European Journal of Pharmaceutics and Biopharmaceutics 71 (2009) 231–242

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

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

Research paper

An ion pairing approach to increase the loading of hydrophilic and lipophilic drugs into PEGylated PLGA nanoparticles

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ARTICLE INFO

Article history:
Received 25 April 2008
26 July 2008
Accepted in revised form 1 August 2008
Available online 17 August 2008

Keywords:
s/o/w
Ion pairing
Microencapsulation
PEGylation
Nanoparticles

ABSTRACT

The aim of this study was to enhance the loading of dalargin (enkephalin derivatives) a hydrophilic drug and loperamide HCl (non-opiate antidiarrheal agent) a lipophilic drug candidates within PEGylated nanoparticles. A novel nanoencapsulation method based on the concept of s/o/w and ion pairing followed by solvent diffusion was adopted. The copolymers with three different mPEG densities (5%, 12% and 17%) were employed separately in combination with two different grades of dextran sulphate (DS) 5000 and 500,000 MW in the preparations. Nanoparticles prepared from copolymers with increasing mPEG densities, showed an insignificant ($p > 0.05$) increasing trend of drug loading, this was however significantly increased when DS5000 was included in the preparations. The particle size remains unchanged after dalargin loading, with no significant ($p > 0.05$) alteration in the neutral zeta potential compared to that of the preparations without DS5000. Considering that a dalargin ion pair could also have a neutral charge, it was not advisable to conclude its incorporation, as the size remain unchanged, which would otherwise increase if an ion pair was incorporated within the core of nanoparticles. Therefore, it was expected that a dalargin ion pair might be located outside the core as a separate particulate entity or reside in the hydrophilic shell of the nanoparticles. A loperamide HCl ion pair showed significant ($p < 0.05$) increase in size when incorporated; at the same time it provided a neutral zeta potential despite adding negatively charged DS5000 in the preparation, hence it seemed encapsulated. Inclusion of DS500,000 in the preparation further increased the drug loading of dalargin and loperamide HCl. However, a significant ($p < 0.05$) negative zeta potential was noted in both cases which suggested that excess charge was still available on the surface of nanoparticles which could trap further amounts of drug on the surface rather than inside the core of nanoparticles. During in vitro evaluation of drug loaded nanoparticles, dalargin released as quickly as free drug, when loperamide HCl showed almost burst free sustained release profile with respect to the release of their free drug solutions, suggested that ion pairing approach was more pronounced for loperamide HCl formulation.

1. Introduction

Biodegradable polymers based on lactide and glycolide monomers have been widely utilised to prepare nanoparticles, however their lipophilic surface is vulnerable for adsorbing serum components (opsonin factors), that induces their rapid clearance from the blood circulation by the cells of the mononuclear phagocytic system (MPS) within the liver and spleen. This affects the in vivo performance, and eventually resulting in very short half life [1]. Such a limitation was also faced by first generation liposomes [2], which was overcome by introducing poly(ethylene glycols) (PEGs) modified lipids to obtain sterically stabilised liposomes, which increased the in-vivo half life by providing a "stealth" effect [3]. Similarly, to overcome such a limitation, polymers were further modified by synthesizing a block copolymer with the PEGs. Nanoparticles generated from such amphiphilic copolymers were expected to provide a similar in vivo advantage over nanoparticles synthesized from a nonPEGylated copolymer that is relatively lipophilic. "Stealth" nanoparticles can be prepared from amphiphilic PEGylated copolymers using common techniques (o/w, w/o/w) [4,5] which are capable of providing a core–shell structure, in which, a hydrophilic PEG layer remains as a shell and a hydrophobic PLGA remains as a core. The existence of PEG imparts greater hydrophilicity to the polymer that makes hydrophilic drug loading difficult. So far many lipophilic drugs have been encapsulated easily into PEGylated PLGA based nanoparticles compared to that of hydrophilic drugs. Only large size hydrophilic molecules (like Bovine Serum Albumin, BSA) have been frequently reported to be encapsulated within PEGylated PLGA based nanoparticles.

Various approaches have been reported to encapsulate a variety of drugs into a PLGA based matrix. The diffusion of neurotensine

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doi:10.1016/j.ejpb.2008.08.004

hexapeptide was retarded by using fatty acid molecules that formed an ion pair with the peptide during encapsulation process. This resulted in the formation of a lipophilic salt of the peptide that favoured partitioning of the drug into the lipophilic core during the encapsulation [6]. The presence of small amounts of negatively charged lipids (dipalmitoyl phosphatidylglycerol or diacetyl phosphate) into the oily phase (acetone), also increased the loading of nafareline acetate (a cationic peptide) by decreasing its diffusion into the external phase [7].

The use of low molecular weight PLGA (oligomers) in the preparation of nanoparticles by solvent evaporation, followed by diffusion (o/w), also enhanced the encapsulation of cationic water soluble peptides by formation of an ion pair by electrostatic attraction between terminal carboxylate and terminal amino groups of the water soluble cationic peptide. This reduced its diffusion into the continuous phase and provided increased drug loading [7].

Besides the use of traditional o/w and w/o/w techniques, relatively novel super critical fluid technology has been also evaluated to obtain drug loaded nano-microparticles from PLGA based copolymers. This also adopted a hydrophobic ion pairing approach to increase the drug loading of a lipophilic cationic drug naltrexone [8,9].

Alternatively, s/o/w (solid/oil/water) techniques have been exploited with PLGA based microspheres, especially for a higher drug loading of large water soluble peptides like insulin [10]. This is a two step solvent evaporation technique, in the first the active peptide is dispersed in an organic phase along with solubilised copolymer or polymer (s/o). This can be achieved by an antisolvent effect of the organic phase on the water soluble drug candidate (by dissolving the peptide in a minimum of water or aqueous solvent that precipitates out when the drug diffuses into an organic phase, s/o). This s/o phase is then added to the aqueous phase containing surfactant (s/o/w) for emulsification followed by evaporation of the solvent. Such an approach has been successfully applied to human insulin, bovine superoxide dimustase (bSOD) and recombinant human growth hormone (rhGH) [11–14].

There are difficulties when a small water soluble peptide is to be considered, since it could potentially diffuse into the external phase (w) due to its high solubility. In the current study, both approaches of ion pairing and (s/o/w) are combined in one method to evaluate drug loading of the water soluble cationic hexapeptide dalargin (an enkephalin derivative). Dextran sulphate (DS) was used as an ion pair to reduce the solubility by coacervating the drug molecules that diffuse slowly and allow the entrapment of the coacervate on instantaneous precipitation of the copolymer. Such an approach has been tried on dalargin (a hydrophilic drug – enkephalin derivative) and loperamide HCl (a biphenyl piperidine derivative), a lipophilic drug; that stimulates opioid receptors in vitro, but posses no in vivo analgesic effect because of its poor penetration through the BBB [15–17]. However, it is always of benefit to achieve a higher drug loading to minimize the amount of excipient to be delivered per unit dose; this simply could minimize the toxicity related to nanoparticle material.

In addition, use of copolymers with increasing PEG density at a constant lactide and glycolide monomer ratio was explored with ion pairing concept. The impact of copolymer composition on the physico-chemical parameters (size and zeta potential) of drug loaded nanoparticles were monitored and their in vitro release performance was evaluated to reveal the reliability of such a drug loading approach to obtain sustain release profiles.

2. Materials and methods

2.1. Material

Polyvinyl alcohol (PVA) (80–89% hydrolysed, MW 9000-10,000, Sigma Aldrich, USA), Nanosep™ (Omega™, 300 K MWCO, Lot No # OD 300C34) purchased from Pall Scientific USA. Dalargin (>95% pure, batch no # P20645, Auspep Ltd., Australia), Loperamide HCl (99% pure, Lot # 103K0611, Sigma–Aldrich, USA), Polyethylene glycol (monomethyl ether) (99% pure, Lot # 44931/1, Fluka, Germany). Glycolide monomer (Purasorb^{G}, 99.9% pure, Lot # 0305000324), D,L-Lactide monomer (Purasorb^{D}, 99.9% pure Lot # 0310000164) purchased from PURAC International, Holland. Stannous octoate (95% pure, Lot # 102K0104, Sigma–Aldrich, USA). Dextran Sulphate MW 5000, Lot # K30054789 O and MW 500,000 Lot # K70050987 M were purchased from Sigma–Aldrich, USA. Ultra pure water (<6 μS) prepared from a Milli-Q purification system was used in all experiments.

2.2. Synthesis of the PLGA copolymer with varying density of mPEG

The synthesis of monomethoxy poly (ethylene glycol) – poly (D,L-lactide-co-glycolide) (mPEG-PLGA) co-polymer is well described [18] and explained [19]. Briefly, 16 g of D,L-lactide, 4 g of glycolide and 4 g of monomethoxy poly (ethylene glycol) (mPEG) was heated at 140 °C under an inert atmosphere (nitrogen) for 6 h with 0.085 g of stannous octoate as a catalyst. The molten polymer matrix was cooled using a cold water bath for 30 min. The matrix was dissolved in a minimum amount of dichloromethane (DCM) and precipitated in excess methanol. The supernatant was discarded and precipitates were redissolved in DCM and treated for second and third precipitations in a similar manner. The final precipitation was dissolved in a minimum amount of dichloromethane and dried as a film at room temperature under vacuum for 48 h. Three copolymers with increasing mPEG composition from 5%, 12% to 17% at constant weight ratio of D,L-lactide and glycolide monomer ratio were synthesized using the above methodology. These copolymers were characterised for glass transistant temperatures using modulated thermal scanning calorimetry (mDSC). The MW weight was determined using solvent based GPC. The identity was performed using Fourier Transformed Infrared spectroscopy (FTIR) and the proportions of monomers were decided from H^1 NMR. The detail procedures for characterisation are described in [20].

2.3. Preparation and characterisation of nanoparticles

2.3.1. Preparation of drug loaded nanoparticles

In the case of dalargin loaded nanoparticles, 0.3 ml of 40 mg/ml dalargin solution in water was added to 10 ml of acetone solution containing 200 mg copolymer, to which a further 0.3 ml of 80 mg/ml dextran sulphate (MW 5000) solution was added. This organic phase was added drop wise to 60 ml of 0.6% w/v aqueous solution of PVA and stirred at ambient condition for 7.5 h.

For loperamide HCl nanoparticle preparation, 200 mg of mPEG-PLGA co-polymer was dissolved in 6 ml of 2 mg/ml loperamide HCl solution in acetone, a further 4 ml of acetone was added for solubilisation followed by the addition of 0.025 ml of DCM. To this organic phase 0.3 ml of 80 mg/ml solution of dextran sulphate (MW 5000) was added. The entire organic phase was added drop wise to 60 ml of 0.6% PVA solution. The solution was stirred for 7.5 h at ambient conditions.

The above two methodologies were adopted for the all three copolymers containing increasing (5–17%) mPEG content. Finally, the copolymer with 17% mPEG was selected further for optimisation of drug loading. This was achieved by (1) increasing the amount of drug per preparation (2) by using a higher MW (500,000) of dextran sulphate in the preparation. The amounts of copolymer and dextran sulphate in preparations were kept constant through out the experiments.

The concentration of the drug (dalargin) was simply increased by doubling the strength of the dalargin solution to 80 mg/ml to keep a constant volume of the aliquots in the preparation.

In the case of loperamide HCl, the concentration was increased up to 24 mg per preparation by taking 12 ml of 2 mg/ml loperamide HCl dissolved in acetone solution.

To evaluate the impact of the dextran sulphate on the drug loading, a set without dextran sulphate was prepared by keeping the same concentration of drugs and copolymer.

Each of the above mentioned preparations were prepared from three weight fractions of a copolymer (each weight fraction equivalent to 200 mg), at the end of process, each was treated individually on three separate Nanosep™ device for drug loading determination as described in Section 2.4.

2.3.2. Characterisation of nanoparticles

Particle size measurements were performed by photon correlation spectroscopy (PCS) at 25 °C with a detection angle of 90° and zeta potential was determined by laser doppler anemometry using a Zeta Sizer 3000HS (Malvern, UK). Statistical comparisons of physical properties and loading parameters were made for all formulations by applying one way ANOVA followed by Scheff'e and Tukey test as well as a paired sample *t* test where appropriate.

2.4. Determination of the drug loading

Nanosep™ 300 K, an ultrafiltration centrifugal device fabricated with Omega™ membrane was used to determine the free drug concentration within the nanoparticle dispersion. A sample of 0.1 ml was added into the reservoir of the device, immediately after preparation of the nanoparticle batch, and spun for 20 min at 6000 rpm. After the centrifugation cycle, 0.1 ml of centrate was obtained, which was diluted with an equal volume of PBS buffer and dalargin contents were analyzed by HPLC as described previously [21]. The retentate was again filled with 0.1 ml of water and the cycle was repeated five times for dalargin and six times for loperamide HCl.

To check for the adsorption and the recovery of the drug onto the device membrane, 0.1 ml of the solublised portion of the drug was filled into the Nanosep™ device and spun under similar conditions followed by HPLC analysis of the centrate obtained after each cycle.

To evaluate the impact of the nanoparticle layer on the free drug removal from the device, a dispersion containing empty nanoparticles (devoid of drug) was prepared as described above, then 6 mg of dalargin was added to final nanoparticle dispersion to imitate existence of free drug. An aliquot of 0.1 ml was treated on Nanosep™ similarly as mentioned above, and recovery of the free drug considered in calculation.

Similarly, the removal of known amount of free loperamide HCl was assessed in presence of empty nanoparticles. This was done using a 0.1 ml of dispersion mix of empty nanoparticles and free loperamide HCl solution, which was placed in the Nanosep™ and treated similarly as mentioned above followed by HPLC analysis of loperamide HCl described in our previous work [21]. The mixture of empty nanoparticles and soluble drug fraction was prepared as described here. Briefly, double strength (400 mg copolymer in 60 ml of PVA solution) empty nanoparticles were prepared by excluding drug from the previous preparation. After preparation, 30 ml of nanoparticle suspension was mixed with 30 ml of 0.25 mg/ml loperamide HCl in 0.6% PVA solution. This provided an equivalent concentration to a single strength (200 mg copolymer in 60 ml) nanoparticle with almost 3.75 mg loperamide HCl in solublised form. This was done to imitate the existence of free drug in the nanoparticle dispersion.

A plot of % drug release from the Nanosep™ device vs. number of centrifugal cycles was prepared for all centrifugal treatments mentioned above. These plots were employed to estimate drug loading parameters. The percentage drug release difference between empty nanoparticle plus free drug mix and loaded nanoparticle samples provided % encapsulation efficiency. Using this, percentage loading was estimated as described previously [21].

2.5. Determination of the solvent (acetone) level during the preparation of nanoparticle

During the preparation of the nanoparticles, 0.1 ml of suspension was taken into the Nanosep™ 300 K, and spun at 6000 rpm for 20 min, centrate was diluted appropriately with water and injected (7683 Series) on to GC system (Agilent™ 6890 N) connected to an Innovax® capillary column (fumed silica) and flame ionisation detector (6890). Helium gas was used as an inert gas carrier at 1 ml/min flow rate. The acetone within the samples was analysed by increasing the temperature at 50 °C per 2 min (hold time) up to 250 °C. A standard acetone solution was used to quantify the unknown amount of solvent within the dispersion. At certain time interval, 0.1 ml of dispersion was analysed and the solvent remained was monitored over a period. Acetone adsorption to the membrane was determined after passing a standard solution of acetone through the Nanosep™ membrane and the resulting factor considered in the calculation. This experiment was performed in duplicate.

2.6. In vitro drug release evaluation from the flow through dialysis device

2.6.1. Preparation of the nanoparticle samples for in vitro release study

Considering the sensitivity of HPLC analytical method, it was decided to prepare more concentrated nanoparticles preparation for release study. For this, a 0.25 ml aliquot of 120 mg/ml dalargin solution in water was added to 10 ml of acetone solution containing 500 mg copolymer, to which further 0.3 ml of 200 mg/ml dextran sulphate solution was added. This organic phase was added drop wise to 30 ml 0.6% PVA solution and stirred at ambient conditions for 7.5 h. Free drug solution of dalargin of 1 mg/ml concentration was prepared using water, and 0.8 ml solution was used for the release study in comparison with the dalargin loaded nanoparticle sample.

Loperamide HCl loaded nanoparticles were prepared by dissolving 1 g of mPEG-PLGA co-polymer in 15 ml of 2 mg/ml loperamide HCl solution in acetone, followed by the addition of 0.025 ml of DCM. To this organic phase 0.3 ml of 200 mg/ml solution of dextran sulphate (MW 5000) was added. The entire organic phase was added drop wise to a 30 ml 0.6% PVA solution. The solution was stirred for 7.5 h at ambient conditions.

For free drug solution testing, loperamide HCl was allowed to saturate in 0.6% PVA at 37 °C over a period of one week. The clear solution was filtered through 0.22 μm GHP Acrodisc™ filter and measured for the concentration using HPLC. This provided 2.82 mg/ml concentration, an aliquot of 0.55 ml was taken and diluted to 0.8 ml with blank 0.6% PVA solution, finally providing approximately 1.0 mg of the loperamide HCl content for *in vitro* release testing.

The drug loaded nanoparticle samples mentioned above were evaluated for *in vitro* release in triplicates.

A continuous flow sink system was used where sample was filled carefully in a dialyser (Spectropore®, Float a lyser™, MWCO 50,000) and capped, then transferred in to a sealed sink compartment tube already filled with 15 ml sink buffer. The sink volume was maintained constant by replacing and collecting sink media at 0.4 ml/min rate via inlet–outlet tubing with aid of peristaltic pump. The release content collected from outlet tubing was as-

sessed by appropriate HPLC method developed in previous study [21].

3. Results and discussions

3.1. Synthesis of copolymer

It is well established that hydroxyl groups initiate the lactone polymerisation; generally, primary alcohols react very quickly with D,L-Lactide and glycolide monomers to induce the ring opening polymerisation. A higher amount of chain initiator in the feed containing constant amounts of the monomers generally results in copolymers with lower molecular weights [22]. A similar trend was observed in this study, when mPEGs (a primary alcohol) were used in increasing amounts at constant ratio of the monomers. On increasing the amount of mPEG in the feed reduced the molecular weight of final copolymer from 50 to 35 K (Table 1). Their Tg consistently was reduced with increasing amounts of the mPEG within the copolymers, mostly because of the plasticizing effect of low molecular weight fractions and mPEG [18] (Table 1). The increasing composition of mPEG was evident in H^1 NMR spectrum while fundamental frequencies within the vibrational spectra of the copolymers remained unaltered (Figures are not presented here). Due to only one available free hydroxyl terminal group of mPEG, the resultant copolymer was most likely to be of AB type copolymer, the other hydroxyl terminal largely remained unreactive as it was blocked with an unreactive and stable methyl ether group of mPEG [18]. Finally the AB type of copolymer expected to have one methoxy ether terminal of mPEG segment and another free hydroxyl terminal of the PLGA segment [23,18]. There was a possibility of some diol impurities within mPEG which could induce ABA type copolymer formation due to two terminal hydroxyl groups available within the diol impurities; however the resulting proportion of such copolymer is expected to be very low.

3.2. Drug loading by ion pairing and s/o/w methodology

The method used for drug loading was a combination of ion pairing and s/o/w techniques. This was a two step solvent evaporation technique, where an aqueous solution of dalargin was added to an acetone solution of the copolymer, followed by a further addition of an aqueous solution of dextran sulphate. As a result of dalargin and dextran sulphate insolubility in acetone, a precipitation was observed with a hazy appearance without any obvious flocculation. Therefore, the resultant solution was defined as a solid in oil (s/o). Because this phase was dominated by organic solvent, the possibility of electrostatic attraction between dextran sulphate (anionic) and dalargin (cationic) was unlikely at this stage. This phase (s/o) was then added with constant stirring to the continuous phase containing 0.6% PVA as surfactant. On evaporation of the organic phase, an instantaneous precipitation of the copolymer was observed; the stirring was continued up to 7.5 h to achieve complete evaporation of the organic solvent. The level of acetone remaining in the dispersion was monitored using gas chromatography and indicated that at the end of 7.5 h stirring, no detectable acetone was left in the nanoparticle dispersion (Fig. 1). With the removal of organic solvent, the ion pairing forces (electrostatic forces) should become dominant at particular stages to form the ion pairing with dextran sulphate and dalargin. The insoluble ion pair has fewer tendencies to diffuse out from the inner phase to the interface of the s/o/w, hence they could be trapped by progressive precipitation of the copolymers on solvent evaporation. This is only possible, if ion pairing is faster than copolymer precipitation or if ion pairing formation is complete well before the all of the copolymer is precipitated.

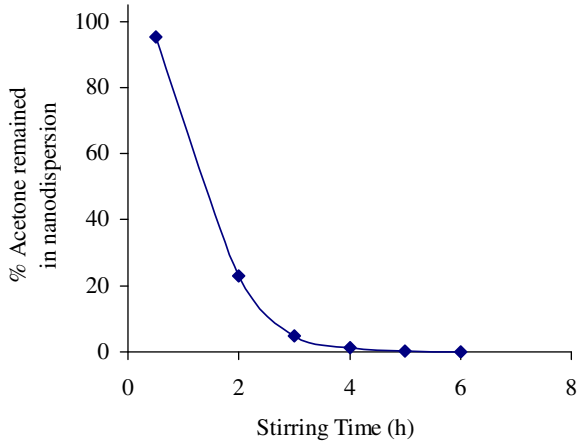

Fig. 1. Level of acetone within nanoparticle dispersion during preparation legend: ♦ level of acetone, mean (n = 2 preparations).

However, if the coacervation is slower than the precipitation of copolymer, then the individual materials (dalargin and dextran sulphate) are likely to diffuse into the continuous phase once they have dissolved into a solution phase and before they are trapped by precipitating copolymer. Such untrapped entities could also possibly form coacervates outside of the nanoparticles, once sufficient organic solvent evaporated from such preparation. Because, the rate of dissolution, diffusion and precipitation remained unknown, it was assumed that all phenomena might have been occurring simultaneously; hence the possibility of encapsulation cannot be neglected. However, the following types of nanoparticles in the population could exist: (1) Empty PEGylated nanoparticles comprised only of PEGylated copolymer; (2) empty nanoparticles with some dextran sulphate entrapped; (3) empty nanoparticle with free dalargin (un-ion paired); (4) encapsulated ion pairs by PEGylated PLGA and (5) un-encapsulated ion pair. However, the relative proportion of these particles was difficult to estimate. Considering all these possibilities, the trapped ion pair, untrapped ion pair and encapsulated drug were likely to be retained within the preparation during the ultrafiltration treatment (using Nanosep™ 300 K) of the sample. Only the amounts of drug which were not ion paired and un-encapsulated were likely to be appeared as a free drug, which was considered in calculating encapsulation parameters.

Table 1
Copolymer composition and physicochemical properties

% weight ratio DL-lactide: glycolide: mPEG		% mPEG	Physicochemical properties	
At the start of reaction	% fraction after purification (H^1 NMR)	H^1 NMR	Glass transition Temperature (Tg)	Average molecular weight (polydispersity)
76.2:19:5	82:13:5	5	45.42 °C	45745 (1.1)
72:18:10	76:12:12	12	31.91 °C	48770 (1.7)
66:17:17	71:12:17	17	24.58 °C	35974 (2.6)

3.3. Optimisation of dalargin loading

3.3.1. Impact of increasing mPEG composition of copolymers on dalargin loading and its physical properties

The percentage free dalargin was significantly (p = 0.017, 0.013) lower for 10% and 17% preparation and insignificantly (p = 0.335) different for 5% sample compared to that of control sample (Fig. 2), which suggested that the copolymer with a lower percentage of mPEG composition was unable to retain substantial amounts of dalargin. The recovery of entire dalargin amount from

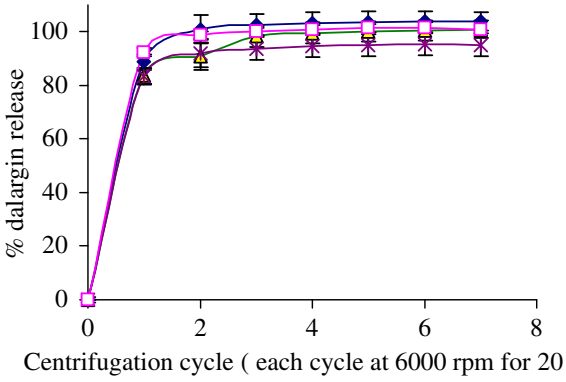

Fig. 2. The impact of mPEG composition of copolymer on percentage free dalargin within the nanoparticle preparation legends: % free dalargin in nanoparticle preparation; □ control (empty nanoparticles containing free drug), ♦ 5% mPEG, Δ 10% mPEG, ×17% mPEG.

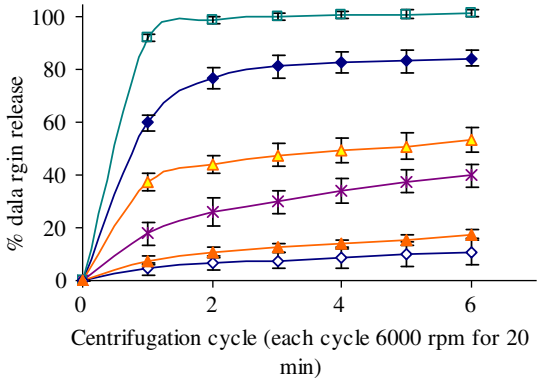

Fig. 3. The impact of mPEG composition of copolymers and molecular weight of DS on percentage free dalargin within the nanoparticle preparation legends: □ Empty nanoparticles containing free drug solution, ♦ 5% mPEG (DS5000), Δ 12% mPEG (DS5000), ×17% mPEG (DS5000), ▲17% mPEG (DS500000, 12 mg dalargin), ◇ 17% mPEG (DS500000, 24 mg Dalargin).

a control preparation indicated no physical barrier imparted by the nanoparticle layer on the Nanosep™ 300 K membrane to retard the drug recovery from the device otherwise encapsulated. When the percentage free dalargin was converted in the percentage loading and encapsulation efficiency, the comparison of median values showed an increasing trend but insignificant ($p = 0.212$, 0.161, 0.997) differences for the nanoparticles prepared from copolymers with increasing mPEG composition (Fig. 4 and Table 2).

This increasing trend may be explained by a weak interaction between the cationic drug and the terminal hydroxyl groups. The copolymer with the higher mPEG composition resulted in a lower molecular weight with higher polydispersity (PD 2.6) due to a higher proportion of the chian initiator (a primary alcohol, here mPEG). This contributed to a higher number of short polymeric chains hence a larger number of terminal hydroxyl groups compared to copolymers with a low proportion of mPEG that showed a higher MW with lower polydispersity (1.67) hence a lower number of terminal hydroxyl groups [22,18]. These groups are believed to be participating in the formation of weak complexes via hydrogen bonding. Other researchers have also exploited the role of terminal functional groups for enhancing drug loading especially using low molecular weight (LMW) fractions in the preparations. Niwa et al. (1993,1994,1995) reported the use of lower molecular weight PLGA (oligomers) to increase the drug loading of nafareline acetate (a cationic drug) in the PLGA based nanoparticles [24,25,7].

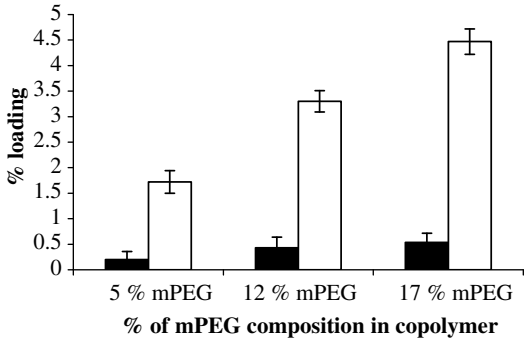

Fig. 4. The impact of DS5000 on percentage dalargin loading within the nanoparticles from the copolymers having increasing composition of mPEG. Legends: ■ without dextran sulphate, □ with dextran sulphate, Mean ± SD, $n = 3$.

This was aimed at providing a higher number of terminal carboxylate/hydroxyl groups that could form hydrogen bond complexes with cationic drugs which eventually reduces the diffusion of soluble drug molecules in the continuous phase [26].

From Fig. 5 it is clear that the particle size obtained from 17% mPEG was significantly lower compared to 5% mPEG copolymer ($p = 0.000$). This could be largely due to the reduction in molecular weight [27,28] and increase in amphiphilic character of the copolymer with increasing mPEG composition [18]. It was obvious that the copolymer with higher mPEG composition provided greater amphiphilic properties, that reduced the interfacial tension to a greater extent to facilitate nanoparticle formation, and hence resulted in a smaller size [27] . This was also supported by the fact that non PEGylated PLGA copolymer required a very high amount of surfactant (PVA) to obtain nanoparticles [29]. The required amount of PVA was drastically reduced during the use of PEGylated PLGA which indicated that amphiphilicity of the copolymer facilitated the formation of nanoparticle by reducing interfacial tension [20]. Thus, the combined effect of MW and amphiphilicity largely contributed to the reduction in particle size. However, this was not linearly co-related with increasing mPEG proportion in the copolymers, as no significant difference ($p = 0.309$) was found between the size of nanoparticle obtained from 12% mPEG and 17% mPEG preparation (Fig. 5, Table 2), which probably indicated a levelling effect of mPEG composition in reducing interfacial tension.

The zeta potential of the loaded nanoparticles remained close to neutral with no significant differences ($p = 0.817$, 0.316, 0.612) among the nanoparticle preparations obtained from the copolymer having 5% to 17% mPEG in composition (Fig. 6, Table 2).

3.3.2. Impact of DS5000 on dalargin loading and physical properties

Inclusion of DS5000 into the preparations significantly ($p = 0.000$) lowered the percentage of free dalargin for all preparations prepared using the copolymer with 5, 12 and 17% mPEG composition compared to those without DS5000 (Figs. 3 and 4). This was largely due to an ion pairing mechanism which was discussed earlier in context of s/o/w methodology for drug loading.

In the presence of DS5000, the decrease in percentage of free dalargin was progressive and significant ($p = 0.000$) as preparations were made from copolymers with increasing mPEG composition. The preparation containing the highest percentage of mPEG (17%) showed the lowest percentage of free dalargin (Fig. 3). As a result, encapsulation efficiency and drug loading were also reflected in a similar manner and were significantly ($p = 0.000$) higher for all mPEG samples (Fig. 4 and Table 3) compared to that of non DS preparations (Fig. 4 and Table 2). In presence of DS 5000, the extent of drug loading was increased from 1.72 ± 0.22 (n=3) to 4.47 ± 0.25% (n=3) as the preparations made from copolymers

Table 2
Loading parameters and physical properties of the dalargin loaded nanoparticles. Formulated without dextran sulphate

Formulation (% wt of mPEG in copolymer)	Concentration of dalargin (mg) per preparation	Encapsulation efficiency (%)	Loading (%)	Zeta (mV)	Size (nm)
(Mean ± SD, n = 3)					
5	12	3.97 ± 3.14	0.20 ± 0.16	−1.26 ± 1.04	141.40 ± 6.77
12		7.12 ± 3.55	0.43 ± 0.21	−1.93 ± 1.32	107.43 ± 3.10
17		8.95 ± 3.00	0.54 ± 0.18	−3.00 ± 1.55	101.50 ± 1.88

% wt of copolymer obtained from H^1 NMR spectra of each copolymer.

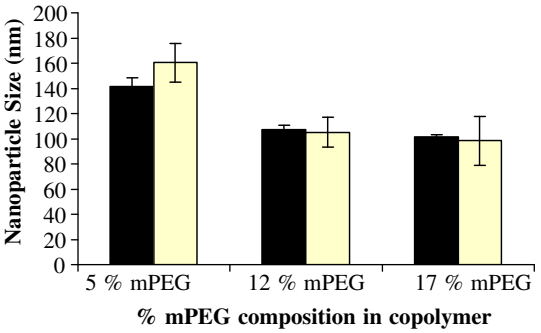

Fig. 5. The impact of DS5000 on particle size of dalargin loaded nanoparticles prepared from copolymers having increased composition of mPEG. Legends: ■ without, □ with DS5000, Mean ± SD, n = 3.

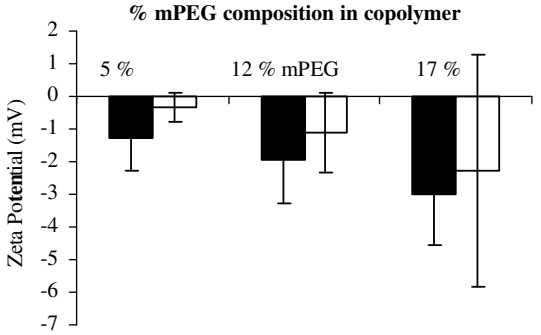

Fig. 6. The impact of DS5000 on zeta potential of dalargin loaded nanoparticles prepared from copolymers having increased composition of mPEG. Legends: ■ without DS5000, □ with DS5000, Mean ± SD, n = 3.

with increasing (5 to 17%) mPEG (Table 3). However, in comparison, during absence of DS5000, the extent of drug loading was much lower (0.2 ± 0.19% to 0.54 ± 0.18%) (Fig. 4, Table 2). This was because, in the presence of DS5000, the interaction was largely govern by a long range electrostatic forces between ion pairing agent DS and drug. When in absence of DS, the interactions between terminal group of copolymer and drug were largely governed by a weak van der Waal's forces.

The impact of increasing hydrophilicity of copolymer with increasing mPEG largely favour electrostatic forces, therefore it may be possible that ion pairing between DS and dalargin favoured to a higher extent when copolymer with higher proportion of mPEG was used and be responsible for showing clear and significant increasing drug loading trend. The adsorption of drug onto hydrophilic and hydrophilic segments of copolymers could significantly affects the drug loading due to various molecular forces depending on the nature of the drug and polymer being used in the preparations [30–32].

Despite the presence of DS5000 in the preparations, the particle size monotonically reduced as nanoparticles were prepared from the copolymers with increasing mPEG composition. This was as a result of reduced interfacial tension and molecular weight of the copolymer with higher amphiphilic properties. When compared with 5% preparation, particles sizes of 12 and 17% preparations were significantly (p = 0.013, 0.007) reduced (Fig. 5). However, the reduction of the particle size with increasing composition was not linear, as no significant difference (p = 0.869) was exist between the size of nanoparticle obtained from 12% and 17% mPEG copolymers (Fig. 5, Table 3), which indicated a levelling effect of mPEG composition on reducing interfacial tension to obtain nanoparticles.

The presence of DS5000 did not significantly (p = 0.078, 0.808, 0.804) increased the particle size, when compared with the size of individual preparations without DS5000 (Fig. 5). This indicated that the additional mass that appeared to be encapsulated may exist as a separate nanoparticle entity outside the core shell structure of nanoparticles.

From Fig. 6, it is clear that despite adding DS5000 in the preparation, the zeta potential values of the dalargin loaded nanoparticles remained close to zero, with insignificant (p = 0.287, 0.621, 0.806) differences compared to the zeta potential values obtained in the relevant preparations without DS5000. This doesn't necessarily indicate that the ion paired dalargin is encapsulated within the core of the nanoparticle leaving a practically neutral surface comprised of mPEG. It is also possible that cationic charge of the dalargin can neutralised the negative charge of DS5000, as a result, the final coacervates had a neutral charge. Thus, from zeta potential and particle size data it remained difficult to conclude the location of the ion paired dalargin within the nanoparticles.

To confirm that DS5000 did not have any negative charge left, the amount of dalargin in the preparation was doubled while amount of DS5000 was kept constant (Table 4) with a view to achieve a higher drug loading by exploiting a remainder negative charge on DS5000. However, the result indicated that at double concentration of dalargin per preparation did not significantly

Table 3
Loading parameters and physical properties of the dalargin loaded nanoparticle formulated with dextran sulphate

Formulation (% wt of mPEG in copolymer)	Concentration of dalargin (mg) per preparation	Encapsulation efficiency (%)	Loading (%)	Zeta (mV)	Size (nm)
(Mean ± SD, n = 3)					
5	12	28.69 ± 3.60	1.72 ± 0.22	−0.33 ± 0.45	160.33 ± 15.50
12		55.08 ± 3.50	3.30 ± 0.20	−1.11 ± 1.23	105.06 ± 12.00
17		74.53 ± 4.10	4.47 ± 0.25	−2.30 ± 3.56	98.43 ± 19.40

Table 4
Optimization of dalargin loading in nanoparticles formulated from copolymer (17% mPEG) in the presence of dextran sulphate

Formulation (% mPEG in copolymer)	Formulation variables per preparations			Encapsulation efficiency (%)	Loading (%)	Size (nm)	Zeta (mV)
	Dextran sulphate		Drug	Mean ± SD $n = 3$			
	Grade (MW)	Conc. (mg)	Conc. (mg)				
17%	N/A	0	12	8.95 ± 3.0	0.53 ± 0.18	101.56 ± 1.88	−3.00 ± 1.5
	5000	24	12	74.53 ± 4.10	4.47 ± 0.25	98.43 ± 19.40	−2.30 ± 3.56
	5000	24	24	38.59 ± 3.47	4.63 ± 0.42	109.26 ± 1.42	−0.40 ± 0.2
	500,000	24	12	84.61 ± 1.96	5.08 ± 0.12	112.76 ± 2.70	−12.06 ± 2.35
	500,000	24	24	87.80 ± 2.28	10.51 ± 0.27	116.16 ± 3.23	−10.90 ± 0.70

($p = 0.630$) increase the drug loading compared to loading of the preparation (Fig. 7, Table 4). The encapsulation efficiency was reduced by almost half from 74.53 ± 4.10 to 38.59 ± 3.47%, n=3, when the dalargin concentration in the preparation was doubled (Table 4). This indicated that the additional amount of dalargin in the preparation did not ion pair further at a fixed concentration of DS, due to lack of further negatively charged sites availability under the proposed preparation conditions. As a result the particle size and zeta potential of the nanoparticle preparations containing double amount of dalargin also remained unchanged (for zeta $p = 0.784$, for size $p = 0.594$) compared to the preparations containing 12 mg dalargin (Table 4, and Figs. 8 and 9).

3.3.3. Impact of DS 500,000 on dalargin loading and its physical properties

To further confirm that an additional negative charge could have trapped an additional dalargin, at 12 mg dalargin concentration per preparation, a higher MW DS 500,000 was used instead DS5000 (low MW) (Table 4). This insignificantly ($p = 0.155$) increased the encapsulation efficiency and induce marginal ($p = 0.086$) increase in loading (Table 4 and Fig. 7) indicated that the additional charges of high MW DS has further entrapped dalargin (Fig. 7). However, the molecular weight of DS 500,000 was higher than the MW of the copolymer itself; therefore, DS chains were likely to protrude outside the nanoparticle surface with excess negatively charged surface that could still accommodate a further amount of dalargin. Under such condition, it was proposed that the nanoparticle may exist in the form of a necklace and allow the DS chain to remain soluble and outside the surface of the nanoparticles [33]. As a result, the zeta potential of this preparation was significantly negative ($p = 0.01$) (Fig. 8, Table 4) with insignificant ($p = 0.348$) increase in particle size compared to the preparation containing DS5000 (Fig. 9, Table 4).

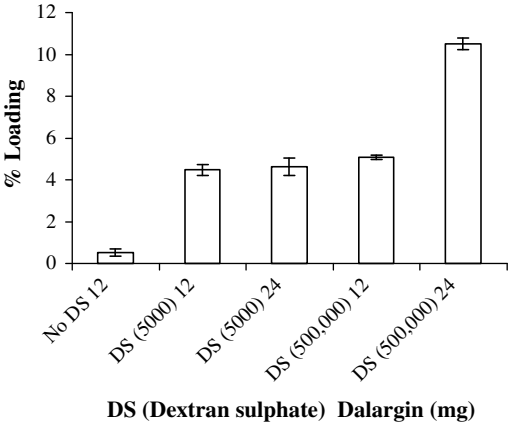

Fig. 7. The impact of DS MW on dalargin loading of the nanoparticles prepared from the copolymer containing 17% mPEG. Legend: *x*-axis legend "DS (MW) (amt of dalargin per preparation)", Mean ± SD, $n = 3$.

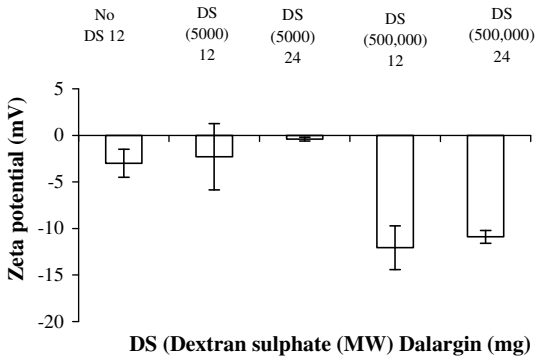

Fig. 8. The impact of DS MW and amount of dalargin on zeta potential of nanoparticles prepared from the copolymer containing 17% mPEG. Legend: *x*-axis legend "DS (MW) (amt of dalargin per preparation)", Mean ± SD, $n = 3$.

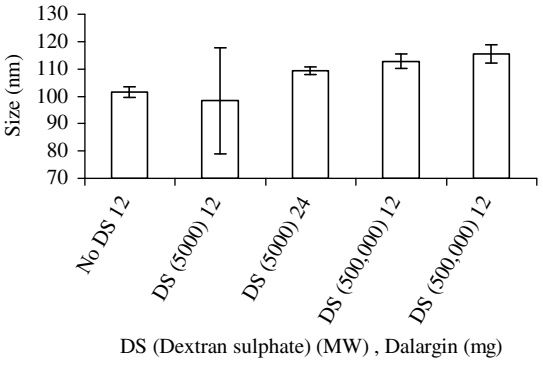

Fig. 9. The impact of DS MW and amount of dalargin on the particle size of nanoparticles prepared from the copolymer containing 17% mPEG. Legend: *x*-axis legend "DS (MW) (amt of dalargin per preparation)", Mean ± SD, $n = 3$.

Considering that DS500,000 remained outside on the surface with excess negatively charged sites still available to trap further drug outside the nanoparticles, amount of dalargin level was doubled (24 mg) in the preparation (Table 4). This did not significantly ($p = 0.945$) change the encapsulation efficiency values compared to the preparation containing 12 mg of dalargin (Table 4). However, this reflected significantly ($p = 0.001$) in terms of loading that doubled in the preparation containing DS500, 000 (Fig. 7, Table 4). As a result, the excess negative charge was only marginally suppressed compared to the preparation containing 12 mg dalargin (Fig. 8, Table 4). There was no significant change in particle size ($p = 0.185$) with increased dalargin concentration in the preparation containing DS500, 000 (Fig. 9, Table 4). The preparation containing DS500,000 had a significantly negative charge on the surface of the nanoparticles compared to the preparations containing DS5000 (Fig. 8). This did not neutralise the zeta potential values despite two fold increase in dalargin in the preparation. This indi-

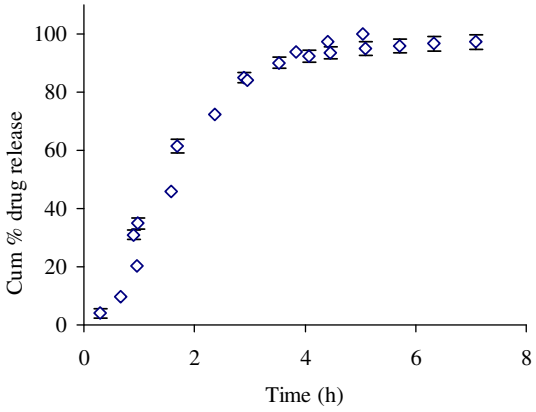

Fig. 10. In vitro drug release evaluation of dalargin loaded nanoparticles legends: Dalargin loaded nanoparticles (Mean ± SD, $n = 3$), Free dalargin solution ($n = 1$).

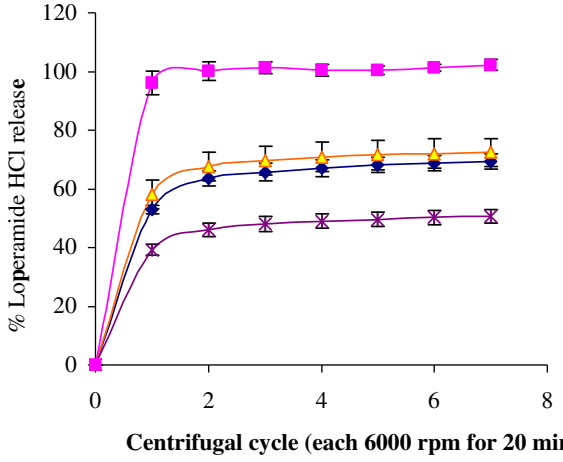

Fig. 11. The impact of mPEG composition of copolymers on percentage free loperamide HCl in the absence of dextran sulphate within nanoparticles preparation. Legends: □ empty nanoparticles and drug solution, Δ 12% mPEG, ♦ 5% mPEG, ×17% mPEG.

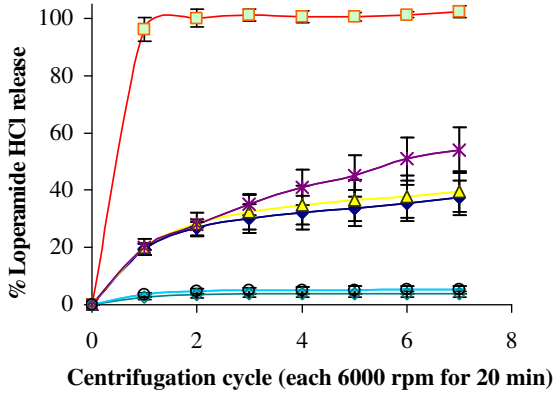

Fig. 12. The impact of mPEG composition of copolymers on percentage free loperamide HCl in the presence of DS 5000 and 500,000. Legends: □ empty nanoparticles and drug solution, ×12% mPEG (DS5000), Δ 5% mPEG (DS 5000), ♦ 17% mPEG (DS5000), ○ 17% mPEG (DS500000, 12 mg loperamide HCl), ◇ (DS500000, 24 mg loperamide HCl).

cated that DS500,000 could still accommodate a much higher amount of dalargin. However, the possibility of trapping dalargin within the core was obviously poor in such a case, as additional dalargin would always be trapped by DS chain protruding from the surface.

3.4. Optimisation of loperamide HCl loadings

3.4.1. Impact of mPEG density of copolymers on loperamide HCl loading and physical properties of nanoparticles

In Fig. 11, the percentage of free loperamide HCl was significantly ($p = 0.04$) lower in nanoparticles prepared from the copolymer containing the highest percentage (17%) of mPEG compared to the preparations made using copolymers containing lower percentages (12% and 5%). A similar trend was observed for dalargin preparations without using DS5000 (Fig. 2). During preparation, the pH of the continuos phase was 5.5, which was capable of dissolving the stated amount of loperamide HCl in the preparation, which remained ionised with a net cationic charge and may form a weak complex with terminal hydroxyl functional groups. Such weak complexation tendency prevailed to a higher extent in the preparation with the highest mPEG composition, owing to a higher number of terminal hydroxyls. However, there was no significant ($p = 0.213$) difference between the percentage free loperamide HCl for the nanoparticle preparations prepared from 5 and 12% mPEG copolymers, probably due to copolymer heterogeneity (Fig. 11). Such a retarded release of lignocaine HCl was reported from PEGylated PLGA nanoparticles prepared using a copolymer with higher PEG density compared to its release from the preparations with a lower density of PEG for unknown reasons [34].

When the percentage of free loperamide HCl values were converted to the percentage loading and encapsulation efficiency, similar differences were noted (Figs. 13, Table 5). The encapsulation efficiency was significantly ($p = 0.03$) higher for 17% mPEG preparation compared to nanoparticle prepared from lower percentages of mPEG (5 and 12%) preparations (Table 5) that showed no significant ($p = 0.275$) difference in encapsulation efficiency and percentage loadings between them possibly due to polymeric heterogeneity.

The particle size progressively and significantly ($p = 0.001$, 0.003) reduced as preparation was made from copolymer with increasing mPEG composition. (Fig. 15, Table 5). Such a trend was also observed in the particle size obtained for dalargin loaded nanoparticles prepared in the absence of DS as discussed before (Fig. 5).

However, the zeta potential of all loperamide HCl containing preparations without DS5000, were near neutral with no significant ($p = 0.896$, 0.293, 0.489) differences among each preparations (Fig. 14, Table 5). This suggested that the encapsulation of the lipophilic loperamide HCl deeper within the core of the nanoparticle

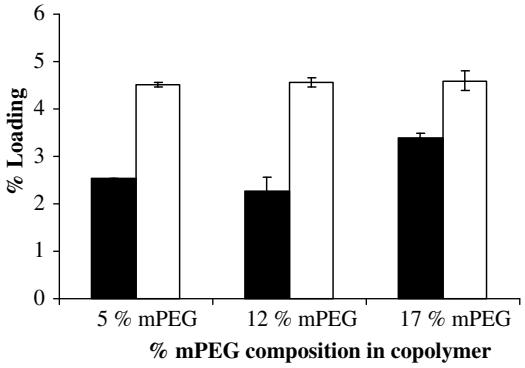

Fig. 13. The impact of DS5000 on loperamide HCl percentage loading within the nanoparticles from copolymers having increasing composition of mPEG. Legends: ■ without DS5000, □ with DS5000, Mean ± SD, $n = 3$.

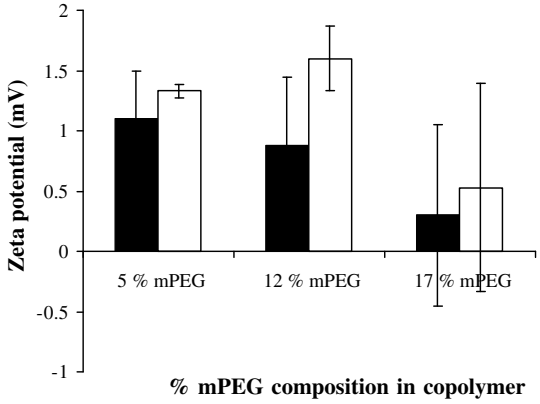

Fig. 14. The impact of DS5000 on the zeta potential of loperamide HCl loaded nanoparticles prepared from copolymers having increasing composition of mPEG. Legends: ■ without DS5000 □ with DS5000, Mean ± SD, $n = 3$.

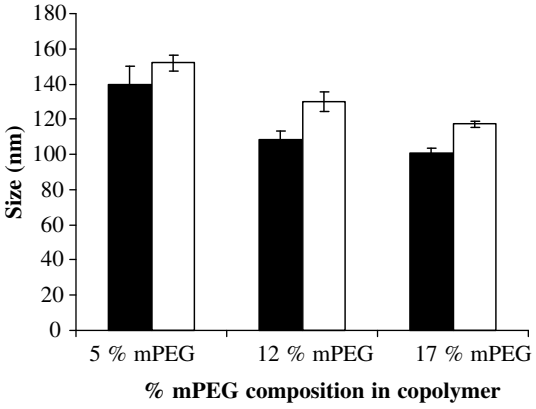

Fig. 15. The impact of DS5000 on the particle size of loperamide HCl loaded nanoparticles prepared from copolymers having increasing composition of mPEG. Legends: ■ without DS5000 □ with DS5000, Mean ± SD, $n = 3$.

and probably leaves a neutral surface comprised of mPEG as a shell.

3.4.2. Impact of DS5000 on the loperamide HCl loading and physical properties of nanoparticles

Inclusion of DS5000 resulted in a significant reduction in percentage free loperamide HCl compared to control and all preparations without DS5000. (Figs. 11 and 12). This significantly ($p = 0.001$, 0.002, 0.000) increased the encapsulation efficiency and loading (Fig. 13 and Tables 5 and 6) compared to without DS5000 preparations. In this case, increasing mPEG composition did not have any differential impact on percentage loading of loperamide HCl, which was seen in the case of dalargin loading using DS5000. Therefore, loperamide HCl loading in 5, 12 and 17% mPEG preparations were not significantly ($p = 0.874$, 0.801, 0.988) different when DS5000 was included (Fig. 13). This may be explained by the different ion pairing tendency of loperamide HCl compared to dalargin with DS5000. The difference in stoichiometry of the drug:DS complex and their final surface were believed to play an important part in their partitioning behaviour with copolymer, and a determinant of their encapsulation trend. It is possible that an ion pair of loperamide HCl sufficiently lipophillic that could favourably partitioned into the lipophilic core of the copolymer during encapsulation. In such a case, encapsulation is largely favoured by hydrophobic forces (lipophilic–lipophilic) and least affected by increasing proportion of mPEG within copolymer. This may be a reason for ineffectiveness of mPEG composition on loperamide HCl loading in the presence of the DS5000. While in the case of dalargin pairing with DS5000, ion pair would be still hydrophilic that favoured the hydrophilic shell layer of mPEG, or had fewer tendencies to be partitioned with the lipophilic core. It is also possible that such multi charged ion pair formation is based on electrostatic forces that generally prevailed to a higher extent as mPEG composition in copolymers increases and imparts greater hydrophilicity. This may be responsible for the differential behaviour of increasing mPEG density on dalargin loading in the presence of DS 5000 (Fig. 3).

In Fig. 15, the particle size of the 5% mPEG formulation was marginally increased when DS5000 was included in the preparation, however this was not significant ($p = 0.268$). Subsequently, with 12% and 17% mPEG preparations, a significant ($p = 0.001$, 0.001, respectively) increase in size was observed in the presence of DS5000 compared to that of preparations without DS5000 (Fig. 15, compare sizes in Tables 5 and 6). Such an increase in particle size in the presence of DS5000 indicated the possibility of encapsulation of the ion paired loperamide HCl. This was evident in the case of loperamide HCl, because it is believed that the hydrophobic nature of the final ion pair imparted miscibility with copolymer that enabled its partitioning deeper within the lipophilic core, thus the additional mass of the ion paired agent within the core resulted in increased particle size. Such an increase is reported in previous studies [28,35]. Oppositely, in the case of dalargin, no significant ($p = 0.279$, 0.811, 0.790) increase in particle size

Table 5
Loading parameters and physical properties of the loperamide HCl nanoparticles formulated without dextran sulphate

Formulation (% wt of mPEG in copolymer)	Amount of loperamide HCl (mg) per preparation	Encapsulation efficiency (%)	Loading (%)	Zeta (mV)	Size (nm)
(Mean ± SD, $n = 3$)					
5	12.0	42.3 ± 0.21	2.53 ± 0.01	1.1 ± 0.40	139.53 ± 10.58
12		38.0 ± 5.00	2.27 ± 0.30	0.88 ± 0.55	108.66 ± 4.44
17		56.9 ± 1.87	3.39 ± 0.10	0.30 ± 0.75	100.96 ± 2.76

Table 6
Loading parameters and physical properties of the loperamide HCl nanoparticle formulated with dextran sulphate

Formulation (% wt of mPEG in copolymer)	Amount of loperamide HCl (mg) per preparation	Encapsulation efficiency (%)	Loading (%)	Zeta (mV)	Size (nm)
(Mean ± SD, $n = 3$)					
5	12.0	75.19 ± 0.87	4.51 ± 0.04	1.1 ± 0.40	151.93 ± 4.33
12		76.32 ± 1.53	4.57 ± 0.10	0.88 ± 0.55	130.10 ± 5.38
17		76.56 ± 3.62	4.59 ± 0.21	−0.30 ± 0.75	117.23 ± 1.72

in the presence of DS5000 suggested poor possibility of ion pair incorporation within the nanoparticle core (Fig. 5).

Comparing the overall trend, the particle size was observed to be significantly decreased ($p = 0.002$, 0.000, 0.020) as the percentage mPEG in the copolymer was increased from 5 to 17%, irrespective of DS5000 presence in the preparations (Fig. 15).

The inclusion of DS5000 within the preparations did not have any significant ($p = 0.465$, 0.270, 0.813) impact on the zeta potential (Fig. 14). Since the zeta potential values remained close to neutral values, the ion pair of loperamide HCl was possibly encapsulated within the core of the nanoparticles and left with a neutral surface of mPEG. In comparison, (Fig. 14), increasing mPEG composition within the copolymer did not had any significant impact on the zeta potential despite the presence of DS5000 in the preparations ($p = 0.717$, 0.295, 0.104 without DS5000 preparations, and p = 0.251, 0.233, 0.150 with DS5000).

The copolymer with the highest percentage of mPEG (17%) was selected for drug loading optimisation. At 12 mg loperamide HCl and 24 mg DS5000 per preparation (weight ratio of Drug:DS equal to 1:2), resulted in significant ($p = 0.000$) increase in encapsulation efficiency and drug loading compared to that without DS5000 (Tables 5 and 6, Fig. 16). This was accompanied by a significant ($p = 0.001$) increased particle size with insignificant ($p = 0.998$) change in the zeta potential values compared to the preparation without DS5000 (Figs. 18 and 17). This indicated that the additional mass of ion paired loperamide HCl with DS5000 was likely to be partitioned inside the core of the nanoparticles hence increase in particle size, with almost neutral zeta potential which possibly suggested undisturbed neutral surface of the mPEG as a nanoparticle shell.

To evaluate the ability of 24 mg DS5000 to accommodate further amounts of loperamide HCl, the concentration of loperamide HCl was raised to 24 mg per preparation (weight ratio of Drug:DS equal to 1:1). This level of loperamide HCl was above the saturation solubility in the continuous phase of the dispersion, and excess drug was crystallised. Therefore, it flawed the particle size measurement and could not be used for comparison (Not mentioned in the Figs. 17 and 18).

3.4.3. Impact of DS500,000 on the loperamide HCl loading and physical properties of nanoparticles

Inclusion of DS500,000 (high MW) at 1:2 weight ratio of drug:DS in the preparation significantly ($p = 0.000$) increased the drug loading from 4.59 ± 0.21, n=3 to 5.55 ± 0.04, n=3 (Fig. 16, Table 7). This was due to a higher negative charge contributed by DS500,000 that induced rather extensive ion pairing compared to that of with DS5000. The particle size was significantly ($p = 0.008$) higher compared to that of the non DS preparation, however no significant ($p = 0.196$) change was observed in the particle size compared to that of the DS5000 preparation (Fig. 18, Table 7). This indicates that, further increase in loperamide HCl loading by DS500,000 was not sufficient to cause further increase in size compared to the increase in size already achieved by the DS5000 over the non-DS5000 preparations. However, the zeta potential remained significantly ($p = 0.000$) negative compared to the zeta potential of nanoparticles obtained using DS5000 (Fig. 17, Table 7). This was due to a higher chain length of DS500,000 than the copolymer itself, which is likely to protrude outside the nanoparticle surface and still carry sufficient negative charge to provide a negative zeta potential. A similar observation was noted when dalargin loading was performed under similar condition using DS500,000.

Considering excess negative charge of the DS500,00 was still available on the surface of the nanoparticles; it could be exploited to entrapped further loperamide HCl. Therefore, in another experiment (Table 7), a higher amount of the loperamide HCl (24 mg per preparation) was used in conjunction with DS500,000 in the preparation (Drug:DS weight ratio was equal to 1:1). This resulted in significant ($p = 0.000$) increase in drug loading (Fig. 16, Table 7) with similar encapsulation efficiency (Table 7) compared to that obtained in the nanoparticle preparations involving 1:2 Drug:DS weight ratio using DS500,000 (Table 7). In the previous experiment at 1:1 weight ratio of drug:DS with DS5000, obvious drug crystallisation was observed due to the solubility limitation of loperamide

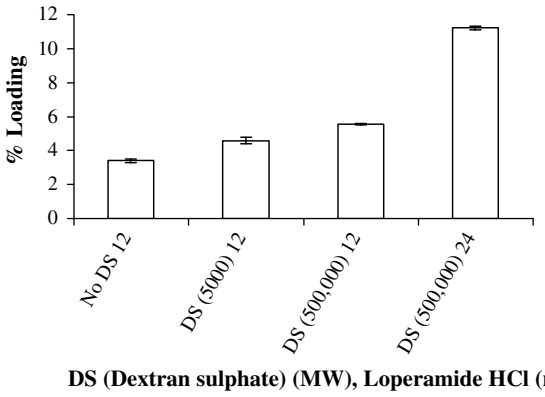

Fig. 16. Impact of DS MW and amount of loperamide HCl on loading within nanoparticles prepared from copolymer containing 17% mPEG. Legend: *x*-axis legend "DS (MW) (amt of dalargin per preparation)", Mean ± SD, $n = 3$.

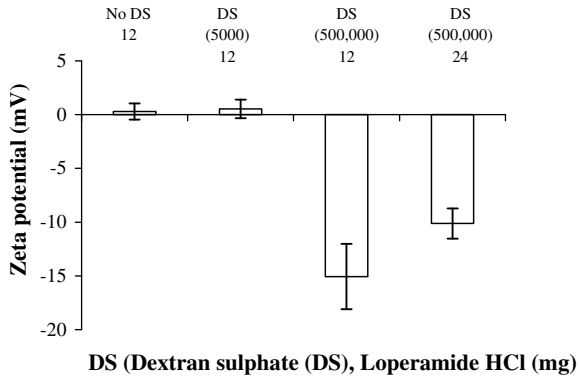

Fig. 17. Impact of DS MW and amount of loperamide HCl on the zeta potential of nanoparticles prepared from the copolymer containing 17% mPEG. Legend: *x*-axis legend "DS (MW) (amt of dalargin per preparation)", Mean ± SD, $n = 3$.

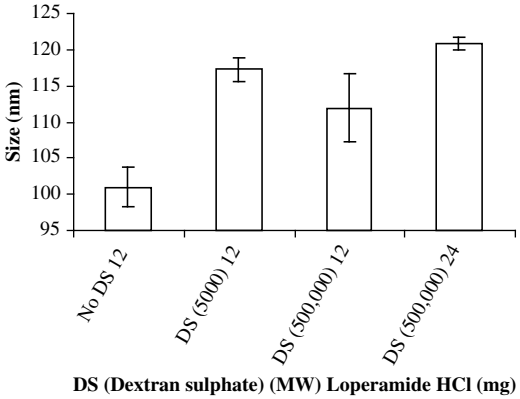

Fig. 18. Impact of DS MW and amount of loperamide HCl on the size of nanoparticles prepared from the copolymer containing 17% mPEG. Legend: *x*-axis legend "DS (MW) (amt of dalargin per preparation)", Mean ± SD, $n = 3$.

Table 7
Optimization of loperamide HCl loading in nanoparticles formulated from copolymer (17% mPEG) in the presence of dextran sulphate

Formulation(% mPEG in copolymer)	Formulation variables per preparation			Encapsulation efficiency (%)	Loading (%)	Size (nm)	Zeta (mV)
	Dextran sulphate		Drug	Mean ± SD $n = 3$			
	Grade (MW)	Conc. (mg)	Conc. (mg)				
17%	N/A	0	12	56.95 ± 1.87	3.41 ± 0.11	100.96 ± 2.76	0.30 ± 0.75
	5000	24	12	76.56 ± 3.62	4.59 ± 0.21	117.23 ± 1.72	0.53 ± 0.86
	5000	24	24	N/A			
	500,000	24	12	92.61 ± 0.71	5.55 ± 0.040	111.93 ± 4.76	−15.06 ± 3.05
	500,000	24	24	93.53 ± 0.81	11.22 ± 0.090	120.83 ± 0.86	−10.13 ± 1.40

HCl in continuous phase. However, 1:1 weight ratio of drug:DS in the preparation containing DS500,000 did not showed any obvious drug crystallisation despite the use of similar conditions of continuous phase in the preparation. It is possible that this water soluble polymer could form a ion to ion, ion to dipole, dipole to dipole, van der Waal's forces and hydrogen bridges with drug molecules, such forces holds the drug molecules and prevent their diffusion to form critical nucleus, thus prevent crystallisation and or retard the formation of crystals. Such inhibitory effect of water soluble polymers on precipitation was reported by many other researchers [36–38]. As a result, a higher drug loading was achieved with a similar entrapment efficiency that significantly ($p = 0.038$) reduced the zeta potential compared to nanoparticles prepared with a 1:2 drug:DS weight ratio using DS500,000 (Fig. 17, Table 7). This was due to further neutralisation of the negative charge on increased ion pairing between loperamide HCl and DS500,000. This was accompanied by significant increased in the particle size (Fig. 18, Table 7) compared to the preparation involving non DS, DS5000, and DS500,000 at 1:2 weight ratios of Drug:DS. Since this increase in size was accompanied by with a significant negative zeta potential value, a chance of additional encapsulation of loperamide HCl within the core of the nanoparticles was poor.

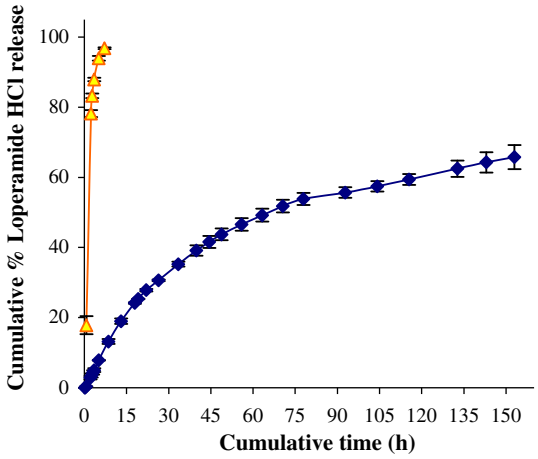

Fig. 19. In vitro drug release evaluation of loperamide HCl loaded nanoparticles legends: ♦ loperamide loaded nanoparticles, Δ free loperamide solution in 0.6% PVA, results are mean ± SD, ($n = 3$).

3.5. In vitro drug release evaluation of drug loaded nanoparticles

From the particle size comparison and zeta potential analysis of the preparation containing DS, encapsulation of the ion-paired dalargin within the core of the nanoparticle was poor. From the hypothesis discussed earlier using s/o/w and ion pairing approaches, a heterogeneous population of nanoparticles was proposed, which was difficult to quantify or separate in to one type of nanoparticles. Therefore, considering the ultimate goal of encapsulating water soluble drug to obtain a sustained release profile, it was decided to evaluate an *in vitro* release performance to prove their usefulness.

When dalargin loaded nanoparticles were evaluated against a dalargin solution, the release profile of the nanoparticles almost overlapped with the recovery profile of the dalargin solution achieved under similar conditions (Fig. 10). This suggests that the proposed method failed to provide an extended release of the water soluble drug, despite an approximate 75% dalargin encapsulation efficiency as shown using the ultrafiltration method (Nanosep™ 300 K). This was largely due to the media used to determine drug encapsulation. During the use of Nanosep™ 300 K, water was used to wash the nanoparticles and to remove free drug which was unable to dissociate the coacervates into water soluble components, despite their position within or outside the nanoparticles. While in the case of the *in vitro* release, a PBS buffer was used as the sink medium which dissociated the coacervates via an ion exchange with the counter ions of the phosphate buffer. This separated the two water soluble components from the coacervates that released from the device as quickly as free drug solution.

The loperamide HCL solution in 0.6% PVA was recovered by 96.81 ± 0.22%, $n = 3$ in just 7.15 h indicated almost complete recovery, this suggested no pH dependent precipitation due to sink buffer, which otherwise could have resulted in incomplete release. The release study profile of loperamide HCl loaded nanoparticles showed an extended release profile compared to free drug solution recovery (Fig. 19). The loperamide HCl loaded nanoparticle showed a release profile over 153 h that cumulatively released 65.76 ± 3.42% $n = 3$. The un-recovered drug from the nanoparticle suspension was assessed and found to be 27.81 ± 1.52% $n = 3$. This showed 93.55% recovery, which was within the HPLC assay accuracy of 94.35 ± 7.63%, ($n = 8$). Stability data indicated that the conditions of exposure were unlikely to induce any degradation [39]. The drug release from the nanoparticles showed little or no burst release events; this was believed to be mostly due to ion pairing that reduced the diffusion of the drug due into continuous phase. Previously evaluated un-ion paired loperamide HCL loaded PEGylated PLGA nanoparticles showed a remarkable burst release, where 70–80% drug was released in just 12 h followed by a prolonged release of the remainder 20% drug in total 36 h [40]. This was significantly improved possibly due to an adoption of ion pairing and s/o/w techniques [11,10,12,9,24,25,7,14,41,6]. From the prolonged release profile of loperamide HCl, it can be considered that loperamide HCl ion pairing with dextran sulphate seemed to be sufficiently lipophilic to promote the partition of the ion pair into the lipophilic core of the nanoparticles.

4. Conclusion

The ion pairing approach to encapsulate a hydrophilic drug was unsuccessful in achieving a controlled release profiles. It is proposed that an ion pair with dalargin was not being encapsulated within the core of nanoparticles, possibly due to its low affinity

with the lipophilic core segment of the copolymer. It is concluded that such an ion pair remained as a separate nanoparticulate entity or only within the shell layer of the nanoparticles. This approach successfully increased the drug loading of loperamide HCl. The DS 5000 was preferred choice of ion pairing, as it allowed a higher drug loading without significantly altering neutral value of zeta potential therefore intact mPEG stealth. The preparation containing 1:2 ratio of drug: DS5000 showed optimal condition for encapsulation, it reduce approximate 22% excipient loading at any dosing level compared to that of the preparation without DS5000. Additionally, such formulation showed an extended release profile of the drug without any burst effect. This indicated the possibility of an ion pair partitioning within the lipophilic segment of the copolymers. It is expected that such nanoparticles would provide better in vivo efficacy profile.

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