Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com

Development and characterization of polo-like kinase 2 loaded nanoparticles—The hypen seems to be elongated in the title of the manuscript.A novel strategy for (serine-129) phosphorylation of alpha-synuclein

C. Rodríguez-Nogales [a, 1], E. Garbayo [a, b, 1], I. Martínez-Valbuena [c], V. Sebastián [d, e], M.R. Luquin [b, c], M.J. Blanco-Prieto [a, b, *]

[a] *Pharmacy and Pharmaceutical Technology Department, University of Navarra, Pamplona, Spain*
[b] *Instituto de Investigación Sanitaria de Navarra (IDISNA), Pamplona, Spain*
[c] *Department of Neurology, Clínica Universidad de Navarra, Pamplona, Spain*
[d] *Chemical & Environmental Engineering Department & Nanoscience Institute of Aragon, University of Zaragoza, Zaragoza, Spain*
[e] *Networking Research Center on Bioengineering, Biomaterials and Nanomedicine, CIBER-BBN, Madrid, Spain*

ARTICLE INFO

Article history:
Received 29 April 2016
Received in revised form 16 June 2016
Accepted 17 June 2016
Available online xxx

Keywords:
Polo-like kinase 2
Alpha-synuclein
Phosphorylation
Nanoparticles
PLGA
TROMS

ABSTRACT

Polo like kinase 2 (PLK2), a serine/threonine serum inducible kinase, has been proposed to be the major factor responsible for phosphorylating alpha-synuclein (α-syn) at Serine-129 (Ser-129) in Parkinson's disease (PD). A suitable strategy to gain insights into PLK2's biological effects might be to increase PLK2 intracellular levels with the aim of reproducing the slow progressive neuronal changes that occur in PD. The goal of this study was to develop and characterize a novel drug delivery system (DDS) for PLK2 cytosolic delivery using Total recirculating one machine system (TROMS), a technique capable of encapsulating fragile molecules while maintaining their native properties. A protocol for nanoparticle (NP) preparation using TROMS was set up. NPs showed a mean diameter of 257 ± 15.61 nm and zeta potential of −16 ± 2 mV, suitable for cell internalization. TEM and SEM images showed individual, spherical, dispersed NPs. The drug entrapment efficacy was 61.86 ± 3.9%. PLK2-NPs were able to enter SH-SY5Y cells and phosphorylate α-syn at Ser-129, demonstrating that the enzyme retained its activity after the NP manufacturing process. This is the first study to develop a DDS for continuous intracellular delivery of PLK2. These promising results indicate that this novel nanotechnology approach could be used to elucidate the biological effects of PLK2 on dopaminergic neurons.

1. Introduction

Polo like kinase 2 (PLK2) is a serine/threonine serum inducible kinase expressed in mammalian cells that play pivotal roles in cell cycle regulation, mitosis and survival (Cizmecioglu et al., 2008; Li et al., 2014). PLK2 has been shown to phosphorylate alpha-synuclein (α-syn) at Serine-129 (Ser-129) (Inglis et al., 2009), this small peptide being closely linked to Parkinson's disease (PD) pathogenesis (Spillantini et al., 1997). In this regard, growing evidence about neurodegenerative disorders suggests that post-translational modifications of α-syn may contribute to the pathogenesis of PD and other synucleinopathies (reviewed in Dehay et al., 2015; Oueslati, 2016; Rodríguez-Nogales et al., 2016). It has been proposed that phospho(Ser-129)-α-syn might promote the formation of α-syn oligomers as the pathogenic species that precede α-syn aggregation. This abnormal aggregation of α-syn finally results into the formation of Lewy bodies (LB), the pathological hallmark of PD. While α-syn aggregation and toxicity remains to be fully understood, the role of PLK2 in the dopaminergic cell death process has yet to be elucidated. Notably, controversial publications have generated considerable debate, suggesting that PLK2 may have a putative toxic or protective attribute, which is probably due to the complex interplay of factors that occur in dopamine neurons. In this sense, Oueslati et al. (2013) demonstrated that PLK2 enhances α-syn clearance and suppresses its toxicity *in vivo* while Looyenga and Brundin (2013) suggested that prolonged periods of PLK2 activity may be detrimental to neurons. There is thus a lack of consensus concerning whether α-syn phosphorylation could lead to neuronal toxicity and LB aggregation.

Some recent studies have attempted to elucidate the biological effects of PLK2, mainly using kinase inhibitors and gene therapy, but their outcomes have been inconclusive (Bergeron et al., 2014; Buck et al., 2015). Since the PLK2 gene is transcriptionally responsive to common events in PD such as mitochondrial damage or reactive oxygen species, gene down-regulation or just the different behavior of the rodent brain could lead to equivocal conclusions. Perhaps a suitable strategy to gain insights into PLK2's biological effects might be to increase intracellular levels of PLK2 in a more physiological manner with the aim of reproducing the pathogenic neuronal changes that occur in PD. However, a major challenge in enzyme overexpression is the difficulty of administering functional proteins in an active conformation given the short half-life of these molecules and their com-

* Corresponding author at: Department of Pharmacy and Pharmaceutical Technology, School of Pharmacy, University of Navarra, C/Irunlarrea 1, 31080 Pamplona, Spain.
Email address: mjblanco@unav.es, maria.blanco@nanomedicinas.es (M.J. Blanco-Prieto)

[1] Contribute equally to this manuscript.

http://dx.doi.org/10.1016/j.ijpharm.2016.06.044
0378-5173/

plex structure, which can be highly sensitive to the environment (Holcenberg, 1982). The use of drug delivery systems (DDS) able to protect enzymes from degradation and to release the drug in a controlled manner over extended time periods might overcome these drawbacks. To date, several DDS such as microparticles (MPs), nanoparticles (NPs) or liposomes encapsulating enzymes have been designed to face not only enzyme deficiencies in metabolic diseases (Genta et al., 2001) but also to take advantage of the defensive role of enzymes such as catalase (Singhal et al., 2013), L-asparaginase (Gasper et al., 1998) and others. However, most of the methods employed to formulate these DDS rely on aggressive processes such as hot emulsifications or energetic sonication that could damage protein integrity and therefore impede its activity. Our group has proven experience in encapsulating growth factors and other labile molecules into polymeric MPs using Total Recirculation One Machine System (TROMS) technology, a technique based on the multiple emulsion solvent evaporation method that avoids shear stress and which is suitable for the encapsulation of labile molecules like proteins (Formiga et al., 2010; Garbayo et al., 2011, 2009; Pascual-Gil et al., 2015). Moreover, TROMS produces very homogeneous batches on a semi-industrial scale, which is of great interest for future industrial manufacturing. Copolymers of lactic and glycolic acids (PLGAs) have been used by our group and others to prepare protein-loaded particles because of their proven safety record and established use in marketed products for the controlled delivery of several peptide drugs (see, among others, Makadia and Siegel, 2011; Shive and Anderson, 1997). We therefore now hypothesize that polymeric NPs for cytosolic PLK2 delivery could be prepared by adapting the TROMS technology manufacturing process. The goal of this study was to develop and characterize a novel DDS for PLK2 cytosolic delivery using TROMS. To this end, first of all, a protocol for NP preparation using TROMS was set up. Then, NP physical characteristics such as morphology, size or surface charge as well as entrapment efficacy were investigated. Furthermore, in order to confirm that there was no loss of kinase activity during the manufacturing process, the biological activity of PLK2-NPs was examined *in vitro* detecting α-syn phosphorylation in SH-SY5Y cells by immunofluorescence. PLK2 was successfully encapsulated into polymeric NPs using TROMS. Moreover, PLK2 released from NPs was able to phosphorylate α-syn *in vitro*, demonstrating that TROMS is a reliable method to entrap active proteins while preserving its integrity. These promising results indicate that PLK2-NPs could be used to elucidate the biological effects of PLK2 on dopamine neurons.

2. Material and methods

2.1. Materials

SH-SY5Y neuroblastoma cells were kindly donated by Prof. J.L. Lanciego, (Area de Neurociencias, Laboratorio de Neuroanatomía de Ganglios Basales.CIMA, University of Navarra). Dubelcco`s Modified Eagle Medium (DMEM) cell medium and Phosphate Buffered Saline (PBS) was purchased from Lonza (Verviers, Belgium). Heat inactivated fetal bovine serum, Trypsin-EDTA and penicillin/streptomycin and MEM Non-essential amino acids were provided by Gibco® (Invitrogen Inc. Carlsbad, USA). Dimethyl sulfoxide (DMSO), and phosphotungstic acid hydrate from Sigma-Aldrich (Barcelona, Spain). Corning 24-well plate was provided by Corning, Inc. (NY, USA) Paraformaldehyde and goat serum were provided by Sigma-Aldrich (Barcelona, Spain). Lysis/binding solution concentrate TRIzol® was obtained from Ambion ThermoFisher, (Waltham, MA, USA). Alpha-synuclein polyclonal IgG antibody from rabbit was provided by Proteintech Group, Inc (USA). Anti-alpha-synuclein (phospho S129) monoclonal antibody from rabbit was provided by Abcam® (Cambridge, UK). Green fluorescent goat anti-rabbit antibody Alexa Fluor® 488 and 4′,6-diamidino-2-phenylindole (DAPI) were obtained from Thermo Fisher Scientific (Massachusetts, USA). Tween® 20 was purchased from Panreac Quimica S.A. (Barcelona, España). PLK2 active GST-tagged human recombinant protein, 685 amino acids length and 105.7 kDa from baculovirus-infected Sf9 cells was provided by Thermofisher life sciences (Carlsbad, USA). Poly (lactic-co-glycolic acid) (PLGA) 502H (Resomer® RG, 502 PLGA 50:50) was purchased from Boehringer Ingelheim (Ingelheim, Germany). Ethyl acetate was obtained from Panreac Applichem (Barcelona, Spain). Human serum albumin (HSA), poly (vinyl alcohol) (PVA) 88% hydrolysed (Mw-124.000), D-(+) Trehalose dehydrate and polyethylenglycol (PEG) 400 were purchased from Sigma-Aldrich (Barcelona, Spain). NuPAGE® Bis-Tris commercial gels were purchased from Novex®. Electrophoresis running buffer MOPS SDS 20%, transference buffer 10X Tris/Glycine and Weight marker Precision plus Protein Standards Kaleidoscope were provided by Bio-Rad (California, USA). Anti-SNK from rabbit primary antibody was purchased from Santa Cruz Biotechnologies, Inc. Secondary antibody Anti-rabbit IgG-Horseradish peroxidase from donkey was purchased from GE Healthcare (Little Chalfont, UK). Loading buffer LDS NuPAGE® was provided by Invitrogen™. Lumi-Light PLUS Western Blotting Substrate was provided by Roche (Mannheim, Germany).

2.2. Nanoparticle preparation

PLK2 was encapsulated into PLGA NPs by the multiple emulsion solvent extraction/evaporation method using TROMS. This method was adapted from (Garbayo et al., 2009, 2008). Briefly, the organic solution composed of 4 ml of ethyl acetate containing the polymer (50 mg of Resomer RG 502H) was added through a needle with an inner diameter of 0.17 mm at 40 ml/min into the inner aqueous phase. The inner water phase contained PLK2 in 200 μl of PBS, 2.5% w/v of HSA and 2.5% v/v of PEG400. Next, the previously formed inner emulsion (W_1/O) was recirculated through the system for 90 s under a turbulent regime at a flow rate of 40 ml/min. After this step, the first emulsion was injected into 20 ml of an aqueous phase (W_2) composed of 0.5% PVA. The turbulent injection through the needle with an inner diameter of 0.17 mm resulted in the formation of a multiple emulsion ($W_1/O/W_2$), which was further homogenized by circulation through the system for 300 s. The resulting $W_1/O/W_2$ emulsion was stirred for at least 3 h at room temperature to allow organic solvent evaporation and nanosphere formation. NPs were washed three times with ultrapure water by consecutive centrifugation at 4 °C (17000*g*, 10 min). Finally, NPs were mixed with D-(+) trehalose 37.5% w/w, frozen and lyophilized for long-term storage. Blank-NPs (drug-unloaded NPs) were prepared in the same manner without adding the PLK2.

2.3. Particle size and zeta potential analysis

The mean particle diameter and polydispersity index (PDI) were measured by dynamic light scattering (Zetasizer Nano, Malvern Instruments, UK). Samples were diluted with ultrapure water until an appropriate concentration of particles was achieved. The particle size analysis of each sample measurement was performed using a quartz cell at 25 °C with a detection angle of 90°. Using the same sample, the surface charge of the NPs was characterized by measuring the zeta potential with laser Doppler velocimetry (Zetasizer Nano, Malvern Instruments, UK) at 25 °C. Measurements were carried out

in triplicate and results were expressed as mean values ± standard deviation. At least three different batches were analyzed to give an average value and standard deviation for the particle diameter, PDI and zeta potential.

2.4. Particle morphology

The transmission electron microscope (TEM) images of the NPs were obtained with a FEI CM200 field emission TEM operating at an accelerating voltage of 200 kV. Sample preparation was performed by dispersing powder in methanol and ultrasounding for a few minutes, after which one drop was pipetted onto a carbon support film on a 3 mm copper grid. Image analysis on the alumina particles was carried out on various TEM images. The processing of the image files was performed on more than 500 particles using standard image analysis software "Soft-Imaging Software GmbH CM-Prof 2.11.002".

The scanning electron microscope (SEM) images of the NPs were obtained with a FE-SEM (Inspect F-50, FEI, Eindhoven) operating at an accelerating voltage of 10–15 kV. Sample preparation was done by dispersing the sample for 30 s in an ultrasonic bath. A drop of this suspension was applied to a glass slide. Then, the NPs were stained with phosphotungstic acid and were air-dried for 30 min at room temperature (RT) The glass slide was mounted on a stub of metal with adhesive, coated with 40–60 nm of Platinum by sputtering to reduce the sample charge during observation.

2.5. PLK2 entrapment efficiency

The PLK2 content in the NPs was quantified using western blot (WB) analysis. PLK2 was extracted from 1 mg of NPs with 25 μl of DMSO in order to perform the electrophoresis. Then, protein was transferred onto nitrocellulose membrane. After 1 h blocking with 5% nonfat dried milk in TBS plus 0.05% Tween 20, nitrocellulose sheets were incubated overnight at 4 °C with primary rabbit antibodies against PLK2 (diluted 1:300). The binding of primary antibodies was performed by incubating membranes with horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (diluted 1:2000). Immunoreactive bands were, after several washes, visualized using LumiLight Plus western blotting substrate. Quantitative analysis of NP extracted PLK2 bands was performed by densitometry using Image Quant RT ECL (GE, Healthcare). Sample values were quantified using a standard curve with ImageJ software.

2.6. In vitro PLK2 bioactivity assay: immunofluorescence against phospho(Ser-129)- α-syn in SH-SY5Y cells

The SH-SY5Y neuroblastoma cell line was cultured in DMEM medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 1% non-essential amino acids at 37 °C under 5% CO2/95% air. Culture media was changed every 2–3 days and the cells were sub-cultured when 80–90% confluency was achieved. For immunofluorescence assay, SH-SY5Y (5×10^4) cells were seeded in 24-well plates in 300 μl of complete growth culture medium. After 24 h, medium was removed and the cells were treated with PLK2 free and PLK2-NPs at 3.2 μg/ml and its proportional amount of blank-NPs (3.1 mg) for 24 h. After washing with PBS, cells were fixed in 4% paraformaldehyde 5 min at 4 °C. Then, cells were treated with blocking solution (PBS with goat serum, Tween® 20 0.05%). Cells were incubated with the anti-α-syn from rabbit antibody (diluted 1:25) and anti-phospho(Ser-129)-α-syn from rabbit antibody (diluted 1:1000) overnight. After washing, cells were incubated for 2 h with secondary goat anti-rabbit Alexa Fluor 488 (diluted 1:200). After washing with PBS, cells were incubated with DAPI for 5 min and observed with a fluorescence microscope (Zeiss Axio Imager M1).

2.7. RNA extraction and real time PCR

Total RNA was extracted from SH-SY5Y (1×10^5) cells after seeding and treating these cells with same conditions as detailed in the previous point with lysis concentrate solution according to manufacturer's protocol. 500 ng of total RNA obtained was used as a template to synthesize cDNAs with the PrimeScript RT-PCR Kit (Takara, Shiga, Japan). To detect the amplification products, RT-PCRs were performed on these cDNAs with Power SYBR® Green (Applied Biosystems, Foster City, CA, USA) and specific primers using an ABI Prism 7300 sequence detector (Applied Biosystems, Foster City, CA, USA). The primer sequences used for quantitative PCR were: for ribosomal protein 36B4 (used as an internal control) forward: TCGACAATGGCAGCATCTAC, reverse: TGATGCAACAGTTGGGTAGC; for PLK2 forward: AGCTGTAGCAGCAGCAGTGA, reverse CTCAGCTGCTCTTTGGGAAT. ΔCT was computed by subtracting CT (the number of cycles to reach threshold) for internal control gene from PLK2 CT.

2.8. Statistical analysis

Results are expressed as mean ± standard deviation of at least three independent determinations. Statistical analyses were performed using Prism 6 (GraphPad, La Jolla, CA, USA). Comparisons between different groups were performed using an Ordinary one way ANOVA. Probability (p) values less than or equal to 0.05 were considered to be statistically significant.

3. Results and discussion

3.1. Nanoparticle preparation

Elucidating the biological effects of PLK2 and the relationship between PLK2, α-syn phosphorylation and PD pathogenesis is essential for validating the potential of PLKs as therapeutic targets for PD and related synucleinopathies. However, its short half-life and physical instability hinder this task. Therefore, the use of NPs might be highly advantageous. Recently, nanotechnology has shown significant promise in enzyme nanoencapsulation (Gasper et al., 1998; Singhal et al., 2013; Vasudev et al., 2011). This technology may result in benefits such as protection from degradation, improvement of enzyme stability and drug release over a long period of time. In the present work, NPs were prepared by multiple emulsion solvent evaporation technique using TROMS. In addition to the fact that multiple emulsion solvent evaporation method is widely used to encapsulate proteins, TROMS technology avoids shear stress produced by sonication and ultraturrax, and consequently is very useful for preserving the bioactivity of an enzyme such as PLK2 during the NP manufacturing process. The structure of TROMS is shown in Fig. 1. The organic solution containing the polymer is injected through a needle into the inner water phase (W_1) containing the PLK2 to form the first emulsion. The first emulsion is homogenized by recirculation through the system, and then is pumped through a needle into the external water phase (W_2) that contains the emulsifier, which results in the formation of the multiple emulsion. Finally, the resulting multiple emulsion is further homogenized by recirculation through the system.

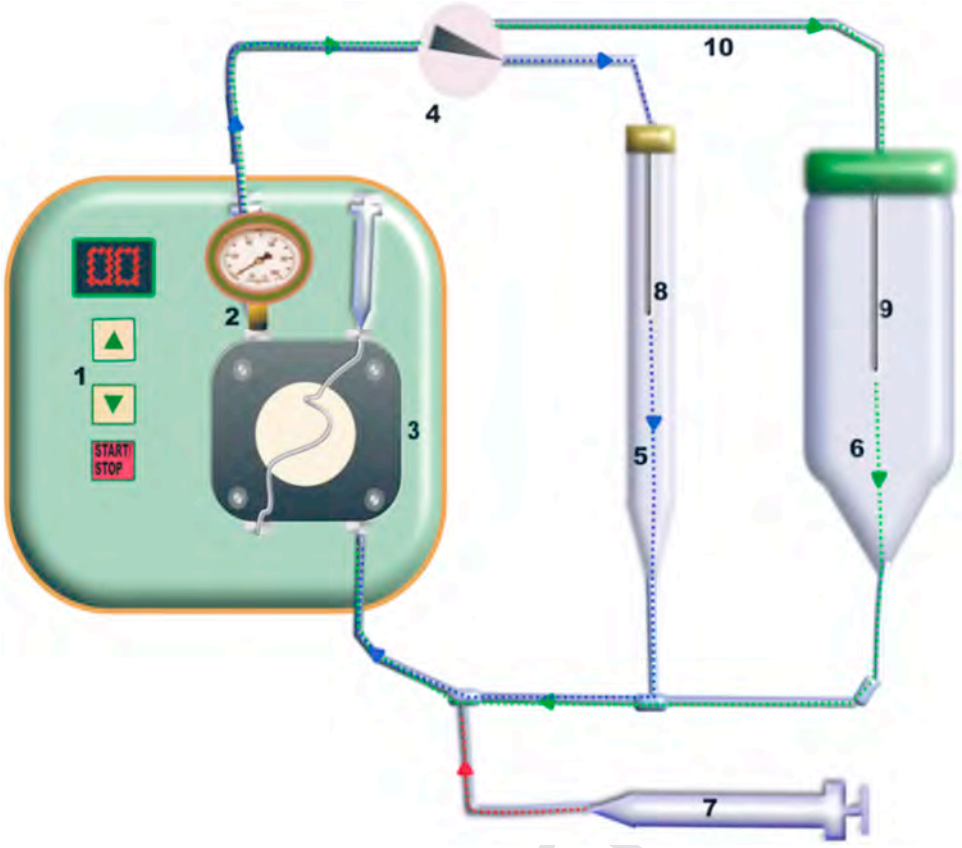

Fig. 1. Scheme of TROMS. 1 Flow panel control, 2 pressure manometer, 3 pump, 4 recirculating valve, 5 first emulsion container, 6 s emulsion container, 7 oleous phase syringe charger, 8 first emulsion needle, 9 s emulsion needle, 10 recirculating system connector.Figure 1 is too large in the pdf version of the proofs. Could be edited in one column style instead of two columns?

Another critical step associated with the retention of the enzyme's bioactivity is the water/organic interface of the emulsion. In this sense, HSA and PEG 400 were co-encapsulated with PLK2 to stabilize the primary emulsion and to reduce PLK2–polymer interactions (Garbayo et al., 2009, 2008). As mentioned above, the previous protocol used to prepare MPs between 5 and 10 μm was adjusted and optimized to prepare NPs using TROMS in order to decrease the size of the particles. Hence, the main goal of this optimization process was to achieve a negative charged polymeric particle of 250 nm in size with suitable re-suspension attributes. Particle size is crucial for delivery and uptake by the cells. Studies about cell uptake have suggested that particle sizes between 100 and 800 nm may achieve better ratios of cell internalization, endocytosis being the predominant mechanism (Qaddoumi et al., 2004). First of all, the flow rate was increased from 20 ml/min to 40 ml/min and a needle with a diameter of 0.17 mm instead of 0.50 mm was used during the double emulsion formation. In turn, particle size decreased sharply from 5 μm to 344 ± 5 nm. As seen in Table 1, other parameters that affected the particle size during the double emulsion formation using TROMS were the second emulsion recirculation time, the composition of the organic phase and the type of PLGA used. Next, particle size was reduced from 344 ± 6 nm to 283 ± 11 nm when the second emulsion recirculation time was increased from 120 s to 300 s, confirming a time-dependent tendency of the recirculation time. Then, the organic phase initially composed of dichloromethane: acetone (ratio 3:1) was replaced by ethyl acetate and a size reduction from 283 ± 11 nm to 273 ± 6 nm was achieved. This is probably due to the different viscosity and density of ethyl acetate. In addition, this organic solvent has been reported to be biocompatible and to not affect enzyme activity (Bilati et al., 2005). Particles formulated with PLGA 502H instead of 503H showed a moderate particle size reduction from 274 ± 6 nm to 261 ± 11 nm. Finally, PLGA 502H was selected for further experiments. The influence of the type of polymer on the size of PLGA-NPs has been previously described in the literature (Haddadi and Jahan, 2015; Mundargi et al., 2008). These studies suggest that an increase in polymer viscosity also results in an increased particle size.

Thus, the above results support the use of TROMS to formulate polymeric NPs of a size around 250 nm. A suitable particle size for cell internalization was obtained in compliance with a technique that does not use sonication, this method becoming optimal to entrap vul-

Table 1
Summary of the parameters that affect NP size prepared using TROMS. Second emulsion recirculation time, organic solvent composition and polymer type are the main parameters that can affect particle size and PDI.

Batch #	Polymer (50 mg)	Organic solvent (4 ml)	Second recirculation time (s)	Particle size (nm)	PDI
1	503H	DCM3:Ac1	120	344 ± 5	0.218 ± 0.004
2	503H	DCM3:Ac1	240	325 ± 10	0.174 ± 0.017
3	503H	DCM3:Ac1	300	284 ± 11	0.135 ± 0.012
4	503H	EtAc	300	271 ± 8	0.099 ± 0.014
5	502H	EtAc	300	261 ± 11	0.105 ± 0.014

Abbreviations: DCM3:Ac1, dichloromethane: acetone (ratio 3:1); EtAc: ethyl acetate; PDI: Polydispersity index.

nerable proteins such as PLK2. This final composition was used to formulate blank and PLK2-NPs.

3.2. Freeze-drying of nanoparticles

After NP preparation, formulations were lyophilized in order to preserve their characteristics for an extended period of time. Transforming the liquid nanodispersion into a dry product facilitates the storage of NPs for future administrations, including *in vivo* studies. In order to avoid particle aggregation during lyophilization and facilitate particle re-dispersions after the freeze-drying process, a cryoprotectant was added to the formulation. D-(+) trehalose has been reported to be one of the most effective cryoprotective agents in preventing particle growth (Bildstein et al., 2009; Jeong et al., 2005) and this is probably due to its low hygroscopicity or to the absence of internal hydrogen-bonds in comparison with other sugars (Abdelwahed et al., 2006). Based on these studies, D-(+) trehalose was chosen to evaluate its influence on the size and PDI after the freeze-drying and subsequent rehydration of NPs. D-(+) trehalose showed a moderate increase in the size of the NPs at low concentrations. Results showed that the addition of 100% w/w (50 mg) of D-(+) trehalose increases by 16.83 ± 5.63% the particle size after lyophilization while 50% (25 mg) w/w increases 20 ± 5%. In line with this trend, 37.5% w/w (18.75 mg) of D-(+) trehalose increases 30 ± 19% the particle size and 25% w/w (12.5 mg) increases by 39 ± 12%. Since the addition of D-(+) trehalose 37.5% w/w was able to provide suitable cryoprotection and complete re-dispersion of the NPs after freeze-drying while minimizing possible undesirable effects, this quantity of cryoprotectant was selected for further studies.

3.3. Particle characterization

Different batches of blank and PLK2-loaded NPs were prepared and particle size was measured by laser diffractometry. Fig. 2A shows Z-average size plot of 9 samples in which 6 were formulated on the same day and 3 on different days. Mean formulation Z-average was 257 ± 15.61 nm and PDI-average was 0.13 ± 0.02. PDI value is an indicator of the homogeneity of the formulation since NPs with PDI values between 0 and 0.3 are considered acceptable according to dynamic light scattering specifications. All the formulations exhibited a monomodal size distribution with only one population peak. In this sense, neither aggregation nor MPs was observed. Particle size was not modified when PLK2 was incorporated in the formulation. The need to determine biological effects based on intracellular changes requires the use of particles of a nanometric size around 250 nm to ensure that the nanocarrier is able to enter the cell predominantly via endocytosis, and to release the entrapped molecule. Reproducibility of the 9 different samples showed a coefficient of variation of 5.67% and standard error of the mean of 5.96. In this study, the coefficient of variation and standard error of the mean were considered acceptable values to confirm the reproducibility and robustness of the method with regard to size. Thus, TROMS has been demonstrated to be a highly feasible semi-industrial method for manufacturing NPs.

Particle surface was analyzed after the freeze-drying process. As shown in Fig. 2B, NPs exhibited a zeta potential of −16 ± 2 mV, which was attributed to the acid residues of PLGA in the polymeric matrix. These results are consistent with previous studies regarding polymeric particles (Panyam et al., 2002; Singhal et al., 2013). Zeta potential is a measure of the magnitude of the electrostatic charge repulsion/attraction between particles, and is one of the fundamental parameters that affect stability. Negative or positive values are characteristic of stable colloidal systems. However, positive charges might provoke a certain degree of toxicity *in vitro* (Xiao-Ru et al., 2015). On the other hand, neutral surface charges or dominant Wan der Waals forces have been reported to lead to particle aggregation (Van Gruijthuijsen et al., 2013.).

In the next stage, TEM and SEM characterization was performed to identify the particle morphology. As shown in Fig. 3A and B, individual, spherical, dispersed particles were observed, with no signs of aggregation. Particle size and distribution were consistent with the results obtained from the dynamic light scattering analysis. NPs were spherical in shape and with a smooth surface. The similarity of these NPs to other PLGA NPs developed using other conventional manufacturing methods (Han et al., 2015; Jaidev et al., 2015), also confirms that TROMS is able to formulate not only MPs but also NPs.

The amount of PLK2 encapsulated in polymeric NPs was quantified by WB. As shown in Fig. 3C, a band around 106 KD due to Glutation-S-transferase tag was detected. Lower molecular weight bands indicative of PLK2 degradation were not observed. These data indicate that the enzyme PLK2 was efficiently encapsulated in NPs. Encapsulation efficacy was found to be 61.86 ± 3.9%, which correspond to a final loading of 309 ± 19.5 ng of PLK2 per mg of polymer. Similar encapsulation efficacy values were found when enzymes such as L-asparaginase were entrapped in PLGA-NPs (Vasudev et al., 2011), as well as by our group when encapsulating glial cell-line neurotrophic factor (Garbayo et al., 2008). Thus, the WB results indicate that TROMS method is appropriate for encapsulating this kinase in NPs, providing a suitable efficacy of encapsulation.

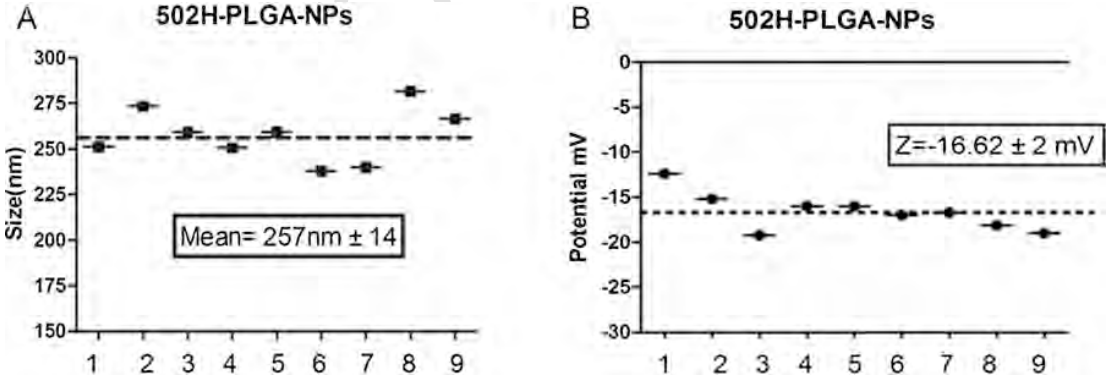

Fig. 2. NP size and zeta potential values of the optimized formulation (A) Particle size plot of 9 fresh formulations of 502H-PLGA-NPs. (B) Zeta potential plot of 9 freeze-dried and reconstituted formulations of 502H-PLGA-NPs.

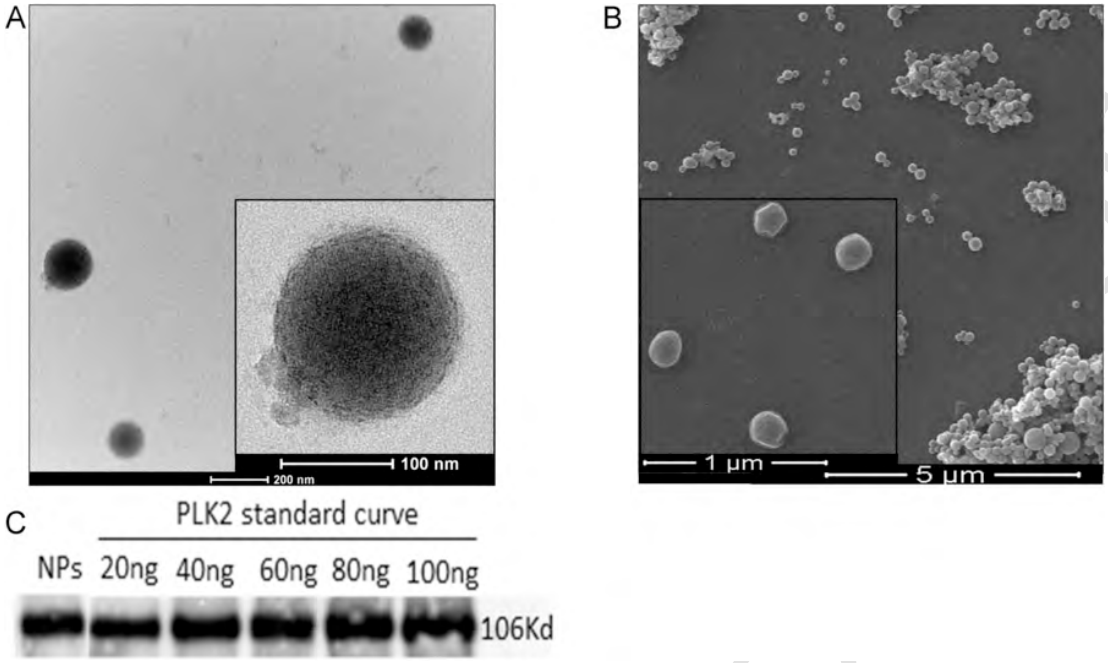

Fig. 3. Characterization of the NPs. (A) Transmission electron micrographs and (B) Scanning electron micrographs of representative PLGA NPs prepared by TROMS. (C) Encapsulation efficiency obtained by WB. Western blot bands corresponding to PLK2 standard curve and PLK2 encapsulated and extracted from NPsPosition of the "A" in Figure 3A seems to be now very close to the edge of the image..

3.4. In vitro bioactivity assay

Since the successful development of a NP DDS for PLK2 requires the preservation of the biological enzyme function through all the manufacturing process, next, the bioactivity of PLK2 released from the NPs was evaluated by assessing the phosphorylation of α-syn at Ser-129 in SH-SY5Y cells. SH-SY5Y human neuroblastoma cell line has been widely studied as an *in vitro* model of dopamine neuron for PD and other neurodegenerative disorders (Liu et al., 2015; Tai et al., 2011). First of all, we verified α-syn expression in the cytoplasm of SH-SY5Y by immunofluorescence against α-syn (Fig. 4). As can be seen in Fig. 4, this cell line possesses this intracellular peptide and so it is a suitable model to determine a hypothetical α-syn phosphorylation triggered by PLK2. According to this, the SH-SY5Y cell line should respond to bioactive PLK2 showing α-syn phosphorylation at Ser-129 in the cytoplasm.

Thus, having shown that SH-SY5Y has cytoplasmic α-syn, we sought to evaluate PLK2 bioactivity using this cell line. After 24 h of incubation of SH-SY5Y cells with the different treatments, immunofluorescence against phospho(Ser-129)-α-syn was performed using an antibody that specifically recognizes α-syn phosphorylated at Ser-129. The results showed that free PLK2 (Fig. 5A) and PLK2-NPs (Fig. 5B) at a concentration of 3.2 µg/ml were able to enter the cell and phosphorylate α-syn at Ser-129 while blank-NPs did not (Fig. 5C). Polymer toxicity was assayed on SH-SY5Y cells. No signs of cell death or toxicity were observed at 24 h with the polymer concentration used in these studies, confirming the biocompatibility of PLGA. A slight staining was observed in the blank-NPs and control (Fig. 5D). This is probably due to baseline phospho(Ser-129)-α-syn or to non-specific interaction of the primary antibody.

Finally, rtPCR was performed to evaluate the expression of PLK2 mRNA in the different SH-SY5Y treatment groups. No significant decrease in mRNA expression levels ($p = 0.348$) for PLK2 was observed in the SH-SY5Y cells treated with PLK2-NPs ($\Delta Ct = 5.67 \pm 0.15$), PLK2 free ($\Delta Ct = 6.53 \pm 0.14$) or Blank-NPs ($\Delta Ct = 6.66 \pm 0.10$) as compared to controls ($\Delta Ct = 6.48 \pm 0.11$) indicating equal expression of PLK2 among groups. The data are ex-

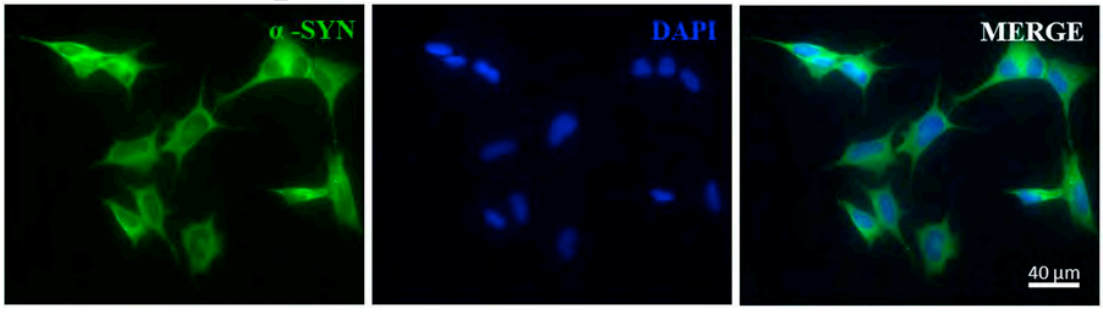

Fig. 4. Presence of α-syn protein within the cytoplasm of SH-SY5Y cells. Representative images showing SH-SY5Y cells stained with antibodies against α-syn and nuclei (DAPI) demonstrating the presence of α-syn in the cytoplasm of this cell line. Bar length 40 µm.

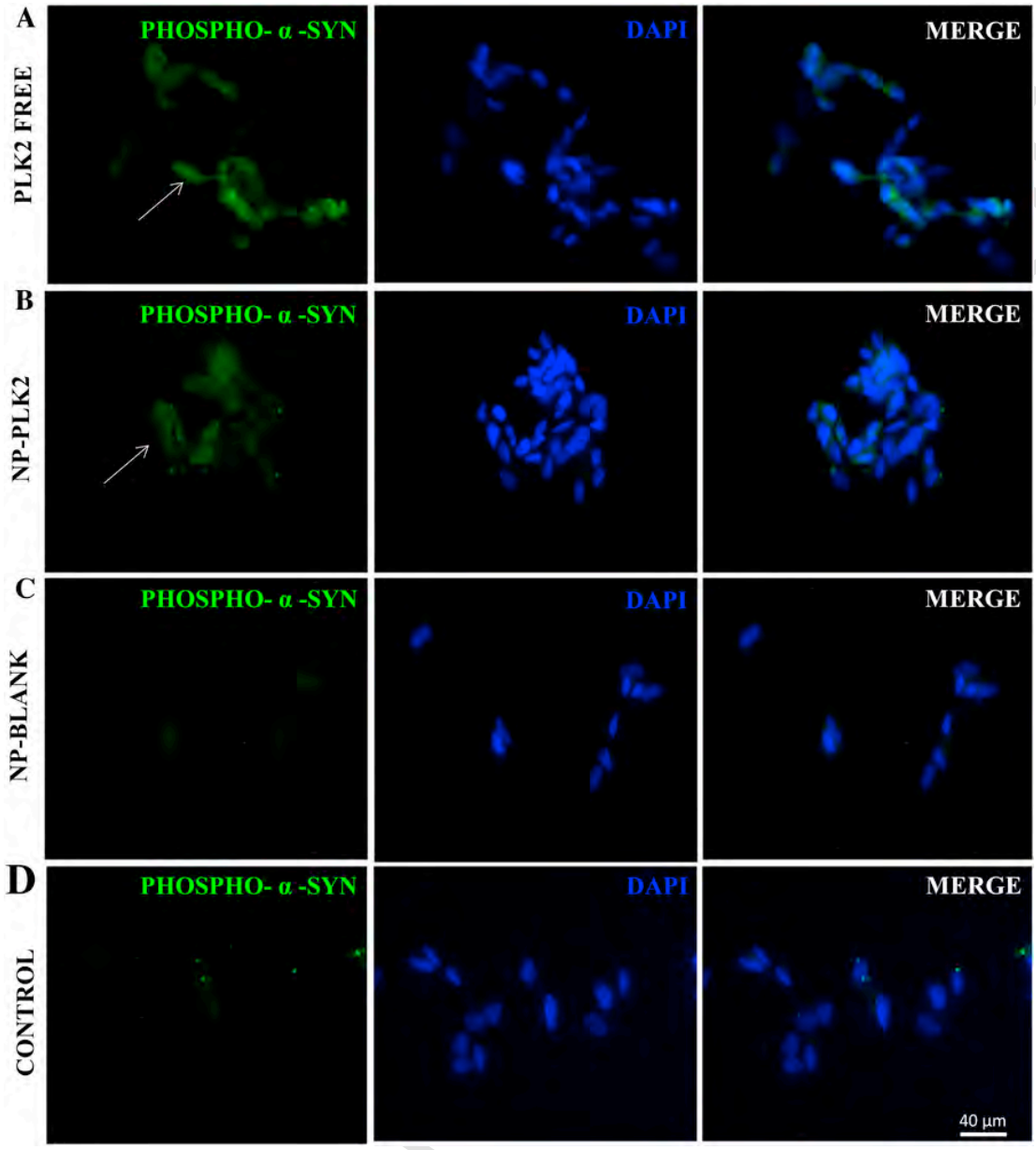

Fig. 5. Bioactivity of released PLK2 in a SH-SY5Y cell culture assay. Immunofluorescence against phospho(Ser-129) α-syn of cells treated for 24 h with (A) PLK2 free, (B) PLK2-NPs, (C) blank-NPs and (D) Control. The phosphorylation of α-syn at Ser-129 in the groups treated with NP-PLK2 and free PLK2 demonstrates that PLK2 remained bioactive after the nanoencapsulation process. Arrows in (A) and (B) indicate the cytoplasm of SH-SY5Y cells showing immunofluorescence signal against phospho(Ser-129) α-syn. Bar length 40 µm.

pressed in ΔCt values from triplicate sample average. This result therefore suggests that the increase in the Phospho(Ser-129)-α-syn found in the PLK2-NPs group was specifically due to the effect of PLK2 released by the NPs and not to different levels of PLK2 expression among groups.

As stated above, attention was focused throughout all the process of encapsulation on preserving PLK2 bioactivity. Using immunofluorescence assays we were able to successfully demonstrate that PLK2 remained bioactive after encapsulation with TROMS. Although PLK2 has the ability itself to enter the SH-SY5Y cells as demonstrated by immunofluorescence, elucidating the direct effect of increasing intracellular levels of PLK2 in the context of PD requires a sustained intracellular release of this protein over the time in order to resemble the physiological conditions of this neurodegenerative disorder. We have therefore developed a novel approach to increase the phospho(Ser-129)-α-syn in neurons based on a kinase cytosolic delivery.

Buck et al. (2015) used a recombinant adeno-associated viral vector to deliver PLK2 and demonstrated that the increase of phospho(Ser-129)-α-syn was not detrimental to cell survival. In this paper, we have developed a novel, more physiological approach for increasing intracellular levels of PLK2 using a non-viral DDS. Interestingly, nanotechnology has the potential to deliver higher payloads in a controlled manner compared to viral vectors without causing immunogenicity or cytotoxicity. Moreover, nanovectors are easier to synthesize than viral ones (Yin et al., 2014). Thus, it is worth noting that the

strategy proposed in this work may be an alternative option to viral vectors in order to increase PLK2 intracellular levels in a more physiological manner. Although no significant changes in SH-SY5Y cell morphology were observed when cells were treated with 3.2 μg/ml of PLK2, it is important to bear in mind that this human neuroblastoma cell line has potential defensive survival mechanisms. Whether this phosphorylation could be pathologic or not, and trigger α-syn abnormal aggregation, should form the subject of further studies. Now that we have demonstrated using the SH-SY5Y cell line that PLK2-NPs are an appropriate DDS, the next step will be to test its efficacy in a more suitable model of neurons, such as induced pluripotent stem cells that have been reprogrammed and differentiated to dopaminergic neurons. The present paper provides a new tool that could be helpful to clarify the role of PLK2 in PD and in an animal model of the disease, which will allow us to develop future strategies to treat this disorder.

4. Conclusions

The results of this study show for the first time that PLK2 can be successfully encapsulated in PLGA NPs using TROMS technology. Moreover, the encapsulated PLK2 remained bioactive and could phosphorylate intracellular α-syn at Ser-129 of SH-SY5Y cells. This may offer an alternative to viral vectors in order to increase PLK2 intracellular levels in a more physiological manner. This nanotechnology-based approach will help to clarify the concrete role of PLK2 in PD.

Acknowledgments

This work was funded by grants provided by the Instituto de Salud Carlos III (PI12/01730) and by FUN (University of Navarra). I. Martínez-Valbuena is beneficiary of a predoctoral fellowship from "Asociación de amigos de la Universidad de Navarra". Authors declare that they have no competing interests.

References

Abdelwahed, W., Degobert, G., Stainmesse, S., Fessi, H., 2006. Freeze-drying of nanoparticles: formulation, process and storage considerations. Adv. Drug Deliv. Rev. 58, 1688–1713.

Bergeron, M., Motter, R., Tanaka, P., Fauss, D., Babcock, M., Chiou, S.S., Nelson, S., San Pablo, F., Anderson, J.P., 2014. In vivo modulation of polo-like kinases supports a key role for PLK2 in Ser129 α-synuclein phosphorylation in mouse brain. Neuroscience 256, 72–82.

Bilati, U., Allémann, E., Doelker, E., 2005. Poly(D,L-lactide-co-glycolide) protein-loaded nanoparticles prepared by the double emulsion method—processing and formulation issues for enhanced entrapment efficiency. J. Microencapsul. 22, 205–214.

Bildstein, L., Hillaireau, H., Desmaële, D., Lepêtre-Mouelhi, S., Dubernet, C., Couvreur, P., 2009. Freeze-drying of squalenoylated nucleoside analogue nanoparticles. Int. J. Pharm. 381, 140–145.

Buck, K., Landeck, N., Ulusoy, A., Majbour, N.K., El-Agnaf, O.M.A., Kirik, D., 2015. Ser129 phosphorylation of endogenous α-synuclein induced by overexpression of polo-like kinases 2 and 3 in nigral dopamine neurons is not detrimental to their survival and function. Neurobiol. Dis. 78, 100–114.

Cizmecioglu, O., Warnke, S., Arnold, M., Duensing, S., Hoffmann, I., 2008. Plk2 regulated centriole duplication is dependent on its localization to the centrioles and a functional polo-box domain. Cell Cycle 7, 3548–3555.

Dehay, B., Bourdenx, M., Gorry, P., Przedborski, S., Vila, M., Hunot, S., Singleton, A., Olanow, C.W., Merchant, K.M., Bezard, E., Petsko, G., a Meissner, W.G., 2015. Targeting α-synuclein for treatment of Parkinson's disease: mechanistic and therapeutic considerations. Lancet Neurol. 855–866.

Formiga, F.R., Pelacho, B., Garbayo, E., Abizanda, G., Gavira, J.J., Simon-Yarza, T., Mazo, M., Tamayo, E., Jauquicoa, C., Ortiz-de-Solorzano, C., Prósper, F., Blanco-Prieto, M.J., 2010. Sustained release of VEGF through PLGA microparticles improves vasculogenesis and tissue remodeling in an acute myocardial ischemia-reperfusion model. J. Control. Release 147, 30–37.

Garbayo, E., Ansorena, E., Lanciego, J.L., Aymerich, M.S., Blanco-Prieto, M.J., 2008. Sustained release of bioactive glycosylated glial cell-line derived neurotrophic factor from biodegradable polymeric microspheres. Eur. J. Pharm. Biopharm. 69, 844–851 (e.V).

Garbayo, E., Montero-Menei, C.N., Ansorena, E., Lanciego, J.L., Aymerich, M.S., Blanco-Prieto, M.J., 2009. Effective GDNF brain delivery using microspheres—a promising strategy for Parkinson's disease. J. Control. Release 135, 119–126.

Garbayo, E., Ansorena, E., Lanciego, J.L., Blanco-Prieto, M.J., Aymerich, M.S., 2011. Long-term neuroprotection and neurorestoration by glial cell-derived neurotrophic factor microspheres for the treatment of Parkinson's disease. Mov. Disord. 26, 1943–1947.

Gasper, M.M., Blanco, D., Cruz, M.E., Alonso, M.J., 1998. Formulation of L-asparaginase-loaded poly(lactide-co-glycolide) nanoparticles: influence of polymer properties on enzyme loading, activity and in vitro release. J. Control. Release 52, 53–62.

Genta, I., Perugini, P., Pavanetto, F., Maculotti, K., Modena, T., Casado, B., Lupi, A., Iadarola, P., Conti, B., 2001. Enzyme loaded biodegradable microspheres in vitro ex vivo evaluation. J. Control. Release 77, 287–295.

Haddadi, A., Jahan, S.T., 2015. Investigation and optimization of formulation parameters on preparation of targeted anti-CD205 tailored PLGA nanoparticles. Int. J. Nanomater. 10, 7371.

Han, J., Michel, A.R., Lee, H.S., Kalscheuer, S., Wohl, A., Hoye, T.R., McCormick, A.V., Panyam, J., Macosko, C.W., 2015. Nanoparticles containing high loads of paclitaxel-Silicate prodrugs: formulation, drug release, and anticancer efficacy. Mol. Pharm. 12, 4329–4335.

Holcenberg, J.S., 1982. Enzyme therapy: problems and solutions. Annu. Rev. Biochem. 51, 795–812.

Inglis, K.J., Chereau, D., Brigham, E.F., Chiou, S.S., Schöbel, S., Frigon, N.L., Yu, M., Caccavello, R.J., Nelson, S., Motter, R., Wright, S., Chian, D., Santiago, P., Soriano, F., Ramos, C., Powell, K., Goldstein, J.M., Babcock, M., Yednock, T., Bard, F., Basi, G.S., Sham, H., Chilcote, T.J., McConlogue, L., Griswold-Prenner, I., Anderson, J.P., 2009. Polo-like kinase 2 (PLK2) phosphorylates α-synuclein at serine 129 in central nervous system. J. Biol. Chem. 284, 2598–2602.

Jaidev, L.R., Krishnan, U.M., Sethuraman, S., 2015. Gemcitabine loaded biodegradable PLGA nanospheres for in vitro pancreatic cancer therapy. Mater. Sci. Eng. C Mater. Biol. Appl. 47, 40–47.

Jeong, Y.-I., Shim, Y.-H., Kim, C., Lim, G.-T., Choi, K.-C., Yoon, C., 2005. Effect of cryoprotectants on the reconstitution of surfactant-free nanoparticles of poly(D,L-lactide-co-glycolide). J. Microencapsul. 22, 593–601.

Li, J., Ma, W., Wang, P.Y., Hurley, P.J., Bunz, F., Hwang, P.M., 2014. Polo-like kinase 2 activates an antioxidant pathway to promote the survival of cells with mitochondrial dysfunction. Free Radic. Biol. Med. 73, 270–277.

Liu, Y., Zhang, R.-Y., Zhao, J., Dong, Z., Feng, D.-Y., Wu, R., Shi, M., Zhao, G., 2015. Ginsenoside Rd protects SH-SY5Y cells against 1-methyl-4-phenylpyridinium induced injury. Int. J. Mol. Sci. 16, 14395–14408.

Looyenga, B.D., Brundin, P., 2013. Silencing synuclein at the synapse with PLK2. Proc. Natl. Acad. Sci. U. S. A. 110, 16293–16294.

Makadia, H.K., Siegel, S.J., 2011. Poly lactic-co-glycolic acid (PLGA) as biodegradable controlled drug delivery carrier. Polymers (Basel). 3, 1377–1397.

Mundargi, R.C., Babu, V.R., Rangaswamy, V., Patel, P., Aminabhavi, T.M., 2008. Nano/micro technologies for delivering macromolecular therapeutics using poly(D,L-lactide-co-glycolide) and its derivatives. J. Control. Release 125, 193–209.

Oueslati, A., Schneider, B.L., Aebischer, P., Lashuel, H. a, 2013. Polo-like kinase 2 regulates selective autophagic α-synuclein clearance and suppresses its toxicity in vivo. Proc. Natl. Acad. Sci. U. S. A. 110, E3945–54.

Oueslati, A., 2016. Implication of alpha-synuclein phosphorylation at S129 in synucleinopathies: what have we learned in the last decade?. J. Parkinsons. Dis. 6, 39–51.

Panyam, J., Zhou, W.-Z., Prabha, S., Sahoo, S.K., Labhasetwar, V., 2002. Rapid endo-lysosomal escape of poly(D,L-lactide-co-glycolide) nanoparticles: implications for drug and gene delivery. FASEB J. 16, 1217–1226.

Pascual-Gil, S., Simón-Yarza, T., Garbayo, E., Prosper, F., Blanco-Prieto, M.J., 2015. Tracking the in vivo release of bioactive NRG from PLGA and PEG-PLGA microparticles in infarcted hearts. J. Control. Release 220, 388–396.

Qaddoumi, M.G., Ueda, H., Yang, J., Davda, J., Labhasetwar, V., Lee, V.H.L., 2004. The characteristics and mechanisms of uptake of PLGA nanoparticles in rabbit conjunctival epithelial cell layers. Pharm. Res. 21, 641–648.

Rodríguez-Nogales, C., Garbayo, E., Carmona-Abellán, M.M., Luquin, M.R., Blanco-Prieto, M.J., 2016. Brain aging and Parkinson's disease: new therapeutic approaches using drug delivery systems. Maturitas 84, 25–31.

Shive, M.S., Anderson, J.M., 1997. Biodegradation and biocompatibility of PLA and PLGA microspheres. Adv. Drug Deliv. Rev. 28, 5–24.

Singhal, A., Morris, V.B., Labhasetwar, V., Ghorpade, A., 2013. Nanoparticle-mediated catalase delivery protects human neurons from oxidative stress. Cell Death Dis. 4, e903.

Spillantini, M.G., Schmidt, M.L., Lee, V.M.-Y., Trojanowski, J.Q., Jakes, R., Goedert, M., 1997. [Alpha]-Synuclein in Lewy bodies. Nature 388, 839–840.

Tai, K.-K., Pham, L., Truong, D.D., 2011. Idebenone induces apoptotic cell death in the human dopaminergic neuroblastoma SHSY-5Y cells. Neurotox. Res. 20, 321–328.

Van Gruijthuijsen, K., Obiols-Rabasa, M., Heinen, M., Nägele, G., Stradner, A., 2013. Sterically stabilized colloids with tunable repulsions. Langmuir 29, 11199–11207.
Vasudev, S.S., Ahmad, S., Parveen, R., Ahmad, F.J., Anish, C.K., Ali, M., Panda, A.K., 2011. Formulation of PEG-ylated L-asparaginase loaded poly (lactide-co-glycolide) nanoparticles: influence of pegylation on enzyme loading, activity and in vitro release. Pharmazie 66, 956–960.
Xiao-Ru, S., Xue-Qin, W., Xu, S., Li-Ying, H., Xiao-Xiao, C., Zhi-Rong, Z., Qiang, P., Yun-Feng, L., 2015. Independent effect of polymeric nanoparticle zeta potential/surface charge, on their cytotoxicity and affinity to cells. Cell Prolif. 48, 465–474.
Yin, H., Kanasty, R.L., Eltoukhy, A.A., Vegas, A.J., Dorkin, J.R., Anderson, D.G., 2014. Non-viral vectors for gene-based therapy. Nat. Rev. Genet. 15, 541–555.