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# Biocompatibility, drug release, and anti-tumor effect of pH -sensitive micelles prepared from poly(2-ethyl-2-oxazoline)-poly( DL -lactide) block copolymers

Yuandou Wang, Baogang Zhang, Xin Shen, Qian Li, Feng Su, S.M. Li

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**Biocompatibility, drug release and anti-tumor effect of pH-sensitive micelles prepared from poly(2-ethyl-2-oxazoline)-poly(DL-lactide) block copolymers**

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10 Yuandou Wang,<sup>a†</sup> Baogang Zhang,<sup>b†</sup> Xin Shen,<sup>c,d</sup> Qian Li,<sup>d</sup> Feng Su,<sup>a,b\*</sup> Suming Li<sup>e\*</sup>  
11  
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13  
14

15 <sup>a</sup> State Key Laboratory Base of Eco-chemical Engineering, College of Chemical  
16 Engineering, Qingdao University of Science and Technology, Qingdao 266042, China  
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18

19 <sup>b</sup> Institute of High Performance Polymers, Qingdao University of Science and  
20 Technology, Qingdao 266042, China  
21  
22

23 <sup>c</sup> CP Pharmaceutical (Qingdao) Co., Ltd., Qingdao, 266426, China  
24  
25

26 <sup>d</sup> Cancer Institute, The Affiliated Hospital of Qingdao University, Qingdao, 266071,  
27 China  
28

29 <sup>e</sup> Institut Européen des Membranes, IEM UMR 5635, Univ Montpellier, CNRS,  
30 ENSCM, Montpellier, France  
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37 Correspondence to: F. Su (E-mail: [sufeng@qust.edu.cn](mailto:sufeng@qust.edu.cn)), S. Li (E-mail:  
38 [suming.li@umontpellier.fr](mailto:suming.li@umontpellier.fr))  
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44 <sup>†</sup>Authors equally contributed to this work and are both co-first authors.  
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## 48 ABSTRACT

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51 Various ratios PEOz-PDLLA diblock copolymers were synthesized through ring-  
52 opening polymerization of DL-lactide initiated by monohydroxyl terminated poly(2-  
53 ethyl-2-oxazoline) (PEOz-OH). The chemical composition and molar mass of the  
54 resulting copolymers were determined by using NMR and GPC. Spherical micelles  
55 with a diameter from 104 to 136 nm were obtained via self-assembly of PEOz-PDLLA  
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4 in water, as evidenced by TEM and DLS. The CMC of copolymers was well below  
5 0.01 mg/mL, and showed a decreasing trend with increase of PDLLA block length. A  
6 two-step procedure was applied to encapsulate curcumin taken as a hydrophobic model  
7 drug. Copolymers with longer PDLLA block exhibit enhanced drug loading capacity.  
8 In vitro drug release was carried out in phosphate buffered saline at physiological and  
9 slightly acidic pH values. A pH-sensitive biphasic drug release behavior was detected  
10 with an initial burst release followed by slower prolonged release. Lower pH or shorter  
11 PDLLA block length leads to higher drug release rate because of faster degradation of  
12 micelles. Hemo- and cytocompatibility studies illustrated the excellent biocompatibility  
13 of PEOz-PDLLA micelles. MTT assay showed a significant cytotoxicity of curcumin-  
14 loaded micelles against A549 lung cancer cells. Therefore, it can be inferred that PEOz-  
15 PDLLA block copolymers with outstanding biocompatibility, controllable drug loading  
16 performance, and prolonged drug release present a good potential as nano-carrier of  
17 hydrophobic drugs.  
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34 **Keywords:** poly(2-ethyl-2-oxazoline); poly(DL-lactide); self-assembly; micelles;  
35 curcumin; nano carrier  
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## 43 1 Introduction

44 Nowadays, cancer is one of the most common causes of death globally. Nearly 10  
45 million of people died of cancer in 2020 according to the World Health Organization.<sup>1-3</sup>.  
46 For patients with different types of solid tumors, the most commonly used first-line  
47 clinical treatment is combination of surgery and chemotherapy. However, one of the  
48 main problems of chemotherapy is the extremely low bioavailability of anti-tumor  
49 drugs and adverse side effects.<sup>4-6</sup> Many nano-platforms such as micelles, liposomes,  
50 vesicles and nanoparticles have been designed to prevent drug deterioration while  
51 increasing effective drug concentration at lesion site.<sup>7-10</sup> Among them, micelles have  
52 attracted much interest due to their advantageous characteristics, including good  
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4 stability in vivo, long circulation in the bloodstream, sustained and adjustable drug  
5 release performance. A number of micelles prepared from amphiphilic block  
6 copolymers, have been brought to the stage of clinical trials.<sup>11-13</sup> Drugs encapsulated in  
7 micelles can be delivered at the target site through drug diffusion and copolymer  
8 degradation<sup>14,15</sup>. Additionally, the chemical structure of micelle material can be  
9 designed to tailor the degradation rate and, thus allowing spatiotemporally controlled  
10 drug release.  
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18 Curcumin is a natural polyphenol extracted from the rhizome of turmeric  
19 (*Curcuma longa*) which exhibits various pharmacological activities because of its  
20 versatile molecular targets.<sup>16</sup> Curcumin has been widely explored in preventing or  
21 treating different types of diseases, such as cancer, gastrointestinal diseases, as well as  
22 gastrointestinal and arthritis-related disorders.<sup>17-20</sup> However, the very low solubility (11  
23 ng mL<sup>-1</sup>), fast metabolism and clearance of curcumin lead to low bioavailability, which  
24 considerably limits its clinical applications. It is thus of major importance to develop a  
25 nano carrier to encapsulate curcumin so as to improve its bioavailability.  
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34 Poly(2-ethyl-2-oxazoline) (PEOz) is a hydrophilic polymer with aliphatic side  
35 chains and a skeleton structure similar to natural polypeptides. It has been approved by  
36 the food and drug administration (FDA) as a food additive.<sup>21</sup> Due to its biosafety and  
37 hydrophilicity, PEOz is considered as an excellent substitute to poly(ethylene glycol)  
38 (PEG) in the field of biomedical research. Micelles prepared from PEOz-based block  
39 copolymers present attractive properties, including long circulation time, sustained  
40 drug release and no accumulation in normal organs.<sup>22,23</sup> Meanwhile, ionization of  
41 tertiary amide groups leads to electrostatic repulsion between PEOz chains that  
42 provides PEOz-based micelles with pH sensitivity. Zhao et al. synthesized a PEOz-  
43 poly(DL-lactide) (PEOz-PDLLA) copolymer composed of a PEOz block with Mn of  
44 6514 and a PDLLA block with Mn of 8200.<sup>24</sup> Doxorubicin loaded micelles were  
45 prepared using dialysis method. Drug release studies were performed at pH 7.4, 6.5 and  
46 5.0 to simulate the blood environment, the tumoral extracellular environment, and the  
47 endo/lysosome mimetic circumstance, respectively. All the designed micelles exhibited  
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4 an accelerated drug release in acidic pH media as doxorubicin was almost totally  
5 released beyond 12 h at pH 5.0.<sup>24</sup> In our previous work, a number of PEOz-poly(L-  
6 lactide) (PEOz-PLLA) copolymers were synthesized as pH-sensitive carrier of  
7 paclitaxel.<sup>25</sup> It was observed that the hydrophilic/hydrophobic balance and hydrophobic  
8 block length of copolymers significantly affect the micelle formation and paclitaxel  
9 release behavior. Spherical micelles were exclusively observed for PEOz-PLLA  
10 copolymers with short PLA blocks, whereas both worm-like and spherical micelles  
11 were obtained for copolymers with long PLA blocks. But drug release was rather slow  
12 with less than 40% of drug released after 30 days.  
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22 In the present work, PEOz-PDLLA diblock copolymers with various compositions  
23 were prepared using monohydroxyl terminated PEOz as macro-initiator. Nano-sized  
24 micelles were obtained by self-assembly of the resulted copolymers in water using  
25 chloroform as a co-solvent, and characterized from dynamic light scattering (DLS),  
26 transmission electron microscopy (TEM), and critical micelle concentration (CMC)  
27 measurements. The cytocompatibility, hemocompatibility and Zebrafish embryo  
28 compatibility of the copolymers were studied in detail. Using curcumin as a drug model,  
29 the drug encapsulation properties and drug release behaviors of micelles were evaluated  
30 under in vitro conditions. Finally, the anti-tumor activity of curcumin loaded PEOz-  
31 PDLLA micelles on lung cancer cells was assessed for their potential applications in  
32 cancer therapy.  
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## 46 2 EXPERIMENTAL

### 47 2.1 Materials

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51 DL-lactide was synthesized by polycondensation of DL-lactic acid, followed by  
52 thermal composition and cyclization according to literature method.<sup>26</sup> Monohydroxyl  
53 terminated poly(2-ethyl-2-oxazoline) was prepared by ring-opening polymerization of  
54 2-ethyl-2-oxazoline.<sup>27</sup> Tin(II) 2-ethylhexanoate ( $\text{Sn}(\text{Oct})_2$ ), curcumin, acetonitrile and  
55 chlorobenzene were supplied by Sigma-Aldrich (Shanghai, China). Chlorobenzene and  
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3 acetonitrile were treated with calcium hydride for water removal. All other organic  
4 reagents were employed without further treatment.  
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## 7 8 2.2 Synthesis of PEOz-PDLLA copolymers 9

10 Monohydroxyl terminated PEOz-OH was first synthesized according to our  
11 previously reported work.<sup>25</sup> The  $M_n$  of obtained PEOz-OH was 2800 g/mol. PEOz-  
12 PDLLA block copolymers were synthesized in chlorobenzene according to the  
13 following procedure. Typically, PEOz-OH (1.0 g), DL-lactide (0.58 g), and stannous  
14 octoate (5 mg) were introduced in a flask, followed by addition of chlorobenzene (15  
15 mL). Ring opening polymerization then occurred at 120 °C with inert gas protection.  
16 After 30 h reaction, the PEOz-PDLLA copolymer was obtained by filtration through  
17 silica gel, precipitation in cold diethyl ether, and vacuum drying in oven at 40°C up to  
18 constant weight.  
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## 28 2.3 Self-assembly of PEOz-PDLLA copolymers 29

30 Co-solvent method was employed to prepare self-assembled PEOz-PDLLA  
31 micelles. Typically, PEOz-PDLLA copolymer (20 mg) was introduced in chloroform  
32 (1 mL), and solubilized. The resulted copolymer solution was then dropped in ultrapure  
33 water (20 mL). Vigorous stirring was applied for 4 h so as to allow self-assembly of  
34 copolymer micelles and evaporation of the solvent. A micelles solution at 1.0 mg/mL  
35 was finally obtained by filtration via a 0.22 µm filter membrane.  
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## 43 2.4 Characterization 44

45 Proton nuclear magnetic resonance (<sup>1</sup>H NMR) was performed using a Bruker  
46 Avance III instrument. 5 mg of sample were dissolved in 0.6 mL deuterated chloroform  
47 (CDCl<sub>3</sub>). And the spectra were recorded at 500 MHz with tetramethylsilane as an  
48 internal reference.  
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53 Gel permeation chromatography (GPC) was carried out on a Shimadzu apparatus  
54 equipped with a Waters 410 refractometer. 10 mg of sample were dissolved in 1 mL  
55 tetrahydrofuran taken as mobile phase. Measurements were made at 25°C, and  
56 calculations were realized based on a calibration curve previously obtained using  
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4 polystyrene standards.

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6 Transmission electron microscopy (TEM) was performed on JEM 1200EX  
7 microscope (JEOL, Japan). A micelle solution (5  $\mu\text{L}$ ) at 1.0 mg/mL was dropped onto  
8 a carbon coated copper grid, followed by negative staining with a phosphotungstic acid  
9 solution at 1.0 wt.%. The sample was air dried, and then examined by TEM at an  
10 acceleration voltage of 80 kV.  
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16 Dynamic light scattering (DLS) was carried out by using a Nano-ZS90 nanosizer  
17 (Malvern, UK). Micelle solutions at 1.0 mg/mL were filtered via a 0.45  $\mu\text{m}$  PTFE  
18 microfilter before measurements made at room temperature. The scattering angle was  
19 fixed at 90°.  
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24 The critical micelle concentration (CMC) of PEOz-PDLLA copolymers was  
25 determined using fluorescence spectrophotometer (F-4600). Pyrene was taken as a  
26 fluorescent probe. First, 1 mL pyrene solution in benzene ( $2 \times 10^{-5}$  M) was poured into  
27 a 10 mL flask, followed by solvent evaporation. Various volumes of micelle solution  
28 and ultrapure water were added to the flask to a total volume of 10 mL. The final  
29 concentration of micelles was in the range from  $1.0 \times 10^{-3}$  to 0.5 mg/mL, and that of  
30 pyrene was  $2.0 \times 10^{-6}$  M. After 24 h equilibrium at 25°C, the excitation spectra of the  
31 solutions were registered in the range from 350 to 450 nm at a wavelength of 334 nm.  
32 The intensity ratio at 375 and 395 nm was plotted vs copolymer concentration, and the  
33 CMC value was derived from the cross-over point of the two regression lines.  
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## 44 2.5 Biocompatibility studies

### 45 2.5.1 Hemolysis test

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47 A micelle solution (10 mL) at 1.0 mg/mL was poured in a beaker which was  
48 previously silanized. The solution was heated at 37 °C for a period of 30 min. 0.2 mL  
49 rabbit blood diluted with acid-citrate-dextrose (ACD) at a dilution ratio of 9/1 was then  
50 added. The mixture was incubated at 37 °C for 30 min, and then centrifuged. The  
51 optical density (OD) of the supernatant was measured at 540 nm. Physiological saline  
52 was taken as negative control, and ultrapure water as positive control. The hemolysis  
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ratio (HR) was obtained using the following equation:

$$HR(\%) = \frac{OD_{test\ sample} - OD_{negative\ control}}{OD_{positive\ control} - OD_{negative\ control}} \times 100\% \quad (1)$$

### 2.5.2 Dynamic clotting time

The dynamic clotting time was determined according to the method previously reported in literature.<sup>28</sup> A micelle solution (25  $\mu$ L) in 0.025 M calcium chloride ( $\text{CaCl}_2$ ) was poured into a siliconized tube. After 5 min incubation at 37°C, ACD solution (200  $\mu$ L) was introduced into the tube and mixed. At preset time points up to 140 min, distilled water (20 mL) was gently poured into the tube to rinse the watch-glass. Subsequently, the absorbance of the rinsing liquid was measured at 490 nm wavelength. Similar analyses were made on siliconized glass as negative control and ordinary glass as positive control. The clotting time of micelle samples was obtained from the OD value vs time plots. Experiments were repeated three times in each case.

### 2.5.3 MTT assay

MTT assay was employed to examine the cytotoxicity of copolymers, using L929 fibroblast cell line. Various micelle solutions at a concentration from 0.05 to 1.0 mg/mL with 10% calf serum were exposed to L929 cells in 96-well plates. At preset time intervals (24, 48 and 72 h), MTT (20  $\mu$ L) at 5 mg/mL was added in the well, and incubated for 6 h. The medium was then taken out, followed by addition of 150  $\mu$ L dimethyl sulfoxide (DMSO). The plates were shaken during 10 min. And the OD value was measured at 570 nm in a microplate reader. A phenol solution (100  $\mu$ L) was taken as positive control, and a fresh medium (100  $\mu$ L) as negative control. The relative growth rate (RGR) of cells was obtained according to the following equation:

$$RGR(\%) = \frac{OD_{test\ sample}}{OD_{negative\ control}} \times 100\% \quad (2)$$

### 2.6 Drug release studies

Curcumin was loaded in the core of micelles using a two-step procedure. Drug free micelles were first prepared as described in Section 2.3. 1 mg curcumin was dissolved in 1 mL methanol, and the solution was then introduced into 10 mL micelle

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4 solution. Vigorous stirring was applied during 4 h to ensure efficient drug loading,  
5 followed by gentle stirring for 24 h to completely eliminate the solvent. The resulting  
6 curcumin-loaded micelle solution was centrifuged to remove unloaded drug, and  
7 filtered through 0.22  $\mu\text{m}$  filter.  
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12 Curcumin release from PEOz-PDLLA micelles was studied under in vitro  
13 conditions according to literature method.<sup>29,30</sup> A dialysis system was employed, which  
14 includes Float-A-Lyzer G2 dialysis devices (MWCO of 3500 Da), an oscillator and a  
15 thermostat. 1 mL curcumin loaded micellar solution was introduced in a dialysis device  
16 which was immersed in a centrifugation tube filled with 40 mL phosphate buffered  
17 saline (PBS). The tubes were fixed in an oscillator thermostated at 37°C. Drug release  
18 then proceeded under constant shaking. Sink conditions were ensured by regular  
19 renewal of the release medium. At each time point, a volume of 25  $\mu\text{L}$  was taken out  
20 and equal volume of PBS was added. The collected samples were lyophilized, and  
21 dissolved in ethanol. The UV absorbance was measured to determine the drug  
22 concentration based on a previously established calibration curve. The drug loading  
23 content (LC) and loading efficiency (LE) were calculated from the following equations:  
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$$35 \quad LC(\%) = \frac{\text{weight of loaded drug}}{\text{weight of drug loaded micelles}} \times 100\% \quad (3)$$

$$36 \quad LE(\%) = \frac{\text{weight of loaded drug}}{\text{theoretical drug loading}} \times 100\% \quad (4)$$

### 41 2.7 In vitro antitumor activity

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44 The anti-tumor activity of free curcumin and curcumin loaded micelles was  
45 determined by MTT assay using A549 lung cancer cell line. A549 cells were suspended  
46 in Dulbecco's Modified Eagle Medium (DMEM) which contains 10% fetal bovine  
47 serum (FBS), 100 U/mL penicillin and 100  $\mu\text{g}/\text{mL}$  streptomycin, and incubated at 37 °C  
48 during 24 h in a humidified environment with 5% carbon dioxide. MTT assay was then  
49 realized according to the experimental protocol described above.  
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## 59 3 Results

### 3.1 Characterization of PEOz-PDLLA copolymers

PEOz-PDLLA copolymers were prepared by ring-opening polymerization of DL-lactide using monohydroxy terminated PEOz-OH with  $M_n$  of 2800 as macro-initiator and  $\text{Sn}(\text{Oct})_2$  as catalyst. The chemical composition of PEOz-PDLLA copolymers was determined by  $^1\text{H}$  NMR, as shown in Fig. 1. Three main signals are detected for the PEOz block. Peak **a** at 1.12 ppm belongs to the methyl protons of ethyl side chain, peak **b** at 2.40 and 2.31 ppm to the methylene protons of ethyl side chain, and peak **c** at 3.46 ppm to the methylene protons of PEOz main chain, respectively. In the case of the PDLLA hydrophobic block, two signals are detected. Peak **d** at 5.17 ppm is attributed to the methine proton, and peak **e** at 1.59 ppm to the methyl protons of lactyl repeat units. The signals **d** and **a** are integrated using MestReNova software, and the integrations are used to calculate the LA/EOz molar ratio of PEOz-PDLLA copolymers. The degree of polymerization (DP) of both blocks and  $M_n$  of PEOz-PDLLA are then obtained according to the following equations :

$$\text{DP}_{\text{PEOz}} = M_{n\text{PEOz}}/99 \quad (5)$$

$$\text{DP}_{\text{PDLLA}} = \text{DP}_{\text{PEOz}} \times (\text{LA}/\text{EOz}) \quad (6)$$

$$M_n = M_{n\text{PEOz}} + \text{DP}_{\text{PDLLA}} \times 72 \quad (7)$$

where 99 designates the molar mass of 2-ethyl-2-oxazoline moieties, and 72 that of lactyl moieties.

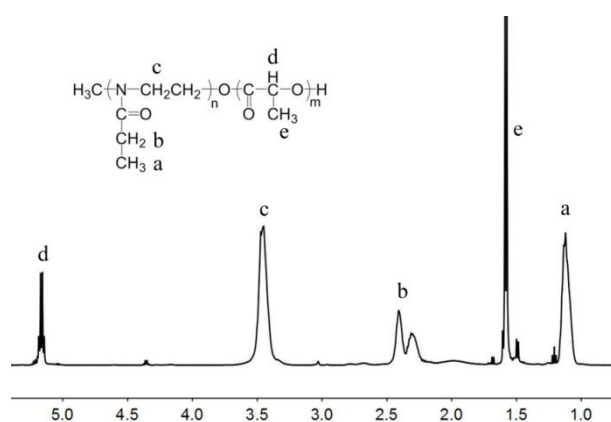


Figure 1.  $^1\text{H}$  NMR spectrum of PEOz-PDLLA copolymer in  $\text{CDCl}_3$

Table 1 presents the characterization results of the three copolymers. The copolymers are abbreviated as  $\text{EOZ}_x\text{DL}_y$  for the sake of simplicity, with the subscripts  $x$  and  $y$  representing the DP of PEOz and PDLLA blocks, respectively. The  $\text{DP}_{\text{PEOz}}$  is 28 for the three copolymers, and  $\text{DP}_{\text{PDLLA}}$  is 13, 29 and 38 for  $\text{EOZ}_{28}\text{DL}_{13}$ ,  $\text{EOZ}_{28}\text{DL}_{29}$  and  $\text{EOZ}_{28}\text{DL}_{38}$ , respectively. The  $M_{\text{nNMR}}$  derived from NMR spectra varies from 3700 to 5500 g/mol. GPC was also used to characterize the copolymers as it allows to determine not only the molar mass, but also the molar mass distribution. As shown in Fig. 2, the GPC chromatograms shift to shorter retention time with increasing PDLLA block length, in agreement with higher molar mass.  $M_{\text{nGPC}}$  values are slightly different from  $M_{\text{nNMR}}$  ones because the former are derived from the hydrodynamic volume of polymers in solution compared to standards (Table 1). The dispersity ( $D$ ) is in the range from 1.15 to 1.39, indicating that the copolymers have a narrow distribution of molar masses.

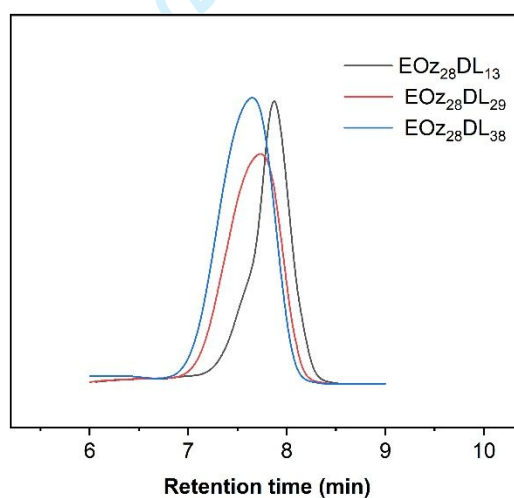


Figure 2. The curves of GPC of PEOz-PDLLA copolymers

The Griffin's method is widely used to determine the hydrophilic-lipophilic balance (HLB) of amphiphilic polymers. High HLB values imply strong hydrophilicity, and *vice versa*. In Table 1 are shown HLB values of the three copolymers which are in the range from 10.2 to 15.1. Copolymers with longer PDLLA block length have lower HLB, in agreement with lower hydrophilicity.

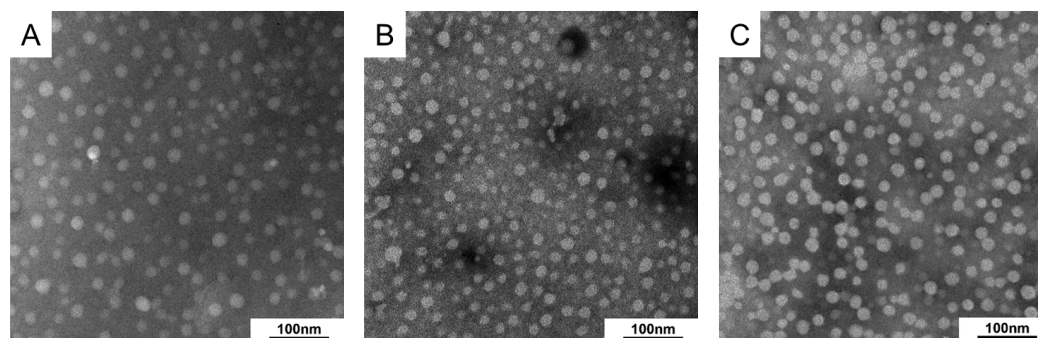
Table 1 Structural characterization of PEOz-PDLLA diblock copolymers

| Copolymer                          | $M_{nPEOz}$ | LA/EOz                   | $DP_{PEOz}$ | $DP_{PDLLA}$ | $M_{nNMR}$ | $M_{nGPC}$ | $\bar{D}$ | HLB  |
|------------------------------------|-------------|--------------------------|-------------|--------------|------------|------------|-----------|------|
| EOz <sub>28</sub> DL <sub>13</sub> | 2800        | 0.45 (0.6) <sup>a)</sup> | 28          | 13           | 3700       | 3500       | 1.15      | 15.1 |
| EOz <sub>28</sub> DL <sub>29</sub> | 2800        | 1.05 (1.2)               | 28          | 29           | 4900       | 5300       | 1.34      | 11.4 |
| EOz <sub>28</sub> DL <sub>38</sub> | 2800        | 1.35 (1.6)               | 28          | 38           | 5500       | 5800       | 1.39      | 10.2 |

<sup>a)</sup> Values in parentheses designate the LA/EOz molar ratios in the feed.

### 3.2 Self-assembly of PEOz-PDLLA diblock copolymers

Amphiphilic PEOz-PDLLA diblock copolymers can spontaneously self-assemble in aqueous medium, leading to formation of nano-sized micelles with a hydrophilic PEOz corona and a hydrophobic PDLLA core. TEM was used to observe the morphology of self-assembled aggregates as shown in Fig. 3A-C. All copolymer micelles exhibit a spherical shape and are uniformly distributed. The diameter of micelles is in the range from 20 to 30 nm as estimated from TEM images. No worm-like micelles were observed, in contrast to PEOz-PLLA copolymers.<sup>25</sup> In fact, polymer chain structure regularity strongly affects the self-assembled morphology of micelles. PDLLA blocks have a disordered chain structure with randomly distributed L- and D-lactidyl units, and PEOz-PDLLA copolymers seem to be more inclined to form spherical micelles as compared to PEOz-PLLA copolymers with rigid stereoregular PLLA blocks.



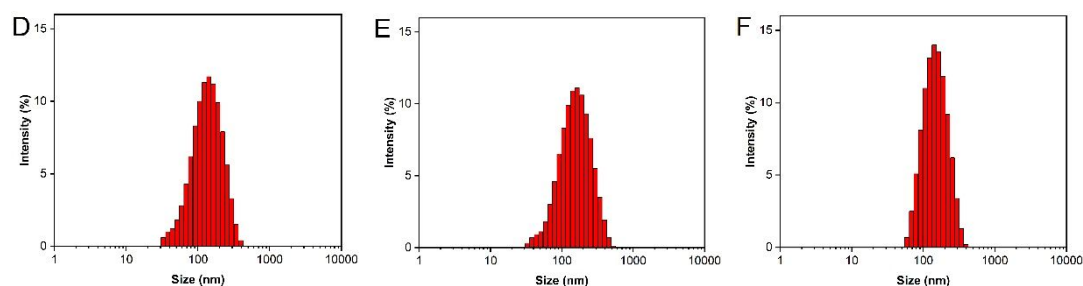


Figure 3. TEM images and DLS graphs of EOz<sub>28</sub>DL<sub>13</sub> (A, D), EOz<sub>28</sub>DL<sub>29</sub> (B, E), and EOz<sub>28</sub>DL<sub>38</sub> (C, F) copolymer micelles.

The average micelle diameter and polydispersity index (PDI) were determined DLS measurements (Fig. 3). A symmetric size distribution is detected for all copolymer micelles, as shown in Fig. 3D-F. Table 2 summarizes the micelle diameter and PDI data of the three copolymers. The diameter of micelles slightly increases with the increase of the PDLLA block length, from 104 nm for EOz<sub>28</sub>DL<sub>13</sub> to 131 and 136 nm for EOz<sub>28</sub>DL<sub>29</sub> and EOz<sub>28</sub>DL<sub>38</sub>, respectively. This finding could be attributed to the increasing hydrophobic block length which could attract more copolymer chains in micelles, as reported in literature.<sup>25</sup> A slight increase of micelle size is observed after encapsulation of curcumin. The micelle diameter slightly increases to 131, 135 and 145 nm for EOz<sub>28</sub>DL<sub>13</sub>, EOz<sub>28</sub>DL<sub>29</sub> and EOz<sub>28</sub>DL<sub>38</sub>, respectively. The PDI values of micelle sizes are below 0.30 in all cases, in agreement with narrow size distribution. It is noteworthy that the diameter values derived from DLS are much larger than those from TEM due to the dehydration or shrinkage of micelles during air drying before TEM observation. Zhao et al. obtained a smaller micelle diameter of 41-43 nm for PEOz-PDLLA copolymer with Mn of 6514 for PEOz and 8200 for PDLLA,<sup>24</sup> which could be assigned to the more compact micelle core due to longer PDLLA block length.

The CMC is one of the key parameters of micelles as it allows to assess their stability *in vivo* after dilution caused by intravenous injection. As shown in Table 2, the CMC value obtained from fluorescence spectroscopy is highly dependent on the PDLLA block length or the HLB of copolymers. The longer the PDLLA block, the lower the HLB, and the smaller the CMC value. This finding is attributed to the fact

that copolymers with longer hydrophobic segments self-assemble more easily in aqueous medium, leading to more stable micelle structures.

Table 2. Self-assembly and drug loading properties of PEOz-PDLLA micelles

| Sample                                  | Diameter<br>(nm) | PDI  | CMC (mg/mL) | LC (%) | LE (%) |
|---|------------------|------|-------------|--------|--------|
| EOz <sub>28</sub> DL <sub>13</sub>      | 104              | 0.29 | 0.0065      | -      | -      |
| EOz <sub>28</sub> DL <sub>29</sub>      | 131              | 0.26 | 0.0043      | -      | -      |
| EOz <sub>28</sub> DL <sub>38</sub>      | 136              | 0.22 | 0.0039      | -      | -      |
| EOz <sub>28</sub> DL <sub>13</sub> -Cur | 131              | 0.22 | -           | 7.0    | 75.0   |
| EOz <sub>28</sub> DL <sub>29</sub> -Cur | 135              | 0.18 | -           | 7.3    | 79.3   |
| EOz <sub>28</sub> DL <sub>38</sub> -Cur | 145              | 0.13 | -           | 7.6    | 82.5   |

### 3.4 Biocompatibility studies

#### 3.4.1 Hemolysis test

As potential drug carrier for intravenous application, hemolysis test is of major importance as it indicates the interaction of biomaterials and red blood cells (RBC). The hemolytic ratio (HR) reflects the dissociation level of hemoglobin after in vitro co-culture of materials with RBC. In Table 3 are summarized the OD and HR values of PEOz-PDLLA micelles, together with the OD values of both positive and negative controls. The HR value of EOz<sub>28</sub>DL<sub>13</sub>, EOz<sub>28</sub>DL<sub>29</sub> and EOz<sub>28</sub>DL<sub>38</sub> micelles is 3.4%, 3.8% and 3.5%, respectively. Therefore, the hemolytic properties of PEOz-PDLLA copolymers are acceptable for medical and pharmaceutical uses as the HR value is lower than 5%, the threshold defined by ISO 10993.

Table 3 Hemolytic properties of PEOz-PDLLA micelles



| Copolymer                                      | OD value    | Hemolytic ratio (%) |
|--|-------------|---------------------|
| Negative control                               | 0.028±0.006 | -                   |
| Positive control                               | 0.795±0.032 | -                   |
| EO <sub>Z</sub> <sub>28</sub> DL <sub>13</sub> | 0.054±0.006 | 3.4±0.8             |
| EO <sub>Z</sub> <sub>28</sub> DL <sub>29</sub> | 0.057±0.004 | 3.8±0.5             |
| EO <sub>Z</sub> <sub>28</sub> DL <sub>38</sub> | 0.055±0.006 | 3.5±0.8             |

### 3.4.2 Dynamic clotting time

Fresh blood in vitro will initiate endogenous coagulation due to foreign bodies activating endogenous coagulation factors. The dynamic coagulation behavior of PEO<sub>Z</sub>-PDLLA micelles and controls was evaluated from time dependent changes of absorbance, as shown in Fig. 4. 0.1 and 0.01 was defined as the initial clotting time (ICT) and the complete clotting time (CCT), respectively. The blood in contact with positive control was the first to undergo endogenous coagulation. The ICT and CCT of positive control were 23 min and 60 min, respectively. EO<sub>Z</sub><sub>28</sub>DL<sub>13</sub>, EO<sub>Z</sub><sub>28</sub>DL<sub>29</sub> and EO<sub>Z</sub><sub>28</sub>DL<sub>38</sub> micelles showed the same trend as the negative control. The OD values gradually decreased over time. The ICT of all PEO<sub>Z</sub>-PDLLA micelles was about 80 min and that of the negative control was 90 min. Furthermore, the CCT of PEO<sub>Z</sub>-PDLLA micelles and the negative control were almost the same (above 140 min), thus suggesting that PEO<sub>Z</sub>-PDLLA micelles present outstanding anticoagulant properties.



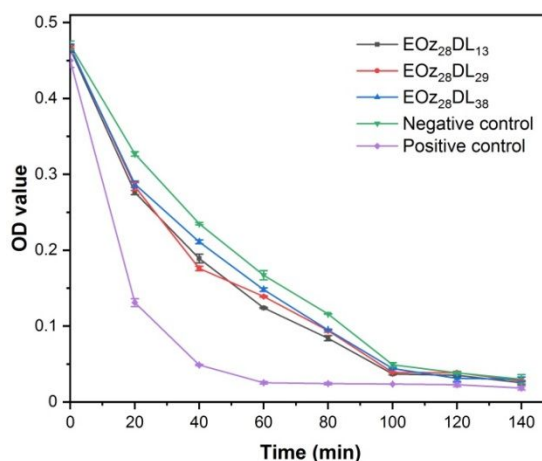
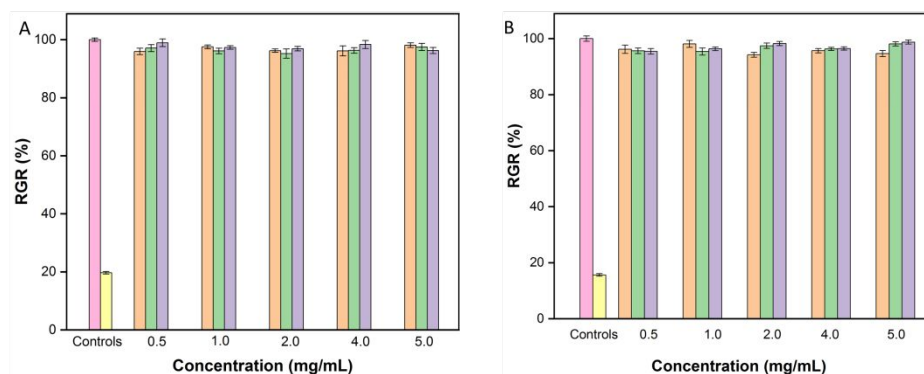


Figure 4. OD value changes of PEOz-PDLLA micelles as a function of time in comparison with the positive and negative controls.

### 3.4.3 MTT assay

Within a certain cell concentration range, the amount of formazan crystals formed by MTT is directly proportional to cell viability. Fig. 5 presents L929 cell viability data after 24, 48 and 72 h co-culture with PEOz-PDLLA micelles at various concentrations from 0.5 to 5.0 mg/mL. The positive control showed very low RGR values which decreased from 24% at 24 h to 12.4% at 72 h. However, the RGR values are over 95% for all micelles at different concentrations throughout the 72 h period, thus showing a cytocompatibility very close to the negative control (100%). Therefore, both hemocompatibility and cytocompatibility data showed that PEOz-PDLLA micelles are biocompatible for potential applications in drug delivery according to the ISO 10993.



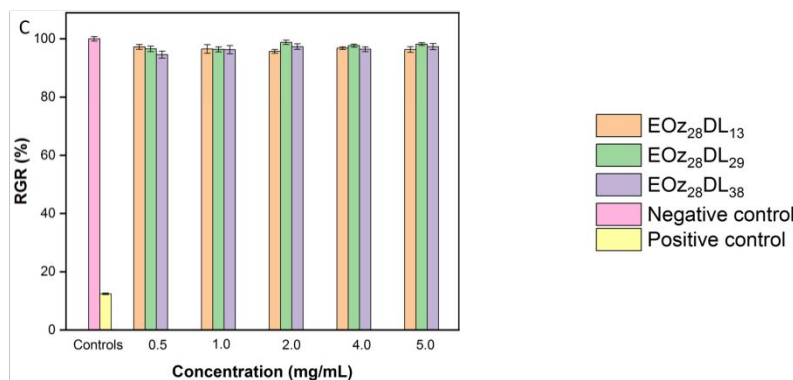


Figure 5. RGR of L929 cells after 24 (A), 48 (B) and 72 h (C) co-culture with PEOz-PDLLA micelles.

### 3.3 In vitro drug release

Due to its excellent anti-tumor activity and unique physico-chemical properties, curcumin was selected as a model of hydrophobic anti-tumor drugs. The nano-drug delivery system containing curcumin was prepared in two steps.<sup>31</sup> Blank micelles were first obtained by using co-solvent evaporation method, and a curcumin solution in methanol was then added into the micelle solution. Drug loaded nano-micelles were obtained under vigorous stirring to remove the solvent.

The LC and LE data of curcumin loaded micelles are summarized in Table 2. EO<sub>z</sub>28DL<sub>13</sub> presents an LE of 75.0% and an LC of 7.0%. Higher LE and LC values are obtained for EO<sub>z</sub>28DL<sub>29</sub> and EO<sub>z</sub>28DL<sub>38</sub>. EO<sub>z</sub>28DL<sub>38</sub> presents the highest LE and LC values of 82.5% and 7.6%, respectively. The increase of LE and LC with increasing PDLLA block length could be attributed to the larger core volume of micelles with longer PDLLA blocks.

Dialysis method was used to determine the drug release behavior of curcumin-loaded micelles at different pH values. Three representative pH values were selected to simulate the environments of micelles entering the body: pH 7.4 when the micelles were intravenously injected into the blood environment, pH 6.5 when the micelles were endocytosed by tumor cells, and pH 5.0 when the micelles were transferred into the

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4 endo/lysosome mimetic environment. As shown in Fig. 6, two distinct release phases  
5 were observed in the release curves of drug loaded PEOz-PDLLA micelles. An initial  
6 fast release of curcumin was detected in the first 12 h, which could be attributed to the  
7 fact that drug molecules are partly located at the interface of the core-corona structure.<sup>32</sup>  
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9 Subsequently, a stable and sustained release behavior was detected up to 144 h. At pH  
10 7.4, the drug release in first 12 h was about 36%, 31%, and 31% for EOZ<sub>28</sub>DL<sub>13</sub>,  
11 EOZ<sub>28</sub>DL<sub>29</sub> and EOZ<sub>28</sub>DL<sub>38</sub>, respectively. At pH 5.0, the initial drug release at 12 h  
12 increased to 56%, 53% and 45% for EOZ<sub>28</sub>DL<sub>13</sub>, EOZ<sub>28</sub>DL<sub>29</sub> and EOZ<sub>28</sub>DL<sub>38</sub>,  
13 respectively. Intermediate rates were obtained at pH 6.5. Beyond 12 h, the three drug  
14 loaded micelle systems showed slower release rates. At 144 h, the final cumulative  
15 release of curcumin at pH 7.4, 6.5 and 5.0 was 59%, 81% and 93% for EOZ<sub>28</sub>DL<sub>13</sub>,  
16 52%, 72% and 85% for EOZ<sub>28</sub>DL<sub>29</sub>, and 48%, 61% and 73% for EOZ<sub>28</sub>DL<sub>38</sub>,  
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29 The drug release behaviors of three micelle systems at the same pH (pH 5) are  
30 comparatively shown in Fig. 6D. In the first 12 h, the cumulative release curves of  
31 EOZ<sub>28</sub>DL<sub>13</sub> and EOZ<sub>28</sub>DL<sub>29</sub> almost overlap, while EOZ<sub>28</sub>DL<sub>38</sub> shows lower release rate.  
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33 In the later stage, drug release continues to increase in all three systems, but at a slower  
34 rate. At pH 5.0, the cumulative drug release was 93%, 85% and 73% for EOZ<sub>28</sub>DL<sub>13</sub>,  
35 EOZ<sub>28</sub>DL<sub>29</sub> and EOZ<sub>28</sub>DL<sub>38</sub>, respectively. Therefore, curcumin release from PEOz-  
36 PDLLA micelles is strongly affected by the environmental conditions and the  
37 composition or PDLLA block length of copolymers.  
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45 The release data of curcumin from PEOz-PDLLA micelles clearly illustrate the  
46 responsiveness of micelles to pH stimulation. Drug diffusion and carrier degradation  
47 are the two main mechanisms that affect drug release behaviors. Jelonek et al. studied  
48 paclitaxel release from PLLA-PEG and PDLLA-PEG micelles at different pH values,  
49 and found that the degradation of micelles strongly affects drug release rate.<sup>33</sup> In our  
50 previous study on PLGA-PEG copolymer micelles, faster degradation was observed for  
51 micelles with shorter PLGA blocks as compared to micelles with longer PLGA  
52 blocks.<sup>34</sup> Thus, the various drug release rates obtained for EOZ<sub>28</sub>DL<sub>13</sub>, EOZ<sub>28</sub>DL<sub>29</sub> and  
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EOZ<sub>28</sub>DL<sub>38</sub> micelles can be directly related to the degradation rate of copolymers. The lower the pH, the shorter the PDLLA block length, the faster the degradation of micelles and the faster the release of curcumin.

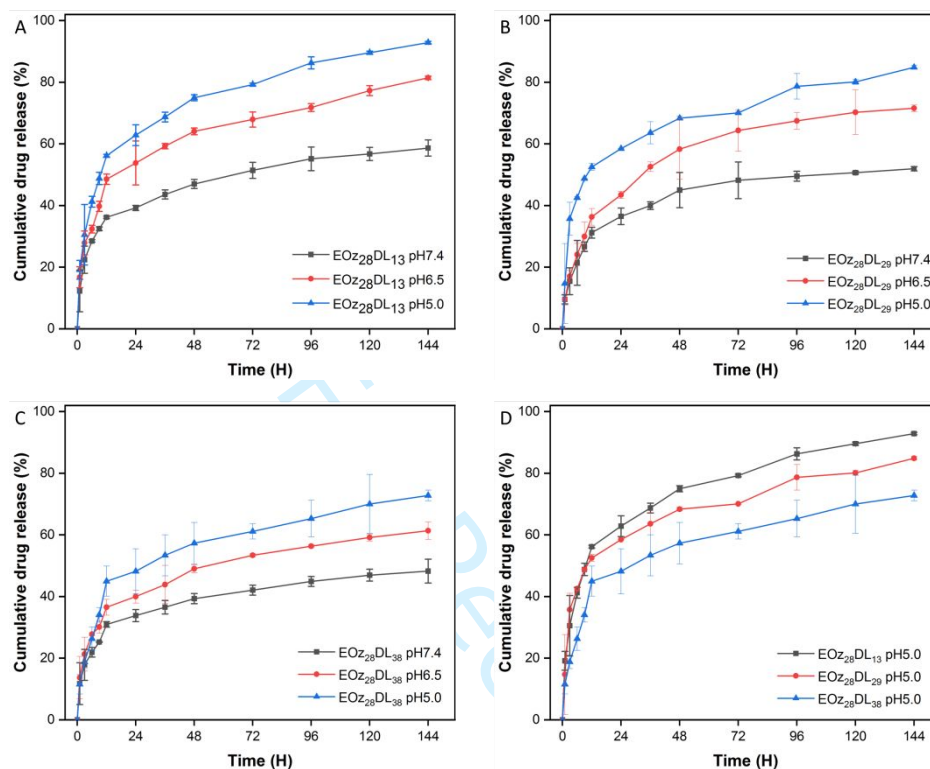


Figure 6. *In vitro* release profiles of curcumin from EOZ<sub>28</sub>DL<sub>13</sub> (A), EOZ<sub>28</sub>DL<sub>29</sub> (B), EOZ<sub>28</sub>DL<sub>38</sub> (C) copolymer micelles in PBS at pH = 7.4, 6.5 and 5.0 at 37 °C; and comparison of the release curves of the three copolymers at pH 5.0 (D). All data are represented as the mean  $\pm$  SD (n = 3).

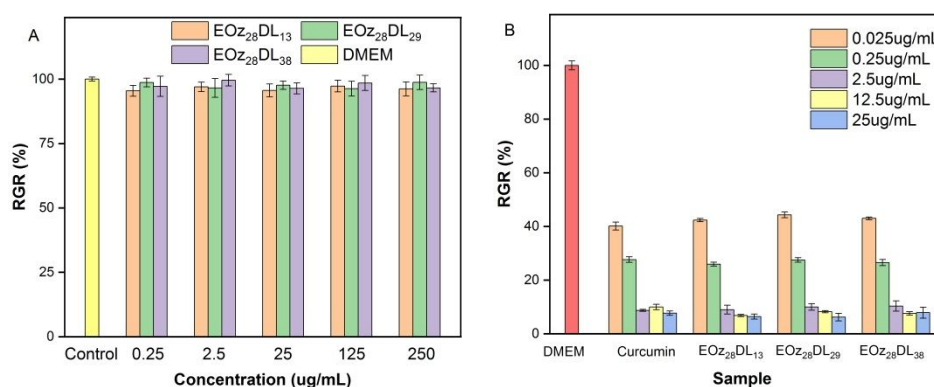
Zhao et al. studied the release of doxorubicin from PEOz-PDLLA micelles at different pH values.<sup>24</sup> The acidic environment promoted the release of doxorubicin because of higher water-solubility of doxorubicin and accelerated degradation of the hydrophobic PDLLA block. Almost total drug release was achieved within 24 h. In our previous work, a series of PEOz-PLLA micelles were evaluated for controlled release of paclitaxel which is one of the most widely studied and most efficace anti-tumor drugs. Within a 30-day period, copolymers with shorter PLLA blocks exhibit faster drug release than those with longer PLLA blocks. Faster drug release is also obtained at

acidic pH than at pH 7.4. Nevertheless, the highest release ratio was only 37.6% which was obtained for EOZ<sub>18</sub>L<sub>5</sub> micelles after 30 days release in PBS at pH 5.0. Therefore, PEOZ-PDLLA micelles present much faster drug release than PEOZ-PLLA ones, which is consistent with the faster degradation rate of PDLLA compared to PLLA.<sup>25</sup>

### 3.5 In vitro antitumor studies

Lung cancer is one of the most deadly malignant tumors for both women and men, making up nearly 25% of all cancer deaths. The inhibitory effect of curcumin loaded micelles on the proliferation of A549 lung cancer cells is studied to evaluate their potential for lung tumor treatment. As shown in Fig. 7A, no cytotoxicity was detected for PEOZ-PDLLA micelles (0.25-250  $\mu$ g) after 72 h co-culture with A549 cells. The relative proliferation rate of A549 cells in micelle solutions was similar to that in fresh medium. This result is consistent with the good cytocompatibility of PEOZ-PDLLA micelles described above.

Fig. 7B presents the inhibitory effect of free curcumin and curcumin loaded micelles on the proliferation of A549 cells. The concentration of free drug is the same as that in the micellar system, taking into account the drug loading content of micelles. For low concentrations of curcumin from 0.025 to 2.5  $\mu$ g/mL, cell proliferation was significantly inhibited and showed a dose-dependent decrease with increasing concentration. In contrast, little RGR changes were detected in the drug concentration range of 2.5-25  $\mu$ g/mL. In the whole drug concentration range, the anti-tumor effect of curcumin loaded micelles was comparable to that of free curcumin.



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4 Figure 7. RGR data of A549 cells after 72 h culture with blank micelles (A), free  
5 curcumin and curcumin loaded micelles (B) in comparison with the control (DMEM).  
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## 10 Conclusion

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13 Amphilic PEOz-PDLLA diblock copolymers with different hydrophobic block  
14 lengths were prepared by ring opening polymerization of DL-lactide in the presence of  
15 hydroxyl terminated PEOz-OH, and characterized by using NMR and GPC analyses.  
16 PEOz-PDLLA copolymers are susceptible to self-assemble in water, yielding  
17 uniformly distributed spherical micelles. The size of micelles is well below 200 nm,  
18 which should allow applications as drug carrier by intravenous injection. The increase  
19 in the hydrophobic block length leads to a decrease in the CMC of copolymers, but to  
20 a increases of the LE and LC of micelles. Curcumin loaded PEOz-PDLLA micelles  
21 exhibit biphasic drug release profile characterized by an initial burst and subsequent  
22 slower release. The release behavior is greatly affected by pH of the medium and the  
23 PDLLA block length. Lower pH or shorter block length leads to faster drug release.  
24 Both blood and cell compatibility experiments demonstrated the excellent  
25 biocompatibility of PEOz-PDLLA block copolymers. Finally the inhibitory effect of  
26 curcumin loaded micelles on the proliferation of A549 cells was evidenced, thus  
27 showing that PEOz-PDLLA copolymer micelles could be a promising nano-carrier of  
28 lipophilic antitumor drugs.  
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