

# Formulating a Sulfonated Antiviral Dendrimer in a Vaginal Microbicidal Gel Having Dual Mechanisms of Action

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**SPL7013 is the sodium salt of a sulfonated dendrimer that has potent antiviral properties. VivaGel<sup>®</sup>, a topical gel containing 3% (wt/wt) SPL7013, is in development as a vaginal microbicide. BufferGel<sup>®</sup> is a Carbopol<sup>®</sup>-based acidic buffering gel that enhances the natural protective action of the vagina to produce a broad-spectrum microbicidal environment. The positive attributes of both gels were combined into a combination vaginal microbicidal gel having dual mechanisms of action. A 3% (wt/wt) SPL7013 combination gel, pH 3.7, was developed and fully characterized and was shown to have more than twofold greater acidic buffering capacity than BufferGel. Ultracentrifugation experiments demonstrated that SPL7013 was not sequestered or entropically trapped in the viscous gel, thereby confirming, along with viral challenge studies, that SPL7013 has sufficient mobility in the viscous gel to exert antiviral properties.**

**Keywords** microbicide; vaginal; dendrimer; ultracentrifugation; diffusion; Carbopol<sup>®</sup>

## INTRODUCTION

The incidence of HIV infection continues to grow rapidly, with the most recent data from the United Nations showing that

33 million people worldwide are HIV positive (Report on the Global HIV/AIDS Epidemic 2008. Joint United Nations Program on HIV [UNAIDS] 2008). Of particular concern is the high percentage of women who have become infected with HIV. In those parts of the world where the primary means of infection is through heterosexual contact, women comprise up to 60% of the total infected population. An additional concern with the increasing number of HIV-infected women is the increased rate of transmission of HIV to the newborn. Thus, there is an urgent need for strategies to prevent HIV infection during sexual contact. One strategy is the use of microbicides, which are chemical entities that can prevent or reduce transmission of sexually transmitted infections (STIs) when applied to the vagina or rectum (Ndesendo et al., 2008). Microbicidal candidates have included, among others, (a) *macromolecular polyanions* such as PRO 2000 (naphthalene 2-sulphonate polymer), cellulose sulfate, dextran sulfate, and carrageenan (Cummins et al., 2007; Maguire, Bergman, & Phillips, 2001; Neurath, Strick, & Li, 2002; Turpin, 2002) and (b) *acidic buffering gels*, which exert their activity by inducing or maintaining low vaginal pH, wherein viruses such as HIV and HSV, cellular vectors such as HIV-infected cells, and pathogenic bacteria are inactivated (Olmsted et al., 2005; Patton et al., 2004; Williams et al., 2007; Zeitlin et al., 2001).

Dendrimers are a relatively new class of macromolecules characterized by highly branched, three-dimensional architectures that offer an alternative to polyanionic microbicidal polymers (McCarthy et al., 2005). The controlled synthesis of

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dendrimers allows the assembly of highly defined structures that radiate out in generations from a central initiator core. Several anionic dendrimers have been synthesized and tested in vitro and in vivo for their antiviral properties. In fact, selected anionic dendrimers are potent inhibitors of a range of STIs, including HIV-1, HSV-1, and HSV-2. One such polyanionic dendrimer being developed by Starpharma Pty Ltd (Melbourne, Australia), SPL7013 (16,581 Da), is built up from a divalent core, the benzhydrylamine amide of L-lysine (Figure 1). Successive addition of four L-lysine layers leads to a dendrimer with 32 amine groups on the surface—16  $\alpha$ -amines and 16  $\epsilon$ -amines from the outer L-lysine layer. The final step in the synthesis involves the last amide-bond-forming reaction to attach 32 sodium (1-naphthylenyl-3,6-disulphonic acid)-oxyacetamido groups to the surface via amide linkers. The  $EC_{50}$  of SPL7013 against HIV-1 (Strain IIIB) was  $<1 \mu\text{g/mL}$  (Bernstein et al., 2003; Gong et al., 2005). In addition, SPL7013 was found to be highly active against CCR5- and CXCR4-tropic HIV-1 infection (Lackman-Smith et al., 2008). A 5% (wt/wt) SPL7013 vaginal gel was found to completely prevent vaginal transmission of SHIV<sub>89.6P</sub> in female pigtailed macaques (Jiang et al., 2005). In June 2003, the SPL7013 Gel (termed VivaGel) was the first submission to the US FDA for a dendrimer-based drug. Initial human trials have shown VivaGel to be safe and well tolerated when administered up to twice daily for 14 days (Rupp, Rosenthal, & Stanberry, 2007), and additional clinical studies are on-going. Roth et al. (2007) recently showed that a SPL7013 Gel significantly reduced the amount of HIV-1 bound to epithelial cells in vitro versus placebo gel. In addition,

Abner et al. (2005) showed that SPL7013 Gel significantly reduced HIV-1 infection in human colorectal explant tissue versus placebo. Rupp et al. (2007) provide an excellent review of the HIV and HSV antiviral activity of SPL7013 and SPL7013-containing gels in in vitro and animal models.

BufferGel is a broad-spectrum microbicide/spermicide gel being developed by ReProtect Inc. (Baltimore, MD, USA) that enhances the protective action of vaginal acidity (Ballagh et al., 2008; Patton et al., 2004; Van De Wijgert et al., 2001; Williams et al., 2007). BufferGel is a nontoxic lubricant gel formulated at pH 3.8, the pH of the healthy vagina. BufferGel is made with a strongly buffering polymer, Carbopol 974P, that releases protons to acidify the ejaculate, thereby enhancing the protective acidity of the vagina and complementing the acidifying action of lactobacilli, the vaginal flora that create the protective acidity of the vagina (Olmsted, Dubin, Cone, & Moench, 2000; Olmsted et al., 2005). Human clinical trials have shown BufferGel to be safe, acceptable, and less inflammatory and tissue-destructive than detergent-based spermicides (Cone et al., 2006; Mayer et al., 2001; Patton et al., 2004; Van De Wijgert et al., 2001), and it is currently being investigated in phase 3 clinical trials.

The overall goal of this study was to combine SPL7013 dendrimer in a Carbopol-based acidic buffering gel to create a microbicidal gel having dual mechanisms of action. Various combination vaginal gels were prepared and tested for pH, osmolality, buffering capacity, and viscosity. The effect of temperature, pH, and SPL7013 concentration on gel viscosity was also determined. Since SPL7013 dendrimer is a polyanionic macromolecule, there was some concern that its diffusion

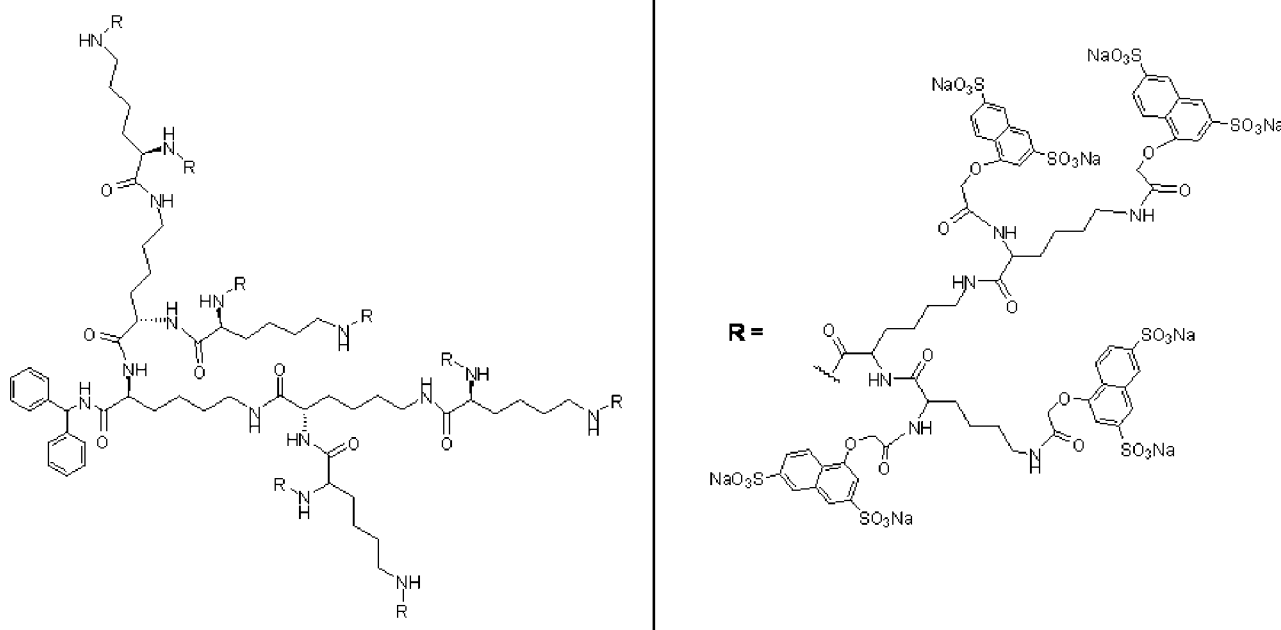


FIGURE 1. Structure of SPL7013 dendrimer (MW 16,581 Da). Successive additions of four L-lysine layers to the divalent core of benzhydrylamine amide of L-lysine leads to a final dendrimer molecule having 32 amide-linked 1-naphthylenyl-3,6-disulphonic acid)-oxyacetamido groups on the surface.

would be constrained in a viscous Carbopol-based gel thereby reducing its antiviral activity. Thus, an ultracentrifugation method was developed to ascertain the tendency of SPL7013 to be sequestered or trapped in the viscous gel.

## MATERIALS AND METHODS

### Materials

Carbopol 974P NF and Carbopol 971P NF were obtained from Lubrizol Corporation (Wickliff, OH, USA). Lactic acid, USP, methyl paraben NF, propyl paraben NF, edetate disodium USP [ethylenediaminetetraacetic acid disodium (EDTA)], glacial acetic acid, potassium hydroxide NF, sodium chloride (0.9%, wt/vol), and sodium hydroxide NF were purchased from Spectrum Quality Products Inc. (New Brunswick, NJ, USA). SPL7013 dendrimer (lot #08-036-026-02) was provided by Starpharma Pty Ltd. The SPL7013 synthetic process is monitored by HPLC and LC/MS in-process controls and delivers SPL7013 as a single molecular entity as determined by HPLC, capillary electrophoresis, and electrospray mass spectral analysis.

### Gel Preparation

The gels were prepared using a modification of a previously published method (Bernstein et al., 2003) using a Caframo Ultrahigh-Torque Stirrer Model BDC1850 (Warton, Ontario, Canada) with a 5.1-cm stainless steel propeller blade. A general formula and method is provided for preparing 200-g batches based on Carbopol 974P. Specifically, MilliQ water (167 g) was added to the stirring vessel with stirring at 800 rpm. Then 300 mg EDTA disodium USP was added and allowed to dissolve (2 min). Then 8.68 g Carbopol 974P was then added slowly over 15–20 min while stirring at 1,000 rpm to allow for homogeneous hydration of the polymer. Then 360 mg methyl paraben and 40 mg propyl paraben were added and allowed to dissolve over 5 min. Lactic acid (1,000  $\mu$ L) and acetic acid (166  $\mu$ L) were then added and allowed to mix for 2 min. SPL7013 dendrimer (6.795 g) was then added and allowed to completely dissolve. An additional 3.72 g of Carbopol 974P was then added while stirring at 1,200 rpm over 15–20 min. Finally, the pH was adjusted to the target pH of 3.7–3.8 using 2 N KOH (~7.3 mL) and the batch was then q.s. to 200 g with MilliQ water. Additionally, to test the effect of final gel pH on viscosity, gels were made having final pHs of 3.4, 3.6, 3.8, 4.0, and 4.2. Finally, to determine the effect of SPL7013 dendrimer on gel viscosity, four gels were made having a final pH 3.7–3.8 with 6.2% (wt/wt) Carbopol 974P and lactic and acetic acids and containing final SPL7013 concentrations of 3, 4, 5, and 6%.

### Gel Characterization Methods

The viscosity of the gels was determined using a Brookfield Cone and Plate Rheometer RVDV-III+ (Brookfield Engineering;

Middleboro, MA, USA) at 25°C for 5 min at an rpm of 1.7 using Spindle CPE-52. For some gel samples, the viscosity was tested at various temperatures (in the range of 4–37°C) to ascertain the effect of temperature on gel viscosity. For these measurements, the rheometer was pre-equilibrated to the desired temperature using a circulating water bath. Osmolality was determined using a FISKE 110 Freezing-Point Depression Osmometer. The pH of the gels was determined using an Orion pH Meter Model 520A. The buffer capacity was determined by measuring the number of mEq of 0.5 N NaOH titrametric standard needed to bring 2.5 g of the formulation from its starting pH of approximately 3.8 to a final pH of 5.0. Specifically, approximately 2.5 g of the sample was deposited in a beaker. The sample was mixed with 20 mL of 0.9% NaCl solution using a stir bar for 5 min at a temperature of 23–25°C. The homogeneously mixed sample was then titrated with standardized 0.5 N NaOH solution until the pH of the mixture was 5.0. The volume of the NaOH needed to raise the pH to 5.0 was recorded and the buffer capacity of the sample was calculated using the following equation:

$$\frac{\text{Volume of NaOH (mL)} \times 0.5 \text{ N}}{\text{Actual weight of sample (mass)}} = \text{buffer capacity (mEq/g)}.$$

### SPL7013 Dendrimer Sequestration Experiments

Experiments were conducted to determine the relative extent of sequestration of SPL7013 in centrifuged prototype combination vaginal gels. One-gram samples of the indicated 3% (wt/wt) gels were ultracentrifuged using a Beckman TL-100 Ultracentrifuge with TLA 100.3 rotor at 25°C at 135,000  $\times$  g for 30 min, along with 1-g samples of a 3% (wt/wt) SPL7013 solution to assess settling and sedimentation, if any, of the SPL7013 in solution, as well as to serve as a control for assaying SPL7013 concentrations. After centrifugation for 30 min, a 100  $\mu$ L aliquot part of the supernatant was carefully aspirated from each sample and diluted in deionized water to a fixed final volume of 20 mL. To determine the concentration of SPL7013 retained in the dense gel layers, the remaining supernatant was carefully decanted, and a 100-mg sample of the gel layer was removed with a spatula, placed in a flask, and similarly diluted. Standard curves of each gel and the control solution were prepared by assaying the UV absorbance (332 nm) of serial dilutions of the 3% (wt/wt) SPL7013 control solution and the two 3% (wt/wt) starting control gels. UV spectroscopy at 332 nm was selected as the analytical method, as the Carbopol polymers showed no absorbance at this wavelength, obviating the need for chromatographic separation of components, whereas SPL7013 displayed an absorption peak at 332 nm. No interference from gel components was noted at 332 nm using this assay, as all three curves were nearly superimposable, with nearly identical linearity and linear equations (data not shown).

## RESULTS AND DISCUSSION

### Background and Target Composition for Prototype Combination Vaginal Gels

The compositions of BufferGel and VivaGel are shown in Table 1. All excipients for both BufferGel and VivaGel are generally regarded as safe (GRAS) when used in the amounts listed for vaginal administration. It should be noted that the concentration of BufferGel excipients is not publicly disclosed. Both gels are currently under human clinical testing for safety and efficacy. The gels are aqueous Carbopol-based gels with the notable difference that BufferGel contains Carbopol 974P while VivaGel contains Carbopol 971P. Carbopol 974P and Carbopol 971P have some important similarities and differences, as shown in Figure 2. While Carbopol 974P is a highly cross-linked, high-viscosity polymer with a consistency and flow like mayonnaise, Carbopol 971P is a lightly cross-linked low-viscosity polymer with a consistency and flow like honey. Carbopol 971P is both more pH- and salt-tolerant, meaning that the viscosity of Carbopol 971P-containing gels is less affected by changes in pH and salt concentration. This fact was considered in the initial development of VivaGel, because the addition of 3% (wt/wt) SPL7013 resulted in a sodium concentration of 115 mM, which significantly affected gel viscosity. Specifically, the addition of 3% (wt/wt) SPL7013 to an otherwise viscous Carbopol-based gel made with 3–5% (wt/wt) Carbopol in the pH range of pH 3–3.8 caused a substantial, if not complete, loss of viscosity for both Carbopol 971P- and Carbopol 974P-based gels. Interestingly, magnesium sulfate is

added to BufferGel to reduce the viscosity of the formulation to that acceptable for vaginal administration and product use.

Carbopol-based gels are known to be comprised of swollen microgel particles having both a macroviscosity and microviscosity. As shown in Figure 2, highly cross-linked polymers such as Carbopol 974P have more rigid gel microparticles (globules) when hydrated, whereas lightly cross-linked polymers such as Carbopol 971P are extended and flexible, thereby creating a more cage-like structure. As a consequence, the release rate of drugs from Carbopol 974P-based gels is usually greater than those from Carbopol 971P-based gels. For example, MacLean-McDavitt, Robertson, and Jay (2003), using X-ray fluorescence, showed that the release rate of iodine-labeled bovine serum albumin from subcutaneously injected Carbopol-based gels was significantly greater for highly cross-linked Carbopol 940 gels as compared to less cross-linked Carbopol-based gels.

As described in the “Introduction,” SPL7013 is a high molecular weight dendrimer, with a multilayered lysine backbone and a polyvalent naphthalene sulfonic acid surface. The size of the dendrimer and the large number of degrees of freedom in the scaffold result in a highly mobile structure comprising both hydrophilic (i.e., lysine moieties and charged sulfonic acid groups) and hydrophobic (i.e., naphthyl rings) regions, in addition to the association of 64 Na<sup>+</sup> ions per molecule. Both Carbopol 971P and Carbopol 974P are formed by crosslinking acrylic acid with allyl ethers of pentaerythritol, giving a saturated structure rich in ether, carbonyl, and hydroxyl moieties. Carbopol might form intermolecular interactions

TABLE 1  
Compositions of BufferGel and VivaGel and Target Composition for Novel Prototype Combination Vaginal Gels  
(Prototypes 1 and 2) Containing SPL7013 dendrimer

Ingredient (All Values in % wt/wt)	BufferGel	VivaGel	Target Prototype Combination Vaginal Gels
Mucoadhesive/thickening/ buffering agent	Carbopol 974P	5.0% Carbopol 971P	Carbopol 974P or 971P
pH adjustment	Dibasic potassium phosphate	9% 2N sodium hydroxide	KOH
Preservative	Sorbic acid	0.18% methyl paraben and 0.02% propyl paraben	0.18% methyl paraben and 0.02% propyl paraben
Osmolality adjustment	Monobasic sodium phosphate	None	None
Antioxidant	EDTA disodium	0.1% EDTA disodium	0.15% EDTA disodium
Emollient	None	1.0% propylene glycol and 1.0% glycerin	None
Viscosity reducer	Magnesium sulfate	None	Not needed due to Na from Na SPL7013
Inactivation of bacterial pathogens (GC and BV)	None	None	Lactic and acetic acids
SPL7013 dendrimer (active)	None	3%	3%

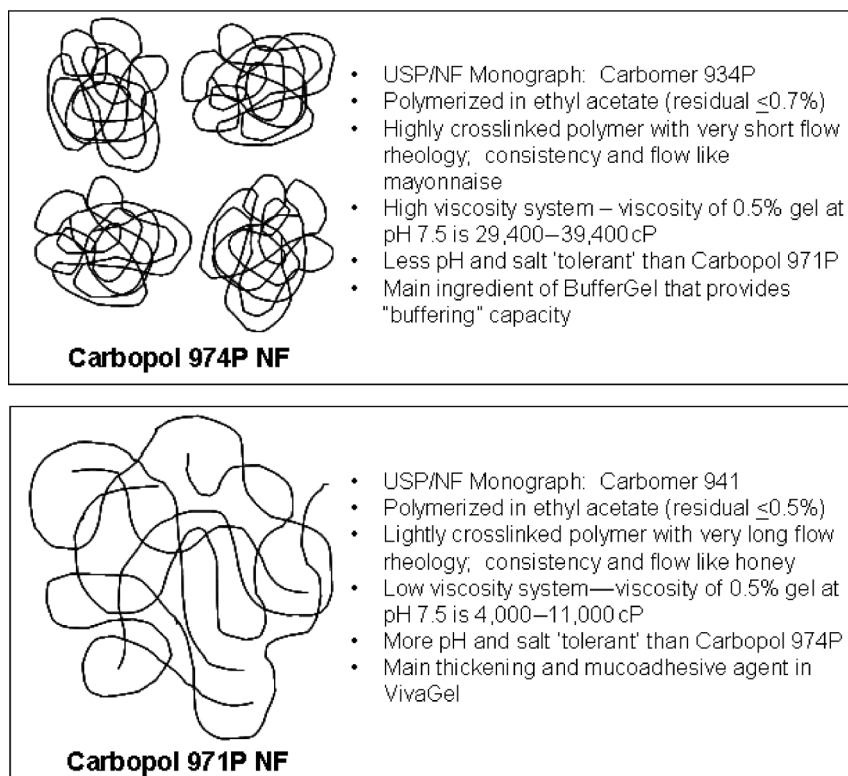


FIGURE 2. Structure and physical/chemical properties of Carbopol 974P and Carbopol 971P. Both polymers are GRAS and strongly mucoadhesive at lower pH because of H bonding of the repeating carboxylic moiety ( $pK_a = 6 \pm 0.5$ ). In addition, aqueous-based gels made of each polymer are non-Newtonian fluids. Differences between the two polymers are listed adjacent to their structures.

with SPL7013 (via hydrogen bonding of dendrimer NH groups with Carbopol carbonyl groups, or dendrimer carbonyls and Carbopol hydroxyls). Additionally, encapsulation of SPL7013 in the polymer matrix might arise because of the size, shape, and flexibility of the compound in solution versus the matrix of polymer chains formed by the Carbopol. Moreover, the differing extent of crosslinking between Carbopol 971P and Carbopol 974P could result in varied sequestration behavior of SPL7013 in these two gelling agents.

To achieve a prototype combination vaginal gel product with desirable product features, a series of Carbopol 974P and Carbopol 971P gels containing SPL7013 were produced and evaluated both qualitatively and quantitatively. Although each of the Carbopol types provide some theoretical advantages, Carbopol 974P-based gels were pursued for the combination vaginal gel as placebo Carbopol 971P-based gels tended to be more "sticky" to the touch with a consistency like "honey."

The assessment of the prototype gels lubricity (or conversely, stickiness) is primarily determined by touch. However, as this evaluation is conducted external to the body, it is acknowledged that internal application of the gel (into a warmer, higher humidity environment) may result in slightly different feel. To simplify the prototype formulations, it was elected to omit any emollient (e.g., the 1% [wt/wt] propylene

glycol or glycerin that are included in VivaGel) unless required to enhance the physical feel. If the prototype formulations were judged to need an emollient, then low levels ( $<10\%$ , wt/wt) would be included to avoid any potential mucosal irritation that has been observed at high concentrations with such excipients and which is caused by the high osmolality of the ensuing gels (Adriaens & Remon, 2008). In addition to the in vivo (slug) evaluation of toxicity related to high glycerol content, the use of a hyperosmolar gel formulation in a phase I clinical trial resulted in unacceptable adverse events (i.e., abnormal transudation of physiologic fluid across the cervico-vaginal mucosae) due to the high glycerin content (Lacey, 2008).

Both lactic and acetic acid were included in the combination vaginal gel product (prototype 1), as these cell-permeant species, naturally occurring in the vagina, have been shown to inactivate both viral and bacterial pathogens (Dimitonova, Danova, Serkedjieva, & Bakalov, 2007). Methyl and propyl parabens were chosen as the preservative in the prototype combination vaginal gels. The levels of the parabens are standard in topical products for providing antimicrobial properties but further confirmed by antimicrobial effectiveness testing on the prototype gel formulations (data not shown).

## Prototype Combination Vaginal Gels

### Gel Composition and Characterization

The composition and characterization of combination vaginal gel prototypes is shown in Table 2. The final prototypes with and without lactic and acetic acids (termed prototype 1 and prototype 2, respectively) were prepared using 6.2% (wt/wt) Carbopol 974P at a pH of 3.7. This relatively high Carbopol content was required to maintain sufficient viscosity of the vaginal gel, because the formulation of 3% (wt/wt) SPL7013 introduced a final sodium concentration of approximately 115 mM, which substantially reduced the gel viscosity at the relatively low pH of the prototype formulations. The reduction of viscosity of Carbopol-based gels by sodium is well known. Moreover, we independently confirmed that the addition of 57.5 mM NaCl to a pH of 3.7 and concentration of 3.8% (wt/wt) Carbopol 974P gel without SPL7013 reduced the viscosity to nearly that of water. In Figure 3, the effect of percent Carbopol 974P on the resulting viscosity of 3% (wt/wt) SPL7013 gels is plotted. There was a linear ( $R^2 = .9796$ ) relationship between Carbopol content and resulting gel viscosity. Interestingly, it was very difficult to produce a gel containing 6.2% (wt/wt) Carbopol 974P in the absence of SPL7013 (and its associated viscosity-reducing sodium ions), as the extremely high viscosity hindered mixing. These findings helped in defining a suitable manufacturing process for the gel wherein ~70% of the Carbopol is added first to make a gel base and the remainder is added after the SPL7013 dendrimer is added. Coincidentally, the necessity of increasing the Carbopol

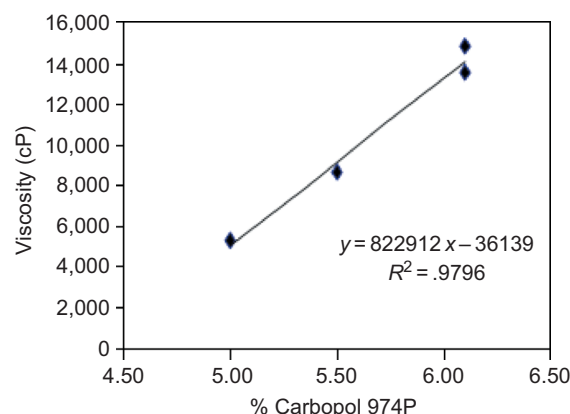


FIGURE 3. The effect of Carbopol 974P concentration on the resulting viscosity (cP) of prototype 1 combination vaginal gel made with 3% (wt/wt) SPL7013 dendrimer.

974P content in the prototype combination vaginal gels due to the inclusion of SPL7013 also resulted in gels having significantly increased acidic buffering capacity as compared with that of BufferGel. While BufferGel and VivaGel had acidic buffering capacity of 0.15 and 0.12 mEq/g, respectively, prototypes 1 and 2 had acidic buffering capacities more than twofold greater than 0.32 and 0.29 mEq/g, respectively. The addition of physiological levels of lactic (0.6% [wt/wt], 67 mM) and acetic acid (0.087% [wt/wt], 15 mM) in prototype 1 resulted in about a 90% increase in the osmolality of prototype 1 versus prototype 2 with an increase in the acidic buffering capacity of 10% (Table 2). In comparison, the inclusion of 1% (wt/wt) propylene glycol and 1% (wt/wt) glycerin in the VivaGel formulation contributed to its increased osmolality of 380 mOsm/kg H<sub>2</sub>O. With reference to the above discussion on hyperosmolar formulations inducing mucosal toxicity (Adriaens & Remon, 2008; Lacey, 2008), it should be noted that adverse toxicological behavior is observed with osmolality values greater than 2,000 mOsm/kg H<sub>2</sub>O, that is, substantially greater than those measured for formulations described here.

## Gel Characterization

### Effect of pH, Temperature, and Percent SPL7013 on Gel Viscosity

To address concerns over the effects of pH and temperature on the viscosity of the combination vaginal gel prototypes, gels containing 5.5% (wt/wt) Carbopol 974P and SPL7013 at various pHs were prepared. As shown in Figure 4A, there was a relatively small increase in viscosity in gels having a pH at or below pH 3.8. However, above pH 3.8, there was an exponential increase in viscosity, which is consistent with what is known about Carbopol-based gels alone in aqueous vehicles. These results suggested that target pH of the gel could be lowered to pH 3.7 and allow for even greater buffering capacity. The effect of temperature on the viscosity of a 3% (wt/wt) SPL7013

TABLE 2  
Final Composition of Prototype Combination Vaginal Gels  
Containing SPL7013 dendrimer

Ingredients	Prototype 1 Vaginal Gel (% wt/wt)	Prototype 2 Vaginal Gel (% wt/wt)
Purified water	q.s. to 100%	q.s. to 100%
Methyl paraben NF	0.18	0.18
Propyl paraben NF	0.02	0.02
EDTA disodium USP	0.15	0.15
Carbopol 974P NF	6.2	6.2
Lactic acid USP	0.6	None
Acetic acid USP	0.087	None
2N KOH NF	q.s.to pH 3.7	q.s.to pH 3.7
SPL7013 dendrimer	3.0	3.0
Characterization (n = 3–5 batches)		
pH	3.7 ± 0.08	3.7 ± 0.03
Viscosity	9484 ± 1344cP	8245 ± 1693cP
Buffering capacity	0.31 ± 0.02mEq/g	0.28 ± 0.01mEq/g
Osmolality	202 ± 13mOsm/kg	102 ± 4mOsm/kg

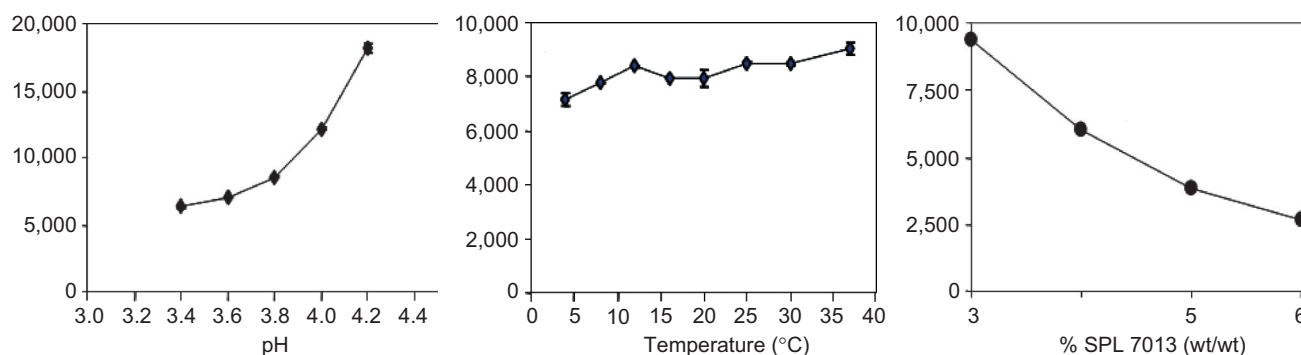


FIGURE 4. The influence of pH (A), temperature (B), and % SPL7013 dendrimer concentration (C) on the resulting viscosity of prototype 1 combination vaginal gel. Y-axis for A–C is viscosity (cP). Gels for (A) and (B) were made with 5.5% (wt/wt) Carbopol 974P and 3% (wt/wt) SPL7013. Gels for (C) were made with 6.2% (wt/wt) Carbopol 974P.

prototype 1 (pH 3.7) was also investigated. As shown in Figure 4B, a plot of viscosity versus temperature demonstrated that the viscosity of prototype 1 varied only modestly across this relatively broad temperature range. The information from these studies should prove extremely useful for large-scale manufacturing of the prototype 1 and subsequent storage. Lastly, the effect of SPL7013 concentration on prototype 1 gel viscosity is plotted in Figure 4C. As expected, as the SPL7013 concentration increased, the resulting gel viscosity decreased in an almost linear manner. It was concluded that a maximum of 4% (wt/wt) SPL7013 could be formulated in the prototype 1 gel allowing the vaginal gel formulation to have sufficient viscosity for administration (i.e., >5,000 cP using this method).

#### Ultracentrifugation of SPL7013 Gels

The phenomenon of “entropic trapping” of macromolecules in the void space of polymer hydrogels has previously been described (Liu, Li, & Asher, 1999). Entropic trapping is influenced by molecular size-dependent diffusion of the macromolecule. The mean (z-average) diameter of SPL7013 as determined by dynamic light scattering measurements (at 1 mg/mL in  $10^{-2}$  M NaCl) (C. Prestidge, I. Ametov, & T. Barnes, Characterization of Starpharma Dendrimers by Dynamic and Static Light Scattering. Private communication, 2005) is 5 nm. This correlates well to the Stokes diameter of approximately 3–4 nm, which may be calculated based on its molecular weight of 16,581 Da. It was therefore hypothesized that Carbopol 974P in the viscous gel might bind or trap SPL7013 by either entropic trapping or hydrogen bonding, thereby leaving some SPL7013 less available for interaction with HIV, HSV, or semen. This would be an obvious concern for a vaginal microbicidal gel product containing an antiviral compound that required the compound to physically interact with the virus to exert activity. Importantly, previous studies have suggested that the rate of diffusion of molecules decreases with increasing size. For example, Flemstrom et al. (1999) showed that the mucus gel layer in the duodenum significantly restricts migration of macromolecules to the duodenal surface.

However, Olmsted et al. (2001) later showed, using improved analytical methods and employing fluorescent recovery after photobleaching and fluorescent imaging, that large proteins (15–650 kDa) and even small viruses such as the human papilloma virus (55 nm) and Norwalk virus (38 nm) could diffuse as rapidly in mucus as in saline. It was demonstrated that the mesh spacing between mucin fibers (20–200 nm) was large enough to allow these macromolecules to diffuse unconstrained. Still, the diffusion of larger proteins as well as that of the herpes simplex virus (180 nm) was hindered by the viscous mucin.

Analytical ultracentrifugation is a common and powerful method employed to quantify the size and shape of macromolecules and their interactions (Howlett, Minton, & Rivas, 2006). This method has many different applications, including the study of molecular associations and reactions under equilibrium conditions, as well as to ascertain macromolecule size and shape. For example, Howlett et al. (2006) reviewed the uses of ultracentrifugation to study protein association and assembly. In addition, Joralemon, O’reilly, Hawker, and Wooley (2005) used analytical ultracentrifugation to study the sedimentation equilibrium of formed nanostructures resulting from polymeric micelles crosslinked with divergently synthesized dendrimers.

In the present studies, we intended to adapt ultracentrifugation to determine whether SPL7013 dendrimer was sequestered in Carbopol 974P- and Carbopol 971P-based aqueous gels. As shown in Figure 5A, tubes containing SPL7013- or SPL7013-containing gels were subjected to ultracentrifugation for 30 min at  $135,000 \times g$  to phase separate the gels into a water-rich band (“water band”) and gel-rich band (“gel band”). Sequestration of SPL7013 by Carbopol 974P would result in a lower than theoretical SPL7013 concentration (e.g., 3%, wt/wt) in the water band as shown in Figure 5C. In contrast, concentrations of SPL7013 in the water band that were close to 3% (wt/wt) (as indicated by Figure 5B) would suggest no discernable binding or trapping of SPL7013 by Carbopol 974P.

The results showed that ultracentrifugation of an aqueous SPL7013 solution (i.e., absent of any Carbopol or other excipients) resulted in no significant sedimentation of SPL7013

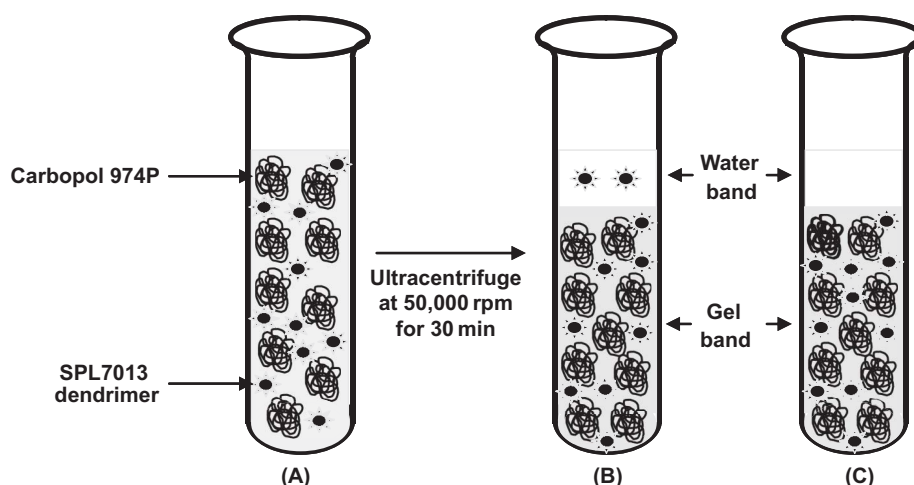


FIGURE 5. Schematic depicting the physical disposition of SPL7013 dendrimer in prototype combination vaginal gels before ultracentrifugation (A) and after ultracentrifugation (B,C). Ultracentrifugation of the viscous Carbopol-based gel produces a water-rich band (“water band”) on top and a gel-rich band (“gel band”) on the bottom of the tube. B shows a water band with equal concentration of SPL7013 in the water band and the gel band as would be expected in the absence of sequestration. C shows a water band with reduced SPL7013 concentration as would be expected if SPL7013 dendrimer were sequestered by the Carbopol-based gel.

under the conditions used for ultracentrifugation (Table 3). The upper layer of a centrifuged SPL7013 solution contained  $99.0 \pm 0.03\%$  ( $n = 6$ ) of the theoretical SPL7013 concentration. Ultracentrifugation of both prototype vaginal gels resulted in the formation of distinct watery supernatants and dense gel-rich bands. Qualitative examination revealed that the water band comprised approximately 20% of the volume of the entire gel sample. The aqueous supernatant was sampled once per vial to avoid puncturing or disturbing the gel band. The total water band volume was not measured, as droplets stuck irregularly to the inside of the vial and to the pipette tips. Thus, a complete mass balance of SPL7013 in the water and the gel bands was not performed. As summarized in Table 3, SPL7013 was not sequestered in the Carbopol-rich band after ultracentrifugation of both the prototype 1 and prototype 2 gels. Instead, the SPL7013 readily diffused from the gel region and concentrated in the watery supernatant. The concentration of SPL7013 in the water band after ultracentrifugation was approximately 50% higher than that in the starting prototype combination vaginal gels and significantly lower in the remaining compressed gel bands.

Interestingly, VivaGel (10,183 cP) and a neutral 2.7% (wt/wt) hydroxyethyl cellulose placebo gel (otherwise known as the “universal” placebo, which is commonly used in topical microbicide development as a comparator formulation) (Tien, 2005), could not form discernible water- and gel-rich layers when ultracentrifuged, even after  $541,000 \times g$  for 45 min. Further examination revealed that viscosity and pH most influenced the ability of the gel to form bands after ultracentrifugation. For example, VivaGel could be compressed into a water band and a gel layer if it was acidified with HCl, or if sodium chloride was added into the gel to yield gels having

viscosities between 3,000 and 4,000 cP. Specifically, when 25 mg 6 N HCl or 50 mg 5 M NaCl was added to 1 g VivaGel, ultracentrifugation at  $135,000 \times g$  for 30 min led to banding and the SPL7013 concentration in the water band was essentially equivalent to that in the initial VivaGel formulation. Interestingly, ultracentrifugation did not lead to banding when only 25 mg 5 M NaCl was added to 1 g VivaGel. This gel had a viscosity of 6,598 cP, which was actually lower than the value measured for either of the prototype combination vaginal gels. However, the pH of this modified VivaGel sample was 4.21, which was still a half pH unit higher than that of the prototype combination vaginal gels. Thus, the influence of lower pH on reduced Carbopol ionization and subsequent reduced electrostatic repulsion in promoting gel compression and SPL7013 diffusion may be particularly important at moderate viscosity levels. In cases of relatively higher pH, where the Carbopol residues would tend to be deprotonated and anionic, such as with the prototype combination vaginal gels or the high salt mixtures, electrostatic repulsion between polymer and SPL7013 may contribute to higher SPL7013 diffusion and subsequent SPL7013 concentration in the water bands. To investigate the possible sequestration of SPL7013 by methods in which the Carbopol 971P Gels were not altered by pH or salt, a filtration method was used. In these filtration studies, SPL7013 Gels containing Carbopol 971P were filtered through a 0.45 micron filter to remove the Carbopol 971P prior to analysis by HPLC. The concentration of SPL7013 in the filtrate was routinely equal to or greater than the original concentration of SPL7013 in the gel. These findings are consistent with SPL7013 not being sequestered in Carbopol 971P Gels (data not shown).

In summary, novel combination vaginal gel formulations have been successfully developed, which aimed to combine

TABLE 3

Concentration of SPL7013 dendrimer in Water Band and Gel Band after Ultracentrifugation of 3% (wt/wt) Prototypes 1 and 2 Vaginal Gels at  $135,000 \times g$  for 30 min

Material	Viscosity (cP)	pH	SPL7013 Concentration (% wt/wt) (n = 6)	
			Water Band	Gel Band
SPL7013				
Aqueous solution, untreated control			$3.00 \pm 0.03$	NA
Aqueous solution, ultracentrifuged			$2.97 \pm 0.01$	NA
Prototype 1 vaginal gel				
Control, untreated	9,484	3.7	$3.03 \pm 0.01$	$3.03 \pm 0.01$
Ultracentrifuged			$4.54 \pm 0.07$	$2.67 \pm 0.06$
Prototype 2 vaginal gel				
Control, untreated	8,245	3.7	$3.00 \pm 0.02$	$3.00 \pm 0.02$
Ultracentrifuged			$4.46 \pm 0.03$	$2.49 \pm 0.01$
VivaGel				
Ultracentrifuged		4.80	No Band	$3.00 \pm 0.05$
Ultracentrifuged				
After diluting 10% with water	8,830	4.47	No Band	$2.74 \pm 0.11$
After diluting 5% with water	9,525	4.37	No Band	$2.87 \pm 0.06$
After diluting 2.5% with water	9,872	4.34	No Band	$2.91 \pm 0.04$
After diluting 10% with 6N HCl	695	0.66	$2.93 \pm 0.10$	$3.56 \pm 0.26$
After diluting 5% with 6N HCl	2,009	1.22	$2.90 \pm 0.08$	$3.48 \pm 0.11$
After diluting 2.5% with water	3,188	1.96	$2.84 \pm 0.07$	$3.29 \pm 0.12$
After diluting 10% with 5M NaCl	2,914	4.27	$3.67 \pm 0.08$	$2.46 \pm 0.16$
After diluting 5% with 5M NaCl	3,237	4.23	$3.50 \pm 0.09$	$2.62 \pm 0.06$
After diluting 2.5% with 5M NaCl	6,598	4.21	No Band	$2.97 \pm 0.11$

design and formulation criteria from two leading topical microbicides currently in human clinical trials. Both prototype gel formulations have been fully characterized and demonstrated to have potential utility as topical vaginal microbicide products. Finally, ultracentrifugation studies have shown that the antiviral macromolecule SPL7013 dendrimer is not entropically trapped in Carbopol-based gels and would in turn be available to physically interact with virus.

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