Published in final edited form as:

J Control Release. 2012 May 10; 159(3): 403–412. doi:10.1016/j.jconrel.2012.01.022.

Polyelectrolyte multilayer nanoshells with hydrophobic nanodomains for delivery of Paclitaxel

Thomas Boudou^{#1}, Prathamesh Kharkar^{#1}, Jing Jing², Raphael Guillot¹, Isabelle Paintrand², Rachel Auzely-Velty², and Catherine Picart^{1,*}

¹Grenoble Institute of Technology and CNRS, UMR 5628, LMGP, 3 parvis Louis Néel, 38 016 Grenoble, France

²Centre de Recherches sur les Macromolécules Végétales (CERMAV-CNRS), affiliated with Université Joseph Fourier, and member of the Institut de Chimie Moléculaire de Grenoble, 601 rue de la piscine, Grenoble, France

[#] These authors contributed equally to this work.

Abstract

Efficient and effective delivery of poorly water-soluble drug molecules, which constitute a large part of commercially available drugs, is a major challenge in the field of drug delivery. Several drugs including paclitaxel (PTX) which are used for cancer treatment are hydrophobic, exhibit poor aqueous solubility and need to be delivered using an appropriate carrier. In the present work, we engineered Taxol-loaded polyelectrolyte films and microcapsules by pre-complexing PTX with chemically modified derivative of hyaluronic acid (alkylamino hydrazide) containing hydrophobic nanocavities, and subsequent assembly with either poly(L-lysine) (PLL) or quaternized chitosan (QCHI) as polycations. The PTX loading capacity of the films was found to be dependent on number of layers in the films as well as on the initial concentration of PTX pre-complexed to hydrophobic HA, with a loading capacity up to 5000-fold the initial PTX concentration. The films were stable in physiological medium and were degraded in the presence of hyaluronidase. The PTX-loaded microcapsules were found to decrease the viability and proliferation of MDA MB 231 breast cancer cells, while unloaded microcapsules did not impact cell viability. All together, our results highlight the potential of hyaluronan-based assemblies containing hydrophobic nanodomains for hydrophobic drug delivery.

Keywords

Polysaccharides; Hyaluronic acid; Chitosan; Hydrophobic drug delivery; Paclitaxel; Layer-by-layer film; Biodegradability, Breast cancer

*corresponding author, Catherine.picart@grenoble-inp.fr; Phone : (33)-456529311; Fax : (33)-456529301.

Supporting information

Supplementary data associated with this article can be found, in the online version DOI:xxx.

1. Introduction

Delivery of hydrophilic and hydrophobic drugs is currently a great challenge in the field of nanobiotechnology, especially for water-insoluble organic compounds, which constitute a great part of the currently available drugs, whether anti-inflammatory or anti-cancer [1, 2]. Indeed, these drugs tend to induce a systemic cytotoxicity due to high affinity towards lipid cell membranes and to spontaneously aggregate in the blood stream, ultimately reducing the bioavailability of the drug. The challenge thus is to improve the therapeutic efficacy and reduce the toxicity profiles of the hydrophobic drug by delivering it using an appropriate carrier. For these reasons, different types of nanoparticulate or microparticulate carriers have been investigated [3]. Besides the well known lipid-based carriers such as liposomes, [4, 5], the development of micelles [6], nanoparticles [7, 8], emulsion-based nanocapsules [9, 10] and more recently polymersomes [11, 12] has gathered increased interest in the pharmaceutical field. However, micellar drug carriers still exhibit limited loading ability as well as a limited control of their release rate whereas emulsion-based systems have limitations associated with the necessity of an oil phase [13]. Despite these recent technological developments, the delivery of Paclitaxel (PTX), one of the most potent chemotherapeutic drug used in cancer treatment [14], is still limited by its hydrophobicity [3]. PTX is an antiproliferative drug, extracted from the bark of the Pacific yew tree, which binds to the β -subunits of tubulin and promotes the formation of extremely stable and non-functional microtubule bundles [15]. As a result, cell replication is blocked in the late G2 and M phase of the cell cycle [16], strongly limiting proliferation. PTX is currently administered in a mixture of Cremophor EL (polyoxyethylated castor oil, CrEL) and dehydrated ethanol to solubilize it, which is believed to be responsible for hypersensitivity reactions, nephrotoxicity and neurotoxicity observed in patients [17]. As CrEL PTX dosage is not a targeted (acting only against the cancer cells) delivery system, it is usually administered at a higher quantitative dosage, increasing the undesirable side effects. Thus, there is a strong need to develop targeted drug carriers which will decrease the body burden of the drug to reduce side effects. Recently, a solvent free injectable formulation of PTX complexed with a protein, albumin (Abraxane[®]) received FDA approval [18] due to improved drug efficacy and improved response as compared to CrEL based formulations. However high cost and life-threatening toxicity caused by drug metabolism seriously limits applicability of Abraxane[®] in cancer chemotherapy treatment [19]. Therefore, there is a need to develop drug delivery system which would not only increase the water-solubility of PTX and decrease the toxic side effects but also be therapeutically efficient and cost-effective. Synthetic and natural polymers are especially interesting candidates due to the versatility of their physico-chemical and biodegradable properties [20]. Depending on their structure, backbone and functional groups, these polymers can be either hydrophilic or hydrophobic and their biodegradability can be finely tuned. Biodegradation can be achieved by simple hydrolysis in the case of synthetic polymers [21, 22] and by enzymatic degradation in the case of natural polysaccharides or polypeptides [23]. Interestingly, the layer-by-layer assembly technique of polyelectrolytes onto a solid or biodegradable template to form polyelectrolyte multilayer films (PEMs) has recently emerged as a promising technique to engineer drug delivery systems [24-27]. Several strategies have already been proposed to trap and release cancer drugs, either by employing the film as a reservoir [28,

29] or by coating preformed drug crystals [1, 30] or porous silica templates containing the drug with PEM films [31]. Until now, few strategies have been reported for incorporation of poorly water-soluble molecules using PEM films. Thierry *et al.* reported prodrug approach in which PTX was covalently coupled to hyaluronan (HA), a natural polymer, via labile succinate ester and embedded inside the multilayer films [32]. A similar approach was adopted by Caruso *et al.* [33] using poly(L-glutamic acid) for coupling PTX and subsequently preparing PTX-loaded polyelectrolyte microcapsules. A drawback of the prodrug strategy is the requirement to chemically modify the PTX molecule for subsequent grafting. Hammond *et al.* used a cyclodextrin-drug complex assembled with a hydrolysable synthetic polymer (a poly β amino ester) to load and subsequently deliver the anti-inflammatory drug Diclofenac [2]. Another strategy for incorporation of hydrophobic molecules within PEMs involved the use of amphiphilic polysaccharides associated with a synthetic polyelectrolyte, such as poly(ethylene imine) [34, 35]. However, this strategy may suffer from problems associated with controlled release of the drug molecules at physiological pH. Other interesting methods of encapsulation of hydrophobic drugs have been lately developed through the loading of water-insoluble drugs to the capsule by introducing lipophilic phases inside the capsules [13] or embedding liposomes within polymer capsule multilayers [36], but these techniques have limitations associated with sample polydispersity and the necessity for an oil phase. To our knowledge, no previous study has attempted to selectively trap a hydrophobic molecule in the nanoshell of polyelectrolyte capsules made from PEM films deposited on a biodegradable core.

Recently, we reported the synthesis of various alkylamino hydrazide derivatives of HA and their ability to form planar PEM when associated with a polypeptide such as poly(L-lysine) (PLL) [37] or a chitosan (CHI) derivative that is soluble at physiological pH [37, 38]. It was found that these PEM assemblies can selectively incorporate the model hydrophobic dye Nile Red (NR) within the PEM film [37] by simple pre-complexation of the dye with the HA derivative. It was also demonstrated that incorporation of NR was dependent on both the degree of substitution and the length of the alkyl chain grafted to HA. Highest amounts of NR were incorporated in the decylamino hydrazide derivative of HA with a degree of substitution of 0.2 (HA20C10). We also demonstrated that the hydrophobic shell can be deposited on a spherical biodegradable template and that mild conditions can be applied to degrade the core [38]. The resulting microcapsules with hydrophobic shell specifically trapped NR in their nanoshell [38].

In the present work, our aim was to use these hydrophobic nanoshells based on chemically modified polysaccharides and polypeptides to deliver the hydrophobic cancer drug paclitaxel to the breast cancer cells. The originality here is to selectively trap the hydrophobic drug in the hydrophobic nanoshell, as the drug can not be trapped in the hydrophilic microcapsule cavity. HA was chosen as polyanion due to its interesting biological properties. Indeed, HA is a non-immunogenic, natural glycosaminoglycan, which is a major ligand for CD44 receptor, and it can be used to target cancer cells having overexpressed CD44 glycoproteins [39-41]. HA can also be degraded by enzyme of the hyaluronidase family [42]. We first compared the ability of PTX-precomplexed HA20C10 paired with either PLL or quaternized chitosan (QCHI) to form (PLL/HA20C10) and

(QCHI/HA20C10) PEM films (Fig. 1). We quantified PTX incorporation in the PEM films as well as its release in a physiological medium and in presence of the hyaluronidase enzyme, which is overexpressed in several breast cancer cell lines [43]. We then show that PTX remain selectively trap in the film when the film is deposited on a degradable template. Finally, we evaluated the potential of the PTX-loaded microcapsules for cancer therapy by assessing viability and proliferation of human breast cancer cells (MDA-MB231) cultured in the presence of unloaded and PTX-loaded microcapsules.

To our knowledge, we demonstrate here for the first time the selective entrapment of a hydrophobic drug (here paclitaxel, PTX) in a planar film as well as in the nanoshell of a PEM microcapsule. We further demonstrate its effectiveness in killing cancer cells.

2. Materials and methods

2.1 Materials

Poly(L-lysine) (PLL) hydrobromide (P2626, 6.8×10^4 g/mol) and poly(ethyleneimine) (PEI, 60 000 g/mol) were purchased from Sigma (USA) and hyaluronan (HA, 3.6×10^5 g/mol) from Lifecore Biomedical (USA). Chitosan (CHI, Protasan, 1.8×10^4 g/mol) with a degree of *N*-acetylation (DA) of 0.09 was kindly provided by FMC BioPolymer AS, Novamatrix (Norway).

Decylamino hydrazide derivative of HA (HA20C10), *i.e.* HA bearing 20 alkyl chain of 10 carbons every 100 repeating disaccharide units (degree of substitution (DS) of 0.2), and quaternized derivative of chitosan (QCHI) were synthesized following a previously published protocol [37, 44]. The substitution degree of CHI was of 1.1. HA20C10 and QCHI solutions were dissolved overnight in pure water at 2 mg/mL under gentle stirring. A 10× concentrated phosphate buffered saline (PBS) solution at pH 7.4 was subsequently added at 1:10 v/v to the polysaccharide solutions to reach a final salt concentration of 0.15 M NaCl. PLL was dissolved at 0.5 mg/mL in 1× PBS (pH 7.4).

Paclitaxel (PTX, 854 g/mol) and Oregon green labeled paclitaxel (OG-PTX, 1319 g/mol) were purchased from Invitrogen (USA). PTX and OG-PTX were dissolved in analytical grade methanol for pre-complexation with HA20C10 and in dimethyl sulfoxide (DMSO) for cell culture experiments. Hyaluronidase type IV (HAase, 1040 units/mg), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxysulfo-succinimide (sNHS) were purchased from Sigma.

2.2. Film buildup followed by UV-visible spectrometry

The films were built directly in a 96-well quartz microplate (Hellma 730.009-QG, Germany). Microplates were cleaned using 10 M NaOH solution, rinsed extensively with water and air-dried afterwards. Film buildup was always initiated by depositing a precursor layer of PEI. 50 μ L of PEI (3 mg/mL in PBS) were introduced into each well and allowed to adsorb for 20 min before rinsing with PBS. 50 μ L of HA20C10 was then added and allowed to adsorb for 8 min. After rinsing twice, 50 μ L of polycationic solutions (PLL or QCHI) were introduced into each well and let adsorbed for 8 min. Wells were then rinsed twice and 50 μ L of HA20C10 was introduced into each well, let adsorbed for 8 min, and subsequently

rinsed. Alternate depositions of polycation and polyanion solutions were repeated until 8 layer pairs have been deposited. During film buildup, all rinsing steps were performed in PBS and the films were never dried. Three wells per condition were prepared. Film buildup was followed by UV-visible spectrometry using a microplate reader (Infinite 1000, Tecan, Austria) and measurements were taken after each deposition step (after rinsing).

2.3 Film characterization by Fourier transform infrared spectroscopy (FTIR)

Film buildup on a ZnSe crystal was investigated by *in situ* Fourier transform infrared (FTIR) spectroscopy in attenuated total reflection (ATR) mode with a Vertex 70 spectrophotometer (Bruker Optic GmbH, Germany). All the experimental details have been previously described [45]. The experiments were performed in a deuterated 0.15 M NaCl solution adjusted to pH 7.4. The spectrum from the bare ZnSe crystal in contact with the 0.15 M NaCl solution was taken as reference. After each polyelectrolyte deposit and rinsing step, a single-channel spectrum from 64 interferograms was recorded between 400 and 4000 cm^{-1} with a 2 cm^{-1} resolution, using Blackman-Harris three-term apodization and the standard Bruker OPUS/IR software v6.5. A previously published procedure was employed to quantify the adsorbed amounts [45, 46].

2.4 Quantification of PTX incorporation and release from the films

The incorporation of PTX in the films was quantified by means of fluorescence measurements, using OG-PTX as a tracer, which was added at fixed concentration (1 μM) to PTX solutions with varying concentrations (0 to 9 μM). The PTX/OG-PTX solutions were pre-complexed with HA20C10 prior to film buildup. The fluorescence of OG-PTX was measured using the same apparatus as for UV-visible spectrometry, after deposition of each pre-complexed HA20C10 layer. Excitation and emission wavelengths were set at 496 ± 10 nm and 524 ± 10 nm, respectively.

To estimate the absolute OG-PTX concentration, a calibration curve for the fluorescence of OG-PTX as a function of its concentration was acquired (Fig. S.I. 1). The effective concentration of PTX in the films was calculated knowing the ratio of unlabeled/labeled PTX, the molecular weight of PTX and OG-PTX and the surface area of the films inside 96-well plate (0.36 cm^2).

2.4. Film stability and enzymatic hydrolysis

The films were cross-linked as described in a previously published protocol, based on the reaction of activated carboxylic groups with primary amine groups in the presence of EDC (30 mg/mL) and sNHS (11 mg/mL), both dissolved in 0.15 M NaCl at pH 5 [47]. The cross-linking was allowed to proceed overnight at 4°C before thorough rinsing with PBS.

To assess the film stability in physiological medium, the PBS solution was replaced with fresh PBS at fixed time intervals of 30 min. Enzymatic hydrolysis of the films was studied by incubating the films for 1 hr at 37°C in 0.15 M NaCl at pH 5 before replacing the solution by 200 μL of hyaluronidase type IV at 250 units/ml in 0.15 M NaCl (pH 5) [47].

2.5. Polysaccharide capsule preparation and quantification of OG-PTX incorporated in capsules

Microcapsules were prepared using calcium carbonate particles as a sacrificial template. CaCO_3 particles were synthesized from solutions of CaCl_2 and Na_2CO_3 as reported in the literature [48, 49]. HA20C10 derivatives were pre-complexed with PTX by adding few μL of a PTX stock solution in the HA20C10 solution in order to have a final total PTX concentration of $60 \mu\text{M}$. The CaCO_3 particles were coated layer-by-layer by incubating them at a concentration 2 % (w/v)[50] in solutions of HA20C10/PTX (at 1 mg/mL) and PLL (at 2 mg/mL), both in 0.15 M NaCl (pH 6.5). After shaking for 10 min, the particles were collected by centrifugation and the residual non-adsorbed polyelectrolyte was removed by washing twice with 0.01 M NaCl (pH 6.5). After the desired number of layers (typically 9 layers) were deposited, the multilayer shell was cross-linked by activation of the carboxylic groups of HA using the water-soluble carbodiimide EDC (400 mM) and sNHS (100 mM) in 0.15 M NaCl to insure the capsule stability after core decomposition [51]. The CaCO_3 core was then removed by treatment with an aqueous solution of EDTA (0.1 M , pH 7.2). To avoid mechanical damages of “soft” polyelectrolyte shells, the dissolved ions resulting from the decomposition of CaCO_3 were removed by dialysis against pure water, using spectra Por dialysis bags with a molecular weight cut off of $3\,500 \text{ Da}$.

The amount of OG-PTX incorporated in the capsules was determined by fluorescence intensity measurements after extraction of PTX from capsules with methanol according to an established procedure [38]. The suspension of capsules ($200 \mu\text{L}$ at $\sim 10^7$ capsules/mL) in PBS was centrifugated (4000 rpm , 4 min , 20°C) and the supernatant was removed. After addition of methanol, the suspension was again centrifugated (4000 rpm , 4 min , 20°C) and the supernatant containing PTX was recovered. This process was repeated two times to ensure maximum extraction of PTX from the microcapsules. Then the solutions were analyzed using the fluorescence microplate reader (see above). The total concentration of PTX in solution was determined using the calibration curve (Fig. S.I. 1).

2.6. Cell Culture

MDA-MB-231 cells were cultured in DMEM (Gibco, Invitrogen, USA) without pyruvate supplemented with 10 % heat-inactivated foetal bovine serum (FBS, PAA Laboratories, France), 100 U/mL penicillin G and 100 mg/mL streptomycin (Gibco, Invitrogen, USA) in a 37°C , 5% CO_2 incubator. Cells were subcultured prior to reaching 60-70% confluence. For all experiments, MDA-MB-231 cells were seeded at $5\,000$ cells per well in 96-well plates in $200 \mu\text{L}$ of medium and were allowed to grow for three days. PTX in solution, unloaded and PTX-loaded microcapsules diluted to low (LD, 5 caps./cell), medium (MD, 10 caps./cell) or high density (HD, 20 caps./cell) were added to the cells 24 h after cell plating.

2.7. Viability and proliferation assays

Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) assay. At given time intervals, $25 \mu\text{L}$ of a MTT solution (1.5 mg/mL in PBS) were added to each well. The plates were then incubated for 75 min, allowing viable cells to reduce the yellow MTT to dark-blue formazan crystals, which were subsequently

dissolved in 200 μ L of DMSO. Absorbance of the plate was measured using the microplate reader at 550 ± 5 nm and at 650 ± 5 nm for the reference wavelength.

Cell proliferation over time was quantified by counting the total number of cells using a Malassez chamber. It was also investigated by the bromodeoxyuridine (BrdU, GE Healthcare) assay. Briefly, 24 hrs after addition of PTX in solution, unloaded and PTX-loaded microcapsules, the cells were incubated 4 hours in presence of BrdU, allowing proliferating cells to incorporate it into replicating DNA. BrdU detection was then performed by indirect immunofluorescence and the percentage of proliferating cells was finally determined by counting the ration between BrdU positive cells over the total cell number using phase contrast microscopy (Axiovert 200M, Zeiss, Germany) with a 10 $\times$ apochromat objective. 5 images per well were acquired with Metaview software using a CoolSNAP EZ CCD camera (Ropper Scientific, France). Experiments were performed in triplicate, with three 3 wells per condition in each experiment.

2.8. Immuno-stainings

Cells were fixed in 3.7 % formaldehyde in PBS for 20 min and permeabilized for 4 min in TBS (0.15 M NaCl, 50 mM Tris-HCl, pH 7.4) containing 0.2% Triton X-100. Slides were blocked in TBS containing 0.1% BSA for 1 h, and were then incubated with monoclonal anti-tubulin (Sigma) (1:200) antibodies in TBS with 0.2 % gelatin for 30 min. AlexaFluor488-conjugated secondary antibody was then incubated for 30 min. Finally, nuclei were stained using Hoechst 33342 (Invitrogen) at 5 μ g/mL for 10 min. The plates were viewed under confocal microscopy (LMS 700, Zeiss) using a 63 $\times$ objective. Images were acquired with Zen 2010 software.

Statistical analysis—All the results are expressed as mean values $\pm$ standard error of the mean (SEM). Statistical comparisons were based on an analysis of variance (ANOVA) for pairwise comparisons ($p < 0.05$ was considered significant).

3. Results and discussion

3.1. Film growth

(PLL/HA20C10)₈ and (QCHI/HA20C10)₈ film growth was followed by FTIR spectroscopy (Fig. 2.A-B) and by UV-visible spectrometry (Fig. 2.C). Specific peaks of the polyelectrolytes CHI and HA were detected by FTIR, especially the saccharide band in the 1150-900 cm^{-1} region [52], the amide I band 1670-1550 cm^{-1} region [45] and the carboxylic peak of HA20C10 at 1608 cm^{-1} . The absorbance increased after deposition of each layer, which qualitatively indicated that film growth was regular. Nevertheless, after the deposition of 4 layer pairs, the PEMs were thick enough to entirely contain the evanescent wave. Hence very low additional signal was observed, though the multilayer was still growing. A calibration procedure applied on the carboxylic peak of COO^- [45, 46] allowed us to quantify the adsorbed mass of polyelectrolyte after each layer deposition. UV-visible spectrometry measurements at 230 nm confirmed that film growth was of exponential type (Fig. 2.C), as previously observed for (PLL/HA) films [53] and (CHI/HA) films [54]. Here again, a superior absorbance level was noted for (PLL/HA20C10) films,

indicating a higher degree of mass deposition than (QCHI/HA20C10) films. Moreover, by fitting the FTIR data by the same exponential function (Fig. 2. C and Fig. S.I. 2), we were able to estimate the total adsorbed mass after the deposition of 8 layer pairs. The adsorbed mass was slightly higher for (PLL/HA20C10)₈ films (42 $\mu\text{g}/\text{cm}^2$) than for (QCHI/HA20C10)₈ films (37 $\mu\text{g}/\text{cm}^2$) (Fig. S.I. 2).

3.2. PTX entrapment in PEM films

In order to load PTX into the films, PTX was pre-complexed with hydrophobic HA20C10, which has a very high affinity for hydrophobic molecules [37]. Indeed, we have previously shown that the fluorescence intensity of aqueous solutions of alkylated HA derivatives containing the hydrophobic dye Nile Red (NR) strikingly depended on the length of the alkyl chain [37], the C10 derivatives exhibiting the highest capacity to form hydrophobic nanodomains. The PTX pre-complexed HA20C10 was deposited as regular layer.

As the absorbance of the PTX molecule itself is low and is masked by the absorbance of the HA20C10 molecules, we quantified the amount of PTX by fluorescence measurements using OG-labelled PTX. Indeed, OG-PTX has been used in previous studies for quantifying PTX and it is assumed not to alter physical behavior and biological activities of PTX [22]. Incorporation of PTX in (PLL/HA20C10)₈ and (QCHI/HA20C10)₈ films was followed step by step using a mixture of unlabeled and labeled PTX/OG-PTX for increasing concentration of PTX, which was initially pre-complexed with the HA20C10, (Fig. 3). Of note, no significant incorporation of PTX was detected in PEM films built with unmodified HA, *e.g.* (PLL/HA)₈ and (QCHI/HA)₈ films (data not shown), indicating that the incorporation of PTX molecules in the hydrophobic nanodomains was specifically due to the presence of alkyl chains, as reported previously [37]. For both (PLL/HA20C10)₈ (Fig. 3.A) and (QCHI/HA20C10)₈ films (Fig. 3.B), the fluorescence intensity steadily increased as a function of the number of deposited layer pairs and the initial PTX concentration. Furthermore, the loaded amounts were about three times higher for (PLL/HA20C10)₈ films as compared to (QCHI/HA20C10)₈ films, most likely due to the greater amount of deposited mass of HA20C10 when combined with PLL (Fig. 2). Of note, the fact that the fluorescence steadily increased with the number of layers also indicates that PTX remains trapped in the hydrophobic nanodomains, with no desorption. These results demonstrate that the amount of hydrophobic drug can be precisely controlled by varying independently the initial concentration of PTX in solution and/or the number of deposited polyelectrolyte layers.

To get insight into the efficiency of PTX loading in the films, we calculated the effective concentration of PTX in the films, using a calibration curve relating the fluorescence to the quantity of OG-PTX in solution (Fig. S.I.1). We observed linear dependence between the initial concentration of pre-complexed PTX and the effective loaded PTX concentration within the films (Fig. 3.C). The ratio between the loaded and the initial concentration of PTX was found to be around 5000 for PLL based films and around 2000 times for QCHI films. These results highlight the exceptional ability of the HA derivative based PEM films to concentrate PTX. Of note, experiments with higher concentrations of pre-complexed PTX up to 100 μM did not show any sign of saturation of the loaded amount (data not shown). We also quantified the mass ratio of PTX over polymer (total mass of the polyelectrolytes in

the films) and obtained about 13 mg/g of polymer in the case of (QCHI/HA20C10)₈ films and 23 mg/g of polymer in the case of (PLL/HA20C10)₈ films (Supp. Info.).

3.3. Release of the loaded PTX and stability of the HA20C10-based films

The stability of PEM films incorporating PTX was investigated in PBS at room temperature (Fig. 4.A,B). First, we observed that the fluorescence intensity of the film was stable when the PBS solution was not refreshed (data not shown), indicating that an equilibrium was reached between the film and the supernatant. Next, we washed the film at regular time intervals to create a concentration gradient between the film and the supernatant, which contains no PTX molecules at time 0 after the change in medium. In this case, we noted that the fluorescence intensity was steadily decreasing. However, absorbance values at 230 nm did not evolve, showing that the film itself was not degraded when submitted to successive washing steps. All together, these results demonstrate that the PTX was released from the film due to the steep gradient of concentration between the two compartments, *i.e.* the hydrophobic film with a very high local concentration of PTX and the PBS medium, which contains no PTX after change of the supernatant. Of note, the release profile of PTX loaded in planar PEM films is very similar to the profile obtained previously with the model hydrophobic molecule NR loaded in HA20C10-based microcapsules (Cui, Adv. Mater. 2011), revealing that planar or spherical geometries do not affect the delivery of the hydrophobic molecule.

In the next step, we investigated the sensitivity of the HA20C10-based PEM films to hyaluronidase (Fig. 4.C,D), an enzyme known to specifically degrade hyaluronan [42]. The concentration of hyaluronidase is known to be elevated in various carcinomas and is often used as a marker for tumor detection [55, 56]. Indeed, overexpression of the hyaluronidase 1 gene has been recently correlated with the malignant behavior of human breast cancer cells MDA MB 231 and MCF 7 [43]. In our previous studies, we demonstrated that (PLL/HA) films were degraded by hyaluronidase [47] and polyelectrolytes microcapsules made of PLL and HA as well [51]. In addition, the alkylation of HA was shown to slow down the enzymatic degradation of hyaluronan [37]. PEM film degradation by enzymatic hydrolysis is also known to depend on several factors such as the ionic pairing between the polyelectrolytes and their molecular weight [52]. Here, we observed that the fluorescence intensity steadily decreased as a function of time and that it was further decreased when the enzyme solution was refreshed, after adding a higher concentration of 433 units/mL (see arrows in Fig. 4.B).

The total absorbance of the film exhibited only a slight decrease, suggesting that the hydrophobic nanodomains were loosened in the presence of the enzyme but that the film was not fully degraded. The number of hydrophobic nanodomains presumably decreased due to the enzymatic attack and PTX was released from these nanodomains by diffusion. Consequently, we anticipate that the PTX-loaded multilayer films could be slightly degraded in the presence of enzyme-producing cells. Thus, PTX may be released at higher rate *in vivo* in the vicinity of tumors where the concentration of hyaluronidases is elevated. This ability of the HA20C10-based PEM films to retain PTX under physiological conditions while

releasing the drug under pathological conditions emphasizes the potential for this approach in cancer therapy.

3.5. Microcapsules assembly and cytotoxicity study

We then investigated the generation of microcapsules made from (PLL/HA20C10) films, their ability of PTX loading and the release of PTX before studying *in vitro* the cytotoxicity of these PTX-loaded microcapsules on MDA-MB-231 breast cancer cells, a cell line known to be sensitive to PTX [57, 58]. (PLL/HA20C10) films were selected due to their superior capacity of PTX-entrapment, as shown in figure 3. The PEM nanoshell was deposited on a CaCO₃ biodegradable template, which was subsequently degraded to form about 5 µm diameter hollow microcapsules. Such procedure has already been recently applied to form (QCHI/HA20C10)-based microcapsules [38, 44] and (PLL/HA)-based microcapsules [51]. The microcapsules exhibited a spherical shape (4.2 ± 0.6 µm measured on three different capsule batches) and selectively incorporated PTX in their hydrophobic nanoshell (Fig. S.I. 3). Furthermore, they were stable in the culture medium for several days.

After extraction of PTX from the nanoshell, the effective concentration of PTX in 200 µL of a microcapsule suspension at 10^7 microcapsules/mL was found to be of 0.168 µM, thus corresponding to ~17 fg of PTX per microcapsules. This value was taken as positive reference for tests using PTX in solution. Unloaded and PTX-loaded capsules at different densities were added to MDA-MB-231 cell culture and observed over three days. The cytotoxicity of the capsules was evaluated by counting the cell number and by measuring the cell viability using a MTT assay [38]. Unloaded microcapsules did not induce any cytotoxic effect, even when added at high density (HD, ~20 caps./cell) to the cell culture (Fig. 5 and S.I. 4). Conversely, the PTX-loaded capsules exhibited a dose-dependent effect on MDA-MB-231 cells (Fig. 5), strongly limiting their proliferation. At day 3, the number of cells cultivated with a low density of PTX-loaded microcapsules (LD, ~5 caps./cell) was 1.7-fold lower than the cells cultivated in the absence of microcapsules or in the presence of unloaded microcapsules. At medium density (MD, ~10 caps./cell) and HD of PTX-loaded microcapsules, the cell number was divided by 4.6 and 7, respectively, compared to the control conditions. A high density of PTX-loaded capsules thus lead to a drastic cytotoxic effect, close to the effect of PTX in solution at 0.17 µM (Fig. 5.G-H).

As PTX is known to drastically affect cell internal structures, especially nucleus and microtubule organization [16], we examined it by immuno-staining β-tubulin and nucleus (Fig. 6). On control cells (no microcapsules or unloaded microcapsules), the nuclei were intact and the microtubule network was well organized (Fig. 6A,B). When PTX was added in the solution, bundles of microtubules were visible and the nuclei were fragmented (Fig. 6C). In the presence of PTX-microcapsules, the fragmentation of the nuclei was particularly visible at high density and bundles of microtubules were also visible after one day of culture (Fig. 6 D-F)). This stabilization of the microtubules resulted in defects in the cell mitosis and the presence of large, multinucleated cells. As a consequence, these deficiencies in cell division lead to a very low cell proliferation, as shown by the BrdU assay (Fig 6.H). We found that PTX-loaded microcapsules at low density reduced the number of proliferating cells from 63 to 53%, whereas medium and high density of PTX-loaded microcapsules

induced a drop to only 37 and 35%, respectively, close to the 37% of proliferating cells cultivated in the presence of PTX in solution (Fig. 6.H).

Of note, we always observed a very close contact of the PTX-loaded microcapsules with the MDA-MB231 cells, but no apparent internalization of entire microcapsules (Fig. 6F). This indicates that, in contrary to phagocytic cells such as RAW macrophages [51], the cancer cells are unable to internalize the microcapsules of $\sim 4.2 \mu\text{m}$ in diameter. This is in line with previous experiments showing that the cellular uptake depends on the size of the object, a maximum limit of $500 \text{ nm} - 1 \mu\text{m}$ being usually accepted for endocytosis by non phagocytic cells [33, 59]. Thus, the mechanism of action of the PTX-loaded microcapsules may be based on the combined action of several phenomena: interactions with cancer cells, passive transfer of PTX from the hydrophobic nanodomains to the hydrophobic lipid membrane of the cells, and local degradation of the microcapsules by hyaluronidase enzyme secreted by these cells.

4. Conclusion

The hydrophobic anticancer drug paclitaxel was successfully loaded in polyelectrolyte multilayer films made of an alkylated derivative of HA as polyanion and of either poly(L-lysine) or quaternized chitosan as polycation. These films exhibited a very high capacity of incorporating the anticancer agent selectively in the hydrophobic nanodomains.

Advantageously, the PTX loaded amount could be varied depending on the number of deposited layers, the initial paclitaxel concentration and the type of polycation (PLL vs. QCHI). Under physiological conditions, the incorporated drug released slowly without initial burst and the films remained stable. Interestingly, enzymatic hydrolysis using hyaluronidase at 37°C accelerates the release of PTX from the films by degrading the film integrity. As tumors are known to secrete high levels of hyaluronidase, this ability of our system to release PTX in the presence of hyaluronidase may represent a real asset for targeting PTX release. We also examined the cytotoxicity of PTX-loaded (PLL/HA20C10) microcapsules on MDA-MB231 breast cancer cells. The PTX-loaded microcapsules were found to inhibit cell growth in a dose-dependent manner, which proved the effective cytotoxicity of PTX to cancer cell proliferation. This cytotoxicity seems to be the result of the enzymatic degradation of the microcapsules in contact with cancer cells, leading to a transfer of the PTX from the hydrophobic nanodomains of the nanoshells to the lipid membrane of the cells, before diffusion of the PTX within the cytoplasm and stabilization of the microtubules. All together, our results open an exciting avenue for the controlled delivery of hydrophobic drugs from PEM films and microcapsules. Furthermore, an additional functionality could be provided to the inner hydrophilic part of the microcapsules to achieve a bi-compartment delivery for both hydrophobic and hydrophilic drugs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Judith Mähner for technical help with the fluorescence measurements and Fabien Dalonneau for assistance with cell culture experiments. This work was financially supported by the “Agence Nationale pour la Recherche” (grant ANR-07-NANO-002 to RAV and CP). P.K. is indebted to the University Joseph Fourier for a pre-doc fellowship. R.A.V. and C.P. are indebted to the Institut Universitaire de France for financial support.

References

- [1]. Agarwal A, Lvov Y, Sawant R, Torchilin V. Stable nanocolloids of poorly soluble drugs with high drug content prepared using the combination of sonication and layer-by-layer technology. *J. Control. Release.* 2008; 128:255–260. [PubMed: 18479772]
- [2]. Smith RC, Riollano M, Leung A, Hammond PT. Layer-by-layer platform technology for small-molecule delivery. *Angew Chem Int Ed Engl.* 2009; 48:8974–8977. [PubMed: 19847838]
- [3]. Singla AK, Garg A, Aggarwal D. Paclitaxel and its formulations. *Int J Pharm.* 2002; 235:179–192. [PubMed: 11879753]
- [4]. Haley B, Frenkel E. Nanoparticles for drug delivery in cancer treatment. *Urol. Oncol.- Semin. Orig. Investig.* 2008; 26:57–64.
- [5]. Park JW. Liposome-based drug delivery in breast cancer treatment. *Breast Cancer Res.* 2002; 4:95–99. [PubMed: 12052251]
- [6]. Oerlemans C, Bult W, Bos M, Storm G, Nijssen JFW, Hennink WE. Polymeric micelles in anticancer therapy: targeting, imaging and triggered Release. *Pharm. Res.* 2010; 27:2569–2589. [PubMed: 20725771]
- [7]. Soppimath KS, Aminabhavi TM, Kulkarni AR, Rudzinski WE. Biodegradable polymeric nanoparticles as drug delivery devices. *J. Control. Release.* 2001; 70:1–20. [PubMed: 11166403]
- [8]. Kumari A, Yadav SK, Yadav SC. Biodegradable polymeric nanoparticles based drug delivery systems. *Colloid Surf. B-Biointerfaces.* 2010; 75:1–18.
- [9]. Huynh NT, Passirani C, Saulnier P, Benoit JP. Lipid nanocapsules: A new platform for nanomedicine. *Int. J. Pharm.* 2009; 379:201–209. [PubMed: 19409468]
- [10]. Mora-Huertas CE, Fessi H, Elaissari A. Polymer-based nanocapsules for drug delivery. *Int. J. Pharm.* 2010; 385:113–142. [PubMed: 19825408]
- [11]. Upadhyay KK, Le Meins JF, Misra A, Voisin P, Bouchaud V, Ibarboure E, Schatz C, Lecommandoux S. Biomimetic Doxorubicin loaded polymersomes from hyaluronan-block-poly(γ -benzyl glutamate) copolymers. *Biomacromolecules.* 2009; 10:2802–2808. [PubMed: 19655718]
- [12]. Sanson C, Schatz C, Le Meins JF, Soum A, Thevenot J, Garanger E, Lecommandoux S. A simple method to achieve high doxorubicin loading in biodegradable polymersomes. *J. Control. Release.* 2010; 147:428–435. [PubMed: 20692308]
- [13]. Sivakumar S, Bansal V, Cortez C, Chong SF, Zelikin AN, Caruso F. Degradable, Surfactant-Free, Monodisperse Polymer-Encapsulated Emulsions as Anticancer Drug Carriers. *Adv. Mater.* 2009; 21:1820.
- [14]. Singh S, Dash AK. Paclitaxel in cancer treatment: Perspectives and prospects of its delivery challenges. *Crit. Rev. Ther. Drug Carr. Syst.* 2009; 26:333–372.
- [15]. Schiff PB, Horwitz SB. Taxol stabilizes microtubules in mouse fibroblast cells. *Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences.* 1980; 77:1561–1565.
- [16]. Yeung TK, Germond C, Chen XM, Wang ZX. The mode of action of Taxol: Apoptosis at low concentration and necrosis at high concentration. *Biochem. Biophys. Res. Commun.* 1999; 263:398–404. [PubMed: 10491305]
- [17]. Weiss RB, Donehower RC, Wiernik PH, Ohnuma T, Gralla RJ, Trump DL, Baker JR, Vanecko DA, Vonhoff DD, Leylandjones B. Hypersensitivity reactions from Taxol. *J. Clin. Oncol.* 1990; 8:1263–1268. [PubMed: 1972736]
- [18]. Gradishar WJ. Albumin-bound paclitaxel: a next-generation taxane. *Expert Opin Pharmacother.* 2006; 7:1041–1053. [PubMed: 16722814]

- [19]. Villano JL, Mehta D, Radhakrishnan L. Abraxane induced life-threatening toxicities with metastatic breast cancer and hepatic insufficiency. *Invest New Drugs*. 2006; 24:455–456. [PubMed: 16505953]
- [20]. Hoare TR, Kohane DS. Hydrogels in drug delivery: Progress and challenges. *Polymer*. 2008; 49:1993–2007.
- [21]. Soga O, van Nostrum CF, Fens M, Rijcken CJF, Schiffelers RM, Storm G, Hennink WE. Thermosensitive and biodegradable polymeric micelles for paclitaxel delivery. *J. Control. Release*. 2005; 103:341–353. [PubMed: 15763618]
- [22]. Westedt U, Wittmar M, Hellwig M, Hanefeld P, Greiner A, Schaper AK, Kissel T. Paclitaxel releasing films consisting of poly(vinyl alcohol)-graft-poly(lactide-co-glycolide) and their potential as biodegradable stent coatings. *J Control Release*. 2006; 111:235–246. [PubMed: 16466824]
- [23]. Oh EJ, Park K, Kim KS, Kim J, Yang JA, Kong JH, Lee MY, Hoffman AS, Hahn SK. Target specific and long-acting delivery of protein, peptide, and nucleotide therapeutics using hyaluronic acid derivatives. *J. Control. Release*. 2010; 141:2–12. [PubMed: 19758573]
- [24]. Sukhorukov GB, Rogach AL, Zebli B, Liedl T, Skirtach AG, Kohler K, Antipov AA, Gaponik N, Susha AS, Winterhalter M, Parak WJ. Nanoengineered polymer capsules: Tools for detection, controlled delivery, and site-specific manipulation. *Small*. 2005; 1:194–200. [PubMed: 17193429]
- [25]. De Geest BG, De Koker S, Sukhorukov GB, Kreft O, Parak WJ, Skirtach AG, Demeester J, De Smedt SC, Hennink WE. Polyelectrolyte microcapsules for biomedical applications. *Soft Matter*. 2009; 5:282–291.
- [26]. Zelikin AN. Drug releasing polymer thin films: new era of surface-mediated drug delivery. *ACS Nano*. 2010; 4:2494–2509. [PubMed: 20423067]
- [27]. Boudou T, Crouzier T, Ren K, Blin G, Picart C. Multiple functionalities of polyelectrolyte multilayer films: new biomedical applications. *Adv Mater*. 2010; 22:441–467. [PubMed: 20217734]
- [28]. Vodouhê C, Guen EL, Garza JM, Francius G, Dejognat C, Ogier J, Schaaf P, Voegel JC, Lavallo P. Control of drug accessibility on functional polyelectrolyte multilayer films. *Biomaterials*. 2006; 27:4149–4156. [PubMed: 16600366]
- [29]. Schneider A, Vodouhê A, Richert L, Francius G, Le Guen E, Schaaf P, Voegel J-C, Frisch F, Picart C. Multi-functional polyelectrolyte multilayer films: combining mechanical resistance, biodegradability and bioactivity. *Biomacromolecules*. 2007; 8:139–145. [PubMed: 17206799]
- [30]. Lvov YM, Pattekari P, Zhang X, Torchilin V. Converting poorly soluble materials into stable aqueous nanocolloids. *Langmuir*. 2011; 27:1212–1217. [PubMed: 21190345]
- [31]. Wang Y, Yan Y, Cui J, Hosta-Rigau L, Heath JK, Nice EC, Caruso F. Encapsulation of Water-Insoluble Drugs in Polymer Capsules Prepared Using Mesoporous Silica Templates for Intracellular Drug Delivery. *Advanced Materials*. 2010; 22:4293–4297. [PubMed: 20564713]
- [32]. Thierry B, Kujawa P, Tkaczyk C, Winnik FM, Bilodeau L, Tabrizian M. Delivery platform for hydrophobic drugs: prodrug approach combined with self-assembled multilayers. *Journal of the American Chemical Society*. 2005; 127:1626–1627. [PubMed: 15700982]
- [33]. Yan Y, Ochs CJ, Such GK, Heath JK, Nice EC, Caruso F. Bypassing Multidrug Resistance in Cancer Cells with Biodegradable Polymer Capsules. *Advanced Materials*. 2010; 22:5398–5403. [PubMed: 20976679]
- [34]. Guyomard A, Nysten B, Muller G, Glinel K. Loading and release of small hydrophobic molecules in multilayer films based on amphiphilic polysaccharides. *Langmuir*. 2006; 22:2281–2287. [PubMed: 16489818]
- [35]. Guyomard A, De E, Jouenne T, Malandain JJ, Muller G, Glinel K. Incorporation of a hydrophobic antibacterial peptide into amphiphilic polyelectrolyte multilayers: A bioinspired approach to prepare biocidal thin coatings. *Advanced Functional Materials*. 2008; 18:758–765.
- [36]. Hosta-Rigau L, Chandrawati R, Saveriades E, Odermatt PD, Postma A, Ercole F, Breheney K, Wark KL, Stadler B, Caruso F. Noncovalent liposome linkage and miniaturization of capsosomes for drug delivery. *Biomacromolecules*. 2010; 11:3548–3555. [PubMed: 21090570]

- [37]. Kadi S, Cui D, Beyma E, Boudou T, Nicolas C, Glinel K, Picart C, Auzély-Velty R. Alkylamino hydrazide derivatives of hyaluronic acid: synthesis, characterization in semidilute aqueous solutions, and assembly into thin multilayer films. *Biomacromolecules*. 2009; 10:2875–2884. [PubMed: 19769354]
- [38]. Cui D, Jing J, Boudou T, Pignot-Paintrand I, De Koker S, De Geest BG, Picart C, Auzely-Velty R. Hydrophobic shell loading of biopolyelectrolyte capsules. *Adv Mater*. 2011; 23:H200–204. [PubMed: 21590815]
- [39]. Luo Y, Ziebell MR, Prestwich GD. A hyaluronic acid-taxol antitumor bioconjugate targeted to cancer cells. *Biomacromolecules*. 2000; 1:208–218. [PubMed: 11710102]
- [40]. Draffin JE, McFarlane S, Hill A, Johnston PG, Waugh DJ. CD44 potentiates the adherence of metastatic prostate and breast cancer cells to bone marrow endothelial cells. *Cancer Res*. 2004; 64:5702–5711. [PubMed: 15313910]
- [41]. Auzenne E, Ghosh SC, Khodadadian M, Rivera B, Farquhar D, Price RE, Ravoori M, Kundra V, Freedman RS, Klostergaard J. Hyaluronic acid-paclitaxel: Antitumor efficacy against CD44(+) human ovarian carcinoma xenografts. *Neoplasia*. 2007; 9:479–486. [PubMed: 17603630]
- [42]. Menzel EJ, Farr C. Hyaluronidase and its substrate hyaluronan: biochemistry, biological activities and therapeutic uses. *Cancer Letters*. 1998; 131:3–11. [PubMed: 9839614]
- [43]. Tan JX, Wang XY, Li HY, Su XL, Wang L, Ran L, Zheng K, Ren GS. HYAL1 overexpression is correlated with the malignant behavior of human breast cancer. *Int J Cancer*. 2011; 128:1303–1315. [PubMed: 20473947]
- [44]. Cui D, Szarpak A, Pignot-Paintrand I, Varrot A, Boudou T, Detrembleur C, Jérôme C, Picart C, Auzély-Velty R. Contact killing polyelectrolyte microcapsules based on chitosan derivatives. *Advanced Functional Materials*. 2010 accepted, 17/06/2010, in pres.
- [45]. Crouzier T, Picart C. Ion pairing and hydration in polyelectrolyte multilayer films containing polysaccharides. *Biomacromolecules*. 2009; 10:433–442. [PubMed: 19199579]
- [46]. Boudou T, Crouzier T, Auzely-Velty R, Glinel K, Picart C. Internal composition versus the mechanical properties of polyelectrolyte multilayer films: The influence of chemical cross-linking. *Langmuir*. 2009; 25:13809–13819. [PubMed: 20560550]
- [47]. Richert L, Boulmedais F, Lavalle P, Mutterer J, Ferreux E, Decher G, Schaaf P, Voegel J-C, Picart C. Improvement of stability and cell adhesion properties of polyelectrolyte multilayer films by chemical cross-linking. *Biomacromolecules*. 2004; 5:284–294. [PubMed: 15002986]
- [48]. Antipov AA, Shchukin D, Fedutik Y, Petrov AI, Sukhorukov GB, Mohwald H. Carbonate microparticles for hollow polyelectrolyte capsules fabrication. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*. 2003; 224:175–183.
- [49]. Volodkin DV, Petrov AI, Prevot M, Sukhorukov GB. Matrix Polyelectrolyte Microcapsules: New System for Macromolecule Encapsulation. *Langmuir*. 2004; 20:3398–3406. [PubMed: 15875874]
- [50]. Petrov AI, Volodkin DV, Sukhorukov GB. Protein-Calcium Carbonate Coprecipitation: A Tool for Protein Encapsulation. *Biotechnology Progress*. 2005; 21:918–925. [PubMed: 15932274]
- [51]. Szarpak A, Cui D, Dubreuil F, De Geest BG, De Cock LJ, Picart C, Auzely-Velty R. Designing Hyaluronic Acid-Based Layer-by-Layer Capsules as a Carrier for Intracellular Drug Delivery. *Biomacromolecules*. 2010; 11:713–720. [PubMed: 20108937]
- [52]. Picart C, Schneider A, Etienne O, Mutterer J, Egles C, Jessel N, Voegel J-C. Controlled degradability of polysaccharides multilayer films in vitro and in vivo. *Advanced Functional Materials*. 2005; 15:1771–1780.
- [53]. Picart C, Lavalle P, Hubert P, Cuisinier FJG, Decher G, Schaaf P, Voegel J-C. Buildup mechanism for poly(L-lysine)/hyaluronic acid films onto a solid surface. *Langmuir*. 2001; 17:7414–7424.
- [54]. Richert L, Lavalle P, Payan E, Stoltz J-F, Shu XZ, Prestwich GD, Schaaf P, Voegel J-C, Picart C. Layer-by-layer buildup of polysaccharide films : Physical chemistry and cellular adhesion aspects. *Langmuir*. 2004; 1:284–294.
- [55]. Lin G, Stern R. Plasma hyaluronidase (Hyal-1) promotes tumor cell cycling. *Cancer Letters*. 2001; 163:95–101. [PubMed: 11163112]

- [56]. Li YJ, Li LL, Brown TJ, Heldin P. Silencing of hyaluronan synthase 2 suppresses the malignant phenotype of invasive breast cancer cells. *Int. J. Cancer*. 2007; 120:2557–2567. [PubMed: 17315194]
- [57]. Frasci G, Comella P, D'Aiuto G, Budillon A, Barbarulo D, Thomas R, Capasso I, Casaretti R, Daponte A, Caponigro F, Gravina A, Maiorino L, Carateni G, Gentile A, Comella G. Weekly paclitaxel-cisplatin administration with G-CSF support in advanced breast cancer. A phase II study. *Breast Cancer Res. Treat.* 1998; 49:13–26. [PubMed: 9694607]
- [58]. Fillmore CM, Kuperwasser C. Human breast cancer cell lines contain stem-like cells that self-renew, give rise to phenotypically diverse progeny and survive chemotherapy. *Breast Cancer Res.* 2008; 10
- [59]. Mitragotri S, Lahann J. Physical approaches to biomaterial design. *Nat. Mater.* 2009; 8:15–23. [PubMed: 19096389]

Europe PMC Funders Author Manuscripts

FIGURE 2.

(PLL/HA20C10)₈ and (QCHI/HA20C10)₈ film growth followed by FTIR spectroscopy in attenuated total reflection mode and by UV-visible spectrometry. Step-by-step acquisition of FTIR spectra during the deposition of a (PLL/HA20C10)₈ film (A) and of a (QCHI/HA20C10)₈ film (B). (C) Evolution of the UV absorbance at 230 nm during film buildup. The experimental data were fitted according to the equation $Abs = a_0 \exp(a_1 i_{bil})$ with Abs the absorbance at 230 nm and i_{bil} the number of layer pairs. The best fits were obtained for $a_0 = 4.4 \times 10^{-3}$ and $a_1 = 0.51$ for (PLL/HA20C10) films (straight line) and $a_0 = 1.4 \times 10^{-3}$ and $a_1 = 0.61$ for (QCHI/HA20C10) films (dashed line). For clarity, only the FTIR spectra and the UV absorbance of the polyanion-ended films obtained after the deposition of an even number of layers are shown. Data from (C) represent mean $\pm$ SEM of three independent experiments.

FIGURE 3.

Loading of PTX in (PLL/HA20C10)₈ and (QCHI/HA20C10)₈ films followed by fluorescence spectroscopy. PTX was pre-complexed to HA20C10 and the fluorescence values were measured after the deposition of each polyanionic layer : (A) (PLL/HA20C10)₈ and (B) (QCHI/HA20C10)₈ films. The initial concentration of pre-complexed PTX in solution was varied between from 1 and 10 μM. (C) Plot of the effective concentration of PTX in (PLL/HA20C10)₈ and (QCHI/HA20C10)₈ films as a function of the initial concentration of PTX in solution, when pre-complexed with the HA derivative. Lines are best-linear regressions with $r^2 = 0.98$ for (PLL-HA20C10)₈ and $r^2 = 0.99$ (QCHI/HA20C10)₈ films. Data represent mean $\pm$ SEM of three independent experiments.

FIGURE 4.

Release of the loaded PTX and stability of the (PLL-HA20C10)₈ and (QCHI-HA20C10)₈ films. (A) Release of the PTX loaded in (PLL-HA20C10)₈ and (QCHI-HA20C10)₈ films measured by fluorescence spectrometry and (B) stability of the films measured by absorbance at 230 nm in function of the number of washes in PBS at room temperature. (C) Release of the PTX loaded in (PLL-HA20C10)₈ and (QCHI-HA20C10)₈ films measured by fluorescence spectrometry and (D) stability of the films measured by absorbance at 230 nm in function of the time of contact with hyaluronidase. Arrows indicate the addition of hyaluronidase solution at 250 and 433 units/mL. Data represent mean ± SEM of three independent experiments.

FIGURE 5.

MDA-MB-231 cell response to unloaded and PTX-loaded (PLL/HA20C10) microcapsules. Brightfield images of MDA-MB-231 cells after 2 days of culture (A) in growth medium, (B) in contact with high density (HD, 20 caps./cell) unloaded microcapsules, (C) in contact with PTX at 0.168 μM in solution, (D) in contact with PTX-loaded microcapsules at low density (LD, 5 caps./cell), (E) medium density (MD, 10 caps./cell) and (F) high density (HD, 20 caps./cell). (G) Cell number per well and (H) cell viability measured by MTT assay in function of the time. Data from (G-H) represent mean $\pm$ SEM of three wells. *, $p < 0.05$

FIGURE 6.

Effect of the PTX on MDA-MB-231 cell proliferation. Immuno-staining of β -tubulin (green) and nuclei (blue) in representative MDA-MB-231 cells after 24 h of culture (A) in growth medium, (B) in contact with high density (HD, 20 caps./cell) unloaded microcapsules, (C) in contact with PTX at 0.168 μ M in solution, (D) in contact with PTX-loaded microcapsules at low density (LD, 5 caps./cell), (E) medium density (MD, 10 caps./cell) and (F) high density (HD, 20 caps./cell). Red arrows indicate bundles of microtubules. (G) 3D reconstruction of MDA-MB-231 cells in contact with PTX-loaded (PLL/HA20C10) microcapsules, red arrows indicate microcapsules. (H) Percentage of proliferating MDA-MB-231 cells after 24 h of culture assessed by BrdU assay. Data from (H) represent mean $\pm$ SEM of three wells. *, $p < 0.005$