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In-Situ Formation of pH-/thermo- Sensitive Nanohybrids via Friendly-assembly of Poly(N-vinylpyrrolidone) onto Laponite

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The development of delivery nanosystems with high payload, desirable release controllability, and cell responsiveness is important for an efficient and safe cancer therapy. In this study, multifunctional nanohybrids are successfully constructed by self-assembling a pH sensitive poly(N-vinylpyrrolidone) (PVP) onto Laponite with a nanodisk structure (25 nm in diameter and 0.92 nm in thickness) in the absence of any organic solvent. The nanohybrids can effectively encapsulate a cationic anticancer drug, doxorubicin (DOX) through its electrostatic interactions with negative-charged Laponite. The hydrophobic component (alkane polymeric chain) of PVP can bind the surface of Laponite, with its hydrophilic components (ketone and tertiary amine residues) as a protective stealth shell for stabilization of the whole systems. The deprotonation/protonation switchability of PVP endows the nanopybrids with good pH- and thermo- dual sensitivity in delivery of DOX drug, as compared to that modified with the polyethylene glycol (PEG, a common hydrophilic polymer for improving stability of nanoparticles). In vitro biological evaluation indicated that the DOX-loaded nanocarriers can be effectively taken up by KB cells (a human epithelial carcinoma cell line), and exhibit uncompromising anticancer cytotoxicity as compared to free DOX, indicating their potential therapeutic delivery applications.

Introduction

As one of top diseases for global mortality, cancer has been posing great challenges for the healthy conditions of human body.[1] In the past few decades, different chemotherapeutics have been widely employed for treatment of different types of cancer (e.g., breast cancer, ovarian cancer, multiple myeloma).[2] However, their clinic application is still limited by the low anticancer efficacy and high side effects associated with multidrug resistance, limited blood circulation period and/or undesirable therapeutic accumulation in heterogeneous tumors.[3-11] To overcome these barriers, extensive investigation has been performed to develop various kinds of nanosystems which displayed some advantages on administration of these therapeutic agents concerning their therapeutic solubility, colloidal stability, drug release controllability, and/or tumor targetability.[12-14] For instance, some kinds of 100-nm stealth liposomal nanoformulations, including DOXil, Genexol-PM and Abraxane, have come into the stage of clinical applications or under clinical trials at different stages.[15-17] However, all these nanosystems are liposomes with a relatively instable structure, and their original structure may be lost during their circulation in the blood, which may lead to unexpected burst drug release, posing a potential risk of high toxicity to human body.[15-18] Recently, taking advantage of tumoral pathological features, various kinds of smart nanocarriers have been developed for anticancer drug delivery, which can be triggered to release drug upon stimuli, such as pH values[19], redox and/or thermo-sensitive signals[20,21]. Although these nanocarriers improved drug delivery targetability in some degree, most of them still present a limited drug loading capacity and a uncontrollable release ability.[20,21]

In order to improve drug loading capacity and release controllability, various kinds of inorganic nanoparticles (NPs) with special structure or dimensional shape, such as nanosphered hydroxyapatite[4], carbon nanotube[6], have been developed. Among these, Laponite (LP) is a kind of synthetic nanoclay with better potentials for biomedical applications concerning its better biocompatibility, loading capacity and controllable release property than conventional ones[4-8]. As a kind of synthetic nanoclay, it can avoid side effect caused by impurity of other natural clays.[22] LP has an empirical formula $Na^{+0.7}[(Si_8Mg_{5.5}Li_{0.3})O_{20}(OH)_4]^{-0.7}$, with components similar to bioactive glasses with biodegradability.[23] Recent reports indicate that LP has good biocompatibility[24] and osteoinductivity.[25] More importantly, LP has a nanodisk shape (25 nm in diameter and 0.92 nm in thickness) and negative-

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charged surface, which make it as a potential candidate for designing nanocarriers for therapeutic delivery.[26] However, investigation on LP-based nanoplatforms for drug delivery has been seldom performed,[27] probably because of their poor colloidal stability under physiological conditions.[24,28,29] There are many reports show that the polymers are better to promote the drug delivery system, particularly the sensitive polymers.[65-67] Our previous study indicates that the stability of LP can be improved by coating its surface with an amphiphilic poly(ethylene glycol)–poly(lactic acid) (PEG-PLA) diblock copolymer in water/ethanol mixture solution. However, the inert structure of PEG-PLA caused a decrease in both drug loading capacity and pH sensitivity in drug release. In addition, the process is still involved in the employment of organic solvent, thus probably causing a high cost and environmental problems.[30,31] In this case, it is preferential to find a simple and effective way to develop a kind of nanocarriers in an environment-friendly approach, which are able to present robust colloidal stability and enhanced drug release sensitivity upon special stimuli existent in microenvironment of solid tumors and/or intracellular compartments.[32]

Compared to PEG-PLA, poly(N-vinylpyrrolidone) (PVP) has better water solubility due to the existence of both oxygen and nitrogen in their structure. Due to the existence of tertirary amino group in its special chemical structure, PVP can undergo protonation/deprotonation switch at different pH values, which has been used to develop PVP-based nanoparticles with pH sensitivity.[33-35] In addition, PVP can be endowed with thermosensitivity by conjugation with different hydrophobic groups. The PVP derivatives have been used to stabilize gold nanoparticles for potential catalyst applications.[36-38] For biomedical perspective, PVP has been proposed as an alternative to PEG for the shell-forming block.[21] For instance, the conjugation of tumor necrosis factor-alpha (TNF-a) with PVP gave a more potent antitumor therapeutic bioactivity than PEGylated TNF-a.[39] Liposomes which were coated with PVP prolonged in vivo circulation period after i.v. administration.[40] Furthermore, PVP has cryo/lyoprotectant effect which can overcome re-suspension problems after lyophilization of nanoparticles.[41] The presence of polar groups like ketone and tertiary amine residues make it hydrophilic, while the alkane polymer main chain maintains their hydrophobicity. The richness of hydroxyl groups on LP as well as its nanodisk structure endow it with an ability to bind PVP via non-covalent interactions, such as "polar-π" effects, hydrophobic effects, hydrogen bonding, and/or other physical adsorption,[24,28,29,42,43] Therefore, it is proposed that functionalization of LP with PVP of high aqueous solubility can improve its colloidal stability. The deprotonation/protonation switchability of PVP and its thermoresposive potentials may better the pH sensitivity[33-35] and thermosensitivity[36-38] of LP in drug release.

In the present work, we developed a new type of Laponite-based nanohybrids with high drug loading capacity and pH sensitivity in sustained release of doxorubicin (DOX, a kind of cationic anticancer drug) through an environment-friendly approach (in aqueous solution) via their strong electrostatic interactions.[24,28,29,42,43]. The process is just involved in one pot: i.e., the addition of PVP aqueous solution into the mixture of LP and DOX in water, which underwent dialysis for purification to obtain LP/DOX/PVP nanohybrids. It was found that the LP/DOX/PVP (LDP) nanohybrids presented a high DOX encapsulation efficiency (above 97%). As comparison, PEG instead of PVP was also employed to modify LP/DOX complexes. Interestingly, compared to LP/DOX/PEG systems, LP/DOX/PVP not only presented a long-term stability under physiological conditions, and but also exhibited good thermo- and pH- sensitivity in DOX release which are able to sustain the DOX release in an acidic-accelerated mode. The DOX-loaded LDP nanocarriers can be effectively taken up by KB cells (a human epithelial carcinoma cell line), and exert uncompromising anticancer cytotoxicity as compared to free DOX.

Results and discussion

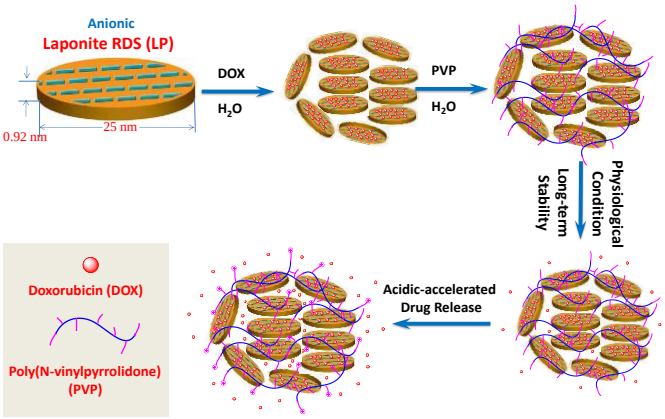
Fabrication and Physical Properties of DOX-loaded Nanoparticles

For anticancer delivery applications, ~100 nm nanosystems are reported to have prolonged circulation period by decreasing their reticuloendothelial system (RES) uptake rate, so that they have more possibility to target tumor site through the enhanced permeation and retention (EPR) effect.[18] Through electrostatic interactions between DOX and LP,[24,28] LP/DOX nanohybrids with a hydrodynamic diameter of 112 ± 24 nm was obtained at the 6:1 weight ratio of LP/DOX (Table 1), which will be used as a core template for further PVP modification.

Table 1 Characterization of DOX-loaded LP and LDP nanohybrids in water.

Sample Identity	Size, nm	Zeta Potential, mV	EE, %[a]
LP	31 ± 4	-38.9 ±0.6	-
LD	112 ± 24	-12.4 ±0	90.0 ± 0.4
LDP_25	368 ± 17	-17.1 ±1.1	99.5 ± 0.1
LDP_50	319 ± 10	-19.6 ±0.7	98.5 ± 0.1
LDP_75	91 ± 4	-11.7 ±0.1	97.3 ± 0.4
LDP_100	80 ± 2	-13.4 ±2.0	97.2 ± 0.6

[a] Encapsulation efficiency (EE) = 100*W_t/W_0, W_0 and W_t are the total DOX weight used for loading and the weight of encapsulated DOX, respectively.



Scheme 1. Technical route to develop LDP nanohybrids. The nanocarriers can maintain long-term stability in physiological conditions and accelerated-drug release behavior under acidic extracellular microenvironment mimicking solid tumor and endo/lysosomal compartments due to protonation of PVP.

Next, the LDP nanocomplexes were optimized concerning their hydrodynamic diameter (size) analyzed by dynamic light scattering (DLS) by variation of PVP content. The addition of PVP seemed firstly to increase the hydrodynamic size of the nanocompexes until PVP/LP ratio is 25%, while the further increasing PVP/LP ratio led to a decrease in LDP nanohybrids. The size change caused by PVP incoporation indicates the existence of strong interactions between PVP macromolecular chain and LP/DOX nanocomplexes. When the PVP/LP ratio is 75%, the formed LDP_75 presented a size around 100 nm (91 ± 4 nm), which is a proper size for circulation in the blood. Therefore, LDP_75 was selected for further study. All DOX-loaded LDP NPs present much higher Zeta potentials than that of LP, indicating their successful loading of cationic DOX. The morphology and size distribution of the nanoparticles were further characterized by transmission electron microscopy (TEM) imaging. LP and LDP had average sizes of 33 ± 6 and 87 ± 11 nm, respectively (Figure 1). An obvious size increase of LDP sample as compared to LP is another proof of the successful PVP coating onto LP nanodisks.

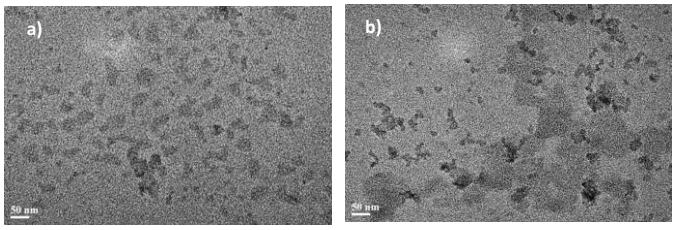


Figure 1. Transmission electron microscope (TEM) images of a) LP, b) LDP_75.

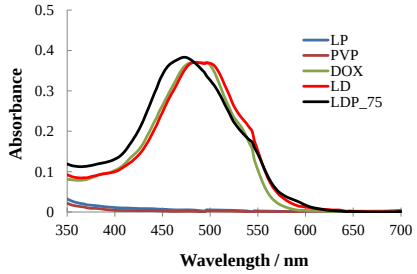


Figure 2. UV–Vis spectra of DOX, LP, PVP, as well as the nanohybrids of LD and LDP_75.

In order to learn more about their microstructure, the LD and LDP_75 nanocarriers were anlyzed by UV-Vis and FTIR spectroscopy. Since DOX presents an absorption peak at around 480 nm, UV-Vis spectroscopy was used to quantitatively investigate its loading capacity in the nanocarriers.[8,24,44] As shown in Figure 2, all the free DOX, LP and LDP nanohybrids presented an absorption peak at around 480 nm, which is absent in LP and PVP spectra, again indicating the successful loading of DOX in the nanohybrids.[24] In general, decoration of LD/DOX with PVP improved the DOX encapsulation efficiency (EE was maintained at around 98%) as compared to LD/DOX themselves (EE was 90%). Since PVP has a tertiary amino group in its structure, it can be more easily under protonation than primary amino groups in DOX, the addition of the former may neutralize doxorubicin hydrogen chloride. That is why there is an obvious blue shift of UV-Vis spectra of DOX after decoration with PVP (Figure 2). The higher loading capacity of LDP may be attributed to the increase of hydrophobicity of DOX due to its neutralization, benefiting its condensation on LP's surface.[45]

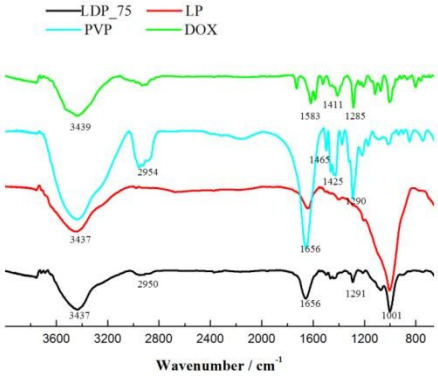


Figure 3. FTIR spectra of DOX, LP, PVP, and of LDP_75 nanohybrids.

It can be seen from Figure 3 that the FTIR spectrum of free DOX presented its own characteristic bands at 1728 (band of C=O), 1583 (bending of NH_2 on aromatic ring), 1411 (bending of NH_2 on aromatic ring), and 1285 cm^{-1} (C–N stretching vibration).[46,47] Both LP and LDP_75 produced spectrum peaks at 1002 and 3437 cm^{-1} of the −Si−O− stretching vibration and the −OH bending vibration in the LP nanodisks.[48] Different from pure LP, the LDP_75 nanohybrids had distinctive bands at 1583, 1411, and 1285 cm^{-1} associated with DOX, again suggesting that DOX was efficiently encapsulated in the nanohybrids. Different from LP, new bands at 2950 and 1290 cm^{-1} (characteristic bands of PVP) appeared for LDP_75 samples, which is an evidence of successful complexation of PVP with LP.

For intravenous injection application, nanomedicines should maintain a sufficient colloidal stability to avoid aggregation of nanoparticles during circulation in the blood. Therefore, their hydrodynamic sizes in water and physiological conditions were investigated using a Zetasizer. As shown in Figure 4a, although LD NPs can be well dispersed at a nanscale level in water, microsized aggregates were formed after transferring them in physiological conditions, probably due to the disturbance of their original state in the presence of ions in PBS buffer.[24] Interestingly, LDP nanohybrids displayed a nanosized state for a long-term period (92 ± 4 nm at 1 day, 70 ± 0 nm at 5 days, 82 ± 13 nm at 10 days), indicating their excellent colloidal stability. This is quite important to use them for intravenous injection as a kind of drug delivery system because their aggregation during blood circulation may result in a fatal problem to human body.[49-51] Since polyethylene glycol (PEG) has been widely employed to improve the stability of nanoparticles, LP was also decorated with PEG for stability study. Strangely, unlike LDP nanohybrids, PEG-functionalized nanocomplexes (LDPEG_75) tended to form aggregates in PBS buffer (346 ± 43 nm in water and 3,842 ± 860 nm in PBS solution up to 5 days). Therefore, the stability of LDP_75 should come from a cooperative effect of PVP which has a distinct structure from neutral-charged

PEG. Since LD can form strong interactions with hydrophobic molecules, it can adsorb and fix the hydrophobic segmental components of PVP on its surface, while the hydrophilic parts (polar groups like ketone and tertiary amine residues) of PVP may extend outside as a layer of protecting shell to prevent their aggregation because of the disturbance of ions present in PBS buffer. In addition, LDPEG_75 had a less EE (95.0 ± 0.3%) as compared to the corresponding PVP modified sample (LDP_75, EE 97.3 ± 0.4%). As comparison, hydrophilic PEG lacks a hydrophobic "anchorage point" on LP and thus has lower ability to bind the surface of LP, so it may be stripped of the LP under the attack of ions from PBS buffer. That is the main reasons why the PVP decoration offered a higher drug loading capacity and maintained a better stability of the whole nanohybrids in PBS than PEG-modified ones (Figure 4b).[52-58]

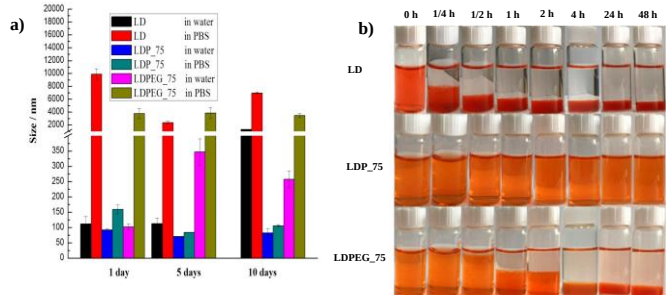


Figure 4. The stability of LD, LDPEG_75 and LDP_75 nanohybrids in water and PBS solution. (a) Hydrodynamic sizes as a function of incubation time; (b) Appearance of solutions of LD, LDPEG_75 and LDP_75 in PBS as a function of incubation time.

Drug Release Behaviors of the LPD Nanoparticles

To achieve optimal therapeutic efficacy, it is very important to deliver the therapeutics to the target site (spatial control) and/or at the right time (temporal control).[8] To ascertain their controllability in drug release, the release kinetics of DOX from the LDP nanohybrids was studied in PBS solution. It is reported that endo/lysosomal compartments (pH 5.0) present a quite acidic environment.[59,60] Therefore, the DOX release from LDP_75 was compared under both physiological (pH 7.4) and acidic (5.0) conditions, with LDPEG_75 as a control. The DOX release efficiency was accelerated under acidic conditions when compared with the physiological pH, revealing that the LDP_75 system were pH sensitive (Figure 5a). Although LDPEG_75 also presented a higher DOX release efficiency at lower pH value, the pH sensitivity in drug release is quite weaker as compared with that of LDP_75 system. For instance, at 24 h, a DOX cumulative release from LDP_75 were 40 ± 4% at pH 7.4, and 60 ± 6% at pH 5.0, while LDPEG_75 gave a DOX cumulative release of 31 ± 1% at pH 7.4, and 34 ± 6% at pH 5.0. The samples at pH 5.0 has a higher cumulative release than at pH 7.4, it's because the presence of proton, the nanocomplexes could accelerate the release of DOX. The more pH sensitivity of LDP_75 may come from deprotonation/protonation switch of PVP under acidic conditions,[61] which increases the aqueous solubility of the outer layer, enhancing the DOX release rate under microenvironments. Since PEG is a neutral-charged polymer, it has no such effect, thus having a less pH sensitivity.[9,24] From Figure5, it shows that the samples seldom reach to 100%. In the process of drug release, dialysis bag inside and outside will reach a dynamic balance in concentration and also the electrostatic interactions between DOX and nanocomposites are also strong, so the drug release seldom close to 100%.

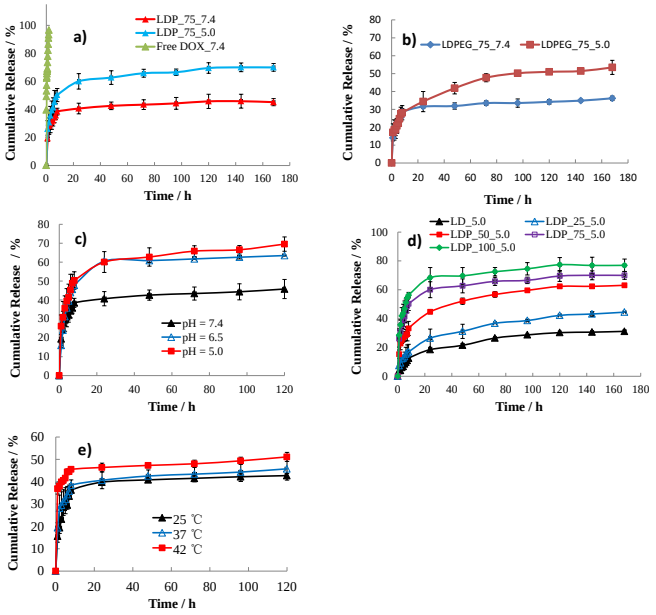


Figure 5. In vitro DOX cumulative release in PBS buffer from (a) LDPEG_75 and (b) LDP_75 under different pH values (7.4 and 5.0) at 37 °C, (c) LDP_75 under acidic conditions mimicking extracellular microenvironment of solid tumor (pH 6.5) and endo/lysosomal compartments (pH 5.0), (d) LD, LDP_25, LDP_50, LDP_75, LDP_100 under acidic conditions mimicking endo/lysosomal compartments (pH 5.0), (e) LDP_75 nanohybrids at pH 7.4 and different temperatures (25, 37 and 42 °C).

Since both the extracellular environment of a solid tumor (pH 6.5) and endo/lysosomal compartments (pH 5.0) present a quite acidic environment.[59,60] We then investigated the release behaviors of DOX under both physiological (pH 7.4) and these acidic (pH 6.5, 5.0) conditions. The DOX release efficiency was accelerated under acidic conditions of solid tumor and endo/lysosomes, when compared with the physiological pH (Figure 5c). This pH sensitivity in DOX release may be associated with its protonation under acidic conditions, which enhances its hydrophicility and makes more easily diffuse from nanohybrids.[62] This means that even if DOX release is limited under physiological conditions (less toxicity to normal tissues), the LDP_75 sample will release more of their drug cargo in the solid tumor extracellular environment and in the endo/lysosomal vesicles, resulting in enhanced anticancer activity with lower side effects.

To design drug delivery systems, it is important to adjust drug release rate in a controllable way via identifying the factors that affect the drug release behavior. In this case, the effect of the PVP/LP ratio on the release behaviors under acidic microenvironments mimicking endo/lysosomal compartments (pH 5.0) was investigated. As shown in Figure 5d, when the PVP/LP ratio was increased, the increase in DOX release rate under intracellular-mimic microenvironment was effectively

mediated. For instance, in the first 24 h, the quantities of drugs released at pH 5.0 from the modified LDP with the PVP/LP ratio of 0, 25, 75% were 18 ± 1, 26 ± 6 and 60 ± 6%, respectively. The adjustable release behaviors are useful to design some nanomedicines with specific release rate, which was reported to play an important role in mediation of cell behaviors.[10,63]

The current developmental technology allows for precise heating of a defined tissue volume up to 43 ℃.[64] The combination of thermosensitivity with hyperthermia can ensure a better controlled release of therapeutic agents at the tumor site either extra- or intracellularly.[65] To determine thermal effect on the release of DOX, the drug release behaviors from the nanohybrids were investigated in PBS (pH 7.4) at different temperatures. As can be seen from Figure 5e, DOX release from LDP_75 was accelerated at higher temperature (42 ℃), compared to those at physiological (37 ℃) and room temperature (25 ℃). For instance, the LDP_75 nanohybrids displayed a cumulative drug release of 34 ± 1, 36 ± 3, 44 ± 1% at 25, 37 and 42 ℃ at 7 h, respectively. The thermosensitivity of the nanohybrids can be used to trigger the drug release through remote manipulation of switchable heating.[66]

Biological Evaluation of DOX-loaded nanoparticles

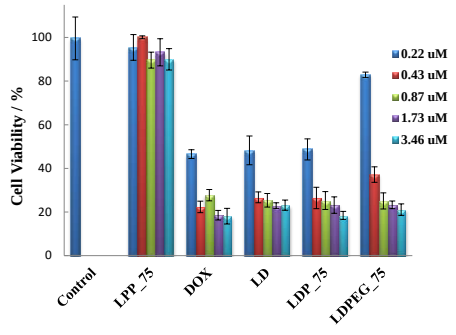


Figure 6. Anticancer cytotoxicity of free DOX, DOX, LD, LDP_75, LDPEG_75 (with equivalent DOX concentration) and LPP_75 (with equivalent weight concentration of the corresponding LDP nanohybrids) after 48 h of cell culture with the KB cells (± standard deviation, n = 3).

If nanomedcines can exert sufficient antitumor bioactivity is a pre-requirement for their biomedical applications in vivo.[67,68] Therefore, the therapeutic efficacy of the DOX upon its release from the LDP samples was quantitatively evaluated using KB cells through the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. It can be seen from Figure 6 that LDP_75 showed a dosage-dependent cytotoxicity towards KB cells, with efficacy comparable to free DOX drug.[3] It has be noted that the blank LP/PVP (LPP_75) did not exert any distinct cytotoxicity, revealing that the cytotoxic effect was only from the drug which was loaded within the nanohybrids. Although LDP_75, together with LD and LDPEG_75, exerted a comparable antitumor cytotoxicity to KB cells, the poor colloidal stability of the latters can be a key problem to block their entrance to bottle line for intravenous injection application in vivo. For *in vivo* anticancer drug delivery, one should expect a higher effect of LDP on the tumor site due to their EPR effect.[18] Therefore, the therapeutic efficacy and excellent stability of LDP nanohybrids make them a promising platform for delivery of anticancer therapeutic agents.

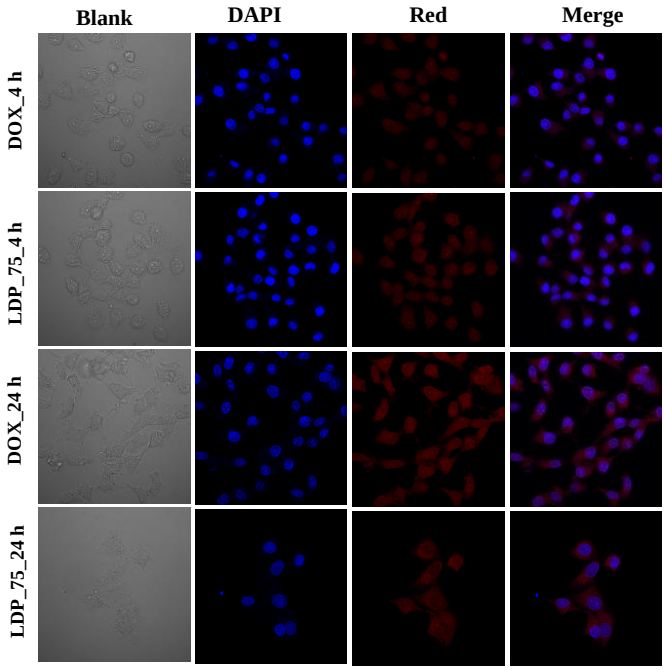


Figure 7. Bright field and fluorescence microscope images of KB cells after 4 and 24 h culture with free DOX (1.73 μM) and LDP_75 nanohybrids with an equivalent amount of DOX (1.73 μM).

It is important that the drug delivery systems should be effectively taken up by cells and be able to tempo-spatially deliver the drug there to exert the necessary therapeutic efficacy.[69] The internalization process of fluorescent DOX drug by KB cells can be tracked by fluorescence microscopy. Figure 7 shows the bright field and fluorescence microscope images of KB cells after 4 and 24 h culture with free DOX (1.73 μM) and LDP_75 nanohybrids with an equivalent amount of DOX diluted in the cell culture medium. The results demonstrated a higher reddish intensity inside both cytosol and nucleus for the cells which were treated with the LDP nanohybrids for 4 h, compared to those with free DOX drug. After 24 h incubation, less amount of cells were observed for cell with 24 h culture of LPD samples as compared to free DOX, indicating the former killed more cells. This higher DOX accumulation may be attributed to the higher cell uptake of the LDP_75, as well as to a facilitated DOX release from the endo-lysosomal compartments to the nucleus.[70]

Experimental

Materials and Cells

Laponite (LP) XLG was friendly offered from Rocwood Additives Limited, UK. Doxorubicin hydrochloride (DOX) was obtained from Dalian Meilun Biology Technology Co., Ltd, China. Polyvinyl pyrrolidone (PVP, molecular weight 40,000 Da) was purchased from Sigma-Aldrich, China. Polyethylene glycol (PEG, molecular weight 20,000 Da) was purchased from

Sinorm Chemical Reagent Co., Ltd, China. KB cells were purchased from cell bank of Chinese Academy of Sciences, Shanghai, China. All other reagents were from Sigma, China, unless otherwise indicated. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) and 4',6-diamidino-2-phenylindole (DAPI) were received from Life Technology, USA. All chemicals were used as received.

Preparation and physical characterization of LP/DOX/PVP (LDP) nanohybrids

Laponite was dispersed in ultrapure water under sonication (BRANSON 2510, 100W) for 30 min to obtain solutions of LP concentration (6 mg/mL). Aqueous solutions of doxorubicin hydrochloride (DOX, 2 mg/mL) and polyvinyl pyrrolidone (PVP, 10 mg/mL)) were prepared using the similar procedure. After that, LP and DOX solutions (2:1 by volume ratio) were mixed under magnetic stirring for 24 h to allow for the complete interaction of DOX with LP to form LP/DOX nanocomplexes. Then, a certain amount (0, 0.3, 0.6, 0.9, 1.2 mL) of the PVP solution was dropwisely added into the LP/DOX solution to maintain the ratio of PVP to LP as 0, 25, 50, 75 and 100%. The mixture was stirred under 400 rpm for 4 h to make sure the complete interaction of LP/DOX and PVP, which was further purified under dialysis (MWCO: 100,000 Da, Shanghai Yuanju Biological Technology Co., Ltd., Shanghai, China) for 12 h to obtain the final LP/DOX/PVP nanohybrids. For abbreviation, the samples with the ratio of PVP to LP as 0, 25, 50, 75 and 100% were named as LD, LDP_25, LDP_50, LDP_75, LDP_100, respectively. The free DOX content in the dialysis medium was determined by measuring the DOX fluorescence (λ_{ex} = 480 nm, λ_{em} = 580 nm) using a microplate reader (SpectraMax M2, Molecular Devices, USA) for calculation of DOX encapsulation efficiency.

The morphology of the nanocomplexes was examined by transmission electron microscope (TEM) (JEOL JEM-2100, Japan) with an accelerating voltage of 120 kV. Before measurement, the samples were dispersed in water (0.1 mg/mL) under sonication. The aqueous suspensions of the samples were dropped onto a 400 mesh copper grid, followed by air-drying before analysis.

Fourier transform infrared (FTIR) The FTIR spectra were recorded in KBr pellets in the range 4000–500 cm^{-1} by using the Fourier transform infrared spectrometer (Nicolet 5700, Thermo Electron, USA). Ultraviolet-visible (UV-Vis) spectroscopy was performed using the Lambda 25 UV/VIS spectrophotometer from PerkinElmer. Free DOX and DOX-loaded nanohybrids were dispersed in water at a DOX concentration of 0.1 mg/mL before measurements. The concentration of LP and LP/PVP were the same as controls. The hydrodynamic diameter of different concentration of LDP nanohybrids in water and phosphate buffer saline (PBS) solution were measured at room temperature using a Zetasizer (Nano ZS, Malvern Instruments, UK). Before measurement, LDP solutions were diluted by ultrapure (UP) water and PBS, followed by sonicatation (BRANSON 2510, 100W) for 10 min.

To investigate their drug release properties, 1.0 mL of LD and LDP containing equivalent DOX concentrations in UP water were put into a dialysis membrane (MWCO: 14, 000 Da), which were placed into 9 mL of PBS solution under 80 rpm stirring in an incubator (Unimax 1010, Heidolph) at 37 ℃. Different pH values (7.4, 6.5, 5.0) and different temperatures (25, 37, 42 ℃) were tested. At a specific time interval, 100 μL of released medium was taken out from each vial, and refreshed with another 100 μL of PBS solution. The released DOX was quantified by measuring the DOX fluorescence using the method above. The cumulative release (*Cr*) of DOX against time was obtained according to the equation

$$Cr = 100 * W_t / W_{tot} \quad (1)$$

where W_t and W_{tot} are the cumulative amount of drug release at time t, and the total drug amount contained in the nanohybrids used for drug release, respectively.

Cell Biological Evaluation

KB cells (a human epithelial carcinoma cell line) were cultured at 37 ℃ in flasks containing Dulbecco's Modified Eagle Medium medium (DMEM) containing 10% fetal bovine serum (FBS) in a humidified atmosphere and 5% carbon dioxide. KB cells were cultured in 25 cm^2 plates in DMEM and 10% FBS under at 37 ℃ in a humidified atmosphere with 5% of carbon dioxide. Afterward, the cells were harvested at 80% confluence, using a trypsin-EDTA solution (buffered saline solution containing 0.25% trypsin and 0.03% EDTA) for the enzymatic detachment of the cells from the plastic substrate.

The cytotoxicity of free DOX, LPP, LD, LDP and LDPEG was evaluated by examining the viability of KB cells using MTT assay[67-69]. Briefly, KB cells were incubated with 100 μL DMEM solution in 96-well plate at a density of 5,000 cells per well. After one day, the DMEM solution was replaced with 200 μL fresh DMEM solutions of free DOX as well as the NPs of LD, LDP and LDPEG containing equivalent DOX concentrations. Cells were then incubated for 48 h at 37 ℃ before the MTT assay. Solutions of DOX-free LPP NPs in PBS pH 7.4 with equivalent concentrations of LDP ones were used as controls for cytobiocompatibility study. For MTT assay, 30 μL MTT solution was added to each well. After further incubation for 4 h at 37 °C, 200 μL DMSO was added to each well to replace the culture medium and dissolve the insoluble formazan crystals. The absorbance at 492 nm was measured by using the UV spectrophotometer. The relative cell viability was demonstrated as $OD_{test}/OD_{control}$ ×100%.

For the cell uptake study, cells were plated for 24 h before incubation with the test solutions, to allow cell attachment. In these experiments, KB cells were incubated with 2 mL DMEM solution at a density of 40,000 cells in Φ20 mm cell culture dish. After one day, the DMEM solution was replaced with 2 mL fresh DMEM solutions of free DOX, LD, LDP NPs at equivalent DOX concentration (1.73 μM), and cells were then incubated for 4 and 24 h at 37 ℃. After that, the cells were washed 3 times with PBS, fixed with glutaraldehyde (2.5%) for 15 min at room temperature, and counterstained with DAPI (2 μg/mL) for 15 min at room temperature. The samples were observed by a Laser Scanning Confocal Microscopy (LSCM, A1R Nikon, Japan). NIS viewer (Nikon) and Photoshop (Adobe

CS5) was used to merge the pictures (all images were treated in the same way).

Conclusions

We have developed Laponite-based nanohybrids with good stability and release controllability in delivery of anticancer agents through a simple and environmental-friendly strategy. The functionalization of DOX-loaded LP nanodisks with poly(N-vinylpyrrolidone) (PVP) improves colloidal stability of LP and drug loading capacity. The protonation/deprotonation switching effect of tertiary amine residues of PVP under acidic conditions enhances the pH- and thermo- sensitivity in drug release as compared to the PEG-modified LP/DOX nanocomplexes. In vitro biological evaluation indicated that the DOX-loaded nanocarriers can enhance their internalization ability by KB cells (a human epithelial carcinoma cell line), resulting in high therapeutic accumulation inside cells and uncompromising anticancer cytotoxicity. Notably, the whole process for fabrication of the nanohybrids is not involved in any organic solvent. This work is expected to provide a new enlightenment to develop a kind of effective and safe nanoplatform with release controllability for therapeutic delivery applications.

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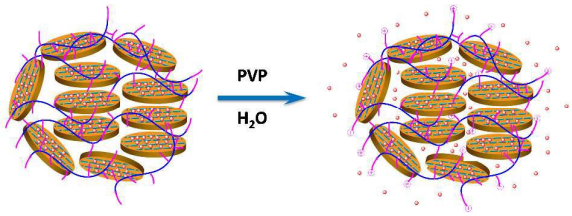

Decoration of silicate nanodisk with pH-sensitive polymer allows for effective delivery of anticancer drug in cancer cells with high efficacy.

Title: In-Situ Formation of pH-/thermo- Sensitive Nanohybrids via Friendly-assembly of Poly(N-vinylpyrrolidone) onto Laponite

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