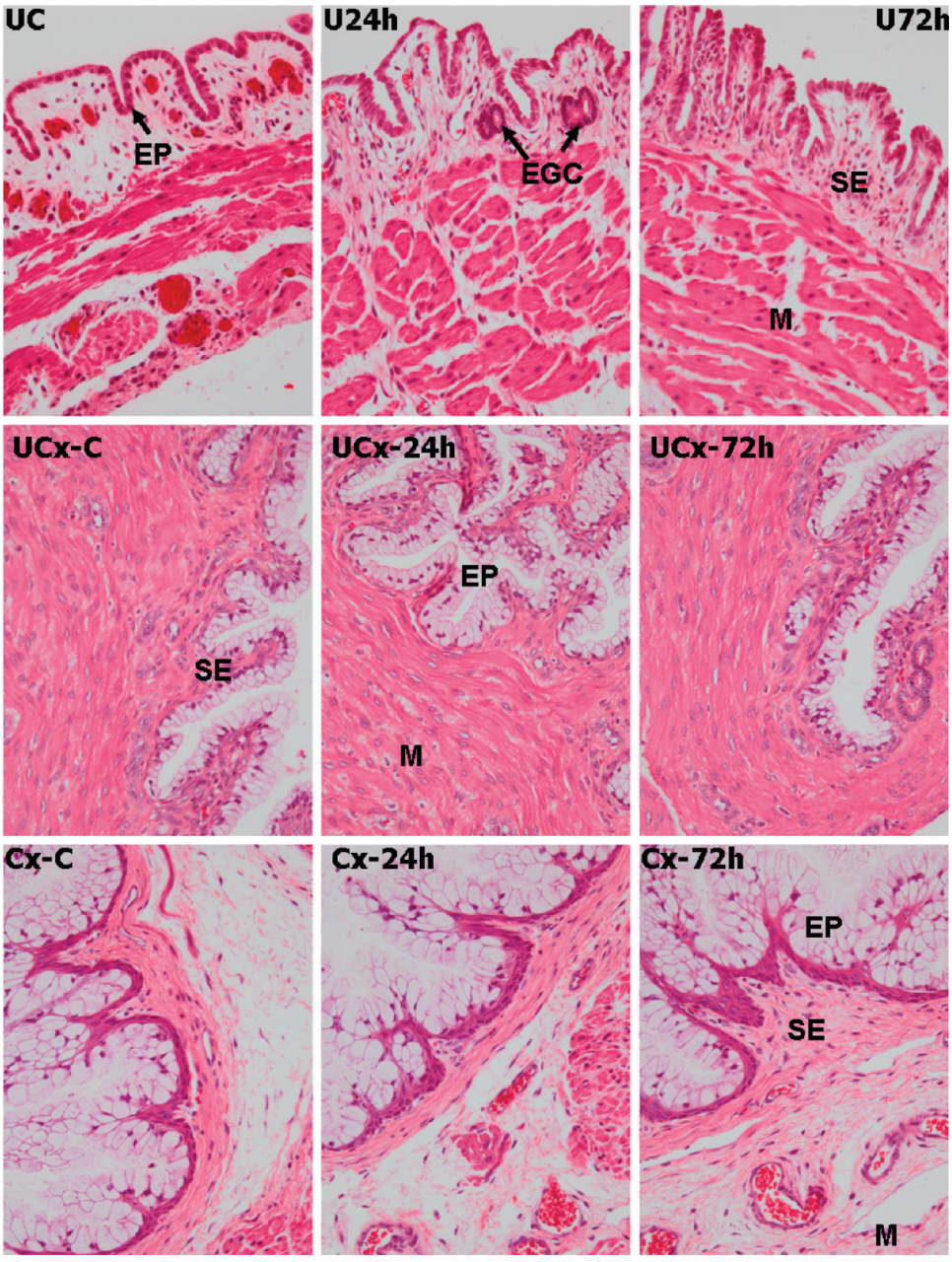


Figure 8. The hemotoxylin and eosin stained histological sections of uterus (U), upper cervix (UCx) and cervix (Cx) of guinea pig treated with the hydrogels for 24 and 72 h (n = 3 per group). The epithelial cell lining in all the tissues is intact and does not show any signs of inflammation and edema. The submucosa of hydrogel treated cervix after 24 and 72 h is comparable to that of the control. None of the tissues showed any signs of epithelial sloughing, necrosis in the submucosa or massive infiltration of inflammatory cells. EP = epithelial cells, SE = subepithelium, M = muscular layer, EGC = endometrial gland cells, UC = uterus control, U24h and U72h = hydrogel treated uterus 24 and 74 h, UCx-C = control upper cervix, UCx-24h and UCx-72h = hydrogel treated upper cervix 24 and 74 h, Cx-C = cervix control, Cx-24h and Cx-72h = hydrogel treated cervix 24 and 74 h (40× magnification).

cells of the cervicovaginal region was further confirmed from the histological evaluation of immunohistofluorescence images (Figure 9). The fluorescent gel comprising $[(NH_2)_{49}$-G4-$(NH\text{-}FITC)_2$-$(NH\text{-}PDP)_{15}]$ cross-linked with 8-arm-PEG-SH was used for this investigation, and the cross sections of the vagina and cervix show the presence of fluorescent gel (green color) on the mucified epithelial layer (red) marked positive with anticytokeratin. The presence of gel is apparent at time points 24 and 72 h respectively. The immunohistofluorescence images of the *fetal* membrane and the uterus at 72 h do not show the presence of the gel across these tissues (Figure 10), as seen by the absence of the fluorescent green. These results confirm that the gel components are primarily located on the epithelial surface of the cervical region and do not cross into deeper tissue. The ascending bacterial infection causes chorioamnionitis, which is associated with development of cerebral palsy, a motor disorder in children due to stimulation of proinflammatory cytokines causing white matter damage and fetal brain injury.[66] The local delivery of antibiotics in the cervicovaginal region is the preferred therapy for the treatment of these infections.

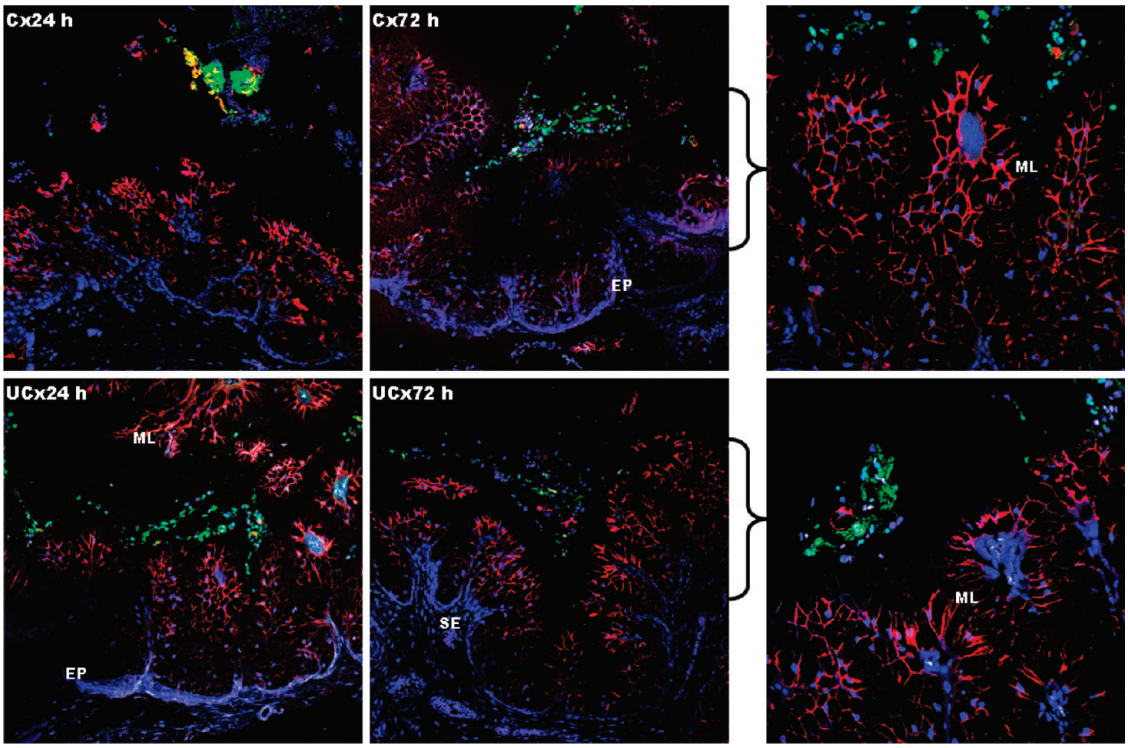


Figure 9. The confocal images of the cervical region of pregnant guinea pigs treated with hydrogels for 24 and 72 h. The *in situ* forming hydrogel comprising $[(NH_2)_{47}$-G4-$(NH\text{-}FITC)_2(NH\text{-}PDP)_{15}]$ cross-linked with 8-arm-PEG-SH was applied to the cervicovaginal region. The hydrogel (green color) is seen on the surface of the mucosal layer (red color). The confocal images after 24 and 72 h confirm the presence of the gel on the tissue surface. The nuclei for all cells are stained blue with DAPI. There is no sign of the degraded gel into the subepithelial or submucosal layers. EP = epithelial layer, SE = subepithelial layer, ML = mucified epithelial layer.

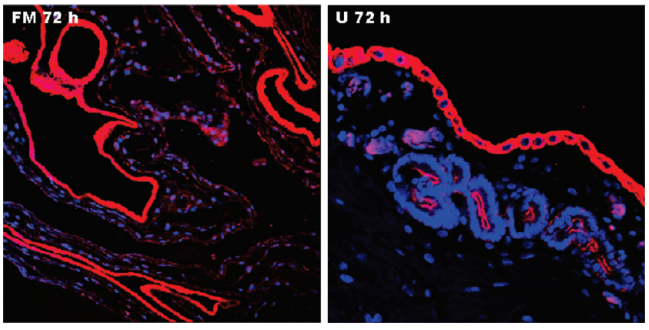


Figure 10. The confocal images of the fetal membrane and uterus of guinea pigs treated with hydrogels for 72 h. The *in situ* forming hydrogel comprising $[(NH_2)_{47}$-G4-$(NH\text{-}FITC)_2(NH\text{-}PDP)_{15}]$ cross-linked with 8-arm-PEG-SH was applied to the cervicovaginal region. The cross section of the uterus and the fetal membrane do not show presence of hydrogel or degraded hydrogel across the tissue.

The hydrogels exhibited long residence times of at least 72 h and were very well tolerated by the tissues. The constituent dendrimer of this hydrogel was not cytotoxic to human cervical epithelial and BV2 mouse microglial cells at the concentrations used in the topical application, as shown before.[67] In addition, PEGylation of the PAMAM dendrimer is expected to reduce the cytotoxicity of the dendrimer significantly.[68] The covalent linking of the dendrimer to the PEG overcomes the cytotoxicity associated with the dendrimer, which is well documented.[17,40] These findings are significant as the dendrimers in the size range 5 to 6 nm do not cross the human fetal membranes which separate the extraamniotic cavity and the fetus, and could be used for local intravaginal delivery to pregnant women, as we have reported recently.[65] The overall findings of the present study suggest that the proposed hydrogels offer an excellent degradable drug delivery system which exhibits sustained local delivery of the antibacterial agents intravaginally to the pregnant mother without transfer to the fetus.

4. CONCLUSIONS

Drug therapy during pregnancy is challenging, and effective ways to selectively treat the pregnant woman without affecting the fetus are always desired. Topical delivery of therapeutic agents is favored to treat ascending genital infections in pregnant women. Biodegradable *in situ* forming hydrogels obtained by cross-linking of $[(NH_2)_{49}$-G4-$(NH\text{-}PDP)_{15}]$ dendrimer and 8-arm-PEG via formation of disulfide bridges is described. Amoxicillin release from these hydrogels (3, 6 and 10% w/v) is sustained for more than 240 h, and the release approaches a Fickian diffusion pattern from the 10% w/v hydrogels. The *in vivo* evaluation of the hydrogels using a pregnant guinea pig model shows that gels are very well tolerated by the animals and no signs of change in vaginal pH and erythema are observed up to 72 h. The gel volume of 100−200 μL was found to sufficiently cover the entire cervicovaginal region as seen by visual examination. The gels exhibited a slow degradation *in vivo* at the vaginal pH, and the degraded gel was retained in the maternal tissues without transfer across the fetal membranes. These results were confirmed by visual and immunohistofluorescence images of tissues

which showed that the gel is largely retained in the superficial mucified epithelial cells. The histopathological evaluation of the vaginal and the cervical tissues showed absence of epithelial cell edema, necrosis and infiltration of inflammatory cells in the subepithelial and submucosal tissues. There were no signs of sloughing of the superficial epithelial cell layer after application of the hydrogels. The morphology of the tissues treated with the hydrogels for 24 and 72 h was comparable to that of the control tissues. The overall results confirm that the gels were very well tolerated by the animals and none of the animals aborted in 72 h after application of gels. The *in situ* forming hydrogels of the present invention offer therapeutic approaches to provide localized selective treatment of the pregnant woman with ascending genital infections without adverse effects to the fetus.

■ ASSOCIATED CONTENT

Supporting Information. Experimental procedures, ^{1}H NMR, ^{13}C NMR, MALDI-TOF, analysis of all products. This material is available free of charge via the Internet at http://pubs.acs.org.

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