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Microparticles prepared from sulfenamide-based polymers

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Abstract

Polysulfenamides (PSN), with a SN linkage (RSNR₂) along the polymer backbone, are a new class of biodegradable and biocompatible polymers. These polymers were unknown prior to 2012 when their synthesis and medicinally relevant properties were reported. The aim of this study was to develop microparticles as a controlled drug delivery system using polysulfenamide as the matrix material. The microparticles were prepared by a water-in-oil-in-water double emulsion solvent evaporation method. For producing drug-loaded particles, FITC-dextran was used as a model hydrophilic compound. At the optimal formulation conditions, the external morphology of the PSN microparticles was examined by scanning electron microscopy to show the formation of smooth-surfaced spherical particles with low polydispersity. The microparticles had a net negative surface charge (−23 mV) as analyzed by the zetasizer. The drug encapsulation efficiency of the particles and the drug loading were found to be dependent on the drug molecular weight, amount of FITC-dextran used in fabricating FITC-dextran loaded microparticles, concentration of PSN and surfactant, and volume of the internal and external water phases. FITC-dextran was found to be distributed throughout the PSN microparticles and was released in an initial burst followed by more continuous release over time. Confocal laser scanning microscopy was used to qualitatively observe the cellular uptake of PSN microparticles and indicated localization of the particles in both the cytoplasm and the nucleus.

Keywords

Polysulfenamide; microparticles; Biocompatible; Biodegradable; Particle size; Controlled release; Cellular uptake

1. Introduction

Microparticles prepared from biodegradable polymers have shown strong potential for sustained and controlled delivery of a wide range of pharmaceutical agents (Egilmez et al., 2000; Ruan and Feng, 2003). For example, biodegradable microparticles have been used as depots for long-term delivery of therapeutic agents and proteins and have found significant potential as vaccine and gene delivery systems (Alonso et al., 1994; Capan et al., 1999). The synthetic biodegradable polymers that have been used for preparing microparticles to date have been primarily focused on polyesters, polyamides, polyanhydrides or polymers that are

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variations of these three functional groups (Park et al., 2005). Despite the successful use of these polymers, limitations in their degradation profiles, the limited ability to functionalize the surfaces of microparticles fabricated from them, and a need to deliver more complex therapeutic agents have led to a desire to integrate more polymers into this field. It is well recognized that new opportunities arise when new functional groups are integrated into polymers used in drug delivery.

We have recently developed a new class of biocompatible, sulfenamide-based polymers. The sulfenamide functional group (RSNR_2) had never been used in the synthesis of polymers prior to recent work by our groups (Fig. 1) (Yoo et al., 2012). The sulfenamide functional group has many chemical properties that make it attractive for use in polymers for pharmaceutical applications. For instance, sulfenamides slowly degrade in water at neutral pH, but they degrade over two orders of magnitude faster than ester bonds under acidic conditions. This property is useful because microparticles fabricated from polysulfenamides (PSN) can be expected to degrade by hydrolysis in physiological environments rather than accumulate in organs and cause a toxic response. Microparticles fabricated from PSN and loaded with pharmaceutical drugs can be expected to undergo a rapid acid-catalyzed degradation that is desired for the release of their drug cargo in the endosome or lysosome of cells. Polysulfenamides also degrade to release different degradation products than esters or anhydrides. Polysulfenamides degrade to release amines and sulfenic acids (Fig. 1). Sulfenic acids (general structure of RSOH) and dimers of sulfenic acids are the active components in garlic and onions and are believed to be the source of their medicinal benefits (Freeman and Koder, 1995; Vaidya et al., 2009). Allicin is composed of a dimer of sulfenic acids, and it is believed to be the active component of garlic that is responsible for its antioxidant properties. In short, sulfenic acids are powerful antioxidants, and PSN degrades to release sulfenic acids.

In prior work we developed methods to synthesize PSN from activated dithiols and secondary diamines. This was the first synthesis of PSN ever reported in the literature. Importantly, these polymers were biocompatible as shown by *in vitro* and *in vivo* experiments in mice. These polymers were unique because they were based on an inorganic functional group that differs remarkably from the organic functional groups that are commonly used for the synthesis of biomedical polymers. The integration of new functional groups into biomedical polymers often opens up new avenues of research that take advantage of their reactions. An important step in the development of new biomedical polymers is a detailed study of a material that contains these polymers. In this article we report such a study by the description of methods to fabricate microparticles from PSN that are loaded with a fluorescently tagged model hydrophilic drug. The location of the drug within the particles and its release over time was studied.

The most common approaches for preparing polymeric microparticles are the single- and double-emulsion solvent-evaporation methods (Alex and Bodmeier, 1990; Flandroy et al., 1990). The single-emulsion evaporation method is used to load hydrophobic molecules whilst the water-in-oil-in-water double-emulsion solvent-evaporation method is primarily used for entrapping hydrophilic molecules. The double-emulsion solvent-evaporation method involves the use of three phases: 1) an inner water phase containing an emulsifying

agent and the hydrophilic drug to be incorporated, 2) an intermediate organic phase consisting of a polymer/organic solvent solution, and 3) an outer water phase containing an emulsifying agent (Alex and Bodmeier, 1990). After fabrication of the microparticles, the organic solvent is removed and the particles are isolated, dried, and thoroughly characterized.

The physicochemical characteristics of microparticles formed from polymers is dependent on the processing conditions including the choice of solvent for the oil phase, the surfactant concentration in the aqueous phase, the polymer concentration in the oil phase, the stirring rate, the molecular weight and lipophilicity of the drug being entrapped and the volume of the internal and external water phases. Obtaining a high entrapment efficiency and loading for a highly hydrophilic drug is often a challenge (Billon et al., 2005). The drug entrapment efficiency of microparticles is governed, mainly, by the solubility of the drug in water, the stability of the primary emulsion during the double emulsification process, and the microparticle formation process as the organic solvent is eliminated from the particles (Yang et al., 2001). For the optimum entrapment of drug in the microparticles, the aqueous solubility of the drug should be relatively low; the organic solvent used should possess relatively high aqueous miscibility and high vapor pressure desirable for the faster diffusion and evaporation rate of the solvent. This facilitates the rapid precipitation of the polymeric emulsion droplets as solid particles. A high solubility of drug in the polymer matrix is an added advantage (Bodmeier and McGinity, 1988; Panyam et al., 2004).

Processing conditions that generate monodisperse, spherical, and smooth poly(lactide-co-glycolide) (PLGA) microparticles do not necessarily produce similar microparticles from alternative polymers including PSN. Developing monodisperse smooth spherical microparticles from new polymers such as PSN therefore requires a systematic evaluation of the processing conditions. As such, one of the aims of this study was to identify the optimal formulation parameters for fabricating microparticles from polysulfenamides. For this purpose, a model hydrophilic compound, FITC (fluorescein isothiocyanate)-labeled dextran (FITC-dextran) was entrapped into PSN microparticles using the water-in-oil-in-water double emulsification method. The effect of various processing parameters involved in formulating microparticles on the physicochemical characteristics of the PSN microparticles was systematically assessed to study their degrees of crystallinity, size, melting temperatures, release of FITC-dextran, encapsulation of FITC-dextran, and internalization into cells that are commonly used in drug and gene delivery development. Many of the key parameters describing the generation of microparticles and their important physical characteristics for drug delivery were studied. In addition, the crystallinity and melting temperature data presented in this study is the first such data for this recently developed class of biodegradable sulfenamide based polymers. The results presented in this study will be critical for the subsequent *in vitro* and *in vivo* studies evaluating these novel PSN microparticles for treatment of a wide range of diseases.

2. Materials and methods

2.1. Materials

A polysulfenamide as shown in Fig. 1 (mol. wt: 6,300 Da, PDI: 1.22) was used in all the formulations studied. FITC-dextran (Mw 4 kDa, 20 kDa and 40 kDa) and polyvinyl alcohol (PVA, Mowiol®, Av Mw ~67 kDa) was obtained from Sigma-Aldrich™ (Sigma Chemical Co., MO). Dichloromethane was purchased from Sigma-Aldrich®, MO. Chloroform and tetrahydrofuran were purchased from Fischer Scientific®, NJ. Deionized distilled water produced by Barnstead Nanopure Diamond™ Water purification Systems, IA, was used throughout. All other chemicals and solvents used were of reagent grade.

2.2. Fabrication of drug-free PSN microparticles and FITC-dextran loaded PSN microparticles

Microparticles were fabricated from PSN using a double emulsion-solvent evaporation method (Alex and Bodmeier, 1990). One hundred microlitres of the surfactant solution containing 1 % PVA in water (W_1) was added dropwise into chloroform containing the dissolved polymer (O) and sonicated for 30 s using a micro-tip probe sonicator (10 watts energy output, Fisher Scientific Sonic Dismembrator Model 100, MA) to form the first emulsion (W_1/O). This primary emulsion was then immediately added dropwise to a second surfactant solution containing 1 % PVA in water (W_2) and sonicated as above to form the second emulsion ($W_1/O/W_2$). The double emulsion was stirred at room temperature in a fume hood until complete evaporation of the organic solvent. The resulting microparticles were harvested by centrifugation ($10,000 \times g$ for 10 min, Fischer Scientific Accuspin™ 400, MA), washed with water and freeze-dried (FreezeZone 4.5, Labconco®, MO). This standard procedure was varied with respect to: 1) polymer concentration, 2) PVA concentration, 3) volumes of W_1 and W_2 phases, 4) volume of O phase, 5) stirring time of the final emulsion, and 6) method of removal of organic solvent. The corresponding effects of these variations on the final particle size and morphology were examined by analysis *in vitro*. Table 1 shows the details of these varying parameters in the microparticle preparation process and their effect on particle shape and external morphology as seen in the referenced SEM images.

FITC-dextran loaded microparticles were prepared as described previously with the following modifications: 200 μ l W_1 phase containing 5 mg FITC-dextran; polymer concentration of 50 mg in 2 mL chloroform; 8 mL of W_2 phase. The double emulsion was immediately added to 32 mL of 1 % PVA. The emulsion was stirred for 1 h and heated at 37 °C for 1 h. This working protocol was varied with respect to: 1) polymer concentration, 2) volumes of W_1 and W_2 phases (W_2 phase = total external water phase), 3) FITC-dextran loading in W_1 phase, 4) Mw of FITC-dextran (4 kDa, 20 kDa and 40 kDa), and 5) addition of a salt (sodium chloride, NaCl) to W_2 phase. The corresponding effects of these variations on the entrapment efficiency and drug loading were examined by analysis *in vitro*.

2.3. Characterization of microparticles

2.3.1. External morphology of microparticles—The shape and the surface characteristics of the microparticles were studied using a scanning electron microscope (SEM, Hitachi Model S-4800, Japan). The particles were mounted on silicon wafers which

were placed on an aluminum specimen stub using adhesive carbon tape. The mount was then coated by ion sputtering with conductive gold set at 10 mA for 2.5 min (K550 Emitech Sputter Coater, TX) and examined using the microscope operated at 3 kV accelerating voltage.

2.3.2. Powder X-ray diffractometry (PXRD)—Powder X-ray diffraction on the microparticles was performed using a Siemens Model D5000 X-ray diffractometer (Bruker AXS Inc., WI). The samples were exposed to CuK_α X-rays (voltage 40 kV, current 50 mA). The scanning angle (diffraction angle, 2θ) was recorded from 4° to 40° at 25°C at a step size of 0.020° . The scanning was obtained in a continuous scan mode.

2.3.3. Differential scanning calorimetry (DSC)—The thermal behavior of the microparticles with respect to the melting temperature (T_m) was studied by analysis of the DSC curves (PYRISTM Diamond Differential Scanning Calorimeter 7, Perkin-ElmerTM, CT). Samples were sealed in a standard aluminum sample pan covered with lids. An empty sealed aluminum pan was used as the reference. Samples were purged with pure dry nitrogen set at a pressure of 20 psi and flow rate of 20 mL/min. DSC thermograms were obtained for both polymer (as a control, to observe the change in T_m) and microparticles by heating the samples from 30°C to 150°C with heating rates of $5^\circ\text{C}/\text{min}$.

2.3.4. Microparticle size and surface charge—The size of microparticles was determined at a concentration of approximately 1 mg particles/mL deionized distilled water. Measurements of suspensions were made using a Zetasizer Nano-ZS (Malvern Instruments, MA) and the mean hydrodynamic diameter of the samples was determined by cumulative analysis. The particle size and particle size distribution by intensity were measured by photon correlation spectroscopy using dynamic laser light scattering (4 mW He-Ne laser with a fixed wavelength of 633 nm, 173° backscatter at 25°C) in 10 mm diameter cells. Zeta potential determinations were based on the electrophoretic mobility of the microparticles dispersed in deionized water at a concentration of 1 mg/mL performed using folded capillary cells in automatic mode of measurement duration.

2.3.5. Determination of entrapment efficiency of microparticles and drug loading—The loading of FITC-dextran in microparticles was determined by a dissolution-precipitation-extraction method (Wang et al., 2004). Five mg of microparticles were dissolved in 500 μL tetrahydrofuran and the polymer was precipitated by adding 2 mL phosphate buffered saline (PBS buffer, pH 7.4). The samples were agitated in an incubator shaker maintained at 37°C for 48 h. The supernatant was analyzed for FITC-dextran loading using a fluorometer (excitation: 490 nm, emission: 520 nm, Spectramax M5, Molecular Devices, CA). The entrapment efficiency (EE) and drug loading were calculated using the following equations:

$$EE \text{ of microparticles}(\%) = \frac{\text{Amount of drug entrapped in particles}(mg)}{\text{initial amount of drug added}(mg)} \times 100 \quad 1$$

$$\text{Drug loading}(\text{drug content}) = \frac{\mu\text{g of drug}(\text{entrapped})}{\text{mg microparticles}} \quad 2$$

2.3.6. Distribution of FITC-dextran within the microparticles—The fluorescent microparticles were redispersed in water, placed on a glass slide and observed under 63 X objective in a confocal laser scanning microscope (CLSM, Carl Zeiss 710, Germany) equipped with Zen 2009 imaging software. The presence of FITC-dextran across microparticles was detected by optical sectioning using an argon laser with an excitation wavelength of 488 nm and a 505–550 nm band-pass emission filter.

2.3.7. Release of FITC-dextran from the microparticles—Ten mg FITC-dextran loaded microparticles were suspended in 1 mL PBS (0.01 M, pH 7.4) containing 0.1 % w/v Tween[®]80 (Fischer scientific, NJ). The microparticles were placed in an incubator shaker (New Brunswick Scientific, NJ) set at 100 rpm and 37±1 °C. At specific time points, 500 µl of release buffer was removed and replaced with fresh PBS. The loading of FITC-dextran was determined using a fluorometer (excitation: 490 nm, emission: 520 nm, Spectramax M5, Molecular Devices, CA)

2.4. Human embryonic kidney-293 (HEK-293) cell culture in vitro

Dulbecco's Modified Eagle Medium (with high glucose 1 X and 4 mM L-glutamine), Trypsin-EDTA (0.25 %, 1 X solution) and Dulbecco's phosphate buffered saline was purchased from Gibco[®] (Invitrogen[™], NY). Fetal bovine serum was obtained from Atlanta Biologicals[®], GA. Gentamycin sulfate (50 mg/mL) was purchased from Mediatech Inc., VA. MTS cell growth assay reagent (Cell Titer 96[®] Aqueous One Solution Cell Proliferation Assay) was purchased from Promega Corporation, WI. Alexa Fluor[®] 568 phalloidin was purchased from Invitrogen, NY. Triton X-100 was obtained from Sigma[®], (Sigma-Aldrich, MO). Vectashield[®], Hardset[™] mounting medium with 4',6-diamidino-2-phenylindole (DAPI) was obtained from Vector Labs Inc., CA. The human embryonic kidney cells (HEK-293) were obtained from the American Type Culture Collection (ATCC[®], VA). HEK-293 cells were maintained in Dulbecco's modified eagle medium (DMEM, supplemented with 10 % fetal bovine serum and 50 µg/mL gentamycin) in a humidified incubator (Sanyo scientific autoflow, IR direct heat CO₂ incubator, CA) at 37 °C containing 95 % air and 5 % CO₂. The cells were cultured as a monolayer in 75 cm² polystyrene cell culture flasks (Corning Incorporated, NY) and subcultured after 80–90 % confluence was achieved.

2.4.1. Investigation of uptake of microparticles in HEK-293 cells—Cells were plated at a density of 50,000 cells/well in clear, flat-bottom, 0.1 % poly-L-lysine-coated 8-chambered glass slide with cover (Lab-Tek, Nunc[™], NY) and allowed to attach overnight. The following day, the cells were treated with FITC-dextran loaded microparticles and further incubated for 24 h. The experiment was terminated by removing the particle suspension. The cells were washed with PBS and fixed with 4 % paraformaldehyde, followed by permeabilization of cells with 0.2 % Triton[®] X-100. The cellular actin was fluorescently tagged with Alexa Fluor[®] 568 phalloidin. The specimen was finally mounted

with Vectashield® Hardset™ mounting medium with 4',6-diamidino-2-phenylindole (DAPI). The cellular uptake of fluorescent microparticles was observed using CLSM (Carl Zeiss 710, Germany) equipped with Zen 2009 imaging software. The images were processed using imageJ® (US National Institutes of Health, MA).

2.5. Data presentation and statistical analysis

Results were reported as means $\pm$ standard deviation (S.D.) from at least three replicate samples of microparticles prepared at the specified conditions. The graphs were generated using GraphPad Prism 5 (GraphPad Software, CA).

3. Results and discussion

In this study, microparticles were fabricated from a novel biodegradable and biocompatible polymer with a sulfenamide based backbone. PSN microparticles were prepared by utilizing the double emulsion solvent diffusion-evaporation method. The formulation parameters employed in the preparation of PSN microparticles were systematically varied and correlated to the physicochemical properties of the PSN microparticles.

3.1. Identification of the optimal formulation parameters for fabricating drug-free PSN microparticles by examination of the external surface morphology

Manufacturing parameters were varied to determine how to fabricate uniform-sized, spherical and smooth-surfaced microparticles from the PSN shown in Figure 1.

SEM images show that the effect of increasing polymer concentration on particle morphology was quite pronounced (Fig. 2A). The lowest polymer concentration (25 mg/mL) resulted in smooth and spherical microparticles with homogenous size distribution (polydispersity index, PDI = 0.073, Fig. 2A i). However, as the polymer concentration increased to 50 mg/mL and 100 mg/mL, with otherwise identical processing conditions, the particles were deformed with irregular precipitates (Figs. 2A ii and 2A iii).

Successful fabrication of the microparticles was achieved by increasing the PVA concentration. With increasing PVA concentration, the shape of the microparticles improved considerably with a decrease in the roughness (Fig. 2B). The particles were spherical in shape without any irregularities. This demonstrated that surfactant concentration was critical for the stability of the emulsion droplets and subsequent microparticle formation. However, with increasing PVA concentration, there was an undesirable increase in the polydispersity of the samples as shown by the inconsistent sizes and shapes of the particles. When the polymer (50 mg) was dissolved in 2 mL of chloroform (vs. 1 mL chloroform used previously, Figs. 2A–2B) and 1 % PVA was used, the microparticles formed were spherical, smooth, non-aggregated (PDI = 0.07) and were homogenous with respect to size (Fig. 2C). This result may be due to the lowered concentration of polymer that results in slower and complete solidification of the emulsion droplets, thus resulting in spherical particles. The microparticles did not differ much in size, shape or surface morphology when heated at 37 °C for 1 h (Fig. 2C i) or 2 h (Fig. 2C ii) during the preparation process. Therefore, the formulation was heated for only 1 h so as to limit the amount of FITC-dextran lost to the outer aqueous phase. Importantly, heating the formulation at 37 °C to aid the rapid removal

of the organic solvent did not affect the morphology of the microparticles. No roughness or any other deformations were observed on the surface of the microparticles. The subsequent experiments were carried out on microparticles prepared using the manufacturing conditions that led to smooth and spherical morphology (manufacturing conditions as utilized in making microparticles shown in Fig. 2C (i)).

3.2. Powder X-ray diffractometry (PXRD) of PSN microparticles

As presented in Fig. 3, the PXRD pattern of PSN exhibited a sharp intense peak at a scattering angle 2θ along with less intense diffraction peaks at 8° , 16° , 19.5° , 23.5° and 26.5° due to the crystalline regions within the polymer. When PSN microparticles loaded with FITC-dextran (formulation batch A, drug loading of $3.1 \mu\text{g}$ per mg of microparticles) were investigated, a nearly identical PXRD pattern was obtained. From both sets of PXRD data, it was concluded that the polymer was semicrystalline. The percent crystallinity for PSN and PSN microparticles fabricated with FITC-dextran was calculated to be 58.7 % and 59.1 % respectively. Clearly, the presence of the drug or the microparticle fabrication process did not significantly affect the crystallinity of the polymer.

3.3. Differential scanning calorimetry (DSC) of PSN and PSN microparticles with and without drug loading

The thermogram of a bulk sample of PSN exhibited an endothermic peak, T_m , at 134°C due to crystalline regions of the polymer (Fig. 4). The PSN microparticles (FITC-dextran loaded, formulation batch A with drug loading of $3.1 \mu\text{g}$ per mg of microparticles and FITC-dextran-free) possessed a slightly lowered T_m of 126°C . The slight lowering of the value for T_m was expected based on the smaller sizes of the microparticles where surface effects were more pronounced than for bulk samples of PSN. These results suggest that formulating microparticles from the polymer did not significantly affect the value for T_m even for particles loaded with FITC-dextran.

3.4. Varying the microparticle size

It is important to be able to control the size of particles used for drug delivery applications. Here the concentrations of polymer and surfactant utilized for preparing microparticles were studied as factors affecting the mean diameter of the final microparticle. Figure 5A shows the effect varying the polymer concentration in the oil phase has on the resultant diameter of the microparticle. The observed relationship between polymer concentration and diameter is similar to those previously reported for PLGA microparticles (Budhian et al., 2007). In these experiments, the PVA concentration was kept constant at 1 %. Increasing the polymer concentration in the oil phase increased the viscosity of the oil phase and also hindered its rapid dispersion into the water phase. As a result, the dispersed oil droplets were large and consequently the microparticles that formed were large. Moreover, as the polymer concentration was increased, while still maintaining 1 % PVA as the surfactant concentration, the amount of surfactant was seemingly insufficient to orient itself at the oil/water interface and reduce the interfacial tension effectively (Song et al., 2008). This led to coalescence and aggregation of the emulsion droplets during emulsification, thereby increasing the microparticle size.

The effect of varying the surfactant (PVA) concentration in the water phase on the size of the microparticles was investigated (Fig 5B). Here, the polymer concentration was fixed at 100 mg/mL and the PVA concentration varied from 0.5 % to 5 %. We observed that when the concentration of PVA increased from 0.5 % to 1 %, the particle size decreased. At 0.5 % PVA, the concentration was insufficient for surfactant activity and emulsion stability which lead to large aggregated particles. As we increased the PVA concentration from 0.5 % to 1 %, the surfactant was able to cover the interface of the emulsion droplets more efficiently. This caused the interfacial tension to be reduced and led to an initial decrease in the particle size due to the breakdown of the larger emulsion droplets into smaller ones which then solidified as small particles (Galindo-Rodriguez et al., 2004; Nandi et al., 2001).

When the concentration of PVA increased from 1 % to 5 % the diameters of the particles increased. Others have proposed that increasing the concentration of PVA would lead to microparticles that are well coated by PVA and thus prevent or reduce the coalescence of emulsion droplets. This effect was predicted to lead to a lowering of the particle size with an increase in the PVA concentration (Fude et al., 2005). Despite this prediction, the diameters of the particles increased as the PVA concentration increased. This increase was predominantly a result of the decrease in the net shear stress with increasing viscosity, at a constant energy output as previously reported (Budhian et al., 2007). The mean surface charge of the FITC-dextran loaded microparticles prepared using 50 mg polymer in 2 mL chloroform and 1 % PVA (formulation batch A, drug loading of 3.1 μg per mg of microparticles) was found to be -22.9 ± 0.03 mV.

3.5. Influence of formulation variables on the entrapment efficiency of FITC-dextran loaded microparticles and drug loading

Figures 6A and 6B illustrate the influence of the polymer concentration in the oil phase on the drug entrapment efficiency (EE) of microparticles and the drug loading, respectively. The EE is the percentage of the amount of drug entrapped in the particles divided by the total amount of drug added to formulate the particles. It is a measure of the success of encapsulating drug in solvent into the particles. The drug loading is the percentage of the amount of drug entrapped in the particles divided by the weight of the particles. An increase in polymer concentration led to an increase in the viscosity of the oil phase and an increase in the particle size. Because the FITC-dextran was loaded into the internal W_1 phase, the increase in polymer concentration lead to a reduced diffusion of FITC-dextran to the external W_2 phase and an increase in the EE (Yang et al., 2001; Ghaderi et al., 1996). The decrease in EE with decreasing polymer concentration can also be ascribed to the larger surface area of smaller microparticles. Small microparticles have an enhanced contact with the external water phase during the emulsification and particle hardening stages which may result in an increase in the drug loss. Reports have also shown that the increasing polymer concentration causes the formation of a compact and dense polymer matrix structure within the microparticles which retards the drug diffusion (Fude et al., 2005).

Table 2 shows the EE and drug loading of the different microparticle preparations. Batch A was a prototype, the formulation parameters of which were then varied, one at a time while keeping other parameters constant. These variations were performed in an attempt to

enhance the drug EE and drug loading properties of the microparticles. The microparticles were prepared using the manufacturing conditions which led to smooth and spherical morphology (manufacturing conditions utilized in making microparticles as shown in Fig. 2C (i)).

Increasing the amount of FITC-dextran used in fabricating FITC-dextran loaded microparticles in the W_1 phase increased diffusion of the drug from the emulsion droplets into the W_2 phase. This led to the loss of drug into the W_2 phase and a corresponding decrease in entrapment efficiency (Batch A vs. Batch G) (Prior et al., 2000). Comparison of Batch A and G also showed that as the amount of FITC-dextran used in fabricating FITC-dextran loaded microparticles increased, the drug loading increased. The decrease in EE may also be attributed to the drug loading into the microparticles reaching its saturation level, where a further increase in the drug amount no longer results in a further increase in the drug loading. In addition, the osmotic pressure difference between W_1 and W_2 phases could possibly increase with increasing the amount of FITC-dextran used in fabricating FITC-dextran loaded microparticles. This phenomenon results in the migration of W_2 phase towards the internal W_1 phase due to less osmotic pressure in W_2 (Alex and Bodmeier, 1990).

NaCl was added to the W_2 phase (Batch B) to increase EE and drug loading. The addition of NaCl should slow the rate of drug diffusion from the double emulsion droplets to the W_2 phase due to increased diffusional resistance to the drug. Using NaCl in W_2 has been studied to produce particles with a relatively denser surface which acts as a barrier to the drug diffusion during the entrapment process. The presence of salt increases the osmotic pressure of the W_2 phase and prevents the inward flow of water to the W_1/O emulsion microdroplets (Rosa et al., 2000).

On increasing the W_1 phase volume, the EE and drug loading decreased (Batch A vs. Batch C). Higher volumes of W_1 possibly results in increased microporosity or channel formation in the polymer matrix which favors rapid drug diffusion as the solvent is being removed (Ghaderi et al., 1996). In addition, the increasing internal water volume decreases the thickness of the polymer solution layer surrounding it (Alex and Bodmeier, 1990). In the case of lower volumes of the W_1 phase, the surrounding oil phase may reduce the rate of diffusion of the entrapped drug into the outer continuous water phase. This situation applies during both the initial emulsification steps and the solvent evaporation stage.

Increasing the W_2 volume (Batch D) allows chloroform to diffuse rapidly into the W_2 phase during the organic solvent evaporation stage. This results in faster polymer precipitation and rapid formation of microparticles along with less time for drug molecules to partition into the W_2 phase (Li et al., 1995). Hence, as the drug loss to the W_2 phase is minimized, the EE and drug loading were found to increase. At the same time, employing a lower volume of the W_2 phase could suppress the rate of diffusion of organic solvent into the external water phase, thereby contributing to low drug entrapment (Batch E).

With an increase in the molecular weight of FITC-dextran (Batches F, G, H), the EE and drug loading decreased which is a phenomenon consistent with other studies on

microparticle preparation (Mao et al., 2007). For FITC-dextran, as the chain length grows with the molecular weight, the drug is incorporated within the polymer as a bulk network. This may create diffusion channels with enhanced water uptake as the particles are being hardened during the organic solvent evaporation stage. This may facilitate the escape of the drug to the W_2 phase (Rothen-Weinhold et al., 1997).

3.6. Drug distribution within the microparticles

Confocal microscopy was employed to detail the distribution pattern of fluorescently tagged dextran in the PSN microparticles (formulation batch A, drug loading of 3.1 μg per mg of microparticles). The green represents the location of the FITC-dextran (Fig. 7). The entrapped FITC-dextran was observed to be distributed throughout the microparticle matrix, with higher concentrations found towards the surface of the microparticles.

3.7. Release of entrapped FITC-dextran from the polymeric microparticles

For the release study, microparticles loaded with FITC-dextran were fabricated using 50 mg PSN in 2 mL chloroform and 1 % PVA (formulation batch A, drug loading of 3.1 μg per mg of microparticles). In accordance with the FITC-dextran distribution data presented previously, the release pattern of FITC-dextran from the microparticles indicated an initial burst release phase (~50 % released within the first 12 h, Fig. 8). A slow, but continuous release followed over the next 2 weeks resulting in almost 100 % release of FITC-dextran. The burst release observed may be due to drug diffusion from a large surface area associated with small-sized microparticles and this is particularly enhanced by the entrapped drug concentrated towards the surface of the microparticles. The observed delayed release phase may result from the combination of 1) diffusion-controlled release of FITC-dextran localized in the core of the microparticles through the water channels formed over time and 2) the eventual slow degradation/bulk erosion of the polymer matrix (Zhang and Feng, 2006).

3.8. Uptake of PSN microparticles by HEK-293 cells *in vitro*

It is critical for drug delivery applications that the microparticles are internalized into cells. In this study human embryonic kidney-293 (HEK-293) cells were selected for evaluation in uptake studies because of their relevance and wide utility in the development of drug and gene delivery vehicles. The ability of HEK-293 cells to internalize microparticles of PSN was studied by confocal microscopy. Briefly, the cells were exposed to microparticles loaded with FITC-dextran (formulation batch A, drug loading of 3.1 μg per mg of microparticles) that appeared green in the confocal microscope. After exposure to the microparticles for 24 h, the solution around the cells was washed to remove excess microparticles and the cells were stained and imaged by confocal microscopy. A confocal image showed the presence of green fluorescent FITC-dextran loaded microparticles mainly in the cell cytoplasm, and to some extent, in the nucleus (Fig. 9). In control experiments, green fluorescence was not observed in cells treated with drug-free microparticles and in untreated cells (data not shown). Thus, there was no autofluorescence from either the polymer or the cells and the observed green fluorescence within the cells was mainly due to the uptake of the FITC-dextran loaded microparticles.

4. Conclusions

This is the first study to develop and optimize a novel PSN based microparticle formulation using the double emulsion solvent diffusion-evaporation method. We are the first to synthesize polysulfenamides and have shown that they possess many desired characteristics for drug/gene delivery systems. In this study, we investigated how to fabricate microparticles from these polymers and many of the important properties of the microparticles. Microparticles with varying formulation parameters were prepared and systematically evaluated. Under the optimal processing conditions, SEM images demonstrated that microparticles with smooth spherical surface morphology and uniform size were fabricated. The particle size, entrapment efficiency and drug loading were found to be a function of the amounts of polymer and surfactant used in the fabrication process. The release profile of FITC-dextran from the microparticles demonstrated gradual and continuous release over two weeks following an initial burst release. FITC-dextran loaded microparticles were readily internalized by cells *in vitro*. PSN based microparticles are a promising new carrier for the controlled delivery of therapeutic drugs, proteins and genes. The results presented here provide a framework for future studies that will include the surface modification of these microparticle formulations with cell specific binding ligands and the evaluation of these novel PSN microparticles for treatment of a wide range of diseases.

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Figure 1.
A polysulfenamide degrades in water to release an amine and sulfenic acids.

A.

B.

C.

Figure 2.

A. Influence of polymer concentration in the oil phase on the morphology of microparticles: Microparticles prepared using 100 μ L W_1 , 1 mL chloroform, (4 mL + 36 mL) W_2 , 1 % PVA, double sonication (30 s/30 s), 1 h stir + 1 h heat at 37 °C (i) 25 mg polymer in 1 mL chloroform (ii) 50 mg polymer in 1 mL chloroform (iii) 100 mg polymer in 1 mL chloroform

B. Influence of surfactant amount in the water phase on the morphology of microparticles: Microparticles prepared using 100 μ L W_1 , 100 mg polymer in 1 mL chloroform, (4 mL + 36 mL) W_2 , double sonication (30 s/30 s), 1 h stir + 1 h heat at 37 °C (i) 0.5 % PVA (ii) 2 % PVA (iii) 5 % PVA

C. Microparticles with optimal morphology: 200 μ L W_1 , 50 mg polymer in 2 mL chloroform, (8 mL + 32 mL) W_2 , 1 % PVA, double sonication (30 s/30 s) (i) 1 h stir + 1 h heat at 37 °C (ii) 1 h stir + 2 h heat at 37 °C

Figure 2. Surface scanning electron microscopy images of microparticles: Influence of different processing conditions on the resulting size, shape and external morphology of microparticles.

Figure 3.
PXRD pattern of PSN and PSN microparticles with FITC-dextran loading.

Figure 4.
DSC thermograms of a bulk sample of PSN and PSN microparticles with and without FITC-dextran loading.

A

Figure 5. Influence of (A) polymer concentration and (B) PVA concentration on the mean diameter of PSN microparticles. Data is shown as mean $\pm$ S.D. (n = 3).

A

B

Figure 6. Effect of polymer concentration on (A) entrapment efficiency (EE) and (B) FITC-dextran loading. Data is shown as mean $\pm$ S.D. ($n = 3$). FITC-dextran loaded microparticles were prepared using 100 μ L W_1 phase containing 5 mg FITC-dextran (20 kDa), 1 mL chloroform, (4 mL + 36 mL) W_2 , 1 % PVA, double sonication (30 s/30 s), 1 h stir + 1 h heat at 37 $^{\circ}$ C.

Figure 7.
Distribution of FITC-dextran in a PSN microparticle. Scale bar, 10 μm .

Figure 8.

In vitro release kinetics of entrapped FITC-dextran from PSN microparticles in PBS (pH 7.4) at 37 °C. Data is shown as mean $\pm$ S.D. (n = 3).

Figure 9. Confocal laser microscopic image of HEK-293 cells (Z-series, magnification 63 X) after 24 h incubation at 37 °C (A) Alexa Fluor 568 phalloidin-stained cellular actin, red (B) DAPI-stained cellular nucleus, blue (C) Cells treated with FITC-dextran loaded PSN microparticles, green (D) Merged image. Scale bar, 20 μm.

Table 1

Summary of the microparticle fabrication conditions

#	Polymer mass (mg)	PVA conc. (%)	W ₁ (μl)	O (ml)	W ₂ volume/Total water volume (ml)	Formulation parameters	Figs.
1	25	1	100	1	4/40	double sonication (30 s/30 s), 1 h stir + 1 h heat at 37°C	2A i
2	50	1	100	1	4/40	double sonication (30 s/30 s), 1 h stir + 1 h heat at 37°C	2A ii
3	100	1	100	1	4/40	double sonication (30 s/30 s), 1 h stir + 1 h heat at 37°C	2A iii
4	100	0.5	100	1	4/40	double sonication (30 s/30 s), 1 h stir + 1 h heat at 37°C	2B i
5	100	2	100	1	4/40	double sonication (30 s/30 s), 1 h stir + 1 h heat at 37°C	2B ii
6	100	5	100	1	4/40	double sonication (30 s/30 s), 1 h stir + 1 h heat at 37°C	2B iii
7	50	1	200	2	8/40	double sonication (30 s/30 s), 1 h stir + 1 h heat at 37°C	2C i
8	50	1	200	2	8/40	double sonication (30 s/30 s), 1 h stir + 2 h heat at 37°C.	2C ii

Table 2

FITC-dextran loaded microparticles prepared from PSN under optimal processing conditions: 50 mg polymer in 2 mL chloroform, 1 % PVA, double sonication (30 s/30 s), 1 h stir + 1 h heat at 37 °C. Data is shown as mean $\pm$ S.D. (n = 3)

Batch	FITC-dextran mol. wt./amount	W ₁ volume(μ L)	W ₂ volume/Total water volume (mL)	EE (%) $\pm$ S.D.	Drug loading* $\pm$ S.D.
A	20 kDa/5 mg	200 μ l	8 mL/40 mL	3.1 $\pm$ 0.07	3.1 $\pm$ 0.07
B	20 kDa/5 mg (0.4 M NaCl)	200 μ l	8 mL/40 mL	3.4 $\pm$ 0.1	3.4 $\pm$ 0.1
C	20 kDa/5 mg	100 μ l	8 mL/40 mL	4.5 $\pm$ 0.03	4.5 $\pm$ 0.03
D	20 kDa/5mg	200 μ l	8 mL/80 mL	5.6 $\pm$ 0.06	5.6 $\pm$ 0.06
E	20 kDa/5 mg	200 μ l	8 mL/20 mL	3.0 $\pm$ 0.1	3.0 $\pm$ 0.1
F	4 kDa/2.5 mg	200 μ l	8 mL/40 mL	5.9 $\pm$ 0.2	2.9 $\pm$ 0.1
G	20 kDa/2.5 mg	200 μ l	8 mL/40 mL	4.0 $\pm$ 0.2	2.0 $\pm$ 0.1
H	40 kDa/2.5 mg	200 μ l	8 mL/40 mL	2.2 $\pm$ 0.1	1.1 $\pm$ 0.08

* Drug loading is expressed as μ g of entrapped FITC-dextran per mg of microparticles