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PEG-PE/clay composite carriers for doxorubicin: Effect of composite structure on release, cell interaction and cytotoxicity

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ABSTRACT

A novel drug delivery system for doxorubicin (DOX), based on organic-inorganic composites was developed. DOX was incorporated in micelles (M-DOX) of polyethylene glycol-phosphatidylethanolamine (PEG-PE) which in turn were adsorbed by the clay, montmorillonite (MMT). The nano-structures of the PEG-PE/MMT composites of LOW and HIGH polymer loadings were characterized by XRD, TGA, FTIR, size (DLS) and zeta measurements. These measurements suggest that for the LOW composite a single layer of polymer intercalates in the clay platelets and the polymer only partially covers the external surface, while for the HIGH composite two layers of polymer intercalate and a bilayer may form on the external surface. These nanostructures have a direct effect on formulation stability and on the rate of DOX release. The release rate was reversely correlated with the degree of DOX interaction with the clay and followed the sequence: M-DOX > HIGH formulation > LOW formulation > DOX/MMT. Despite the slower release from the HIGH formulation, its cytotoxicity effect on sensitive cells was as high as the “free” DOX. Surprisingly, the LOW formulation, with the slowest release, demonstrated the highest cytotoxicity in the case of Adriamycin (ADR) resistant cells. Confocal microscopy images and association tests provided an insight into the contribution of formulation-cell interactions vs. the contribution of DOX release rate. Internalization of the formulations was suggested as a mechanism that increases DOX efficiency, particularly in the ADR resistant cell line. The employment of organic-inorganic hybrid materials as drug delivery systems, has not reached its full potential, however, its functionality as an efficient tunable release system was demonstrated.

Statement of Significance

DOX PEG-PE/clay formulations were design as an efficient drug delivery system. The main aim was to develop PEG-PE/clay formulations of different structures based on various PEG-PE/clay ratios in order to achieve tunable release rates, to control the external surface characteristics and formulation stability. The formulations showed significantly higher toxicity in comparison to “free” DOX, explained by formulation internalization. For each cell line tested, sensitive and ADR resistant, a different formulation structure was found most efficient. The potential of PEG-PE/clay-DOX formulations to improve DOX administration efficacy was demonstrated and should be further explored and implemented for other cancer drugs and cells.

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1. Introduction

The ultimate goal of chemotherapeutic nanocarrier agents is to achieve therapeutic concentrations of the drug at the target site

while drug concentrations at healthy tissues are kept at safe levels [1,2]. Moreover, nanocarriers must be of a specific size range, stable in the blood and inert to serum components [3]. Drug accumulation at optimal concentrations constitutes a major clinical challenge mainly for resistant cell lines.

A wide range of materials have been used as drug carriers including, natural and synthetic polymers, lipids and surfactants [4–6]. A group of drug delivery systems based on inorganic minerals has recently drawn much attention due to its ability to adsorb

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the drug and release it in a controlled manner. This group includes minerals such as mesoporous silica [7–10], halloysite [11], layered double hydroxides [12,13], and montmorillonite (MMT) [14–16].

MMT is a natural layered clay-mineral with an exceptionally high surface area (756 m²/g), considered safe for both oral and intravenous application and considered biocompatible [17]. MMT has a negative charge (76 meq/100 g), derived from isomorphous substitutions between Al³⁺ and Si⁴⁺, which are naturally compensated by inorganic cations. The dispersion/flocculation of MMT depends on particle size, the compensating cations, surface charge and solution chemistry. Physiological systems are characterized by relatively high salt and protein concentrations which can induce flocculation by reduction of the double electrostatic layer or by cementation, respectively. In addition to instability of clay suspensions in physiological systems, incomplete release of drugs from MMT-drug formulations has been reported [18]. These disadvantages of employing clays as nanocarriers were addressed in the current study, by modifying the clay surface with organic components [19,20].

Tailored modification of the clay surface with an organic phase to form organic-inorganic composite materials, is of great interest and pursued in many fields [21]. Different interactions such as electrostatic, van der Waals and hydrogen bonds can occur between the organic modifiers and MMT [22–24]. Composites can be designed to increase the solubility of poorly soluble drugs [25,26], stabilize incorporated drugs [27,28], control drug release [29–32] and to increase formulation stability in the blood [29]; hence, they are beneficial as drug delivery systems.

In the current study, polyethylene glycol-phosphatidylethanolamine (PEG-PE) was employed as an organic modifier for the design of doxorubicin (DOX) formulations. PEG-PE, widely used in drug delivery systems, [33] is an amphiphilic molecule. When added to water solution above its critical micelle concentration (CMC), it forms micelles with a hydrophobic core (PE) and hydrophilic shell (PEG). Hydrophobic molecules can be solubilized into the PE core while the PEG shell hinders interactions with blood components, reduces binding to plasma proteins and increases circulation time [34–38].

The incorporation of DOX in PEG-PE micelles has been reported [30] and the contribution of the electrostatic interactions between the cationic DOX and the negatively charged phosphate group of PEG-PE was found to be dominant. Tang et al. [39] demonstrated that incorporation of DOX in PEG-PE micelles increases cancer-cell internalization, enhancing cytotoxicity. Despite the advantages of drug-PEG micelles, in many cases, the micelle systems are unstable under physiological conditions such as high dilution, different blood components and high salt concentrations, [2,40,41].

The clinical use of DOX, an anti-cancer drug, in chemotherapy is limited due to its low specificity which, among other side effects, causes cardio toxicity [42]. Indeed, many approaches to form DOX nanocarriers were developed in order to achieve this goal; among them: liposomes [43,44], micelles [45–47] and inorganic minerals [48,49]. The unspecific toxicity intensifies if the cancer cells develop Adriamycin (ADR) resistance which reduces the drug's intracellular accumulation [47]. The ADR resistance is based on several mechanisms, but the most known and investigated one is the over expression of ABC-transporter P-glycoprotein (P-gp), which acts as a cell surface efflux pump, reducing drug intracellular accumulation and its cytotoxicity effect [47,50,51]. Therefore, in the case of ADR resistant cells, the need to develop nanocarriers that improve drug uptake [2,47] is even more pronounced.

In the current study we designed novel formulations based on incorporation of DOX in PEG-PE micelles followed by the adsorption of these micelles to MMT. The merging between PEG-PE and MMT, in which MMT anchors both micelles and DOX, is a new approach which synergistically improves the release profile and

the bio-effectiveness of the drug delivery systems. The direct and indirect interactions (via incorporation in PEG-PE) of DOX with the clay surface were investigated. We aimed to design different structures based on various PEG-PE/MMT ratios in order to achieve tunable release rates and to control the external surface characteristics of the formulations. Finally, in vitro experiments were conducted on sensitive and ADR resistant cell lines to assess the bioactivity of the formulations. To the best of our knowledge, this is the first report on improved DOX uptake in sensitive and resistant ADR cells by composites based on MMT.

2. Material and method

2.1. Materials

Doxorubicin hydrochloride (DOX) was purchased from Sigma-Aldrich, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-750/5000] (ammonium salt) (PEG750/5000-PE) were purchased from Avanti-Polar lipids. Wyoming Na-montmorillonite SWy-2 (MMT) was obtained from the Source Clays Repository of the Clay Mineral Society (Columbia, MO); cation exchange capacity (CEC) and specific surface area are 76.4 meq/100 g and 756 m²/g, respectively. MCF-7 (human breast cancer) cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). A2780 (Human ovarian carcinoma) and A2780 ADR (doxorubicin resistant derivative of A2780) were purchased from Sigma Aldrich/ECACC (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), 0.25% Trypsin-EDTA and penicillin/streptomycin 100X stock solution were purchased from Mediatech, Inc. (Manassas, VA). Heat-inactivated fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Flowery Branch, GA). A2780 and MCF-7 cells were grown in DMEM and A2780 ADR cells were grown in RPMI supplemented with 10% (v/v) FBS, 50 units/ml penicillin and 50 µg/ml streptomycin (DMEM complete media). Cell cultures were maintained in a humidified atmosphere of 5% CO₂ at 37 °C. Cell Titer Blue® cell viability assay reagent was purchased from Promega (Madison, WI). Hoechst 33342 was purchased from Invitrogen/Molecular Probes, Inc (Eugene, OR). All other chemicals and solvents were purchased as analytical grade reagents and were used without further purification.

2.2. Formulation size measurements

2.2.1. Reduced MMT particle size

The average size of Wyoming Na-montmorillonite SWy-2 (MMT) is 1–2 µm. In order to utilize MMT for medical applications clay particle size was reduced. The clay was suspended (10 g/L) and precipitation was induced by centrifugation (5000g for 0.5 h). The supernatant, a suspension of small clay particles, was re-centrifuged (11,000g for 2 h), dried in an oven (105 °C for 24 h) and then re-suspended in DW at defined concentration using sonication. The size of the re-suspended (by sonication) solution of MMT was on average 90 nm (see method below).

2.2.2. Colloid size measurements

The size (hydrodynamic diameter) and size distribution of composites and DOX formulations in complete media were measured by dynamic light scattering (DLS) using a N4 Plus Submicron Particle System (Coulter Corporation, Miami, FL, USA). For size analysis, the stock solutions of the formulations were prepared at DOX concentration of 30 µM in distilled water. The formulations were then diluted to 10 µM in complete media for measurement, to provide a light scattering intensity of 5 × 10⁴ to 1 × 10⁶ counts/s. The particle size distribution of all samples was measured in triplicate.

In addition, Particle size average of DOX formulations in distilled water and in phosphate buffer 0.14 M was measured using the Zetasizer Nano system (Malvern Instruments, Southborough, MA). The measurements were taken for PEG-PE micelles (0.1 mM), MMT after size fractionation, DOX/MMT and M-DOX/MMT formulations (0.6 g clay/L).

2.3. Analysis

2.3.1. PEG-PE

PEG-PE concentrations in solution were calculated by measuring phosphate concentration by Inductively Coupled Plasma Atomic Emission Spectrometer (ICP-AES), (ARCOS end on plasma), limit of detection was 3E^{-3} g/L and 7.5E^{-3} g/L for PEG5000-PE and PEG750-PE, respectively.

2.3.2. DOX

DOX (0.01–56 mg/L) was analyzed by HPLC (Agilent Technologies 1200 series) equipped with a fluorescence detector. HPLC column was LiChroCART 250–4 Purospher STAR RP-18 ($5\text{ }\mu\text{m}$), the flow rate was 1.0 mL/min; the excitation wavelength was 475 nm and emission was collected at 580 nm. Measurements were carried out isocratically. A mobile phase of acidic water (0.1% formic acid, 0.1% ammonium solution 25%)/acetonitrile 70/30 was used. Limit of quantification was 0.01 mg/L.

2.4. Formulation fabrication and characterization

2.4.1. PEG-PE adsorption to MMT

An MMT suspension (0.6 g/L) was added to PEG(750 or 5000)-PE solutions (0.05–0.56 g/L) (Table S1). The clay-polymer suspensions were agitated (overnight, reaching equilibrium), centrifuged (25,000g for 30 min, $20\text{ }^{\circ}\text{C}$) and supernatant was separated. Polymer concentration in the supernatant was measured (see subsection 2.3.1) and polymer adsorption was calculated accordingly. The low loading composite (0.07 g/g) was denoted LOW and the high polymer loading composite (0.46 g/g) was denoted HIGH.

2.4.2. DOX incorporation

DOX was added to PEG5000-PE (0.07 mM) at 0–1.5 M ratio in poly propylene tubes. The samples were agitated for 24 h (equilibrium reached within 0.5 h [52]), 0.5 ml of solutions were placed into centrifugal filter units, (regenerated cellulose, 30000 MWCO, MILIPORE) and centrifuged for 10 min, 12,000g at $4\text{ }^{\circ}\text{C}$. The filtrated DOX, which wasn't incorporated into the micelle structure, was measured (see subsection 2.3.2) and compared to the filtrated “free” DOX at the same concentration to avoid overestimation of DOX incorporation which was calculated accordingly.

2.4.3. DOX/MMT preparation

DOX (7–56 mg/L, 2.5–20% of the MMT CEC) was added to MMT (0.6 g/L). After reaching equilibrium, samples were centrifuged (25,000g for 30 min, $20\text{ }^{\circ}\text{C}$) and the supernatants were separated. DOX concentrations in the supernatants were measured (see subsection 2.3.2) and adsorption was calculated accordingly. The resulting formulation was denoted DOX/MMT (Table S3).

2.4.4. M-DOX/MMT preparation

DOX was incorporated in micelles, at 1:1 DOX: PEG5000-PE molar ratio (0.012–0.096 mM, Table S2) and was denoted M-DOX. M-DOX was added to MMT (0.6 g/L) and samples were agitated for 24 h (reaching equilibrium). After reaching equilibrium, samples were centrifuged (25,000g for 30 min, $20\text{ }^{\circ}\text{C}$) and the supernatants were separated. DOX and PEG-PE concentrations in the supernatants were measured (see subsection 2.3) and adsorp-

tion was calculated accordingly. The resulting formulations were denoted M-DOX/MMT.

2.4.5. X-ray diffraction (XRD) measurements

The diffractions of PEG5000-PE (0.84 g/L), PEG5000-PE/MMT composites (0.07–0.46 g/g clay) and of DOX incorporated in PEG5000-PE micelles which were adsorbed to the clay (M-DOX/MMT formulations, 0.1–0.48 g/g clay) were collected by X-ray diffractometer (Philips PW1830/3710/3020) with Cu KR radiation, $\lambda = 1.526\text{ }\text{\AA}$. On a glass slides, 1–2 mL of the suspensions were left to sediment (oriented sample) for 24 h. The diffractions were measured before and after heat treatment at $360\text{ }^{\circ}\text{C}$.

2.4.6. Thermal measurements

Thermal gravimetric analysis (TGA) of air equilibrated freeze dried PEG5000-PE/MMT composites (0.07–0.46 g/g clay), before and after rinsing the composites with tap water, was carried out by a Q500 Thermogravimetric Analyzer (TA instruments Inc.). The high resolution-dynamic program (sensitivity-1, resolution-4) includes a heating rate of $25\text{ }^{\circ}\text{C}/\text{min}$, from 30 to $800\text{ }^{\circ}\text{C}$, nitrogen flow rate was 60 mL/min. The adsorption of PEG-PE was calculated excluding the weight loss under $150\text{ }^{\circ}\text{C}$ and above $550\text{ }^{\circ}\text{C}$ associated with adsorbed and structural water, respectively.

2.4.7. FTIR measurements

FTIR spectra were obtained for MMT, PEG5000-PE and a PEG5000-PE/MMT composite (0.46 g/g clay). Pellets were prepared from dried polymer or composite mixed with KBr (ratio of 2:98). FTIR spectra were recorded at room temperature in the range of $500\text{--}4000\text{ cm}^{-1}$ using a FTIR spectrometer (Nicolet Magna-IR-550, Madison WI).

2.4.8. Zeta potential measurements

Zeta potentials of PEG5000-PE (0.048 mM) and M-DOX (up to 1:1 DOX:PEG5000-PE molar ratio), DOX/MMT formulations (Table S3), and formulations of M-DOX/MMT (Table S2) were measured using a Zetasizer Nano-system (Malvern Instruments, Southborough, MA).

2.4.9. RT-TEM and Cryo-TEM images

RT-TEM. A $3\text{ }\mu\text{L}$ drop of the sample was applied to a TEM grid (ultrathin carbon film on Lacey carbon support film, 400 mesh, copper, Ted Pella, Ltd.) following a short treatment of the grid by glow discharge. The excess liquid was blotted off using a filter paper and the grid was left to dry in air. The dry samples were examined using FEI Tecnai 12 TWIN TEM operated at 120 kV and the images were recorded by a $4\text{K} \times 4\text{K}$ FEI Eagle CCD camera.

Cryo-TEM. A $5\text{ }\mu\text{L}$ drop was applied to a TEM grid (300 mesh Cu Lacey substrate, Ted Pella, Ltd.) following a short treatment of the grid by glow discharge. The excess liquid was blotted off and the specimen was vitrified by rapid plunging into liquid ethane precooled by liquid nitrogen using a vitrification robot system (Vitrobot mark IV, FEI). The vitrified samples were examined at $-177\text{ }^{\circ}\text{C}$ using FEI Tecnai 12 TWIN TEM operated at 120 kV and equipped with a Gatan model 626 cold stage. The images were recorded by a $4\text{K} \times 4\text{K}$ FEI Eagle CCD camera in low dose mode.

2.4.10. Vis-spectrophotometer measurements

DOX (0.012–0.096 mM, 7–56 mg/L), M-DOX (DOX:PEG5000-PE in 1:1 M ratio), DOX/MMT formulations (Table S3) and M-DOX/MMT formulations (Table S2) were prepared as mentioned above. UV-vis spectra (400–700 nm) were collected in quartz cuvettes by a UV-vis spectrophotometer (Thermo- evolution 300). In addition, the stability of DOX/MMT and M-DOX/MMT formulations was assessed by comparing the absorption of the aliquot at 480–500 nm immediately after shaking and after 2 h.

2.5. DOX release from the formulations

2.5.1. DOX desorption at equilibrium

DOX/MMT and M-DOX/MMT formulations were prepared as mentioned above. The wash cycle included addition of 1.5 ml of distilled water to the precipitates (in 1.5 ml Eppendorf vials). The solutions were vigorously agitated until resuspension was achieved. After 24 h the samples were centrifuged (25,000g for 30 min, 20 °C) and DOX and PEG-PE concentrations in the supernatant were measured.

2.5.2. Kinetics of DOX release

Two concentrations of DOX (0.012 and 0.096 mM), M-DOX (at 1:1 M ratio, and two respective loadings of DOX in DOX/MMT and M-DOX/MMT formulations (11.3 ± 0.15 and 84 ± 4 mg/g clay) were added to dialysis tubes (Spectra Pore 6, MWCO 50000). The dialysis bags were inserted to a phosphate buffer solution (0.14 M pH 7.4) reaching an internal/external volume ratio of 1:10 (formulation suspension–4.5 ml, buffer solution 45 ml). DOX release increased with an increase in micelle concentration in solution (Fig. S1). To avoid this effect, PEG-PE concentration in the external solutions was monitored and kept below the CMC (0.036 g/L, 6.2×10^{-6} M). The release of each case was compared to the release rate of “free” DOX from the dialysis bag at the relevant concentration. Aliquots from the external solution were transferred to glass HPLC inserts (150 μ L) (volume removed was about 6% from the total volume) and DOX concentrations were measured with time by HPLC. No relative release differences were observed for M-DOX and DOX/MMT formulations in different DOX concentrations.

2.6. Bioassays

2.6.1. Formulations and composites preparation

In order to work with constant DOX concentration, the preparation procedure was adjusted. HIGH formulation was prepared by incorporating 30 μ M of DOX into access concentrations of PEG-PE (0.096 mM) reaching equilibrium; consequently, MMT (0.6 g/L) was added to the micelles solution. (No significant cytotoxicity or size differences were observed when different preparation procedure that includes removal of the non-adsorbed PEG-PE was applied). LOW formulation was prepared by preliminary adsorption of PEG-PE to MMT (0.07 g/g clay) and consequently adsorption of DOX (30 μ M). M-DOX and DOX/MMT formulations were prepared as described above at relevant concentration of DOX (30 μ M). Almost complete DOX adsorption was obtained in all cases (95–99%). Composites were prepared in the same procedure without the addition of DOX.

2.6.2. Cell association studies: interaction of formulations with cells

Cells were plated at a density of 8×10^4 cells per well in 12-well plates, and allowed to adhere for 24 h in a humidified atmosphere at 37 °C and 5% CO₂. After incubation, the cells were washed once with media and incubated with various formulations containing DOX at a concentration of 3.33 μ M for 2, 6 or 24 h. Following this incubation, the formulation containing medium was removed from the wells and cells were washed twice with media. Cells were detached from the plates by addition of 0.2 mL trypsin-EDTA, and 1 ml complete media (containing FBS) was added to cells to neutralize trypsin. Cells were then collected in 1.5 ml centrifuge tubes and spun at 0.3g for 5 min to obtain a pellet. The pellet was washed twice with cold PBS, pH 7.4 and finally resuspended in 0.15 mL cold PBS, pH 7.4. The fluorescence of formulation-treated cells was then analyzed immediately using a BD FACSCalibur™ (Becton Dickinson, Franklin, NJ). Cells were gated upon acquisition using forward vs. side scatter to eliminate the dead cells and debris.

10,000 gated events were collected for each sample. The red fluorescence of DOX was detected in the FL-2 channel at an emission wavelength of 580 nm. Analysis was performed using the CellQuest™ Pro software (Becton Dickinson, Franklin, NJ). The mean fluorescence intensity (MFI) was plotted for all groups on a bar graph.

2.6.3. Cytotoxicity studies

Cells were plated at a density of 3×10^3 cells per well in 96-well plates (Corning, Inc., NY, USA). They were incubated for 24 h in a humidified atmosphere at 37 °C and 5% CO₂. After incubation, the medium was replaced with free DOX or various DOX formulations containing DOX in a concentration range of 0.1–10 μ M. The cells were incubated with the formulations for 24 and 48 h. Cells treated with only cell culture medium were used as controls. After incubation, cells were washed twice with media and cell survival was measured using CellTiter-Blue® cell viability assay (Promega, Madison, WI) as per the manufacturer's protocol. The fluorescence of the plates was read at a wavelength of 530 ex/590 nm using a Bio-Tek Synergy HT multi-detection micro-plate reader (BioTek, Winooski, VT). The data were analyzed using the Gen5 software (BioTek, Winooski, VT).

2.6.4. Statistical analysis

The results of cell interaction and cytotoxicity tests are expressed as mean $\pm$ SD (3 repetitions). One-way ANOVA and two-way ANOVA tests were used to determine the statistical differences among groups and considered statistically significant when $p < 0.05$.

2.6.5. Confocal Imaging to study the internalization of DOX formulations into cells

Cells were grown on glass cover-slips in 12-well plates to 60–70% confluency. After 24 h of seeding, cells were treated with DOX formulations containing DOX at a concentration of 3.33 μ M for 2 or 6 and 24 h. After incubation, the cells were washed three times with DMEM to remove the unbound formulations and then fixed with 2% paraformaldehyde (15 min at RT). Following fixation, the cells were washed twice with PBS and incubated with Hoechst 33342 (5 μ g/ml) containing PBS for 10 min at room temperature. The cover-slips were mounted on glass slides with Fluoromount-G® medium and sealed using nail lacquer. The slides were observed with Zeiss LSM 700 inverted confocal microscope (Carl Zeiss Co. Ltd., Jena, Germany) equipped with a 63×7 , 1.4-numerical aperture plan-apochromat oil-immersion objective. The images were analyzed using the Fiji software [53]. The laser power and gain settings as well as the brightness and contrast values were kept constant in all images during the acquisition and analysis. The mean gray area values were calculated by Image J software with Fiji package (NIH, Bethesda, MD, version 1.51 h) for DOX signal after background correction. First, the nuclei of the cells that were given in Figs. 4b and 5d were selected using the Hoechst signal in the blue channel. Then the DOX signal in the same regions of interest was measured in the red channel. Although the contrast and brightness settings were kept constant in the confocal images, it should be noted that mean gray area values cannot be affected by these settings.

3. Results and discussion

3.1. PEG-PE/MMT composites

The adsorption of PEG5000-PE to MMT was significantly higher than the adsorption of PEG750-PE in terms of g polymer/g clay (Fig. 1a) or when expressed as mol polymer/g clay (Fig. S2). The

adsorption of the longer PEG-PE polymer with the negatively charged MMT (-43 mV), is driven by lower electrostatic repulsion. As the length of the PEG segment increases, the negative charge of PEG-PE micelles is screened out [33] with zeta potentials of -36 and -17 mV for PEG750-PE and PEG5000-PE micelles, respectively. The interactions of the polymer with the clay surface are mainly through direct interactions of the PEG oxygen with the exchangeable cations (ion-dipole) and indirectly through the hydration shell of the exchangeable cation (H-bonds) [54]. This can explain the higher adsorption of micelles with longer PEG chains (apparent hydrodynamic diameter of PEG750-PE and PEG5000-PE micelles is 15 vs. 35 nm).

Indeed, upon adsorption, a change in PEG5000-PE conformation was supported by FTIR measurements (Fig. S3). The polymer, switched from a helix (1061 , 1113 and 1148 cm^{-1}) to a more extended configuration (1118 cm^{-1}) [54], exposing its oxygens and enabling interactions with the clay. A vibration at 1118 cm^{-1} is also obtained for the bare clay, however, the clay has also an indicative Si-O vibration at 1046 cm^{-1} [55] therefore, the increase in the $1118/1046$ ratio for the composite (in comparison to the bare clay) strengthens the adsorption of PEG-PE in an extended configuration.

In addition to higher adsorption, a longer PEG chain also correlates to lower non-specific binding to plasma proteins and to longer systemic circulation [56]. Therefore, PEG5000-PE was chosen for formulation fabrication.

The adsorption of PEG5000-PE at low concentrations is nearly complete and reaches a plateau at higher concentrations indicating two phases of adsorption (Fig. 1a). Polymer desorption from all PEG5000-PE/MMT composites was negligible reaching less than 5% desorption for the highest polymer loading emphasizing the

cumulative nature of the interactions. The low loading composite (0.07 g/g) was denoted LOW and the high polymer loading composite (0.46 g/g) was denoted HIGH.

To unveil the nano-structural differences between the LOW and HIGH composites, the composites were characterized by XRD and TGA measurements. For the non-adsorbed polymer, three indicative diffractions (0.46 , 0.38 and 0.29 nm) of its crystalline structure were obtained as reported for other PEG surfactants [54]. These diffractions were not obtained for the adsorbed polymer implying that adsorption of the polymer on the clay surfaces induces polymer structural changes. The basal spacing of MMT, 1.24 nm, [57] was increased as a function of PEG-PE loading (Fig. 1b and 2c). For the LOW and HIGH composites, basal spacings of 1.4 and 1.79 nm were obtained, respectively. An intercalation of one and two parallel polymer layers can explain the increase in basal spacing for LOW and HIGH composites [58]. An intermediate basal spacing of 1.6 nm was reported for an intermediate PEG loading of 0.22 g/g indicating a more extended monolayer conformation [58]. PEG-PE intercalation was confirmed by heating the samples to 360 °C and obtaining higher basal spacings (1.07 – 1.1 nm) than the non-hydrated spacing (0.97 nm) of MMT. The size and PDI of MMT and composites in complete media were measured. Size was 144 ± 54 , 140 ± 46 and 161 ± 55 nm and PDI was 0.34 , 0.28 and 0.22 for MMT, LOW and HIGH composite, respectively. The particle sizes was confirmed by TEM images, for both MMT and HIGH composite particle sizes were between 100 and 200 nm and diverse morphology can be observed (Fig. S4a and S4b). Although the similarity between MMT and the composite, it looks like the composite platelets have less of a tendency to sediment one on top of the other, implying that the composite has a different surface, evolved from the PEG-PE cover. The distinction between

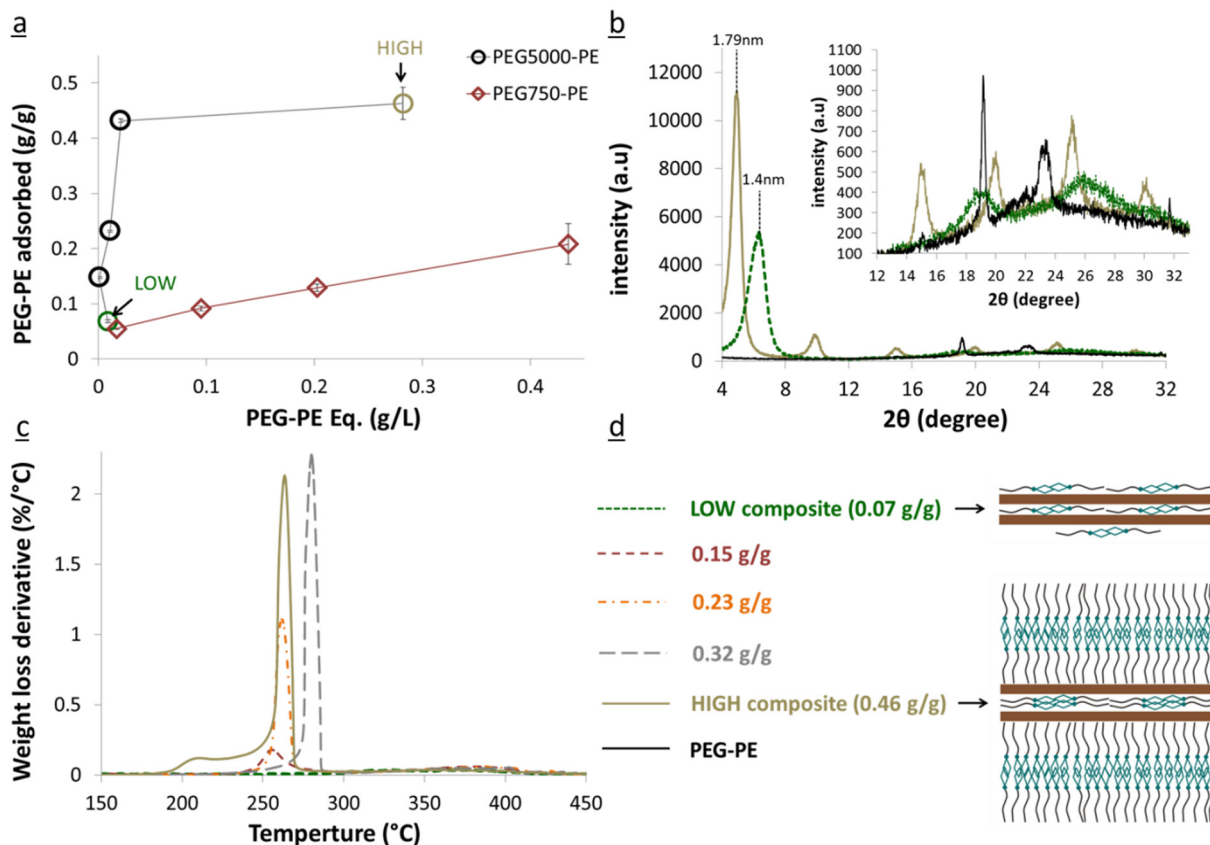


Fig. 1. a The adsorption of PEG750-PE and PEG5000-PE on MMT (0.6 g/L). b. Diffractograms of PEG-PE and of the LOW and HIGH composites. c- DTG measurements of MMT and PEG-PE/MMT composites. d. Suggested conformations of LOW and HIGH composites.

the two structures is limited using RT-TEM since it involves drying [59]. To better characterize suspensions, a Cryo-TEM image of the HIGH composite was obtained (Fig. S4c). In contrast to the reported dispersed mono layer structure of Na-montmorillonite, similar condition (Distilled water) [59], HIGH composite exhibited more complexed structure with small clusters of clay platelets.

Polymer intercalation for the LOW and HIGH composites was further supported by DTG measurements of the composites. For PEG-PE (non-adsorbed) two weight losses were obtained at 210 and 270 °C (Fig. S5). Upon polymer adsorption, the weight loss was obtained at higher temperatures of 260–280 °C and at a very high temperature of 380 °C supporting thermo-stabilization of the polymer upon adsorption [60] (Fig. 1c). The weight loss at the higher temperature (380 °C) can be attributed to polymer intercalation. Polymer weight loss at 380 °C was 0.071 and 0.114 g/g for the LOW and HIGH composites, respectively, supporting intercalation of one or two polymer layers. The weight loss at 260–280 °C was affected by polymer loading and can be attributed to external polymer adsorption [61]. For the LOW composite polymer weight loss at 260–280 °C was negligible indicating low adsorption on the external clay surface. As polymer loading in the composites increased (0.15–0.32 g/g), the temperature of polymer weight loss increased (260–280 °C) (Fig. 1c). However, for the HIGH composite, the weight loss was obtained at the lower temperature (260 °C) and an additional loss was obtained at even a lower temperatures (200–260 °C), similar to the non-adsorbed polymer. The increased weight loss temperature as function of polymer loading can evolve from intrinsic interactions between PEG-PE chains and the clay. The weight loss at the low temperatures for the HIGH composite suggests a structure in which a polymer layer is relatively remote from the clay surface. For the

adsorption of PEG based surfactants on hydrophilic surfaces (silica) two structures were suggested; globular and bilayer [62]. However, High affinity between the surfactant and the surface leads to a flattening of the micelle, increasing the contact between the head groups and the surface which results in a bilayer structure [63]. Since the PEG has a high affinity to the clay surface we suggest the formation of bilayer on the external clay surfaces.

Finally, we suggest that for the LOW composite a mono layer of polymer intercalates in the MMT platelets in a parallel orientation and the external clay surfaces are only partially covered. For the HIGH composite two layers of polymer intercalate in a parallel orientation and a bilayer may form on the external surface (Fig. 1d). Such different structures may affect both the stability of PEG-PE/MMT formulations and the rate of DOX release. These two issues along with formulation design were further explored.

3.2. M-DOX /MMT formulations

3.2.1. Formulation fabrication

The design of DOX formulations was based on DOX incorporation in PEG-PE micelles (M-DOX) and the adsorption of these micelles to MMT. For the sake of comparison (structure, stability and performance) formulations based on the direct adsorption of DOX to the clay were prepared as well.

DOX adsorption to the clay (denoted DOX/MMT) was nearly complete, above 94%, since all added concentrations were well below the clay's cation exchange capacity. Zeta potential of MMT was only slightly reduced, from –41 to –31 mV, upon DOX adsorption. However, DOX adsorption to MMT resulted in a clear red shift in its Vis absorption spectrum (Fig. 2d) indicating strong interactions including electrostatic and H-bonds (discussed below).

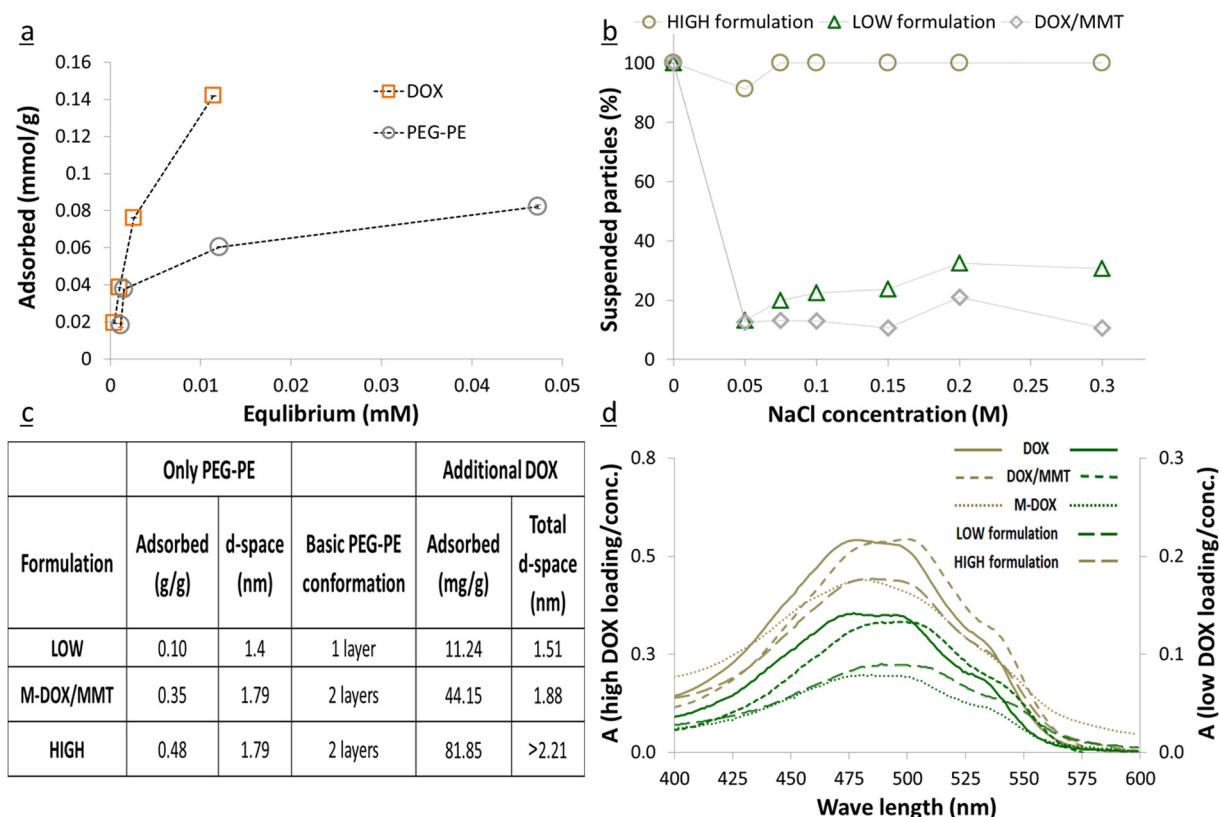


Fig. 2. a Adsorption of DOX and PEG-PE from M-DOX (1:1 M ratio) to MMT (0.6 g/L). b. The stability of DOX formulations as function of NaCl concentrations after 2 h. c. DOX and PEG-PE effect on MMT basal-spacing. d. Absorption of DOX formulations at low (0.012 mM), (green), and high (0.096 mM), (Khaki), added concentrations of DOX, the respective loadings are described in Table S2 and S3.

Complete incorporation of DOX in PEG5000-PE micelles (denoted M-DOX), (0.07 mmol/L) was obtained up to a DOX/PEG-PE molar ratio of 0.5:1. The degree of incorporation slightly decreased at higher ratios of 1:1 (85%) and reached 75% at 1.5:1 ratio. Wang et al. (2009) studied DOX incorporation in smaller PEG-PE micelles and concluded that DOX is located between the PEG and PE segments, dominated by electrostatic interactions between the cationic DOX and the negatively charged phosphate group. These interactions can explain the complete DOX incorporation, up to 1:1 M ratio, reached in these smaller micelles which bare a more negative zeta potential than the PEG5000-PE micelles (−37 vs. −17 mV).

Neither the adsorption of DOX nor the adsorption of PEG-PE was significantly reduced upon the adsorption of PEG-PE and DOX as M-DOX to MMT (1:1 ratio) relative to the adsorption of the sole components (Fig. 2a). Meaning that the adsorption of PEG-PE micelles incorporated with DOX reached similar polymer loadings as the adsorption of the “empty” micelles (Fig. S2). In the case of M-DOX/MMT formulations, PEG-PE adsorption was only partial, while, DOX adsorption was nearly complete. Since DOX is equally distributed between the micelles in solution, the adsorbed state represents a higher DOX/polymer ratio and perhaps a rearrangement of PEG-PE and DOX on the MMT surfaces [64].

Based on the LOW and HIGH composites (Fig. 1d), two DOX formulations, denoted LOW and HIGH formulations, were fabricated.

3.2.2. Formulation characterization

The effect of the structural differences between the LOW and HIGH formulations (Fig. 1d) on their interactions with DOX, on the formulations' stability and on the rate of DOX release was explored.

The stability and size of the DOX/MMT (different DOX loadings) (Table S3), and LOW and HIGH formulations was measured (Table S2). All of the suspensions were stable in distilled water and their size ranged between 90 and 130 nm. In complete cell culture media, the LOW formulation was not stable and an increase in size was observed, due to aggregation (Fig. S6). However, the suspensions of the HIGH formulation and of DOX/MMT (0.2 g clay/L, 10 mM DOX) were stable (tested for 50 h) and their apparent hydrodynamic diameters were 230–300 nm and 420–480 nm, respectively. Despite the less negative zeta potential of the HIGH formulation, in comparison to the LOW formulation, (−21 mV vs. −36 mV) its suspension was more stable, which may suggest a steric stabilization due to high PEG-PE coverage in the HIGH formulation.

A common aggregation mechanism of colloids, such as clays, can be explained in terms of reduction in the electric double layer due to the higher ionic strength of the medium [65]. To emphasize the steric contribution to suspension stability, the stability of the formulations as a function of NaCl concentrations in solution was measured. The LOW formulation displayed negligible stability improvement in comparison to DOX/MMT formulation while the HIGH formulation was completely stable at all concentrations tested; supporting the presence of bilayer on the external surfaces compared to a partial coverage in the case of LOW formulation.

The different external polymer structures affect the stability of DOX formulations. To unveil the role of the intercalated polymer in the formulation performance, the location of DOX within the intercalated polymer layers was assessed (Fig. 2c). Upon direct adsorption of DOX to the clay (44 and 142 mg/g), an additive increase in basal spacing was observed (1.59 and 1.71 nm, respectively). The intercalation was confirmed by post heat treatment (360 °C), as basal spacing of the 142 mg/g composite was reduced to 1.35 nm in comparison to 0.97 nm for MMT (not shown). As demonstrated above (Fig. 1d), PEG-PE intercalates as a monolayer (basal spacing of 1.4 nm) and as a bilayer (basal spacing of 1.79 nm) in the LOW

and HIGH composites, respectively. Above a loading of 0.35 g PEG-PE/g clay the polymer adapts a bilayer and the basal spacing of 1.79 nm does not further increase with polymer loading.

For all cases of M-DOX/MMT formulations, the basal spacing was higher than the basal spacing of the corresponding PEG-PE/MMT composites and the increased basal spacing was correlated to the degree of intercalated DOX (Fig. 2c). In the case of the LOW formulation the XRD results imply that both the PEG-PE layer and the DOX directly adsorb to the internal clay surface. However, in the case of the HIGH formulation the DOX may be located between the intercalated PEG-PE bilayer and/or directly adsorbed to the internal clay surface.

DOX Vis spectra were collected in order to explore whether DOX adsorbed directly to the clay or is hosted by the adsorbed polymer. DOX has a wide peak at about 480–500 nm (Fig. 2d). As expected, DOX spectrum was not affected by the presence of PEG-PE below its CMC. At PEG-PE concentrations above its CMC (M-DOX) a decrease in DOX absorption was observed (Fig. 2d), supporting incorporation. In addition, a small red shift at high wave lengths >540 nm, characteristic of the formation of DOX dimers [66,67], was obtained which obviously occurs upon incorporation.

DOX adsorption on MMT surfaces (at low and high loadings), resulted in a significant red shift (Fig. 2d), explained by direct DOX interactions with the clay surface [68,69]. However, such a shift was only preserved in the case of the LOW formulation while the spectra of the HIGH formulation resembled that of the M-DOX; indicating that for the HIGH formulation DOX is most likely located between the intercalated polymer bilayer.

The differences may be explained in terms of PEG-PE coverage on the clay surfaces; while in the LOW formulation DOX interacts directly with the clay due to low polymer coverage (mono interlayer of PEG-PE), in the case of the HIGH formulation, DOX interactions with the clay are mediated by the intercalated or external PEG-PE bilayer. The different degree of interactions between DOX and MMT undoubtedly affect the rate of DOX release from the formulations [70].

To assess the effect of formulation nanostructure on DOX release, a comparison between its release from DOX/MMT and M-DOX/MMT formulations with increasing DOX/MMT ratios (DOX/PEG-PE molar ratio was 1:1) was conducted (Fig. 3a). As expected, DOX release from clay surfaces was low (1.2–2.9%). Its release from M-DOX/MMT formulations was significantly higher for all cases tested although the active ingredient (% weight) within these formulations was lower. In addition, in most cases (2.5–10 DOX:CEC ratio), release increased as function of PEG-PE loading. Differences in release between the two higher PEG-PE loadings (0.352 and 0.475 g/g) were not observed, indicating that their intercalated bilayer structure governs the higher release rate.

The kinetics of DOX desorption from the LOW and HIGH formulations was monitored (Fig. 3b). DOX release from M-DOX micelles was the highest and fastest reaching complete desorption within 7 h. As expected, the release from DOX/MMT was limited and reached low values (8%). The release from M-DOX/MMT formulations exhibited intermediate values at equilibrium; reaching 28% and 48% release for LOW and HIGH formulations, respectively.

The release trends remained similar (32% and 55% release of residuals DOX for LOW and HIGH formulations, respectively) in a second release cycle, indicating that the unique formulation structure remains intact, governs the release and emphasizes the effect of subsequent dilution. Treating the release of DOX from the surfaces of M-DOX/MMT formulations using first order equation (up to 30 h), (Fig. S7) yields rate constants of 0.0076 and 0.0191 h^{−1}, representing a release rate 2.5-fold higher for the HIGH formulation.

Finally, the effect of the formulations' nanostructures on DOX release was correlated to the degree of DOX-clay interactions

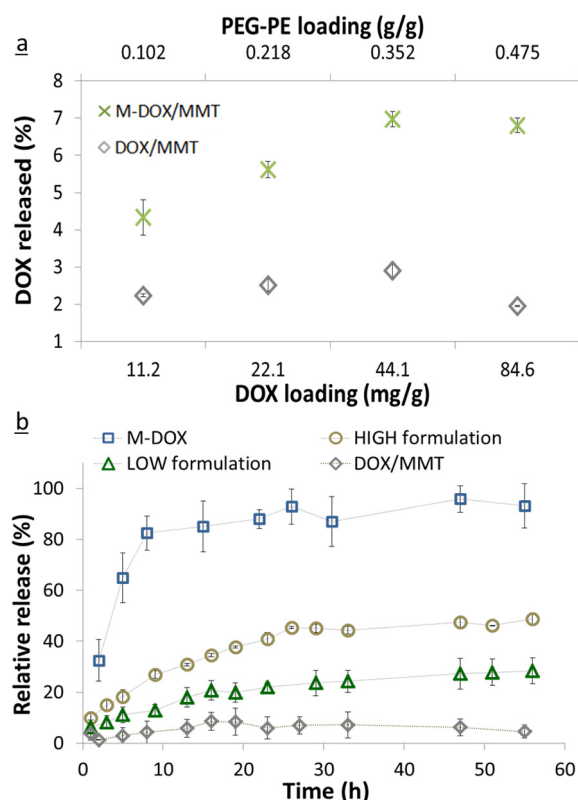


Fig. 3. a. DOX release from DOX/MMT and M-DOX/MMT formulations at different DOX:MMT ratio, (PEG-PE loading (g/g) refers merely to the M-DOX/MMT formulations). b. DOX release from M-DOX, DOX/MMT and LOW and HIGH formulations (relative to “free” DOX) as function of time.

[70]; the weaker the interactions were (due to polymer mediation) the higher and faster the release was. The effect of the formulations’ nanostructures and the release rate on the association and toxicity of DOX with MCF-7 and A2780 ADR cell lines was investigated.

3.3. Bioassays – in vitro experiments

The association of DOX applied as “free” DOX, M-DOX, DOXIL or LOW and HIGH formulations with MCF-7 cells was measured after 2 and 6 h (Fig. 4a). The association of DOX applied as “free” DOX or as M-DOX was the highest, while DOXIL exhibited the lowest association. All three MMT based formulations displayed intermediate association values with the trends coinciding with their release rate: HIGH formulation > LOW formulation > DOX/MMT. In contrast to these results, Feng et al. (2009b) reported that cellular uptake of coumarin 6 loaded nano-particles by MCF-7 cells was enhanced in the presence of MMT, due to high affinity between MMT and cells. However, in our case, since the association of “free” DOX with MCF-7 cell line is high (Fig. 4a) and the release of DOX from MMT formulations is gradual (Fig. 3b), the association of DOX, released from the formulations, with MCF-7 cell-line, was reduced.

In order to define the location of DOX associated with the cells, confocal images were obtained after 2 (Fig. 4b) and 6 h (Fig. S9). “Free” DOX was completely inside the nucleus within 2 h, while, significant amounts of DOX can be seen on the external cell membranes when applied as DOX/MMT or as LOW formulations. When applied as the HIGH formulation most of the DOX is localized inside the nucleus. Fluorescence intensity of the DOX internalized by cells were quantitatively evaluated and confirms the visual

interpretation (Fig. S8a). After 6 h, for all formulations applied, DOX is prominently positioned in the nucleus (Fig. S9) indicating the delaying effect of M-DOX/MMT formulations on DOX internalization.

Cytotoxicity tests for different DOX formulations as a function of DOX concentrations and for control samples, were performed after 24 (Fig. 4c and S10) and 48 h (Figs. S12 and S13). Respective values of the IC_{50} of the formulations are specified in Fig. 4d. The cytotoxicity of the control samples, MMT and LOW and HIGH composites after 24 h was negligible (Fig. S10), [72]. For the formulations, the cytotoxicity of DOXIL was low, in agreement with the association test. In addition, the cytotoxicity trends of the formulations correlated with the release rate trends and association values; i.e., the formulations’ efficiencies are in the order of HIGH formulation > LOW formulation > DOX/MMT. However, despite the lower release rate of DOX from the HIGH formulation, lower association and delayed entrance to the nucleus, in comparison to the “free” DOX, the HIGH formulation’s efficiency was higher. The time gap between the different experiments tested may partially explain the apparent discrepancy. The association and confocal measurements were taken after a short time; hence significantly higher levels of “free” DOX can be seen associated with the cells and inside the nucleus, respectively (Fig. 4a and b). However, after 24 h, when the cytotoxicity was measured, these differences decreased dramatically. The higher cytotoxicity of the HIGH formulation may suggest that the presence of both components, composites and DOX, induces synergetic effect.

The MCF-7 in vitro experiments showed that the release trends of DOX from the various formulations had a dominant effect on its association with the cells, amount of DOX entering the cells and on the degree of cytotoxicity, however, the mechanism of DOX internalization is not yet clear.

To test whether DOX is released on the outer membrane and internalized as “free” molecule and/or internalized as a formulation and released inside the cell, the formulations were tested with Adriamycin (ADR) resistant cell line. The cytotoxicity measurements for A2780 ADR were obtained after 24 (Figs. S14 and S15) and 48 h (Figs. 5a and S11), in both cases the trends were similar; However, after 48 h the differences were more substantial. As for the control samples after 48 h, the cytotoxicity of MMT (control) was negligible, but LOW and HIGH composites caused an intermediate and similar effect (30%) irrespective of their concentrations (Fig. S11).

DOXIL displayed the lowest toxicity (at all concentration) as was found for MCF-7 cells. In contrast to the MCF-7 results, DOX followed DOXIL and exhibited relative low cytotoxicity effect due to DOX removal by the P-gp pumps [51]. All formulations (HIGH, LOW and DOX/MMT) had a better cytotoxicity effect in comparison to the “free” DOX (at all concentrations). In this case (A2780 ADR) the LOW formulation was more efficient than the HIGH one as demonstrated by IC_{50} values after 24 and 48 h (Fig. 5b). After 48 h the IC_{50} values of LOW, HIGH and DOX/MMT formulations were 4.93, 3.26 and 3.05 folds lower than of free DOX value, enabling the use of lower DOX doses. The increased differences after 48 h indicate a slow internalization process in ADR resistant cells which was also demonstrated in other DOX nanoparticles [47].

The advantage of the LOW formulation in comparison to the HIGH formulation (similar cytotoxicity of the LOW and HIGH controls) along with the advantage of the DOX/MMT formulation compared to “free” DOX, (negligible cytotoxicity of bare MMT) (Fig. S11), imply that the MMT surface contributes to the cytotoxicity of DOX.

Since the toxicity trend does not follow the release trend we suggest that the degree of interaction, governed by the external surfaces of the formulations, may promote formulation internalization and thereby affect the toxicity as was demonstrated by

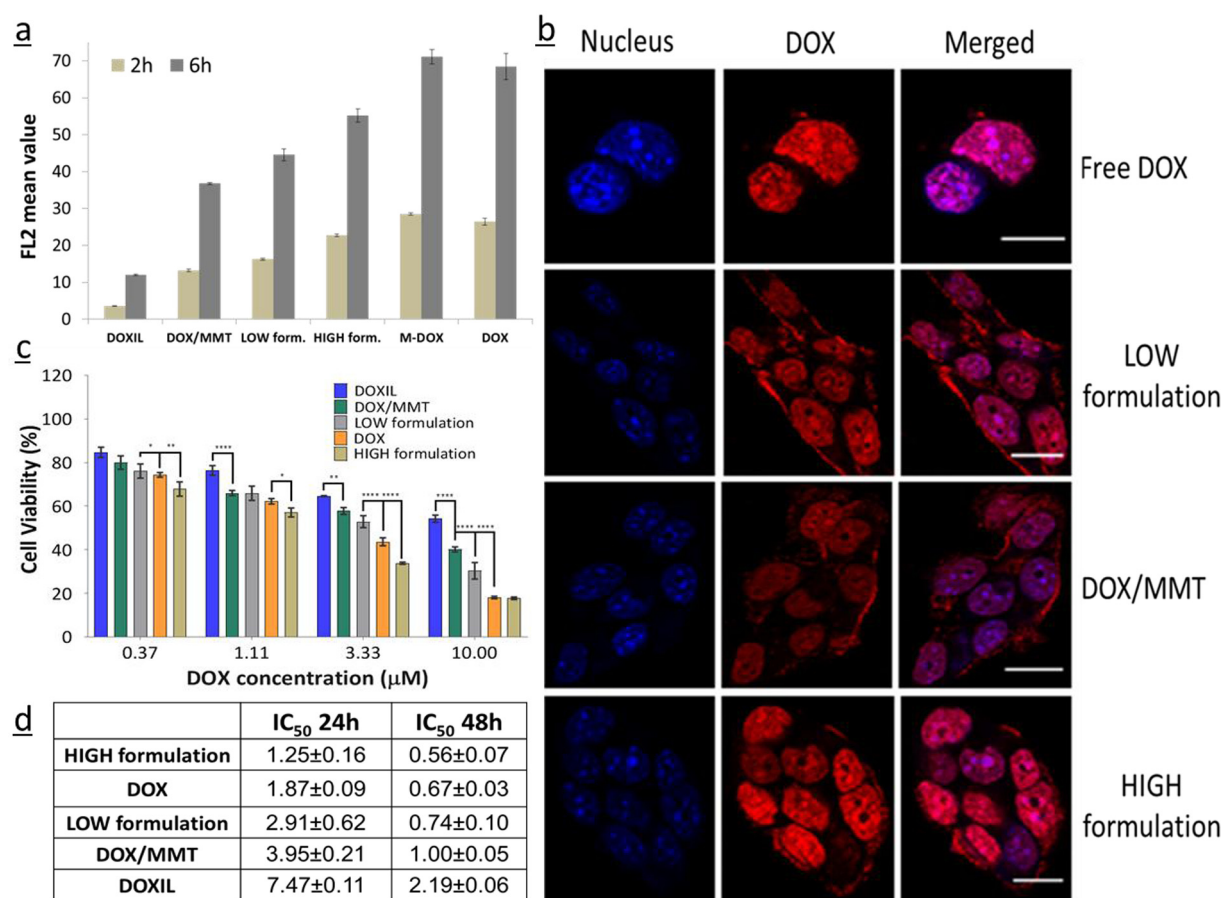


Fig. 4. a Association test for DOX formulations (3.33 μM) with MCF-7 cells after 2 and 6 h, tested by flow cytometry. b. Images of MCF-7 cells' nucleus (blue) and DOX (red) upon 2 h incubation with DOX formulations (3.33 μM), gained by confocal microscopy, scale bars=75 μm . c. Cytotoxicity test for DOX formulations (0.37–10 μM) on MCF-7 cells after 24 h. d. IC₅₀ for DOX and DOX formulations with MCF-7 cell line.

other inorganic platforms [48,73,74]. Omelyanenko et al. (1998) followed the mechanism of cellular uptake and found that DOX conjugated to targetable copolymer was internalized through endocytosis to A2780 ADR resistant cells and accumulated in perinuclear regions. They suggested that the subsequent release affects ADR concentration gradient, increases its availability to the nucleus and reduces its exposure to P-gp activity.

The suggested interaction-internalization mechanism is further supported by association tests (Fig. 5c) and by the confocal microscopy images (Fig. 5d). At short reaction times (two h, results not shown) the differences between the formulations were negligible. At longer reaction times, after 6 and 24 h (Fig. 5c), the advantage of the LOW formulation compared to the “free” DOX was significant. After 24 h the association level is in the order of LOW formulation > DOX/MMT > HIGH formulation > free DOX. The higher interactions of MMT related formulations with these cells can be attributed either to the increased viscosity of the particle suspension or to direct hydrogen and London van der Waals interactions with the cells [71,76].

The more efficient internalization of the LOW formulation is reflected by confocal microscopy measurements (Fig. 5d). When applied as “free” molecule, DOX was not accumulated in the nucleus due to the activity of the P-gp pumps and was concentrated mostly at the external cell membrane. While administered as LOW formulation, DOX displayed higher concentrations in the cytosol and nucleus rather than on the cell membrane (Fig. S8b). In contrast, for HIGH formulations, despite its higher release rate,

lower DOX concentrations were observed in the cytosol and nucleus; suggesting that the presence of PEG-PE on the outer formulation surface delays formulation internalization. The location of DOX release might be a crucial factor in the case of ADR cells, if the release occurs outside the cells, “free” DOX is pumped out by P-gp pumps. However, if the formulation can be internalized (as it is suggested for LOW and DOX/MMT formulations) DOX can be released inside the cells and the cytotoxicity effect can be enhanced.

Finally, the toxicity efficiency of the formulations is a function of two main factors: the rate of DOX release from the formulation and formulation association with the cell membrane which may induce internalization. The formulations' nanostructure affects both factors, but while the release rate is cell-line independent the interactions are obviously cell dependent. The effect of DOX formulations on cell viability was more dominant in the case of ADR resistant cells. Likewise, Thierry et al. (1993) reported that DOX transport via encapsulation in liposomes may alter the intracytoplasmic vesicle transport in ADR resistant cells. Accordingly, it appears that in the case of the sensitive cells the rate of DOX release is the dominant factor that controls toxicity. Whereas, in the case of the resistant cell line, formulation interactions with the cells are predominant; however, the (intra cellular) release rate may also contributes to the cytotoxicity effect. We suggest that DOX entrance by formulation internalization reduces DOX removal by P-gp pumps and enables DOX release in perinuclear regions [75].

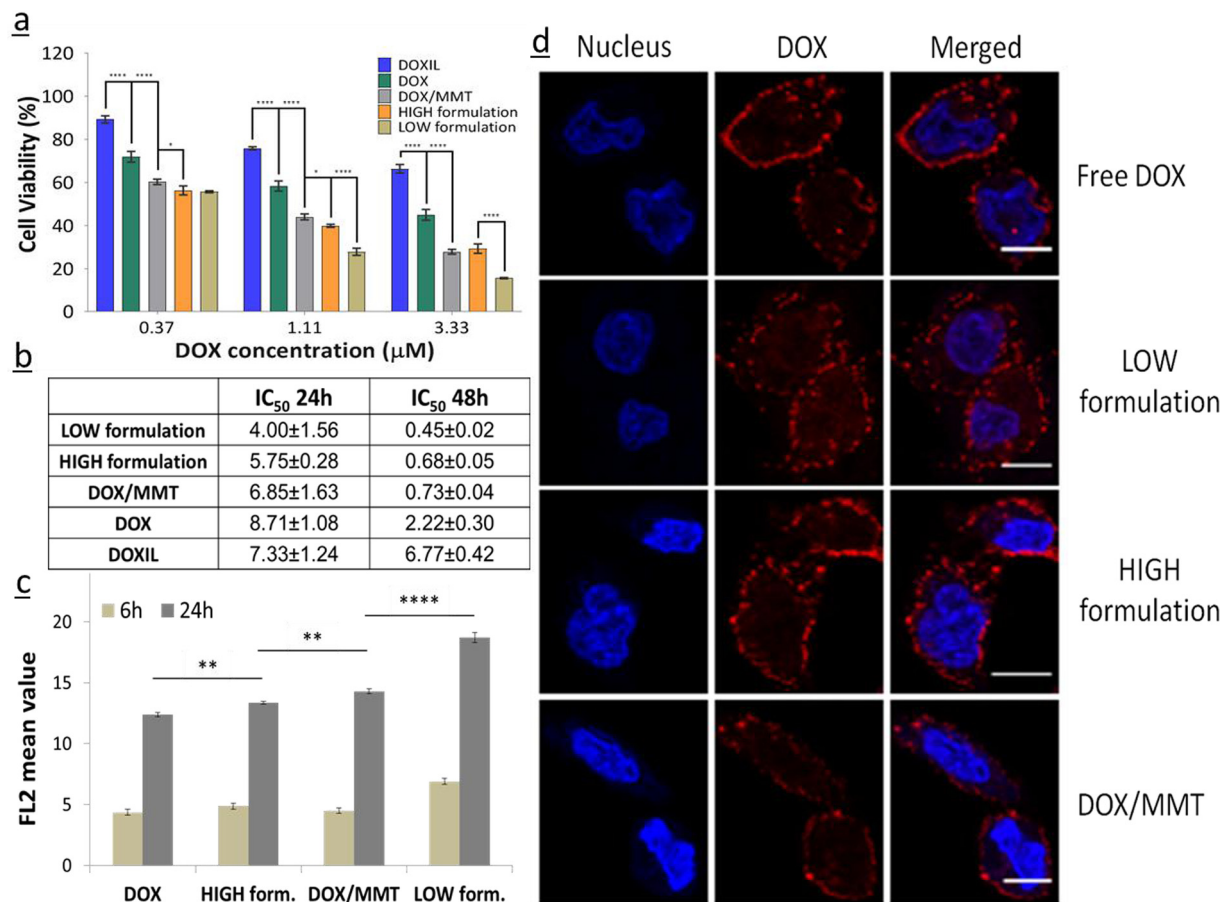


Fig. 5. a Cytotoxicity test for the DOX formulations (0.37–10 μM) on A2780-ADR cells after 48 h. b. IC₅₀ for DOX and DOX formulations with A2780-ADR cell line. c. Association test for DOX formulations (3.33 μM) with A2780-ADR cells after 6 and 24 h, tested by flow cytometry, scale bars–10 μm . d. Images of A2780-ADR cells' nucleus (blue) and DOX (red) upon 24 h incubation with DOX formulations (3.33 μM) gained by confocal microscopy.

4. Conclusions

Two DOX formulations based on different PEG-PE/MMT ratios, (LOW and HIGH) were designed and characterized. For the LOW composite, a mono layer of polymer intercalated in the MMT platelets and the polymer partially covered the external clay surfaces. For the HIGH composite two layers of polymer intercalated and a bilayer formed on the external surface. We demonstrated the correlation between these different structures and both the stability of the formulations and the rate of DOX release. The release trend followed the order of HIGH formulation > LOW formulation > DOX/MMT. On sensitive cells (MCF-7), despite its slower release, HIGH formulation exhibited higher cytotoxicity effect in comparison to the “free” DOX. In the case of Adriamycin resistant cell line (A2780-ADR), LOW formulation demonstrated the highest cytotoxicity. A better understanding of the contribution of formulation-cell interactions vs. the contribution of DOX release rate was reached by confocal microscopy images and association tests. Internalization of the formulations was suggested as a mechanism that reduces DOX removal by P-gp pumps and increases its efficacy in ADR cells. The mechanism of clay internalization as a function of PEG-PE coverage and the effect of targetable sites on the formulations specificity are two subjects to be further investigated.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.actbio.2017.04.008>.

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