Research Article

Influence of Surface Chemistry on Cytotoxicity and Cellular Uptake of Nanocapsules in Breast Cancer and Phagocytic Cells

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Abstract. The present work tests the hypothesis that stabilizers have a critical role on nanocarrier stealthiness and anticancer drug efficacy. Two different types of docetaxel (Doc)-loaded nanocapsules (NCs) stabilized with polysorbate 80 (NC_{T80}) and polyvinyl alcohol (NC_{PVA}) were synthesized using the emulsion solvent diffusion method. These NCs were characterized for particle mean diameter (PMD), drug content, morphology, surface composition, and degree of crystallinity. Furthermore, the cytotoxicity and cellular uptake of the NCs were investigated in MDA-MB 231 cells, THP-1 monocytes, and THP-1-derived macrophages. The optimized spherical NC_{T80} had 123.02±14.6 nm, 0.27±0.1, and 101±37.0% for PMD, polydispersity index, and drug encapsulation efficiency, respectively. Doc release kinetics from NC_{T80} and NC_{PVA} mostly provided better fit to zero-order and Higuchi models, respectively. Powder X-ray diffraction (PXRD) and X-ray photoelectron spectroscopy (XPS) results revealed the presence of amorphous stabilizers on the surface of the NCs. At high drug concentration, the cytotoxicity of NC_{T80} was substantially improved (1.3–1.6-fold) compared with that of NC_{PVA} in MDA-MB 231 cells. The uptake of both NCs was inhibited by latrunculin A and dynasore, indicating an actin- and dynamin-dependent endocytosis in MDA-MB 231 cells. This occurred *via* a multifaceted mechanism involving clathrin, caveolin, cytoskeleton, and macropinocytosis. Interestingly, the uptake of NC_{PVA} was 2.7-fold greater than that of NC_{T80} and occurred through phagocytosis in monocytes and macrophages. This study demonstrates the potential impact of the surface chemistry on the cytotoxicity and phagocytic clearance of nanocarriers for a subsequent improvement of the efficacy of Doc intended for breast cancer chemotherapy.

KEY WORDS: cancer chemotherapy; cytotoxicity; nanocapsules; phagocytosis; surface chemistry.

INTRODUCTION

The pharmacological effect of an anticancer agent can be drastically affected by the phagocytic clearance of its nanocarriers. An increased phagocytic clearance of nanocarriers can reduce its systemic half-life before reaching the tumor sites. Tumor accumulation represents only 1–10%

of the total injected dose of nanomaterials (1). Currently, research has been shifted towards the optimization of the nanocarrier surface chemistry for improved cancer diagnosis and therapy. In addition, polymeric nanoparticles (NPs) offer numerous benefits for improving drug stability, providing controlled release patterns, and reducing drug toxicity and side effects (2,3). However, the number of nanoparticulate systems approved by the US Food and Drug Administration (FDA) is still limited. This limitation has been attributed to the toxicity of the nanocarrier, its physicochemical instability, environmental impact, and extensive uptake by the mononuclear phagocyte system (4–7).

In order to address the above unwanted features, various stabilizing agents including polyvinyl alcohol (PVA), polyvinylpyrrolidone (PVP), polysorbate 80 (Tween® 80), poloxamer 407, and poloxamer 188 have been used (8–13). So far, PVA and Tween® 80 are the most extensively used stabilizers in the preparation of NP-based emulsions (14,15). Both stabilizers can adsorb on the surface of nanocapsules (NCs) leading to a change in the particle cytotoxicity and cellular uptake. To fill the gap related to the lack of fundamental knowledge in the nano-bio interactions, it is important to understand how the physicochemical properties

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of NPs govern its interaction with biological systems. Particle–cell interactions have been introduced and emphasized as a key process for the internalization of nanocarriers in mammalian cells (16). Besides the nanocarrier's size and shape, the surface chemistry plays a critical role in the particle–cell interactions. In this respect, nanocarriers' with well-characterized surface composition have been engineered and tested *in vitro* to improve our understanding of particle–cell interactions.

In this study, docetaxel (Doc), a lipophilic anticancer agent, was chosen as a model drug for its high antitumor activity in various types of human cancers including prostate, lung, ovary, and breast cancer (17). A randomized phase III study reveals the effectiveness of Doc compared to paclitaxel for the treatment of metastatic breast cancer (18).

The current work is focused on the impact of the surface properties of NCs on their cytotoxicity and phagocytic clearance. In that respect, effort was devoted to understand the mechanism underlying the particle endocytic pathways and its toxicity in breast cancer and phagocytic cells.

Care must be taken in studying the relationship between the surface properties of NPs and their potential toxicity. Hence, we hypothesized that the stabilizer might have a pivotal role on the nanocarrier's stealthiness, toxicity, and anticancer drug efficacy. The present study is divided into three sections. The “SECTION 1” is focused on the preparation and optimization of Doc-loaded NCs (NC_{T80}) using the Box–Behnken design (19). The “SECTION 2” is dedicated to the physicochemical characterization of the newly synthesized NCs stabilized with Tween 80 in comparison with our previously engineered oily core NCs stabilized with PVA (20). The “SECTION 3” is designed to investigate the influence of Tween 80 and PVA on the drug-loaded nanocarrier cytotoxicity and cellular uptake. Despite the number of contemporary works in particle–cell interactions, a complete understanding of this process is still needed. Advanced outcomes from the current study would significantly impact the development of nanomedicine in metastatic cancer chemotherapy.

MATERIALS AND METHODS

Materials

Poly(D,L-lactic acid) Resomer® R208 (PLA, Mw = 250 kDa) was purchased from Boehringer Ingelheim Inc (Ridgefield, CT). Polysorbate 80 (Tween® 80, Mw 1.3 kDa), polyvinyl alcohol (PVA, Mw 30–70 kDa), phosphate buffered saline (PBS), dichloromethane (DCM), dimethyl formamide (DMF), chloroform, boric acid, and ethyl acetate were obtained from Sigma-Aldrich (St. Louis, MO). Docetaxel (Doc) was provided by LC Laboratories from PKC Pharmaceuticals Inc (Woburn, MA). Labrafac® CC oil was kindly given by Gattefossé Corp. (Saint-Priest, France). Acetonitrile and methanol were purchased from Fisher Scientific (Pittsburgh, PA). Dylight 488 NHS ester was obtained from Thermo Fisher Scientific Inc (Rockford, IL). Sodium hydroxide (NaOH) was obtained from Fisher Scientific Company Inc (Hanover Park, IL). Anhydrous ether was purchased from Acros Organics (Morris Plains, NJ). All the reagents were of analytical grade and used without further purification.

Preparation of Docetaxel-Loaded Oily Core Nanocapsules

Doc-loaded oily core NCs were prepared by an oil-in-water (O/W) emulsion solvent diffusion method (21). Known amounts of Doc and PLA were dissolved in 8 ml of water-saturated ethyl acetate. The resulting solution was poured dropwise into 40 ml of ethyl acetate-saturated water containing 1% Tween 80 (v/v) or 5% PVA (w/v) (20) and homogenized (IKA, Ultra-Turrax T-25, Staufen, Germany) at 8,000 rpm for 10 min. To promote the diffusion process, a volume of 100 ml of deionized (DI) water was added to the previous emulsion under constant stirring (model Ro 15, IKA-Werke GmbH & Co, Staufen, Germany). After 1 h, the organic solvent was evaporated at 40°C for 30 min under reduced pressure (Rotavapor® RII, BUCHI Labor technik AG, Flawil, Switzerland). The NCs were washed three times using DI water *via* ultracentrifugation (L8-70M ultracentrifuge, Beckman Coulter Inc. Brea, CA) at 20,000 rpm for 30 min at 5°C. The purified NCs were finally resuspended in DI water containing 1% (w/v) of trehalose (Sigma Aldrich, Saint-Louis, MO) and lyophilized (Labconco Corp., Kansas City, MO). Fifteen formulations including three center points were prepared and optimized using the Box–Behnken design (Table S1 in Supplemental data).

Particle Mean Diameter and Polydispersity Index Analysis

The particle mean diameter (PMD) and the polydispersity index (PDI) of Doc-loaded oily core NCs were measured using dynamic light scattering technique (DLS, Malvern Instruments Inc, Houston, TX). A volume of 100 µl of colloidal suspensions was diluted ten times with DI water and sonicated for 2 min (Sonicator 3000, Misonix Inc, Farmingdale, NY). Sample measurements were performed at a scattering angle of 90° in triplicate at 25°C. According to the National Institute of Standards and Technology (NIST), monodispersity is considered when the PDI is below 0.05 (22).

Determination of Drug Loading and Percent Drug Encapsulation Efficiency

The freeze-dried NCs (3 mg) were dissolved in 2 ml of dichloromethane (DCM) and sonicated (Model 150D-bath sonicator, VWR, Darmstadt, Germany). After 2 min, DCM was evaporated under vacuum at 40°C for 30 min, and the pellet redissolved in 1.5 ml of the mobile phase. The resulting solution was sonicated at 25°C for 2 min, filtered through an organic membrane filter (Whatman International Ltd, Maidstone, UK), and transferred into an HPLC vial. The amount of Doc was determined by reverse-phase high performance liquid chromatography method (RP-HPLC, Waters Corp., Milford, MA). The analytical method was validated using a XBridge™, C18 column (4.6×150 mm, 3.5 µm particle size). The mobile phase consisted of methanol/water/acetonitrile (30:30:40, v/v/v) at a flow rate of 1 ml/min. The analyte detection was carried out by UV at 230 nm using a Waters 2487 Dual λ absorbance detector system.

The experimental loading of Doc was quantified using the peak area from each formulation-containing drug. The theoretical drug loading (DL%) and percent drug encapsulation

efficiency (EE%) were calculated using the following formulas (1) and (2), respectively:

$$DL(\%) = \frac{\text{Amount of drug}}{\text{Total amount of polymers} + \text{Amount of drug}} \times 100 \quad (1)$$

$$EE(\%) = \frac{\text{Experimental drug loading}}{\text{Theoretical drug loading}} \times 100 \quad (2)$$

Transmission Electron Microscopy (TEM) Analysis

TEM analysis was performed to determine the PMD and morphology of NCs using 100 kV with a JEOL JEM-1200 EX II Electron Microscope (JEOL Ltd., Tokyo, Japan). Samples were suspended in DI water and sonicated for 2 min at room temperature. A drop of the colloidal suspension was mounted on 300 mesh solid carbon grids (Ted Pella Inc, Redding, CA) and stained with 2% uranyl acetate (Sigma-Aldrich, St. Louis, MO) for 2 min at room temperature. TEM negatives were scanned at 508 dpi (Eurocore, Heidelberg, Germany), processed with ColorBrain 3.1 software (Eurocore), and coupled with Adobe Photoshop 5.0 software (Adobe, San Jose, CA). Furthermore, a light scattering microscope (LSM) coupled with a charge-coupled device camera, also known as nanoparticle tracking analysis, was used to visualize and record particles in aqueous phase.

Nanoparticle Tracking Analysis (NTA)

NTA was performed using a NanoSight LM20 (Malvern Instruments Inc, Houston, TX) equipped with a sample chamber, a 640-nm laser, and a Viton fluoroelastomer O-ring. NTA version 2.3 Build 0017 software was used for the capture and the data processing. Samples were injected in the sample chamber with sterile pipettes and analyzed at room temperature (23).

Powder X-ray Diffraction (PXRD) Analysis

PXRD analysis was carried out to determine the crystallinity of the formulation components of the NCs and the drug-loaded NCs as well. The MiniFlex-automated X-ray diffractometer (Rigaku, The Woodlands, TX) was equipped with Ni-filtered Cu K α radiation operating at 30 kV and 15 mA at room temperature. The diffraction angle was covered from $2\theta=5^\circ$ to $2\theta=40^\circ$ with a step size of $0.05^\circ/\text{step}$ and a counting time of 2.5 s/steps ($1.2^\circ/\text{min}$) for 30 min. The diffraction patterns were processed using Jade 8+ software (Materials Data, Inc., Livermore, CA).

X-ray Photoelectron Spectroscopy (XPS) Analysis

An analysis of the core level photoemission intensity as a function of the binding energy was conducted to identify the elemental and chemical composition of the surface of the NCs. X-ray photoemission spectra were acquired at a base pressure of 8×10^{-10} Torr using a Kratos Axis HS in hybrid mode with an Al K α anode ($h\nu=1,486.6$ eV) at a pass energy of 80 eV. Photoelectrons

were collected (after interaction with the sample surface) in angle-integrated mode. The electron energy analyzer's relative kinetic energy scale was calibrated as per ASTM E 2108-10 (24). The absolute binding energy was corrected for minor sample charging by referencing to the Ar 2p $_{3/2}$ core level at 241.3 eV with an accuracy of ± 0.1 eV. The calibration of the binding energy scale was performed using the Fermi level crossing of clean gold. Gold was chosen for its high density of states near the Fermi edge. A charge neutralizer was employed under constant potential with an anode current of 15 mA. Elemental quantification was completed by acquiring survey scans from -1000 to +5 eV of binding energy. The integrated area of the signal intensity (number of electrons detected) was divided by element-specific relative sensitivity factor (RSF) values and normalizing over all elements detected (25). Core level spectra were deconvoluted by using a Casa XPS software version 2.3.15. (Neal Fairly, UK) with 70%:30% Gaussian/Lorentzian functions fitted against a Shirley background (26). All spectra were normalized to a maximum intensity.

In Vitro Release Kinetics of Docetaxel from Oily Core Nanocapsules

In vitro drug release of the selected formulations was performed using a dialysis method (20). The powdered NCs (2 mg) were dispersed in a dialysis bag (Spectra/Por Float-A-Lyzer G2, MWCO 3.5–5 kDa, Spectrum Laboratories Inc, Rancho Dominguez, CA) containing 5 ml of PBS (pH 7.4) at 37°C as donor chamber. The agitation speed of the water bath (BS-06, Lab. Companion, Des Plaines, IL) was set at 50 rpm. A volume of 200 μl of the samples were collected from the receptor chamber containing 40 ml of PBS at appropriate time intervals of 0, 24, 48, 72, 96, 120, and 144 h and replaced with an equal volume of the fresh dissolution medium. The withdrawn samples (in triplicate) were diluted four to ten times with the mobile phase, filtered through a membrane filter (pore size 0.2 μm , Whatman nylon membrane) and transferred into HPLC vials. The amount of Doc in the release medium was determined by RP-HPLC as described above. Then, the average of the cumulative percentage of the released drug from NCs was calculated. To predict the drug release mechanism and kinetics, the *in vitro* release data were applied to various kinetics models including zero order, first order, Hixon–Crowell, Higuchi, and Korsmeyer–Peppas (27).

Model Validation

A theoretical optimum condition was obtained by setting the maximum desirability of minimum PMD, minimum PDI, and maximum EE%. Polynomial equations of response values for PMD (Y_1), PDI (Y_2), and EE% (Y_3) were derived from the total result of 15 runs, including three center points in three-factor, three-coded levels. An analysis of variance (ANOVA) was performed to evaluate the validity of the model. Formulation variables that significantly affect the PMD, PDI, and EE% were identified through a Pareto chart.

Furthermore, a checkpoint analysis was performed to validate the model for PMD (Y_1) and EE% (Y_3) of Doc-loaded NCs. A center point (0; 0; 0) and three random points, including C_1 (−0.5; 0; +0.5), C_2 (0; +0.5; −0.5), and C_3 (+0.5; −0.5; 0) were selected. Each checkpoint was prepared in triplicate and characterized for Y_1 and Y_3 .

Preparation of Dylight 488-Conjugated PLA-Based Nanocapsules

The DL488-labeled NCs were synthesized in three steps. First, amine modification of PLA was made by conjugating ethylenediamine (EDA, Sigma-Aldrich, St. Louis, MO) to PLA as previously published (28). Briefly, 0.28 μmol of PLA was dissolved in 5 ml of DMSO in the presence of 1.5 μmol of ethyl (dimethylaminopropyl) carbodiimide (EDC) and 2.24 μmol of N-hydroxysuccinimide (NHS) for 1 h at pH 5.5. Then, the activated PLA (PLA-NHS) was precipitated in 10 ml of anhydrous ether. Furthermore, the precipitate was put in 10 ml of cold ether/methanol mixture (1:1, v/v) and centrifuged at 8,000 rpm at 5°C for 20 min to remove the excess of the EDC and NHS. For the coupling reaction, the activated PLA was co-dissolved with 1.4 μmol of EDA in chloroform and maintained under stirring for 12 h at room temperature. To remove untreated EDA, the obtained conjugate (PLA-ethylene amine) was precipitated in 10 ml of cold methanol and centrifuged (14,000 rpm at 5°C for 20 min). The conjugate (PLA-ethylene amine) was dried under reduced pressure for 40 min at 50°C (rotavapor, BUCHI Labor technik AG, Flawil, Switzerland) and stored at 4°C.

Second, PLA-ethylene amine was conjugated with Dylight 488 NHS ester (DL488, 1:5 mol/mol) using the EDC chemistry adapted from the above method (28). DL488 is an amine reactive fluorescent dye. NHS esters react with primary amines to form a stable and covalent amide bond. Briefly, an appropriate amount of DL488 (0.31 mg) was transferred in a 15-ml tube containing 15 mg of PLA amine and dissolved in 10 ml of 0.05 M sodium borate buffer at pH 9. The obtained mixture was maintained under constant stirring at room temperature for 2 h in continuous darkness. The nonreacted dye was removed by overnight dialysis (Spectra/Por Float-A-Lyzer G2, MWCO 3.5–5 kDa). The obtained conjugate (PLA-DL488) was freeze-dried and stored at 4°C until further use. The degree of labeling was calculated according to the manufacturer's instruction (<http://www.piercenet.com/instructions/2161963.pdf>). The same procedure was used for the preparation of PLA-FITC in the presence of EDC (3.5 mg) and NHS (4.5 mg). The final molar ratio between PLA and FITC was 1:5 mol/mol.

Third, the labeled NCs were synthesized using a mixture of PLA and PLA-DL488 in a 10:1 ratio (w/w) to minimize any changes in the particle zeta potential. Two types of DL488 conjugated NCs were prepared using Tween 80 and PVA following the above NC preparation method. A calibration curve was constructed by plotting the fluorescence intensity *versus* the corresponding dye concentration (0.03–125 $\mu\text{g/ml}$, Fig. S1 in Supplemental data). This analysis was performed using a microplate reader at 485 $\pm$ 20 nm (excitation) and 525 $\pm$ 25 nm (emission). Furthermore, the association efficiency (AE%) of DL488 in the freeze-dried NCs (NC_{T80} and NC_{PVA}) was estimated using the following Eq. (3):

$$AE(\%) = \frac{A_C - A_P}{A_C} \times 100 \quad (3)$$

where A_C is the amount of DL488 associated with PLA/DL488 conjugate. A_P is the amount of DL488 associated with PLA after physical mixture and separation of PLA and DL488.

Determination of the Particle Recovery (Percent)

The percent of the particle recovery was indirectly determined by DLS from the difference between the initial (percent) and the final concentration of particles (percent) in the supernatant from cell lysate after 24 h of incubation with cells estimated from count rate. For this purpose, a standard curve was constructed by plotting the count rate of standard polystyrene NPs at concentration 4.1% (w/v) (190 nm, Invitrogen, Eugene, OR) *versus* the particle concentration (percent, w/v) in saline solution. Seven different concentrations of polystyrene NP (4×10^{-9} , 4×10^{-8} , 4×10^{-7} , 4×10^{-6} , 4×10^{-5} , 4×10^{-4} , and 4×10^{-3} (percent, w/v)) were prepared by suspending an appropriate amount of particles in 10 mM sodium chloride (NaCl). The colloidal suspensions were sonicated for 2 min and analyzed for the determination of the particle count rate (kilocycles per second) at position 1.25 mm. The initial and final concentrations of NC suspensions were estimated from the linear equation of the standard curve.

A simple linear relationship between the concentration of the NCs and the fluorescence value was established. The standard curve was obtained by spiking 100 μl of particles in complete media (0–300 $\mu\text{g/ml}$) into each well ($n=6$). The limit of detection (LOD) is the lowest concentration level that can be determined to be statistically different from a blank (99% confidence), while the limit of quantification (LOQ) is defined as equal to ten times the standard deviation of the results for a series of replicates to determine a justifiable limit of detection (<http://dnr.wi.gov/regulations/labcert/documents/guidance/-LODguide.pdf>).

Stability Test of Free Dylight 488 and Labeled Nanocapsules in Cell Culture Medium

For the stability of free DL488, experiments were run in triplicate in 96-well plates filled with 100 μl of complete growth medium containing different amounts of dye (12.5, 25, 50, 75, 100, and 125 $\mu\text{g/ml}$). At different time points (0, 2, 4, and 6 h), the fluorescence was read by a microplate reader at 485 $\pm$ 20 nm (excitation) and 535 $\pm$ 25 nm (emission). Furthermore, the stability of the DL488-loaded NCs was performed using a microplate reader over 6 h following the above method. Briefly, an amount of 7.5 mg of NCs was suspended in 15-ml centrifuge tubes (Sigma-Aldrich, St. Louis, MO) containing 6 ml of the complete growth medium and incubated in a water bath at 37°C for 6 h. At different times point (0, 2, 4, and 6 h), a volume of 100 μl of nanosuspension was withdrawn in triplicate and diluted ten times with 0.1 M NaCl. Data are expressed as scaled values with respect to their initial value and as averages of the replicates $\pm$ standard deviation (SD).

Cell Culture Conditions

Human breast carcinoma (MDA-MB 231) and THP-1 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA). MDA-MB 231 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) high glucose supplemented with 1% Minimum Essential Medium Non-Essential Amino Acid solution (MEM-NEA), 2 mM glutamine (Sigma-Aldrich, St. Louis, MO), 10% fetal bovine serum (FBS, ATCC, Manassas, VA), 1% penicillin-streptomycin (Gibco BRL, Grand Island, NY), and 1.7% MEM-Vitamin (Thermo Scientific, Rockford, IL). THP-1

monocytes and THP-1-derived macrophages were cultured in suspension, RPMI 1640 (Sigma-Aldrich, St. Louis, MO) containing 20% FBS. Cells were maintained in a humidified incubator (5% CO₂ at 37°C).

In Vitro Cytotoxicity of Blank and Docetaxel-Loaded Oily Core Nanocapsules

In vitro cytotoxicity was assessed using the MTS [(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium)] (29) assay according to the manufacturer's protocol. Briefly, MDA-MB 231 cells within 11 passages were seeded in 96-well plates (5×10⁵ cells/ml) and cultured in a complete growth medium to reach 80% confluence. To avoid any interference due to the absorbance of NCs at the same wavelength of the formazan (MTS assay dye) that can lead to erroneous results, Blank NCs (NC_{T80} and NC_{PVA}) were tested in the same conditions as well. The NC concentrations were estimated by subtracting the equivalent drug loading from the total weight of the NCs. For treated samples, the cells were incubated with NC_{T80} and NC_{PVA} at different concentrations (3–12%) for 48 h. Subsequently, 20 µl of Cell titer 96® Aqueous Non-Reactive cell proliferation assay kit was added to each well. After 4 h, the 96-well plate was placed on a DTX 800 multimode microplate reader (Beckman Coulter, Brea, CA), and the absorbance of the formazan product was measured at 490 nm. The percentage cell viability was determined using Eq. 4.

$$\text{Cell viability (\%)} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \times 100 \quad (4)$$

For treatment with Doc-loaded oily core nanocapsules, cells were incubated with samples at equivalent concentrations of Doc (0–100 µM) for 48 h. Two different types of NCs including Tween 80-stabilized NCs (NC_{T80}) and PVA-stabilized NCs (NC_{PVA}) were used at different concentrations of Doc. One column of the 96-well plate containing 100 µl of complete medium, cells, and blank NCs was used as background. The corrected fluorescence intensity was obtained by subtracting the background fluorescence intensity from the average value of that of the tested samples. Triton™ X-100 (1%, Sigma-Aldrich, St. Louis, MO) in 0.1 M sodium hydroxide (NaOH, Fischer Scientific, Hanover Park, IL) was used as positive control. The percentage of cell viability was evaluated after treatment with different concentrations of Doc-loaded NC_{T80} or NC_{PVA}. The acceptable cut-off criterion for cell viability was set to be 80% (30). Untreated cells were taken as reference to 100% cell viability. The percentage of cell viability was determined using the above Eq. 4.

Differentiation of THP-1 Monocytes into Monocyte-Derived Macrophages

Human leukemic cells (THP-1) within five passages were cultured in 96-well plates (2×10⁵ cells/ml) following the above conditions. Cells were treated with 200 µl of phorbol 12-myristate 13-acetate (200 nM, PMA, Sigma-Aldrich, St. Louis, MO) to mimic the monocytes differentiation into

macrophage phenotype (31). Adherent THP-1 cells were obtained after treatment with 5 nM of PMA. After 24 h of incubation, the medium was discarded and replaced with 200 µl of fresh cell culture medium without PMA for an additional 24 h (32).

Quantitative Measurement of the Particle Cellular Uptake

MDA-MB 231 cells (5×10⁵ cells/ml), THP-1 monocytes, or THP-1 monocyte-derived macrophages (2×10⁵ cells/ml) were separately incubated in each well with 100 µl of FITC-labeled NC_{T80} or NC_{PVA} suspensions (37.5, 75, and 150 µg/ml) at 37°C, 5% CO₂ for 24 h. After three-step washing with PBS solution, cells were resuspended in 100 µl of fresh culture medium. The extracellular fluorescence was quenched with 0.2% Trypan Blue (Gibco, BRL, Grand Island, NY) (33). This step was followed by three-step washing using a PBS solution. The quenching percentage (Quenching %) was calculated according to the following equation:

$$\text{Quenching (\%)} = \frac{F_{\text{without}} - F_{\text{with}}}{F_{\text{without}}} \times 100 \quad (5)$$

where F_{with} and F_{without} are the mean fluorescence of the control with and without the quenching solution, respectively.

Triton X-100 (1%) in 0.1 M NaOH was used to permeabilize the cellular membranes. The fluorescent intensity of FITC was measured using a microplate reader (Beckman Coulter) at excitation 485±20 nm and emission band pass 505–530 nm. Untreated cells were considered as negative controls. One column containing cells and NCs (NC_{T80} or NC_{PVA}) containing 150 µg/ml of FITC was considered as positive control.

Confocal Imaging

THP-1 monocytes and THP-1-derived macrophages (2×10⁵ cells/ml) were seeded in an eight-well chambered coverglass (NalgeNunc Inc., Rochester, NY). Both cells were incubated with DL488-labeled NC suspensions at different concentrations (37.5, 75, and 150 µg/ml) at 37°C. After 24 h of incubation, the extracellular fluorescence was quenched with 0.2% Trypan blue in PBS. After 10 min, cells were washed twice with PBS and fixed with a PBS solution containing 4% (v/v) paraformaldehyde (Sigma-Aldrich, St. Louis, MO) for 15 min. After three-step washing with PBS, the cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, Invitrogen Corp., Carlsbad, CA) for incubation. After 30 min, cells were rinsed twice with PBS and resuspended in 200 µl of complete growth medium. Confocal imaging was performed using a Leica TSC SP5 inverted confocal microscope (Leica, Wetzlar, Germany) with a ×40 objective. Emission profiles were recorded at 493–543 nm (argon laser, excitation at 485±20 nm). DAPI signals were collected using a 405-nm diode laser with a detection window set at 412–492 nm. LAS AF Lite 2.4.1 (Leica Microsystems CMS GmbH) and ImageJ 1.44 p (National Institute of Health, USA) were used for the image processing.

Identification of the Particle Cellular Uptake Pathways

Prior to the identification of the particle cellular uptake pathways, the authors aimed to investigate three critical points. First, the intrinsic cytotoxicity of the selected chemical inhibitors on the cell viability was determined by MTS assay (34). The concentration and the incubation time of the selected pharmacological inhibitors were obtained from the literature (35–37). Second, the differential fluorescent interference between the complete medium containing cells and the selected pharmacological inhibitors (at their working concentrations) was determined using a microplate reader at emission wavelength of 485 ± 20 nm and excitation of 525 ± 25 nm. Third, molecules or particles that are known to selectively enter cells *via* a specific pathway were used as positive controls to confirm the activity of a given endocytic pathway. The positive controls included transferrin (Tf, Sigma-Aldrich, St. Louis, MO), cholera toxin B (CT-B, Invitrogen Corp., Carlsbad, CA), FITC-albumin (ALb, Sigma-Aldrich, St. Louis, MO), low-density lipoprotein (LDL, Lee Biosolutions, Inc, St. Louis, MO), and latex beads ($\varnothing$ 2.4 μ m, LTx, Sigma-Aldrich, St. Louis, MO) for clathrin, caveolae, cytoskeleton, macropinocytosis, and phagocytosis-mediated endocytic pathways, respectively (29,38–40). Tr and LDL were conjugated to DL488 in a 1:1 ratio (mol/mol) (see method in [Supplementary material](#)). The final concentrations of the positive controls were as follows: Tr (0.1 mg/ml), CT-B (0.005 mg/ml), ALb (0.05 mg/ml), LDL (1.25–3.00 mg/ml), and latex beads (0.12% w/v). Cells were exposed to different positive controls in complete medium for 2 h at 37°C with or without their respective pharmacological inhibitor. The cells were then washed twice in PBS, pH 7.4, and lysed in 0.1 M of NaOH containing 0.1% Triton X-100 at 37°C for 40 min. The fluorescence of the different positive controls was determined using a microplate reader at 485 ± 20 nm (excitation) and 525 ± 25 nm (emission) excepted for latex beads where the excitation and emission wavelengths were 360 and 420 nm, respectively. The percentage of fluorescence uptake of each positive control was adjusted for background.

For the particle uptake inhibition, several pharmacological inhibitors were used to identify the underlying endocytic pathways in MDA-MB 231, THP-1 monocytes, and THP-1-derived macrophages. Actin polymerization was inhibited by incubating cells with 1 μ M latrunculin A (LAT-A, Tocris Bioscience, McKinley Place NE, MN) for 30 min (35). In parallel, another group of cells was pretreated with 80 μ M dynasore (DYN, Tocris Bioscience), a noncompetitive inhibitor of dynamin GTPase activity that can block the cellular dynamin-dependent endocytosis (35). Cytoskeleton reorganization was prevented by incubating cells with 10 μ g/ml cytochalasin D (CCD) for 30 min. Macropinocytosis was promoted through cell pretreatment with 1 μ M of phorbol myristate acetate (PMA) for 30 min. To disturb the clathrin-dependent endocytosis, cells were pretreated with 10 μ g/ml chlorpromazine (CPZ) for 30 min. To perturb caveolin-mediated pathways, cells were treated with 200 μ g/ml genistein (GNT) for 30 min (36). Additionally, THP-1 monocytes and their derived macrophages were pretreated with 100 μ M sodium azide (NaN_3) for 30 min to impair the

active uptake of NCs through phagocytosis. The above five chemical inhibitors were purchased from Sigma-Aldrich (St. Louis, MO). After treatments, cells were washed twice with PBS and incubated with FITC-loaded NCs (150 μ g/ml) for 24 h. After incubation, cells were washed thrice with PBS. The following steps are the same as described above.

Data Analysis

Experiments were repeated at least three times and data were expressed as mean $\pm$ standard error of the mean (SEM). A two-tailed Student's *t* test was performed to test the significance of the particle uptake compared to control (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

RESULTS

Optimization of the Formulation of Docetaxel-Loaded Tween 80-Coated Nanocapsules

Results of NC_{PVA} formulation have been published in our previous work (20). The obtained particle mean diameters (PMD, Y_1) were in the range of 119.9 to 192.4 nm (Table I). The standardized effect of the independent variables on the dependent variables and their interactions are shown in the Pareto chart (Fig. 1a). As shown, PLA, oil contents, and their interaction exerted a positive and significant effect on the PMD ($p < 0.05$). A significant curvilinear effect of Doc on the PMD ($p = 0.0044$) was also depicted in Fig. 1a. The predicted and observed values of PMD were also shown (Table S2 in Supplemental data). The initial polynomial equation (full model) for PMD (Y_1) is as follows:

$$Y_1 = 138.84 + 11.95X_1 - 0.97X_2 + 20.89X_3 + 4.13X_1X_2 + 12.03X_1X_3 + 7.39X_2X_3 + 5.15X_1^2 + 16.81X_2^2 + 0.14X_3^2 \quad (6)$$

Positive and negative signs, respectively, mean increasing and decreasing effects of the formulation variables on the PMD. From Eq. 6, the coefficient of determination (r^2) value was 0.97, indicating a statistically significant fit to the tested model. Interestingly, the lack of fit test was not significant ($p = 0.48$), indicating the adequacy of the model.

The PDI (Y_2) of the 15 runs varied (Table I), with a non-negligible polydispersity and an inadequate data fit ($p = 0.846$), as shown in the following formula:

$$Y_2 = 0.206 + 0.006X_1 + 0.004X_2 - 0.018X_3 - 0.004X_1X_2 - 0.001X_1X_3 + 0.005X_2X_3 + 0.040X_1^2 - 0.007X_2^2 + 0.036X_3^2 \quad (7)$$

The meaning of positive and negative signs is the same as stated above. Also, the coefficient of determination (r^2) was 0.46, indicating a dramatic difference between predicted and experimental data, thus supporting the above conclusion.

Table I. Box–Behnken Experimental Design of Independent Variables with Measured Responses

Formulation	X_1	X_2	X_3	Y_1 (nm)	Y_2	Y_3 (%)
F_1	0	0	0	132.0	0.274	84.7
F_2	-1	-1	0	156.4	0.188	13.1
F_3	0	1	1	182.1	0.227	91.0
F_4	1	0	-1	127.5	0.252	92.3
F_5	-1	0	1	136.7	0.242	86.2
F_6	0	1	-1	124.6	0.269	92.0
F_7	0	0	0	140.6	0.154	88.6
F_8	1	1	0	173.5	0.209	92.0
F_9	-1	0	-1	119.9	0.258	91.9
F_{10}	0	-1	1	172.2	0.191	13.8
F_{11}	0	0	0	143.9	0.189	86.5
F_{12}	-1	1	0	149.1	0.184	93.0
F_{13}	1	0	1	192.4	0.230	92.1
F_{14}	0	-1	-1	144.3	0.252	13.6
F_{15}	1	-1	0	164.3	0.228	92.3

The coded independent factors are X_1 for the PLA amount (milligram), X_2 for the Doc amount (milligram), and X_3 for the volume of Labrafac CC oil (milliliter); Y_1 as NC_{T80} mean diameter (nanometer) Y_2 as PDI and Y_3 as EE%

An increasing amount of Doc seemed to significantly induce higher EE% (Y_3 , $p=0.0041$, Fig. 1b). However, the EE% did not significantly increase when the amount of PLA was increased ($p=0.1317$). Similarities between predicted and observed values were found with residual EE% (Table S3 in Supplemental data). The initial polynomial equation is as follows:

$$Y_3 = 86.57 + 10.57X_1 + 29.40X_2 - 0.84X_3 - 20.06X_1X_2 + 1.34X_1X_3 - 0.28X_2X_3 + 12.02X_1^2 - 26X_2^2 - 7.98X_3^2 \quad (8)$$

The meaning of positive and negative signs is the same as stated above. The coefficient of determination (r^2) from Eq. 8 was found to be 0.92, indicating a significant fit of the data to the model being tested. In addition, the lack of fit was not significant ($p=0.081$), confirming the above conclusion. High values of the coefficient of interaction terms indicated that the response fluctuation can be related to a simultaneous change of two independent variables (Eqs. 6 and 8).

The optimized formula was found to be 36 mg for X_1 , 6.16 mg for X_2 , and 0.2 ml for X_3 corresponding to a formulation with 123.02 ± 14.63 nm as Y_1 , 0.27 ± 0.10 as Y_2 , and $101.06 \pm 37.00\%$ as Y_3 values (Fig. 2a). By using a two-tailed t table at degree of freedom (df) equals the number of runs minus the number of terms minus 1 ($df=15-9-1=5$), the obtained value of the critical t (t_{critical}) was 2.571 for Y_1 and Y_3 , $p=0.025$.

The effects of the independent variables on the response variables are shown in Fig. 2b, c. As shown in the response surface plot, an increase of the PLA amount was related to an increase of PMD (Fig. 2b). The increases of PLA and Doc amounts could also lead to higher EE% (Fig. 2c). Results from ANOVA of the corresponding F_{ratio} are shown in Table S4 in Supplemental data).

a Sorted Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob> t
Oil (0.2, 0.6)	20.88	2.32	9.01	0.0003*
PLA (4, 36)	11.95	2.32	5.16	0.0036*
DOC * DOC	16.81	3.41	4.93	0.0044*
PLA * Oil	12.03	3.28	3.67	0.0144*
DOC * Oil	7.39	3.28	2.26	0.0738
PLA * PLA	5.15	3.41	1.51	0.1913
PLA * DOC	4.13	3.28	1.26	0.2638
DOC (2, 10)	-0.97	2.32	-0.42	0.6939
Oil * Oil	0.14	3.41	0.04	0.9697

b Sorted Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob> t
DOC (2, 10)	29.40	5.87	5.01	0.0041*
DOC * DOC	-26.00	8.65	-3.01	0.0298*
PLA * DOC	-20.06	8.31	-2.42	0.0605
PLA (4, 36)	10.57	5.87	1.80	0.1317
PLA * PLA	12.02	8.65	1.39	0.2230
Oil * Oil	-7.98	8.65	-0.92	0.3981
PLA * Oil	1.34	8.31	0.16	0.8784
Oil (0.2, 0.6)	-0.84	5.87	-0.14	0.8914
DOC * Oil	-0.28	8.31	-0.03	0.9744

Fig. 1. Pareto chart showing the standardized effect of the formulation variables and their interaction on: **a** particle mean diameter and **b** docetaxel percent encapsulation efficiency (EE%). Oil (0.2, 0.6) refers to low and high levels of oil volume in milliliter, PLA (4, 36) refers to low and high amounts of PLA in milligram, DOC (2, 10) refers to low and high levels of docetaxel amount in milligram; DOC*DOC, PLA*PLA, and OIL*OIL expressed the eventual curvilinear effects of docetaxel, PLA, or oil on the model, respectively. The two vertical lines represented the t_{critical} values. Any bar exceeding these lines indicated statistical significance for the corresponding effect

Checkpoint Analysis

To test the validity of the model, four specific formulations including the center point (CP₀, CP₁, CP₂, and CP₃) were prepared according to the full model polynomial. Results indicated that the predicted and observed Y_1 values were similar ($p=0.5$), thus confirming the null hypothesis as shown in Table S5 in Supplemental data. On the other hand, the suitability of the model prediction of Y_3 was also confirmed using the same model of comparison ($p=0.4$) as shown in Table S5 in Supplemental data.

Transmission Electron Microscopy (TEM) Analysis

TEM images show smooth spherical NCs with an average size of 120 nm for both nanocarriers (NC_{T80} and NC_{PVA}; Fig. 3a, b), which is consistent with the DLS data (Fig. 3c, d). However, the NTA data show at least two populations of particles for both NCs (Fig. 3e(1)). For NC_{PVA}, the results from NTA indicated a broad range of PMD (112–180 nm) covering the DLS and TEM data.

Fig. 2. **a** Prediction and desirability plot showing the effect of poly(lactic acid) or PLA amount (X_1), docetaxel amount (X_2), and oil volume (X_3) on the particle mean diameter (PMD), polydispersity index (PDI), and docetaxel percent encapsulation efficiency (EE%). **b, c** Response surface plot showing the effects of docetaxel amount (X_1) and PLA amount (X_2) on particle mean diameter (PMD) (Y_1 , **b**) and drug percent encapsulation efficiency (EE%, Y_3 , **c**)

Figure 3e(2, 3) (a 3D plot of the relative intensity *versus* particle size plot) showed several populations of particles for both NC_{T80} and NC_{PVA}, thus supporting the polydispersity data obtained by DLS for these samples.

Powder X-ray Diffraction Analysis

Typically, the PXRD data showed the presence of a hemihydrated crystalline form of Doc with its

characteristic diffraction peaks at 7.3, 8.8, 13.7, 17.2, and $20.2 \pm 0.2^\circ$ two-theta (41) (Fig. 4a). The presence of amorphous PVA and Tween 80 in the NCs was also noticeable in Fig. 4a. The characteristic intense peak of the semicrystalline PLA remained apparent at 38° two-theta. Interestingly, the intensity of the characteristic peaks of Doc was attenuated in both NC_{PVA} and NC_{T80}, indicating a negligible crystal content of Doc when encapsulated in the oily core NCs.

Fig. 3. Transmission electron microscopy images of docetaxel-loaded PLA stabilized with **a** Tween 80 (NC_{T80}; F_{12}) and **b** polyvinyl alcohol (NC_{PVA}, Fig. S1, in Table S7 in Supplemental data) size distribution from DLS analysis; **c** NC_{T80} and **d** NC_{PVA}; **e 1** Size distribution from NTA of NC_{T80} (yellow) and NC_{PVA} (red), 2 and 3, tridimensional plot of the relative intensity versus the NCS (NC_{T80} and NC_{PVA}) mean diameter

X-ray Photoelectron Spectroscopy Analysis

XPS analysis reveals the presence of oxygen (O) and carbon (C) from Tween 80 in the NC_{T80} (Fig. 4b(1)). These two chemical elements were in the form of aliphatic carbon (C–H), (C–O) bonds, and carbonyl (C=O) groups at 284.98, 287.47, and 289.34 eV, respectively (Fig. 4b(2)). The C/O atomic ratio of Tween 80 was found to be 3.8. Moreover, the spectrum of Tween 80 shows the presence of O–C bond formation at 532.76 eV (Fig. 4b(3)).

Figure 4c(1, 2) shows the spectrum of PVA indicating the presence of oxygen and carbon with a C/O atomic ratio of 3.5 in NC_{PVA} formulation. The majority of elements was comprised of C–C formation, followed by the C–O group. Figure 4c(3) indicates O–C as the major species, which is consistent with the expected chemical composition of PVA.

Drug Release Kinetics

Drug release kinetics data from NC_{T80} formulations mostly provided a better fit to zero order than Higuchi and Korsmeyer–Peppas equations ($r^2 > 0.95$, Fig. S2 and Table S6 in

Supplemental data). Moreover, the drug release kinetics data from NC_{PVA} formulations were mostly consistent with the Higuchi equation ($r^2 > 0.95$; Table S7 in Supplemental data).

Cytotoxicity of Blank and Docetaxel-Loaded Nanocapsules in MDA-MB 231 Cells

Blank NCs treated wells exhibited a percent of cell viability above 80%. This suggested a minor toxicity for blank NC_{T80} and NC_{PVA} in the cell suspensions (Fig. 5a(1, 2)). The cytotoxicity levels of the two NCs were similar ($p > 0.05$, t test) at low and medium levels of Doc concentrations (5 to 25 μ M), as shown in Fig. 5b. Interestingly, a gradual decrease of the cell viability with increasing concentrations of Doc (50–100 μ M) was observed in Fig. 5b. At this range of concentrations, NC_{T80} appeared to be more cytotoxic than NC_{PVA} with a 1.3–1.6-fold increase ($p < 0.001$).

Preparation of Dylight 488-Labeled Nanocapsules

DL488-labeled NCs were successfully synthesized and the particle mean diameter was 145.0 ± 5.8 and 150.6 ± 7.0 nm for

Fig. 4. **a** Powder X-ray diffraction pattern for (1) docetaxel, (2) Tween 80, (3) PVA, (4) PLA, (5) NC_{PVA}, and (6) NC_{T80}; **b** 1–3 XPS signals of carbon and oxygen from poly(lactic acid)-based NCs coated with Tween 80; and **c** 1–3 from poly(lactic acid)-based NCs coated with polyvinyl alcohol (PVA)

labeled NC_{T80} and NC_{PVA}, respectively (Table S8 in Supplemental data). As shown, the averages of zeta potential were -20.4 ± 1.6 and -22.3 ± 0.8 mV for labeled NC_{T80} and NC_{PVA}, respectively. The degree of DL488 labeling was found to be 1.8 and its AE% with the NCs was $65.2 \pm 2.3\%$ and $72.1 \pm 1.5\%$ for NC_{T80} and NC_{PVA}, respectively (Table S8 in Supplemental data).

Determination of the Limit of Detection and Limit of Quantification of the Nanocapsules

The concentration of NCs was first determined from a standard calibration curve obtained from DLS (Fig. S3 in Supplementary data). Secondly, the LOD and LOQ of the labeled NCs were obtained from a calibration curve where the particle fluorescence intensity was plotted as a function of the particle concentration (Fig. S4 in Supplementary data). The results indicated that the LOD of the NCs was 4.05 and 4.55 ng/ml for NC_{PVA} and NC_{T80}, respectively, while the LOQ was found to be 0.34 μ g/ml for NC_{PVA} and 0.38 μ g/ml for NC_{T80}. In addition, the average of

percent recovery of the NCs after 24 h of incubation and cell lysis was $80.2 \pm 5.7\%$ ($n=7$) for NC_{T80} and $85.6 \pm 4.3\%$ for NC_{PVA} ($n=6$).

Stability of Free Dylight 488 and Labeled Nanocapsules in Culture Medium

Whatever the selected concentration, the free DL488 was quite stable over the timescale of the experiments (Fig. S5-a in Supplementary data). However, the PMD of DL488-grafted NCs was significantly decreased after 4 h of incubation ($p < 0.05$) for NC_{T80} versus 6 h for NC_{PVA} (Fig. S5-b, in Supplementary data).

Safety, Interference, and Functionality Test of the Particle Cellular Uptake Pathways

The safety of the pharmacological inhibitors was tested in MDA-MB 231 cells, THP-1 monocytes, and THP-1-derived macrophages, respectively, using the above MTS method. The selected pharmacological inhibitors exhibited a minimal toxicity and interference in fluorescence at the working condition (Fig. 5c,

Fig. 5. *In vitro* cytotoxicity of blank **a** 1 NC_{T80} and 2 NC_{PVA} and **b** MDA-MB 231 cells viability after 48 h of incubation with docetaxel-loaded nanocapsules stabilized with Tween 80 (NC_{T80}) in comparison with docetaxel-loaded nanocapsules stabilized with polyvinyl alcohol (NC_{PVA}) at equivalent doses of docetaxel ($n=6$). **c** *In vitro* cytotoxicity of pharmacological inhibitors in MDA-MB 231, THP-1 monocytes, and THP-1-derived macrophages, mean $\pm$ SD, $n=6$; **d** intrinsic fluorescence of the pharmacological inhibitors in the complete growth media, mean $\pm$ SD, $n=6$

d). Furthermore, the functionality of different particle cellular uptake pathways was tested in the presence of particles or

substances known to be internalized in a specific pathway. Results revealed a significant decrease of the percentage of

fluorescence in the presence of the corresponding pharmacological inhibitors in MDA-MB 231 cells, THP-1 monocytes, and THP-1-derived macrophages (Fig. 6a–c).

Quantitative Measurement of the Particle Cellular Uptake in MDA-MB 231 Cells

The particle cellular uptake efficiency was analyzed using the percentage of fluorescence intensity at 4°C and 37°C. Typical profiles of particle uptake as a function of the concentration of FITC-loaded NCs at 4°C and 37°C are shown for both types of NCs (Fig. 7a–c). This suggested that the particles were taken up by MDA-MB 231 cells in a concentration-dependent manner. Compared to that of 37°C, the particle cellular uptake decreased significantly at 4°C ($p < 0.05$). This indicated that the particles were taken up by MDA-MB 231 cells through an energy-dependent pathway (Fig. 7a, b). Moreover, the uptake of NC_{T80} was considerably increased at 37°C from 1.5- to 1.75-fold compared to that of NC_{PVA} (Fig. 7c). Furthermore, the particle cellular uptake mechanism underlying the endocytic pathways was investigated. As shown, the uptake of NC_{T80} was inhibited by latrunculin A and dynasore (down to 40%, Fig. 7d) indicating the involvement of actin and dynamin filaments in the internalization of NC_{T80} in MDA-MB 231 cells. The data suggests that the cellular internalization of NC_{T80} occurred through the caveolae-mediated pathway. Also, clathrin- and cytoskeleton-mediated pathways might be involved to a lesser extent. For NC_{PVA}, a decrease of the particle uptake (down to 40%) was noticed for most of the treated samples ($p > 0.05$, Fig. 7e). This particle internalization occurred *via* a

multifaceted mechanism involving clathrin, caveolin, cytoskeleton, micropinocytosis, actin, and dynamin-mediated pathways.

Quantitative Measurement of the Particle Cellular Uptake in Phagocytic Cells

Confocal Images

The results show that NC_{T80} and NC_{PVA} cellular uptakes were slightly increased with the concentration of the labeled NCs in monocytes (Fig. 8a). Interestingly, higher amounts of NCs were taken up by macrophages compared to monocytes, thus supporting the above results (Fig. 8b). In addition, the uptake of NC_{PVA} by macrophages was higher than that of NC_{T80} (Fig. 8b(1–3) vs. (4–6)).

Microplate Reader

Results from Fig. 9a, b indicated that the internalization of both NCs occurred in a concentration-dependent manner in THP-1 monocytes and its derived macrophages. The results show that both NCs (NC_{T80} and NC_{PVA}) are more taken up by macrophages compared to monocytes (Fig. 9a, b). In THP-1 monocytes, the internalization of NC_{T80} appeared to mainly occur by phagocytosis (Fig. 9c). However, the endocytic pathways of NC_{PVA} exhibited another pattern in THP-1 monocytes. As shown in Fig. 9d, except NaN₃, the percentage of NC_{PVA} taken up by monocytes was also inhibited by CCD, CPZ, GEN, PMA, LAT-A, and DYN (down to 80–70%). In THP-1-derived macrophages, NC_{T80} was exclusively taken up

Fig. 6. Levels of inhibition of the uptake of positive controls denoted with (+) in **a** MDA-MB 231, **b** THP-1 monocytes, and **c** exposed to different inhibitors denoted with (+) in respect to the uptake in untreated cells denoted with (-). Results are expressed as mean $\pm$ SD, $n=6$, * $p < 0.05$ and ** $p < 0.01$

Fig. 7. Cellular uptake of fluorescein isothiocyanate (FITC)-loaded oily core nanocapsules in MDA-MB 231 cells. **a** Tween 80-coated nanocapsules (NC_{T80}) at 4°C and 37°C; **b** FITC-loaded oily core polyvinyl alcohol-coated nanocapsules (NC_{PVA}) at 4°C and 37°C; **c** comparative cellular uptake of NC_{T80} versus NC_{PVA} at 37°C after 24 h of incubation; **d**, **e** determination of the cellular endocytic pathway of **d** NC_{T80} and **e** NC_{PVA} in MDA-MB 231 cells. For Fig. 7a-c each value is the mean the mean of six measurements, and the error bars represent the standard deviation. *p<0.05, after pairwise comparison between equivalent samples. For Fig. 7d-e, treated samples are compared to control

via phagocytosis, while the uptake of NC_{PVA} obeyed to a multifaceted pathways including, actin, caveolin, clathrin, macropinocytosis-mediated pathway, and phagocytosis (Fig. 9c, d).

DISCUSSION

Besides the particle size and shape, surface properties may play a crucial role in the cytotoxicity and phagocytic clearance of drug-containing nanocarriers. As a consequence, the drug concentration in the bloodstream and, subsequently, its therapeutic effect may be dramatically affected. A wide range of stabilizers has been extensively used for the preparation of nanocarrier systems (8–10). Unfortunately, less information is available regarding the potential effect of stabilizers on the cytotoxicity, phagocytic clearance, and pharmacological effect of drug-containing nanocarriers.

In this study, the Box–Behnken design was used to investigate the influence of Doc, PLA, and oil contents on PMD, PDI, and EE% during the formulation of NC_{T80}. Overall, results showed NC_{T80} with mean diameter less than 200 nm that are suitable for passive tumor target (42) and low uptake by the reticulum endoplasmic system (43). A significant increase in the mean diameter of NCs was consistently correlated with the curvilinear effect of Doc (*p*=0.0044), indicating the model suitability. As reported earlier, a large volume of lipophilic oil might contribute to an increase of the PMD (44). Also, an increase of PLA amount had a greater effect on the PMD. Increasing PLA amount has been shown to increase the viscosity of the organic phase, leading to a less effective mixing of the emulsion (8). The interactions between the amount of Doc and the oil/PLA ratio were also shown to increase the PMD. One possible explanation is that, by adding oil (viscosity 25–35 mPa s at 20°C (45)), the viscosity of the internal phase can be increased, leading to an increase of the PMD (44). The addition of Tween 80 in the external phase appeared to

Fig. 8. Confocal microscopy images showing the uptake of Tween 80-coated nanocapsules in THP-1 monocytes (a, 1–3) and THP-1-derived macrophages (b, 1–3). Uptake of PVA-coated nanocapsules in THP-1 monocytes (a, 4–6) and THP-1-derived macrophages (b, 4–6). Nanocapsules and cell nuclei are labeled with Dylight-conjugated NCs (green) and DAPI (blue), respectively

be related to a decrease of the PMD in comparison with PVA, which was consistent with previous findings (20). This might be attributed to the higher molecular weight of the PVA compared to Tween 80 (30–70 vs. 1.3 kDa, respectively).

Higher EE% values may be attributed to the hydrophobic interaction between oil and PLA, which is in favor of the high encapsulation of Doc ($\log P=4.1$) (46). This may also arise from a synergistic effect between the surfactant and the oil ratio, thus increasing the emulsion viscosity in the presence of ethyl acetate residue within the dispersed phase (47). The lower EE% was related to minimal amounts of Doc used in the formulation. This phenomenon can be explained by the solubility of PLA in ethyl acetate (48), delaying its precipitation during the solvent diffusion that leads to drug loss (49).

Similar negative zeta potential values (ζ) from NC_{T80} and NC_{PVA} (ζ average = -35.9 ± 3.7 mV for NC_{T80} and -36.5 ± 9 mV for NC_{PVA}) were observed. High absolute values of zeta potential may contribute to improved the stability of the NCs in increasing the repulsive potential (V_r) of adjacent particles (50) (Eq. 9).

$$V_r = 2\pi\epsilon a\zeta^2 \exp(-\kappa D) \quad (9)$$

where D is the particle separation distance, a is the particle radius, ϵ is the dielectric permittivity, and κ , the inverse Debye length, is a function of the ionic composition.

Several kinetic factors including polymer swelling, polymer erosion, drug dissolution/diffusion characteristics, drug/polymer ratio, and system geometry are able to affect the drug release kinetics (51). For NC_{T80}, the *in vitro* drug release mechanism could be explained by the zero-order model ($R^2 > 0.95$). For F_3 and F_7 , the release exponent (n) is higher than 0.45, indicating a non-Fickian diffusion which is a combination of erosion and controlled release rate. For F_{10} and F_{15} , the drug release kinetics follow the zero-order equation indicating that the drug release rate is independent of its concentration (Table S6 in Supplemental data).

For NC_{PVA}, it appeared that the drug release occurred mainly by diffusion *via* the Higuchi equation, when the percentage of the drug released is plotted *versus* the square root of time ($r^2 > 0.95$). The polymer erosion might have occurred after slow hydrolytic degradation of PLA monomers (52). The drug diffusion rate from NCs could be related to an unequal drug partition between the oil core and the aqueous release media (53). In a nanoscale range, this phenomenon can be considered as a summation of individual particle behavior. Therefore, the transfer rate constants of a drug (k) from an oily compartment of a single particle to an aqueous phase limited by an interfacial barrier (polymeric shell) can be extended using the following Eq. 10:

$$\ln(1-M_T/M_0) = -\left(\frac{-3kt}{(d/2)^2}\right) \quad (10)$$

where M_t/M_0 is the fraction of released drug at time t , and d is the particle mean diameters. $3k/(d/2)^2$ is the slope obtained after plotting $\ln(1-M_t/M_0)$ *versus* time (t).

TEM images show small spherical particles with PMD around 120 nm (Fig. 3a, b). However, for NC_{PVA}, the PMD values obtained from NTA were not consistent with those given by TEM and DLS analyses (Fig. 3c–e(3)). For example, NTA results revealed the existence of at least two populations for both NCs. For polydispersed samples, NTA has much higher resolving power ($<1:1.33$) compared to DLS ($\sim 1:4$) (23). In other words, NTA is likely to discriminate 300 nm particles from 400 nm with higher precision and accuracy compared to DLS analysis.

Results from PXRD analysis show characteristic peaks of native Doc, which is consistent with Doc crystallographic pattern (54). Results implied also that Doc was present in its amorphous form inside the NCs. In addition, Tween 80 and PVA remained in their amorphous state on the surface of the NCs.

Quantitative XPS analysis reveals that the aliphatic carbon is the major component (81%) of Tween 80 ($C_{64}H_{124}O_{26}$) on the NC_{T80} surface and consistent with the carbon aliphatic chain of Tween 80 (55). The peak at binding energy 284.98 eV is shown as the C–O group of PEG portion containing Tween 80. XPS analysis of NC_{PVA} indicates the presence of oxygen and carbon with a majority comprised of C–C formation, followed by C–O, both consistent with the chemical composition of PVA: $(C_2H_4O)_n$ (56). However, the photoemission intensity indicates the presence of carboxyl groups (C=OO) which might be ascribed to the PLA and Doc residues on the surface of particles. The peaks at binding energy 289.34 (for Tween 80) and 289.69 eV (for PVA) are an indicator of high negative charges of the surface of NCs.

Fig. 9. Cellular uptake of Tween 80 and PVA-coated nanocapsules in **a** THP-1 monocytes and **b** THP-1-derived macrophages. Determination of the particle cellular endocytic pathway of **c** FITC-loaded NC_{T80} and **d** FITC-loaded NC_{PVA}

For both NCs, the blank NCs exhibited minor toxicity when compared to control ($p > 0.05$, Fig. 5a(1, 2)). As shown in Fig. 5b, Doc-loaded oily core NCs decreased the cell viability in a dose-dependent manner. Due to its sustained release profile, the drug cytotoxicity was more effective at higher drug concentration. However, the NC_{T80} were more cytotoxic than NC_{PVA} in MDA-MB 231 cells at high-dose exposure of Doc. Bearing in mind the similarity between NC_{T80} and NC_{PVA} in terms of PMD, EE%, and zeta potential, the highest cytotoxicity of NC_{T80} could be explained by high intracellular drug concentration in MDA-MB 231 cells (57). Both intracellular particle and drug concentration could contribute to an increase of the intracellular drug concentration. To support the above assumption, the particle uptake was investigated.

The most important requirements for the determination of the mechanism underlying the particle cellular uptake included the intrinsic cytotoxicity and the fluorescence interference of the pharmacological inhibitors used in the experiments. The criteria for an effective inhibition included the intrinsic safety of the selected pharmacological inhibitor and its statistically significant effect (p value < 0.05) on the particle cellular uptake when compared to untreated samples used as controls. This ongoing work aimed to determine whether the observed decrease of the particle cellular uptake was due to chemical inhibitor-induced cell death or not. As shown in Fig. 5c, d, the selected pharmacological inhibitors exhibited minor toxicity and interference at their respective working concentration and time exposure. This also implies that the results are not affected by the presence of pharmacological inhibitors in the culture media for MDA-MB 231 cells, THP-1

monocytes, and THP-1-derived macrophages. The lack of specific positive controls for each of the distinct endocytic pathways has been highlighted as a major weakness of such study (58). To address this concern, in this study, the uptake of molecules or particles known to specifically enter cells in a specific pathway (positive marker) has been used to fully confirm the functionality of the selected pathways. The use of these makers in such a study is still under debate. For example, cholera toxin has been found to be internalized in Caco-2 cell *via* different endocytic mechanisms (58). However, their potential ability to selectively enter cells in a specific pathway is continuing to gain more interest in research. Chlorpromazine (CPZ) and genistein (GEN) were successfully used to inhibit clathrin- and caveolin-mediated endocytosis, respectively. Phorbol myristate acetate (PMA) was used to stimulate the microtubules cytoskeleton reorganization, while cytochalasin D (CCD) and sodium azide (NaN₃) were used to respectively inhibit the polymerization of actin and the phagocytosis. A functional link between clathrin-mediated endocytosis and the actin cytoskeleton in mammalian cells, either directly or indirectly, was pointed out in a previous study (36), indicating a possible interference between CPZ and CCD in the particle endocytosis. However, these claims are strongly dependent on the cell line and the selected pharmacological inhibitor. In this study, the positive markers strongly confirmed the activity of the selected endocytic pathways. More interestingly, the selectivity of each pharmacological inhibitor on the assigned pathway was also proven.

Despite the predicted unfavorable interaction between the particles and the negatively charged cell membrane, the uptake of negatively charged particles was evidenced (Fig. 7).

In both NCs, the highest particle uptake at 37°C compared to 4°C indicated that the particle internalization in MDA-MB 231 cells was energy dependent, with a decrease of the average cellular fluorescence down to 5–15% (Fig. 7a, b). It is well known that several proteins and enzymes are sensitive to temperature; thus, lowering temperature leads to their inhibition (59). Interestingly, at similar drug loading, the relative percentage of NC_{T80} uptake was higher than that of NC_{PVA} at 37°C (Fig. 7c). In comparison with PVA (HLB: 18) (60), the relative lesser hydrophilicity of Tween 80 (HLB: 15) (61) may enhance the binding affinity of NCs and its subsequent cellular uptake (62).

As shown in Fig. 7d, e, both NC_{T80} and NC_{PVA} were predominantly taken up by MDA-MB 231 cells through a multifaceted pattern of endocytosis. These results were not consistent with a previous report, where clathrin-mediated endocytosis was the main entry route for particles up to 200 nm and the caveolin pathway for particles of 500 nm in size (63). In addition to the hydrophilic nature of the particle surface, their small size might enhance the drug efficacy *via* the enhanced permeability and retention (EPR) effect (64).

Results using the confocal microscope indicated that both NCs (NC_{T80} and NC_{PVA}) were more taken up by macrophages compared to monocyte THP-1 cells (Fig. 8a, b). This can be explained by the higher phagocytic activity of macrophages in comparison with monocytes (65). The particle uptake mostly decreases with increasing hydrophilicity of their surface. The two identical hydrophilic groups of Tween 80 are able to reduce the cell adhesion and phagocytosis *via* steric stabilization leading to a decrease of the particle cellular uptake. Indeed, it has been demonstrated that polaxamer 88 can suppress phagocytosis in macrophages (66). Reversely, nanoparticles formulated with 5% PVA have been shown to exhibit lower uptake compared to the one formulated with 2% PVA. However, the relationship between the lower uptake and the surface hydrophobicity remains unclear (14).

The internalization of NC_{T80} in monocytes implies the involvement of clathrin, caveolin, actin-mediated pathway, and phagocytosis. Unexpectedly, the uptake of NC_{T80} in these cells was not inhibited by LAT-A indicating the non-involvement of the actin polymerization in the internalization of NC_{T80}. Indeed, the role of the actin polymerization in the particle engulfment is to stabilize the ligand–receptor bond. However, the particle might be internalized in an actin-independent pathway *via* a Fcγ receptor-mediated uptake named zipper mechanism (67). In this case, the ligand (L)–receptor (R) bonds are robust enough to maintain the R–L binding and internalize the particles.

Interestingly, the uptake of NC_{T80} in THP-1-derived macrophages was only inhibited by NaN₃, indicating that the internalization of NC_{T80} occurred through phagocytosis, that is the specialty of macrophages (40), while NC_{PVA} was internalized in a multifaceted manner involving clathrin, caveolin, actin, macropinocytosis, dynamin-mediated pathways, and phagocytosis.

Overall, the study demonstrated also that the uptake of both NCs in THP-1-derived macrophages was significantly impeded after treatment with sodium azide. These results are in disagreement with previous results using a less concentrated sodium azide (15 μM) (68). This is probably due to inefficient concentration of sodium azide for the blockade of the uptake of NCs in THP-1 monocytes and macrophages.

So far, the particle size and zeta potential have been considered to be the main factors involved in the particle cellular

uptake (32). In this present work, the nature and chemical composition of the particle surface appear to significantly impact the particle cellular uptake and cytotoxicity. Hence, considerations must be given to a thorough selection of stabilizers for the formulation of NCs. In future works, the authors planned to investigate a possible contribution of the complement system in the uptake of such NCs in THP-1 monocytes and its monocyte-derived macrophages.

CONCLUSIONS

Data set from the formulation prediction provided a suitable model for the optimization of such NCs in terms of PMD and EE%. Morphological and light scattering analyses confirmed the formation of spherical Doc-loaded NCs with an approximate average diameter of 120 nm. XPS analysis confirmed the presence of stabilizers on the surface of NCs, while the PXRD spectra revealed the presence of amorphous Doc in both types of NCs. Cytotoxicity and particle cellular uptake data showed greater antitumor effect of NC_{T80} with a reduced phagocytic clearance compared to NC_{PVA}. The uptake of NC_{PVA} revealed a multifaceted mechanism involving clathrin, caveolin, cytoskeleton, and micropinocytosis in MDA-MB 231 cells. In macrophages, the uptake of NC_{T80} occurred through a monofacetic pattern *via* phagocytosis, while the internalization of NC_{PVA} obeyed to a multifacetic pattern in macrophages. The present work has raised an important issue regarding the critical role of the surface properties of NCs in their cytotoxicity and endocytic pathways.

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