Salt-Leaching Synthesis of Porous PLGA Nanoparticles

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***Abstract*—Poly(lactic-co-glycolic acid) (PLGA) nanoparticles are widely used for controlled delivery of bioactive agents in therapeutic applications. These nanoparticles show bioavailability, better encapsulation, controlled release, biocompatibility, and *in vivo* biodegradability. This paper reports a novel approach to synthesize porous PLGA nanoparticles and their use as controlled release vehicles. Bovine serum albumin (BSA) loaded PLGA nanoparticles (porous and nonporous) were synthesized using *water-in-oil-in-water* double emulsion method. Specifically, PLGA nanoparticles were prepared using chloroform and polyvinyl alcohol, and freeze drying was employed for the phase separation to obtain the nanoparticles. The porous nanoparticles were prepared through the salt-leaching process where sodium bicarbonate was used as an extractable porogen. *In vitro* drug release behavior of porous and nonporous nanoparticles was monitored over a period of 30 days. A much more enhanced BSA release was observed in case of porous polymeric nanoparticles when compared to nonporous nanoparticles. The characterization was done using laser scattering, zeta potential analysis, and scanning electron microscopy. The drug loading efficiencies for BSA in porous and nonporous PLGA nanoparticles were 65.50% and 77.59%, respectively. Over a period of 30 days, the cumulative BSA released from PLGA porous and nonporous nanoparticles were measured to be 87.41% and 59.91%, respectively. The synthesis of porous nanoparticles with this novel, rapid, and inexpensive method opens a new horizon of using a wide range of cheap and easily-accessible water-soluble salts that can be extracted through leaching process to introduce porous morphology on the nanoparticle surfaces. The porous nanoparticles can have useful applications in controlled drug delivery systems.**

***Index Terms*—Drug delivery, drug loading efficiency, nanoparticles, nanoporous particles, porogen, salt-leaching.**

I. Introduction

OVER the last few decades, there has been increased interest in developing biodegradable micro/nanoparticles for drug delivery applications. Among the new drug delivery systems, the polymeric nanoparticles have emerged as promising carriers for sustained release of bioactive agents. The increased surface area offered by small particles makes these exceedingly effective for targeted drug delivery [1]–[3]. It has also been reported that nanoparticles can offer elevated drug content inside the neoplastic cells by prevailing over the multidrug resistance [4], [5]. The increased focus on deploying nanoparticles as drug delivery systems is due to the ease of their preparation with well-defined biodegradable polymers like poly(lactic-co-glycolic acid) (PLGA), which are low cost and show highly stable behavior in the biological fluids [6], [7]. The destiny of nanoparticles in any drug delivery operation is also an important concern during the development process.

The controlled and targeted release eliminates the possibility of both under and overdosing. The control of the concentration of released drug in the therapeutic range significantly improves the effects and quality of the drug. The PLGA offers many possibilities to accurately control the drug release kinetics over periods of days to months and easy administration using standard intramuscular or intravenous routes. But drug stability and accelerated polymer degradation due to autocatalytic effects are the major concerns in nonporous PLGA-based particles [8]. The porous drug delivery systems can overcome these autocatalytic effects by increasing the diffusivity of the molecules [9]. Thus, PLGA-based porous nanoparticles can be very helpful to optimize the therapeutic efficiency of medical treatments and to reduce serious side effects [10]. The porous nanoparticle systems also exhibit much better flow and aerosolization efficiency during pulmonary administration when compared to nonporous nanoparticles [11], [12]. The porous and nonporous PLGA nanoparticles are widely studied for their drug release behaviors. The pore characteristics have significant impact on drug release and by controlling pore morphology, it is possible to design highly controlled drug release systems [10]. In this paper, we describe the salt-leaching technique to prepare porous

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polymeric drug delivery system with prolonged and controlled drug release properties. PLGA is a biocompatible and biodegradable copolymer which undergoes hydrolysis in the body to produce the biodegradable metabolites; glycolic acid and lactic acid. The biodegradability, biocompatibility, and tissue reaction of PLGA have been documented before [5], [13], [14]. Given all these exquisite properties, it has been approved by Food and Drug Administration in USA and European Medical Agency for use in parenteral drug delivery systems. Numerous polymeric objects like microspheres, microcapsules, and nanoparticles have been synthesized using PLGA to deliver variety of drugs [15]. These PLGA nanoparticles are prepared either by using single emulsion or double emulsion methods. The single emulsion method is straightforward where only one water phase solution is emulsified with an oil phase solution. The water-in-oil-in-water (*w*/*o*/*w*) double emulsion method incorporates dispersed oil beads containing smaller aqueous globules. The nanoparticles obtained by this technique provide a great opportunity for the controlled release of chemical species initially entrapped in the internal globules [16], [17].

Bovine serum albumin (BSA) is a hydrophilic drug model which finds its use in a wide range of applications due to its stability, low-cost, and minimal side effects in most of the biochemical reactions. Similarly, chloroform ($CHCl_3$) is also widely used organic solvent because it is fairly unreactive, conveniently volatile, and effortlessly miscible with other organic solutions.

Nanoparticles have been identified as important drug carrier nanovehicles where therapeutic and diagnostic agents are encapsulated, covalently attached, or adsorbed on to the surface of such nanocarriers. The research into rational and controlled drug delivery has resulted into hollow nanospheres and porous nanoparticles. The porous nanoparticle systems can be made of various materials, prepared in a variety of different ways, and designed to deliver drugs to specific parts of the body. The porous nanoparticles can vary from 25 nm to several hundred nm of diameter [11]. Owing to large effective surface areas, porous nanoparticles are widely used as inorganic carriers for biological reagents [18]. The porous nature has proven to facilitate a controlled release of encapsulated drugs of varying chemistry and molecular weights.

Various approaches have been developed before to synthesize porous micro/nanoparticles which incorporate the use of poly(acrylic acid), Pluronic F-127, or ammonium bicarbonate as the porogen reagent [19]–[21]. These chemical species are either limited by their physicochemical properties, high price, or need for thermal curing. We offer a simple and straightforward method, which may empower us to use a wide range of cheap, easily obtainable, and water-soluble salts as extractable porogens. Such a rapid, inexpensive, and simple technique with potential for a variety of alternative materials as porogen, can improve the controlled drug delivery systems to a large extent.

In this work, porous and nonporous PLGA nanoparticles containing BSA were prepared by the *w*/*o*/*w* double emulsion method using sodium bicarbonate as the extractable porogen. The porous nanoparticles showed increased and sustained drug release than the nonporous ones. The nanoparticle size, size distribution, porosity, stability, drug loading efficiency, and drug release behavior were characterized. The BSA release behavior was characterized using bicinchoninic acid (BCA) protein assay. Standard curves were plotted to determine the amount of the drug loaded and released.

II. Materials and Methods

A. Chemicals

All the chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted. There were three major experimental goals achieved in succession. The first section details the synthesis of drug loaded porous and nonporous nanoparticles. The second step focuses on drug loading efficiency. The third and the most important part demonstrates the drug release behavior of the nanoparticles.

B. Synthesis of BSA Loaded Porous and Nonporous PLGA Nanoparticles

Twenty milligrams of the hydrophilic BSA was dissolved in 0.2 ml of deionized (DI) water to make the water phase of the solution (w_1). In parallel, 90 mg of PLGA was dissolved in 3 ml of chloroform to make 3% PLGA solution, forming the oil phase solution (o). Then, 1 gm of polyvinyl alcohol (PVA) was dissolved in 20 ml of warm (50 °C) DI water to make 5% aqueous PVA solution that formed the second water phase solution (w_2). All the solutions were vortexed to ensure that the reagents were mixed well. Now, the BSA solution (w_1) was added to the PLGA solution and vortexed for 30 s to make water-in-oil phase. In order to prepare porous nanoparticles, 2 mg of sodium bicarbonate was mixed in 1 ml of DI water to form 2% (*w*/*v*) porogen solution. This solution was immediately added to the above water-in-oil solution to introduce pores on the particles. These pores would be formed at a later stage due to salt-leaching. This step of adding sodium bicarbonate solution was skipped for the preparation of nonporous nanoparticles as shown by the schematic flowchart in Fig. 1. The solution was then sonicated for 1–2 min at 40 W, and then added drop-wise to the stirring beaker of aqueous PVA solution. This formed the *w*/*o*/*w* phase. The resultant solution was then emulsified by sonication for 2 min at 40 W. Next, the mixture was gently stirred overnight in a chemical hood allowing the chloroform to evaporate. Once the chloroform evaporated completely, the solution was transferred into a 50 ml tube and centrifuged at 4000 rpm for 15 min. The supernatant was collected and frozen for calculating the loading efficiency at later stage. The pellet was resuspended in 5 ml PBS and vortexed for 1 min. The dynamic light scattering (DLS) was used to measure the size and polydispersity of nanoparticles using a ZetaPALS DLS detector (Brookhaven Instruments, Holtsville, NY, USA). The solutions were then freeze dried to –80 °C.

In order to get the porous nanoparticles, the freeze-dried nanoparticles were taken in a plastic tube and 3 ml of DI water was added to perform salt leaching. The tube was vortexed again and centrifuged at 4000 rpm for 15 min. This step was repeated three times to completely remove the trapped salts.

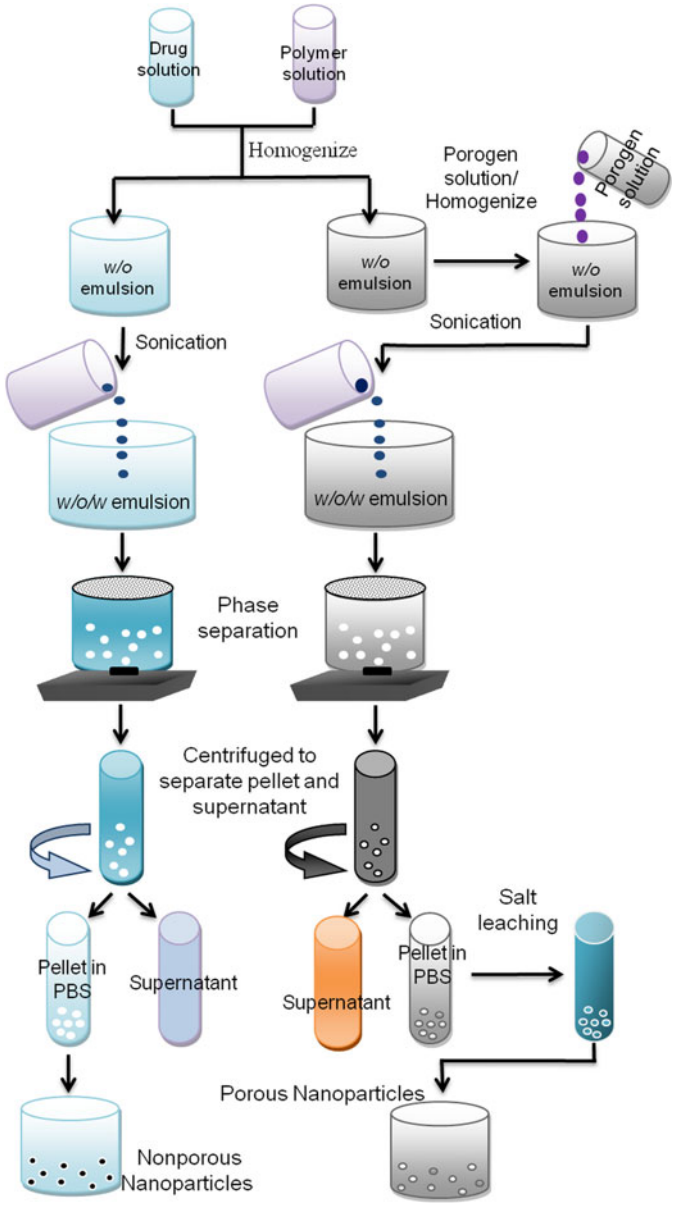

Fig. 1. Schematic flowchart demonstrates the procedure to synthesize porous and nonporous nanoparticles.

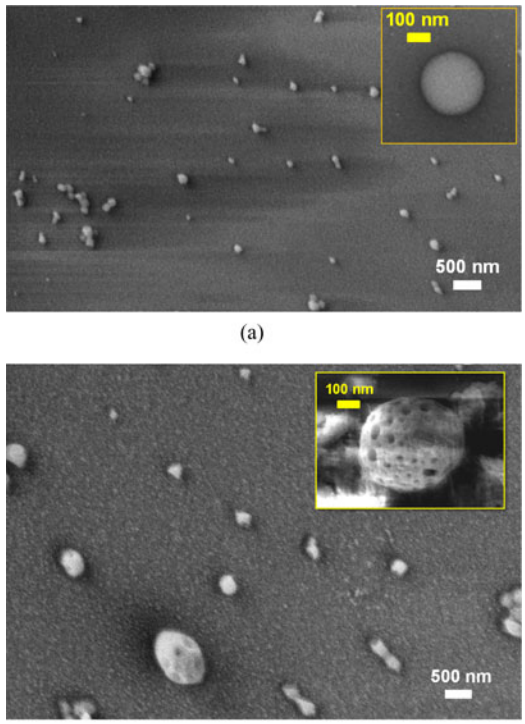

Fig. 2. SEM micrographs of BSA-loaded PLGA nanoparticles. (a) Nonporous nanoparticles with average diameter of 321.8 nm. The inset shows a magnified view of a nonporous nanoparticle. (b) Porous nanoparticles with average diameter of 525.2 nm. The inset shows a magnified view of a porous nanoparticle to illustrate the surface topography and estimated pore sizes of 30–60 nm.

Iterative washing in DI water after centrifugation is known to completely remove the water-soluble salts [22], [23]. The supernatant during salt-leaching was also collected and frozen to study the possible drug released during leaching as the loaded drug (BSA) was hydrophilic.

C. Preparation of PLGA Nanoparticles for Scanning Electron Microscope Imaging

The freeze-dried nanoparticles were suspended in DI water and diluted 10X to reduce their density on the substrate surface used for imaging. DI water was used instead of PBS to avoid crystallization and imaging artifacts stemming from the salts of PBS. The solution was then poured on the top of autoclaved glass cover slips and left overnight to dry in nitrogen ambient. The samples were then sputter coated with 50 Å thick gold to prevent the surface from getting charged during electron microscopy. The thin layer of gold coating made sure that the cover slip surfaces were conductive enough to image with scanning electron microscope (SEM). Fig. 2 shows the SEM micrographs of porous and nonporous nanoparticles.

D. Indirect Calculation of Drug Loading Efficiency

The drug loading efficiency was determined using an indirect method. The supernatant samples collected and frozen in the previous steps were used to calculate the loading efficiency. The indirect method relied on the protein present in the supernatant solution. Instead of direct investigation of the amount of drug loaded in the nanoparticles, the amount of drug present in the supernatant was measured using the standard curves. The drug present in supernatant solution reflected the amount of unloaded drug which was subsequently used to calculate the loaded drug. Loading Efficiency was calculated using the formula given below

$$\text{Loading Efficiency}\,(\%) = \frac{A-B}{A} \times 100 \qquad (1)$$

Where A represents the amount of original drug dissolved in DI water and B is the amount of drug present in the supernatant (unloaded drug).

E. BSA Release from Nanoparticles

In order to monitor drug release from BSA loaded nanoparticles, these were resuspended in PBS and placed in a 100 kDa molecular weight cutoff dialysis bag (Spectrum Laboratories Inc., Rancho Dominguez, CA, USA). Equal concentrations of porous and nonporous nanoparticles (2 mg each) were separately mixed in 2 ml of PBS and were put in different bags. Each dialysis bag was immersed in 10 ml of PBS solution which was the dialysate. Three replicates for both types of particles were prepared to study the drug release behavior. Drug release studies were performed by measuring BSA release at predefined time points: 1, 5, 7, 11, 14, 18, 22, and 30 days. At each time point, the PBS solution was replaced by fresh PBS solution keeping the volume unchanged and the collected samples were stored at $-20\ ^{\circ}$C for further analysis.

At each time point, three samples (one from each replicate) were collected to get reliable release data. After collecting all the samples for porous and nonporous PLGA nanoparticles, Pierce BCA protein assay (Fisher Scientific, Hampton, NH, USA) was used for quantitative analysis of the drug release. The working reagent consisted of reagent A and reagent B at the ratio of 50:1, where BCA was used as reagent A and copper sulphate as reagent B. A 200 μl volume of the working reagent and 25 μl of PBS (containing BSA obtained from collected samples) were added to each well for respective measurements of porous and nonporous nanoparticles' drug release behavior. After adding all the samples, which were collected at predetermined time points, to the BCA containing wells, standard solutions were also added on the same well plate, with gradually reducing concentrations of BSA to determine the standard curve. The well plate was then covered with the aluminum foil and kept in the incubator at 37 °C for 30 min. In the presence of protein, the color of the solution changed from blue to purple. Absorbance was recorded using spectrophotometry (Tecan Infinite M200 Plate Reader, Durham, NC, USA) at wavelength of 562 nm. Absorbance values for the standard concentration values provided a standard curve that was then used to calculate the concentration of BSA released. The percentages of protein release were calculated using the following equation:

$$\text{Percentage of protein release} = \frac{X}{Y} \times 100 \qquad (2)$$

where X indicates the amount of released BSA and Y represents the total amount of loaded BSA.

With the obtained values, percentage of cumulative protein release was calculated and plotted against the administered time points. The standard deviations of the absorbance at each time point were also plotted on the same graph to demonstrate the precision of the data.

TABLE I
CHARACTERISTICS OF BSA-LOADED POROUS AND NONPOROUS PLGA NANOPARTICLES

Property	Non-porous PLGA Nanoparticles	Porous PLGA Nanoparticles
Porogen concentration (% w/v)	0	0.2
Average diameter (nm)	321.8 ± 7.4	525.2 ± 7.4
Polydispersity	0.132 ± 0.031	0.314 ± 0.006
Zeta potential, ζ (mV)	−17.09 ± 0.25	−21.67 ± 1.5
Loading efficiency	77.59%	65.5%
Cumulative release	59.91%	87.41%

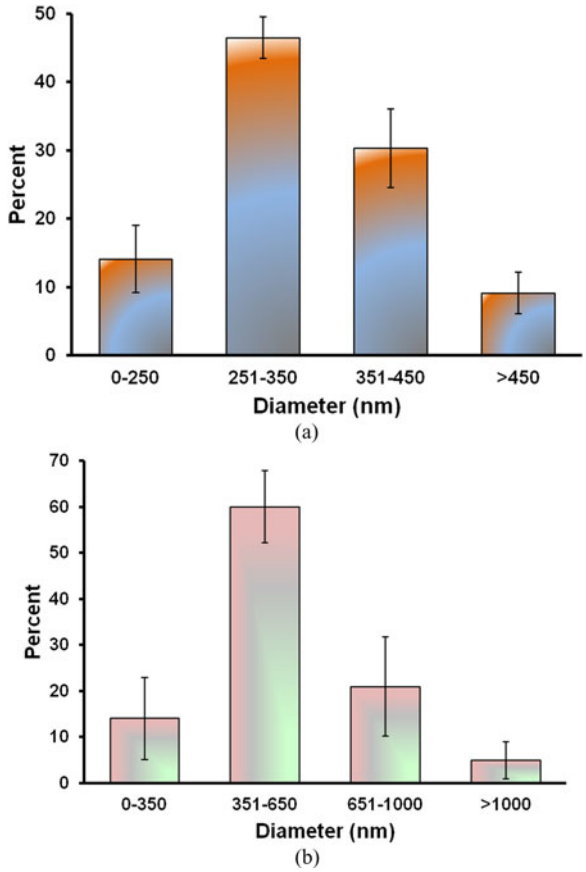

Fig. 3. Size distribution of BSA-loaded (a) nonporous and (b) porous PLGA nanoparticles.

III. RESULTS AND DISCUSSION

The morphology of synthesized nanoparticles was found to be spherical from SEM micrographs with average diameters for porous and nonporous nanoparticles to be 525.2 and 321.8 nm, respectively (see Table I). The size distribution was also observed to be more uniform in case of nonporous nanoparticles as shown in Fig. 3.

The porous nanoparticles were expected to be larger than the nonporous nanoparticles in line with previous reports [11]. The larger sizes of the porous nanoparticles were due to sodium bicarbonate incorporation which later leached out, leaving porous features on the nanoparticle surfaces. The porosity, pore sizes, and distribution of pores on the porous nanoparticles could be controlled by using different concentrations of the porogen [21]. Here, a single concentration of porogen was used because the main focus was to establish salt-leaching as a novel technique to prepare the porous nanoparticles. The pore sizes were seen to range between 30–60 nm from SEM data.

Polydispersity index, a dimensionless number, is used to gauge the particle size distribution. Its value may vary from 0.01 (monodispersed particles) to 0.7 (particles with fluctuating size distribution) [5]. Particles with broadly varying size distribution indicate polydispersity index >0.7 [24]. Polydispersity of both types of nanoparticles (given in Table I) clearly shows that both types of nanoparticles had polydispersity index much less than 0.7. Porous PLGA nanoparticles exhibited polydispersity higher than nonporous ones which might have stemmed from the uncontrolled vortexing of porogen in *w/o* phase solution while synthesizing the nanoparticles.

The stability of the nanoparticles in solution is very important for biological applications that incorporate colloidal suspensions. The particle composition and the medium in which these nanoparticles are dispersed determine the zeta potential (ζ) of the particles. High surface charges ($\zeta > \pm 30$ mV) prevent aggregation and thus make the nanoparticles highly stable in the colloid suspension [25]. Particles with surface charges ($\zeta > \pm 10$ mV) are also relatively stable whereas $\zeta < \pm 10$ mV allows the particles to flocculate due to small repulsive effect [26]. In this work, we used PLGA with carboxylic acid end groups (–COOH) on it and the presence of carboxyl groups on the PLGA nanoparticle surfaces passed on negative charges which was confirmed by the ζ values recorded for both types of nanoparticles. Though the zeta potential for the nanoparticles was less than –30 mV, but it was still high enough to give reasonably good colloidal properties.

The drug loading efficiency was determined by using the indirect method because BSA is hydrophilic drug and can degrade in organic solvent. The loading efficiency was found to be 65.50% and 77.59% for BSA loaded porous and nonporous nanoparticles, respectively. The drug loading efficiency was sufficiently high which showed the viability of those nanoparticles in controlled drug delivery systems. High loading efficiency is always desired for an efficient drug delivery system so that less quantity of the nanoparticles would be needed to administer a particular dose. The nonporous nanoparticles had higher loading efficiency as compared to porous nanoparticles. The porous structures on the surface of nanospheres allowed some drug to easily flow out whereas nonporous nanoparticles incorporated more drug [21]. The salt-leaching process, on one hand, caused the formation of porous structures on the nanoparticles surface but it also reduced the loading efficiency of the nanoparticles.

To investigate the BSA drug release from porous and nonporous PLGA nanoparticles, BCA protein assay was used. The cumulative BSA release for porous and nonporous PLGA nanoparticles was found to be 87.41% and 59.91%, respectively, over a period of 30 days. For the first few days, more than 30% of BSA was released, while after that relatively less amount of BSA release was observed as shown in Fig. 4. The release behavior of both types of nanoparticles is plotted to show the comparison over the same period of time.

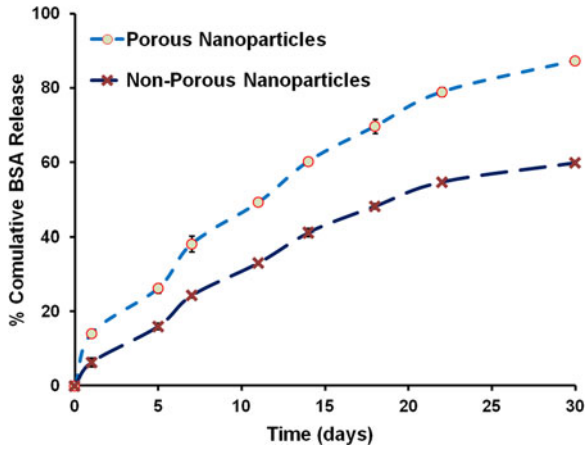

Fig. 4. Comparison of BSA release from porous and nonporous PLGA nanoparticles shows a sustained release behavior. The porous nanoparticles show higher release dose over the course of a month than nonporous ones. Error bars represent the standard deviation in percentage protein release ($n = 3$).

Comparison of BSA release from the porous and nonporous PLGA nanoparticles showed a faster but sustained release from porous nanoparticles. The porous nanoparticles offered better release behavior owing to their increased surface area and better flow of fluid through leaky structures [27], [28]. Generally, reports show that larger particles show lower drug release rate when compared to smaller particles because the length of diffusion pathway is higher for larger particles and drug concentration gradient is smaller [10]. Here, porous nanoparticles were larger in size but exhibited higher release rate than smaller nonporous nanoparticles. This nonintuitive behavior clearly stemmed from the porosity. As the porosity increased, the percent of cumulative drug release also increased [21]. Moreover drug release from porous nanoparticles would also depend on the pore characteristics and the nature of host guest interactions [29]. These data show nanoparticles that are not only larger but also have higher release of drug.

The drug release data for 30 days showed a sustained release which was reflected by a good amount of BSA. A burst release for the first few days was observed which stemmed from the release of drug adsorbed on or near the surface of nanoparticles and thus ensured immediate availability of the therapeutic drug. The PLGA nanoparticles are employed for oral and intravenous drug delivery routes but these are not selective to specific cells. Selectivity can be imparted by making the nanoparticles functional. A number of functional groups, antibodies, and aptamers can be employed to make these selective to certain cells, proteins, or genes. These functional groups can bind to the target cells and the payload of nanoparticle-based drugs can be delivered selectively [30], [31].

IV. Conclusion

This paper demonstrates that BSA-loaded porous PLGA nanoparticles provide sustained and enhanced drug release, even though these are larger in size. The salt-leaching is a novel approach to synthesize porous nanoparticles where sodium bicarbonate is used as the porogen. Moreover, the nonporous nanoparticles are very uniform in their size distribution but the porous nanoparticles show a larger distribution over a specific range, due to the trapped salts that lead to the pore formation after leaching. Drug loading efficiency of nonporous nanoparticles is calculated to be higher than that of porous nanoparticles which is due to easy release through the features on the porous nanoparticles. The drug release behavior of both types of nanoparticles is monitored over a period of 30 days and porous nanoparticles, although larger in size, show faster sustained release. Owing to the enhanced surface area, better release behavior and their stability over varying temperatures, porous nanoparticles are thus recognized as much better drug carriers.

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