



# Biodegradable nano-micro carrier systems for sustained pulmonary drug delivery: (I) Self-assembled nanoparticles encapsulated in respirable/swellable semi-IPN microspheres

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## ABSTRACT

Design of appropriate inhaled carriers with adequate aerodynamic properties, drug release, biodegradation and evasion of macrophage uptake is a major challenge for controlled release pulmonary drug delivery. In this study, PEG graft copolymerized onto N-phthaloyl chitosan (NPHCs) was synthesized then characterized using FTIR, EA, DSC and 2D-XRD. The resulting PEG-g-NPHCs copolymers were self-assembled into drug-loaded nanoparticles and encapsulated in respirable/swellable sodium alginate semi-IPN hydrogel microspheres as novel biodegradable carriers for controlled release pulmonary drug delivery. The developed nano-/microspheres carrier systems were formed via spray drying followed by ionotropic crosslinking in mild aqueous medium. The size of the developed self-assembled nanoparticles and the microspheres was measured using dynamic light scattering and laser diffraction, respectively. Morphology, moisture content, *in vitro* biodegradation and dynamic swelling studies were also investigated for the developed carriers. A model protein was entrapped and the *in vitro* release profiles were determined in PBS, pH 7.4 at 37 °C. A dry powder aerosolization study was conducted using a Next Generation Impactor (NGI). The developed microspheres had suitable aerodynamic diameters (1.02–2.63 μm) and an excellent fine particle fraction, FPF of 31.52%. The microspheres showed also a very fast initial swelling within the first 2 min and started to enzymatically degrade within the first 2 h. Moreover, the microspheres entrapped up 90% of the model drug and showed promising *in vitro* sustained release profiles as compared to the control formulation.

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## 1. Introduction

One of the major shortcomings in pulmonary drug delivery is the present inability to control the pharmacokinetics of inhaled therapeutics beyond a few hours. To date, there are no effective sustained release pulmonary drug delivery systems available due to the efficient clearance of therapeutics from the deep lung either through phagocytosis in the alveolar region or via the rapid absorption of delivered therapeutics (Smyth and Hickey, 2005; Grenha et al., 2007). Therefore, development of an appropriate drug-loaded vehicle system with adequate aerodynamic characteristics that can confer sustained release of drug once deposited in the lung is considered one of the major challenges in inhalation therapy (Ahsan et al., 2002). In particular, the drug-loaded carrier particles, targeted to the deep lung, should be aerodynamically small enough to

pass through the mouth, throat and conducting airways and reach the deep lung, but not so small that they fail to deposit and are exhaled again (Grenha et al., 2007). The most appropriate range of aerodynamic diameters of these particles to be respirable is around 0.5–5 μm. However, microparticles of this aerodynamic respirable size range are also of a size that is cleared most rapidly from the lung by alveolar macrophages. Ahsan et al. and others (Ahsan et al., 2002; Makino et al., 2003; Martonen et al., 2005) have reported that increasing the size of microparticles tends to reduce their clearance by macrophages. However, increasing microparticle size is generally impractical for pulmonary drug delivery as this would minimize the respirable fraction that can be delivered. Larger low density particles can be delivered in this way but controlling drug release from these particles may be problematic. Therefore, in our earlier studies (El-Sherbiny et al., 2010a; El-Sherbiny and Smyth, 2010), a promising new strategy was suggested. This new strategy involves the developing of new swellable microparticles that have respirable aerodynamic sizes when dry but large geometric sizes when swollen after deposition in the moist lung. This enables the delivery systems to evade macrophage uptake and at same

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time confer sustained drug release through a controlled polymeric architecture.

Ultra small particles with geometric diameters less than a couple hundred nanometers (nanoparticles, NPs) have showed an obstinate residency in the lungs (Oberdorster, 2001). Once deposited, these NPs may stay in the lung lining fluid until their dissolution, evading phagocytosis and mucociliary clearance (Krenis and Strauss, 1961; Kawaguchi et al., 1986; Rudt and Muller, 1992). In addition, drug-loaded NPs embedded within hydrogels can allow for additional control of drug release (Cascone et al., 2002; Kim and Martin, 2006). Therefore, using NPs embedded in swellable and respirable microparticles may represent a promising approach for controlling drug action and release, not only in alveolar region where macrophage clearance occurs but also throughout the lumen of the lungs. NPs must be encapsulated or formulated into microparticles for pulmonary drug delivery due to their small aerodynamic size which, as discussed above, leads to lower deposition in the airways and their extensive exhalation from the lungs following inspiration (Heyder and Rudolf, 1984). In addition, NPs-based carrier systems may aggregate in both dry powder and liquid forms causing them to form microparticles suitable for rapid phagocytosis and clearance (Kabbaj and Phillips, 2001; Tsapis et al., 2002).

In this contribution, a new carrier system was developed for controlling pulmonary drug delivery and combines the benefits of both NPs and the respirable/swellable hydrogel microparticles suggested in our previous studies (El-Sherbiny et al., 2010a; El-Sherbiny and Smyth, 2010) while avoiding their drawbacks. The developed carrier system comprised of semi-interpenetrating polymeric network (semi-IPN) microspheres. This semi-IPN is based on spray-dried ionotropically crosslinked alginate microspheres containing homogeneously entrapped self-assembled NPs. The NPs were prepared from the N-phthaloyl chitosan (NPHCs) graft copolymerized with poly(ethylene glycol) (PEG).

Sodium alginate is a natural non-toxic biodegradable polyanionic copolymer. It consists of 1,4-linked  $\beta$ -D-mannuronic acid (M) and  $\alpha$ -L-guluronic acid (G) residues arranged either as consecutive blocks or in a random distribution. Alginate has a unique ability to form hydrogels via ionotropic crosslinking in presence of divalent cations such as calcium and barium ions (El-Sherbiny, 2010) and the hydrogels based on calcium-crosslinked alginate have been widely investigated for drug delivery purposes (Zahoor et al., 2005; Murata et al., 2009; El-Sherbiny, 2010; El-Sherbiny et al., 2010b).

Chitosan (Cs) is a cationic biopolymer obtained through alkaline N-deacetylation of chitin. Cs has numerous desirable characteristics including biodegradability, non-toxicity and biocompatibility (Majeti and Kumar, 2000). Also, Cs showed a good ability to improve drug absorption via protection of the drug against enzymatic degradation (Parka et al., 2006). Moreover, Cs was found to have a significant role in enhancing the drug absorption in lung tissues which was attributed to its effect in opening the intercellular tight junction of the lung epithelium (Parka et al., 2006). Graft copolymerization of various synthetic polymers onto Cs and its derivatives can further enhance their characteristics and consequently expands their potential applications. One of these synthetic polymers that commonly grafted onto Cs is poly(ethylene glycol) (PEG). PEG is a water soluble biocompatible and non-toxic polymer (Ohya et al., 2000). Owing to the characteristics of PEG, development of polymeric vehicles in form of micro- and nano- hydrogel particles based on PEG graft copolymerized onto either Cs or the N-phthaloyl derivative of Cs (NPHCs) have received recently a growing interest as novel carriers for drugs (Maoa et al., 2005; Opanasopit et al., 2006; Prego et al., 2006; Opanasopit et al., 2007; Yao et al., 2007; Zhang et al., 2008).

In this study, PEG-g-NPHCs was synthesized and characterized then self-assembled into NPs. The suspensions of these NPs in aqueous sodium alginate solutions were spray dried to produce

microspheres followed by ionotropic crosslinking in mild aqueous medium to form semi-IPN hydrogel microspheres. The resulting respirable/swellable microspheres were then evaluated as novel biodegradable carriers that can confer sustained release of drugs once deposited in the lung.

## 2. Materials and methods

### 2.1. Materials

Chitosan (medium MW, %N-deacetylation; about 76.4%, as determined by elemental analysis and FTIR spectroscopy), monomethoxy-poly(ethylene glycol) (m-PEG, Mn 5000 Da), 1-hydroxybenzotriazole (HOBt) and succinic anhydride were purchased from Aldrich (USA). 4-Dimethylaminopyridine (DMAP) was obtained from Sigma (St. Louis, MO, USA). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC.HCl) was provided by Fluka Chemical Corp. (Milwaukee, WI, USA). Sodium alginate (low viscosity; 250 cps for a 2% solution at 25 °C), triethyl amine, phthalic anhydride, dioxane and dimethyl formamide (DMF) were obtained from Sigma-Aldrich, SIAL (St. Louis, MO, USA). Phosphate buffer saline (PBS pH 7.4), absolute ethanol and all other reagents were of analytical grade and used as received.

### 2.2. Methods

#### 2.2.1. Preparation of PEG/NPHCs graft copolymer

The copolymer of PEG grafted onto NPHCs was prepared by a modified method reported in our earlier study (El-Sherbiny et al., 2010a) and described briefly as follow.

Firstly, monohydroxy-terminated monomethoxy-poly(ethylene glycol) macromer (m-PEG) was converted into carboxyl-capped precursor by reacting with succinic anhydride. Briefly, m-PEG (100 g, 20 mmol), succinic anhydride (2.4 g, 24 mmol), DMAP (2.44 g, 20 mmol) and triethylamine (2.02 g, 20 mmol) were dissolved in dry dioxane (350 ml). The reaction mixture was stirred at room temperature for 48 h under a dry nitrogen atmosphere. The dioxane was evaporated under vacuum and the residue was taken up in CCl<sub>4</sub>, filtered and precipitated by diethyl ether to produce a white powder of m-PEG-COOH (yield: 98%). Secondly, NPHCs was synthesized by reaction of 10 g Cs with 44.8 g phthalic anhydride (5 mol equivalent to pyranose rings) in presence of 200 ml of DMF at 130 °C under dry nitrogen atmosphere for 8 h. The reaction mixture was left to reach room temperature before pouring it onto ice-water. The precipitated product was collected by filtration, washed with methanol, and dried at 40 °C under vacuum to give a pale brown product (NPHCs). PEG-g-NPHCs copolymer was then prepared by stirring of 37.9 g of m-PEG-COOH with 5.0 g of dried NPHCs (0.4 mol equivalent to m-PEG-COOH) in 75 ml of DMF. After that, HOBt (3.4 g, 3 mol equivalent to m-PEG-COOH) was added as a catalyst and the reaction mixture was stirred at room temperature until the solution became clear. Subsequently, EDC.HCl (4.25 g, 3 mol equivalent to m-PEG-COOH) was added and the reaction was continued overnight under stirring at room temperature. The mixture was dialyzed against distilled water and washed with ethanol to remove impurities and unreacted macromere and to obtain the white product of PEG-g-NPHCs (5.47 g).

*m-PEG-COOH*: FTIR ( $\nu_{\max}$ , cm<sup>-1</sup>) 3496 (OH stretching), 2882 (C–H stretching), 1733 (C=O of carboxylic group), and 1102 (C–O–C stretching); EA, (C<sub>23</sub>H<sub>46</sub>O<sub>11</sub>). Anal. calculated (%): C, 54.35 and H, 9.02, found (%): C, 56.8 and H, 9.19.

*NPHCs*: FTIR ( $\nu_{\max}$ , cm<sup>-1</sup>) 3281 (OH stretching, NH bending and intermolecular H-bonding), 2961 (C–H stretching), 1775 and 1698 (C=O anhydride), 1395 (C=C, phthaloyl), 1058 (C–O, pyranose) and 732 (aromatic ring of phthaloyl); EA, found (%): C, 60.31; H, 4.83; and N, 4.92.

PEG-g-NPHCs: FTIR ( $\nu_{\max}$ ,  $\text{cm}^{-1}$ ) 3423 (OH stretching, NH bending and intermolecular H-bonding), 2879 (C–H stretching), 1736 (C=O ester and anhydride), 1703 (C=O anhydride), 1096 (C–O–C stretching) and 723 (aromatic ring of phthaloyl). EA, found (%): C, 56.16; H, 4.69; and N, 5.15.

### 2.2.2. Characterization

The elemental analysis for m-PEG-COOH, Cs, NPHCs and PEG-g-NPHCs was carried out using Costech ECS4010 elemental analyzer coupled to a Thermo-Finnigan Delta Plus isotope ratio mass spectrometer. FTIR characterization of the synthesized polymers was performed using Nicolet 6700 FTIR spectrometer and the differential scanning calorimetry (DSC) was carried out using DSC 2920 (Modulated DSC, TA Instruments) in a nitrogen atmosphere from  $-40$  to  $400^\circ\text{C}$  at a heating rate of  $10^\circ\text{C}/\text{min}$ . Samples of weight 10–12 mg were placed in aluminum sample pans and sealed. An empty aluminum pan of approximately equal weight was used as a reference. All peaks were determined and the areas were converted into enthalpy values. The crystallography patterns of powdered polymer samples were investigated by X-ray diffraction (XRD) using a Scintag Pad V diffractometer with Data-Scan 4 software (MDI, Inc.) for system automation and data collection. Cu K $\alpha$  radiation (40 kV, 35 mA) was used with a Bicorn Scintillation detector (with a pyrolytic graphite curved crystal monochromator). Data were analyzed with Jade Software (Ver 9.0 from MDI, Inc.). The samples were run between  $2\theta$  values of  $5^\circ$  and  $45^\circ$  at a continuous-scan data collection rate of  $0.5^\circ/\text{min}$ .

### 2.2.3. Preparation of the semi-IPN hydrogel microspheres

The semi-IPN hydrogel matrices were developed via spray drying of PEG-g-NPHCs NPs dispersed in sodium alginate solution followed by ionotropic gelation using divalent cations ( $\text{Ca}^{2+}$ ). In a typical procedure, homogenous solutions of the synthesized copolymer, PEG-g-NPHCs (1%, w/v) and sodium alginate (3%, w/v) were prepared using 0.06% acetic acid and distilled water as solvents, respectively. The self-assembled PEG-g-NPHCs NPs were prepared by sonication of the copolymer solution (1%, w/v) using a probe type sonicator (Misonix ultrasonic processor, S-4000, Misonix Inc., CT, USA) at 60 W for 2 min. Sonication step was repeated three times until the desired size was obtained. The sonication was performed in an ice-water bath with applying pulse function (pulse on 5 s and pulse off 5 s) in order to avoid the heat built-up of the polymer solution during the sonication. After that, a predetermined volume of PEG-g-NPHCs NPs suspension was added dropwise with homogenization (15,000 rpm) to a calculated volume of 3% aqueous alginate solution (Table 1). The polymer mixture was completed with distilled water until obtaining same final concentration (1.5%, w/v) in all the prepared formulations. Subsequently, the homogenized polymer mixtures were spray-dried with a 0.7 mm two-fluid pressurized atomizer at a feed rate of 25% (6 ml/min) in a Büchi Mini spray dryer B-290 (Büchi, Switzerland). The atomizing air flow rate was 500–600 NL/h. The inlet temperature was adjusted at  $125^\circ\text{C}$  and the outlet temperature was varying between 60 and  $65^\circ\text{C}$ . The obtained microspheres powder was collected and the spray drying yield (%) was calculated. The semi-IPN hydrogel microspheres were obtained via soaking a part of the collected powder in 10 ml of 0.1 M  $\text{CaCl}_2$  solution for 10 min with slow stirring. The crosslinked powder was filtered through 0.2  $\mu\text{m}$  filter paper followed by washing with 10 ml of distilled water, freeze dried and stored in dry conditions until further investigation.

### 2.2.4. Entrapment of a model drug and determination of entrapment efficiency

Semi-IPN hydrogel microspheres loaded with bovine serum albumin (BSA), as a model for protein drugs, were prepared in the

**Table 1**  
The composition of the developed semi-IPN hydrogel microspheres and some of their characteristics.

Sample code	Alg (W%)	PEG-g-NPHCs (W%)	Moisture content (%)		<sup>a</sup> E% $\pm$ SD	<sup>b</sup> VMD ( $\mu\text{m}$ )		<sup>c</sup> Span		Bulk density		<sup>d</sup> $d_a$	
			Plain	BSA-loaded		Plain	BSA-loaded	Plain	BSA-loaded	Plain	BSA-loaded	Plain	BSA-loaded
SIPN0	100	00	10.4 $\pm$ 1.7	7.5 $\pm$ 0.4	78.7 $\pm$ 2.8	2.2 $\pm$ 0.12	2.8 $\pm$ 0.21	2.9 $\pm$ 0.06	2.5 $\pm$ 0.07	0.22	0.71	1.0 $\pm$ 0.09	1.5 $\pm$ 0.13
SIPN1	80	20	10.2 $\pm$ 1.0	6.5 $\pm$ 0.4	90.3 $\pm$ 0.9	2.6 $\pm$ 0.10	4.0 $\pm$ 0.10	2.5 $\pm$ 0.03	1.7 $\pm$ 0.09	0.25	0.36	1.3 $\pm$ 0.12	2.4 $\pm$ 0.68
SIPN2	60	40	8.7 $\pm$ 1.3	5.8 $\pm$ 0.4	85.2 $\pm$ 1.9	2.2 $\pm$ 0.02	3.4 $\pm$ 0.03	2.4 $\pm$ 0.01	1.8 $\pm$ 0.11	0.30	0.31	1.2 $\pm$ 0.17	1.9 $\pm$ 0.51
SIPN3	50	50	8.3 $\pm$ 1.5	5.5 $\pm$ 0.3	49.6 $\pm$ 2.2	2.9 $\pm$ 0.08	4.4 $\pm$ 0.02	2.4 $\pm$ 0.02	1.8 $\pm$ 0.32	0.34	0.37	1.7 $\pm$ 0.06	2.6 $\pm$ 0.79

<sup>a</sup> E%: entrapment efficiency %.

<sup>b</sup> VMD: Volume median diameter, VMD.

<sup>c</sup> Span =  $(D_{90} - D_{10})/D_{50}$ .

<sup>d</sup>  $d_a$ : aerodynamic diameter.

same manner described in the previous section. Predetermined volumes of aqueous BSA solution (10%, w/v) were added, with stirring, to the PEG-g-NPHCs solution before sonication. The initial percentage of added BSA was 40% (w/w) based on the total weight of polymer mixture (PEG-g-NPHCs and alginate) before the spray drying. After collecting the spray-dried powder, a certain amount of it was soaked in 10 ml of 0.1 M CaCl<sub>2</sub> solution for 10 min with stirring then filtered through 0.2 µm filter paper, washed with 10 ml of distilled water and freeze dried.

Both the washings (10 ml) and the CaCl<sub>2</sub> solution (10 ml) were collected and filtered. The content of BSA was estimated using the Bradford assay (Bradford, 1976) from the absorption at λ<sub>max</sub> 595 nm with aid of UV–vis spectrophotometry (Infinite M 200 fluorophotometer, TECAN, USA). The difference between the amount of BSA initially added to the microspheres and that estimated in the 20 ml (washings plus CaCl<sub>2</sub>) was taken as a measure of the amount of BSA entrapped. The mean values for three replicate determinations ±SD were reported and the entrapment efficiency (E%) of BSA was calculated as follows:

$$E\% = \left( \frac{m_r}{m_i} \right) \times 100 \quad (1)$$

where  $m_i$  and  $m_r$  are the amounts (mg) of the BSA initially uploaded and remained in the microspheres respectively.

#### 2.2.5. Determination of particle size

The size of the developed PEG-g-NPHCs self-assembled NPs was estimated using Dynamic Light Scattering, DLS. Copolymer solutions of different concentrations (0.1%, 0.2% and 1%) were

$$\begin{aligned} \text{RF}(\%) &= \left( \frac{\text{Amount of drug – loaded particles with aerodynamic diameters} < 4.46 \mu\text{m}}{\text{Total amount of drug – loaded particles}} \right) \times 100 \\ \text{FPF}_{<4.46 \mu\text{m}}(\%) &= \left( \frac{\text{Amount of drug – loaded particles with aerodynamic diameters} < 4.46 \mu\text{m}}{\sum \text{amounts of drug – loaded particles collected from all NGI stages + throat + adapter}} \right) \times 100 \end{aligned} \quad (4)$$

sonicated three times for 2 min each at a power of 60 W. Then, the size of the resulting NPs was estimated using Wyatt Technology Corporation Dyna Pro-titan DLS. The size of the hydrogel microspheres and particle size distribution were determined using laser diffraction (SYMPATEC, Sympatec GmbH, System Partikel-Technik, Germany with a He–Ne laser beam 5 mW max at 632.8 nm) using WINDOX 5.1.2 software. The measurements were carried out in the range 0.5–175 µm for the suspension of the microspheres in acetone. Volume median diameters (VMD, µm) were calculated from the particle size distribution curves for both plain and drug-loaded microspheres. VMD is the particle diameter at which half the particles volume is contributed by particles larger than the VMD and half by particles smaller than the VMD. Moreover, the particle densities were approximated from tapped density experiments then the aerodynamic diameters of the microspheres were calculated using the following relationship:

$$d_a = d_g \left( \frac{\rho_p}{\rho_0 \chi} \right)^{0.5} \quad (2)$$

where,  $d_a$  is the microsphere aerodynamic diameter (µm),  $d_g$  is the geometric diameter (VMD, µm),  $\rho_p$  is the microspheres density (g/cm<sup>3</sup>),  $\rho_0$  is the standard density (1 g/cm<sup>3</sup>) and  $\chi$  is the dynamic shape factor ( $\chi = 1$ , assuming that the particles developed in this study are spherical).

#### 2.2.6. In vitro aerosolization study

An *in vitro* dry powder aerosolization study was carried out for the BSA-loaded SIPN2 microspheres, as example, using a Next Generation Impactor (NGI, MSP Corporation, MN, USA). In a typical procedure, a size 3CS capsule (Capsugel®, MA, USA) was packed

with 10–15 mg of hydrogel microspheres powder. The capsule was then placed in a handheld, dry-powder, breath-activated inhaler device (Handihaler®). The capsule was punctured prior to inhalation and a pump (Copley HCP5, Nottingham, UK) was actuated to simulate an inspiration (air flow rate of 60 ± 5% l/min for duration of 6 s). Three capsules were emptied, one in each run. The powder emitted from each capsule was deposited on different stages of the NGI. Each stage was containing a specific volume (5–15 ml) of sodium acetate buffer, pH 3.4 as a collecting solvent. Moreover, the powders deposited on the inhaler, capsule and the adapter were also collected in appropriate volumes of the collecting solvent. The amount of BSA-loaded-powder deposited on each stage was then determined by Bradford method (Bradford, 1976) with measuring the absorbance at λ<sub>max</sub> of 595 nm using Infinite M-200 fluorophotometer (TECAN, USA). The emitted fraction (EF%) that is corresponding the percent of total loaded powder mass exiting the capsule was determined gravimetrically and it can be expressed as follow:

$$\text{EF}(\%) = \left( \frac{m_{\text{full}} - m_{\text{empty}}}{m_{\text{powder}}} \right) \times 100 \quad (3)$$

where  $m_{\text{full}}$  and  $m_{\text{empty}}$  are the weights (mg) of the capsule before and after simulating the inhalation and  $m_{\text{powder}}$  is the initial weight (mg) of the powder in the capsule.

Also, the analysis of drug deposition in the device, capsule, adapter, throat and NGI stages allows determination of different deposition parameters (respirable fraction, RF% and the fine particle fraction, FPF%) according to the following relationships:

In addition, the mass median aerodynamic diameters (MMAD, µm) of the aerosolized hydrogel particles were estimated experimentally from analysis of drug deposition in the different NGI stages.

#### 2.2.7. Scanning electron microscopy analysis

The morphology of the developed drug-free and drug-loaded hydrogel microspheres was investigated by SEM (Hitachi S-800 field emission scanning electron microscope operated in secondary electron mode with a Robinson backscatter detector and with a Hitachi PCI system for digital image capture). Dry microspheres were mounted on aluminum stubs with double-sided conducting carbon tapes and coated with a 50/50 mixture of Au/Pd to minimize surface charging. The samples were scanned at an accelerating voltage of 20 kV.

#### 2.2.8. Determination of moisture content

The moisture percentages of the developed microspheres were determined using HR83 Halogen Moisture Analyzer (Mettler-Toledo GmbH, Switzerland). The samples weights were in the range of 120–200 mg and the applied drying temperature was 120 °C for 2 min. The moisture content was obtained as a weight loss (%) and calculated as the average ± SD from three independent measurements.

#### 2.2.9. Dynamic swelling study

The swelling behavior of drug (BSA)-loaded hydrogel microspheres in PBS, pH 7.4 was studied by determining the increase in both volume median diameter (VMD, µm) and medium diameter ( $X_{50}$ , µm) of the microspheres with time using laser diffractometer (SYMPATEC, Sympatec GmbH, System Partikel-Technik, Germany).



### 2.2.10. *In vitro* biodegradation study

A biodegradation study of the microspheres was carried out in presence of lysozyme. Typically, lysozyme was dissolved in PBS (pH 7.4) to prepare a solution with a concentration of 2 mg/ml (Hirano et al., 1989). Predetermined weights of microspheres (20–25 mg) were transferred to microcentrifuge tubes and incubated with 1.0 ml of lysozyme solution in a shaking (100 rpm) water bath at 37 °C for 1 h until the equilibrium swelling of these microspheres almost attained. Then, the samples were centrifuged for 3 min at a speed of 14,500 rpm and the supernatant was removed. The weights of the swollen microspheres ( $W_0$ ) were determined. Then 1 ml of fresh lysozyme solution was added to the swollen microspheres. At certain intervals, starting from determination of  $W_0$ , the steps of centrifugation and weighing were repeated and the final weights ( $W_t$ ) of microspheres at these intervals were determined. The percent weight remaining ( $W_r\%$ ) of the samples due to enzymatic degradation were determined according to the following equation:

$$W_r(\%) = 100 - \left( \frac{(W_0 - W_t)}{W_0} \times 100 \right) \quad (5)$$

where  $W_0$  is the weight of sample after 1 h swelling in lysozyme solution and  $W_t$  is the weight of the sample after incubation with lysozyme for a given time,  $t$ .

### 2.2.11. *In vitro* release studies

The *in vitro* release profiles of the uploaded BSA were determined by transferring a certain amount (40–50 mg) of BSA-loaded microspheres to a vial containing 2 ml of PBS, pH 7.4. Then, all samples were maintained at 37 °C in an incubator with shaking (100 rpm). At predetermined intervals, 100  $\mu$ l aliquot was withdrawn and analyzed by the Bradford method (Bradford, 1976) at  $\lambda_{\max}$  595 nm using a UV–vis spectrophotometry. The withdrawn sample was replaced with an equal volume of fresh buffer, to keep the volume of the release medium constant. It has been found prior the studies that no absorbance interference from the plain microspheres under the same conditions. The amounts of BSA released (mg) from hydrogel microspheres were then calculated using a standard curve of BSA in PBS, pH 7.4. The results were calculated in terms of cumulative release (% w/w) relative to the actual initially entrapped weight of BSA in the microspheres. The data points represent mean  $\pm$  SD from three independent experiments.

### 2.2.12. Statistical analysis

The results were analyzed and expressed as mean  $\pm$  SD. Effects of various parameters on the properties of the developed semi-IPN hydrogel microspheres were statistically analyzed by one-way

ANOVA using Excel (Microsoft Office 2007). Differences were considered significant at the level of  $p < 0.05$ .

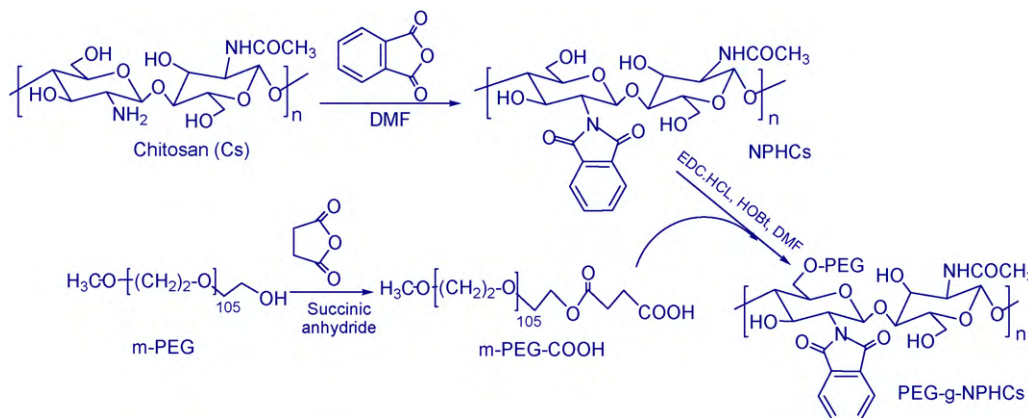
## 3. Results and discussion

### 3.1. Synthesis of PEG-g-NPHCs copolymers

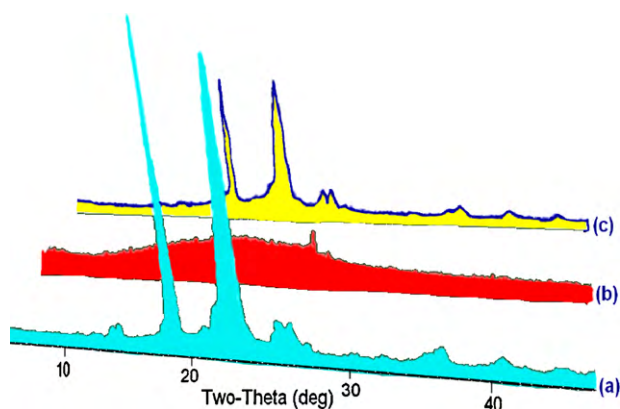
The copolymer of m-PEG grafted onto NPHCs was successfully synthesized through a modified method reported in our earlier study (El-Sherbiny et al., 2010a). The copolymer was prepared through a systematic sequence (Scheme 1) started with converting of m-PEG into carboxyl-capped m-PEG precursor (m-PEG-COOH) upon reaction with succinic anhydride. The prepared m-PEG-COOH was obtained in a very good yield then characterized using FTIR and EA. The free  $\text{NH}_2$  groups of Cs were masked via phthaloylation process upon reaction with phthalic anhydride in DMF to produce phthaloyl Cs (NPHCs). Synthesis of NPHCs was confirmed by FTIR through appearance of bands at 1395 and 732  $\text{cm}^{-1}$  which correspond to the “aromatic C=C” and “aromatic C–H” bonds of the phthaloyl groups, respectively. Then the PEG-g-NPHCs copolymer was synthesized upon reaction of m-PEG-COOH with NPHCs in DMF. The grafting % of PEG-g-NPHCs was found to be 9.3%. The obtained PEG-g-NPHCs copolymer was characterized by EA and FTIR. The copolymer synthesis was also confirmed by studying its thermal characteristics in comparison with that of starting materials, PEG-COOH and NPHCs. For instance, the DSC thermogram of PEG-COOH showed an endothermic peak at about 65 °C which corresponds to its melting process. This endothermic peak appeared also in the thermogram of the copolymer, PEG-g-NPHCs at 58 °C due to the melting of the grafted PEG side chains. The PEG-g-NPHCs showed also an exotherm at 231 °C which was ascribed to the decomposition of the graft copolymer. In addition, the XRD patterns of the prepared PEG-g-NPHCs, as compared to that of PEG-COOH and NPHCs (Fig. 1) represent another experimental proof on the synthesis of the copolymer. From the figure, the diffractogram of NPHCs reflects an amorphous nature of it with a broad hump over a range of approximately 18° and centered at  $2\theta$  value of 23°. In case of m-PEG-COOH and PEG-g-NPHCs, they showed development of very similar crystalline patterns with a pronounced greater peak intensity and indicative of more crystalline material in case of m-PEG-COOH.

### 3.2. Preparation of semi-IPN hydrogel microspheres

One of the most commonly used strategies to enhance the physicochemical characteristics of a polymer upon using it in preparation of a hydrogel is the incorporation of this polymer in an interpenetrating polymeric network (IPN). In these IPNs, the



Scheme 1. Synthesis of PEG-g-NPHCs copolymer.



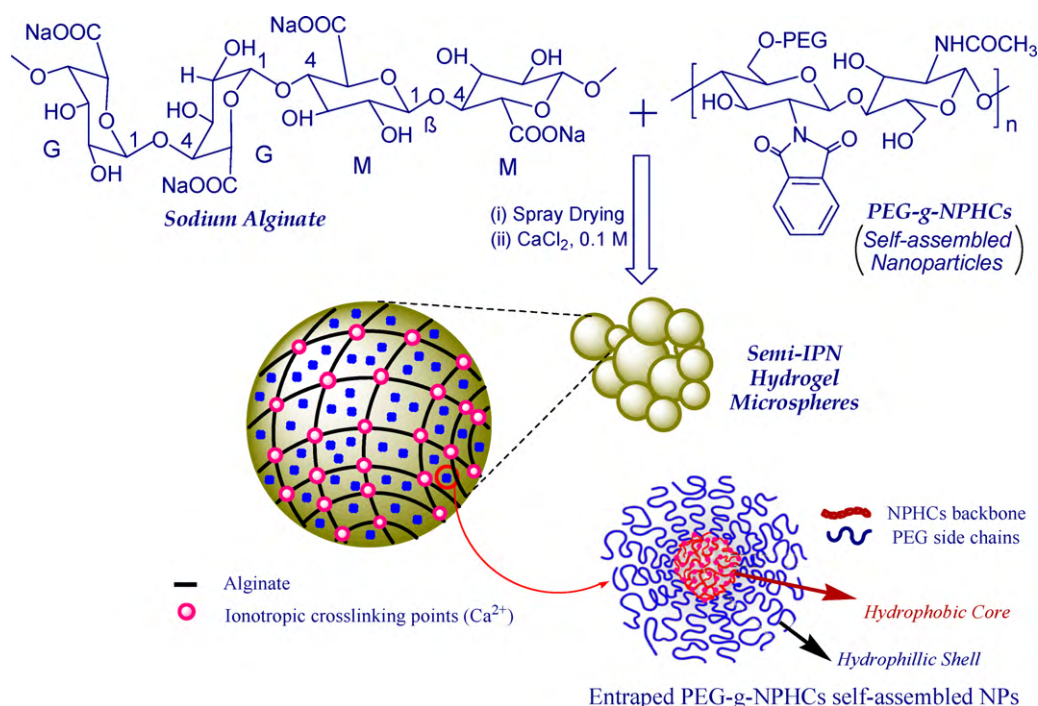
**Fig. 1.** A 3D overlay of the XRD patterns of (a) m-PEG-COOH, (b) NPHCs and (c) PEG-g-NPHCs copolymer.

polymer is incorporated with either hydrophilic polymer(s) or with hydrophilic monomer(s) treated to bring about in situ copolymerization in the presence of a suitable crosslinking agent. In general, an IPN can be defined as a combination of two or more chemically distinct polymers held together ideally and solely by their permanent mutual entanglements. The IPNs have two basic characteristics: first, one or more of the polymers must be synthesized and/or crosslinked, in the immediate presence of the other(s), and second, the combination produces an advanced multicomponent polymeric system, with new desired property profiles (Klempner, 1994; Kim et al., 2004). Generally, when only one polymer undergoes crosslinking, the resulting hydrogel matrix is called semi-IPN. In this study, the synthesized PEG-g-NPHCs was self-assembled into NPs. Then respirable/swellable semi-IPN hydrogel microspheres were developed via incorporating these self-assembled PEG-g-NPHCs NPs within a calcium-crosslinked spray-dried alginate matrix (Scheme 2). The main purpose was to investigate these nano-/microspheres biodegradable semi-IPN hydrogel matrices as new carrier systems for sustained pulmonary drug delivery

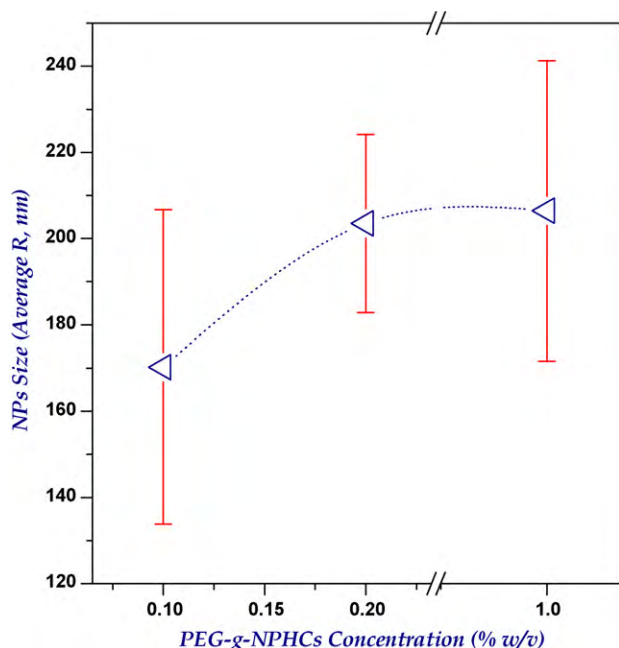
that combines the benefits of both NPs and the swellable hydrogel microspheres suggested in our previous studies (El-Sherbiny et al., 2010a; El-Sherbiny and Smyth, 2010) while avoiding their shortcomings. As shown in Table 1, three different formulations (SIPN1–SIPN3) were prepared in addition to a control formulation based on alginate alone (SIPN0).

### 3.3. Particle size

Designing of a suitable respirable drug carrier system with adequate aerodynamic characteristics represents a critical issue in inhalation therapy. These carriers should be small enough to reach the deep lung, but not so small that they fail to deposit and are exhaled again. The size of the developed PEG-g-NPHCs self-assembled NPs was found to be  $206.4 \pm 34.8$  nm (for 1% solution) as determined by DLS. A trial was also made to investigate the effect of PEG-g-NPHCs concentration onto size of the resulting NPs. It was found (as shown in Fig. 2) that, increasing the concentration of copolymer solution from 0.1% to 0.2% has increased the NPs size from  $170.0 \pm 36.5$  nm to  $203.5 \pm 20.7$  nm, respectively. This increase in size continued upon further increasing the concentration to 1% ( $206.4 \pm 34.8$  nm). Although Fig. 2 appears to indicate that there is little or no differences between the mean sizes, statistical analysis of the data ( $n = 114$  separate measurements at each concentration) indicates significant differences ( $p = 1.93E-18$ ). In case of microparticles, there are different types of diameters depending on the method of their determination. Volume median diameter, VMD is a commonly determined parameter when employing laser diffraction technique and it represents the cumulative diameter of 50% of microparticles and it can provide an appropriate estimation of particle size for dry-powder inhalation purposes. From the size data in Table 1, the VMDs of the developed plain and BSA-loaded microspheres are in the range  $2.2 \pm 0.12$  to  $2.9 \pm 0.08$  and  $2.8 \pm 0.21$  to  $4.4 \pm 0.02$   $\mu\text{m}$ , respectively. It was noted that uploading BSA in the developed microspheres increases their sizes significantly. It seems also from the obtained results that increasing the percentage of alginate in the microspheres tends, mostly, to decrease the size. This can be attributed to increasing the crosslinking extent.

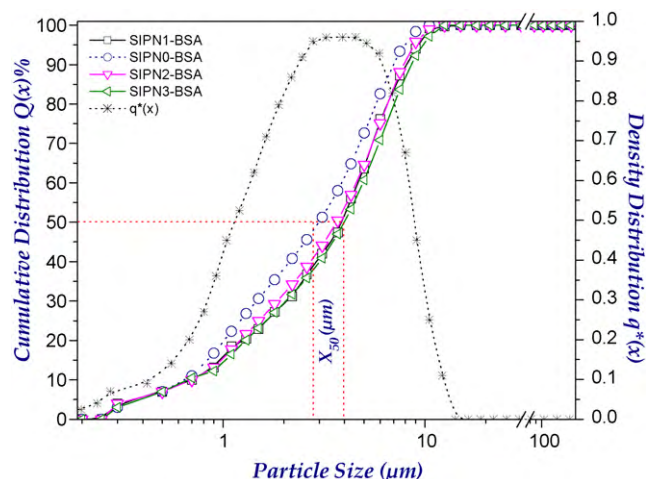


**Scheme 2.** A schematic illustration for preparation of the semi-IPN hydrogel microspheres.



**Fig. 2.** Dependence of the size ( $R$ , nm) of the synthesized self-assembled PEG-g-NPHCs nanoparticles on the concentration of copolymer solution (%), as determined by DLS ( $n = 114$ ).

However, this effect of alginate content onto particle size was found to be statistically insignificant. Moreover, as both aerosolization performance and lung deposition efficiencies are determined by aerodynamic diameters ( $d_a$ ), the densities of the developed microspheres were also determined because aerodynamic diameters are related to both density and geometric diameters (VMD) as described earlier in Eq. (2). As apparent in Table 1, the bulk densities of the microspheres are relatively low (in the range 0.22–0.34 and 0.31–0.71 g/cm<sup>3</sup> for plain and BSA-loaded microspheres, respectively). Therefore, the aerodynamic diameters of the microspheres are likely to be significantly less than the geometric diameters (in the range  $1.0 \pm 0.09$  to  $1.7 \pm 0.06$  and  $1.5 \pm 0.13$  to  $2.6 \pm 0.79$   $\mu\text{m}$  for plain and BSA-loaded microspheres, respectively). This explains the relatively high respirability (high RF values) of the developed microspheres as demonstrated in the aerosol dispersion study (Section 3.9). The cumulative size and density distributions of the developed semi-IPN microspheres are shown in Fig. 3. The mass median aerodynamic diameters (MMAD) of the developed microparticles were also determined experimentally and found to be in the range 5.2–6  $\mu\text{m}$ . These values are significantly higher than

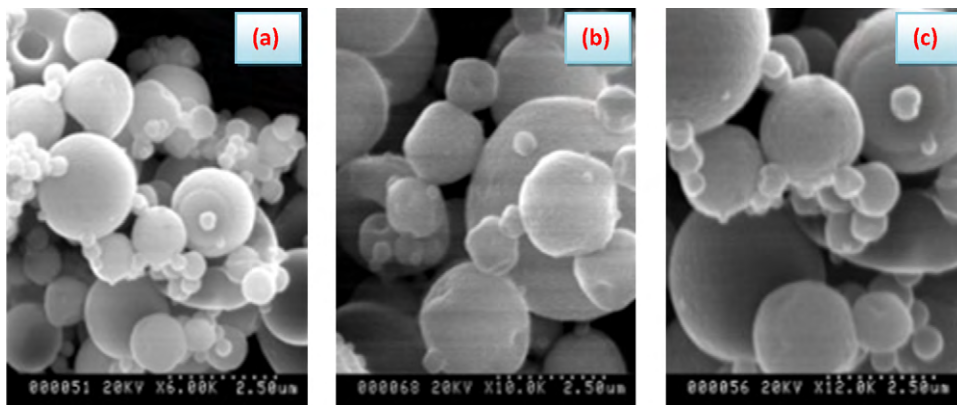


**Fig. 3.** The cumulative size and density distributions of the developed semi-IPN microspheres.

those calculated with aid of the laser diffraction analysis of the primary particles using tapped density. These deviations likely arise from strong aggregation states of micronized powders that are very commonly observed for dry-powder inhaler formulations. Despite the low density of the particles, particle-particle cohesive interactions can still exceed the dispersion forces. In future studies we will assess the optimization of the DPI formulation using interactive blends of the developed microparticles with standard excipients such as lactose to improve particle deaggregation and dispersion performance.

#### 3.4. Scanning electron microscopy

Fig. 4 shows the scanning electron micrographs of some plain and BSA-loaded semi-IPN hydrogel microspheres. The developed hydrogel particles have, in general, spherical shapes with different extents of regularity and surface smoothness. For instance, the drug-free spray-dried microspheres (Fig. 4a) showed a regular spherical nature with highly smooth, dense and integrated surfaces. This regular spheres turned to show a relatively distorted spheres with a kind of surface roughness upon crosslinking of these drug-free spray-dried microspheres with  $\text{Ca}^{2+}$  (Fig. 4b). In case of the BSA-loaded  $\text{Ca}^{2+}$ -crosslinked spray-dried microspheres (Fig. 4c), the shape seems more regular than the BSA-free  $\text{Ca}^{2+}$ -crosslinked spray-dried microspheres and their surfaces appear also relatively smoother and integrated.



**Fig. 4.** Surface morphology of the developed semi-IPN hydrogel microspheres: (a) drug-free spray-dried microspheres; (b) drug-free  $\text{Ca}^{2+}$ -crosslinked spray-dried microspheres and (c) BSA-loaded  $\text{Ca}^{2+}$ -crosslinked spray-dried microspheres.



### 3.5. Moisture contents

Table 1 illustrates the moisture contents (%) of the plain and BSA-loaded hydrogel microspheres developed in this study. As noted from the results, the moisture percentages fall in the ranges of  $(8.3 \pm 1.5$  to  $10.4 \pm 1.7)$  and  $(5.5 \pm 0.3$  to  $7.5 \pm 0.4)$  for plain and BSA-loaded microspheres, respectively. From the table, plain microspheres tend to have higher percentages of moisture as compared to BSA-loaded microspheres. Although the differences in moisture contents of different formulations are not statistically significant, it seems that moisture percentages decrease with increasing the PEG-g-NPHCs content in the microspheres.

### 3.6. Dynamic swelling study

The swelling patterns of the BSA-loaded hydrogel microspheres in PBS, pH 7.4 are shown in Fig. 5. Swelling behavior was estimated via determining the increase in both volume median diameter (VMD,  $\mu\text{m}$ ) and medium diameters ( $X_{50}$ ,  $\mu\text{m}$ ) of the microspheres with time using laser diffractometer. As appeared from the figure,

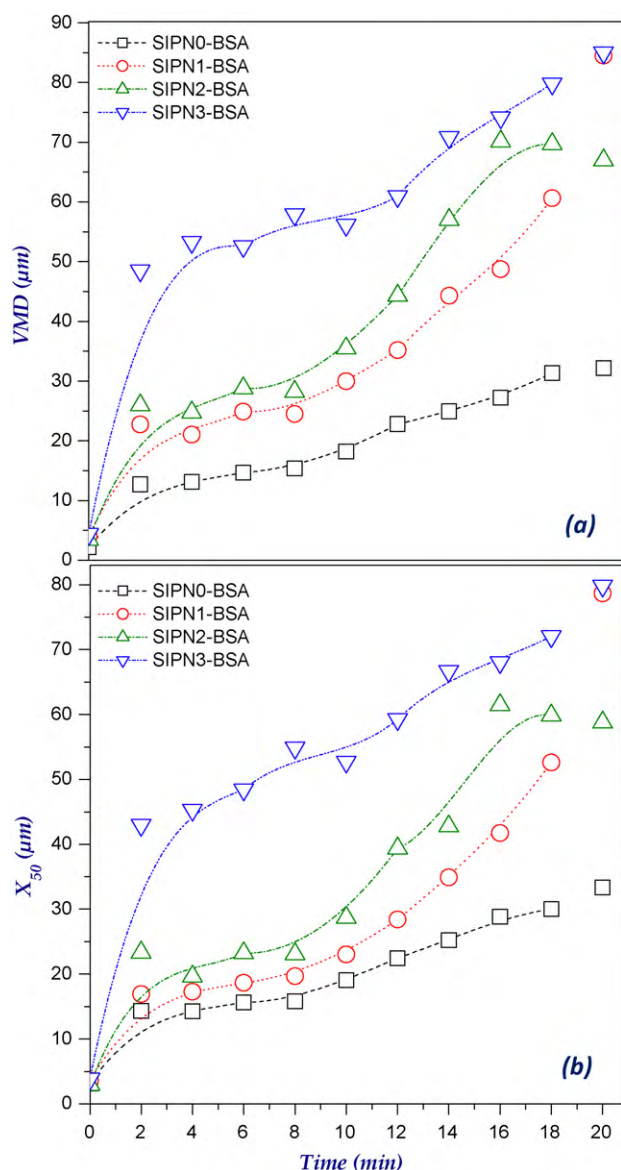


Fig. 5. Volume median diameter (VMD,  $\mu\text{m}$ ) and medium diameter ( $X_{50}$ ,  $\mu\text{m}$ ) of swelled BSA-loaded semi-IPN hydrogel microspheres.

the developed formulations showed a fast initial swelling within the first 2 min. For instance, the VMD of SIPN1, SIPN2 and SIPN3 microspheres has increased from about  $4 \mu\text{m}$  when dry to 23.6, 26.0 and  $48.1 \mu\text{m}$ , respectively. This swelling continues regularly with time to reach 84.1, 66.3 and  $84.2 \mu\text{m}$  at 20 min for SIPN1, SIPN2 and SIPN3, respectively. It can be noted also from Fig. 5 that, increasing the percent of alginate upon moving from SIPN3 (50% alginate) to SIPN2 (60% alginate) and SIPN1 (80% alginate) tends to decrease the swelled sizes of the microspheres. This behavior may be attributed to increasing the crosslinking extent of the semi-IPN hydrogel microspheres upon increasing the alginate percent due to the interaction with  $\text{CaCl}_2$ . This speculation can be confirmed also by comparing the swelling profiles of the microspheres formulations SIPN1–SIPN3 with that of the control, SIPN0 (100% alginate) which attained only a VMD value of  $31.7 \mu\text{m}$  after 20 min. The same conclusion was also obtained from the  $X_{50}$  ( $\mu\text{m}$ ) values of swelled microspheres (Fig. 5b).

### 3.7. In vitro biodegradation study

The *in vitro* enzymatic degradation study of the developed microspheres was carried out in PBS, pH 7.4 in presence of 2 mg/ml lysozyme as has been the protocol in previous studies (Hirano et al., 1989). In this study, the percent weight remaining of the microspheres as a function of time was determined and taken as a measure of degradation. Fig. 6 shows the enzymatic degradation profiles of the developed microspheres. As shown in the figure, degradation of the three prepared formulations was started to occur within the first 2 h. It seems also that, in general, increasing the percent of PEG-g-NPHCs copolymer in the microspheres tends to reduce the degradation rate (high remaining weights). For instance, at 6 h, the remaining weights of SIPN1 (20% PEG-g-NPHCs), SIPN2 (40% PEG-g-NPHCs) and SIPN3 (50% PEG-g-NPHCs) are  $65.9 \pm 2.5$ ,  $74.3 \pm 0.2$  and  $82.4 \pm 3.3$ , respectively. This effect of copolymer percent became relatively non significant beyond 144 h. The retarding effect of the PEG-g-NPHCs for the enzymatic degradation of microspheres is in agreement with the results obtained by our group in an earlier study (El-Sherbiny et al., 2010a) with other formulations containing PEG-g-NPHCs. This influence of PEG-g-NPHCs can be attributed to both hydrophobicity and the steric hindrance arises from their bulky phthaloyl groups. These two effects tend to impede the diffusion of enzyme through the microspheres matrices and consequently retard their degradation. The

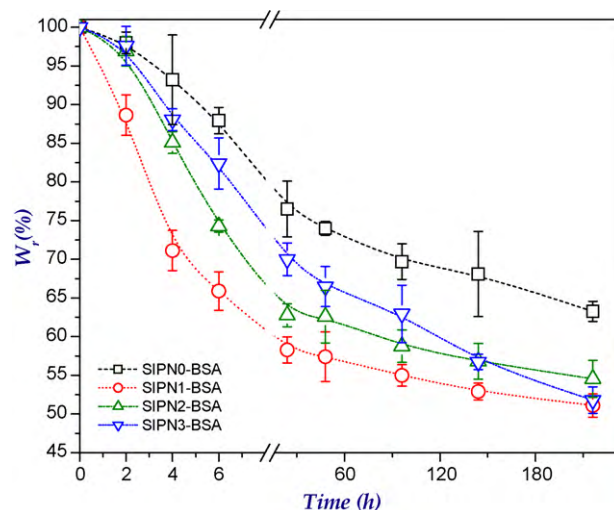
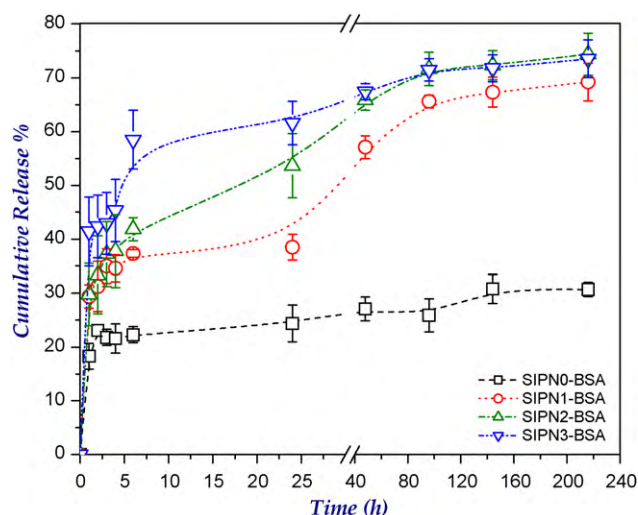


Fig. 6. *In vitro* degradation profiles of the developed semi-IPN hydrogel microspheres as investigated in PBS, pH 7.4 at  $37^\circ\text{C}$  in presence of lysozyme.





**Fig. 7.** The cumulative release profiles of the model protein, BSA from the developed semi-IPN hydrogel microspheres in PBS, pH 7.4 at 37 °C.

SIPN1–SIPN3 microspheres showed better biodegradation rates as compared to that of the control formulation, SIPN0 which is attributed to the high crosslinking extent in SIPN0.

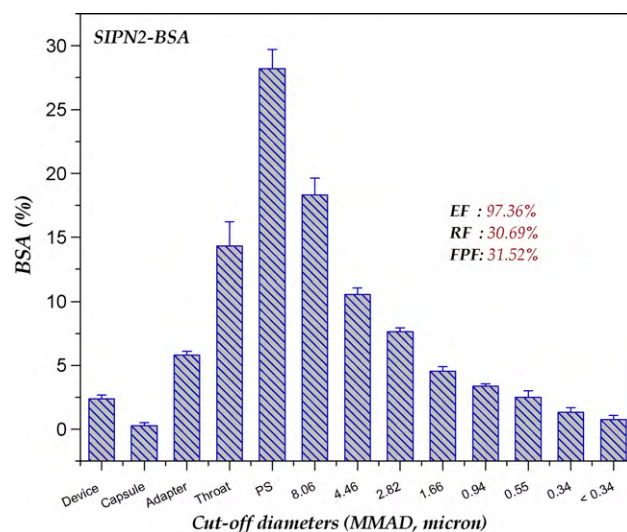
### 3.8. *In vitro* cumulative release studies

The entrapment efficiency (E%) of the model protein, BSA in the developed hydrogel microspheres were estimated as shown in Table 1. As noted from the table, the E% values are in the range  $49.6 \pm 2.2$  to  $90.3 \pm 0.9$ . The partial loss of the entrapped drug takes place particularly during both the crosslinking in  $\text{CaCl}_2$  solution and washing of the microspheres after crosslinking. Moreover, it seems that the E% tends to decrease, in most cases, with decreasing the alginate content in the developed microspheres. Decreasing the alginate content leads to a reduction in the extent of ionic crosslinking and consequently facilitates loss of more drug during washing.

The cumulative release profiles of BSA from the developed hydrogel microspheres in PBS, pH 7.4 at 37 °C is shown in Fig. 7. As apparent from the figure, the SIPN1–SIPN3 microspheres showed enhanced release profiles of BSA than that of the control formulation (SIPN0). The BSA release from the microspheres showed three different phases. Initially, there was a burst release within the first 2 h. This burst release can be attributed to the fast initial swelling of the hydrogel microspheres (see Fig. 5). Then, the microspheres showed a slow sustained release of BSA up to 4 days. The drug released in this stage may be the drug that is physically entrapped in the microspheres. In the final phase, a slower release was observed which may be attributed to the release from the NPs upon starting degradation of the microspheres (see Fig. 6). However this BSA release observed in the last phase is not a quantitative reflection of the extent of biodegradation. Moreover, the figure shows that increasing the alginate content in the microspheres tends to decrease the percentage of BSA released which can be attributed to increasing the crosslinking extent and consequently decreasing the swelling (Fig. 5).

### 3.9. *In vitro* aerosolization study

The powder aerosolization characteristics of the formulation SIPN2-BSA, as an example for the semi-IPN hydrogel microspheres developed in this study, were assessed *in vitro* using a dry-powder, breath-activated inhaler device (Handihaler® DPI) with aid of NGI at air flow rate of  $60 \pm 5\%$  l/min. The percentage of drug deposition



**Fig. 8.** Dry-powder aerosolization study using NGI with a flow rate; 60 l/min and using a Handihaler® as inhaler device.

on each stage of the NGI, adapter, capsule and the device was calculated and plotted as in Fig. 8. The average emitted fraction, EF (%) of the investigated powder (SIPN2-BSA) was found to be relatively high (97.36%). It was found also that, for the formulation SIPN2-BSA, the average respirable fraction, RF (%) of the powder is 30.69% and its fine particle fraction, FPF% (cut-off diameters,  $d_a < 4.46 \mu\text{m}$ ) is 31.52%. The FPF value represents the fraction of the dry powder that would likely deposit in the deep lung. Although this value of FPF% is not exceptional, it does exceed or match almost all marketed dry-powder inhaler systems currently available. In addition, the investigated powders were not formulated in anyway. Further optimization of the aerosol formulation via creating an interactive mixture with carrier particles such as lactose as is standard with most DPI systems is expected to enhance the aerosolization performance. In conclusion, the data obtained from this preliminary powder aerosolization study implies that the semi-IPN hydrogel microspheres developed in this contribution could be suitable for inhalation and drug delivery to the deep lung.

## 4. Conclusion

In this contribution, PEG-g-NPHCs self-assembled nanoparticles were synthesized then used in a combination with sodium alginate to prepare respirable semi-IPN hydrogel microspheres as novel biodegradable carriers for pulmonary sustained drug delivery. The developed nano-/microspheres carrier systems were obtained via spray drying technique followed by ionic crosslinking in mild aqueous medium. The carrier systems were designed in such a way to display appropriate morphological and aerodynamic characteristics. The preliminary *in vitro* evaluation of these carriers showed that they can be used as good potential carriers for pulmonary sustained drug delivery.

### Conflict of interest

The authors report no declarations of interest.

### Acknowledgements

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