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Development of polypeptide-based zwitterionic amphiphilic micelles for nano drug delivery

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Abstract

Protein molecules, which typically have hydrophobic core and zwitterionic shell with a polypeptide backbone, could be an ideal material for nano drug vehicle (NDV) with low side effect. Here, we synthesized poly(L-aspartic acid(lysine))-b-poly(L-lysine(Z)) (PAsp(Lys)-b-PLys(Z)) (PALLZ), a novel amphiphilic block polypeptide with the key structures of protein to investigate the possibility for NDV. This polypeptide can spontaneously self-assemble into micelles in aqueous solution with a zwitterionic brush (the PAsp(Lys) part) to provide the nonfouling shell and a hydrophobic core (the PLys(Z) part) for loading hydrophobic drug. The Doxorubicin (DOX) loaded PALLZ micelles showed excellent resistance to nonspecific protein adsorption in FBS, which leads to very low internalization. Moreover, PALLZ micelles showed no cytotoxicity to MCF7, HeLa and HepG-2 cells up to 500 μg/ml. All these results indicated that zwitterionic amphiphilic block polypeptide could be a promising material for NDVs.

Key words: Zwitterionic; Polypeptide; Drug delivery.

1. Introduction

Nanoparticles, like micelles and liposomes, modified with nonfouling shells have attracted lots of attention as drug carriers, due to their prolonged circulation time by evading the clearance of reticuloendothelial system (RES) and enhanced accumulation of drugs in the tumor site via the EPR effect.[1-7] As a biocompatible

material, polyethylene (PEG) with its excellent antifouling property and biocompatibility[8-10] has been utilized to protect NDVs for decades. However, amphiphilic PEG is subject to oxidation, and induces PEG antibodies in animals after repeated injections.[11-13] Moreover, PEG could not be metabolized naturally. Thus, the PEG shell on NDVs is far beyond as an ideal one. On the other hand, hydrophilic zwitterionic polymers including poly(phosphobetaine), poly(sulfobetaine), poly(carboxybetaine), and mix charged peptides have also showed excellent nonfouling properties and biocompatibilities.[14-23] Poly(carboxybetaine acrylamide) (polyCBAA) coated gold nanoparticles have superior performance in undiluted blood serum over gold nanoparticles modified with other conventional coatings.[24] Poly(carboxybetaine) (PCB)–poly(lactic-co-glycolic acid) (PLGA) block copolymers with sharp contrast in polarity can form extraordinarily stable NDVs.[25] Cyclic Arg-Gly-Asp-d-Tyr-Lys [c(RGDyK)] modified zwitterionic cross-linked micelles possessing high stability in blood circulation and enhanced cellular uptake showed improved therapeutic efficacy *in vivo* through active-targeting.[26] All these works show great potential of zwitterionic polymer protected NDVs. However, these zwitterionic polymer are polymethacrylate materials, which also faces the metabolization problem.

To make delivery materials more compatible with metabolization systems, peptide based block polymer could be ideal one for both hydrophobic and nonfouling block. In this work, poly(L-aspartic acid(OBzl))-b-poly(L-lysine(Z)) (PAsp(OBzl)-b-PLys(Z)) was first synthesized by two-step ring opening

polymerization to obtain a block copolymer with controllable ratio of two different amino acids. Then the PAsp(OBzl) part was modified into zwitterions by aminolysis reaction,[27-29] to obtain PAsp(Lys)-b-PLys(Z)(PALLZ), which can self-assemble into micelles with a zwitterionic shell. PALLZ micelle maintains the major structure of proteins, which have a polypeptide backbone with hydrophobic core and zwitterionic shell. And the sharp polarity difference between these two parts made this block copolymer extraordinarily stable, and maintained its structure after lyophilization without any cryoprotectant additives[25]. DOX·HCl was loaded in PALLZ, and the drug release, cell uptake behavior, stability in FBS were investigated. The fine particle size, good stability and low cell uptake of DOX loaded PALLZ micelles suggest that zwitterionic amphiphilic block polypeptides lay an ideal foundation for future improvement.

2. Materials and Methods

2.1 Materials and Characterizations

L-Aspartic acid β-benzyl ester (H-Asp(OBzl)-OH), Nε-Carbobenzyloxy-L-Lysine (H-Lys(Z)-OH) and Nα-(tert-Butoxycarbonyl)-L-lysine (Boc-Lys-OH) were purchased from GL Biochem (Shanghai) Ltd. N-carboxyanhydride (NCA) of β-Benzyl-L-Aspartate and NCA of Nε-Carbobenzyloxy-L-Lysine were synthesized as described previously in the literature.[29, 30] Trifluoroacetic acid (TFA), triethylamine (TEA)， butylamine, 2,3,4,6,7,8,9,10-Octahydropyrimidol[1,2-a]azepine (DBU) and triphosgene were

purchased from Aladdin Reagent Co., Ltd. 33 wt% HBr/HOAc solution was purchased from Sigma-Aldrich Co. LLC. Anhydrous dimethylformamide (DMF) were purchased from Alfa Aesar. Tetrahydrofuran (THF) and n-hexane were purchased from Sinopharm Chemical Reagent Co.,Ltd and dried by refluxing over Na metal under argon atmosphere and distilled immediately before use. All other reagents were used as received.

^{1}H Nuclear Magnetic Resonance (^{1}H NMR) spectra were measured by Bruker ADVANCE2B/400MHz apparatus. The solvents were DMSO-d_6 or CF_3COOD, and the internal reference was Tetra-methylsilane (TMS). Gel permeation chromatography (GPC) was used to analyze the molecular weights of polymers. Samples were dissolved in DMF and the flow rate was 0.8 mL/min. The critical micelle concentration was obtained by spectrofluorometer (RF-5301pc, shimadzu) with pyrene as a fluorescent probe at 25℃. The average size of all of these micelles was measured by dynamic light scattering (DLS) (Zetasizer Nano-ZS, Malvern Instruments).

2.2 Synthesis of PAsp(OBzl)-b-PLys(Z) block copolymers by using ring-opening polymerization

The copolymers were synthesized by two steps. PAsp(OBzl) was firstly synthesized by ring-opening polymerization (ROP) in DMF using butylamine as initiator. Briefly, NCA of β-Benzyl-L-Aspartate (1.75 g, 7 mmol) was dissolved in 9 mL DMF and stirred under N_2, followed by the addition of butylamine (14.6 mg, 0.2

mmol). This reaction was kept at room temperature (RT) for 48 h. Then the solution was precipitated in diethyl ether for 3 times. The precipitate was filtered and dried in vacuum overnight. PAsp(OBzl)-b-PLys(Z) block copolymers were synthesized by ROP of NCA of Lys(Z) using PAsp(OBzl) as initiator. The length of PLys(Z) block was controlled by the ratio of NCA of Lys(Z) and initiator. This reaction was also kept at RT for 48 h, and then precipitated in diethyl ether for 3 times.

2.3 Synthesis of PAsp(BOC-Lys)-b-PLys(Z)

Boc-Lys-OH (221 mg, 0.9 mmol) dissolved in 1 ml DMF with DBU (0.14 mL, 0.9 mmol) was added to the solution of PAsp(OBzl)-b-PLys(Z) (100 mg) in 1 mL DMF. The solution was stirred at RT for 24 h and precipitated in diethyl ether for twice, then precipitated in hydrochloric acid (0.1 M). The precipitate was washed with water twice to remove benzyl alcohol and unreacted BOC-Lys, and then was dried in vacuum overnight.

2.4 Synthesis of PAsp(Lys-CF_3COOH)-b-PLys(Z) (PALLZ- CF_3COOH)

PAsp(BOC-Lys)-b-PLys(Z) (100 mg) was dissolved in 2 mL DCM/TFA (2/3, v/v) under stirring to deprotect the BOC group for 4 h. Then the solution was precipitated in diethyl ether. The precipitate was filtered and dried in vacuum overnight.

2.5 Preparation of PALLZ micelle and PALLZ/DOX micelle

The precipitate of PALLZ-CF_3COOH (50 mg) dissolved in DMF was added to 100 mL water dropwise under stirring. After stirring for 4 h, the solution was dialyzed against PB (pH=7.4, 0.02 M) for one day and against deionized water for another day

using a 3500 Da cutoff membrane. The micelle was lyophilized to afford white solid.

To prepare DOX loaded micelle, PALLZ micelle (10 mg) was dissolved in 10 mL water, then the solution of DOX·HCl (2 mg) in 0.5 mL DMF with 2.4 μL TEA was added dropwise under stirring. After stirring for 4 h, the solution was dialyzed against PBS (pH=7.4) for one day and against deionized water for another day using a 3500 Da cutoff membrane. The solution was divided into several packs, each contains 1 mL solution, and then the solution was lyophilized to afford red solid.

Drug loading content (DLC) and drug loading efficiency (DLE) were determined by Fluorescent spectrometer. A calibration curve was constructed using different concentrations of DOX·HCl in DMSO/H_2O (4/1, v/v). DLC and DLE were calculated from the following equations.

DLC (%) = (weight of loaded DOX / weight of DOX loaded NPs) × 100%

DLE (%) = (weight of loaded DOX / weight of DOX in feed) × 100%

2.6 Critical micelle concentration (CMC) determination

The CMC of PALLZ-2 block copolymer in water was determined using pyrene as a fluorescence probe. A solution of pyrene in acetone (1.6×10^{-6} M) was dropped into tubes, and then the acetone was allowed to evaporate. The PALLZ-2 block copolymer aqueous solution was serially diluted with deionized water starting with a concentration of 0.4 mg/mL down to 5×10^{-5} mg/mL. Each polymer solution was added to a tube containing pyrene and the mixtures were equilibrated by shaking for 24 h at room temperature. The final pyrene concentration in polymer solutions

reached 6×10^{-7} M. The excitation wavelength range from 300 nm to 360 nm and the detection wavelength was fixed at 390 nm. The CMC was determined by plotting I_{338}/I_{333} ratio against the polymer concentration.

2.7 In vitro drug release

The release of DOX was tested at pH 7.4 and 5.0. 2 mL PALLZ-2/DOX solution sealed in a dialysis bag (3500 Da cutoff) was incubated in 20 mL buffer maintaining at 7.4 or 5.0 at 37℃ under shaking. At predetermined time intervals, 1 mL buffer was withdrawn for testing and replaced with equal volume fresh buffer. The amount of released DOX was determined by detecting the fluorescent intensity of DOX.

2.8 Cell culture

HepG-2 (ATCC® HB-8065™), HeLa (ATCC® CCL-2™), MCF7 (ATCC® HTB-22™) and HUVEC (ATCC® CRL-1730™) cell lines were cultured in Dulbecco's modified eagle's medium (DMEM) (HepG-2, HeLa) or Roswell Park Memorial Institute-1640 (RPMI 1640) (MCF7, HUVEC) respectively. The media were supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Cells were maintained at 37℃ and 5% CO_2 in humidified atmosphere.

2.9 Cell cytotoxicity assays

HepG-2, HeLa, MCF7 and HUVEC cells were seeded into a 96-well plate at a density of 8×10^3 cells per well in 200 μL of growth medium and incubated for 24 h. Then blank micelles at concentrations ranging from 10 to 500 μg/mL were added and

incubated for another 24 h. Then all media were removed and replaced with 200 μL MTT solution (0.5 mg/mL). 4 h later, all media were discarded carefully and 200 μL of DMSO was added to each well. The plate was gently shaken. The absorbance was measured at 570 nm on a 96 plate-reader (Molecular Devices, US). The results were expressed as the mean percentage of cell viability relative to untreated cells. The cytotoxicity of DOX-loaded micelles was carried out in a similar way. Free DOX and the DOX-loaded micelles were added with DOX content ranging from 0.1 to 10 μg/mL.

2.10 In vitro cellular uptakes

Flow cytometry (FCM) and confocal laser scanning microscopy (CLSM) were employed to investigate the cellular uptake and intracellular drug release behaviors of the DOX-loaded micelles. For FCM test, HepG-2 cells were seeded in 6-well plates supplemented with DMEM and 10% fetal bovine serum (FBS) under 5% CO_2 at 37℃ for 24 h. Then the medium was removed, free DOX and PALLZ-2/DOX solution were added respectively at the equivalent DOX concentration. The cells were cultured for another 6 h, then washed with fresh PBS for three times. Cells were collected by trypsin and centrifuged at 900 rpm for 5 min, and washed with fresh PBS. Finally, the cells were suspended in PBS, and analyzed for fluorescent intensity with a BD Bioscience FACSCalibur (BD US). For CLSM test, HepG-2 cells were seeded in confocal dishes and incubated under the same condition as in the FCM test for 24 hours. Fresh medium with free DOX and PALLZ/DOX was added. After 6 h

incubation, cells were washed three times with PBS and the cell nucleus was stained with hochest 33258 for 20 min. Cells were washed three times with PBS, and fixed with fresh 4% paraformaldehyde for 30 min at room temperature, then washed three times with PBS, stored at 4□ before CLSM test.

3. Results and discussion

3.1 Synthesis and characterization of PAsp(Lys)-b-PLys(Z)(PALLZ)

PALLZ was synthesized by the post modification of block copolymers of PAsp(OBzl)-b-PLys(Z). First, three PAsp(OBzl)-b-PLys(Z) block copolymers with different lengths of PLys(Z) were prepared using NCA ring opening polymerization, by which the molecular weight can be well controlled with a low molecular weight distribution PDI (Scheme 1). The GPC results showed that the molecular weight increased with the longer Lys(Z) segment formation while the PDI values kept relatively low (Table 1). And the molecular weight calculated by ^{1}H NMR spectra showed similar values. Then, the benzyl ester group of PAsp(OBzl)-b-PLys(Z) was quantitatively aminolyzed by the primary amine of BOC-Lys as illustrated by Kataoka's group[29]. After the aminolysis, the intensity of CH_2 (d) and C_6H_6 (e) clearly decreased and a peak C(CH_3) at 1.4 ppm (j) appeared in the ^{1}H NMR spectroscopy (Fig. 2), which indicated the success of aminolysis. To evaluate the quantitative aminolysis ability of the primary amine of BOC-Lys, the reaction of PAsp(OBzl) with 1 fold BOC-Lys in DMSO-d_6 at 25℃ was investigated to eliminate the distraction of overlapped peaks of PLys(Z) in ^{1}H NMR spectroscopy (Fig. S1). After 6 h, the sharp

peak of CH_2 (d) of the leaving benzyl alcohol appeared at 4.5 ppm and the peak of CH_2 (c) at 5.0 ppm almost disappeared, with a shift of the peak of CH (a) at 4.6 ppm to CH (e) at 5.5 ppm showed the success of the amibolysis reaction. DBU was used to increase the solubility of BOC-Lys, so the chemical shift was a little different from the one in Fig. 2 in which DBU was removed. Last, the BOC group was removed by TFA to get the amphiphilic zwitterionic block copolymers (Fig. 3).

3.2 Preparation and Characterization of PALLZ micelle and PALLZ/DOX micelle

PALLZ micelles with three different lengths of hydrophobic parts were prepared, and details of the micelles were listed in Table 2 and Fig. 4. The results showed that the micelle formed by PALLZ-1 was not stable, and a large size peak of aggregation was shown in Fig. 4. With the increase of the length of PLys(Z), the micelles became more stable with increased particle size. When loaded with drugs, the PALLZ-1 micelle became stable because of the stronger hydrophobic and π-π interaction of loaded DOX with OBzl groups in the micelles. It is reported that the nonfouling nanoparticles with a diameter range from 30 to 200 nm could have prolonged circulation time and accumulate into the tumor tissues more preferentially through EPR effect.[31] All these three DOX loaded micelles, with the size from 51.29 nm to 134.9 nm are good candidates for drug delivery. Among the three micelles, PALLZ-2 showed a better drug loading content (DLC, 8.7%) and drug loading efficiency (DLE, 46%), so it was chosen for the following investigation.

The PALLZ-2/DOX micelles were in uniform size and close to spherical shape in TEM image (Fig. 5). The diameter was slightly smaller than the one obtained from DLS measurements, which was mainly caused by the dehydration of micelles during the sample preparation. It is believed that the particles away from perfect spherical shape might be caused by the peptide chains since peptide chain is less flexible than most poly vinyl chain and poly ester chain used in most other NDVs.

Moreover, CMC is an important property of micelles because it should be low enough to maintain the micelle structure to against dilution by blood when NDVs are injected into body. Using pyrene as fluorescence probe, the CMC of PALLZ-2 was determined to be 4×10^{-3} mg/mL (Fig. 6), which is low enough for clinical usage.

Lyophilization is one of the most convenient methods for drying and improving the stability of various pharmaceutical products including: vaccines, proteins, peptides, and colloidal carriers. However, lyophilization could destabilize the nanoparticles by the stresses like freezing and dehydration.[32-35] Most polymer-based nanoparticles needs cryoprotectant additives to survive lyophilization, even for PEGylated nanoparticles.[25] PALLZ-2/DOX micelles showed remarkable stability after lyophilization without any cryoprotectant additives (Fig. 7). One explanation of lyoprotectants for stabilizing nanoparticles during drying steps is the water replacement hypothesis, which suggested the lyoprotectants preserve the native structures of nanoparticles by serving as water substitutes. The stability of PALLZ-2/DOX micelles may attribute to the sharp polarity difference between

polypeptide segments or the water molecules that strongly bounded to zwitterionic polymers during lyophilization.[25] The zwitterionic shell provides a well protective layer to keeps the hydrophobic core apart even in highly dehydrated environment due to the sharp contrast in polarity between the two blocks.

Serum stability is also a very important character of NDVs for long blood circulation time without inducing clotting and clearance by phagocytosis. The incubation of the mixture of 50 μL PALLZ-2/DOX micelles (5 mg/ml in PBS) and 950 μL FBS at 37℃ was used to evaluate the serum stability of the new NDV through monitoring the size change by DLS. No obvious change of particle size during 48-hour incubation indicated the good serum stability of PALLZ-2/DOX micelles (Fig 8). This result showed the zwitterioinc PAsp(Lys) segment can form an excellent coating to resist nonspecific protein adsorption, which is agree with our observation of similar zwitterioinc peptides.[22, 36]

3.3 In Vitro and Intracellular Drug Release

The in vitro drug release of PALLZ-2/DOX micelles was carried out at pH=7.4 and 5.0. The results showed a slight increase of release at pH=5.0 compared to pH=7.4 (Fig. 9) since the protonation of $-NH_2$ group of DOX in low pH can improve the solubility of DOX. However, the release rate of DOX was very slow in 6 days in both pH=7.4 and 5.0 buffers. This may be explained by the ultra-stable structure of PALLZ/DOX that prevents the release of DOX. This slow release process provides the necessary time to accumulate in tumor tissue by EPR effect, and avoids the side

effect of DOX by premature release.

The cellular uptake behavior of PALLZ-2/DOX micelles was investigated by incubation the NDVs with HepG-2 cell lines. As shown in Fig. 10, the red fluorescence of DOX observed by confocal laser scanning microscopy (CLSM) has been used to visualize the uptake of PALLZ-2/DOX micelles and the release of DOX. Both free DOX and PALLZ-2/DOX micelles showed an increase of cell uptake with the increase of incubating time. However, PALLZ-2/DOX micelles showed much less DOX cell uptake compared to free DOX. Moreover, almost all DOX mainly located in the cell nucleuses, which suggested that PALLZ-2/DOX micelles may effectively avoid the endocytosis because of its nonfouling property and weak interaction with cell membrane. Generally, free DOX was transported into cells by a passive diffusion mechanism and DOX loaded micelles were taken up by endocytosis.[37-40] The lack of red fluorescence of PALLZ-2/DOX micelles in cytoplasm is very largely different from the observation of the most NDVs after uptake. Usually, large amount NDVs can be found in cytoplasm while DOX in cell nucleus is rather low.[41] In fact, the low DOX concentration in cell nucleus is not a preferred condition since DOX are mainly used to intercalate DNA in cell nucleus. At this stage, we believe that the low amount DOX in cell nucleus should mainly be from the released DOX in the incubation solution from PALLZ-2/DOX micelles although the quick release of DOX after endocytosis is possible. These results are consistent with the ones of flow cytometry (FCM) (Fig. 11). After 6 hours' incubation, the fluorescence intensity of HepG-2 cells

incubated with free DOX was much higher than PALLZ-2/DOX micelles, which was 512 and 60, respectively. To further confirm the low cell uptake during blood circulation, the cell uptake experiment was investigated on RAW264.7 cells (macrophage). Macrophages are a type of white blood cell that engulfs and digests foreign substances, microbes and anything else that does not have the types of proteins specific of healthy body cells on its surface. During 6 hours' incubation, the results (Fig. s2) showed that PALLZ-2/DOX can still effectively reduce the cell uptake by macrophage cells. These results indicated that the zwitterioinc PAsp(Lys) segments on PALLZ-2/DOX micelles can effectively reduce the cell uptake, which could elongate the circulation time of NDVs.

3.4 In Vitro Cytotoxicity Assay

The cytotoxicity of DOX-free and DOX loaded PALLZ-2 micelles to HeLa, HepG-2, MCF7 and HUVEC cell lines were evaluated. As shown in Fig. 12, DOX-free micelle showed no obvious cytotoxicity to all investigated cell lines. Even when the concentration was up to 500 μg/mL, the cell viability was all above 90% which indicated the well compatibility of the micelles. Furthermore, PALLZ-2/DOX micelles (Fig. 13) showed 10 times less cytotoxicity than free DOX to all investigated cell lines, due to the less cell uptake and slow drug release.

4. Conclusion

In this work, a new way of synthesizing zwitterionic block polypeptide was established. The block polypeptide PALLZ with sharp contrast in polarity between

two blocks was obtained by post modification of PAsp(OBzl)-b-PLys(Z). The new NDV, PALLZ-2/DOX micelle, showed excellent resistance to nonspecific protein adsorption in FBS and ultralow cell uptake due to the zwitterionic polypeptide shell, which provide excellent base for further modification of targeting motifs to achieve tumor site targeting. Furthermore, the block polypeptide shows extremely low cytotoxicity. The good stability in lyophilization of PALLZ-2/DOX micelle also gives an advantage in NDV preparation and storage. In short, block polypeptide with zwitterionic segment could be promising materials for NDVs, which can perform as other synthetic polymers and also solve the disadvantages, such as no nature metabolizing route, of other synthetic polymers.

Supporting information

Time-trace of ^{1}H NMR spectra of PAsp(OBzl) reacting with 1 fold BOC-Lys; Inverted fluorescence microscope images of RAW264.7 cells after incubation with free DOX and PALLZ-2/DOX for 1, 3 and 6 h.

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Table 1. Structure and Composition of PAsp(OBzl)-b-PLys(Z) Copolymers

	Composition ratio				
sample	Asp(OBzl)/ Lys(Z)[a]	Asp(OBzl)/Lys(Z)[b]	M_n[b] (Da)	M_w[b] (Da)	M_w/ M_n[b]
P-1	1/0.23	1/0.24	8793	10370	1.179
P-2	1/0.43	1/0.36	9866	12060	1.223
P-3	1/0.87	1/0.59	11880	15140	1.274

[a] Calculated by ^{1}H NMR spectra. [b] Calculated by GPC.

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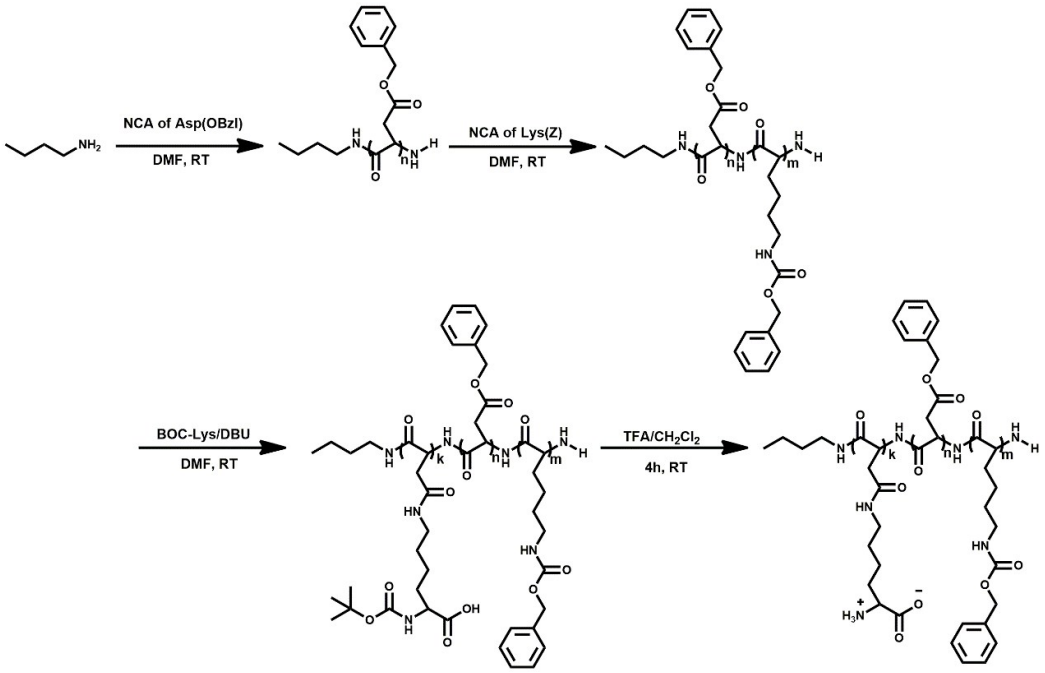
2 Table 2. Characteristics of PALLZ and PALLZ/DOX micelles

	PALLZ			PALLZ/DOX		
sample	Size/nm	PDI	Zeta/mv	Size/nm	PDI	Zeta/mv
PALLZ -1	33.56/212.7	0.469	-5.98	51.29	0.243	-5.12
PALLZ -2	57.55	0.236	-5.82	70.23	0.205	-5.46
PALLZ -3	117.9	0.212	-5.67	134.9	0.178	-4.94

3



Scheme 1. Schematic illustration of the synthesis approach for PALLZ

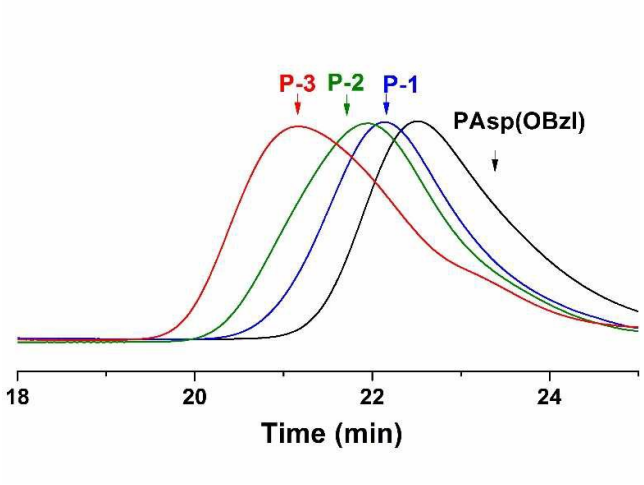


Figure 1. GPC retention time of different polymers in DMF.

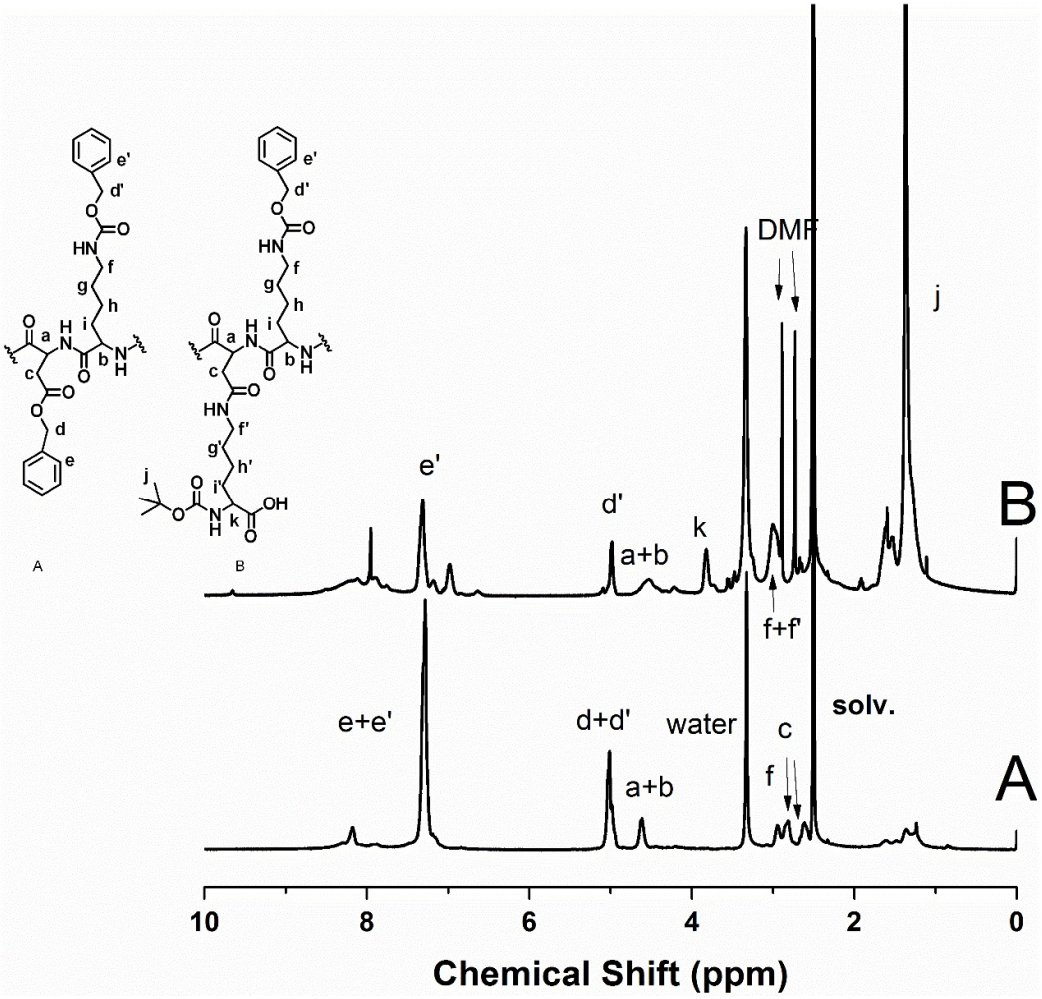


Figure 2. ^{1}H NMR spectra of (A) PAsp(OBzl)-b-PLys(Z) and (B) PAsp(BOC-Lys)-b-PLys(Z) in DMSO-d_6.

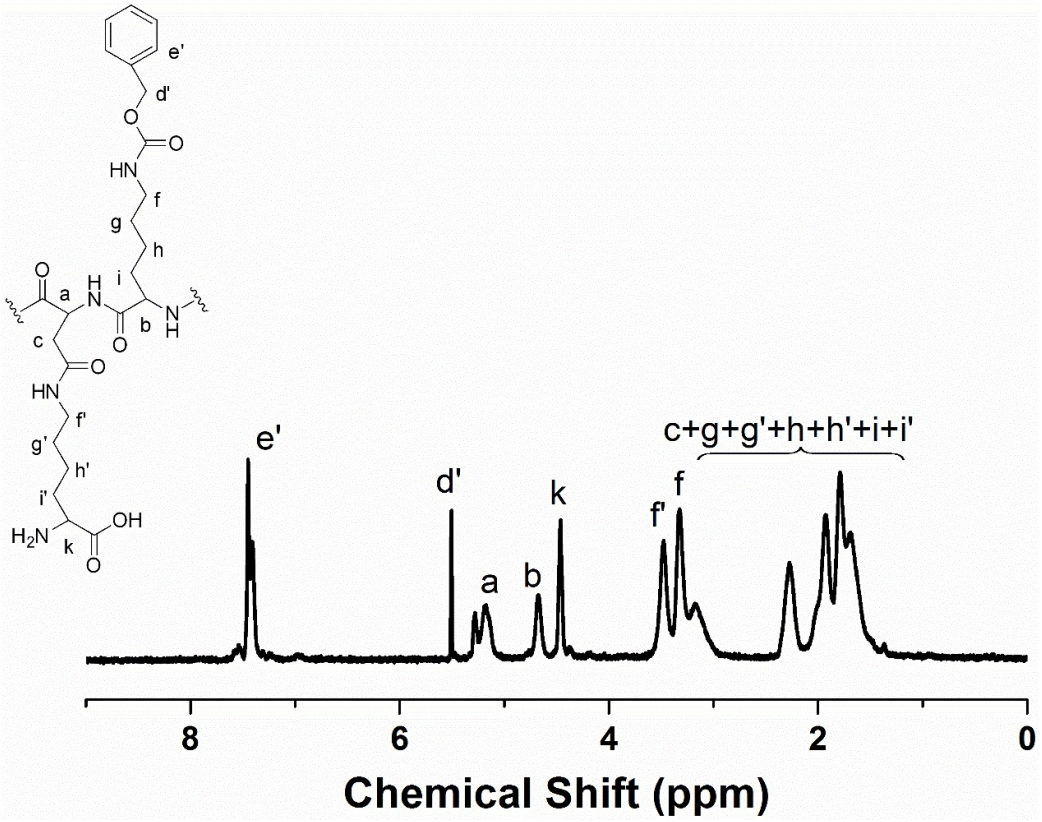


Figure 3. ^{1}H NMR spectra of PALLZ in CF_3COOD

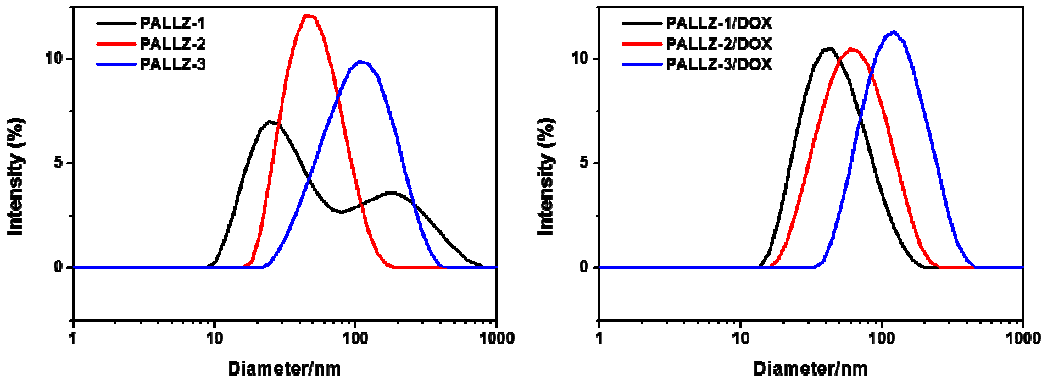


Figure 4. Size distribution of blank micelles and DOX-loaded micelles

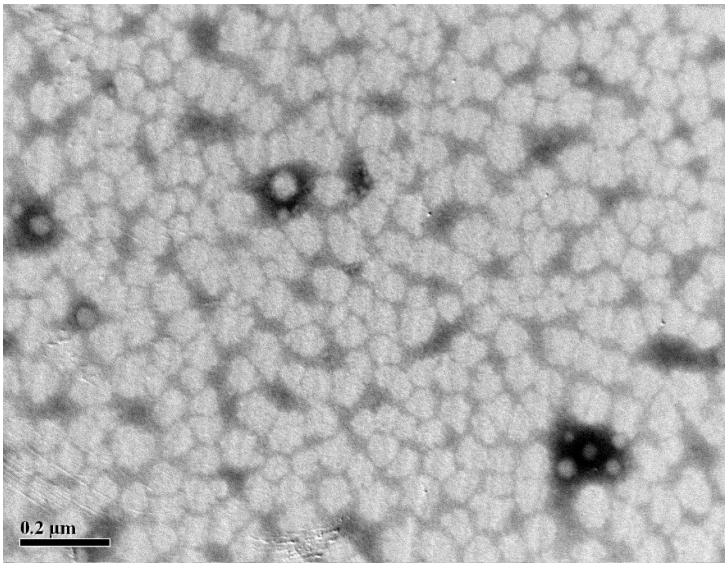


Figure 5. TEM micrograph of PALLZ-2/DOX micelles.

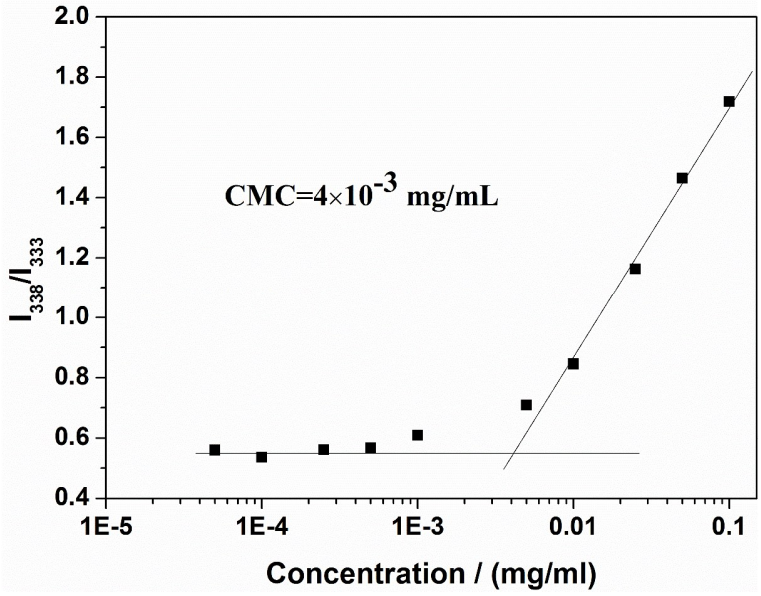


Figure 6. The intensity ratio I_{338}/I_{333} in the fluorescence excitation spectra of pyrene as a function of concentration of PALLZ-2 solution.

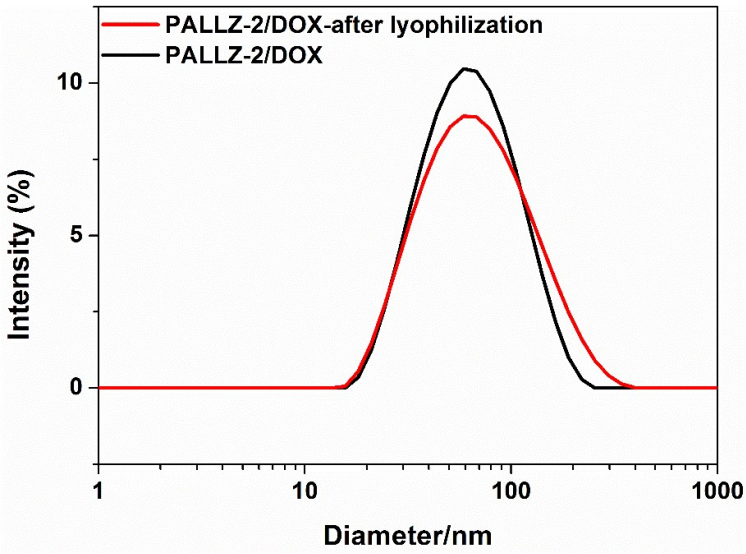


Figure 7. The size distribution of PALLZ-2/DOX before and after lyophilization.

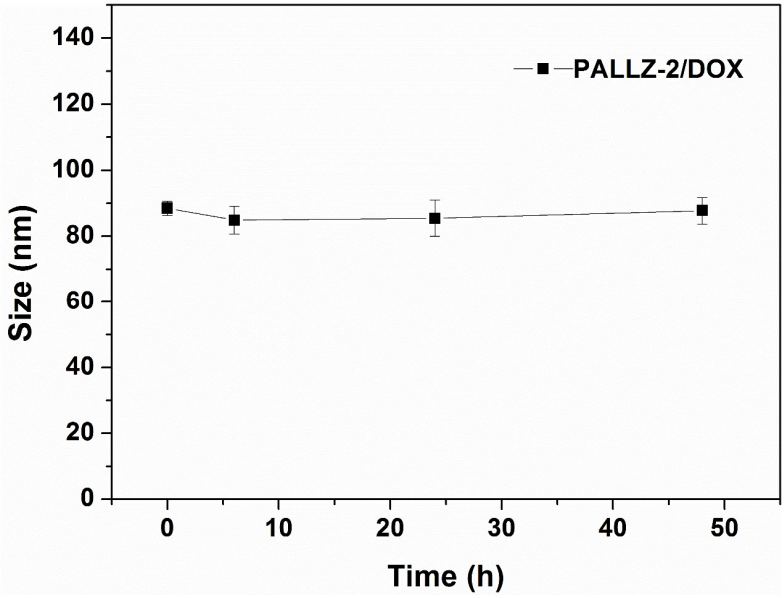


Figure 8. Particle size of PALLZ-2/DOX incubated with 95% FBS at 37℃ at various time.

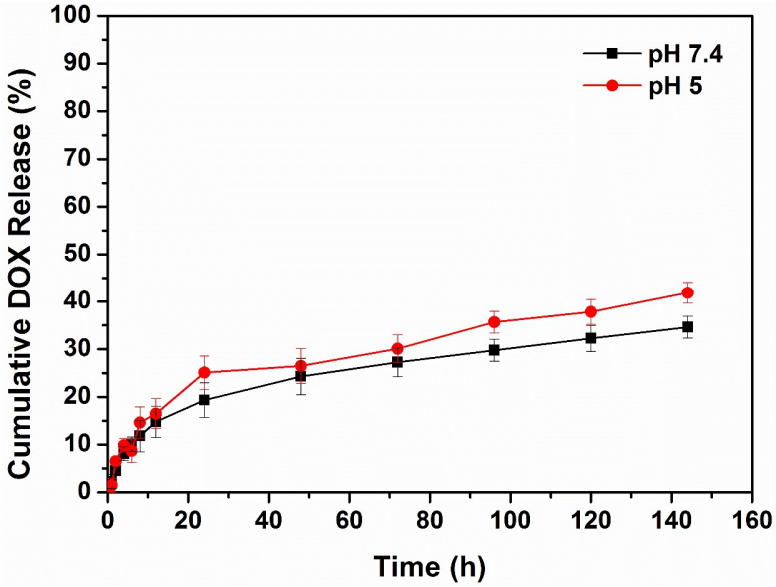


Figure 9. In vitro drug release of PALLZ-2/DOX at 37℃ in pH 5 and pH 7.4 PBS.

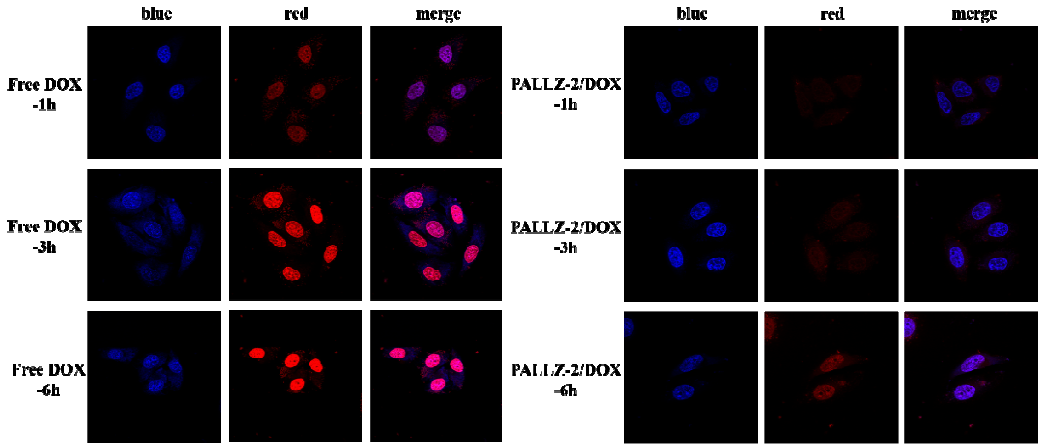


Figure 10. Confocal laser scanning microscopy images of HepG2 cells after incubation with free DOX and PALLZ-2/DOX for 1, 3 and 6 h.

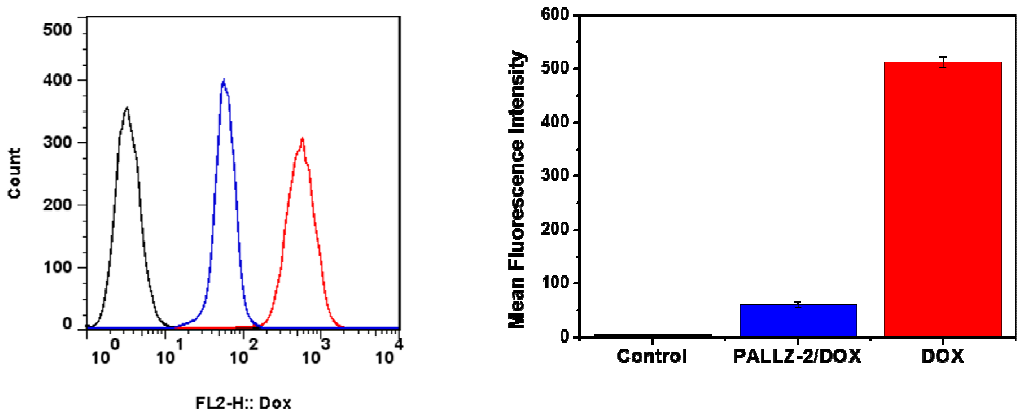


Figure 11. HepG-2 cellular uptake after 6 h of incubation at pH 7.4 and 37 ^{o}C, as analyzed by flow cytometry with a minimum of 1×10^{4} events. A) Flow cytometry histograms of cellular uptake. B) Mean DOX fluorescence intensity in HepG-2 cells treated with DMEM (black), PALLZ-2/DOX (blue), free DOX·HCl (red).

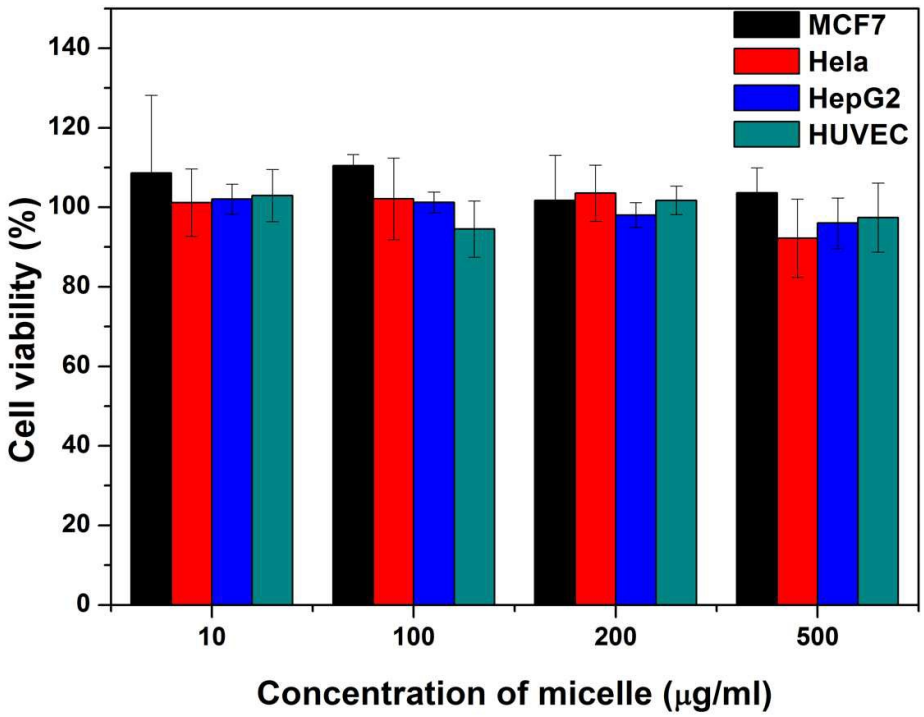


Figure 12. Relative cell viability of MCF7, HeLa, HepG-2 and HUVEC cells after 24 h incubation with PALLZ-2.

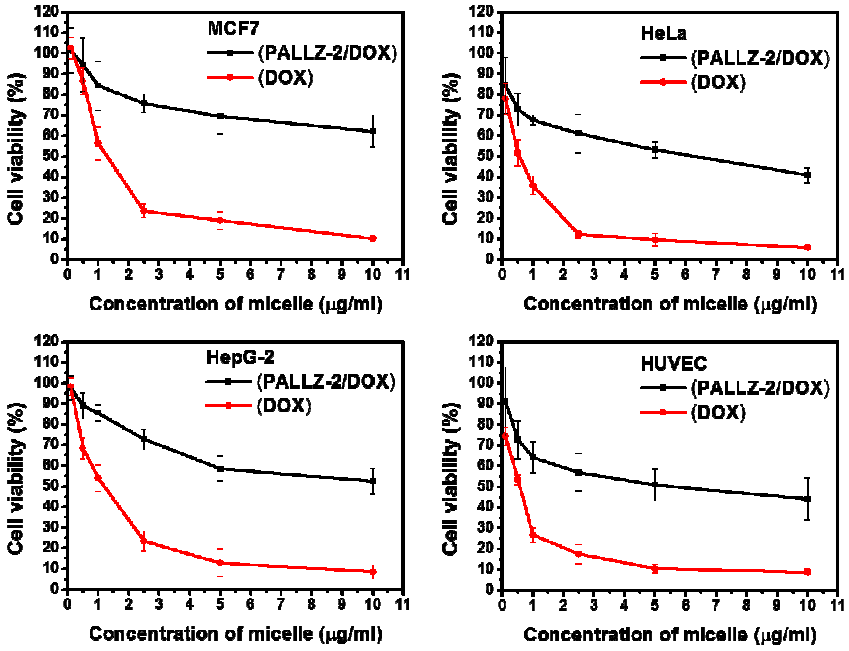


Figure 13. Relative cell viability of MCF7, HeLa, HepG-2 and HUVEC cells after 24 h incubation with free DOX and PALLZ-2/DOX at the equivalent amount of DOX.

TOC

Stable, protein molecules mimicking zwitterioinc amphiphilic micelles based on polypeptide.