THE CYCLODEXTRIN INCLUSION COMPLEX CONTAINING BIODEGRADABLE POLYMERIC SYSTEMS AS DRUG CARRIER

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Abstract - This article shows that the supramolecular micelle assemblies from PCL-b-P4VP block copolymers with α-CD via self-assembly of inclusion complexes in an aqueous solution. Dox encapsulation and the release at different pH of supramolecular micelle assemblies from poly (*ε*-caprolactone-*block*-4-vinylpyridine) (PCL-*b*-P4VP) block copolymers with α-CD showed excellent cytocompatibility. Dox was successfully loaded into the micelles with a loading content of 14.4% and loading efficiency of 28.9% by using UV-Vis spectroscopy (UV). The Dox loaded micelles showed lower cytotoxicity than free drugs, and could efficiently deliver and release the drug into human hepatocellular carcinoma (Hep-G_2) cells as confirmed by confocal laser scanning microscopy (CLSM). These properties make the polymer micelles attractive as drug carriers for pharmaceutical applications.

Key words - micelles; inclusion complex; drug carrier; cyclodextrin; biodegradable polymeric.

1. Introduction

Structures with well-defined architectures and tailored physical properties can be produced via supramolecular self-assembly of polymeric inclusion complexes (ICs) consisting of cyclodextrins (CDs). Recently, there has been significant interest in the use of polymers to design novel supramolecular nanostructures as polymers micelles under inclusion complexation [1, 2]. Cyclodextrins have the ability to spontaneously form complex structures with guest molecules [3]. In this contribution, the main strategies exploited to design a broad range of CD-containing polymeric systems have been described. It is clear that the use of CDs has opened opportunities for designing highly versatile materials with often improved properties compared to conventional (non-CD) polymeric systems. The complexation of polymers with CD induces self-assembed polymers which can be useful in various fields [4, 5]. This article mainly focuses on the application of a novel cyclodextrin inclusion complex containing biodegradable polymeric systems as a new drug carrier. Thanks to the unique capability of CDs to form inclusion complexes in the inner cavities and many other favourable physicochemical and biological properties, they are closely related to many interesting topics [6, 7]. The aim of this article is to synthesize and characterize a novel cyclodextrin inclusion complex containing biodegradable polymeric systems, moving towards some practical applications such as drug delivery. Efforts have been made to develop CD-based micelle vehicles for sustained drug release for controlled and targeted drug delivery. In this work, we fabricated micelles obtained from poly(ε-caprolactone-block-4-vinylpyridine) (PCL-*b*-P4VP) copolymer and α-CD. The micelle system reported in this work is a new approach for the development of micelle vehicles. In order to investigate the potential of hydrophobic drug delivery from micelles, we prepared supramolecular micelle assemblies from PCL-*b*-P4VP block copolymers with α-CD via self-assembly of inclusion complexes in an aqueous solution. Self-assembled aggregates, such as micelle-like supramolecular structures, are constructed from a one-dimensionally well-defined crystal structure in an aqueous solution, and then examined using dynamic light scattering (DLS). Dox encapsulation and the release at different pH of supramolecular micelle assemblies from poly (*ε*-caprolactone-*block*-4-vinylpyridine) (PCL-*b*-P4VP) block copolymers with α-CD were evaluated. Moreover, the cytotoxicity of Dox-loaded pseudopolyrotaxane-*b*-P4VP micelles was investigated in Hep-G_2 cells by MTT assays.

Based on this work, we hope to gain a new insight into the relative contributions made by the cyclodextrin inclusion complex containing biodegradable polymeric systems as drug carrier.

2. Experiment

2.1. Materials

ε-Caprolactone (ε-CL, 99.5%), 4-vinylpyridine (4-VP, 99.5%) and α-Cyclodextrin (α-CD) were purchased from Acros. PCL-*b*-P4VP block copolymers with two different molecular weights (PCL_{175}-*b*-$P4VP_{75}$ and PCL_{175}-*b*-$P4VP_{117}$) were prepared using methods described elsewhere [8]. Cell culture medium Eagle's Minimum Essential Medium (EMEM) with Earle's Balanced Salt Solution, trypsin-EDTA (0.25%), penicillin G, streptomycin sulfate, fetal bovine serum (FBS) and trypan blue stain were purchased from Invitrogen Corporation (Grand Island, NY, USA). Bovine serum albumin (BSA), and Doxorubicin hydrochloride (Dox) were purchased from Sigma-Aldrich. The solvent acetone was purified by distillation and de-ionized water was used in this study. All other solvents and analytical reagents were purchased from commercial suppliers and used as received.

2.2. In vitro cytotoxicity of Dox-loaded micelles

2.2.1. Cell culture maintenance

Human hepatocellular carcinoma (Hep-G_2) cells were maintained in Eagle's Minimum Essential Medium (EMEM) with Earle's Balanced salt solution, supplemented with 1.5 g/l sodium bicarbonate, 2 mM l-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% (v/v) fetal bovine serum (FBS) and grown at 37°C in a humidified atmosphere and in the presence of 5% CO_2. The culture medium was changed and the cells were tripsinized when confluence reached approximately 80-90%.

2.2.2. In vitro cytotoxicity of Dox-loaded micelles

The quantification of the cytotoxic effect of the free Dox, Dox-loaded micelle based systems containing pseudopolyrotaxane-*b*-P4VP and Dox-loaded pseudopolyrotaxane-*b*-P4VP micelles against Hep-G_2 cells was investigated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, the cells were seeded on 96 microplates at concentration of 5×10^4 cells/well in 100 µl of EMEM media and were grown in a humidified incubator at 37°C with 5% CO_2 for 24h. Then the culture medium was replaced by EMEM without increasing the FBS containing concentrations, then incubated at 37°C. After 24h, when the cells had adhered, hydrogel solutions with or without Dox, micellar solutions including pseudopolyrotaxane-*b*-P4VP or pseudopolyrotaxane-*b*-P4VP-Dox (0, 1, 10, 100, 1000 µg/ml) and free Dox at different concentrations (0, 0.3, 3, 30, 300 µg/ml) were incubated separately with the cells for 24h. Following incubation, the cells were washed twice with the culture medium and 100 µl of EMEM medium added with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (10 µl) 5.0 mg/ml in sterile-filtered PBS to each well, and the plates were re-incubated for another 2.5 h. The yellow MTT was reduced to purple formazan in the mitochondria of living cells. The formazan crystals were dissolved in DMSO and then the MTT solution was removed by aspiration, after which MTT crystals were solubilized with 100 µl dimethyl sulfoxide (DMSO). The optical density (OD) of each sample was assessed at 540 nm using a dual wavelength reader (Dynatech, Denkendort, Germany). The data were presented as multiples of control. The untreated cells were used as the control. The values were the mean of 8 replications for each treatment. The absorbance of each well was normalized with their respective controls. The cell viability was calculated as follows:

$$\text{Cell viability (\%)} = \frac{B}{A} \times 100$$

where A is the absorbance of the cells incubated with the culture medium (control) and B is the absorbance of the cells incubated with the drug-loaded micelles or free drug (treatments).

2.2.3. Cell image studies

To confirm the toxicity of the Dox-loaded pseudopolyrotaxane-*b*-P4VP and pseudopolyrotaxane-*b*-P4VP micelles against Hep-G_2 cells, we studied the cell images after the treatment of free Dox, pseudopolyrotaxane-*b*-P4VP and Dox-loaded pseudopolyrotaxane-*b*-P4VP micelles for 24 hrs in the 24 well plates (10^5 cells/well). The drug-treated cells were washed three times with PBS pH 7.4, the cells were fixed with parapolyformaldehyde in PBS for 30 mins, and then the nucleus was counterstained with propidium iodide (final concentration of 50 µg/ml) for 30 mins at 37°C. All specimens were washed twice with distilled water and cell images were examined.

2.3. Preparation of pseudopolyrotaxane-b-P4VP

Both PCL_{175}-*b*-$P4VP_{75}$ and PCL_{175}-*b*-$P4VP_{117}$ block copolymer solutions were made up by dissolving them in THF at 30°C. The inclusion complexes (pseudopolyrotaxanes) of block copolymers were prepared by mixing the solutions of PCL-*b*-P4VP (0.25g PCL-*b*-P4VP dissolved in 25 ml THF). They were then separately added to α-CD solutions (3.75g α-CD dissolved in 50 ml H_2O). The contents of the mixtures were stirred ultrasonically at 40°C for 2 hours and then stirred again for 12 hours at room temperature in a magnetic stirrer. The solution contents were left to stand overnight at room temperature without further stirring. During the preparation of the pseudopolyrotaxanes, the solutions gradually became turbid after mixing with the α-CDs, finally producing white precipitates, apparently indicating the formation of pseudopolyrotaxanes. The above pseudopolyrotaxane solutions were freeze-dried and then washed with THF and water several times to remove free PCL-*b*-P4VP and un-complexed α-CD, respectively.

2.4. Preparation of Dox-loaded micelles and in vitro release

Dox was loaded during the micelles formation and the weight ratio of pseudopolyrotaxane-*b*-$P4VP_{75}$ to drug was kept at ca. 2.0. Before loading the Dox into the pseudopolyrotaxane-*b*-$P4VP_{75}$ micelles, the hydrochloride of Dox was removed by triethylamine (TEA), making the drug hydrophobic, with a mole ratio of Dox.HCl/TEA is 1.2/1. Then, 3 mg of pseudopolyrotaxane-*b*-$P4VP_{75}$ was dissolved in 4.5 ml H_2O and 1.5 ml DMSO. Then 1.5 mg Dox.HCl was dissolved in 2 ml DMSO, together with 0.3 µl TEA. The mixture of pseudopolyrotaxane-*b*-$P4VP_{75}$ solution and Dox solution was dialyzed against 1000 ml distilled water at 30 °C for 24h, with the distilled water being replaced 6 times. After dialysis, the solution of micelles was collected and frozen using a freeze dryer system to obtain dried micelles. Weighed amounts of mixed micelles were dissolved in DMSO at room temperature for 12 hours, after which ultrafiltration (membrane filtration, MWCO 6000-8000, MFPI, USA) was performed to extract samples. The Dox content of the mixed micelles was determined by UV/Vis spectrometry at 482 nm. The drug loading content (DLC) and drug loading efficiency (DLE) were calculated according the following formula:

DLC (%) = weight of drug in mixed micelle/weight of pseudopolyrotaxane-*b*-P4VP x 100%;

DLE (%) = weight of drug in mixed micelle/weight of drug in feed x 100%;

The particle size of Dox-mixed micelle was determined by DLS.

In vitro release of Dox from the micelles was determined as follows. The phosphate buffered saline (PBS) solution with drug loaded micelles (30 µg/ml) was introduced into a dialysis tube (MWCO 6000-8000) which was placed in 10 ml of the PBS solution release media with pH 5.0 or pH 7.4 and the media were stirred at 37°C. At predetermined time intervals, 2 ml aliquots of the aqueous solution were withdrawn from the release

media and another 2ml fresh PBS were added into the release media. Then the concentration of Dox released was measured using a UV spectrometer at 482 nm.

2.5. Dynamic light scattering (DLS)

The DLS measurements of the micelles solutions (1mg/1ml) were obtained at a scattering angle of 90° and a temperature of 25°C using the Malvern series-MAL1039696 apparatus equipped with a 7132 multiple-τ auto correlator recording on 128-channels. A He-Ne ion laser acted as a light source, operating at a power of 4 mW with a wavelength of 633 nm.

3. Results and discussion

3.1. In vitro cytotoxicity of Dox-loaded micelles

To guesstimate the potential toxicity of polymeric materials for drug delivery applications is extremely important. The MTT method was used to determine the viability of Hep-G_2 cells in the culture medium after 24 hours of incubation with the pseudopolyrotaxane-*b*-P4VP and Dox loaded pseudopolyrotaxane-*b*-P4VP micelles, as shown in Figure 1. Overall, free Dox was shown to be dose-dependent with the most pronounced cytotoxic effect at 30 and 300 µg/ml effective than other doses which we have taken in our studies for 24 hours incubation time. We fixed the amount at 300 µg/ml for later experiments due to its potential effect at this dose on this cell line.

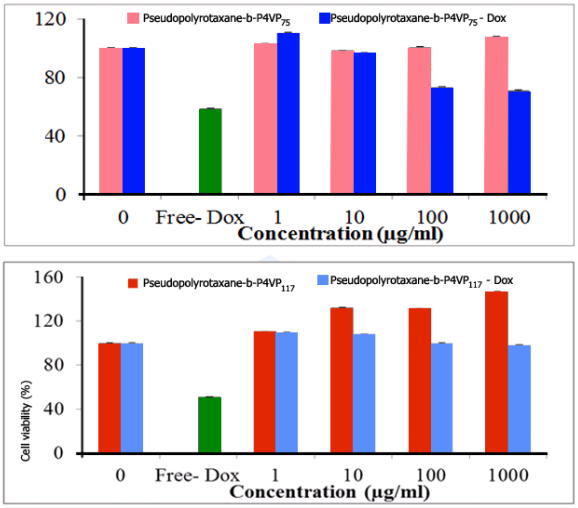

Figure 1. *In vitro cell viability of (a) pseudopolyrotaxane-b-P4VP75 (b) pseudopolyrotaxane-b-P4VP117 and Dox loaded polymeric micellars - Dox formulations against Hep-G_2 cells after 24 h of incubation*

In Figure 1, it can be seen that Dox loaded pseudopolyrotaxane-*b*-$P4VP_{75}$ micelles showed higher cytotoxicity than free pseudopolyrotaxane-*b*-$P4VP_{75}$ micelles at high concentration after 24h. More interestingly, neither free form of pseudopolyrotaxane-*b*-P4VP alone showed any sign of cytotoxicity, even with high concentrations. On the other hand, the cell viability of the pseudopolyrotaxane-*b*-$P4VP_{117}$ with high concentration was relatively higher than 100%. It seemed that the high concentration of pseudopolyrotaxane-*b*-$P4VP_{117}$ favoured cell proliferation. In addition, for both pseudopolyrotaxane-*b*-$P4VP_{75}$ and pseudopolyrotaxane-*b*-$P4VP_{117}$ concentrations, the viabilities in Hep-G_2 cells were comparable to those of controls. The results showed that these micelles were nontoxic even in high concentrations. It is believed that the excellent biocompatibility can be attributed to the following possible reason: that the Dox may affect the threading of α-CD molecules onto the PCL chains during the micelle formation. Between these two micelles, high pseudopolyrotaxane-*b*-$P4VP_{75}$ concentrations appeared to be toxic.

These results were corroborated by the cell imaging studies. Figures 2 and 3 show confocal microscopy photographs of Hep-G_2 cells incubated with Dox, and Dox loaded micelles in different concentration after 24 h. For the first three concentrations there could be uptake by the Hep-G_2 cells. The difference is the red fluorescence of the Dox loaded micelles observed in both the cytoplasm and the nucleus. However, the high concentrations of pseudopolyrotaxane-*b*-$P4VP_{75}$ and pseudopolyrotaxane-*b*-$P4VP_{117}$ loaded with Dox have considerable cytotoxicity as shown by the appearance of cells with apoptotic bodies.

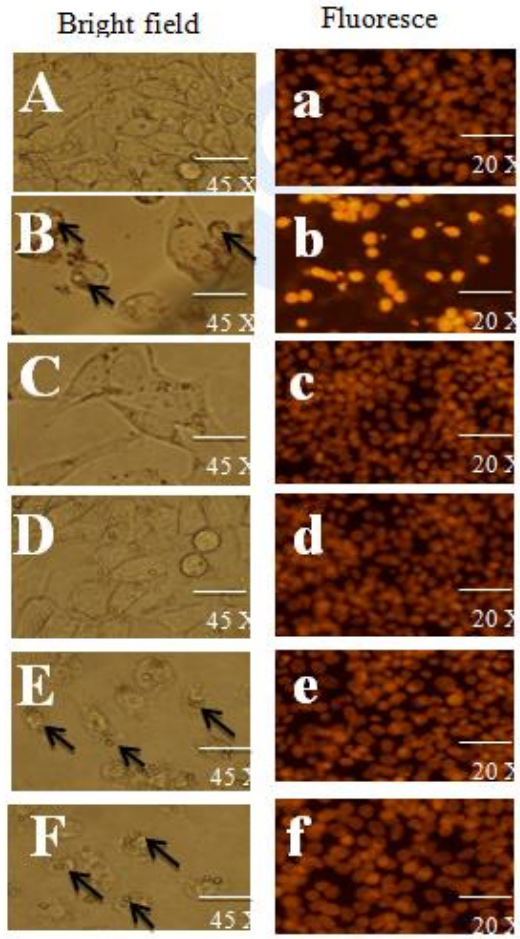

Figure 2. *Change in morphology of attached Hep-G_2 cells treated with free Dox or Dox-loaded pseudopolyrotaxane-b-P4VP75. Confocal laser scanning microscopy images of(left) bright field and (right) fluorescence images of control (A, a), or exposure of free Dox (300 ug/ml) (B, b), Dox-loaded pseudopolyrotaxane-b-P4VP75 micelles 1 µg/ml (C, c), 10 µg/ml (D, d), 100 µg/ml (E, e) and 1000 µg/ml (F, f) for 24h*

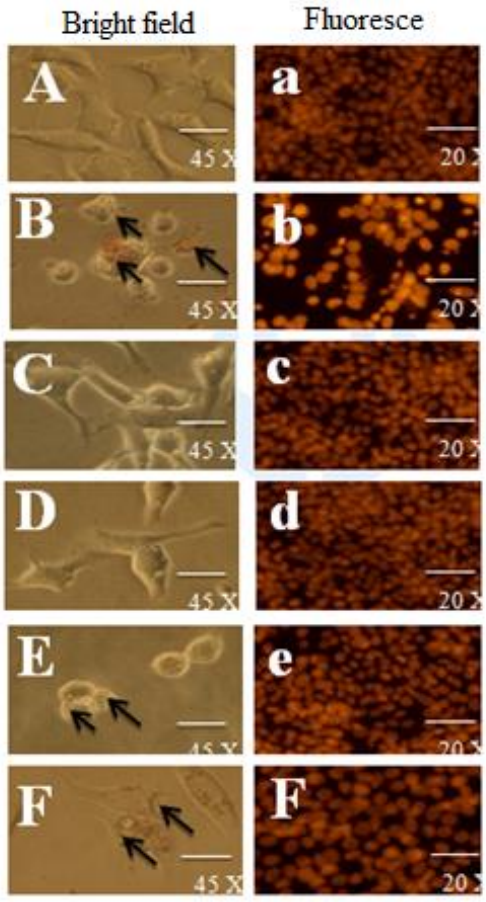

Figure 3. *Change in morphology of attached Hep-G2 cells treated with free Dox or Dox-loaded pseudopolyrotaxane-b-P4VP117. Confocal laser scanning microscopy images of (left) bright field and (right) fluorescence images of control (A, a), or exposure of free Dox (300 ug/ml) (B, b), Dox-loaded pseudopolyrotaxane-b-P4VP117 micelles 1 µg/ml (C, c), 10 µg/ml (D, d), 100 µg/ml (E, e) and 1000 µg/ml (F, f) for 24 h*

3.2. Loading and in vitro release of Dox from pseudopolyrotaxane-b-P4VP micelles

Pseudopolyrotaxane-*b*-P4VP$_{75}$ shows less cytotoxicity for the higher concentration and size of the micelles formed from pseudopolyrotaxane-*b*-P4VP$_{75}$ smaller than from pseudopolyrotaxane-*b*-P4VP$_{117}$. Smaller size micelles can be effective in drug delivery; hence, we selected pseudopolyrotaxane-b-P4VP$_{75}$ for Dox delivery application.

To estimate the potential of pseudopolyrotaxane-b-P4VP$_{75}$ micelles in drug delivery, we used Dox as the model drug and loaded during formation of the micelles. The content of drug loaded and efficiency were estimated to be 14.4% and 28.9%, respectively. According to the DLS measurements shown in Figure 4, the average hydrodynamic diameter of the blank pseudopolyrotaxane-*b*-P4VP$_{75}$ micelles in an aqueous solution was about 130.2 nm, the size of the Dox-loaded pseudopolyrotaxane-*b*-P4VP$_{75}$ micelles was about 218.6 nm, and the size of the blank pseudopolyrotaxane-*b*-P4VP11$_{117}$ micelles in an aqueous solution was about 159.4 nm with a broader size distribution (PDI = 0.24 ± 0.02). The increament in average size of the Dox-loaded pseudopolyrotaxane-*b*-P4VP micelles, as compared to the blank micelles, was caused by the drug molecules becoming entrapped in the hydrophobic cores.

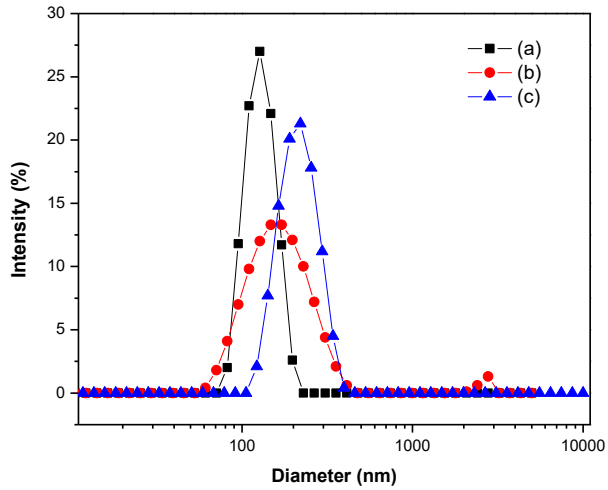

Figure 4. *Size distributions of (a) pseudopolyrotaxane-b-P4VP$_{75}$, (b) pseudopolyrotaxane-b-P4VP$_{117}$ and (c) Dox loaded pseudopolyrotaxane-b-P4VP$_{75}$*

The release of the Dox from the pseudopolyrotaxane-*b*-P4VP$_{75}$ micelles was investigated using the dialysis method as a function of pH dependence. The release of Dox carried out in pH 7.4 and pH 5 PBS at 37 °C was monitored using a UV spectrometer at 482 nm, which is the characteristic maximum absorbance of Dox in a solution. We compared the release behavior of the drug loaded pseudopolyrotaxane-*b*-P4VP$_{75}$ micelles in pH 7.4 and pH 5, demonstrated by plotting the relative release percentages of drug versus time in Figure 5.

As shown in Figure 5, there was a slow release of about 36% of the incorporated Dox within 48 h in PBS at pH 7.4. However, the release rate of Dox from pseudopolyrotaxane-*b*-P4VP$_{75}$ micelles at pH 5 was much faster, with a 56% release in the first 12 h and up to 67% release after 48 h. Combined with the data in the TEM results, this reveals that the Dox-loaded pseudopolyrotaxane-*b*-P4VP$_{75}$ micelles preserved their core shell structures given the physiological pH, resulting in the small drug molecules diffusing from the core to maintain a slow and sustained release. However, when the drug-loaded micelles were dispersed in an acidic environment, the drug molecules entrapped in the pseudopolyrotaxane-*b*-P4VP$_{75}$ core would be quickly released with quick demicellization, for the protenation of the nitrogen group of the P4VP and pseudopolyrotaxane construction being demolished by the lower pH. Interestingly, the change in pH from 7.4 to 5 corresponds to the change in pH from the extracellular pH to early endosomal pH, suggesting that these Dox-loaded pseudopolyrotaxane-*b*-P4VP$_{75}$ micelles may be suitable for intracellular drug delivery. In addition, slow drug release is very important for drug delivery so it is better for inclusion complex carriers to be delivered to the targeted tissue through the bloodstream over a long time with the circulation, and less drug leakage can result in successful drug targeting due to the passive enhanced

permeability and retention effect. Our experimental results suggested that micelles prepared from pseudopolyrotaxane-*b*-P4VP$_{75}$ in pH 7.4 could be used as a candidate for injectable drug delivery carrier.

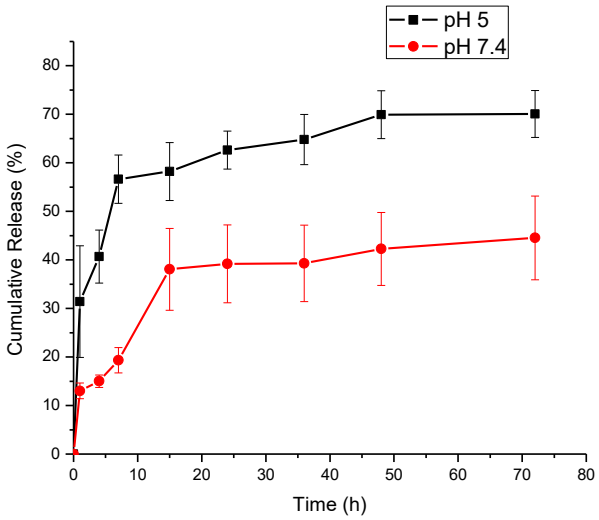

Figure 5. *In vitro release profiles of Dox from pseudopolyrotaxane-b-P4VP$_{75}$ micelles under acidic (pH 5) and neutral (pH 7.4) condition at 37°C, n=3*

4. Conclusion

This article has demonstrated that Dox was successfully loaded into the pseudopolyrotaxane-*b*-P4VP micelles. The Dox loaded pseudopolyrotaxane-*b*-P4VP micelles showed lower cytotoxicity than the free drug and could efficiently deliver and release the Dox. Furthermore, the results described in this study provide a novel method that potentially widens the options for the self-assembly of block copolymers and shows prospects for the development of a potential intracellular anticancer drug delivery system.

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