Supporting Information for

Rapidly *in Situ* Forming Polyphosphoester-based Hydrogels for Injectable Drug Delivery Carriers

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Experimental

Materials

2-Chloro-2-oxo-1, 3, 2-dioxaphospholane (COP) was synthesized by a method described previously and distilled under reduced pressure before use.[S1] 2-Hydroxyethyl methacrylate (HEMA, TCI) and ethanol (Sinopharm Chemical Reagent Co., China) were purified by the reported methods.[S2] Briefly, HEMA was firstly dissolved in deionized water and extracted with *n*-heptane to remove ethylene glycol dimethacrylate. Subsequently, sodium chloride was added into the water phase to salt out HEMA, which was then dried by anhydrous magnesium sulfate and distilled under reduced pressure. Tetrahydrofuran (THF) was initially dried over potassium hydroxide for at least two days and then refluxed through sodium wire with benzophenone as indicator until the color turned to purple. 2-(Dimethylamino)ethyl methacrylate (DMAEMA, Wuxi Xinyu Chemical Reagent, China) was dried over CaH_2 and distilled under reduced pressure before use. Methylene chloride (CH_2Cl_2) was purchased from Sinopharm Chemical Reagent Co., and used after distillation. *N, N′*-Dicyclohexyl-carbodiimide (DCC) and 4-dimethylamino-pyridine (DMAP) from Shanghai Medpep Co., ammonium persulfate (APS) (Sinopharm Chemical Reagent Co.), and doxorubicin hydrochloride (DOX, 99%, Beijing ZhongShuo Pharmaceutical Technology Development Co.) were used as received. Stannous 2-ethyl hexanoate [$Sn(Oct)_2$] was obtained from Sigma-Aldrich and used after purification by vacuum distillation. L929 fibroblasts was obtained from American Type Culture Collection (ATCC) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenylte-trazolium bromide (MTT) was purchased from Sigma-Aldrich. Other solvents and chemicals were used as received.

Synthesis of 2-Ethoxy-2-oxo-1, 3, 2-dioxaphospholane (EOP)

2-Ethoxy-2-oxo-1, 3, 2-dioxaphospholane (EOP) was synthesized by esterification of 2-chloro-2-oxo-1, 3, 2-dioxaphospholane with ethanol by following previous literature procedures (Scheme S1).[S3] In brief, ethanol (10.1 g, 0.217 mol) and TEA

(20.1 g, 0.198 mol) were dissolved in 100 mL of dry THF in a 250 mL fresh dried and argon-purged three-necked flask equipped with a dropping funnel and a magnetic bar. After cooling at -20 °C for 20 min, 2-chloro-2-oxo-1, 3, 2-dioxaphospholane (COP, 28.2 g, 0.198 mol) in 30 mL of dry THF was added dropwise to the stirred solution over a period of 30 min, and the mixture was maintained at -20 °C overnight. The precipitates were filtered off and the filtrate was concentrated under reduced pressure. The residue was distilled under vacuum to give 24.2 g of EOP as colorless liquid (yield 80%, b.p. 118~120 ºC/5 mmHg). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.36 ppm (t, 3H, -O-CH_2-C***H***$_3$), δ 4.2 ppm (m, 2H, -O-C***H***$_2$-CH_3), δ 4.4 ppm (m, 4H, -O-C***H***$_2$-C***H***$_2$-O-); ^{13}C NMR (400 MHz, $CDCl_3$): δ 15.8 ppm (-O-CH_2-***C***H_3), δ 64.7 ppm (-O-***C***H_2-CH_3), δ 65.8 ppm (-O-***C***H_2-***C***H_2-O-); ^{31}P NMR (400 MHz, $CDCl_3$): δ 18.0 ppm (Fig. S1).

Synthesis of *α*-Methacryloyloxyethyl *ω*-Acryloyl Poly(ethyl ethylene phosphate) (HEMA-PEOP-Ac) Macrocrosslinker

The macrocrosslinker was synthesized using the same procedure as we recently reported,[S4] and the synthesis route is shown in Scheme S2.

Hydrogel Preparation

HEMA-PEOP-Ac, 2-(dimethylamino)ethyl methacrylate (DMAEMA), and ammonium persulfate (APS) with a particular weight ratio were dissolved in deionized water (pH ~6.5) in a small vial, which was immersed in water bath at 25 °C, resulting in the hydrogels within several minutes depending on the weight ratios of components. For some characterizations, the dry hydrogels were obtained by the following procedures. The rapidly formed hydrogel was transferred into a 100 mL of beaker filled with deionized water, and the water was refreshed four times in the first day and then three times one day for the following three days to leach out the possible impurities. The dry hydrogels were then obtained by drying the swelling hydrogels at 50 °C and stored in sealed vials. The sample numbers of the hydrogels corresponding to the feed compositions of different components are listed in Table S1.

Characterizations

Fourier transform infrared spectroscopy (FT-IR) spectra were recorded on a Nicolet Avator 6700 Fourier transform infrared spectrometer using the KBr disk method. ^{1}H NMR and ^{31}P NMR spectra were recorded on a 400 MHz NMR spectrometer (INVOA-400) using $CDCl_3$ as solvent and tetramethylsilane (TMS) as the internal standard. Phosphoric acid (85%) was used as the external standard for ^{31}P NMR analysis. The molecular weights and polydispersity indexes (PDIs) of the polymers were determined with a Waters 1515 gel permeation chromatographer (GPC) instrument equipped using 500 Å, 10^3 Å and 10^4 Å MZ-Gel SD plus columns and a differential refractive index detector (RI 2414). DMF with 0.05 mol L^{-1} of LiBr was used as the eluent with a flow rate of 0.8 mL min^{-1} operated at 40 °C. The calibration was carried out with polystyrene standards. Thermogravimetric analysis (TGA) was examined on a Pyris 1 TGA (Perkin Elmer, USA) with a heating rate of 20 °C/min from 80 to 600 °C under a nitrogen atmosphere. All the samples were dried under vacuum at 35 °C for 24 h before measurements.

Rheological Analysis

Rheological analysis was carried out with RS6000 rheometer (Thermo Haake) with parallel plate geometry (PP20H, 20 mm diameter) at 25 °C in the oscillatory mode. HEMA-PEOP-Ac, DMAEMA and APS aqueous solutions in deionized water (pH ~6.5) were quickly mixed and applied to the test platform. The evolution of storage modulus (G') and loss modulus (G'') was recorded as a function of time. The dynamic strain sweeps was first carried out at a constant frequency of 1 Hz in order to determine a suitable strain value, under which the measurements were ensured within the linear viscoelastic range. A gap of 0.5 mm, a frequency of 1 Hz and a strain of 1% were applied to maintain the linear viscoelastic regime. A solvent trap was used to avoid water evaporation. For the study of effects of different components on the gelation, the time to form a gel was denoted as gelation time and determined using the vial tilting method.[S5,S6] Absence of flow within 20 s after inverting the vial indicated the formation of the gel state.

Swelling Kinetics

Swelling kinetics of the gels were analyzed by measuring swelling ratio of the gels. Before tests, the dry hydrogel samples were further dried in a vacuum oven at 50 °C for 24 h till the sample reached constant weight and weighed as W_d. The hydrogels were then immersed in PBS buffer solutions with predetermined pH values (2.2, 7.4, and 12) and the same ionic strength (0.2 M) at 25 °C. The swollen hydrogels were taken out from the PBS buffer solution at regular time intervals. After wiping off the water on the surfaces of the hydrogel samples with moist filter papers, the hydrogels were weighed and recorded as W_t. The swelling ratio was then defined as: swelling ratio = $(W_t-W_d)/W_d$. All the experiments were carried out in triplicate, and the average values were reported. Here, in order to equilibrate the influence of ionic concentration on swelling of hydrogels, the sodium chloride was used to adjust the ionic strength (I) of all the buffer solutions to 0.2 M. The ionic strength and the needed amount of sodium chloride (NaCl) was calculated by the corresponding equation $I = (\Sigma C_i Z_i^2)/2$, where C_i is the ionic concentration (mol L^{-1}) and Z_i is the amount of electric charge.

Morphology Observation by Scanning Electron Microscopy (SEM)

To visually examine the interior structure of the hydrogels in swollen state, the swollen hydrogel samples equilibrated in deionized water (pH ~6.5) for 24 h were quickly frozen in liquid nitrogen and further freeze-dried in a freeze drier at -40 °C for 3 days until all the solvent was sublimed. The freeze-dried hydrogels were then fractured carefully and then loaded on the surface of an aluminum SEM specimen holder and sputter coated with gold before observation, and then the interior morphologies of the hydrogels were visualized by using a scanning electron microscope (Hitachi S-4700).

Cytotoxicity Tests

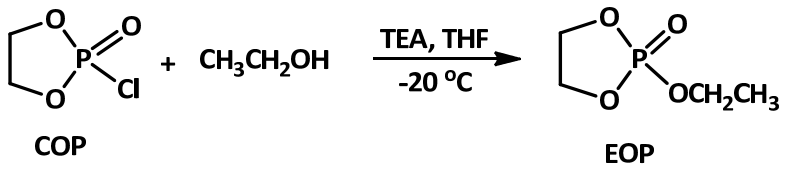
The relative cytotoxicity of the hydrogels was assessed with a methyl tetrazolium (MTT) assay against L929 fibroblasts. The weighed dry hydrogels were sterilized by

Gamma radiation and then immersed in PBS solution (pH 7.4, I = 0.2 M) until an equilibrium state. On the basis of our experimental measurements, after the treatment of radiation sterilization, the equilibrium swelling ratio of the hydrogel was somewhat decreased compared with that before sterilization. Subsequently, 100 μL of L929 fibroblasts in RPMI 1640 culture medium at a density of 5000 cells per well was added to each well in a 96-well plate. Cells were incubated for 24 h in an incubator (37 °C, 5% CO_2) followed by replacing the culture medium with 100 μL of RPMI 1640 containing the hydrogels with dry hydrogel concentrations ranging from 0.5 to 1.5 mg mL^{-1}. Following another 48 h of incubation, 25 μL of MTT stock solution (5 mg mL^{-1} in PBS) was added to each well of the plate. After incubation for an additional 4 h, the purple formazan produced by active mitochondria was solubilized using 100 μL of DMSO. The optical density (OD) at 570 nm in each well was measured on a microplate reader (PowerWave XS, Bio-Tek, USA). The absorbance values were normalized to wells in which cells were not treated with hydrogels. Therefore, the cell viable rate was calculated by the following formula: viable rate (%) = $(OD_{hydrogel}/OD_{blank}) \times 100$, where $OD_{hydrogel}$ is the value obtained in the presence of hydrogel, and OD_{blank} is the optical density that acquired in the absence of hydrogel. In order to visually observe the cell morphologies, the cells were quickly observed by an inverted microscope (Axiovert 40 CFL) before MTT analysis.

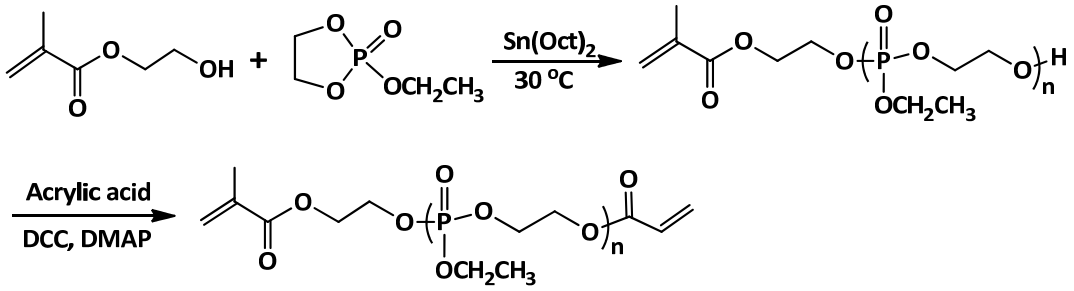
In Vitro Drug Loading and Release

To evaluate the drug loading capacity and release property, doxorubicin hydrochloride (DOX), a potent anti-cancer drug, was used as a model anticancer agent and encapsulated into hydrogels by the following steps. DOX was first dissolved in a preweighed amount of deionized water (pH ~6.5) in a small vial, and then HEMA-PEOP-Ac, DMAEMA and APS were added in the solution. The mixture was fully mixed by a vortex mixer and put in a water bath at 25 °C resulted in drug-loaded hydrogels. The loading concentration of DOX was fixed with 2 mg mL^{-1} and it was found that the addition of drug has little effect on the gelation time. The *in vitro* drug release was performed in a shaking water bath at 37 °C. About 0.45 mL of gels were

suspended in 20 mL of PBS (pH 7.4, I = 0.2 M), provided a reservoir into which drug could be released from the gel complex and subsequently measured. At a predetermined time, 5 mL of the buffer solution was removed from the vials and replenished with 5 mL of fresh buffer solution to maintain a constant volume. The concentration of DOX was determined by using a UV-vis spectrophotometer (Shimadzu 3150) measured at 490 nm. A standard plot was plotted by measuring the absorbance at 490 nm of a series of DOX solutions with known concentrations prepared under identical conditions, which was used to calculate the amount of released drug. The results were presented in terms of cumulative release as a function of time, and the cumulative DOX release (%) was calculated as following formula: cumulative release (%) = $(W_t/W_0) \times 100$, where W_t is the amount of DOX released from the hydrogel at time t, and W_0 is the initial amount of DOX loaded in the hydrogel as described above. All the loading and release experiments were carried out in the darkness, and the data from duplicate experiments were reported.



Scheme S1. Synthesis of 2-ethoxy-2-oxo-1, 3, 2-dioxaphospholane (EOP).



Scheme S2. Synthesis of *α*-methacryloyloxyethyl *ω*-acryloyl poly(ethyl ethylene phosphate) (HEMA-PEOP-Ac) macrocrosslinker.

Table S1. Feed composition and sample numbers of the hydrogels.

Components		**Sample No.**			
		gel-1	**gel-2**	**gel-3**	**gel-4**
HEMA-PEOP-Ac	mg	125	150	175	200
	mmol	5.36×10^{-2}	6.44×10^{-2}	7.51×10^{-2}	8.58×10^{-2}
DMAEMA	mg	325	300	275	250
	mmol	2.06	1.91	1.75	1.59
APS/mg		22.5	22.5	22.5	22.5
H_2O/mL		3.0	3.0	3.0	3.0

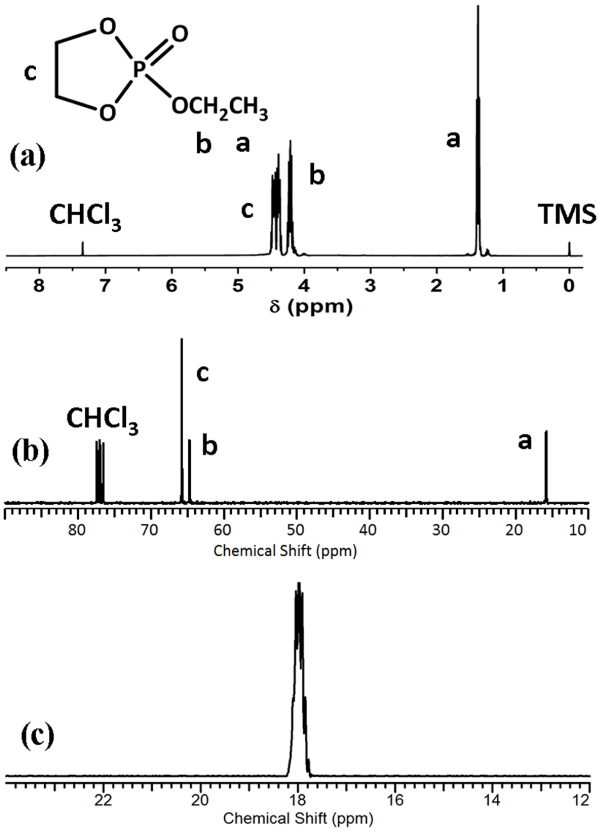


Fig. S1. ^{1}H NMR (a), ^{13}C NMR (b) and ^{31}P NMR (c) of 2-ethoxy-2-oxo-1, 3, 2-dioxaphospholane (EOP) monomer.

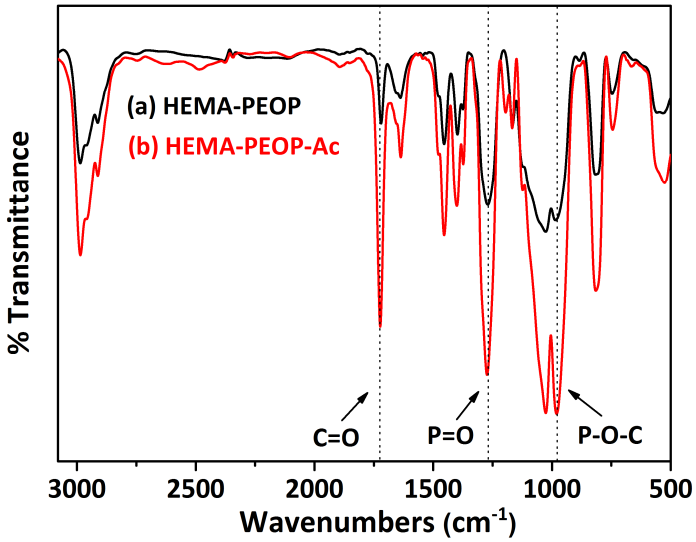


Fig. S2. FT-IR spectra of the polyphosphoesters for (a) HEMA-PEOP and (b) HEMA-PEOP-Ac.

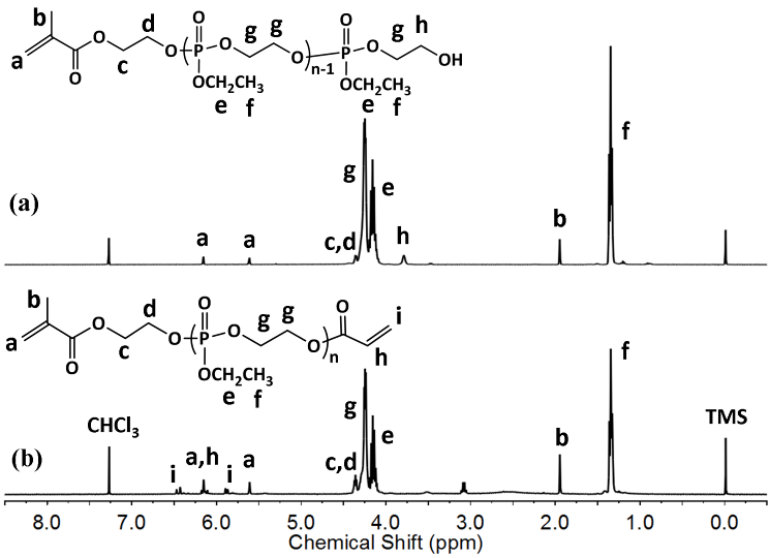


Fig. S3. ^{1}H NMR spectra of polyphosphoesters for (a) HEMA-PEOP and (b) HEMA-PEOP-Ac in $CDCl_3$.

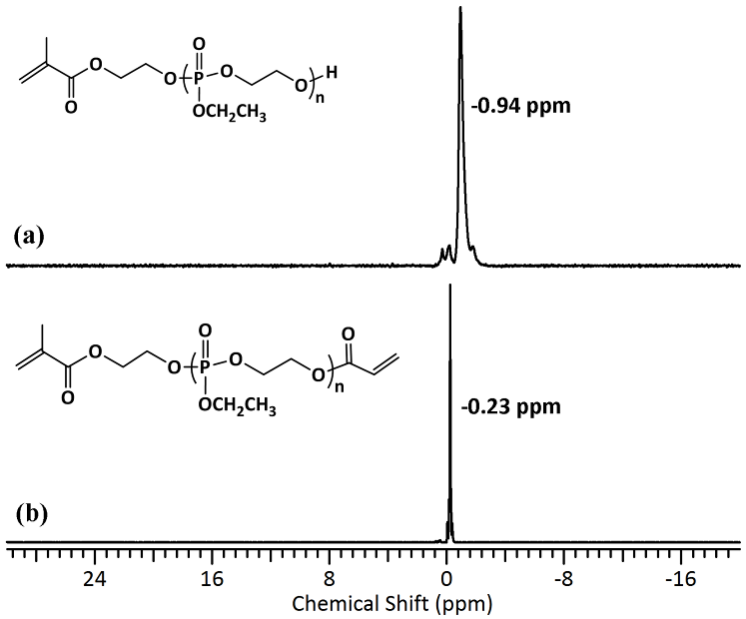


Fig. S4. ^{31}P NMR spectra of polyphosphoesters for (a) HEMA-PEOP and (b) HEMA-PEOP-Ac in $CDCl_3$. Phosphoric acid (85%) was used as the external reference.

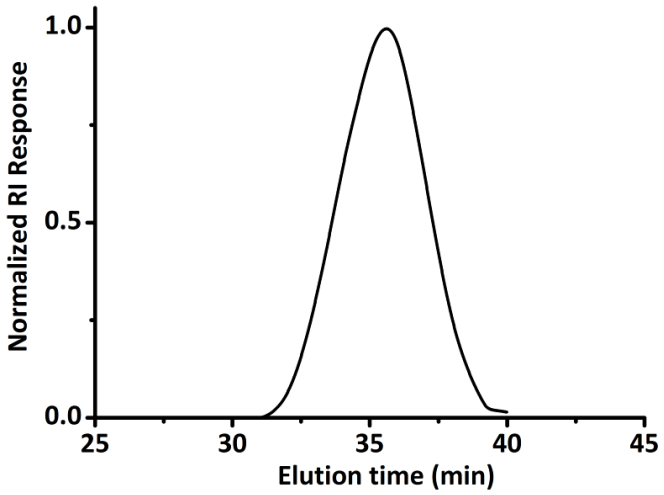


Fig. S5. GPC profile of the HEMA-PEOP-Ac macrocrosslinker, $\bar{M}_n$ = 6520 g mol^{-1}, PDI = 1.12.

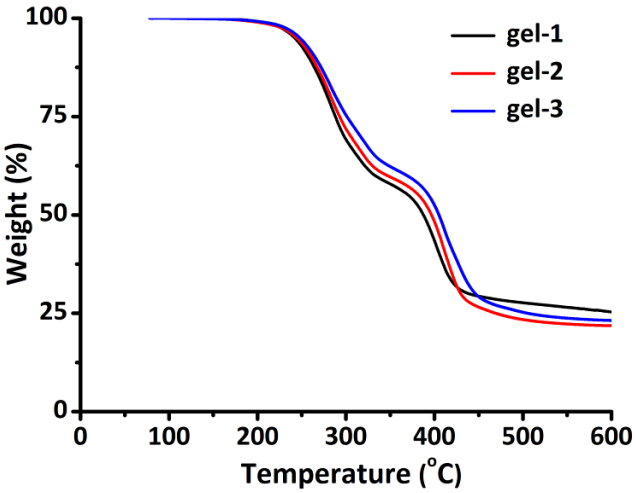


Fig. S6. TGA curves of the dried hydrogels for gel-1, gel-2, and gel-3.

References

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