Enzymatically-Triggered, Isothermally Responsive Polymers: Re-programming poly(oligoethylene glycols) to respond to Phosphatase

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Abstract

Polymers which can respond to externally applied stimuli have found much application in the biomedical field due to their (reversible) coil-globule transitions. Polymers displaying a lower critical solution temperature are the most commonly used, but for blood-borne (i.e. soluble) biomedical applications the application of heat is not always possible, nor practical. Here we report the design and synthesis of poly(oligoethylene glycol methacrylate)-based polymers whose cloud points are easily varied by alkaline phosphatase-mediated dephosphorylation. By fine-tuning the density of phosphate groups on the backbone, it was possible to induce an isothermal transition: A change in solubility triggered by removal of a small number of phosphate esters from the side chains activating the LCST-type response. As there was no temperature change involved, this serves as a model of a cell-instructed polymer response. Finally, it was found that both polymers were non cytotoxic against MCF-7 cells (at 1 $mg.mL^{-1}$), which confirms promise for biomedical applications.

Introduction

Responsive polymers provide a valuable tool in drug delivery with common stimuli including pH, light, and electric fields.[1-3] Thermoresponsive polymers are by far the most common with those displaying a lower critical solution temperature (LCST), such as pNIPAM (poly(*N*-isopropyl acrylamide)) being widely studied, along with poly(oligoethylene glycol methacrylates),[4, 5] poly(dimethylamino ethyl methacrylate)[6, 7] or poly(vinyl alcohol) copolymers.[8] However, for them to be applied biomedically a temperature response is not always desirable or even possible, meaning the incorporation of alternative responsive components is essential.[9] Enzymes are a particularly attractive stimulus where specific responses are desired given their highly specialised nature and unique localisation to certain tissues or even cells. For example, azoreductase is produced by the microbial flora present in the colon;[10] pepsin is present at high levels in the stomach[11] and a variety of digestive enzymes exist throughout the gastrointestinal tract.[12] They can also provide valuable biomarkers with a range of stress and disease states characterised by imbalances in enzyme expression and activity.[13] For instance, hepsin is a protease overexpressed in the early stages of prostate cancer,[14] cathepsins are released at inflammatory sites[15] and widely used as a release mechanism in polymer-drug conjugates and matrix metalloproteinases have been linked to vascular disease and tumour growth.[16, 17] The use of enzymes to trigger a specific response and hence manipulate the structures and pharmacokinetics of polymer-based materials has received increasing attention.[18-20] Rao and Khan prepared polymeric micelles containing an azoreductase-susceptible azobenzene linkage at the copolymer junction of an amphiphilic diblock copolymer. Treatment of the aqueous-assembled micelle with this enzyme, in the presence of the coenzyme NADPH, cleaved the junction and disrupted the micellar assembly.[21] A similar approach has been employed by Harnoy *et al.* who used penicillin G amidase to disrupt phenyl acetamide-containing micelles.[22] Thayumanavan and

co-workers have prepared dendrimer-based amphiphilic nanocontainers containing ester units which are cleaved to release encapsulated guests in the presence of porcine liver esterase.[23] Fuchs *et al.* have prepared nanoparticles from polystyrene-peptide-polystyrene triblock copolymers with the peptide susceptible to different enzymes depending on its sequence: Trypsin or hepsin could be used depending on whether a Gly-Phe-Phe or Arg-Gln-Leu-Arg-Val-Val-Gly-Gly sequence was present respectively.[24] Enzymes have also been shown to promote material assembly. For example, Hu and Messersmith have used transglutaminase as a gelation agent in the assembly of hydrogels[25] whilst peroxidases[26] and tyrosinases[27] have also been used.

A particularly interesting class of enzymes are the phosphatases which catalyse the hydrolysis of phosphate monoesters.[28] Within this family, alkaline phosphatase (ALP) is found in the liver and bone, as well as in the intestinal lining, placenta and the kidney, playing an important role in mineralisation and osteogenesis.[29, 30] Elevated levels of the enzyme can therefore be implicated in Paget's bone disease,[31] whilst depleted levels have been linked with Wilson's disease[32] and in a range of cancer types.[33] Xu and co-workers used ALP to trigger hydrogelation by neutralising an ionic group on an amino acid derivative, rendering it a small-molecular hydrogelator.[34] Wang *et al.* have prepared a phosphatase-responsive system using electrostatic interactions between a double hydrophilic block copolymer comprising poly(ethylene glycol), poly(L-lysine hydrochloride) and adenosine 5'-triphosphate (ATP) to form a superamphiphile in aqueous solution. Addition of ALP hydrolyses ATP to single phosphates and a neutral adenine group prompting aggregate disassembly.[35] Zelzer and co-workers have prepared a peptide-based surface whose chemical properties change upon the catalytic action of ALP.[36]

The ability to combine the specificity of an enzymatic action with other complex responses holds great promise to develop multi-responsive systems.[37] For example, thermally-

responsive systems typically undergo an abrupt solubility change with an aqueous polymer solution either phase separating (LCST), or a phase-separated mixture re-solubilising (UCST, upper critical solution temperature) upon heating. To fine-tune the LCST, the introduction of hydrophobicity/philicity to the system decreases/increases the transition temperature respectively.[38, 39] It is therefore reasonable to assume that the phosphatase-driven dephosphorylation of a phosphate to hydroxyl functional group will decrease the hydrophilicity of the system and hence affect the LCST, allowing an isothermal[9] change in polymer solubility without the need for a change in temperature. Gibson and co-workers have demonstrated glutathione[40, 41] and Fe^{3+} isothermal responses[42] and there are othrt examples of ion[43] and even bacteria- responsive polymers.[44]

It has been observed that upon passing through the LCST, polymers can gain entrance to cells due their increased lipophilicity and membrane permeability.[45, 46] For example, Alexander and co-workers have prepared chemo-therapy loaded nanoparticles which display enhanced uptake and cytotoxicity upon heating above the particle thermal transition temperature.[47] In addition to polymer nanoparticles,[45] other structures which have employed this property to enhance cell uptake include liposomes,[48] hydrogel submicron particles[49] and elastin-like polypeptides.[50, 51] An attractive extension of this concept would be to trigger cell uptake using localised biochemical gradients (such as local enzyme concentration) to enable cell uptake guided by the cellular physiology rather than external heating, and provide an alternative approach to the more traditional, receptor-mediated uptake pathways.[52-56]

Considering the potential for using an LCST-type transition to drive cellular uptake,[45, 47, 49] this manuscript describes the synthesis and evaluation of phosphate-containing polymers and their elaboration into isothermally responsive polymers, capable of undergoing their coil-globule transition at constant temperature. Preliminary cytotoxicity evaluation is also presented.

Materials and Methods

Phosphorus (V) oxychloride (≥ 99.0 %), 2-hydroxyethyl methacrylate (97.0 %), triethylamine (≥ 99.0 %), 4-cyano-4-(phenylcarbonothioylthio) pentanoic acid (> 97.0 %), 4,4'-azobis(4-cyanovaleric acid) (≥ 98.0 %), mesitylene (analytical standard), 2-mercaptoethanol (≥ 99.0 %), tribasic potassium phosphate (reagent grade, ≥ 98.0 %), carbon disulfide (≥ 99.0 %) and benzyl bromide (98.0 %) were purchased from Sigma-Aldrich. Oligo(ethylene glycol, average M_n = 300 g.mol^{-1}), methyl ether methacrylate and di(ethylene glycol) methyl ether methacrylate (95.0 %) were also purchased from Sigma-Aldrich and inhibitors removed by passing through a column of basic alumina prior to polymerisation. Hostasol methacrylate was kindly donated by the Haddleton group at the University of Warwick. 2-(dodecylthiocarbonothioylthio)-2-methylpropanoic acid was synthesised as previously described.[41] Calf Intestinal Alkaline Phosphatase (10,000 U/mL) was purchased from New England Biolabs Inc. The enzyme was packaged with a 10x NEBuffer 3. This was diluted 10-fold prior to use with the resulting 1x NEBuffer 3 containing 100 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.9 at 25 °C. The phosphate colorimetric assay kit was purchased from BioVision. A standard curve was prepared by diluting 10 µL of the supplied 10 mM Phosphate Standard to 990 µL dH_2O to give a 100 µM working standard. Desired standard concentrations were prepared by mixing the 10 mM standard with deionised water to give a total volume of 200 µL. The colorimetric phosphate reagent was used as received.

MCF-7 (ER + ve breast cancer cell line, wild type) was donated by Tenovus centre for Cancer research (Cardiff, UK). Tissue culture media and reagents were from Lonza, UK. Reagents for phosphate buffered saline (PBS) for cell culture were purchased from Sigma Aldrich, UK and PBS (pH 7.4) was prepared in house using UHQ water. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, powder) and Dimethyl sulfoxide

solution (DMSO) were purchased from Sigma Aldrich, UK. MTT solution (5 $mg.mL^{-1}$) was prepared in PBS (pH 7.4).

Physical and Analytical Methods

SEC analysis was performed on one of two systems:

(i) Dimethylformamide: Varian 390-LC MDS system equipped with a PL-AS RT/MT autosampler, a PL-gel 3 µm (50 x 7.5 mm) guard column, two PL-gel 5 µm (300 x 7.5 mm) mixed-D columns using DMF with 5 mM NH_4BF_4 at 50 °C as eluent at a flow rate of 1.0 $mL.min^{-1}$. The SEC system was equipped with ultraviolet (UV)/visible (set at 280 and 461 nm) and differential refractive index (DRI) detectors. Narrow molecular weight PMMA standard (200 - 1.0 x 10^6 $g.mol^{-1}$) were used for calibration using a second order polynomial fit.

(ii) Aqueous: Varian 390-LC MDS system equipped with a PL-AS RT/MT autosampler, an aquagel-OH (50 x 7.5 mm) guard column, one PL-aquagel-OH 40 8 µm (300 x 7.5 mm) and one PL-aquagel-OH 30 8 µm (300 x 7.5 mm) column using a buffer comprising 0.2 M $NaNO_3$ and 0.01 M $NaH_2PO_4.2H_2O$ adjusted to pH 8.2 with 1 M NaOH at 30 °C as eluent at a flow rate of 1.0 $mL.min^{-1}$). The SEC system was equipped with a differential refractive index (DRO) detector. Narrow molecular weight PEO standards (100 – 1.0 x 10^5 $g.mol^{-1}$) were used for calibration using a second order polynomial fit.

NMR spectroscopy (^1H, ^{13}C, ^{31}P) was conducted on a Bruker DPX-300, Bruker DRX-500 or Bruker AV III 600 spectrometer using deuterated chloroform or deuterated methanol as solvent. All chemical shifts are reported in ppm (δ) relative to the solvent used. FTIR spectra were acquired using a Bruker Vector 22 FTIR spectrometer with a Golden Gate diamond attenuated total reflection cell. A total of 64 scans were collected on samples in their native

state. High resolution mass spectra were recorded on a Bruker Electrospray Ultra-High Resolution tandem TOF mass spectrometer using electrospray ionisation (ESI) in either positive or negative mode on samples prepared in methanol. Cloud points were determined by turbidimetric analysis using either an Optimelt MPA100 system (Stanford Research Systems) or an Agilent Cary 60 UV/visible spectrophotometer with the absorbance monitored at 650 nm. A heating rate of 1 °C.min^{-1} was used in both cases and the recorded turbidimetry curve was normalised between values of 0 and 1. The cloud point was defined as the temperature corresponding to a normalised absorbance of 0.5.

Methods

Cell viability (MTT-Assay). MCF-7 cells were cultured in RPMI 1640 growth medium supplemented with 5% fetal bovine serum under standard tissue culture conditions (37 °C, humidified 5% CO_2 atmosphere). Stock solutions of the polymer (4 mg.mL^{-1}) were prepared in complete growth medium, sterile filtered (0.2 μm) and then further diluted with complete growth medium. MCF-7 cells were seeded (4×10^4 cells per mL) in a 96-well plate and incubated for 24 h to allow attachment. Then, fresh complete growth medium containing the polymers was added (concentrations 0 – 1 mg.mL^{-1}. After treatment, cytotoxicity was assessed by MTT assay as previously reported.[57] Briefly, MCF-7 cells were incubated with the polymer solutions. After 67 h, MTT solution (20 μL) was added to each well and the cells were incubated for further 5 h after which 100 μL of DMSO was added to dissolve the purple crystals formed. The plates were read at 570 nm using a SPECTRA max UV spectrometer (Bio-Rad). Data represent the mean of at least three independent experiments and cell viability is expressed as % of control (complete growth medium without polymers).

Example polymerisation of 2-(methacryloyloxy)ethyl phosphate using 4-cyano-4-(phenylcarbonothioylthio) pentanoic acid

2-(methacryloyloxy)ethyl phosphate (1.00 g, 4.76 mmol), 4-cyano-4-(phenylcarbonothioylthio) pentanoic acid (13.30 mg, 47.61 µmol, 0.01 equiv) and 4,4'-azobis(4-cyanovaleric acid) (2.70 mg, 9.63 µmol (0.002 equiv) were added to a vial fitted with stir bar and rubber septum and dissolved in methanol (4 mL). Mesitylene (200 µL) was added as internal reference and the mixture stirred (5 mins). An aliquot of this starting mixture was removed for ^{1}H NMR spectroscopic analysis. The mixture was degassed by bubbling through nitrogen gas for 30 mins and placed in an oil bath thermostated at 65 °C for 7 hrs. The reaction was quenched in liquid nitrogen, an aliquot removed and conversion determined by ^{1}H NMR spectroscopy. The product was purified three times by precipitation from methanol into cold diethyl ether, the solid isolated by centrifugation and dried to yield a pale pink solid. Conversion (NMR): 54.7 % M_n (theoretical): 11500 g.mol^{-1}; M_n (SEC): 38000 g.mol^{-1}; M_w/M_n (SEC): 2.20.

Example polymerisation of 2-(methacryloyloxy)ethyl phosphate using 2-(dodecylthiocarbonothioylthio)-2-methylpropanoic

2-(methacryloyloxy)ethyl phosphate (1.00 g, 4.76 mmol), 2-(dodecylthiocarbonothioylthio)-2-methylpropanoic acid (17.35 mg, 47.58 µmol, 0.01 equiv) and 4,4'-azobis(4-cyanovaleric acid) (2.70 mg, 9.63 µmol, 0.005 equiv) were added to a vial fitted with stir bar and rubber septum and dissolved in methanol (4 mL). Mesitylene (200 µL) was added as internal reference and the mixture stirred (5 mins). An aliquot of this starting mixture was removed for ^{1}H NMR spectroscopic analysis. The mixture was degassed by bubbling through nitrogen gas for 30 mins and placed in an oil bath thermostated at 65 °C for 10 hrs. The reaction was quenched in liquid nitrogen, an aliquot removed and conversion determined by ^{1}H NMR

spectroscopy. The product was purified three times by precipitation from methanol into cold diethyl ether, the solid isolated by centrifugation and dried to yield a pale yellow solid. Conversion (NMR): 67.0 % M_n (theoretical): 14100 g.mol^{-1}; M_n (SEC): 27700 g.mol^{-1}; M_w/M_n (SEC): 2.17.

Polymerisation of oligo(ethylene glycol) methyl ether methacrylate using 4-cyano-4-(phenylcarbonothioylthio) pentanoic acid

Oligo(ethylene glycol) methyl ether methacrylate (average M_n = 300 g.mol^{-1}) (1.00 g, 2.5 mmol), 4-cyano-4-(phenylcarbonothioylthio) pentanoic acid (13.97 mg, 50.00 µmol, 0.02 equiv) and 4,4'-azobis(4-cyanovaleric acid) (2.80 mg, 10.00 µmol, 0.004 equiv) were added to a vial fitted with stir bar and rubber septum and dissolved in dioxane (3 mL). Mesitylene (100 µL) was added as internal reference and the mixture stirred (5 mins). An aliquot of this starting mixture was removed for ^{1}H NMR spectroscopic analysis. The mixture was degassed by bubbling through nitrogen gas for 30 mins and placed in an oil bath thermostated at 70 °C for 4 hrs. The reaction was quenched in liquid nitrogen, an aliquot removed and conversion determined by ^{1}H NMR spectroscopy. The product was purified three times by precipitation from dioxane into cold diethyl ether, the solid isolated by centrifugation and dried to yield a waxy pink solid. Conversion (NMR): 44.2 %; M_n (theoretical): 8800 g.mol^{-1}; M_n (SEC): 11300 g.mol^{-1}; M_w/M_n (SEC): 1.18.

Example co-polymerisation of 2-(methacryloyloxy)ethyl phosphate and diethylene glycol methyl ether methacrylate using 4-cyano-4-(phenylcarbonothioylthio) pentanoic acid

Diethylene glycol methyl ether methacrylate (1.03 g, 5.47 mmol), 2-(methacryloyloxy)ethyl phosphate (0.13 g, 607.92 µmol), 4-cyano-4-(phenylcarbonothioylthio) pentanoic acid (17.00 mg, 60.79 µmol, 0.01 equiv) and 4,4'-azobis(4-cyanovaleric acid) (3.41 mg, 12.16 µmol, 0.002 equiv) were added to a vial fitted with stir bar and rubber septum and dissolved in methanol (4 mL). Mesitylene (200 µL) was added as internal reference and the mixture stirred (5 mins). An aliquot of this starting mixture was removed for ^{1}H NMR spectroscopic analysis. The mixture was degassed by bubbling through nitrogen gas for 30 mins and placed in an oil bath thermostated at 65 °C for 6.5 hrs. The reaction was quenched in liquid nitrogen, an aliquot removed and conversion determined by ^{1}H NMR spectroscopy. The product was purified three times by precipitation from methanol into cold diethyl ether, the solid isolated by centrifugation and dried to yield a waxy pink solid. Conversion (NMR): 23.3 %; M_n (theoretical): 4400 g.mol^{-1}; M_n (SEC): 6200 g.mol^{-1}; M_w/M_n (SEC): 1.30.

Phosphatase Assays

General assay conditions for colorimetric determination of phosphate release

A 105 µg.mL^{-1} stock solution of polymer in water was prepared. In a 96-well plate, in triplicate was added 188 µL of 1x NEBuffer 3 solution (diluted from the 10x concentrated solution provided by supplier), 10 µL of polymer solution and 2 µL calf intestinal alkaline phosphatase (10,000 U/mL from supplier = 20 U. U = '1 unit of activity as specified by the supplier. 1 unit should hydrolyse 1 µmol in 1 mL in 1 minute. In this assay 20 Units were added to 0.1 µmol phosphate groups and therefore was in excess). The plate was then covered and incubated at 37 °C. At a given time-point, 30 µL of the phosphate reagent was mixed into each well, incubated at 37 °C for 90 mins and the absorbance at 650 nm measured. The molar concentration and hence percentage of phosphate released was determined by comparing to a known calibration curve.

Assay determining the effect of phosphatase on polymer cloud point

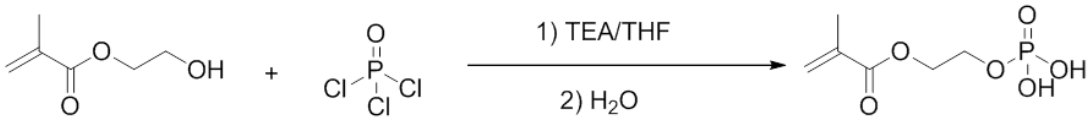
Polymer was dissolved in 1x NE Buffer 3 and calf intestinal alkaline phosphatase added to give a final polymer concentration of 5 $mg.mL^{-1}$. This mixture was stirred at 37 °C for 24 hrs. After this time, an aliquot of the mixture was transferred to a melting point tube and the cloud point determined at a heating rate of 1 $°C.min^{-1}$.

Turbidimetric assay for phosphatase-mediated isothermal polymer response

A 5.56 $mg.mL^{-1}$ solution of polymer in 1x NEBuffer 3 was prepared. 2 x 90 µL aliquots of this stock were transferred into individual wells of a 96-well plate. The plate was left to incubate at 34 °C in the plate reader for 35 mins. The plate was then removed and the well volume made to 100 µL with distilled water or calf intestinal alkaline phosphatase. The plate was re-incubated at 34 °C and the absorbance at 650 nm was recorded for an additional 25 mins.

Results and Discussion

To access a polymer which would undergo a switch in its cloud point (LCST) upon loss of a phosphate group, triggered by (the enzyme) alkaline phosphatase, the monomer ethylene glycol methacrylate phosphate (Phos-HEMA) was synthesised by reaction of phosphorus oxychloride with hydroxyethyl methacrylate, Scheme 1. This reaction produced the desired monomer in good yield, with negligible traces of the undesired diene impurity. ^{1}H NMR spectroscopic analysis revealed vinyl proton peaks at 6.17 and 5.67 ppm, together with two CH_2 groups and one CH_3 group at 4.37, 4.21 and 1.97 ppm respectively (Figure 6.3A). Doublets at 64.1 and 63.5 ppm observed in the ^{13}C NMR spectrum on account of coupling with the phosphorus atom and the single peak observed by ^{31}P NMR spectroscopic analysis further confirmed monomer structure and purity (see ESI).



Scheme 1 Synthetic scheme for the preparation of Phos-HEMA.

With phosphorylated monomer to hand, phosphate-functional polymers were prepared by the RAFT process. Given the few reports on this, preliminary experiments were performed to better understand the polymerisation rate, and to confirm the controlled nature. Two chain transfer agents, 4-cyano-4-(phenylcarbonothioylthio) pentanoic acid (CPADB) and 2-(dodecylthiocarbonothioylthio)-2-methylpropanoic acid (DDMAT) were employed in a reaction mixture comprising [M]:[CTA]:[Initiator] = 100:1:0.2, and an initial monomer concentration of 1.19 M. Mesitylene was used as an internal standard and conversion was monitored by ^{1}H NMR spectroscopy, following the change in integral of vinyl proton peak relative to this standard. Polymerisation was observed to be faster using the trithiocarbonate compared to the dithiobenzoate, with approximately 85 % conversion reached after 10 and 24

hours respectively (Figure 1). First order kinetics were observed with both RAFT agents indicating a constant number of propagating chain ends; a key characteristic of controlled radical processes.[58] This indicated that both RAFT agents were suitable.

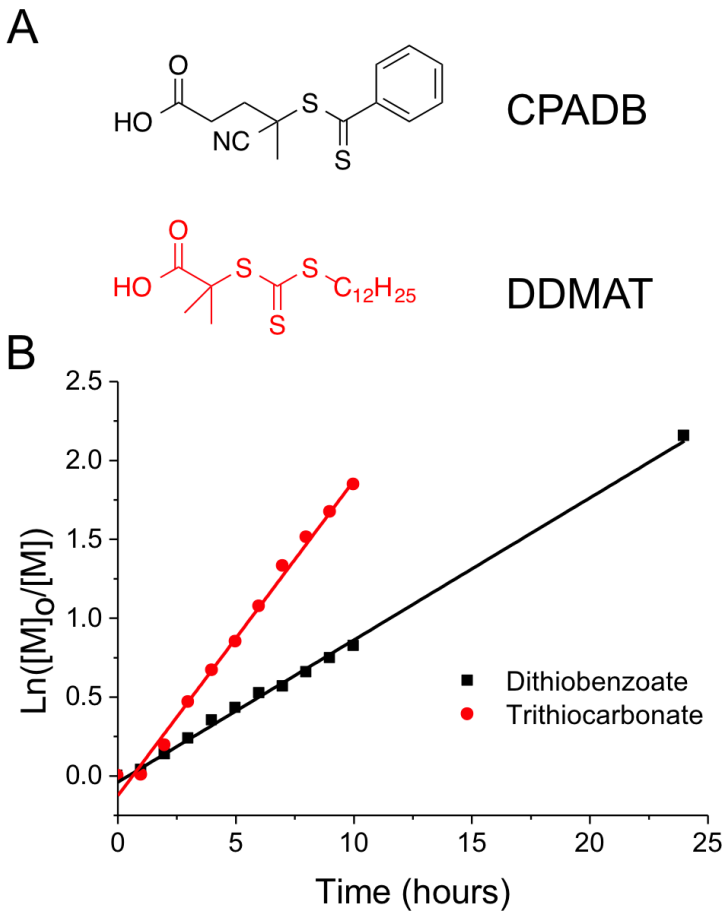


Figure 1 Kinetic analysis of the RAFT polymerisation of Phos-HEMA: (A) RAFT agents used; (B) pseudo first order kinetic plot.

A library of poly(Phos-HEMAs) were prepared using the dithiobenzoate RAFT agent and the results of this summarised in Table 1. At higher molecular weights, the dispersity becomes broader than typically desired for controlled radical methodologies, with a high molecular weight shoulder beginning to develop. This may be an artefact of the SEC system used – for instance, partial deprotonation of the phosphate functionality may influence the hydrodynamic volume of some polymer chains or promoting interaction with the column

materials. For our purpose however, RAFT was simply used as a facile route towards the preparation of phosphate-functional polymers. To confirm the presence of this functional group, ^{31}P NMR analysis of the purified material was performed and revealed a single phosphorus peak, confirming the presence of a phosphate group and hence the suitable application of the RAFT technique. Moreover, the use of a diene-free monomer ensured the poly(diester) impurities observed in previous reports[59] was not seen in our case.

Table 1 Characterisation of pPhos-HEMA samples prepared using 4-cyano-4-(phenylcarbonothioylthio) pentanoic acid in this study.

Polymer	**[M]:[CTA]**	**Conversion (%)[a]**	$M_{n(th)}$ **(g.mol^{-1})[a]**	$M_{n(SEC)}$ **(g.mol^{-1})[b]**	M_w/M_n [b]
pPhos-HEMA-1	10	41.2	870	9400	1.24
pPhos-HEMA-2	75	50.8	8000	30900	2.00
pPhos-HEMA-3	100	54.7	11500	38000	2.20

[a]Determined by ^{1}H NMR spectroscopy relative to an internal standard (mesitylene). Theoretical molecular weight is determined by the feed ratio, and conversion, assuming a linear increase in molecular weight with conversion; [b]Determined by SEC (aqueous) relative to PEO standards.

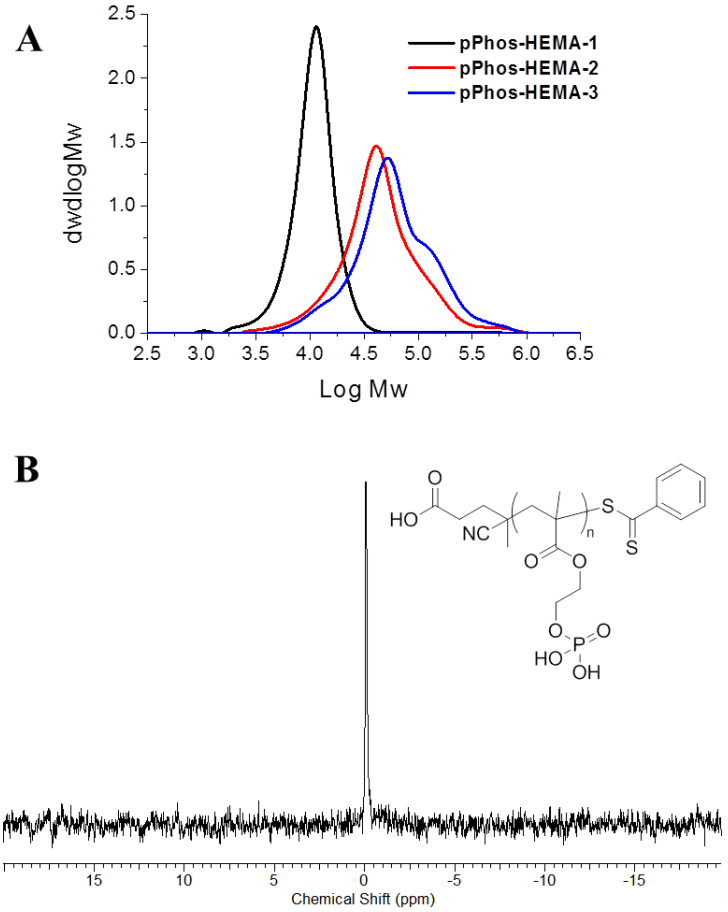


Figure 2 Characterisation of pPhos-HEMA. (A) **Aqueous** SEC of **pPhos-HEMA-1/2/3**; (B) ^{31}P NMR spectrum of **pPhos-HEMA-1** in MeOD.

With the panel of polymers to hand, the ability of calf intestinal alkaline phosphatase (CIALP) to cleave phosphate groups from the chain was investigated. A colorimetric assay was employed to monitor this reaction; briefly this assay uses malachite green and ammonium molybdate which, in the presence of a phosphate ion, forms a chromogenic complex with an intense absorption band around 650 nm, which can be calibrated against known phosphate solutions. To ensure biological relevance, all experiments were performed at 37 °C. **pPhos-HEMA-3** was added to buffer followed by the enzyme and the absorbance at 650 nm measured after a 90 minute incubation time (Figure 3). Phosphate release was observed to be rapid with 38 % released after 30 min before plateauing at approximately 60 % after 8 hours, either due to unwanted polymer interactions or due to the protein losing

catalytic activity. The produced poly(hydroxyethyl methacrylate), pHEMA is known to be water swellable, rather than water-soluble at chain lengths beyond approximately 20 repeat units and therefore will be less soluble.[60, 61] Whilst no polymer precipitation was observed during the assay, the increasing hydrophobic character obtained upon dephosphorylation may result in the formation of globule-type micro-domains which could limit enzyme access to the remaining phosphate functional groups. A similar observation has been made by Amir *et al.* who used acid phosphate to dephosphorylate a diblock copolymer containing PEG-derived and phosphorylated styrene blocks. Incomplete dephosphorylation was observed which was attributed to the beginnings of a self-assembly process which shielded some phosphate groups from being accessed by the enzyme.[62]

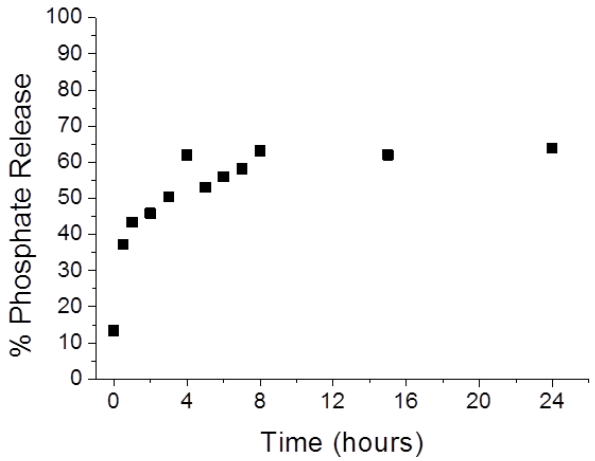


Figure 3 Percentage phosphate release from **pPhos-HEMA-3** upon incubation with CIALP.

To investigate whether the polymer chain length could influence the degree of phosphate release, the same assay was performed on polymers **pPhos-HEMA-1** ($M_{n(theo)}$ = 870 g.mol^{-1}) and **pPhos-HEMA-2** ($M_{n(theo)}$ = 11500 g.mol^{-1}). In each case, the mass of polymer used was fixed meaning despite the difference in chain length the number of phosphate groups in each sample is identical. All samples were incubated for 24 hours after which phosphate release

was measured, with all polymers exhibiting similar release profiles between 55 and 60 % (Figure 4). This observation suggests enzyme activity or access to dephosphorylation is independent of molecular weight (in the limited range tested), which makes the design of isothermally responsive polymers simpler using this motif (*vide infra*). Several controls were also performed to confirm the validity of the assay. Firstly, the absorbance of the kit in buffer was shown to be minimal confirming no interaction between the chromogenic complex and the solution salts. Polymer **pPhos-HEMA-3** was also tested in the absence of enzyme (pPhos-HEMA-3 – CIP in Figure 4). Less than 10 % release (due to hydrolysis) was observed indicating the measured absorbance was due to the dephosphorylation of phosphate groups by CIALP, rather than by other non-specific mechanisms such as aqueous phosphate hydrolysis (Figure 4). Finally, a non-phosphate containing, water-soluble polymer was also tested – poly[oligo(ethylene glycol)$_{300}$ methyl ether methacrylate], **pOEGMA$_{300}$** (ESI for details). A small change in absorbance which equated to approximately 10 % phosphate release was also observed suggesting the polymer may interfere with the colorimetric assay in some way though this was still significantly less than that observed for **pPhos-HEMA-1/2/3** in the presence of the enzyme (Figure 4).

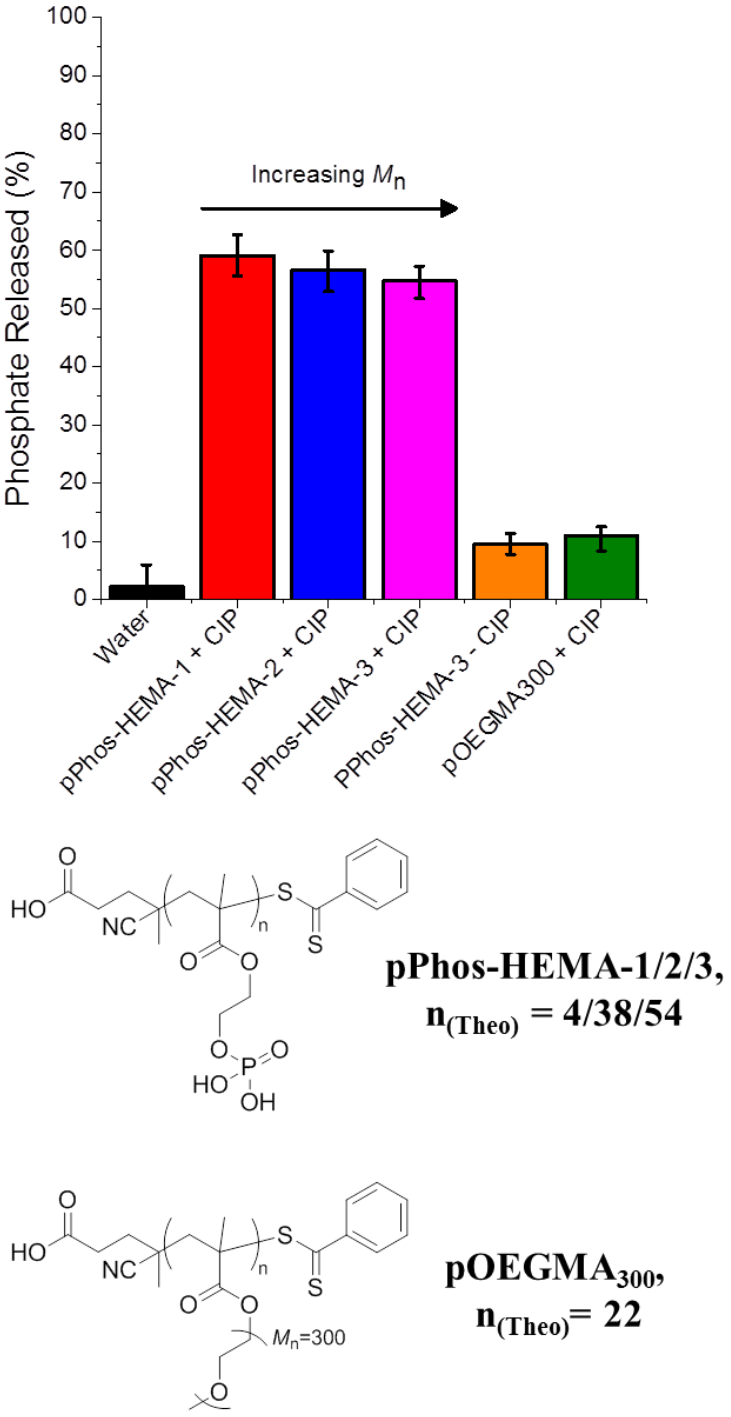


Figure 4 Phosphate release as a function of polymer structure. A) Phosphate release in presence of CIALP. Error bars represent standard deviation from minimum of 3 repeats; (B) Chemical structures of polymers employed.

The main aim of this study was to induce an isothermal switch based on a known thermo-responsive polymer, triggered by CIALP. To achieve this, co-polymers of a known responsive polymer using poly(phosHEMA) as the enzymatic trigger were required. Diethylene glycol methyl ether methacrylate (DEGMA) was selected for this. It is known to have similar reactivity ratios to HEMA ensuring random co-polymers are obtained and it has a cloud point of around 26 °C in aqueous solution.[63, 64] We therefore reasoned that addition of Phos-HEMA units would raise the cloud point, but upon enzymatic cleavage, reduce it, such that an isothermal transition at 37 °C could be obtained. To assess the effect of Phos-HEMA on the cloud point of the DEGMA-based co-polymers, a series of copolymers was prepared (Table 2).

Table 2 Characterisation of poly(DEGMA-*co*-Phos-HEMA) samples prepared using 4-cyano-4-(phenylcarbonothioylthio) pentanoic acid in this study.

Polymer	**[DEGMA]:[phos-HEMA]:[CTA]**	**Conversion (%)[a]**	$M_{n(th)}$ **(g.mol^{-1})[a]**	$M_{n(SEC)}$ **(g.mol^{-1})[b]**	M_w/M_n [b]
P_{co}1%	99:1:1	42.7	8000	7300	1.23
P_{co}2.5%	97.5:2.5:1	45.9	8600	7200	1.21
P_{co}5%	95:5:1	46.4	8800	6400	1.21
P_{co}10%	90:10:1	23.3	4400	6200	1.30

[a]Determined by ^{1}H NMR spectroscopy relative to an internal standard (mesitylene) assuming equal conversion of DEGMA and Phos-HEMA (Phos-HEMA vinyl proton peaks too poorly resolved for analysis); [b]Determined by SEC (DMF inc. 5 mM NH_4BF_4) relative to PMMA standards.

SEC analysis indicated a controlled polymerisation, supporting our earlier (*vide supra*) arguments about column interactions of phosphate-rich polymers. The obtained molecular weights were in agreement with the monomer feed ratio for **P_{co}1%**, **P_{co}2.5%** and **P_{co}5%,** but **P_{co}10%** (most phosphate) gave lower conversion and hence lower molecular weight. Cloud point analysis of these polymers at 5 mg.mL^{-1} revealed an increase from 35 °C to 45 °C upon increasing the Phos-HEMA ratio from 1 % to 10 % (Figure 5), providing evidence of the successful incorporation of the monomer in the co-polymer structure. The very hydrophilic nature of Phos-HEMA means only small proportions are required to produce a cloud point with physiological relevance, which is desirable to ensure minimal enzymatic interaction are needed to induce the change.

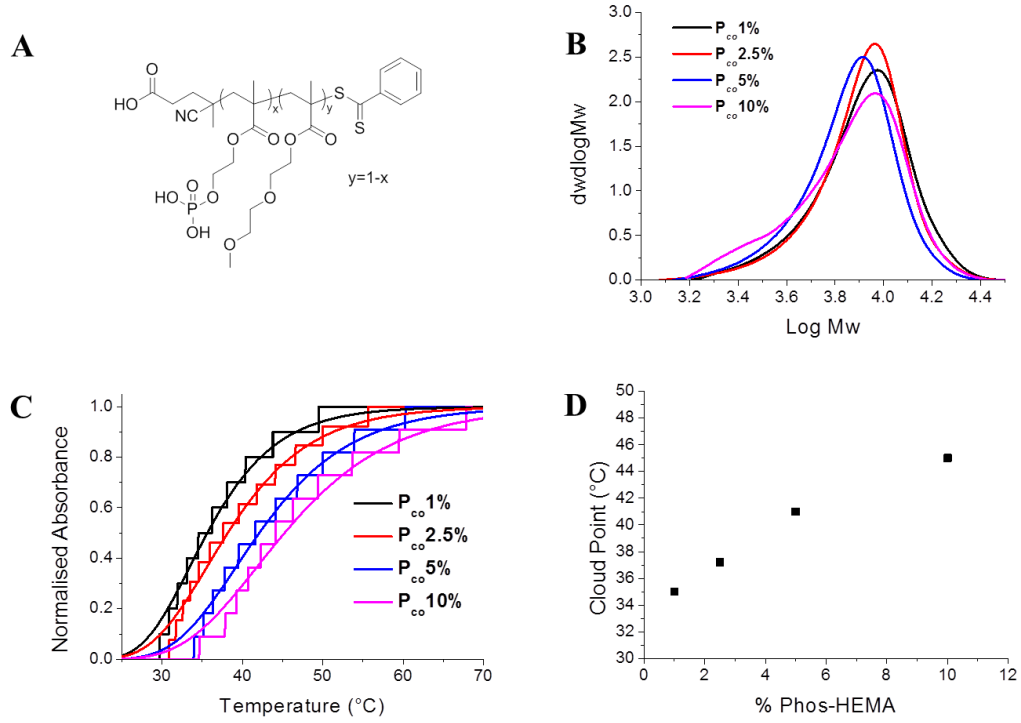
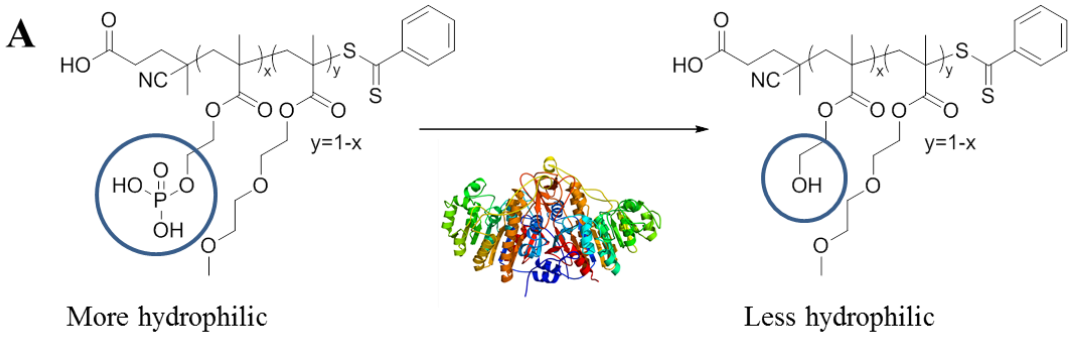


Figure 5 Characterisation of pDEGMA-*co*-Phos-HEMA: (A) Co-polymer chemical structure; (B) SEC characterisation; (C) Cloud point traces at a polymer concentration of 5 mg.mL^{-1} (solid curves added to guide the eye only); (D) Cloud point as a function of co-polymer composition.

It was anticipated that the action of phosphatase on this polymer would trigger a decrease in cloud point by converting phosphate groups to less hydrophilic hydroxyl moieties (Figure 6). To test this hypothesis, a 5 mg.mL^{-1} solution of **P_{co}5%** was incubated with CIALP and the sample stirred at 37 °C for 24 hours. This polymer concentration was selected to ensure the change in turbidity (precipitation) observed upon passing through the cloud point would be sufficiently strong for detection by the instrument used (i.e. lower concentrations would respond, but not give such a large response). After this time, the sample was analysed by turbidimetry and a decrease in the cloud point by 4 °C and 8 °C was observed in the presence of 2 µL (2 units) and 10 µL (10 units) of enzyme respectively confirming the isothermal response (Figure 6B).



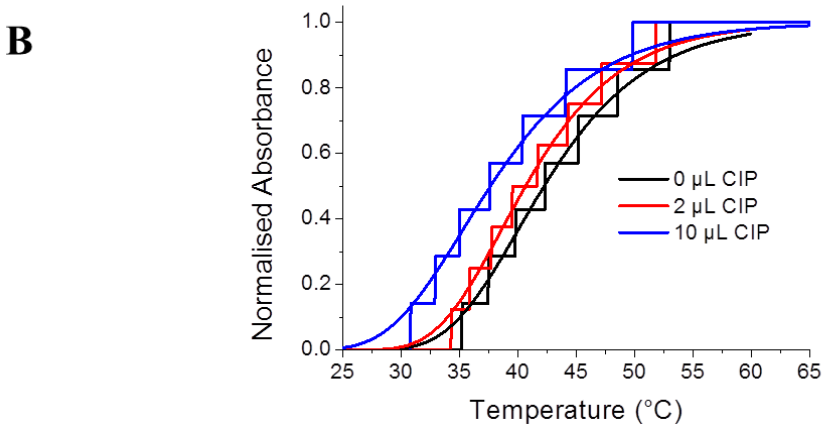


Figure 6 Dephosphorylation of co-polymers by CIALP. (A) Change in chemical structure of poly(DEGMA-*co*-Phos-HEMA) upon incubation with CIALP; (B) Effect of CIALP on cloud point of **P_{co}5%** (solid curves are to guide the eye).

To confirm these observations were due to phosphate removal, two controls were performed. Firstly, the polymer was incubated without enzyme for a period of 24 hours and negligible change in cloud point was observed, confirming any shift is not due to direct hydrolysis of either the phosphate groups or the methacrylate backbone. Moreover, to verify the shift was not simply due to the additives present in the buffer used for analysis the non-phosphate-containing **pOEGMA$_{300}$** was also analysed following 24 hour incubation with 10 µL CIALP (Figure 7). Minimal change was observed in this case with the cloud point actually increasing slightly, in contrast to the behaviour expected and observed if dephosphorylation was occurring (Figure 6B). In comparison to the assays performed on the homopolymers where a phosphate concentration of 25 µM was employed, the phosphate concentration in this test was nearly 50 x higher (~ 1.2 mM) and hence the effect of a larger enzyme volume is perhaps unsurprising.

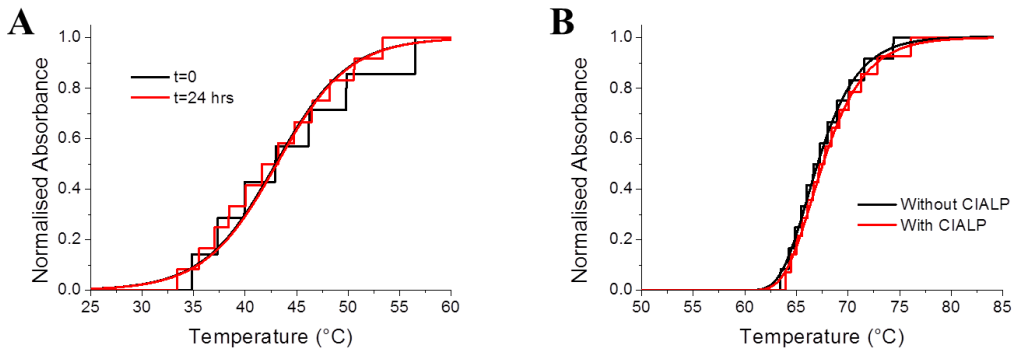


Figure 7 Effect of CIALP on POEGMAs. (A) Cloud point of **P$_{co}$5%** in 1x NEBuffer 3 after 24 hours; (B) Cloud point of **pOEGMA$_{300}$** with and without 10 µL CIALP (solid curves are to guide the eye).

The change in cloud point observed upon the addition of CIALP provided a window for an isothermal transition to be achieved and was tested using a turbidimetric assay (Figure 8). **P$_{co}$5%** was held at 34 °C, below its cloud point but above that observed in the presence of 10

μL CIALP. No increase in absorbance was observed until the enzyme was added, after which time a rapid and significant increase in absorbance, concomitant with polymer precipitation was observed. Altering a material's solubility without needing a temperature change is potentially attractive for biomedical applications providing, for example, a route towards improved, controlled targeting of therapeutic delivery vehicles.[65, 66]

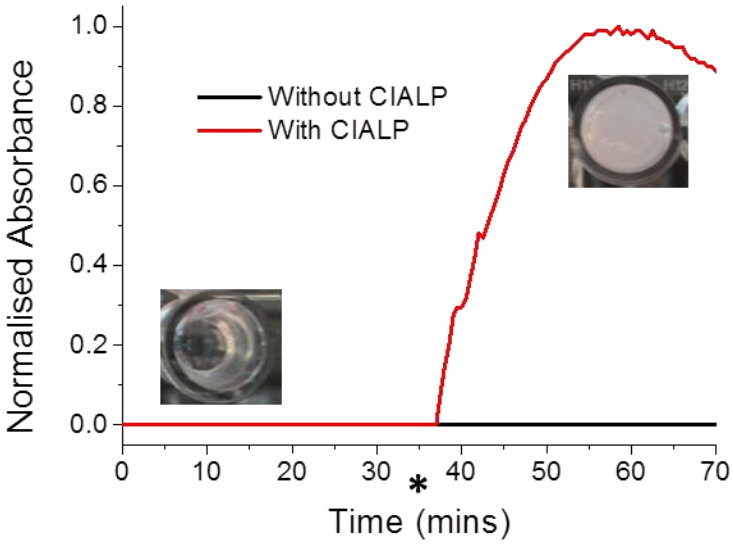


Figure 8. Isothermal turbidimetry data for $\mathbf{P_{co}5\%}$: Polymer concentration = 5 mg.mL^{-1}; Temperature = 34 °C; CIALP added at time indicated by asterisk. Black = control (polymer without enzyme); red = polymer with 10 μL CIALP. Inset = representative photo of wells before and after experiment.

Finally, the impact of incorporation of the phosphate groups on the cytocompatibility of the polymers was evaluated in MCF-7 cells. Cells were incubated with the polymers at concentrations ranging from 0.01 to 1 mg.mL^{-1} for 72 hours and their cell viability determined using the MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide) assay. No cytotoxicity was observed for either polymer at the concentrations tested (Figure

9). This finding is important as it confirms applicability to biological systems and future translational applications.

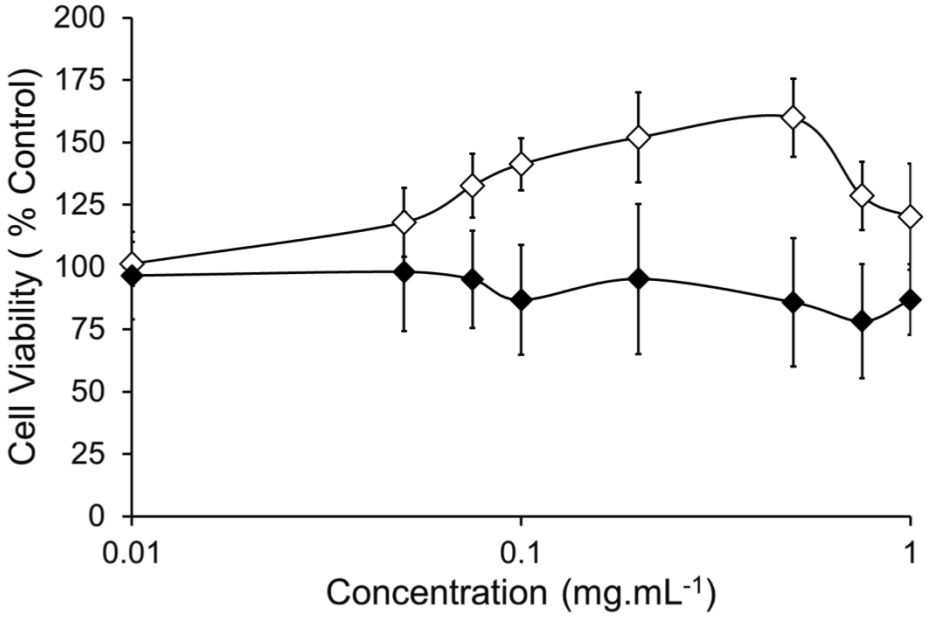


Figure 9. Cell viability of MCF-7 cells after incubation with polymers for 72 h. **pOEGMA$_{300}$** (black diamond) and **poly(DEGMA-*co*-Phos-HEMA) 5%** (hollow diamonds). Data are shown as mean ± standard error of the mean from a minimum of 3 replicates.

The above results demonstrate that by selecting a thermally responsive polymer that is 'primed' to undergo its coil-globule (LCST) transition it is possible to introduce enzyme-responsivity but only by incorporating a very low loading of the responsive monomers. Such an approach is appealing to take advantage of the known advantages and applications of thermally responsive polymers (such as selective delivery) but by re-programming them to respond to a biological, rather than thermal trigger. We anticipate such materials will find use in cellular delivery and imaging.

Conclusions

Here we describe the synthesis of a phosphorylated methacrylate (Phos-HEMA) which is well tolerated by the RAFT methodology to produce phosphate-functional polymers. The susceptibility of these polymers to the action of the enzyme calf intestinal alkaline phosphatase was also demonstrated with no obvious effect of polymer molecular weight on the phosphate release profiles, in all cases a maximum of ~ 60 % of phosphate release was observed. Guided by this, PhosHEMA was co-polymerised with diethylene glycol methyl ether methacrylate (DEGMA) to produce thermally-responsive materials. The cloud point of these polymers was tuneable by changing the percentage of Phos-HEMA; the inclusion of between 1 and 10 % resulting in a cloud point increase from 35 °C to 45 °C. An isothermal response was shown, as the cloud point of these polymers was observed to decrease in the presence of calf intestinal alkaline phosphatase due to enzyme-mediated dephosphorylation to the more hydrophobic hydroxyl functional group. This switch was used to modulate the polymer solubility isothermally, enabling cloud point activation by the enzyme, without a temperature change. Preliminary cytotoxicological evaluation showed no toxicity, confirming the polymers potential for biomedical applications. This work demonstrates a practical method to introduce enzymatic response into synthetic polymers with the aim of enabling them to respond to extra-cellular cues, rather an externally applied stimuli, and may find application in drug delivery and biomedicine.

Supporting Information Available

Supporting information available. This includes additional characterization of the PhosHema monomer and additional details of the phosphatase assay. This material is available at free of charge via the Internetat http://pubs.acs.org

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