International Journal of Pharmaceutics 403 (2011) 262–267

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

pH-sensitive Eudragit nanoparticles for mucosal drug delivery

Jin-Wook Yoo [1], Namita Giri, Chi H. Lee *

Division of Pharmaceutical Sciences, School of Pharmacy, University of Missouri, Kansas City, MO 64110, USA

ARTICLE INFO

Article history:
Received 25 August 2010
Received in revised form 11 October 2010
Accepted 18 October 2010
Available online 31 October 2010

Keywords:
pH-sensitive nanoparticles
Eudragit
Intravaginal delivery

ABSTRACT

Drug delivery via vaginal epithelium has suffered from lack of stability due to acidic and enzymatic environments. The biocompatible pH-sensitive nanoparticles composed of Eudragit S-100 (ES) were developed to protect loaded compounds from being degraded under the rigorous vaginal conditions and achieve their therapeutically effective concentrations in the mucosal epithelium. ES nanoparticles containing a model compound (sodium fluorescein (FNa) or nile red (NR)) were prepared by the modified quasi-emulsion solvent diffusion method. Loading efficiencies were found to be 26% and 71% for a hydrophilic and a hydrophobic compound, respectively. Both hydrophilic and hydrophobic model drugs remained stable in nanoparticles at acidic pH, whereas they are quickly released from nanoparticles upon exposure at physiological pH. The confocal study revealed that ES nanoparticles were taken up by vaginal cells, followed by pH-responsive drug release, with no cytotoxic activities. The pH-sensitive nanoparticles would be a promising carrier for the vaginal-specific delivery of various therapeutic drugs including microbicides and peptides/proteins. Published by Elsevier B.V.

1. Introduction

Assorted mucosas have long been explored as a promising route for delivery of therapeutic compounds including proteins or peptides, which suffer from poor bioavailability owing to harsh conditions of the oral administration. Of those mucosal routes, the vagina has been studied as an alternative route of various classes of drugs, such as microbicides, antimicrobials, sexual hormones and proteins or peptides (Gavini et al., 2002; Richardson and Illum, 1992; Veazey et al., 2003; Yoo et al., 2006). A dense network of blood vessels in the vaginal wall and by-pass of the hepatic first pass metabolism have made the vagina as a potential route for a systemic as well as topical drug (e.g. antifungal agents, hormones or microbicides) delivery of therapeutically active compounds (Hussain and Ahsan, 2005).

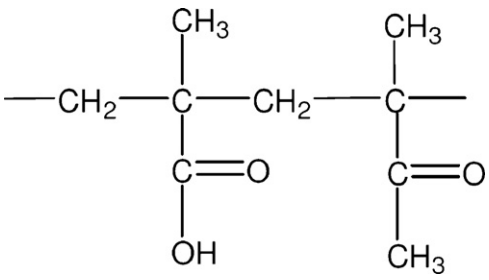
The extent of drug absorption through vaginal epithelium is, however, limited by biochemical and enzymatic activities in the vagina because the vaginal epithelium contains exo-/endopeptidases which stimulate digestion of peptides and proteins (Lee, 1988). For instance, the vaginal absorption of macromolecules like polypeptides and proteins was substantially hindered by enzymatic degradation (Richardson and Illum, 1992). The vaginal application of acid labile compounds, such as erythromycine (Durfee et al., 1979) and nitric oxide donors (Yoo et al., 2010), was also constrained due to their instability in acidic environments. Moreover, a burst release of a drug may cause mucosal irritation in the vaginal tissue. Therefore, it is integral to develop an advanced drug carrier which lengthens the stability of therapeutic agents and facilitates their delivery into vaginal epithelium, thus achieving enhanced bioavailability, optimal pharmacological efficacy and high patients' compliance.

Nanoparticulate systems have been used as an advanced drug delivery carrier due to their unique features, such as capability to protect therapeutic compounds, versatility to control the release profiles of loaded drugs and tunable surface properties. Nanoparticulates with thixotropical properties make them even more suitable to mucosal application and free from any influences caused by environmental pH changes (Lee et al., 2009). Since most pores in mucosa including cervicovaginal mucus range 50–1800 nm with an average pore size of 340 nm (Lai et al., 2010), nanoparticles with a size of about 300 nm or smaller can penetrate through mucosal membranes and overcome the existing barriers to success (Mallipeddi and Rohan, 2010).

Eudragit S-100 (ES) composed of methacrylic acid and methyl methacrylate (1:2, Mw = approx. 135,000) (Scheme 1), was chosen as a pH-sensitive polymer owing to its unique dissolution behavior above pH 7.0. Eudragits have been used as pH-sensitive polymers in various applications including enteric coating materials and drug delivery vehicles (Gupta et al., 2001; Khan et al., 1999; Khan et al., 2000), and exhibited plastic deformation and significant speed sensitivity (Tatavarti et al., 2008). Eudragit in combinations with other polymers, such as hydroxypropyl methyl-

* Corresponding author at: 2464 Charlotte St., HSB # 4242, Division of Pharmaceutical Sciences, University of Missouri at Kansas City, Kansas City, MO 64108, USA. Tel.: +1 816 235 2408; fax: +1 816 235 5779.
E-mail address: leech@umkc.edu (C.H. Lee).
[1] Current address: Department of Chemical Engineering, University of California, Santa Barbara, CA 93106-5080, USA.

0378-5173/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.ijpharm.2010.10.032



Scheme 1. Chemical structure of Eudragit S-100. The ratio of the free carboxyl groups to the ester groups is 1:2 (Mw = approx. 135,000).

cellulose and talc, stabilized loaded drugs and provided a controlled release of them (Maghsoodi and Esfahani, 2009; Raffin et al., 2007). Nano-/microparticles made from Eudragit polymers have been utilized for protein drug delivery through oral route (Jain et al., 2005; Leroux et al., 1996; Morishita et al., 1993). Since little attention has been given to nanoparticles made from Eudragit for their mucosal applications, we attempted to develop the pH-sensitive Eudragit nanoparticles as an intravaginal drug delivery carrier.

ES nanoparticles were loaded with a hydrophilic (FNa) or a hydrophobic (nile red) model drug. The characteristics of ES nanoparticles including particle size distribution, morphology and drug loading efficiency were examined. The release profiles of model compounds from nanoparticles were evaluated under various environmental pHs. The cellular uptake of ES nanoparticles by human vaginal epithelial cell line was examined using a confocal microscope. The vaginal cell viability upon exposure to ES nanoparticles was determined for cytotoxicity assessment.

2. Materials and methods

2.1. Materials

Eudragit S-100 (ES) was a gift from Röhm (Germany). Polyvinyl alcohol (PVA, Mw 30,000–70,000), sodium fluorescein (FNa) and nile red (NR) were purchased from Sigma Aldrich (St. Louise, MO). All other reagents and solvents were of analytical grade.

2.2. Preparation of ES nanoparticle

ES nanoparticles were prepared by modified quasi-emulsion solvent diffusion methods (Kawashima et al., 1989; Pignatello et al., 2002). For a hydrophobic compound (NR), 2.5% (w/v) of ES was dissolved in acetone and NR (0.05%, w/w) was added to the polymer solution. For a hydrophilic compound (FNa), 2.5% (w/v) of ES was dissolved in methanol and FNa (0.1%, w/w) dissolved in distilled water was added to the polymer solution. For blank nanoparticles, ES was dissolved without any drug. The solution was slowly injected (0.33 ml/min) into 100 ml citrate buffer (pH 5.0) containing PVA (1%, w/v, emulsifier) using a syringe pump connected to a thin Teflon tube. During the injection process, the mixture was agitated at 600 rpm. The mixture immediately turned into the biphasic solution with the polymer–organic solvent solution in the external aqueous phase and drug-containing pseudo-emulsion in the internal phase. The solution was left under a slow magnetic stirring at room temperature for 24 h to remove the organic solvent. The nano-suspensions were centrifuged at 1500 rpm for 5 min to remove polymer aggregations. After centrifuging, the supernatant, which contains ES nanoparticles, was carefully collected and centrifuged at 40,000 × *g* for 30 min and washed with distilled water three times. The collected nanoparticles were freeze-dried for 24 h.

2.3. Morphology of nanoparticles

The examination of morphology and surface property of nanoparticles was performed using scanning electron microscopy (SEM; FEG ESEM XL 30, Hillsboro, OR). ES nanoparticles were mounted on a double-sided tape and spray-coated with gold palladium at 0.6 kV prior to inspection by electron microscope.

2.4. Assessment of loading efficiency

A known amount of ES nanoparticles was dissolved in 1 M sodium hydroxide and the suspension was kept in a bath sonicator for 10 min. The amount of a model drug was measured and analyzed by a plate reader (DTX 880, Beckman Coulter, CA). The analyses were performed at excitation and emission wavelengths of 494 nm and 521 nm for FNa and those of 535 nm and 625 nm for NR. For the calibration curve, the standard solutions were prepared by mixing blank nanoparticles and proper amounts of a model drug in 1 M sodium hydroxide. Samples were prepared in triplicate and loading efficiency (%) was calculated using the following equation:

$$\text{Loading efficiency (\%)} = \frac{\text{Amount of remaining drug in the nanoparticles}}{\text{Amount of initially added drug}} \times 100$$

2.5. Size distribution of ES nanoparticles

The particle size distribution of the freshly prepared ES nanoparticles was analyzed using dynamic light scattering (DLS) (Brookhaven 90 Plus, Brookhaven Instruments Limited, Holtsville, NY) with a photon counting rate of at least 400 kcps. The sample solutions at the concentration of approximately 2 mg/ml in citrate buffer (pH 4.0) were diluted by distilled water.

2.6. In vitro drug release study

50 mg of ES nanoparticles was added to 10 ml of phosphate buffer (pH 7.4) or citrate buffer (pH 4.0) at 37 °C. For the release study, Tween 80 (5%, v/v) was added to the buffer solutions to facilitate the solubilization process of NR released from nanoparticles. At a predetermined time interval, an aliquot (0.5 ml) of the sample was withdrawn and subsequently replaced with the fresh buffer solution. The sample was centrifuged and the supernatant was analyzed by a plate reader as described above. The samples were analyzed in triplicate.

2.7. Evaluation of cellular uptake of ES nanoparticles

Normal vaginal epithelial cell line, VK2/E6E7 (ATCC), was used for evaluation of the cellular uptake profiles of ES nanoparticles. VK2/E6E7 cells were harvested with keratonized serum-free medium (K-SFM) (Gibco, Carlsbad, CA) containing bovine pituitary extract (BPE) (50 μg/ml), human recombinant epidermal growth factor (EGF) (0.1 ng/ml), $CaCl_2$ (0.4 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml). They were incubated at 37 °C under 5% CO_2 and 95% humidity. Cells were incubated with phosphate buffer (pH 6.5) containing NR-loaded ES nanoparticles (equivalent to 0.05 mg NR) for 1 h. The NR solution containing 0.05 mg of NR was diluted with the same buffer solution from stock solution (0.5 mg/ml in DMSO) and was used as a control. The cells were fixed with 2% paraformaldehyde and the cellular uptake of ES nanoparticles was examined using Nikon TE-2000U scanning fluorescence confocal microscope (Nikon Inc., Melville, NY). The digital images

Table 1

Formulation	Effective particles size (nm)	Polydispersity index	Yield[a] (%)	Entrapment Efficiency (%)
Blank ES NP	211.5 ± 10.4	0.07 ± 0.02	92.3 ± 5.4	–
ES-F NP[b]	241.9 ± 8.2	0.09 ± 0.04	86.4 ± 6.1	26.2 ± 2.7
ES-NR NP[c]	331.7 ± 25.0	0.23 ± 0.06	89.4 ± 4.7	71.4 ± 3.8

[a] All were expressed as mean ± S.D. ($n = 3$).
[b] ES-F NP: fluorescein sodium loaded ES nanoparticle.
[c] ES-NR NP: nile red loaded ES nanoparticle.

were processed using Image-J software (National Institute of Mental Health, Bethesda, MA).

2.8. Cytotoxicity assessment study

Two vaginal cell lines, VK2/E6E7 and HeLa cells, were used to assess the cytotoxicity of ES nanoparticles. The cells were seeded onto 96-well plate. After 2 days, cell culture media were replaced with fresh media confining various amounts of blank ES nanoparticles. After 24 h of incubation, cell viability was measured using Cell Titer 96 AQueous assay kit (Promega).

3. Results

3.1. Characterization of ES nanoparticles

The formulation characteristics of ES nanoparticles are summarized in Table 1. The morphology of the particles was studied using SEM. As shown in Fig. 1, ES nanoparticles displayed a solid spherical shape having a smooth surface. Nanoparticles did not have any porous surfaces or internal porous structure, suggesting that the matrix rupture process was not a fundamental component in determining the release profiles of loaded compounds from nanoparticles.

The varying sizes of ES nanoparticles as low as 150 nm were produced to find the most suitable mucosal formulation for candidate compounds. The size of ES nanoparticles was affected by the polymer concentration and injection rate. The particles size was also affected by the encapsulation status of compounds, showing a greater size of the nanoparticles incorporated with drugs than that of blank particles (Table 1). The average particle size of nanoparticles incorporated with model compounds ranged from 242 to 332 nm. The size of the particles encapsulated with hydrophobic compound (NR) is greater than those with hydrophilic compound (FNa).

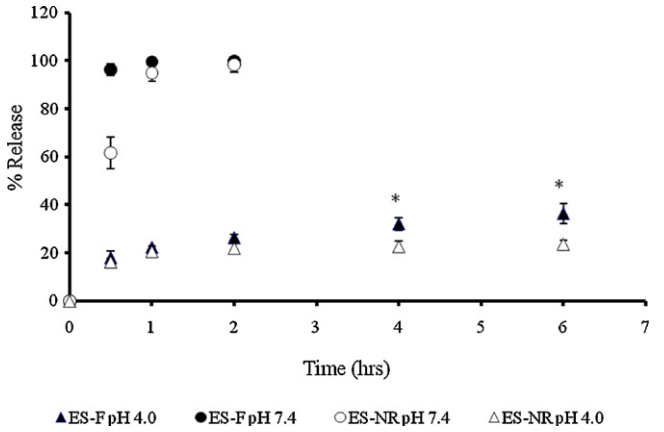


Fig. 2. In vitro drug release profiles from ES nanoparticles. At acidic pH (4.0), both drugs showed slow release profile, whereas nearly 100% of drugs were released within 1 h at pH 7.4. Data were expressed as mean ± S.D. ($n = 3$). *$p < 0.05$ versus ES-NR pH 4.0.

The loading efficiency of the model compounds depends on their physical properties, especially hydrophobicity. Loading efficiency for hydrophobic NR was 71%, whereas it was 26% for hydrophilic sodium fluorescein. The lower entrapment efficiency of hydrophilic drug can be explained by its greater solubility in the external aqueous phase than in the internal lipid phase (i.e. polymer emulsion phase). Hydrophilic compounds are present at a greater concentration in external water phase, thus having a tendency to diffuse out to the water phase, whereas hydrophobic drug is more likely to remain in the particles. The fabrication parameters, such as a concentration, flow rate and PVA concentration, were optimized in the initial formulation development step to achieve the highest efficiency of both hydrophilic and hydrophobic compounds.

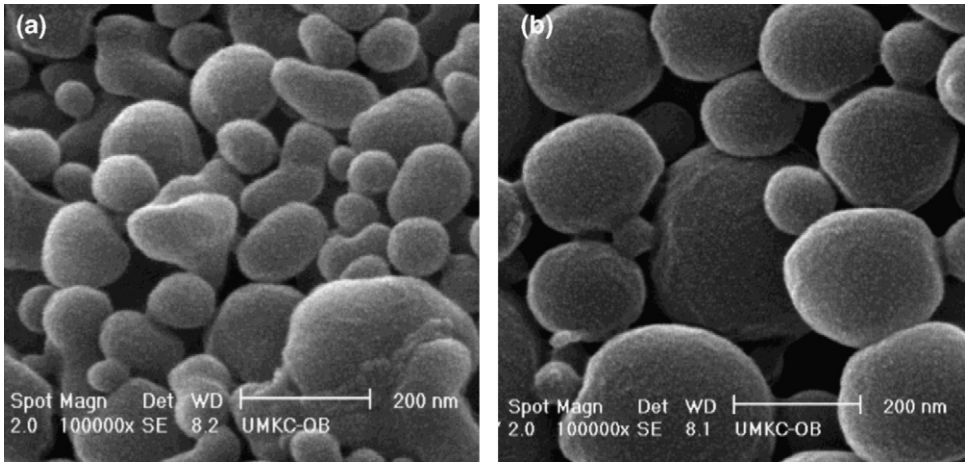


Fig. 1. SEM images of ES nanoparticles containing FNa (a) and NR (b).

3.2. pH-sensitive release profiles of model compounds from ES nanoparticles

The release profiles of the model compounds from nanoparticles were evaluated at acidic pH (4.0) and physiological pH (7.4) (Fig. 2). The environmental pH significantly affected the release profiles of the model compounds from nanoparticles. At pH 4.0, no significant differences in the release profile between two model drugs were observed at the early stage, but the amount of FNa released from nanoparticles was significantly greater than that of NR at 6 h. The cumulative amount of FNa released from ES nanoparticles at pH 4.0 gradually reached about 30% of the loading dose as the hydrophilic FNa entrapped within the nanoparticles was diffused out for 6 h, whereas almost no more NR was released up to 6 h after the initial burst release of ~20% in first 30 min. The release rate of both model compounds distinctively increased, as pH of the fluid changed from 4.0 to 7.4 at which ES nanoparticles quickly dissolved, achieving 100% of the release amount of the loaded model compounds (both FNa and NR) within 1 h. FNa was released more quickly from the nanoparticles owing to their higher hydrophilicity than NR. These results indicated that both model compounds were retained by nanoparticles at acidic vaginal pH, but they were rapidly released from nanoparticles at physiological pH.

When the amounts of drug released from nanoparticles at pH 7.4 at the initial stage after being normalized with the loading efficiency and initial loading amount were compared between two model drugs, the amount of FNa released from nanoparticles was significantly smaller than that of NR (Fig. 2). The results of this study clearly supported that ES nanoparticles can be used as an intravaginal carrier for both hydrophilic and hydrophobic compounds and the loading dose can be adjustable according to their physicodynamic characteristics.

3.3. Cellular uptake of ES nanoparticles by vaginal cells

The uptake study of ES nanoparticles incorporated with NR was performed on vaginal cells. NR, which was not formulated with ES nanoparticles, bound on cell membranes and no NR was found inside the cells (Fig. 3a), indicating that NR is not able to penetrate the cells. The ES nanoparticles containing NR, however, stained at most part of cells (Fig. 3b), indicating that ES nanoparticles were internalized into vaginal cells and released NR. The results of this study clearly supported that ES nanoparticles can be used for the systemic delivery of both hydrophilic and hydrophobic compounds.

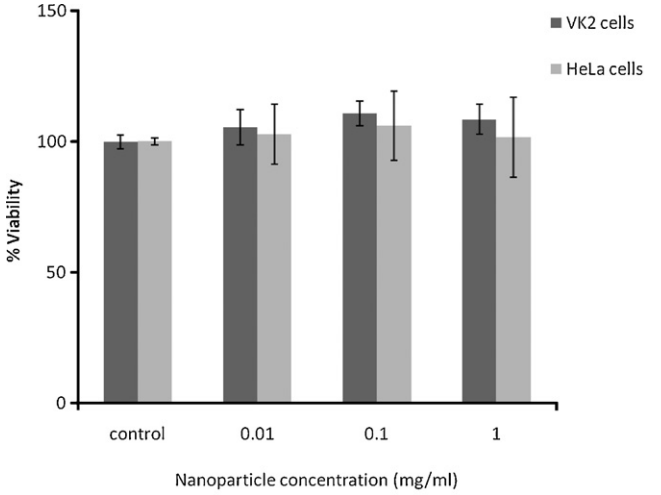


Fig. 4. Cytotoxicity study of ES nanoparticles. ES nanoparticles were incubated in two vaginal cell lines. No cytotoxic activities of ES nanoparticles were observed in both vaginal cells. Data were expressed as mean ± S.D. ($n = 6$).

3.4. Cytotoxicity study

Cell viability study was performed to evaluate whether ES nanoparticles exert cytotoxic activity on vaginal cells. As shown in Fig. 4, ES nanoparticles at various concentrations caused no cytotoxicities to vaginal cells upon exposure for 24 h, demonstrating its safeness in mucosal applications and relieving compliance concern from patients.

4. Discussion

The majority of women acquiring sexual transmitted disease (STD) today are infected by mucosal exposure to virus through sexual contact [21]. Women may need help from barrier devices containing exogenous compounds including microbicides for self protection until an arrival of the first generation of vaccines. This uncertainty provides a rationale to pursue an advanced form of nanoparticles which are capable of carrying multiple compounds with different pharmacological activities and physicodynamic properties as a means for protection against STD.

Nanoparticles made of ES were herein utilized for the vagina-specific delivery of two physically different model drugs with a simultaneous delivery pattern and a controlled release rate. Since

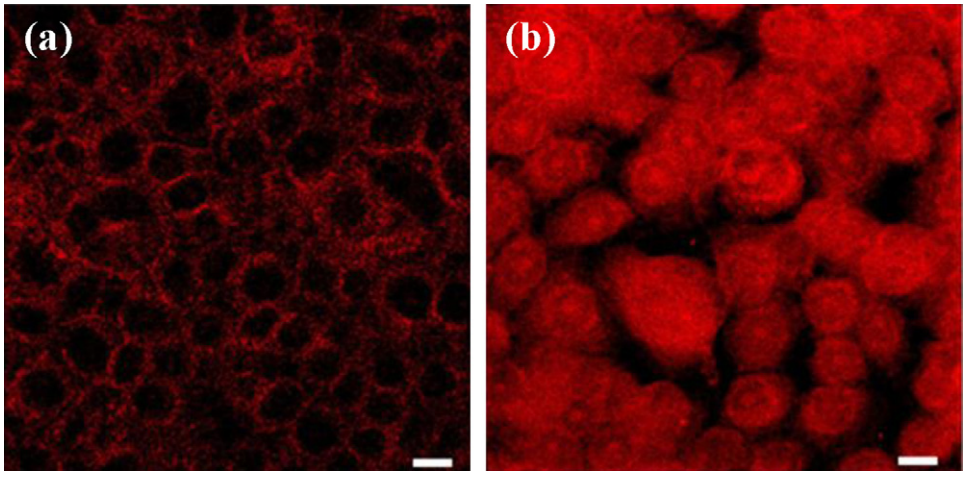


Fig. 3. Cellular uptake profiles of ES nanoparticles in vaginal cells. (a) NR solution. NR bound on the cell membrane, but not crossing the membrane. (b) NR-loaded ES nanoparticles. The entire vaginal cells were evenly stained with NR, implying that the cells internalized NR-loaded nanoparticles and released NR in cytosol. Scale bar = 10 μm.

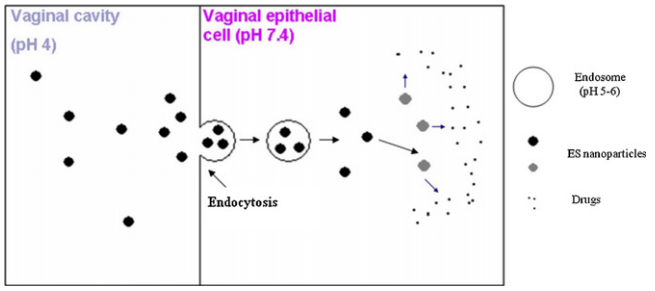


Fig. 5. Schematic presentation of ES nanoparticles uptake through vaginal cells. It was hypothesized that ES nanoparticles containing a drug are internalized by vaginal epithelial cells via endocytosis and escape from endosomes, followed by drug release in cytosol.

ES is a pH-sensitive anionic copolymer, these polymeric carriers can serve as a novel drug delivery system achieving both spatial and temporal control in conjugation with localized pH (i.e. 4.0 for the vagina). The following beneficial properties of ES have been considered for the development of nanoparticles for the dual delivery of exogenous compounds including topical microbicides. They are (1) accepted as a pharmaceutical excipient (e.g. enteric coating material), (2) generally regarded as non-toxic, (3) pH-sensitive polymer and dissolved at above pH 7.0, dissolution occurs as a result of structural changes of the polymer associated with ionization of the carboxylic functional group, and (4) have advantages in mucosal uptake of the compounds loaded in nanoparticles.

Various combinations of solvents were tested to optimize the fabrication procedure of ES nanoparticles. For encapsulation of NR, acetone was used as a solvent due to its high solubility of the drug in it and its ability to produce uniform particle formation. Acetonitril was not suitable as it produces aggregations of ES polymer. To encapsulate FNa, methanol was used as a solvent as it is readily miscible with the drug solution in water. Other solvents, such as isopropanol or ethanol, produced severe aggregations during the nanoparticles fabrication process. Injection rate of the solvent was optimized to prevent aggregation of ES nanoparticles. The rate higher than 0.33 ml/min of polymer–drug solution to external aqueous phase resulted in a greater particle size with partial aggregation. Further optimization of the fabrication parameters would be required depending on the physical properties of drugs to be encapsulated. In both methods, the pH of external aqueous medium was maintained at pH 5.0, as the polymer starts dissolving in the medium above pH 7. In vitro drug release studies revealed that both hydrophilic and hydrophobic model drugs were retained in nanoparticles under acidic conditions, but they were immediately released from nanoparticles as their environment pH changed from acidic to physiological pH.

We here hypothesize that ES nanoparticles are internalized by endocytosis and release the drug in cytosol (Fig. 5). Fig. 3 clearly reveals that NR bound to the cell surface, but not penetrated the cell membrane (Fig. 3a), whereas NR was entirely stained in the vaginal cells when they were incubated with NR-loaded nanoparticles (Fig. 3b). Since NR is not able to enter the cells (Fig. 3a), the presence of NR inside the cells can be attributed to cellular uptake of NR-loaded ES nanoparticles (Fig. 3b). Considering endosomal pH (pH 5–6), it is unlikely that NR is released from ES nanoparticles in endosomes, rather implying that ES nanoparticles were escaped from endosomes and subsequently release NR in cytosol whose pH is 7.4. Further studies seem to be needed to elucidate the mechanisms of cellular uptake and intercellular trafficking process of ES nanoparticles.

The ES nanoparticles described here will show a distinctive merit in intravaginal applications. While vagina in human is acidic (pH 4–5) with a low buffering capacity under normal conditions, semen from the male partner is neutral (~pH 7.5) with a higher buffering capacity (Owen and Katz, 2005), thus altering vaginal pH during intercourse (Tevi-Benissan et al., 1997). Therefore pH dependent conformational changes of nanoparticles upon exposure to semen have great advantages in achieving optimal release rate and pharmacological efficacy of intravaginally delivered formulations (Lee et al., 2009).

Since the release pattern of both model drugs at pH 4.0 was sustained, ES nanoparticles in conjunction with mucoadhesive polymers would be advantageous in long term delivery of pharmaceuticals inside vaginal cavity. Due to cellular uptake by vaginal cells and subsequent pH-sensitive drug release, the ES nanoparticles developed in this study have a potential to be used not only for topical delivery but also for systemic delivery of loaded drugs. Further optimization and application of these pH-sensitive nanoparticles will lead to self-controlled female drug delivery systems (Wang and Lee, 2002) carrying various therapeutic compounds including peptides/proteins, siRNA and microbicides for various clinical applications including prevention against STD.

5. Conclusions

ES nanoparticles loaded with hydrophilic or hydrophobic model compounds were successfully fabricated by a modified emulsion solvent diffusion method. The nanoparticles had homogeneous surface morphology with spherical nature and uniformed texture. In vitro release profile showed that model compounds were retained by the nanoparticles at vaginal pH, but they were rapidly released from particles at physiological pH. The nanoparticles also showed the cellular uptake by vaginal cells and subsequent drug release. No cytotoxicity of the nanoparticles was detected in vaginal cell lines. The pH-sensitive ES nanoparticles presented in this study would be a potential carrier for not only topical delivery but also systemic delivery of therapeutically active compounds.

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