Supplementary Information

Encapsulation of Nor-β-Lapachone into Poly(D,L)-lactide-co-glycolide (PLGA) Microcapsules: Full Characterization, Computational Details and Cytotoxic Activity against Human Cancer Cell Lines

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1. MATERIALS AND METHODS

Poly(D,L-lactide-co-glycolide) (PLGA 50:50, Mw 30,000-60,000) was purchased from Sigma Aldrich Co. Nor-β-Lapachone (NβL) was synthesized following the procedure described by da Silva Júnior *et al.*[1] Poly(vinyl alcohol) (Mw 13,000-23,000, 98% hydrolyzed), polyethylene glycol (PEG, Mw 4000 Da), dichloromethane, D(+) trehalose dehydrate, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], Alamar Blue and Doxorubicin were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). RPMI 1640 medium, penicillin and streptomycin were purchased from Gibco (Invitrogen, Carlsbad, CA). Fetal bovine serum was purchased from Cultilab (Campinas, SP). All the other chemicals employed in this study were of analytical grade and used as indicated by their manufacturers.

2. PREPARATION

2.1. Preparation of Nor-β-lapachone-loaded PLGA microparticles

PLGA microparticles were prepared by the conventional simple (oil/water) and double (water/oil/water) emulsion-solvent evaporation method.[2] NβL was dissolved in a polymeric solution of PLGA in dichloromethane and emulsified (Ultra-Tturrax T25, IKA, Germany) with aqueous PVA solution for 2 min at 11,000 rpm using an ice bath **(Figure S1, Table S1)**. By the way, in order to prepare the double emulsions, NβL was dissolved in a solution of PLGA in dichloromethane and emulsified (Ultra-Turrax) with aqueous PEG solution in an ice bath. Afterwards, this simple emulsion was added to a continuous aqueous phase, constituted of PVA, and emulsified again (Ultra-Turrax), resulting in a water/oil/water emulsion **(Table S1)**. In both processes, stirring was performed for another 4 hours after the last emulsification to evaporate the dichloromethane and produce a suspension of microparticles which were recovered by centrifugation and washed twice with deionized water. Following each washing step, the particles were recovered by centrifugation. Finally, the particles were suspended in trehalose aqueous solution after the third washing stage and frozen-dried to obtain lyophilized particles. Lyophilized microparticles were weighed, and the yield was calculated in percentages using the equation: percentage yield = [(weight of particles)/(weight of polymer + weight of NβL)] × 100.[3]

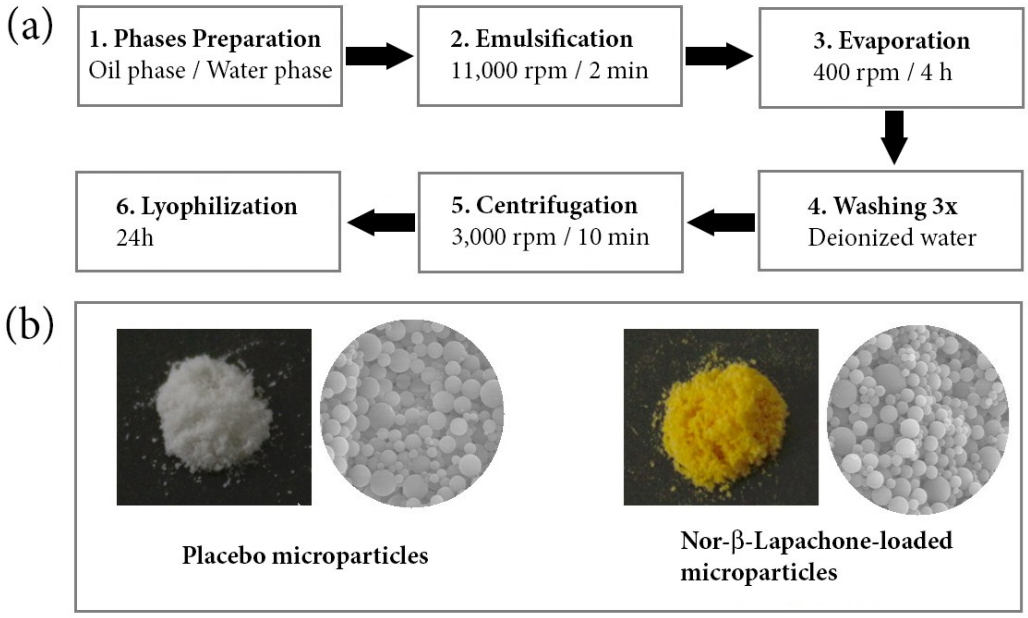

Figure S1. (a) Schematic representation of the preparation stages of PLGA microparticles obtained by simple emulsion (o/w). (b) Left: Empty PLGA microparticles as a lyophilized white powder. Right: PLGA microparticles containing the drug (NβL) as a lyophilized orange powder.

Table S1. Composition of PLGA microparticles formulated through the emulsion (o/w or w/o/w) process.

	Formulations (No) – O/W method									
Composition	**1[b]**	**2[b]**	**3[b]**	**4[b]**	**5[b]**	**6[b]**	**7[b]**	**8[b]**	**9[b]**	**10[b]**
PLGA 50:50 (mg)	200	200	100	100	100	150	100	50	150	100
Nor-β-Lapachone (mg)	10	10	10	5	8.5	10	5	2	5	8.5
Dichloromethane (mL)	20	10	10	10	10	10	10	10	10	10
2.0 % PVA solution (mL)	40	40	40	40	40	40	25	25	25	25
	Formulations (No) – W/O/W method									
Composition		**11[a]**	**12[a]**	**13[a]**	**14[a]**	**15[b]**	**16[a]**	**17[b]**	**18[a]**	**19[b]**
PLGA 50:50 (mg)		450	250	200	300	300	300	300	100	100
Nor-β-Lapachone (mg)		40	20	10	20	20	20	20	8.5	8.5
Dichloromethane (mL)		12	12	12	12	12	12	12	12	12
PEG 1 mg.mL^{-1} (mL)		5	5	5	-	-	-	-	-	5
PEG 1.5 mg.mL^{-1} (mL)		-	-	-	5	5	-	-	-	-
PEG 3.0 mg.mL^{-1} (mL)		-	-	-	-	-	5	5	-	-
0.5 % PVA solution (mL)		50	50	50	80	80	80	80	-	-
2.0 % PVA solution (mL)		-	-	-	-	-	-	-	40	40

PLGA indicates poly(d,l-lactic-co-glycolic acid); PVA indicates polyvinyl alcohol; PEG indicates polyethylene glycol 4000; (a) 7000 rpm; (b) 11000 rpm.

3. ANALYSIS

3.1. Microparticles morphology and size distribution.

The shape and surface morphology of PLGA microparticles were acquired and analyzed using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). To capture the images on the scanning electron microscope (SEM), samples suspended in deionized water were sonicated (15 min), placed on a cleaned silicon surface, and dried under vacuum. Subsequently, the microcapsules were coated with a conductive layer of gold in Baltec, MED 020 Coating System (Boeckeler Instruments, Tucson, AZ) in high vacuum. The scanning electron microscopes JEOL JSM 6360 LV (JEOL Europe SA, Croissy-sur-Seine, France) and FEI QUANTA 200F (FEI Company, USA) were used to carry out the measurements. For transmission electron microscope (TEM) images, the samples were dropped on a copper grid and dried at room temperature. Images were generated in a transmission electron microscope FEI model TecnaiTM G2 (FEI Company, USA). To examine their internal morphology using SEM, the microparticles were deposited on a silicon surface, dried under vacuum, frozen (-20°C), cut in aluminum foil and processed. Also, in order to follow the degradation over time, microparticles were dispersed in 1 mL of distilled water and incubated at 37°C. At different time intervals (t = 24, 48 and 120 h), some samples were collected, dried under vacuum and processed to be submitted to scanning electron microscopy. The diameters of 500 particles in several different fields were measured using the ImageJ software, and the average particle size was calculated afterwards following the procedure of Shahani and Panyam.[4]

3.2. Zeta potential determination.

The Zeta potential of the empty PLGA microparticles and PLGA microparticles containing NβL were determined using Zetasizer Nano ZS (Malvern Instruments Ltd, Worcestershire, UK). The lyophilized formulation was reconstituted in distilled water. This suspension was then rapidly dispensed in an electrophoresis cell to measure zeta potential values. The experiment was repeated three times, and the average zeta potential with the standard deviation was calculated.

3.3. UV absorption spectra.

Experimental measurements of the UV absorption spectra were carried out using the Varian Cary 5000 UV-visible NIR spectrophotometer (Varian Inc, USA), equipped with a solid sample holder. All measurements were done on pellets made from KBr mixed with the sample powder (proportion of 2 mg of sample to 100 mg of KBr). Absorption spectra were recorded by transmittance in the 200-800 nm (50000-12500 cm^{-1}) wavelength (wavenumber) range, background removal being performed by comparison with the absorption spectrum of pure KBr pellets. Baseline corrections were implemented when necessary.

3.4. FT-IR parameters.

FT-IR spectra of the pure NβL, empty PLGA microparticles and PLGA microparticles containing NβL were run on a KBr pellet (100 mg, 1 wt %). The spectra were collected over 40 scans at a resolution of 2 cm^{-1}. The absorption measurements were all performed in a FTLA 2000 series laboratory ABB Bomem. Baseline corrections were made in the spectral window from 400 to 4000 cm^{-1} for a better comparison of the obtained data. All vibrational modes in the obtained spectra were analyzed and compared.

3.5. FT Raman Parameters.

The FT Raman spectrum of the pure NβL, empty PLGA microparticles and PLGA microparticles containing NβL were recorded on a Bruker Vertex 70/RAM2 with Raman attachment that uses a 1064 nm Nd-YAG laser and a liquid-nitrogen cooled Ge detector. The line of the excitation source for recording the Raman spectrum was in the 80-4000 cm^{-1} region. The samples were measured in the hemispheric bore of an aluminum sample holder with 512 scans at a laser power of 500 mW and spectral resolution of 4 cm^{-1} .

3.6. Drug content and encapsulation efficiency.

Both the drug content and loading efficiency were determined in triplicate using a Varian Cary 5000 UV-visible NIR (Varian Inc, California, USA) spectrophotometer. To extract the NβL, known weights (~2 mg) of the lyophilized microparticles were broken with a mixture of dichloromethane and methanol (3:2) under ultrasound agitation for 30 min to ensure the complete polymer dissolution and release of the drug. The solubilized drug was quantified at 450 nm. A calibration curve spanning a NβL concentration range of 5.0–125.0 μg mL^{-1} was used to determine the drug content. The drug content of NβL in PLGA-microcapsules was calculated expressing the actual amount of entrapped drug as a percentage of the total microparticle mass. The encapsulation efficiency of NβL in PLGA-microcapsules was calculated taking into account the amount of encapsulated drug as a percentage of the NβL amount used in the process.[5]

3.7. Drug release.

In vitro release experiments were performed using a modified dialysis method.[5] Briefly, approximately 150 mg of NβL-loaded PLGA microparticles were put into a dialysis bag and suspended in 200 mL of release medium (PBS, pH 7.4). The suspensions were incubated in glass tubes in an incubation shaker Cientec, CT712RTN (Cientec, SP, Brazil) under stirring at 100 rpm at 37°C. A two millilitre sample of the solution was removed at predetermined time intervals and the concentration of NβL was analysed using a DTX880 Multimode Detector (Beckman Coulter Inc, California, USA) spectrophotometer at 450 nm. Releasing profiles were calculated in terms of cumulative releasing percentage of NβL (%) over the incubation time. Each experiment was carried out in triplicate.

3.8. Thermal analysis.

Pure NβL, PLGA microparticles with or without NβL were analyzed via differential scanning calorimetry (DSC) using a DSC-60 (Shimadzu Corporation, Kyoto, Japan) thermal analyser. Samples were heated from 25 to 220°C at a heating rate

of 10°C/min under azote atmosphere (50 mm/mim). Thermogravimetric analysis (TGA) was performed using a DTG-60H (Shimadzu Corporation, Kyoto, Japan). The samples were heated from 25 to 700°C at 10°C/min (azote atmosphere) and compared.

4. CELL LINES AND CELL CULTURES.

The human cell lines used in this work were SF-295 (glioblastoma), OVCAR-8 (ovarian), HCT-116 (colon), DU145 (prostate), PC3 (prostate) and PC3M (prostate), all obtained from the National Cancer Institute (Bethesda, MD, USA). The cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 $U.mL^{-1}$ penicillin, and 100 $\mu g\ mL^{-1}$ streptomycin at 37°C with 5% CO_2 atmosphere. Peripheral blood mononuclear cells (PBMC) were also tested. Briefly, PBMC were obtained from the peripheral blood of healthy volunteers after centrifugation on a Ficoll gradient. Afterwards, the cells were removed, washed with phosphate buffered saline (PBS) and resuspended in a RPMI 1640 medium supplemented with 20% of fetal bovine serum, 100 U mL^{-1} penicillin and 100 $\mu g.mL^{-1}$ of streptomycin to a final concentration of 3×10^5 cells mL^{-1}. Phytohemagglutinin (3 %) was added to induce cell proliferation.[6] The cells were treated with pure NβL, empty PLGA microparticles and NβL-loaded PLGA microparticles after 24 h of culture.

4.1. Cytotoxicity against tumor cell lines.

The biological evaluation of the pure NβL, empty PLGA microparticles and NβL-loaded PLGA microparticles started by studying the *in vitro* antiproliferative activity against six human tumor cell lines (SF-295, OVCAR-8, HCT-116, DU145, PC3M, PC3). The cell viability was determined by the reduction of a yellow dye 3-(4,5-dimethyl-2-thiazolyl)2,5-diphenyl-2H-tetrazolium bromide (MTT) to a blue formazan product, as described by Mosmann.[7] Briefly, the cells were distributed in 96-well plates (SF-295, OVCAR-8, DU145, PC3M and PC3 for 0.1×10^6 cells/well in 100 µL of medium and $0.7x10^5$ cells/well for HCT-116 in 100 µL of medium). After 24 h, NβL dissolved in DMSO, empty PLGA microparticles and NβL-loaded PLGA microparticles suspended in PBS pH 7.4 at concentrations in the 1–10 µM range were added to each well. Doxorubicin was used as positive control (0.02-8.6 µM). Control groups received

the same amount of DMSO. After 72 h of incubation, the plates were centrifuged and the medium was replaced with a fresh medium (150 µL) containing MTT (0.5 mg mL^{-1}). Only the prostate cancer cell lines (DU145, PC3 and PC3M) were evaluated three times (within 24, 72 and 96 h). Three hours later, the plates were centrifuged, and the MTT formazan product was dissolved in 150 µL DMSO. The absorbance was measured using a multiplate reader (DTX880 Multimode Detector, Beckman Coulter Inc, California, USA). Free and encapsulated drug were quantified as percentages of the control absorbance at 595 nm.

4.2. Inhibition of PBMC proliferation.

To investigate the selectivity of pure NβL and NβL-loaded PLGA microparticles towards normal proliferating cells, an Alamar Blue assay was performed with PBMC after 72 h of treatment.[8] Briefly, PBMC were plated in 96-well plates (2×10^4 cells/well in 100 µL of medium). After 24 h, pure NβL or NβL-loaded PLGA microparticles (1–10 µM) suspended in PBS pH 7.4 were added to each well, and the cells were incubated. Control groups received the same amount of PBS. Doxorubicin (0.02–8.6 µM) was used as a positive control. Twenty-four hours before the end of incubation, 10 µL of a stock solution (0.436 mg ml^{-1}) of Alamar Blue reagent was added to each well. The absorbance was measured using a multiplate reader (DTX 880 Multimode Detector, Beckman Coulter, Inc., California, USA). The drug effect was quantified as the percentage of the control absorbance at 570 nm and 600 nm.

5. MICROSCOPY IMAGES.

Adherence and internalization of PLGA microcapsules containing NβL were observed by optical and confocal microscopy. DU145 cells were seeded in 6-well plates at 3×10^4 cells/well, pre-incubated for 24 h, and further incubated with suspended PLGA microcapsules containing NβL (1µM) for 10, 20, 30 or 60 min. After the incubation period, the cells were processed (washed, fixed and stained or marked) for visualization using an optic microscope (Olympus, Tokyo, Japan) and a confocal microscope (Zeiss LSM 410, Germany). Hematoxylin/eosin-stained PC3M cells treated with NβL free and encapsulated were analyzed by light microscopy (400×) after 24, 48 and 72 h.

6. STATISTICAL ANALYSIS

All statistical analyses were carried out using GraphPad Prism software (Intuitive Software for Science, San Diego, CA, USA). The IC_{50} values for the MTT assay and Alamar Blue were obtained by nonlinear regression from three independent experiments performed in triplicate. The data are presented as mean ± S.D. from three independent experiments. Differences between experimental groups were compared by one-way ANOVA followed by Tukey's test for multiple comparison ($p < 0.05$).

7. CLASSICAL MOLECULAR DYNAMICS, ANNEALING AND DFT CALCULATIONS

In order to simulate the atomic structure of a typical PLGA nanoparticle surface, 100 units of glycolic acid ($C_2H_4O_3$) and 100 units of lactic acid ($C_3H_6O_3$) were employed to build a PLGA linear polymer with 1512 (904 non-hydrogen) atoms. Geometry optimization of the polymer was carried out using the polymer consistent force field (PCFF) with the following convergence parameters: energy variation less than 2×10^{-5} kcal mol^{-1}, maximum force per atom less than 0.001 kcal.mol^{-1}.$Å^{-1}$, and maximum atomic displacement less than 1×10^{-5} Å. After that, classical molecular dynamics and classical annealing simulations were performed to improve the quality of the molecular geometry of the polymer. We have chosen an initial temperature of 1000 K and a mid-cycle temperature of 2000 K to carry out 40 annealing cycles with 10 heating ramps per cycle and 500 dynamic steps per ramp and a time step of 1 fs. The PLGA geometry with the smallest total energy was then adopted to carry out the adsorption annealing of a previously-optimized NβL molecule, as the initial stages of drug release by the microparticules are due to the adsorbed drug. In this process, low-energy adsorption sites were sought after successive Monte Carlo searches across the configuration space of the substrate-adsorbate system with decreasing temperature. A set of 50 adsorption configurations were obtained to estimate the range of adsorption (binding) energies between PLGA and NβL, and three of them were selected to perform quantum density functional theory (DFT[9]) geometry optimizations: the configuration with largest binding energy (MAX), the configuration with smallest binding energy (MIN) and a configuration with intermediary binding energy value (MED). The generalized gradient approximation (GGA) for the exchange-correlation functional was adopted according

with the Perdew-Burke-Ernzerhof (PBE) parametrization[10] together with a dispersion correction scheme proposed by Tkatchenko and Scheffler.[11] The DMOL3 code[12] was used to carry out the computations using a double numerical plus polarization (DNP) basis set. Convergence thresholds along successive optimization steps were set to 10^{-5} Ha for the total energy variation, 0.002 Ha/Å for the maximum force per atom, and 0.005 Å for the maximum atomic displacement. The self-consistent field energy tolerance was adjusted to 10^{-5} Ha and a smearing scheme for the orbital occupancy was adopted to speed up the computations with smearing energy of 0.005 Ha.

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