Conjugation of phosphatidylethanolamine to poly(*N*-isopropylacrylamide) for potential use in liposomal drug delivery systems

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Solutions of poly(*N*-isopropylacrylamide) (polyNIPAAm) become turbid at their lower critical solution temperature (*LCST*) of 33°C. In this study we have conjugated a phospholipid, 1-acyl-2-{12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanol}-phosphatidylethanolamine, to polyNIPAAm. Conjugation increases the *LCST* of polyNIPAAm by ~2°C and broadens the temperature range over which turbidity changes. Conjugation of phospholipids to polymers in general and to thermally reversible polymers in particular may allow some novel applications that relate to their potential for insertion into liposomes, micelles or cell membranes and hydrophobic interfaces.

(Keywords: poly(*N*-isopropylacrylamide); lower critical solution temperature; phospholipid; conjugation)

Introduction

Poly(*N*-isopropylacrylamide) (polyNIPAAm) is a non-ionic, water-soluble polymer that exhibits unusual thermally reversible behaviour[1]. That is, aqueous solutions of polyNIPAAm have a lower critical solution temperature (*LCST*) around 33°C[2,3]. PolyNIPAAm is hydrophobic and insoluble in water when the temperature is above its *LCST*, but becomes hydrophilic and dissolves when cooled below the *LCST*. The thermal reversibility of soluble polymers and hydrogels of polyNIPAAm has been the basis of an immunoassay[4], bioseparations[5], controlled drug release[6–8] and enzyme or cell bioreactors[9].

Proteins have been conjugated to polyNIPAAm for potential use in diagnostic testing[4], affinity separations[10] and an enzyme bioprocess[11]. A single-chain lipid, *N*-hexadecylamine, has also been conjugated to polyNIPAAm[12]. However, until now there has been no report of the conjugation of phospholipids to polyNIPAAm, although phospholipids have been conjugated to polyethylene glycol (PEG) in the preparation of PEG-coated liposomes[13,14]. Liposomes coated with PEG have prolonged *ex vivo* circulation times[13,14]. Other kinds of lipids, such as palmitic acid and cholesterol, have also been coupled to conventional polymers such as polysaccharides[15,16]. Polymer-borne lipid hydrophobic groups have been anchored to lipid vesicles to build up artificial cell-wall-like structures for stabilizing liposomes[17,18].

We report here the conjugation of a phospholipid (PL) to the backbone of polyNIPAAm. Such polymer–PL conjugates may be useful for insertion into liposomes for thermal control of drug delivery from liposomes as shown in *Figure 1*.

Methods

The PL was conjugated to the backbone of a copolymer of NIPAAm (from Eastman Kodak Co.) and *N*-acryloxysuccinimide (NAS, from Eastman Kodak Co.) via amino groups on the PL. A copolymer of NIPAAm and NAS (polyNIPAAm-*co*-NAS) was synthesized by following the protocol of Yang *et al.*[19] with a weight feed ratio of NIPAAm to NAS of 50 to 3. Next, the fluorescently labelled lipid, 1-acyl-2-{12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanol}-phosphatidylethanolamine (C_{12}NBDPE, from Avanti Polar Lipids, Inc.) (0.3 mg) was reacted with polyNIPAAm-*co*-NAS (1 mg) in chloroform for 2 days. Excess C_{12}NBDPE was used to assure conjugation to all active *N*-hydroxysuccinimide ester groups on polyNIPAAm-*co*-NAS. The conjugation chemistry is shown in *Figure 2*. After reaction, the chloroform was removed by evaporation and evacuation. The dried sample was hydrated with deionized water. After hydration, the sample was centrifuged twice, once below and once above the *LCST* of polyNIPAAm to separate polymer–PL conjugate from unreacted lipid. Fluorescence measurements were made on both supernatants and precipitates to assay the conjugation. All fluorescence measurements were done at a concentration low enough that the fluorescent intensity of the PL was linearly proportional to its concentration. After purification, the polymer–PL conjugate was examined by thin layer chromatography (t.l.c.). Controls were run on the same plate with pure

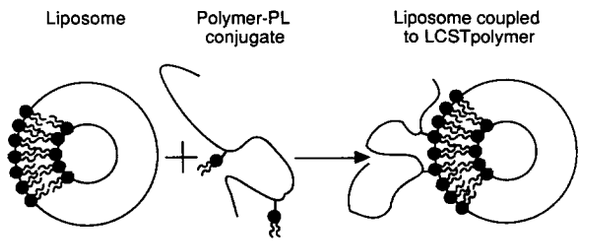

Figure 1 Schematic diagram of the insertion of polyNIPAAm–PL conjugate into liposomes for possible applications in thermally controlled drug delivery from liposomes

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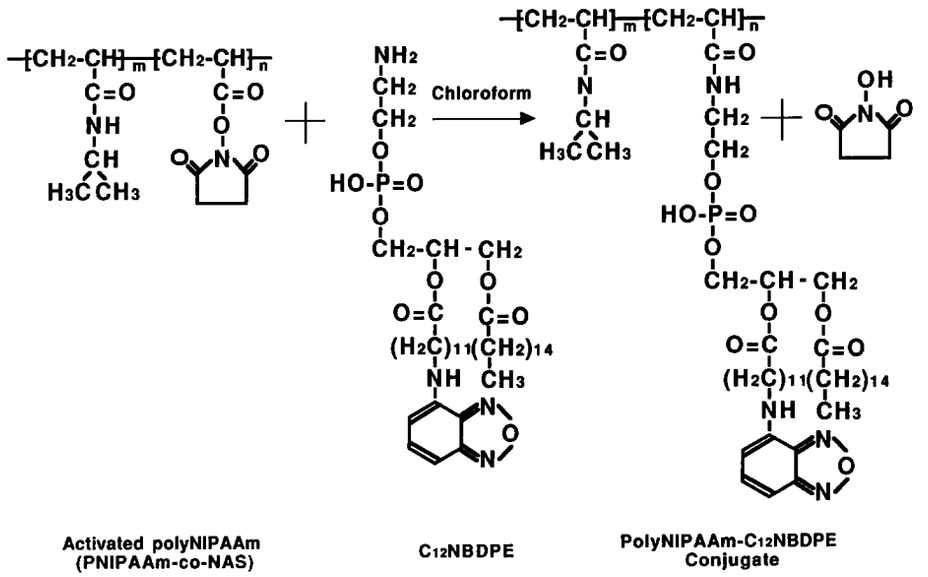

Figure 2 Chemistry of the conjugation of phosphatidylethanolamine to polyNIPAAm

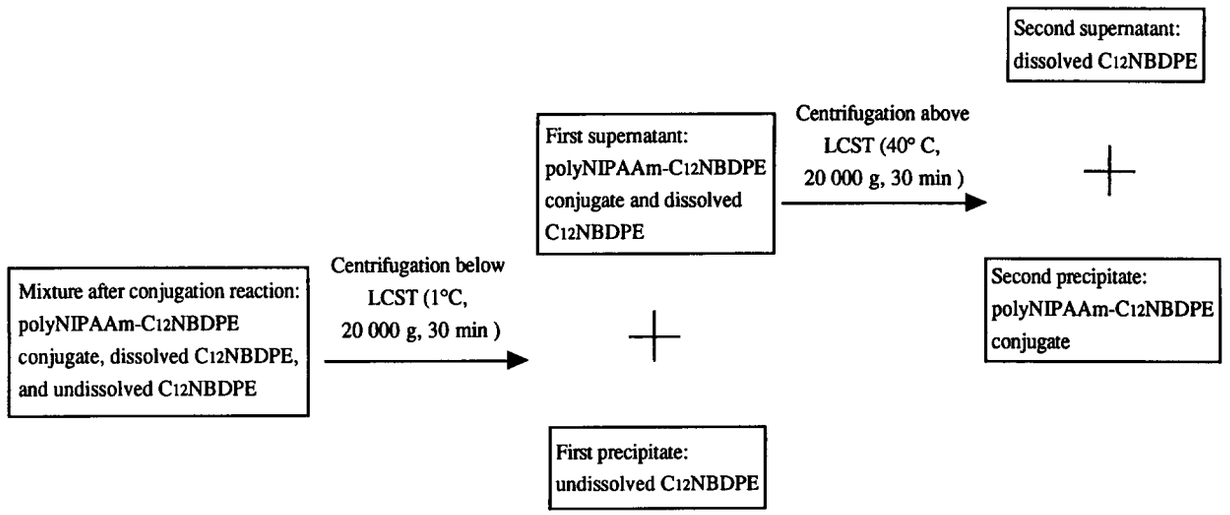

Figure 3 Schematic diagram of the separation of the polyNIPAAm–C_{12}NBDPE conjugate from unreacted C_{12}NBDPE (PL)

C_{12}NBDPE, pure polyNIPAAm-*co*-NAS and a mixture of polyNIPAAm and C_{12}NBDPE that had been mixed immediately before spotting. A mixture of chloroform, methanol and water (64:5:1 v/v) was used as the mobile phase and the plates were developed in iodine vapour.

The *LCST* of a 0.1 wt% solution of the polymer–PL conjugate in deionized water was determined spectrophotometrically by measuring the temperature dependence of its turbidity at 600 nm. The temperature was raised at a rate of 0.5°C min^{-1}. The *LCST* or cloud point is defined here as the temperature at which the transmittance at 600 nm drops to 90% of its value at room temperature (22°C).

Results and discussion

N-hydroxysuccinimide esters are excellent electrophilic reagents that can react with nucleophiles such as primary amines to give biologically stable amide linkages[20,21]. Therefore, *N*-hydroxysuccinimide esters have been widely used as active groups to conjugate proteins to synthetic supports through amide bond formation[4,10,22]. Conjugation of proteins to polymers has to be done in aqueous media such as buffers since proteins are usually denatured in organic solvents. Unfortunately, when *N*-hydroxysuccinimide esters are employed to conjugate proteins in aqueous media there is a competition between the reaction of *N*-hydroxysuccinimide esters with primary amines and hydrolysis of the *N*-hydroxysuccinimide ester group[22]. This competition limits the use of *N*-hydroxysuccinimide esters for this purpose. However, the conjugation of phospholipids with polymers through the *N*-hydroxysuccinimide esters need not be limited by competition with hydrolysis, since phospholipids dissolve in organic solvents such as chloroform. Phosphatidylethanolamine has a primary amino group that allows it to be coupled to polymers through the *N*-hydroxysuccinimide ester group, and both polyNIPAAm and phosphatidylethanolamine dissolve very well in chloroform. Therefore, *N*-hydroxysuccinimide esters can be widely employed to conjugate phospholipids to polymers.

Figure 3 schematically shows the separation of the polyNIPAAm–C_{12}NBDPE conjugate from the unreacted C_{12}NBDPE. *Table 1* gives the results of the fluorescence

Table 1 Fluorescence intensity of mother and daughter solutions centrifuged above the *LCST*

Components	Mother solution (free PL and polyNIPAAm–PL conjugate)	Supernatant (free PL)	Precipitate (redissolved) (polyNIPAAm–PL conjugate)
Fluorescence (relative intensity per ml solution)	60.5 ± 1.2	39.9 ± 3.1	26.4 ± 2.7

measurement of the polymer–PL conjugate and the unreacted C_{12}NBDPE. Since C_{12}NBDPE was in excess in the conjugating reaction, all the activated polyNIPAAm (polyNIPAAm-*co*-NAS) should have been converted to the polymer–PL conjugate. Therefore, in the hydrated sample there should have been only polymer–PL conjugate and free (unreacted) C_{12}NBDPE, which in turn occurred in two states: lipid precipitate and dissolved lipid. When the hydrated sample was centrifuged at temperatures below the *LCST* of polyNIPAAm, polymer–PL conjugate should have remained in the supernatant. When the supernatant was re-centrifuged above the *LCST* (40°C), the polymer–PL conjugate should have precipitated, but free C_{12}NBDPE and *N*-hydroxysuccinimide, which are by-products of the reaction, should have remained in the solution. Therefore, the precipitate of the second centrifugation must have been either polymer–PL conjugate or polyNIPAAm. A fluorescent precipitate must be polymer–PL conjugate because polyNIPAAm is not intrinsically fluorescent. *Table 1* shows that the second precipitate was indeed fluorescent. *Table 1* also shows that the sum of the fluorescent intensity of the solution of the second supernatant and the redissolved precipitate was nearly equal to that of the mother solution (first supernatant). The supernatant and the redissolved precipitate were diluted to the same volume as the mother solution used for the second centrifugation in order to compare their fluorescent intensity. Therefore, the results of *Table 1* indicate that PL has been conjugated to polyNIPAAm through either covalent bonding or physical association such as hydrogen bonding and/or hydrophobic interactions.

T.l.c. can distinguish whether the polymer–PL conjugate is a covalently bonded compound or a physical associate. *Figure 4* shows that the PL was in fact covalently bonded to polyNIPAAm. Lanes b and c have only one spot that is nearly at the same position as the polyNIPAAm-*co*-NAS control. But lane d, a control, in which only non-covalent bonding between polyNIPAAm and PL could occur, yields two spots. The upper spot must be polyNIPAAm-*co*-NAS (probably partially hydrolysed) since it is at the same position as the polyNIPAAm-*co*-NAS control. The lower spot is at the same position as the PL control. The unclear spot right below the PL spot in lane d may be *N*-hydroxysuccinimide. If the 'polymer–PL conjugate' were a physical associate, the lanes of the 'polymer–PL conjugate' (or lanes b and c) would have two spots instead of one.

It is believed that the *LCST* phenomenon for thermally phase-separating polymers in water is due to a hydrophilic-to-hydrophobic balance of the polymer structure[23]. Conjugation of water-insoluble PL molecules would be expected to increase the hydrophobicity of polyNIPAAm, which should, in turn, decrease its *LCST*.

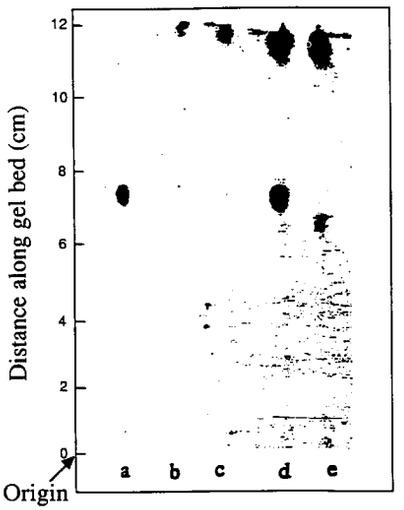

Figure 4 Thin layer gel chromatogram of polyNIPAAm–PL conjugate and related compounds on silica gel: (a) PL; (b) polyNIPAAm–PL conjugate (15 μg); (c) polyNIPAAm–PL conjugate (30 μg); (d) polyNIPAAm-*co*-NAS and PL mixed just before spotting; (e) polyNIPAAm-*co*-NAS

However, after incorporation of <4 mol% PL (calculated from the feed ratio) into polyNIPAAm, the *LCST* of the polyNIPAAm–PL conjugate increased by ~2°C to 35°C (*Figure 5*). The temperature range over which the polymer precipitated also broadened. The explanation may be that the head group of phosphatidylethanolamine is zwitterionic in water at pH 7 but, after conjugation to polyNIPAAm via an amide bond (*Figure 2*), only the negative charge of the phosphate group remains. The polymer therefore acquires a negative charge at every conjugation site. The PL used in this study (C_{12}NBDPE) has a fluorescent group on one of the hydrocarbon chains (*Figure 2*) that is more polar than a normal hydrocarbon chain. Therefore, conjugation of C_{12}NBDPE to polyNIPAAm probably slightly increases the net hydrophilicity of the polymer. A full interpretation of the small increase in *LCST* and the small reduction in the sharpness of the polyNIPAAm precipitation after conjugation with PL must await more thorough characterization of this conjugate.

Conclusions

Phospholipid was successfully conjugated to polyNIPAAm through *N*-hydroxysuccinimide active ester groups that were incorporated into the polyNIPAAm chain. Aqueous solutions of polymer–PL conjugates still have a *LCST*. The conjugation of PL to polyNIPAAm raises the *LCST* of polyNIPAAm ~2°C and decreases the sharpness of the precipitation. Conjugation of phospholipids with polymers in general and with thermally reversible polymers in particular may allow

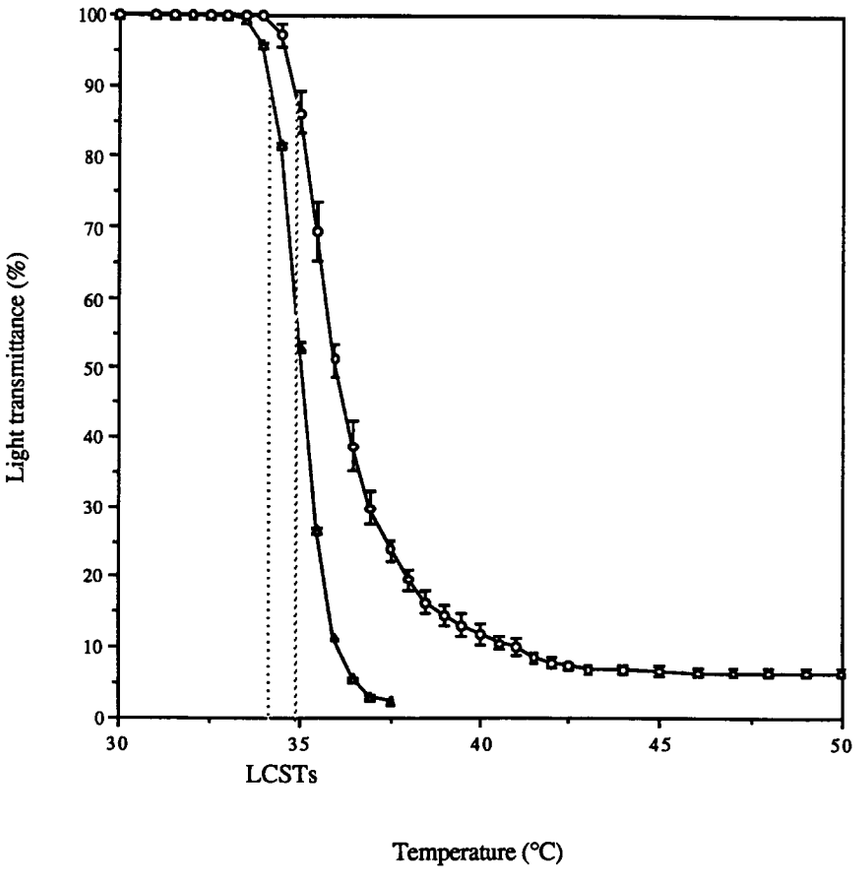

Figure 5 Temperature dependence of the light transmittance of aqueous solutions of polyNIPAAm (△) and polyNIPAAm–PL conjugate (○)

some novel applications involving insertion into liposomal or cell membranes and interfaces[17].

Acknowledgement

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