



# A new double emulsion solvent diffusion technique for encapsulating hydrophilic molecules in PLGA nanoparticles

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## ARTICLE INFO

### Article history:

Received 17 July 2008

Accepted 14 September 2008

Available online 24 September 2008

### Keywords:

Nanoparticles formulation

Hydrophilic

Double emulsion

PLGA

Macrophages restenosis

## ABSTRACT

The commonly utilized techniques for encapsulating hydrophilic molecules in NP suffer from low encapsulation efficiency because of the drug rapid partitioning to the external aqueous phase. We hypothesized that combining the double emulsion system with a partially water-soluble organic solvent, could result in better encapsulation yield of hydrophilic molecules in nano-sized NP, and the utilization of both biocompatible surfactants and solvents. As a model drug we used alendronate, a hydrophilic low MW bisphosphonate. The new NP preparation technique, double emulsion solvent diffusion (DES-D), resulted in improved formulation characteristics including smaller size, lower size distribution, higher encapsulation yield, and more biocompatible ingredients in comparison to classical methods. The utilization of partially water-miscible organic solvent (ethyl acetate) enabled rapid diffusion through the aqueous phase forming smaller NP. In addition, the formulated alendronate NP exhibited profound inhibition of raw 264 macrophages, depletion of rabbit's circulating monocytes, and inhibition of restenosis in the rat model. It is concluded that the new technique is advantageous in terms of smaller size, lower size distribution, higher encapsulation yield, and more biocompatible ingredients, with unaltered bioactivity.

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## 1. Introduction

Nanoparticles (NP) fabricated from the biodegradable and biocompatible polymers, polylactide (PLA), polyglycolide (PLG) or their copolymer (PLGA), are the most intensively investigated polymers for drug delivery systems [1–3]. The choice of a particular method of encapsulation is mainly determined by drug solubility and molecular stability considerations. The commonly utilized techniques for NP preparation are the nanoprecipitation technique [4], the emulsification solvent evaporation (ESE) [5], emulsification solvent diffusion (ESD) [6,7], and the double emulsion solvent evaporation (DES-E) [8,9].

In the DES-E technique developed for hydrophilic compounds [8–10], the hydrophilic compound is initially dissolved in the inner aqueous phase with subsequent polymer precipitation upon removal of the water-insoluble organic solvent (dichloromethane or chloroform). However, this method still suffers from low encapsulation efficiency of hydrophilic low MW compounds for manufacturing of nano-sized NP [11]. Thus, in contrast to lipophilic drugs [12,13], encapsulation of hydrophilic agents remains a challenge because of the drug's rapid partitioning to the external aqueous phase [14].

We hypothesized that combining the double emulsion system of the DES-E technique with the partially water-soluble organic solvent utilized in the ESD technique, could result in better encapsulation

yield of hydrophilic molecules in nano-sized NP, and the utilization of both more biocompatible surfactants (tween and pluronic rather than PVA) and solvents (ethyl acetate vs. dichloromethane or chloroform). In this work we describe a novel NP preparation technique, double emulsion solvent diffusion (DES-D), in comparison to the DES-E technique. As a model drug we used alendronate (ALN), a hydrophilic low MW bisphosphonate, which was shown to inhibit restenosis when encapsulated in particulate delivery systems [15,16]. We characterized the new DES-D technique, the effect of formulation variables, and the mechanism of PLGA NP formation. In addition, we demonstrated the biological bioactivity of the manufactured NP on monocytes depletion and restenosis inhibition.

## 2. Materials and methods

### 2.1. ALN NP preparation

For a typical formulation of the DES-D technique, 1 ml of 20 mg/ml ALN (Unipharm, Tel-Aviv, Israel) solution, pH=7, adjusted by ammonia solution, was emulsified in 3 ml of ethyl acetate (EtAc, JT Baker, The Netherlands) containing 3% PLGA 50:50 (MW 40,000–75,000, Sigma-Aldrich, Israel) and 3% Pluronic F68 (Sigma-Aldrich, Israel), by sonication over an ice bath using a probe sonicator (Microson XL, Misonix, USA), at 20 W output for 90 s. The resulting primary emulsion was added to 10 ml double distilled water (DDW) containing calcium chloride (calcium, Sigma-Aldrich, Israel) at a 2:1 molar ratio of calcium to ALN, and was

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sonicated for 90 s at 50% amplitude over an ice bath to form the double emulsion. EtAc was eliminated by evaporation under reduced pressure using a rotary evaporator (Buchi, Switzerland), and the pH was adjusted to 7. When polyvinylalcohol (PVA, MW 30,000–70,000, Sigma-Aldrich, Israel) was used as a stabilizer, it was dissolved in both, the inner and the external aqueous phases, 2.8% w/v and 2% w/v, respectively.

Drug-free NP (empty NP) were prepared by the same procedure omitting the drug. Fluorescent NP were prepared by replacing 10% of the polymer with rhodamine-labeled PLGA synthesized in our lab. To visualize the encapsulation of a hydrophilic molecule, 10% of ALN was replaced with hydroxypyrene trisulfonic acid (HPTA, Sigma-Aldrich, Israel) as a fluorescent drug marker. The effect of formulation variables on the NP characteristics was examined, including surfactant type, polymer and surfactant concentration, sonication time periods, ALN:calcium ratios, final pH of the formulation and pH adjustment reagents.

## 2.2. ALN NP physicochemical characterization

### 2.2.1. Drug load, size, zeta potential and morphology

ALN concentration was determined by a fluorimetric assay of its complex with fluorescamine. The free drug was separated by ultra-filtration using centricon tubes (30,000 Da, Millipore, Germany). NP were degraded with sodium hydroxide (1 M) and the solution neutralized with hydrochloric acid (1 M). ALN was reacted with fluorescamine (Sigma-Aldrich, Israel) at pH 10 (borate buffer) and was quantified fluorimetrically (FLUOstar, BMG, Germany) against a suitable calibration curve.

NP size was measured by dynamic light scattering, ALV (NIBS/HPPS GmbH, Langen, Germany). Zeta potential was measured by the laser Doppler velocimetry (LDV) technique by means of a Nano ZS (Malvern, UK). NP morphology was observed utilizing transmission electron microscopy (TEM CM 12 Philips, Eindhoven, The Netherlands).

### 2.2.2. In vitro release

ALN NP (1 ml suspension) were diluted in DDW (50 ml) and incubated at 37 °C under magnetic stirring (Lab Line Instruments, Melrose Park, IL, USA). At predetermined time points, a sample of the NP suspension (0.45 ml) was withdrawn, centrifuged in an amicon tube (30,000 Da), and analyzed for the amount of ALN.

### 2.2.3. In vitro visualization of NP uptake

Cells ( $1 \times 10^4$ /chamber) were seeded in a Lab-Tek chambered cover glass system (Nalge Nunc International Corp., USA) and incubated for 24 h. Fluorescent NP (PLGA-rhodamine) loaded with ALN and the hydrophilic fluorescent marker (HPTA) were added to the cells. Following incubation for 60 min, cells were washed with PBS ( $\times 3$ ), fixed with absolute methanol for 6 min at  $-20$  °C, washed ( $\times 3$ ) with PBS, and mounted with fluorescence microscopy mounting media (Sigma). NP and markers uptake and localization was observed and recorded by means of confocal microscopy (Zeiss LSM 410, Germany), in comparison to NP loaded with ALN and HPTA, and to ALN NP with added free HPTA.

## 2.3. Cell growth inhibition

A murine macrophage cell line (RAW 264, ATCC, Rockville, MD, USA) was utilized. Cells were grown in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin and 100  $\mu$ g/ml streptomycin in 5% CO<sub>2</sub> atmosphere at 37 °C. Unless otherwise noted all materials for cell cultures were purchased from Biologic Industries (Beit Haemek, Israel) and from Sigma. The cells were plated at  $2 \times 10^4$  cells per well on 24-well plates and allowed to grow overnight. Inhibition of RAW 264 proliferation by ALN loaded NP prepared by the DES-D method was determined by incubating the cells with the ALN NP or free ALN for 48 h followed by counting the number of viable cells by means of a Coulter counter (Coulter

Corporation, Miami, FL, USA). ALN NP were diluted with empty NP for reducing ALN dose keeping equivalent polymer concentrations.

## 2.4. Depletion of monocytes in vivo

Animal care and procedures were in accordance with the standards for care and use of laboratory animals of the Hebrew University of Jerusalem conforming to NIH regulations. New Zealand white rabbits (Harlan Laboratories), weighing 2.5–3.5 kg were randomly assigned to treatment or control groups. Blood was extracted before and 48 h after IV injection of 3 mg/kg of ALN NP ( $n=5$ ), 3 mg/kg free ALN ( $n=3$ ) or equivalent volumes of empty NP ( $n=5$ ) or MES-HEPES buffer ( $n=3$ ). Monocyte level in rabbit's blood was assayed by Fluorescence Activated Cell Sorting (FACS) as previously described [17]. In short, anticoagulated blood (100  $\mu$ l) was incubated for 30 min (4 °C, in the dark) with mouse anti-human RPE-conjugated anti-CD14 (DAKO, Denmark). FACS lysing solution (1:10 dilution, BD Biosciences, NJ USA) was added for 10 min. The residual cells were washed ( $\times 1500$  RPM, 5 min) in FACS medium (PBS, 1% BSA, 0.02% sodium azide) and suspended in 1 ml FACS medium for flow cytometry. Monocytes were identified according to their relative size, side scattering, and fluorescence.

## 2.5. Rat model of vascular injury

Male Sabra rats (Harlan Laboratories), weighing 350–420 g were randomly assigned to treatment or control groups. Animals were fed standard laboratory chow and tap water ad libitum, and experiments were performed under general anesthesia achieved with ketamine (IP, 80 mg/kg, Fort Dodge, Animal Health, USE) and xylazine (IP, 5 mg/kg, V.M.D.N.V, Belgium). The rat carotid injury model was performed as described previously [18,19]. In short, the distal left common and external carotid arteries were exposed through a midline incision in the neck. The left common carotid artery was denuded of endothelium by the intraluminal passage of a 2F balloon catheter (Baxter, Irvine, CA, USA) introduced through the external carotid artery. The balloon was sufficiently distended with saline to generate a slight resistance and passed three times. At the time of surgery, animals were injected IV via the tail vein with 3 mg/kg ALN NP ( $n=8$ ), 3 mg/kg free ALN ( $n=7$ ), MES-HEPS buffer ( $n=16$ ) or empty NP ( $n=10$ ) at equivalent volumes.

### 2.5.1. Morphometric analysis

Slides were examined microscopically by an investigator blinded to the type of treatment. Eight to ten sections on each slide were evaluated by computerized morphometric analysis (NIH Image). The residual lumen, the area bounded by the internal elastic lamina (original lumen) and the area circumscribed by the external elastic lamina ('total arterial area') were measured directly. The degree of neointimal thickening was expressed as the ratio between the neointimal area and the area of the media (N/M), and as the ratio between the area of the neointima and the original lumen (% stenosis). The medial area, an indirect index of SMC viability, was determined as the difference between the total arterial area and the original lumen area.

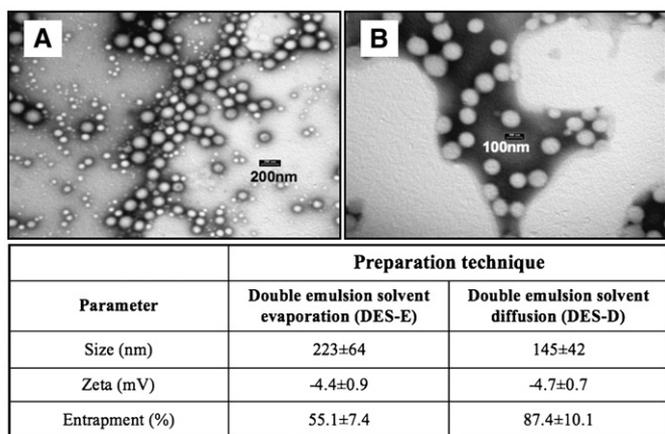
## 2.6. Statistical analysis

Results were expressed as mean  $\pm$  SE. Statistical differences between groups were tested using one-tailed unpaired Student's *t* test and the 2-way ANOVA analysis followed by Tukey-test. Differences were termed statistically significant at  $P < 0.05$ .

## 3. Results

### 3.1. Comparison between the DES-E and the DES-D techniques

The features of ALN loaded NP prepared by DES-D and DES-E techniques with PVA as the surfactant are summarized in Fig. 1. The NP



**Fig. 1.** Transmission electron micrographs of ALN loaded NP prepared by the double emulsion solvent evaporation (DES-E, A) and double emulsion solvent diffusion (DES-D, B) techniques. Size, zeta and entrapment of NP formulations prepared by DES-E and DES-D using PVA as the stabilizer (data±SD).

obtained by both methods were spherical and in the nano size range. NP prepared by the DES-D technique exhibited smaller particles than those prepared by the DES-E technique,  $145 \pm 42$  nm and  $223 \pm 64$  nm, respectively. Entrapment efficiency was also dependent on the method,  $55.1 \pm 7.4\%$  and  $87.4 \pm 10\%$  encapsulation yield, in the DES-E and DES-D techniques, respectively. The zeta potential of the NP obtained by both methods was similar.

Prolongation of the sonication time from 30 s to 90 s resulted in reduced NP size by 126 nm (36%) and 18 nm (10%) in the DES-E and DES-D methods, respectively (Table 1). Lowering polymer concentration resulted in reduced NP size; 36% and 7% size reduction was obtained at a 9-fold lower polymer concentration, DES-E and DES-D techniques, respectively (Table 1).

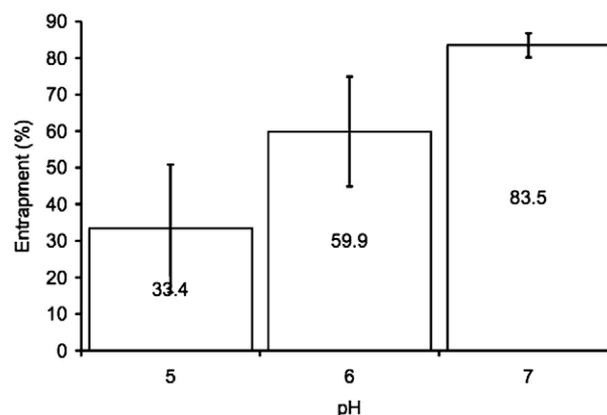
### 3.2. Effect of formulation variables

The properties of NP prepared by the DES-D technique utilizing different surfactants are summarized in Table 2. NP prepared with Pluronic F68 or Tween 80 were statistically insignificantly smaller than those prepared with PVA ( $123 \pm 36$  nm,  $121 \pm 50$  nm and  $145 \pm 42$  nm, Pluronic F68, Tween 80 and PVA, respectively). Similar entrapment efficiency was obtained with the 3 surfactants,  $83.5 \pm 3.3\%$ ,  $81.5 \pm 8.1\%$  and  $87.4 \pm 10.1\%$ , Pluronic F68, Tween 80 and PVA, respectively. The zeta potential of the NP was,  $-13.8 \pm 1.74$  mv,  $-19.6 \pm 1.98$  mv and  $-4.7 \pm 0.7$  mv, Pluronic F68, Tween 80 and PVA, respectively. Pluronic F68 was found suitable for formulating ALN NP since preliminary *in vitro* experiments demonstrated over 90% cells growth inhibition at 25  $\mu$ M in comparison to 100  $\mu$ M and 50  $\mu$ M required for Tween 80 and PVA,

**Table 1**

The effect of sonication time and polymer amount on the size of NP prepared by double emulsion solvent evaporation (DES-E) and double emulsion solvent diffusion (DES-D) techniques

Formulation variable		NP size (nm)	
		DES-E	DES-D
Sonication time	30 s	349±158	178.8±46
	90 s	223±47	223±36
Polymer amount	30 mg	233±79	158±66
	45 mg	213±59	145±44
	90 mg	232±83	150±44
	150 mg	268±115	155±42
	180 mg	309±147	166±27
	270 mg	361±191	170±36



**Fig. 2.** The effect of pH on the entrapment efficiency of ALN in NP formulations prepared by the double emulsion solvent diffusion (DES-D) technique using Pluronic F68 as the stabilizer.

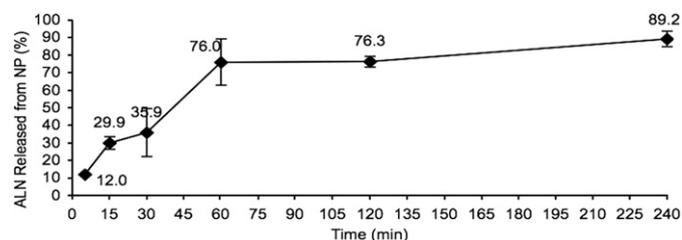
respectively (data not shown). Therefore, the effect of formulation variables on the characteristics of NP prepared by the DES-D technique was examined with Pluronic F68 as the surfactant. Polymer and surfactant concentrations had no significant effect on NP size formulated with Pluronic F68 (Table 3).

$\text{Ca}^{2+}$  was used as a counter ion for ALN in order to reduce its solubility in water (Table 4). The introduction of calcium appears essential for efficient ALN entrapment, since only a low entrapment efficiency of 4.5% was achieved in its absence. The minimal ALN:calcium ratio required was 1:2; increasing the amount of  $\text{Ca}^{2+}$  reduced the entrapment efficiency (83.5%, 72.7% and 65.6% for 1:2, 1:3 and 1:4, respectively). The size was found to be similar for all ALN:calcium ratios, with a lower size distribution in the 1:2 ratio (PDI values of 0.008, 0.102 and 0.154, with ALN:calcium ratios of 1:2, 1:3 and 1:4, respectively). Formulating calcium in the internal aqueous phase and ALN in the external aqueous phase yielded similar results to those obtained when ALN was formulated in the internal aqueous phase and calcium in the external. The final pH of the formulation affected the encapsulation efficiency,  $83.5 \pm 3.3\%$ ,  $59.9 \pm 15.0\%$  and  $33.4 \pm 17.5\%$ , at pH 7, 6 and 5, respectively (Fig. 2). The agent used for pH adjustment was found to affect NP formation and entrapment efficiency (Table 5). At an ALN:calcium ratio of 1:2 and the pH adjusted with NaOH, NP were not formed, and a relatively low entrapment efficiency of 19.8% was obtained at a 1:3 ratio. Ammonia yielded NP with a relatively high entrapment efficiency at both ratios ( $83.5 \pm 3.3\%$  and  $72.7 \pm 3.9\%$ , at 1:2 and 1:3 ALN:calcium ratio, respectively).

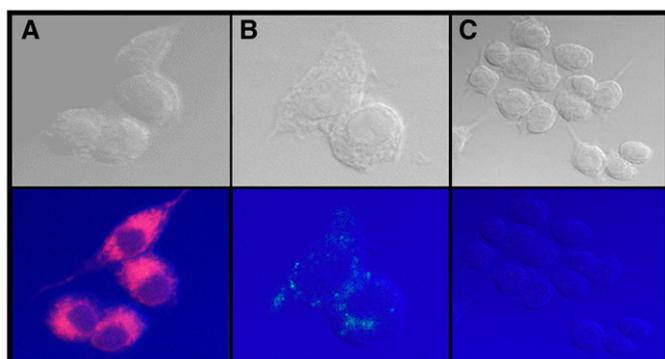
A rapid drug release rate was observed, 12%, 36% and 76% of the drug were released, after 5 min, 30 min and 60 min, respectively. The release was almost completed (90%) after 4 h (Fig. 3).

### 3.3. NP cellular uptake and effect on RAW 264

NP were shown to internalize and accumulate in the cytoplasm of the macrophage-like RAW264 cells and demonstrated strong signals of the



**Fig. 3.** Cumulative release of alendronate from NP *in vitro* (suspension of NP was diluted 1:50 in DDW, pH 7, 37 °C).



**Fig. 4.** Confocal microscopy photomicrographs of Raw 264 cells 30 min after incubation with fluorescent marker (hydroxy pyrene trisulfonic acid, HPTS). Labeled NP (rhodamine-PLGA) loaded with ALN and fluorescent marker (A), non-labeled NP loaded with fluorescent marker (B) and free fluorescent marker (C).

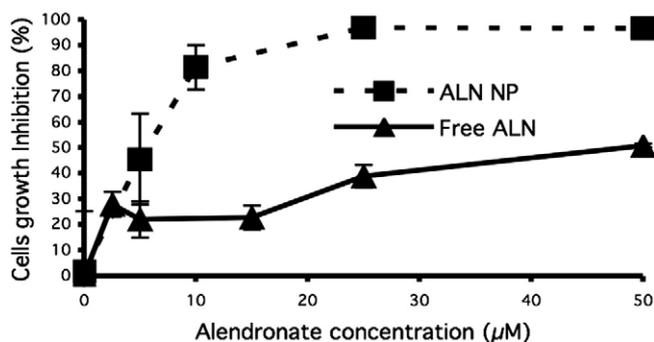
entrapped hydrophilic fluorescent marker (Fig. 4A and B). In contrast, the free fluorescent marker did not penetrate the cells (Fig. 4C). Treatment of macrophage-like RAW 264 cells with ALN NP resulted in a significant reduction of viable cell numbers, and in a dose–response relationship (Fig. 5). Cells treated with ALN NP at a 10  $\mu$ M drug concentration resulted in reduction of cell numbers by 84.1%, and over 95% inhibition was obtained at 25  $\mu$ M. Treatment with free ALN was found to be less potent than ALN NP; cells proliferation was inhibited by 22%, 39% and 51%, at 5  $\mu$ M, 25  $\mu$ M and 50  $\mu$ M, respectively. Empty NP treatment exhibited no effect on cells growth.

### 3.4. Inhibition of blood monocytes

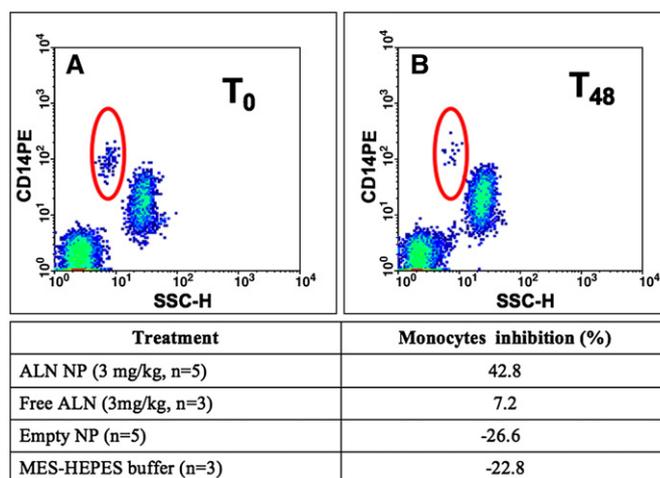
ALN NP treatment resulted in 42.8% inhibition of blood monocytes 48 h after IV injection of a 3 mg/kg dose ( $n=5$ ,  $p<0.05$ , Fig. 6). In contrast, monocyte numbers were insignificantly reduced (7.2%) following treatment with free ALN (3 mg/kg,  $n=3$ ). An increased number of monocytes was observed following treatment with empty NP ( $n=5$ ) and MES-HEPES buffer ( $n=3$ ), 26.6% and 22.8%, respectively (Fig. 6).

### 3.5. Inhibition of neointimal proliferation

Fourteen days following arterial injury no significant differences were found among the control groups, blank NP ( $n=10$ ), buffer ( $n=16$ ) and free ALN ( $n=7$ ). Luminal stenosis was  $50.3\pm 3.69\%$ ,  $40.7\pm 3.64\%$  and  $49.1\pm 5.36\%$ , in blank NP, buffer and free ALN treatment groups, respectively (Fig. 7). In contrast, treatment with ALN NP (3 mg/kg) significantly inhibited neointimal proliferation resulting in a stenosis ratio of  $27.5\pm 5.15\%$  (Fig. 7). Similarly, a significant reduction in the neointima/media ratio was detected in the ALN NP group ( $0.79\pm 0.11$ ) vs.

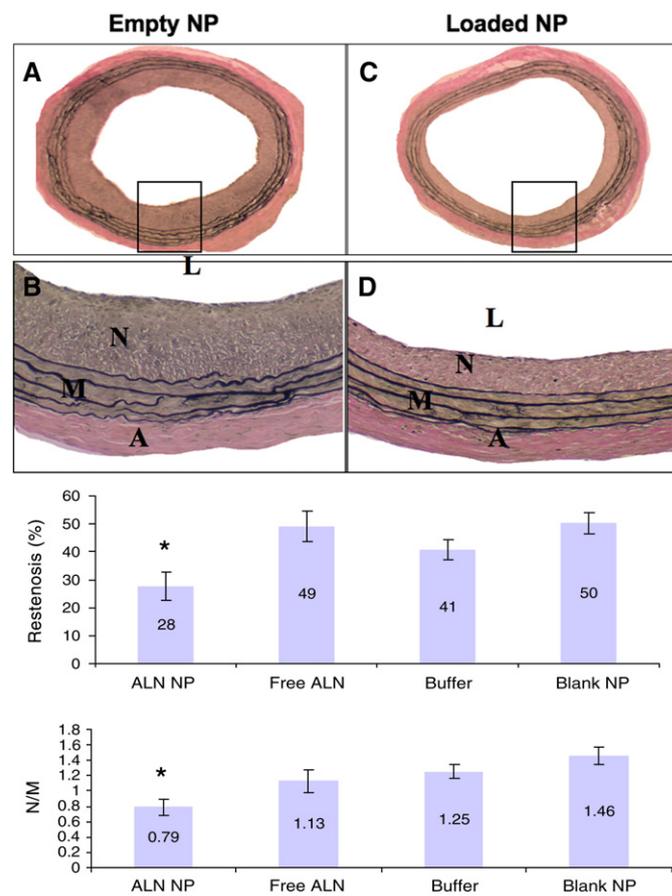


**Fig. 5.** Inhibition of raw 264 cells growth by ALN NP prepared by the DES-D method in comparison to free ALN.



**Fig. 6.** Representative FACS analysis demonstrating CD14<sup>+</sup> monocytes (circled) in peripheral blood of rabbits before (A) and 48 h after (B) I.V. injection of alendronate NP (3 mg/kg). Monocytes inhibition (%) 48 h after treatments of alendronate NP (3 mg/kg,  $n=5$ ), free alendronate (3 mg/kg,  $n=3$ ), empty NP ( $n=5$ ), and MES-HEPES buffer ( $n=3$ ). Monocytes are expressed as % of total white blood cells (WBC); SSC, side scattering and FL2, fluorescence intensity.

the blank NP and buffer control groups ( $1.46\pm 0.12$ ,  $1.25\pm 0.079$ , respectively). The N/M ratio in the free ALN control group was found to be statistically insignificantly higher than in the ALN NP treatment group ( $1.13\pm 0.15$ ).



**Fig. 7.** Representative photomicrographs of explanted sections of arteries (Verhoeff's tissue elastin staining) 14 days after balloon injury and treatment with empty NP (A, B) and alendronate NP (3 mg/kg, IV; C, D). Bar graphs demonstrate the extent of stenosis and the neointima to media area ratio in rat carotid arteries treated by alendronate NP in comparison to control groups, free ALN, MES-HEPES buffer and empty NP.

#### 4. Discussion

The choice of a suitable NP formulation technique is dependent on the physicochemical properties of the drug to be encapsulated. While several methods have been successfully developed and applied for the incorporation of lipophilic compounds into biodegradable NP [20,21], formulation of hydrophilic drugs involving these carriers is more problematic, since the drug is expelled from the hydrophobic matrix into the dispersing water phase during NP preparation [20].

The developed new DES-D method combines the advantages of the two well-established techniques, DES-E and ESD. The double emulsion system minimizes escape of the hydrophilic drug to the aqueous core, and EtAc is more pharmaceutically acceptable and considered to be less toxic than DCM (class III and II according to the ICH specifications, respectively). A comparison between the size of NP formulations prepared by the DES-E and DES-D techniques shows that although the amounts of the polymer, drug and surfactant were similar, the novel DES-D method yielded smaller particles (Fig. 1). In order to examine the mechanism by which NP are formed we studied the effect of the sonication time and polymer amount on NP size. Prolongation of the sonication time resulted in a marked reduction of NP size prepared by the DES-E technique (36%) but not by the DES-D technique (Table 1). In addition, the size of the NP prepared by the DES-E technique could be modulated by the polymer amount, while NP size was found independent on the polymer amount in the new technique. These findings can be explained by the different NP formation mechanisms. DCM utilized in the DES-E method is of relatively low water solubility and the solvent does not diffuse extensively through the aqueous phase. NP are formed during evaporation once the critical concentration for polymer precipitation is reached, with a single particle formed from each emulsion droplet. On the other hand, EtAc used in the DES-D method is partially water-miscible, and the solvent diffuses freely via the aqueous phase creating regions of local supersaturation near the interface [7]. As a result of the phase transformations and polymer aggregation that take place in these regions each emulsion droplet forms several NP [22]. Therefore, the effect of initial droplet size reduction (by prolonged sonication or reduced emulsion viscosity) on NP size is more pronounced in the DES-E technique than in the DES-D technique.

NP size is a significant determinant of the formulation safety and efficacy. Large particles are known to cause adverse effects after injection when taken up by various organs [23], whereas particles smaller than 100 nm are capable of penetrating non-phagocytic cells and escaping the MPS [24]. Particles larger than 100 nm are most likely to be taken up by monocytes. Although NP prepared by the DES-E technique are suitable for effective monocytes inhibition [25], the DES-D technique produces smaller NP enabling sterilization by filtration, the method of choice for PLGA NP [26–28].

The entrapment efficiency of ALN in NP formulated by the DES-D technique was better in comparison to NP prepared by the DES-E technique, probably because the partial miscibility of EtAc in water enabled a slight mutual solubility of the organic and aqueous phases and created a temporary phase in which the polymer and ALN were dissolved. EtAc, being less toxic than DCM, also enables the use of surfactants other than PVA, such as Pluronic F68 and Tween 80. NP prepared using Pluronic F-68 and Tween 80 were about 20 nm smaller on average in comparison to the PVA stabilized NP, which may be attributed to their different structure including separate hydrophilic and

**Table 2**

Size, zeta and entrapment of several NP formulations prepared by the double emulsion solvent diffusion technique (DES-D) using different surfactants (data±SD)

Surfactant	PVA	Pluronic F68	Tween 80
Size (nm)	145±42	123±36	121±50
Zeta (mV)	-4.7±0.7	-13.8±1.74	-19.6±1.98
Entrapment (%)	87.4±10.1	83.5±3.3	81.5±8.1

**Table 3**

The effect of polymer and surfactant amount on the size of NP prepared by the double emulsion solvent diffusion (DES-D) technique using pluronic F68 as the surfactant

Formulation variable		Size (nm)
Polymer amount	50 mg	111±38
	70 mg	112±36
	90 mg	123±35
	110 mg	118±39
	130 mg	124±40
	150 mg	134±43
Surfactant amount	180 mg	138±44
	50 mg	132±44
	70 mg	134±44
	90 mg	123±35
	110 mg	134±49
	130 mg	140±53
	150 mg	144±55
	180 mg	150±57

lipophilic ends as opposed to PVA, resulting in a more efficient emulsification and stabilization of the emulsion droplets.

We further examined the effect of formulation variables on the properties of NP prepared by the DES-D (Tables 3–5). Polymer and surfactant concentrations had no effect on the size of NP prepared by the DES-D method using pluronic F68 as the surfactant (Table 3), which correlates with the results obtained with PVA (Table 1) and with the suggested NP formation mechanism for DES-D. pH adjustment to reduce the solubility of the hydrophilic drug was utilized in the ESD and nanoprecipitation techniques in order to improve the encapsulation of both large [14] and small [29] hydrophilic molecules. The entrapment of ALN in NP was enabled by utilizing calcium as a counter ion in order to reduce ALN solubility [30] and its escape to the aqueous phase. Calcium was found to be essential for the entrapment of ALN since negligible entrapment efficiency was obtained in its absence (Table 4). NP properties were found to be affected by the ratio between ALN and Ca<sup>2+</sup> (Table 4). While a minimal ratio of 1:2 between ALN and calcium was necessary for the creation of ALN:calcium complex, the optimal molar ratio between calcium and ALN was found to be 2:1 providing small NP with a low size distribution (123±36 nm, PDI=0.008), and high encapsulation efficiency (83%, Table 4). Since the inversion in the positions of ALN and calcium positions (calcium and ALN in the internal and external aqueous phases, respectively) yielded similar encapsulation results (Table 4), it seems that the complex is located on the particles surface, rather than entrapped inside the particles. This together with the observation that ALN was not entrapped without calcium suggest that the importance of the double emulsion system in the specific case of ALN and calcium, is only for separating them in different phases prior to particles formation, thereby preventing their early precipitation.

The solubility of the ALN-calcium complex is known to be pH dependent [30]. The encapsulation of ALN based on its complexation with calcium was found to be pH dependent as well. ALN encapsulation decreased with pH (Fig. 2); the highest encapsulation was achieved at pH 7 at which the ALN-calcium complex is 2000 times less soluble in water than ALN itself. The agent used to adjust the pH is of no less

**Table 4**

The effect of ALN:Calcium ratio on the size, size distribution and entrapment of NP prepared by the double emulsion solvent diffusion (DES-D) technique using Pluronic F68 as the surfactant (in batch No. 6, calcium was in the internal phase)

Batch	ALN:Ca molar ratio	Size (nm)	PDI	Entrapment (%)
1	Na Ca	126	0.097	4.5±6
2	1:1	<sup>a</sup> Agg	Agg	Agg
3	1:2	123	0.008	83.5±3.3
4	1:3	126	0.103	72.7±3.9
5	1:4	120	0.154	65.6±1.7
6	1:3	128	0.076	77.1±1.24

<sup>a</sup> Aggregation, no NP.

**Table 5**

The effect of pH adjusting agent and ALN:Ca ratio on the size, size distribution and entrapment of NP prepared by the double emulsion solvent diffusion (DES-D) technique using Pluronic F68 as the surfactant

Batch	pH adjusting agent	ALN:Ca molar ratio	Size (nm)	Entrapment (%)
1	Ammonia	1:2	123	83.5±3.3
2	Ammonia	1:3	125.9	72.7±3.9
3	NaOH	1:2	<sup>a</sup> Agg	Agg
4	NaOH	1:3	110.3	19.8±3.0

<sup>a</sup>Aggregation, no NP.

importance than the final pH value (Table 5). At a molar ratio of 2:1 (calcium:ALN) aggregation occurred when NaOH was used for pH adjustment. At a molar ratio of 3:1 (calcium:ALN), pH adjustment with ammonia yielded better entrapment results in comparison to NaOH. It is possible that the ammonium ALN salt has a higher affinity for ethyl acetate than the sodium salt, thus enabling a better entrapment.

ALN NP treatment resulted in a significant inhibition of macrophage-like RAW 264 cells proliferation in a dose-dependent relationship (Fig. 5). The inhibition of Raw 264 cells by ALN NP prepared by the new DES-D method is slightly superior to the inhibition obtained with ALN NP prepared by the DES-E technique [25]. Treatment of rabbits with ALN NP (3 mg/kg) significantly depleted monocytes 48 h following IV injection (Fig. 6). The NP enable the penetration of the hydrophilic drug into the cells, as demonstrated with the hydrophilic fluorescent marker, HPTA, which was transported into the cells only when encapsulated in NP (Fig. 4). The in vivo monocytes depletion by ALN NP in comparison to free ALN indicates that ALN was successfully delivered into circulating monocytes by the NP and released at an appropriate rate to achieve the desirable effect. A similar impact on circulating monocytes was obtained by both liposomes and by NP prepared by the DES-E technique [16,25]. Examination of rats' arteries 14 days after balloon injury showed that IV injection before surgery of ALN NP (3 mg/kg) resulted in a significant inhibition of neointimal proliferation ( $P<0.05$ ) in the carotid artery rat model. The results are comparable to those obtained for ALN NP prepared by the DES-E technique [25]. The release profile of ALN from the NP (Fig. 3) was found suitable for the purpose of monocyte depletion. The drug release rate from the NP successfully addressed the aim of protecting the drug in circulation for a sufficient period of time to be taken up by monocytes, while still rapidly and sufficiently releasing the drug inside the cells.

## 5. Conclusion

The new DES-D preparation technique described here for the encapsulation of hydrophilic molecule in PLGA NP resulted in improved formulation characteristics including smaller size, lower size distribution, higher encapsulation yield, and more biocompatible ingredients in comparison to the classical DES-E technique, with unaltered bioactivity.

## Acknowledgments

This work was supported in part by grants from The Israel Science Foundation (126/00) and Biorest Ltd., Israel. GG is affiliated with the David R. Bloom Center for Pharmacy at The Hebrew University of Jerusalem.

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