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Abstract

Polymeric nanoparticles offer advantageous characteristics as gene-delivery vectors such as biocompatibility and biodegradability. With this aim, a smart and innovative strategy was followed here: Cationic PLGA nano-emulsions, prepared by a low energy method, were used as templates to obtain cationic nanoparticles (NPs) able to easily complex with nucleic acids (i.e. plasmid DNA) by electrostatic interactions. The strategy employed to produce stable positively-charged nanoparticles was the use of non-ionic/cationic surfactant mixtures to stabilize template nano-emulsions. This methodology allowed to obtain nanoparticles with reproducible nanometric sizes and positive zeta potential values, appropriate to successfully complex with nucleic acids, resulting in nanometric spherical polyplexes. Nanoparticles, plasmids and polyplexes proved to be biocompatible at the optimal concentration. Therefore, we can conclude that we have designed a novel strategy to efficiently obtain cationic polymeric nanoparticles that can be a promising approach to act as novel non-viral gene-delivery vectors, useful for many applications in gene therapy, such as gene vaccines.

Keywords	Polymeric nanoparticles, cationic nanoparticles, nano-emulsion templating, low energy methods, gene vaccines
Manuscript category	Regular Paper
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Dear Editor,

Please find attached the manuscript entitled ***PLGA cationic nanoparticles, obtained from nano-emulsion templating, as potential DNA vaccines*** by Eduard Soler Besumbes, Cristina Fornaguera, Marta Monge, María José García-Celma, Javier Carrión, Conxita Solans and Aurora Dols-Perez for its consideration to be published *European Polymer Journal* as Original research paper. The manuscript addresses a novel strategy to prepare cationic polymeric nanoparticles by nano-emulsion templating and the posterior conjugation with plasmid DNA to act as novel non-viral gene delivery vectors.

Polymeric nanoparticles offer advantageous characteristics as gene-delivery vectors such as biocompatibility and biodegradability. With this aim, a smart and innovative strategy was followed here: Cationic PLGA nano-emulsions, prepared by a low energy method, used as templates to obtain cationic nanoparticles (NPs) able to easily complex with nucleic acids (i.e. plasmid DNA) by electrostatic interactions. The strategy employed to produce stable positively-charged nanoparticles was the use of non-ionic/cationic surfactant mixtures to stabilize template nano-emulsions. This methodology allowed to obtain nanoparticles with reproducible nanometric sizes and positive zeta potential values, appropriate to successfully complex with nucleic acids, resulting in nanometric spherical polyplexes. Nanoparticles, plasmids and polyplexes proved to be biocompatible at the optimal concentration. Therefore, we can conclude that we have designed a novel strategy to efficiently obtain cationic polymeric nanoparticles that can be a promising approach to act as novel non-viral gene-delivery vectors, useful for many applications in gene therapy, such as gene vaccines.

We believe that the results obtained in this manuscript are of major interest and appropriate for the readership and scope of *European Polymer Journal* and we hope that you will consider it for publication.

Sincerely,

On behalf of the authors

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Highlights

- PLGA cationic nanoparticles were prepared using nano-emulsions (NE) as template
- Cationic surfactants used in the NE confer the surface charge to the NP
- PLGA cationic nanoparticles were successfully complex with DNA HisAK70 plasmids

PIC method

1 PLGA cationic nanoparticles, obtained from nano-emulsion 2 templating, as potential DNA vaccines

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1 **ABSTRACT**

2 Polymeric nanoparticles offer advantageous characteristics as gene-delivery vectors
3 such as biocompatibility and biodegradability. With this aim, a smart and innovative
4 strategy was followed here: Cationic PLGA nano-emulsions, prepared by a low energy
5 method, were used as templates to obtain cationic nanoparticles (NPs) able to easily
6 complex with nucleic acids (i.e. plasmid DNA) by electrostatic interactions. The strategy
7 employed to produce stable positively-charged nanoparticles was the use of non-
8 ionic/cationic surfactant mixtures to stabilize template nano-emulsions. This
9 methodology allowed to obtain nanoparticles with reproducible nanometric sizes and
10 positive zeta potential values, appropriate to successfully complex with nucleic acids,
11 resulting in nanometric spherical polyplexes. Nanoparticles, plasmids and polyplexes
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13 that we have designed a novel strategy to efficiently obtain cationic polymeric
14 nanoparticles that can be a promising approach to act as novel non-viral gene-delivery
15 vectors, useful for many applications in gene therapy, such as gene vaccines.

16

Graphical abstract: Schematic representation of the innovative methodology used to prepare cationic nanoparticles, from template nano-emulsions, and their resulting properties.

Keywords: Nano-emulsion templating, polymeric nanoparticles, gene-delivery vector, polyplexes, non-viral gene therapy.

INTRODUCTION

Nanomedicine is a promising field of study that takes advantage of the singular properties of nanomaterials to overcome the limitations of conventional pharmaceutical formulations[1-10]. Poor solubility in water, low bioavailability, non-effective targeting of the desired tissue or potential cytotoxicity are factors that limit the therapeutic benefits that can be obtained through classical drug and gene therapies. Nanotechnologies developed in the last years have raised expectations to overcome most of these drawbacks. One of the most promising approaches is the use of nanoparticles[11-16].

1 Nanoparticles are solid colloidal disperse systems that can have different properties
2 depending on the nature of their components[17, 18]. Lipids, carbon structures or
3 polymers as well as inorganic components can be used to prepare them for different
4 purposes. Most of the nanoparticles characteristics, such as surface charge or particle
5 size, can be modulated in a relatively easy way either by changing the components of
6 the formulation or by choosing a specific production process[15, 19]. In
7 pharmacotherapy, nanoparticles are outstanding devices as novel drug or gene delivery
8 systems. They can overcome the limitations of conventional carriers in terms of specific
9 targeting, sustained release, reduced toxicity or lower production costs. Moreover, these
10 properties allow the reduction of active component dose which in turn means a
11 minimization of undesired side-effects [11-16].

12 Polymeric nanoparticles have been widely investigated for biomedical purposes given
13 the benefits regarding biocompatibility and biodegradability of many polymers, both from
14 natural or synthetic origin, and the versatility in their production and use[10, 20-22].
15 Polymeric nanoparticles have proved to be useful for the delivery of small molecules,
16 both hydrophobic and hydrophilic, that can be entrapped, adsorbed, attached or
17 encapsulated[1-4]. Among different polymers, poly(lactic-co-glycolic) acid (PLGA) is one
18 of the most used synthetic polymers for the preparation of polymeric nanocarriers,
19 thanks to the approval of its use for medical applications by the FDA[23]. Consequently,
20 PLGA nanoparticles have been extensively investigated for sustained and targeted
21 delivery of proteins or peptides[16, 24-27].

22 In recent years, nanoparticles are being considered as gene delivery systems for gene
23 vaccines with improved properties such as the decrease of the immunization frequency

1 but, at the same time, the reduction of the unspecific immune response, which takes
2 place when using viral vectors [9, 26, 28]. Not long ago, DNA vaccines (also called *third*
3 *generation* vaccines) appeared as carriers of DNA encoding for specific proteins from a
4 pathogen[28-34]. One example is HisAK70 DNA-vaccine, where HisAK70 enhances
5 anti-leishmanial immunity at various stages [35, 36]. To improve the properties of these
6 vaccines, different types of vectors were used to facilitate cell internalization, and among
7 them, viral vectors were the most widely described and used, thanks to their efficiency
8 infecting cells[9, 37-39]. However, they do not show an adequate safety profile; hence,
9 non-viral gene-delivery systems have been studied lately and nanoparticles possess
10 properties to serve this purpose such as high biocompatibility and possibility to be easily
11 tuned to obtain desired functionalities. In addition, non-viral vectors such as polymeric
12 nanoparticles can circumvent the drawbacks that viral vectors present, such as the
13 difficulty to codify large genes, the low loading capacity and immunogenicity problems
14 related to repeated dosages (making viruses non-effective for people previously
15 exposed to environmental viruses of the same family) [38]. Interestingly, polyplexes,
16 including PLGA nanoparticles are efficiently taken up by macrophages and other
17 antigen-presenting cells (APCs). Moreover, PLGA-based nanoparticles, as advanced
18 delivery systems of plasmid DNA, induced a stronger and more sustained immune
19 response after immunization compared to naked DNA. These properties allow for
20 targeted DNA delivery and consequently, the potential therapeutic and preventive
21 applications in infectious diseases[40, 41]. Following this rising field and future
22 applications, our purpose is the preparation of cationic polymeric NPs by nano-emulsion
23 templating for their use as HisAK70 plasmid carriers that could be applied to the
24 development of anti-leishmanial vaccines.

1 Nano-emulsions are emulsions with small nanometric droplet sizes, typically between 20
2 and 200 nm, with a translucent to transparent visual aspect[42, 43]. The fact that the
3 interfacial area generated is big and the entropy increment small are the reasons why
4 nano-emulsions (as conventional emulsions) require an energy input to be prepared[42,
5 43]. The main emulsification methods described to produce nano-emulsions use highly
6 disruptive forces (high-energy methods) to achieve droplet sizes within the nanoscale
7 range resulting in expensive procedures[42, 44]. Low-energy emulsification methods
8 have gained increasing attention recently as they use the intrinsic chemical energy of
9 the system, thus producing nano-sized droplets with low polydispersity, avoiding the use
10 of high disruptive forces and expensive equipment. Moreover, low-energy emulsification
11 can be produced under mild process conditions avoiding the materials to be exposed to
12 extreme (temperature, pH, etc.) conditions[42, 43]. The Phase Inversion Composition
13 (PIC) method is the low-energy emulsification method more suitable for pharmaceutical
14 applications, when the system components commonly involve labile molecules. It
15 consists on the formation of nano-emulsions by changing the surfactant spontaneous
16 curvature from negative to positive (or *vice versa*) at constant temperature (e.g. at room
17 temperature)[42, 45]. The fact that this process is not performed under harsh conditions
18 makes it promising for further biomedical applications [24, 46-50]. Polymeric
19 nanoparticles can be prepared from nano-emulsion templating by two different methods:
20 the polymerization of a monomer or using preformed polymers. The last method is
21 advantageous because the polymer properties are well defined, no residual toxic
22 products from the polymerization are produced and no cross-reactions can take
23 place[18, 42, 44] .

1 In this context, the main objective of this work was to prepare stable cationic PLGA
2 nanoparticles with controlled particle nanometric size and stability to serve as carriers of
3 DNA HisAK70 plasmids. To accomplish this goal, cationic polymeric nanoparticles were
4 prepared by nano-emulsion templating using a cationic surfactant in the initial nano-
5 emulsion template formulation. This approach allows to create nanoparticles based on
6 PLGA and without additional steps of coating by cationic polymers, usually toxic.
7 Plasmid DNA was electrostatically attached to the surface of the nanoparticles, thus
8 forming polyplexes. Particle size, zeta potential and toxicity of the formulations was
9 assessed in order to confirm that these PLGA-based nanoparticles are suited to genetic
10 immunization strategy in a potential mammalian host.

MATERIALS AND METHODS

Materials

Poly(lactic-co-glycolic) acid (PLGA) 75:25 lactic:glycolic ratio and MW~10kDa (Resomer 752H, Evonik) was used as a biocompatible and biodegradable FDA approved polymer for the fabrication of nanoparticles[23]. Sodium chloride, di-sodium hydrogen phosphate and sodium di-hydrogen phosphate (Merck, Darmstadt, Germany) and MilliQ ultrapure water were used to prepare phosphate buffer saline (PBS) pH=7.4. Hepes (Sigma-Aldrich, Steinheim, Germany) MW~240g/mol and MilliQ ultrapure water were used to prepare 20mM Hepes buffer. Ethyl acetate (Fischer Chemical, Strasbourg, France) was used as organic solvent for nano-emulsion formation. Polyoxyl 35 Castor Oil (Kolliphor® EL, BASF SE, Ludwigshafen, Germany) and Varisoft® RTM 50 (Evonik Industries, Ruhr, Germany) were used as surfactants to stabilize the water/oil interface. Dialysis procedures were performed using Float-A-Lyzer®G2 Dialysis device MWCO: 3.5-5 kD (Spectrum Laboratories, Rancho Dominguez, California) and Spectra/Por® Dialysis Membranes MWCO: 3.5 kD (Spectrum Laboratories, Rancho Dominguez, California). Two different DNA plasmids, the control plasmid pVAX and the therapeutic plasmid pVAX-HisAK70, of 2916bp and 7342bp respectively, were used[35, 36]. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) from Sigma-Aldrich were used to determine cell culture viability. The human cell line HeLa was used to test the cytotoxicity of the nanoparticles. Dulbecco's modified eagle medium (DMEM) supplemented with fetal bovine serum (FBS, 10%) and 1 wt% of antibiotics (penicillin and streptomycin) was used as cell culture medium. Uranyl acetate was used to perform the negative staining of the samples to be imaged by Transmission

1 Electron Microscopy (TEM). Carbon coated grids (Cu with Formvar 0.5%, 200 mesh)
2 were used as substrates to support the TEM samples.

3 ***Polymeric nano-emulsion preparation***

4 Nano-emulsion preparation was the first step to obtain cationic polymeric NPs. Oil-in-
5 water (O/W) nano-emulsions were all prepared by the Phase Inversion Composition
6 (PIC) method at 25°C: The aqueous solution was added step-wise to a mixture of oil,
7 (4wt%PLGA in ethyl acetate), and the non-ionic and cationic surfactant mixture (NI:C) at
8 different ratios (Polyoxyl 35 Castor Oil and Varisoft RTM 50, respectively) at a constant
9 flow rate of approximately 1 ml/min under continuous stirring to obtain O/W structures
10 (Figure 1), as previously described[42, 44]. The total content of aqueous solution,
11 consisting on PBS, was 90 wt% in all formulations and pH and osmolality maintained at
12 7.4 and 300mOsm/kg respectively. Oil/surfactant ratio was 70/30, 80/20 or 90/10. Nano-
13 emulsion formation was assessed visually, by their transparent or translucent
14 appearance (a milky aspect indicates too large droplets).

Fig. 1. Schematic representation of nano-emulsion preparation by the PIC method and the subsequent formation of polymeric nanoparticles by solvent evaporation.

Polymeric nanoparticle preparation

Polymeric nanoparticles were prepared by solvent evaporation method, using polymeric nano-emulsions as templates[18, 44]. This procedure was performed under reduced pressure using a R-215V Rotavapor (Büchi, Germany) at 43 mbar and 25°C for 60 minutes at continuous rotation speed of 150 rpm. Subsequent to the solvent evaporation step, the volume of the nanoparticle dispersion was adjusted with MilliQ water as to

1 maintain the pH and osmolality of the precursor nano-emulsion (7.4 and 300 mOsm/kg
2 respectively).

3 ***Characterization of nano-emulsions and nanoparticles***

4 The mean hydrodynamic droplet size of the nano-emulsions and nanoparticles was
5 determined by dynamic light scattering (DLS) using a photon cross-correlation
6 spectrometer (Malvern, 4700PSMW) equipped with an Ar laser. The measurements
7 were performed at a scattering angle of 90° at 25°C. The values regarding droplet
8 diameters and polydispersity index (PDI) shown in the results section are the mean
9 values +/- the standard deviation values of at least three different measurements.

10 The zeta potential of the nano-emulsions and nanoparticles was assessed by
11 electrophoretic mobility measurements using a Zetasizer NanoZS instrument (Malvern
12 Co. Ltd., UK) equipped with a laser ($\lambda = 633$ nm). The zeta potential was calculated
13 applying the Hückel-Onsager equation (Eq. 1)[51]

$$14 \quad \mu = \frac{3 \cdot \zeta \cdot \epsilon_r \cdot \epsilon_0}{2 \cdot \eta} \quad (1)$$

15 where, ζ is the zeta potential, μ is the electrophoretic mobility, η represents the viscosity
16 of the media and ϵ_r and ϵ_0 stand for the relative dielectric constant of water and the
17 vacuum permittivity respectively. The values regarding the zeta potential of the samples
18 shown in the results section are the mean value of at least three different measurements
19 and the standard deviation values are also presented.

1 The long-term stability of the nanoparticle dispersions was assessed by determining
2 zeta potential and droplet size as a function of time both at 25° and 37°C for several
3 days.

4 DLS and zeta potential measurements were performed at the ICTS *NANB/OSIS* by the
5 Nanostructured Liquids Unit (U12) of the CIBER in Bioengineering, Biomaterials &
6 Nanomedicine (CIBER-BBN), located at the IQAC-CSIC.

7 ***Complexation of polymeric nanoparticles with plasmid DNA***

8 Prior to the complexation process, plasmid aliquots were diluted in Hepes buffer to a
9 concentration of 7.5 µg/ml. The complexation of cationic polymeric nanoparticles with
10 plasmid was achieved by electrostatic interaction. Cationic nanoparticles were mixed
11 with plasmid solutions, followed by their sonication (Ultrasounds-H Bath model, P-
12 Selecta, power = 200 W) for 5 min at 25°C. After the sonication process, the mixture was
13 incubated at 37°C during 30 min as described in literature[52] [45]. To assess the
14 formation of polyplexes, zeta potential was determined. Complexation is described to
15 take place in the range of 0 mV value where the charges are equilibrated[52] [45].
16 Various nanoparticle/plasmid DNA ratios (N/P) were assayed to find out the appropriate
17 complexation range.

18 ***Transmission electron microscopy (TEM)***

19 TEM micrographs of negatively stained samples were obtained with Jeol1010 TEM (Jeol
20 Korea Ltd., Tokyo, Japan) equipped with an accelerating voltage of 100 kV and a CCD
21 Megaview 1kx1k (Münster, Germany). Samples were prepared as following, a Formvar®
22 coated grid was placed Formvar side-down on top of a drop of sample for 10 min, 5 min

on uranyl acetate and 3min on MilliQ water, eliminating the excess of solution between steps. Later, the grid was placed in a Petri dish and let dry at room temperature overnight.

In vitro viability tests

In vitro cytotoxicity was assessed by measuring cell metabolic activity in contact with selected formulations. The NP samples tested were NP 1:1 (nonionic:cationic surfactant weight ratio 1:1), NP1:1+pVAX and NP1:1+pVAX-HisAK70, with a polymer concentration of 3mg/ml, 2.74mg/ml and 2.8mg/ml, respectively, and a DNA concentration of 320µg/ml and 241µg/ml. Tests were carried out with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay using HeLa cells, as previously described[53]. Cells were seeded in a concentration of $5 \cdot 10^3$ cells per well on 96-well plates in 200 µl of culture medium and cultured for 24h at 37°C under a 5% CO₂ atmosphere, to achieve 70 – 90% confluence. After that, culture medium was replaced by the samples at desired concentration (50µg sample/ml solution well) and incubated for 4 hours under the same conditions previously reported. Then, samples were replaced by culture medium and incubated overnight. The following day, MTT reagent was added to the wells at a final concentration of 0.5 mg/ml and incubated for 2 more hours under the same conditions. Subsequently, the medium was removed and 100 µl of DMSO were added to dissolve the formazan crystals obtained from the metabolic degradation of the MTT reagent. The plate was placed under agitation at 60 rpm for a period of 15 min at room temperature. Absorbance of the formazan was measured at $\lambda = 560$ nm using a SpectraMax M5 spectrophotometer (Molecular

Devices). Dilution with DMSO were performed to avoid the saturation of absorbance when needed.

RESULTS AND DISCUSSION

Preparation and characterization of polymeric nano-emulsions and nanoparticles

Nano-emulsions consisting of Hepes 20 mM (W)/nonionic:cationic mixture (S)/[4 wt.% PLGA in ethyl acetate](O) were prepared by the PIC method (detailed in the experimental part). As described previously, cationic surface charge is needed in order to further electrostatically attach DNA but, at the same time, low contents of surfactants are desirable to reduce their potential cytotoxic effect[2, 54, 55]. For this reason, a compromise is required between droplet size and stability on one hand and presence of cationic surfactant on the other hand. Thus, cationic surfactant is suspected to induce more irritant effect and cytotoxicity than nonionic due to its structure[56]. Different ratios of oil/surfactant (O/S), 70/30, 80/20 and 90/10, and different ratios of nonionic:cationic surfactants (NI:C), 1:1, 1:2, 2:1, were assayed in order to obtain the best compromise between small particle size and low cationic surfactant content as well as positive surface charge. Nano-emulsions with 90 wt% of aqueous phase exhibited translucent visual appearance, indicating small particle size and relative stability against sedimentation or creaming (data not shown). Hydrodynamic diameter and zeta potential results as a function of O/S and NI:C ratio are shown in Figure 2.

Nanoparticles prepared using a 90/10 O/S ratio presented the biggest sizes (higher than 500 nm), followed by the 80/20 samples. 70/30 O/S nanoparticles presented diameters

Fig.2. Hydrodynamic diameter (nm) and Zeta Potential (mV) of polymeric NPs for the different formulations.

1 lower than 200 nm. Small sizes are required to administer nanoparticles through a
2 parenteral route, in order to avoid embolization of blood vessels, decrease the
3 recognition of the immune system (i. e. less phagocytosis) and facilitate the crossing of
4 biological membranes, including plasmatic membrane[2, 57, 58]. The system that
5 showed most appropriate characteristics, small size, higher zeta potential and lower
6 cationic surfactant content, is the 70/30 1:1, showing zeta potential values higher than
7 10 mV and particle sizes of around 125 nm in diameter. Formulation 70/30 1:2,
8 containing the same O/S ratio and Ni:C molar ratio 1:2 showed also good properties with
9 zeta potential values up to around 19 mV and similar particle size (130 nm). Therefore,
10 both formulations, in addition to surface charge required for the final application,
11 accomplish the size requirements to be safely administered through parenteral route[2,
12 59]. These small sizes could be attributed to the use of an optimized nano-emulsion

templating method by PIC for the further formation of nanoparticles[42, 44]. It is also important to remark that both

selected formulations showed PDI 0.280 and 0.545 respectively. Low polydispersity of a nanoparticle dispersion is always desired. Similarities in structure homogenize nanoparticle behaviour making easier to predict the performance of the whole population in a physiological environment.

Concerning surface charge, previous studies claimed that positive surface charges negatively affected plasmatic membranes, since these are mainly anionic and cationic nanoparticles could electrostatically attach them, thus hampering their structure and physiological activity[2, 60]. However, other authors did not agree with this fact, since they argue that the positive charge is a must for the nanoparticle cell penetration, which is required to enable the expression of the genes codified. However, the release of the drug inside the cell is not always required, since some drugs affect cells from the extracellular matrix or surrounding components[61, 62]. Therefore, the most appropriate surface charge to enable cell penetration but avoiding cell toxicity would be a slightly positive nanoparticle[41] such are those selected in the current study. Thus, concerning surface charges, these nanoparticles are also appropriate for their use in biological systems.

The stability is another important parameter in terms of nanoparticles storage and use. Therefore, stability of both formulations, 70/30 with NI:C ratios of 1:1 and 1:2, was studied following the evolution in time of particle size and zeta potential, at 25 °C to identify the appropriate formulation (*Fig. 3*). Both formulations were stable during the

1 experimental time of 21 days after formation, as they do not exhibit considerable
 2 variation within this time. Therefore, since both formulations showed equivalent
 3 properties, the composition containing less content of cationic surfactant) was chosen to

Fig.3. Zeta potential (A) and hydrodynamic diameter (B) of NPs after preparation. Black squares represent NPs with NI:C 1:1 whereas blue circles represent NPs with NI:C 1:2 Max time represented 3 weeks. Values correspond to mean values $\pm$ standard deviation ($n = 3$)

perform further experiments, due to its expected low cytotoxicity. In the following we will refer to them as NPs 1:1.

Complexation of polymeric nanoparticles with plasmid DNA

Two plasmid DNA, pVAX and pVAX-HisAK70, were used with different total lengths, ~3000bp and ~7000bp, to study the model plasmid (the shorter one) and a therapeutic plasmid (with longer length). The *control plasmid* (pVAX) corresponds to a vector without any insert having a length of 2916 base pairs (bp), while the *therapeutic plasmid* (pVAX-HisAK70) corresponds to the control vector and an additional codifying fragment to have a total length of 7342 bp. The chosen formulation of NPs, 70/30 O/S and 1:1 NI:C, designated as (NPs 1:1), was used for the complexation with both plasmids by means of electrostatic interaction. To find out the most appropriate ratio of complexation, different ratios of NPs/plasmid (NP/P) were tested for both plasmids. NPs showed complexation ratios close to 5/1 and 10/1 when complexation was done with pVAX and pVAX-HisAK70 respectively, as can be seen in *Fig. 4*. As expected, the use of a longer plasmid required a higher nanoparticle amount, since it has a higher anionic charge that must be counteracted with a higher amount of the cationic component (the nanoparticles). Also, as expected, the ratio is proportional to the bp length. It is important to take into account that the active is a plasmid, a non-toxic compounds, thus, potential toxicity could come from the surfactant of the NPs. So, it is appropriate to reduce the NP to the minimum without affecting the complex stability.

Fig. 4. Zeta potential of complexes as a function of the NP/plasmid mass ratio for complexes of both plasmids. Black is used for complexes with pVAX and red for complexes with pVAX-HisAK70. Magnification of the complexation region is presented. Values correspond to mean values $\pm$ standard deviation ($n = 3$)

1 Long term stability was assessed for the complexes of both plasmids with NPs to
 2 evaluate the changes in zeta potential and particle size of the complexes with time.
 3 Stability was assessed at 25°C to obtain indications about storage conditions. Results of
 4 the long-term stability at 25°C are presented in *Fig. 5* showing zeta potential and particle
 5 size mean values for the experimental time of 21 days. Results showed small variations
 6 of zeta potential values, within the range of ± 1 mV, for both complexes during the period
 7 of 21 days, which indicate that plasmids remain attached to the nanoparticles. This study
 8 at 25°C is important to know the colloidal stability of the complexes.

Fig. 5. Zeta potential (A) and hydrodynamic diameter (B) of the complexes of NPs of the with plasmids at 25°C. Black is used for complexes with pVAX whereas red is for complexes with pVAX-HisAK70. Values correspond to mean values $\pm$ standard deviation ($n = 3$).

1

2 It is worth remarking the long stability of these plasmid-carrying nanoparticles.

3

1 **Transmission electron microscopy (TEM)**

2 Regarding nanoparticle size and shape, transmission electron microscopy analysis was
3 performed. Three samples were chosen: nanoparticles without plasmid DNA (NP 1:1)
4 and with each plasmid (NP 1:1/pVAX 5/1, NP 1:1/pVAX-HisAK70 10/1). Samples were
5 characterized at different magnifications and representative micrographs of each sample
6 are presented in *Figure 6*.

7 NPs 1:1 (*Fig. 6A*) showed diameters of around 130 nm and size polydispersity in
8 accordance with results obtained in DLS (*Figure 2*). While these nanoparticles had a
9 perfectly defined spherical shape, nanoparticles with plasmid as in *Fig. 6B and 6C*
10 showed rough surfaces that were associated with the presence of the plasmid
11 molecules. The bigger size of pVAX-HisAK70 plasmid compared with pVAX is
12 suspected to be the cause of the fibres of free DNA that were seen in *Fig. 6C* suggesting
13 that the plasmid is not fully packed around the cationic NP. Depending on the roughness
14 of the outside layer, DLS and TEM particle sizes could vary[63]. It is worth mentioning,

Fig 6. TEM images of negatively stained preparations. A) NP 1:1. B) NP 1:1/pVAX 5/1. C) NP 1:1/pVAX-HisAK70 10/1. D) Zoomed region from each sample (A-C) (All scale bars represent 100 nm)

1 that although the plasmid interacts with the outer part of the NP and it could induce to
2 think that is not an stable configuration, it has been demonstrated that the complexes
3 presented a good colloidal stability (Figure 5).

4 ***In vitro cytotoxicity tests***

5 Testing *in vitro* cytotoxicity is necessary in any formulation designed for biomedical
6 applications. Even though *in vitro* behaviour may not correlate directly with *in vivo*
7 results, it provides a quite reliable approach to identify toxicity associated with a
8 formulation at a given concentration. Many different tests are available nowadays to
9 assess the cell viability of a given sample; in this case MTT colorimetric assay, the most
10 used assay to assess cytotoxicity was selected [64, 65]. It measures cell viability by
11 means of mitochondrial activity and can give precise data concerning cellular metabolic
12 state.

13 MTT assay was performed to test the cytotoxicity of polyplexes and their components:
14 NPs and plasmid DNA. Results are shown in *Fig. 7*. All samples present an optimal
15 viability, higher than 70% [66], except concentrated (as prepared) cationic nanoparticles,
16 as expected, due to the cytotoxic potential of cationic surfactants. It is important to
17 mention that just a dilution to a third of the as-prepared concentration (3mg/ml to
18 1mg/ml), being polyplexes still more concentrated than the expected concentration of
19 administration, represents an increment of the cell viability to values greater than the
20 70%. It is important to mention that both model plasmids, pVAX and pVAX-HisAK70,
21 present good cell viability. Complexes formed with nanoparticles and plasmids, with
22 concentrations of plasmid around 1µg/ well (1.16 and 0.86, for NP/pVAX and NP/pVAX-

1 HisAK70) exhibited some differences showing better viability, slightly lower than 100%
 2 viability, the complexes formed with shorter plasmid. In contrast, polyplexes formed with
 3 pVAX-HisAK70 presented values around 73% of viability. Both present values of viability
 4 in the desired range. Authors would like to remark that this slight cytotoxicity is not a
 5 drawback for the further use of these nanoparticles for in vitro and even in vivo efficacy
 6 studies and application. In previous studies, it was demonstrated that with
 7 concentrations of 0.3 μ g plasmid/ well or even lower, the transfection efficiency is
 8 sufficient enough to produce a significant expression of the proteins encoded in
 9 encapsulated plasmids[41, 61, 67]. Therefore, these nanoparticles are not hypothesized
 10 to produce any toxicity at the concentration of use.

Fig. 7. In vitro cytotoxicity results of MTT assay represented as cell viability percentage. Control was only treated with DMEM culture medium. Samples correspond to plasmid solutions (pVAX and pVAX-HisAK70), NPs of the formulation 1 (NP 1:1 and diluted 1/3), NPs with plasmids (NP 1:1+pVAX, NP 1:1+pVAX-HisAK70). Values correspond to mean values $\pm$ standard deviation ($n = 3$)

CONCLUSIONS

The objective of this work was to obtain polymeric non-toxic nanoparticles with the best properties to form polyplexes with plasmid DNA. This objective was completely achieved. The best characteristics, 11.5 mV and 125 nm hydrodynamic diameter, were shown by the formulation with 90 wt% Hepes 20mM, 70/30 oil/surfactant ratio and 1:1 nonionic:cationic surfactant ratio. NPs were complexed with genetic material; specifically, plasmid DNA molecules, by means of electrostatic interactions in order to obtain formulations with potential use as non-viral gene-delivery vectors. The stability exhibited by the complexes formed with NPs was a remarkable feature, since it was longer than most dispersed nanoparticles. In addition, these nanoparticles, at therapeutic equivalent concentrations, showed no toxicity in cell culture models.

With all the results obtained we can conclude that polymeric nanoparticles can be useful gene delivery systems as plasmid DNA vaccines showing small sizes and easy complexation conditions as well as low cytotoxicity. Therefore, the generated nanoparticles could represent novel promising vaccine carriers and HisAK70–PLGA NPs should be evaluated in future studies on *in vivo* anti-leishmanial activity, in an attempt to develop control alternatives against leishmaniosis.

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