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Preparation and Evaluation of Radioiodinated Thermoresponsive Polymer Based on Poly(*N*-isopropyl acrylamide) for Radiotherapy

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ABSTRACT: A thermoresponsive polymer based on poly *N*-isopropylacrylamide (PNIPAM) was synthesized and radioiodinated to explore its potential use in localized radiotherapy. The synthesized PNIPAM polymer was functionalized with L-tyrosinamide to facilitate radioiodination. The content of tyrosinamide groups in the polymer was assayed spectrophotometrically ($\lambda = 275$nm). The functionalized polymer showed a cloud point temperature of 29–31°C and phase separation at 35°C, as revealed by Differential Scanning Calorimetry (DSC) and Dynamic Light Scattering (DLS). The phase transition temperature is conducive for preferential localization of the polymer at the site of injection due to changes in the polymer conformation at body temperature. For *in vivo* demonstration, the biodistribution studies of radioiodinated polymer were carried out in Swiss mice bearing fibrosarcoma tumor. Biodistribution studies showed a retention of 30% of the injected labeled polymer, PNIPAM-^{125}I-tyrosinamide, in the solid tumor tissues after 2 h of intratumoral injection. Although the activity decreased with time, 3–4% of the injected dose (i.d) was found to be retained in the tumor on 5 d post injection. The results suggest the potential use of thermosensitive polymer based on poly *N*-isopropylacrylamide for locoregional radionuclide therapy. © 2013 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 130: 860–868, 2013

KEYWORDS: biomedical applications; applications; drug delivery systems; light scattering; phase behavior

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INTRODUCTION

Thermoresponsive polymers are special class of polymers which undergo physical change when exposed to external thermal stimuli. The ability to undergo such changes under easily controlled conditions makes this class of polymers qualify in the category of "Smart Materials"[1]. The physical changes in the polymer upon external stimuli can be exploited for biomedical applications including drug delivery and tissue engineering.[2–4] There are many objectives for using polymeric carriers in drug delivery, viz. (a) To maximize the bioavailability of a therapeutic agent in a target tissue, (b) to optimize the onset, rate and duration of drug delivery, (c) to maximize the steady state plasma drug level within a therapeutic range as long as required for an effective treatment, and (d) to minimize adverse side-effects of a therapeutic agent on other organs. Amongst many polymers, poly *N*-isopropylacrylamide (PNIPAM) has generated wide interest among researchers due to its thermoresponsive nature. PNIPAM is a special kind of polymer which contains both, carbonyl and amide groups, along its long and hydrophobic backbone chain. In aqueous media, slightly crosslinked PNIPAM forms a soft gel, which can undergo a discontinuous, reversible volume phase transition in response to changes in temperature, salt content, solvent composition, pH and electric field. It is hydrophillic and water-soluble below its lower critical solution temperature (LCST) (~ 32°C), undergoing a reversible phase transition within a narrow temperature range to an insoluble aggregate above the LCST.[1–5] This transition has substantial importance from medical, technological, and scientific points of view. Drugs encapsulated within water-soluble thermoresponsive polymers based on poly (*N*-isopropylacrylamide), poly (*N*-isopropylmethacrylamide), elastin like peptides, substituted polyphosphazenes and similar polymers are increasingly gaining importance in both chemotherapy and radiotherapy of cancer.[6–12] The reversible phase transition properties of thermoresponsive polymer-based drug delivery systems allows the drug molecules to be targeted directly to tumor cells by direct intratumoral administration. The site specific delivery of anti-tumor agents reduces the negative side effects of intravenous applications, and

can overcome the multiple drug-resistant phenotypes. Regional drug delivery, however, will not be effective at treating distant sites or deep seated tumor metastasis unless the drug is targeted to each known site. Locoregional and intra-arterial radiotherapy or chemotherapy can avoid first-pass effect and permit a higher local concentration of the radiotherapeutic or chemotherapeutic agent in the tumor. More and newer drug delivery modalities for cancer therapy are an urgent need and towards this objective thermoresponsive polymers are widely investigated. Many other systems such as low-molecular weight prodrugs, liposomes, and micro or nanoparticles have also been developed[12–16] for targeted drug therapy, but many of these delivery vehicles have exhibited limited efficacy in actual patient trials.[12]

There is an unmet medical need for new targeted treatments for solid tumors. It is reported that approximately 60–84% of patients diagnosed with solid tumors develop bone metastases[17] as they do not get effective treatment at the first stage. The major obstacle in such cases is the poor accessibility through conventional drug administration routes such as i.v. path. Locoregional administration of therapeutic drug is an attractive option for accessible solid tumors and local application of radionuclide in synovectomy is well known.[18–20] In this context, a polymer carrying active chemical or radioisotope moiety has been pursued by different research groups over the past few years.[6–12,21–24] Additionally, labeling of various synthetic polymers with radionuclides is also gaining importance in life sciences research.[22–29] One of the attractive features of polymeric system is the possibility to synthesize a polymer coupled to a suitable moiety or chelator for facilitating radiolabeling with desired radioisotopes, which would have the additional advantage of delivering the therapeutic radioisotope or radiopharmaceutical at the site compared with the system based on physical property such as transdermal devices or osmotic pump deliveries. Local application of radionuclide-carrying thermoresponsive polymers with cloud point temperature between the room temperature and body temperatures offers a unique advantage for local radiotherapy, as the polymer can be radiolabeled and applied by injection at a target site in aqueous solution. At body temperature, it precipitates at the site of application. The radionuclide carried by the polymer thus remains at the application site and cause effective radiotherapy. The effective amount of radionuclide to be attached to polymer for required therapy would be extremely small (typically in nanogram scale) which is much lower than chemical drug (typically in milligram scale). Furthermore, the radionuclides attached to the polymer need not be released for exerting their action.[27,30–32]

The aim of this study is to exploit the combined properties of both, Poly (*N*-isopropyl acrylamide) (PNIPAM) and that of radioiodine. PNIPAM can undergo a sharp and reversible phase transition at body temperature, thus helping it to be retained at the site of injection, while radioiodine is known to be effective for treatment of cancer. Radioiodination of PNIPAM was facilitated by the introduction of L-tyrosinamide groups through an azo-initiator that couples both, PNIPAM through the availability of methyl group on one side, and L-tyrosinamide on the other side through $-N<$ group in the azo-initiator. This could now be radioiodinated conveniently to obtain PNIPAM-^{125}I-tyrosinamide polymer. The conformational change of the polymer above its LCST is conducive for passive targeting to tumors and is confirmed from biodistribution studies. Although the route of preparation of PNIPAM –L-tyrosinamide through the azo-initiator was originally shown by M. Hruby et al.,[25] its potential use as a therapeutic radiopharmaceutical was yet to be evaluated. Hence, in this paper, we describe the preparation and characterization of PNIPAM polymer coupled to L-tyrosinamide groups, its radioiodination procedure optimization and the biodistribution of the radioiodinated polymer in fibrosarcoma tumor bearing Swiss mice. Also, the procedure for the synthesis of the polymer and its radioiodination thus optimized can be scaled up for production purposes.

EXPERIMENTAL

Materials and Equipments

N-isopropylacrylamide (NIPAM), L-tyrosinamide, 4,4′-azobis (4-cyanopentanoic acid), thiazolidine-2-thione, 4-dimethylaminopyridine (4-DMAP), tetrahydrofuran (THF), dicyclohexylcarbodimide (DCC), were purchased from Sigma Aldrich (USA). NIPAAm was purified by recrystallization from benzene. Carrier-free Na^{125}I solution [Specific activity 15–17 mCi/μg, 100 mCi/mL (3700 MBq/mL)] was procured from Izotope, Hungary.

Dialysis tubing Spectra / Por 3 (Molecular weight cut off 3500Da) was purchased from Arthur H. Thomas, Philedelphia, USA. Sephadex G-25 was obtained from Pharmacia, Sweeden. Whatman 3 mm chromatography paper was purchased from Whatman, England.

Thermal degradation of the polymer was determined using MR-VIS from LABINDIA, India. IR spectrum and spectrophotometer used were from JASCO, Japan. Mettler Toledo DSC 822 was used for thermal measurements of the samples, with an empty aluminium pan as a reference at an external pressure of 180 kPa. Scans were performed from 0 to 350°C at heating and cooling rate of 10°C. Malvern 4800 Autosizer employing a 7132 digital correlator, coupled with 633 nm wavelength He-Ne laser at an output power of 15 mW as a light source was used to measure the diffusion coefficient and calculate the average molecular weight distribution of the polymer.

Methods

Synthesis of Azo-Initiator and Its Use for PNIPAM-L-Tyrosinamide Polymer Preparation. The synthesis of the polymer was carried out in three steps modifying the method described by M. Hruby et al.[25].

Step 1: Synthesis of ***azo-initiator:*** *(3, 3′-azobis (4-cyano-4-methyl-1-oxobutane-1-4-diyl) bis (thiazolidine-2-thione):*

The synthesis of the azo-initiator involved the coupling of thiazolidine-2-thione to 4, 4′ azobis (4-cyanopentanoic acid) using dicyclohexylcarbodimide (DCC) and 4-dimethylaminopyridine (4-DMAP) as the acylation catalyst. Nearly equal molar ratio of 4, 4′-azobis (4-cyanopentanoic acid) and thiazolidine-2-thione, and proportionately half the molar ratio of 4-DMAP, were dissolved in cold 20 mL THF. To this solution,

5 mL of DCC (~ 4 g) dissolved in cold THF was mixed and the reaction was carried out for 18–24 h with stirring at 4°C. The reaction mixture was acidified by the addition of glacial acetic acid (100 μL) to facilitate the precipitation of dicyclohexylurea which was filtered off and the filtrate was evaporated in vacuum to remove THF. The residue thus obtained was dissolved in dichloromethane and crystallized from a dichloromethane-diethylether mixture to obtain pure azo-initiator.

This azo-initiator thus synthesized was characterized by determination of melting point, elemental analysis, and molar absorption coefficient at 305 nm. IR spectrum was studied in KBr pellets to ascertain the structure of the azo-initiator.

Step 2: Synthesis of poly N-isopropylacrylamide (PNIPAM) polymer:

The azo-initiator (~ 100 mg) prepared in Step 1 and *N*-isopropylacrylamide (NIPAM) (500 mg) (1:5 ratio) were dissolved in 5 mL of THF and allowed to polymerize overnight at 60°C under nitrogen atmosphere using a balloon filled with nitrogen at the mouth of reaction flask. No purification of the polymer was carried out at this stage.

Step 3: Synthesis and Purification of the PNIPAM-L-tyrosinamide polymer:

L-tyrosinamide (180 mg) was added to the reaction mixture in step 2 and the mixture was stirred for another 3–4 h at 60°C. The mixture was filtered through a G-4 glass column under vacuum and the PNIPAM-L-tyrosinamide polymer was precipitated from the filtrate using diethylether.

The precipitate which contained the crude polymer was purified in two steps, initially through dialysis of the product. The product dissolved in a mixture of methanol: water (1 : 2) was transferred to spectra/por 3 dialysis tubing and dialyzed against fresh distilled water at room temperature, changing the water five times at 24-h intervals. The aqueous solution of partially purified polymer was passed through Sephadex G-25 column (40 cm × 1.5 cm) using distilled water as mobile phase to isolate the purified PNIPAM-L-tyrosinamide polymer. Fractions of 3 mL each were collected in tubes and those fractions which showed absorption at $\lambda = 275$ nm indicating the presence of the tyrosinamide groups were pooled. The aqueous solution was freeze dried (lyophilized) to obtain the pure PNIPAM-L-tyrosinamide polymer, which was then characterized.

Characterization of the PNIPAM-L-Tyrosinamide Polymer. Thermal degradation of the polymer was determined using a Visual Melting Range Apparatus MR-VIS, from LABINDIA, India. The purified polymer was characterized by IR spectra. The IR spectra were recorded using samples pellatized with KBr for azo-initiator and the PNIPAM-tyrosinamide polymer. Tyrosinamide content of aqueous solution of PNIPAM-L-tyrosinamide polymer was determined spectrophotometrically in comparison with the absorbance obtained for a standard solution of pure tyrosinamide at $\lambda = 275$ nm. The lower critical solution temperature (LCST) of the polymer was determined using Differential Scanning Calorimeter (DSC). Data was corrected for instrument response time and analyzed using the software supplied by the manufacturer. The polymer concentration of 3.4 mg/mL in water as well as 0.9% NaCl was used to study the effect of ionic strength on phase transition. The LCST transition of the polymer was also confirmed by DLS where the scattering intensity and the apparent size of the polymer particles with an increase in temperature were measured. Dynamic light scattering (DLS) measurements were carried out using Malvern 4800 Autosizer employing 7132 digital correlator and a He-Ne laser. A vertically polarised light at 632.8 nm is used as the source and the scattered intensity is measured at a scattering angle of 90°. Synthesized PNIPAM polymer is normally very broadly distributed with respect to molar mass. The distribution of the size or the average molecular weight of the purified polymer was thus determined by the diffusion coefficient of the polymer and this was estimated by DLS.

Radioiodination of PNIPAM-L-Tyrosinamide Polymer with $Na^{125}I$ and Its Purification [PNIPAM-^{125}I-Tyrosinamide Polymer]. Radiolabeling procedure of PNIPAM-L-tyrosinamide with ^{125}I using Chloramine-T as the oxidant and further its purification was optimized in-house.[33,34] A mixture of pure PNIPAM-L-tyrosinamide polymer dissolved in 0.05*M* Phosphate buffer, pH 7.4 [300 μL (10.5 μg)], 0.5*M* phosphate buffer, pH 7.4 (35 μL) and $Na^{125}I$ [10–20 μL (1.0 mCi/37MBq)] were taken in a clean and dry glass tube. Chloramine-T [10 μL (50 μg)] was added to this mixture and the radioiodination reaction was stopped after 15 min by the addition of sodium meta-bisulphite [10 μL (300 μg)]. All the reagents were dissolved in 0.05*M* phosphate buffer, pH 7.4. The radioiodination yield and specific activity of the tracer were determined by paper electrophoresis. A small aliquot of the reaction mixture was applied on Whatman paper No. 1 and the electrophoresis was run for 1.5 h in 0.025*M* phosphate buffer, pH 7.4.

The reaction mixture was purified by gel-filtration over a glass column (40 cm × 1.5 cm) of Sephadex G-25 to separate the unreacted iodide from the radiolabeled polymer. The column of Sephadex G-25 was pre-equilibrated with 1% bovine serum albumin (BSA) to block the non-specific sites of Sephadex. The reaction mixture was loaded on the column and eluted with 0.05*M* Phosphate buffer, pH 7.4. One mL fractions were collected in polystyrene tubes containing 0.1 mL (0.65 mg) of ascorbic acid solution (free radical scavenger). The radioactivity in each fraction was measured in a NaI (Tl) scintillation counter and the elution profile was constructed. The radiochemical purity of each fraction with high radioactivity content forming the elution peak was determined by paper electrophoresis. The fractions with high radiochemical purity were pooled together to obtain pure PNIPAM-^{125}I-tyrosinamide polymer, which was lyophilized and used for biodistribution.

Biodistribution of PNIPAM-^{125}I-Tyrosinamide Polymer. The usefulness of the synthesized radioiodinated PNIPAM polymer (PNIPAM-^{125}I-tyrosinamide) for application as a radiopharmaceutical for loco-regional administration was evaluated by carrying out biodistribution studies in tumor bearing mice. Fibrosarcoma tumor was developed by injecting ~ 10^6 tumor cells in 150 μL sterile normal saline into the left lateral thigh muscles of male Swiss mice (6–8 weeks age). The animals with tumor of ~ 1 cm diameter were selected for the study. Three animals were taken for each time point and for control.

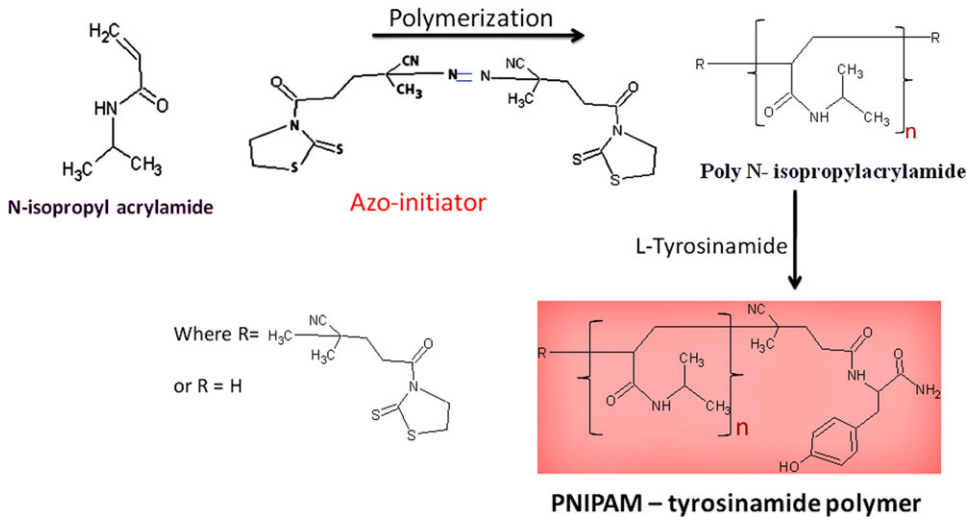

Figure 1. Diagrammatic representation of the synthesis of PNIPAM-L-tyrosinamide polymer. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

PNIPAM-^{125}I-tyrosinamide polymer [10 μCi/50 μL/animal] ($\sim$ 1,00,000 cpm/50 μL) was administered intratumorally to the mice with fibrosarcoma tumor. At 2 h, 1, 3, and 5 days post injection (p.i), the mice ($n = 3$) in each group were sacrificed under anesthesia, dissected and various organs were counted for radioactivity. The activity in blood, liver, intestine, stomach, heart, lungs, spleen, bone (tibia), muscles, kidneys, tumor (injected site), thyroid, excreta, and carcass were measured in a well type NaI (Tl) scintillation counter. In order to compare the *in vivo* deiodination of the radiolabeled polymer with that of plain iodide (Na^{125}I), a control group of mice ($n = 3$) with fibrosarcoma tumor were injected with Na^{125}I ($\sim$ 1,00,000 cpm/50 μL) intratumorally. These mice were sacrificed and dissected at 1d p.i. to evaluate the retention of injected activity in all the organs, mainly in that of the thyroid and tumor and compared them with that of the activity obtained in the experimental animals at 1d p.i. This study was primarily aimed at ascertaining that the radioiodinated polymer was less prone to *in vivo* deiodination and thus proving its *in vivo* stability.

All the animal experiments were performed in compliance with the national laws on animal ethics and welfare.

RESULTS AND DISCUSSION

Synthesis and Characterization of the Azo-Initiator (3,3′-azobis (4-cyano-4-methyl-1-oxobutane-1-4-diyl) bis (thiazolidine-2-thione) and Its Use in Preparation of PNIPAM-L-tyrosinamide Polymer

PNIPAM polymer with suitable groups for radioiodination could be prepared by the polymerization of *N*-isopropylacrylamide using the synthesized azo-initiator. The azo-initiator was prepared by acylation of thiazolidine-2-thione with 4,4′-azobis(4-cyanopentanoic acid) using DCC as the condensation agent and 4-DMAP as the acylation catalyst. The thiazolidine-2-thione activated carboxyl reactive groups serve as an acylation agent, reacting with amines in the presence of water and alcohols.[33,34] To avoid undesired hydrolysis of the thiazolidine-2-thione amides during separation and purification, the reactive polymeric intermediate was not isolated, but was immediately aminolyzed with tyrosinamide to a corresponding stable polymer amide.

Characterization of azo-initiator: Yield obtained: 1 g (30%), melting point: 128.3°C, Elemental analysis calculated/found: **C** 50.79/51.62, **H** 9/8.45, **N** 17.41/15.95, **S** 26.57/25.32. Molar absorption coefficient, ϵ_{305nm} = 28,641 L mol^{-1} cm^{-1} (methanol). IR spectra in KBr confirmed the presence of various functional groups, such as **C** $\equiv$ N (2243 cm^{-1}), C—H asymmetric stretching (2851–2944 cm^{-1}), >C=O (1715 cm^{-1}), —CH$_2$-CO-N< (1360−1180 cm^{-1}),

>N-CH$_2$-H stretching (2958−2944 cm^{-1}), —CH$_2$-CO- (1435−1405 cm^{-1}). This azo-initiator helps in obtaining a functional group, —CH$_2$-CO-N< (1380−1180 cm^{-1}) in the NIPAM polymer, where tyrosinamide moiety can be attached through an amide bond. The diagrammatic representation of the synthesis of the PNIPAM polymer, when NIPAM undergoes polymerization by the presence of azo-initiator, and further PNIPAM-L-tyrosinamide polymer preparation when reacted with L-tyrosinamide possessing phenolic —OH group, which is essentially required for carrying out radioiodination using Na^{125}I or Na^{131}I, are depicted in Figure 1.

Characterization of the PNIPAM-L-Tyrosinamide Polymer

The synthesized crude PNIPAM-L-tyrosinamide was obtained in $\sim$ 70% yield. Visual inspection of this polymer in aqueous solution at room temperature (25°C) and at body temperature (37°C) showed the formation of precipitate within seconds (Figure 2), which makes it an appropriate agent for its use as local radiotherapeutic application. The purification of crude polymer is an essential requirement for carrying out its radioiodination. Dialysis against distilled water ensures the removal of low-molecular weight impurities from the synthesized polymer. To remove any traces of free tyrosinamide groups present with the PNIPAM-tyrosinamide polymer, the partially purified polymer was passed through Sephadex G-25. The elution pattern of the purified PNIPAM-tyrosinamide polymer as a function of the

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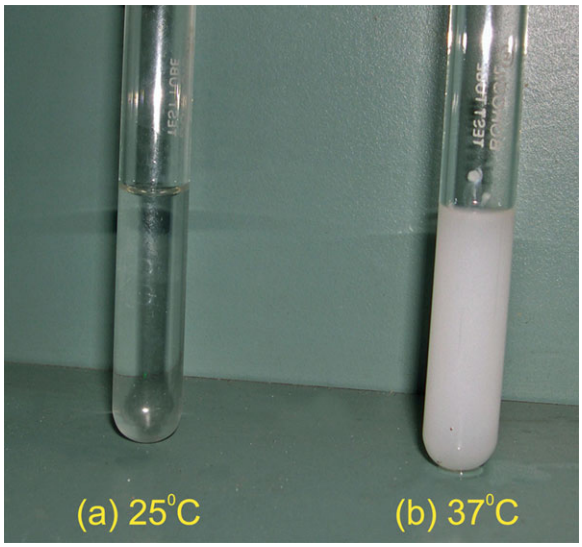

Figure 2. Visual Inspection of the PNIPAM-tyrosinamide polymer at room temperature and body temperature. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

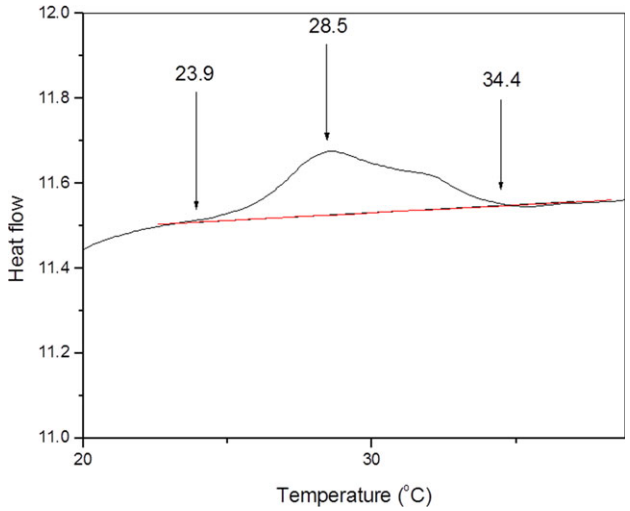

Figure 4. Differential scanning calorimetry (DSC) thermogram of PNIPAM-tyrosinamide polymer indicating the phase transition temperature. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

absorbance measured at $\lambda = 275$nm is shown in Figure 3. The content of tyrosinamide groups in the purified PNIPAM-tyrosinamide polymer fractions when pooled together and assayed as against the standard solution of pure tyrosinamide at $\lambda = 275$ nm was calculated as 35 μg/mL.

The thermal degradation of the polymer was found to be 175−178°C. Formation of PNIPAM-tyrosinamide of the polymer was ascertained by examining the FTIR spectra. Characteristic vibrational bands of amide bonds (-C=O stretching of amide – 1646 cm^{-1}, —NH of amide stretching – 3292 cm^{-1}), Phenolic —OH (3423 cm^{-1}), aromatic —CH of tyrosinamide group (3082 cm^{-1}), —CH_3 asymmetric and symmetric stretching (2973 cm^{-1} and 2934 cm^{-1}, respectively) can be seen in the IR spectra. These amide bonds between polymers and tyrosinamide (protein) are highly hydrolytically stable and anticipated to maintain blood stability of polymers. Also, possessing a tyrosine ring in the molecule facilitates the probability of the molecule getting radioiodinated. The phase separation behaviour of PNIPAM-tyrosinamide polymer was studied by DSC. The cloud-point temperature (CPT) of the polymer in water and in physiological solution (0.9% NaCl) was found to be 35.4°C and 34.4°C, respectively (Figure 4). This is in accordance with the effect of ionic strength on phase separation behaviour, wherein as the ionic strength increases from 0 (distilled water) to 0.15 mol L^{-1} (saline), the phase separation temperatures of PNIPAM decreases.[25] Dynamic light scattering measurements were carried out on aqueous polymer solutions and the intensity correlation function was analyzed by using the CONTIN algorithm to extract the diffusion coefficient distribution. Kubota et al., Fujishige et al., and Zhou et al.[33–35] carried out detailed light scattering studies on PNIPAM polymer solutions of different molecular weights and found that the diffusion coefficient of the polymer follows the relation, $D = K.M_w^a$; the value of K being 2.42×10^{-4} and a is $= -0.56$. Using the above values of K and a, the molecular weight distribution of the polymer was obtained. The average Mw of the PNIPAM-tyrosinamide polymer as estimated from the diffusion coefficient of the polymer by DLS was found to be ~ 48 kDa (Figure 5). This relationship between D and M, i.e. $D = K.M_w^a$, is used to characterize the polymer chains in the solution, where "a" is directly linked to the chain conformation. This linkage has been theoretically predicted and experimentally proven.[35] Phase separation that is exhibited by the polymer was also evident from the variation in the apparent or averaged hydrodynamic diameter of PNIPAM particles as calculated by Stokes-Einstein equation, $R_h = k_BT/6\pi\eta D$, where k_B, η and T are the Boltzmann constant, solvent viscosity and absolute temperature respectively and these were calculated by the software provided with the instrument. The turbidity (scattered intensity) and the apparent hydrodynamic diameter of PNIPAM-tyrosinamide polymer

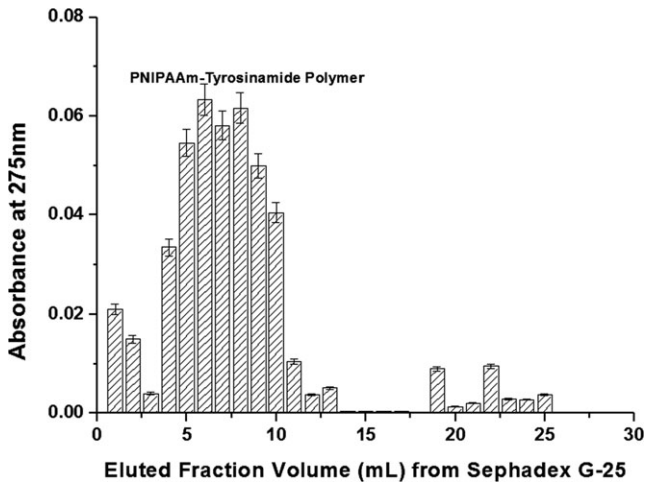

Figure 3. Elution pattern of Sephadex purified PNIPAM-tyrosinamide Polymer determined by Spectrophotometric measurement at $\lambda = 275$ nm.

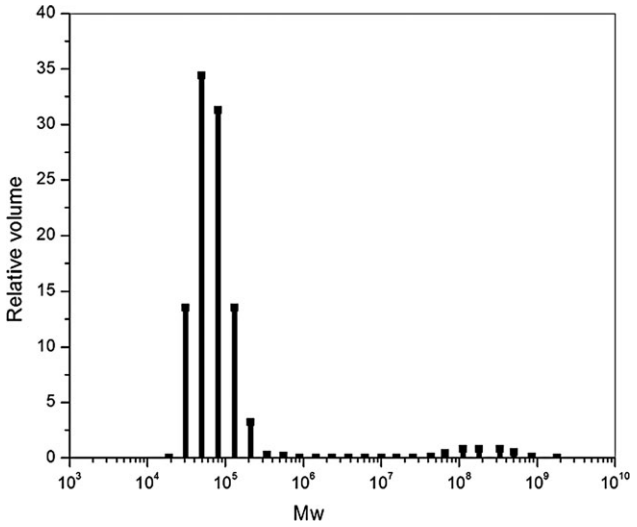

Figure 5. Average molecular weight distribution of PNIPAM-tyrosinamide polymer, as estimated from the diffusion coefficient of the polymer measured by DLS.

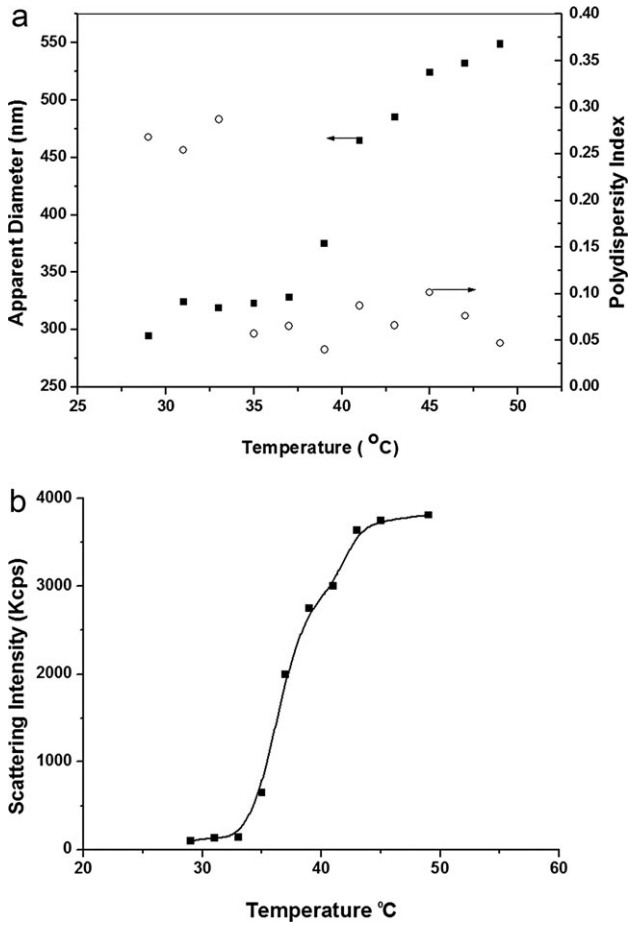

Figure 6. (a) Variation of apparent hydrodynamic diameter of PNIPAM-tyrosinamide polymer and PDI as a function of temperature. (b) Variation in the light scattering intensity of PNIPAM-tyrosinamide Polymer with temperature, indicating the phase transition.

Table I. Optimized Protocol for Radioiodinated of PNIPAM-Tyrosinamide (PNIPAM-^{125}I-tyrosinamide) Polymer

Reagents	Amount
Aqueous PNIPAM-L-tyrosinamide polymer	300 μL (10.5 μg tyrosinamide) (500 mg purified polymer)
0.5*M* Phosphate buffer, pH – 7.4	35 μL
$Na^{125}I$	1mCi
[a]Chloramine – T (oxidizing agent)	10 μL (50 μg)
[a]Sodium meta-bi-sulphite (reducing agent)	10 μL (300 μg)
[a]Potassium Iodide (carrier)	10 μL (100 μg)

[a]All the solutions prepared in 0.05*M* Phosphate buffer, pH-7.4.

particles (nm) increases drastically as the temperature increases above the CPT of the polymer [Figure 6(a)]. The variation of scattering intensity of the polymer as a function of temperature follows a sigmoidal behavior [Figure 6(b)]. It is observed that the polydispersity index (PDI) decreases significantly above the LCST and remains more or less constant. This change in PDI arises from the conformational changes of the polymer above its LCST where highly scattering colloidal particles are formed. Below LCST, the soluble form of the polymer drastically decreases the scattering intensity and hence contributions from any small fraction of large molecular weight polymers become significant. This results in an increase in the PDI of the formulation. These studies thus confirm the formation of precipitate at body temperature.

Radioiodination of PNIPAM-L-Tyrosinamide Polymer with $Na^{125}I$ and Its Purification to Obtain Pure PNIPAM-^{125}I-Tyrosinamide Polymer

Since the thermoresponsive polymer based on PNIPAM was synthesized because of its appropriate characteristics as carriers for radiodiagnostics/radiotherapeutics and since PNIPAM without suitable functional moieties cannot be directly radiolabeled, we introduced a phenolic moiety that can be radiolabeled into the polymer. Phenol is a highly activated aromatic moiety towards electrophilic substitution with iodine radioisotopes suitable for radiodiagnostics (^{123}I, ^{124}I) or radiotherapy (^{131}I) (Figure 2). Here, for experimentation, we radiolabeled the synthesized thermoresponsive poly (*N*-isopropylacrylamide)-L-tyrosinamide with ^{125}I by electrophilic substitution using by Chloramine-T[36,37] and used the product for evaluation in animal tumor model.

Optimized radioiodination procedure flow chart is shown in Table I. Paper electrophoresis of the radioiodinated but unpurified PNIPAM-^{125}I-tyrosinamide reaction mixture indicated a radioiodination yield of 82−85% [Figure 7(a)]. The figure shows two peaks. The first peak, which is at the point of spotting, is the purified PNIPAM-^{125}I-tyrosinamide. Unreacted free iodide, which is negatively charged moves to the anode and appears as

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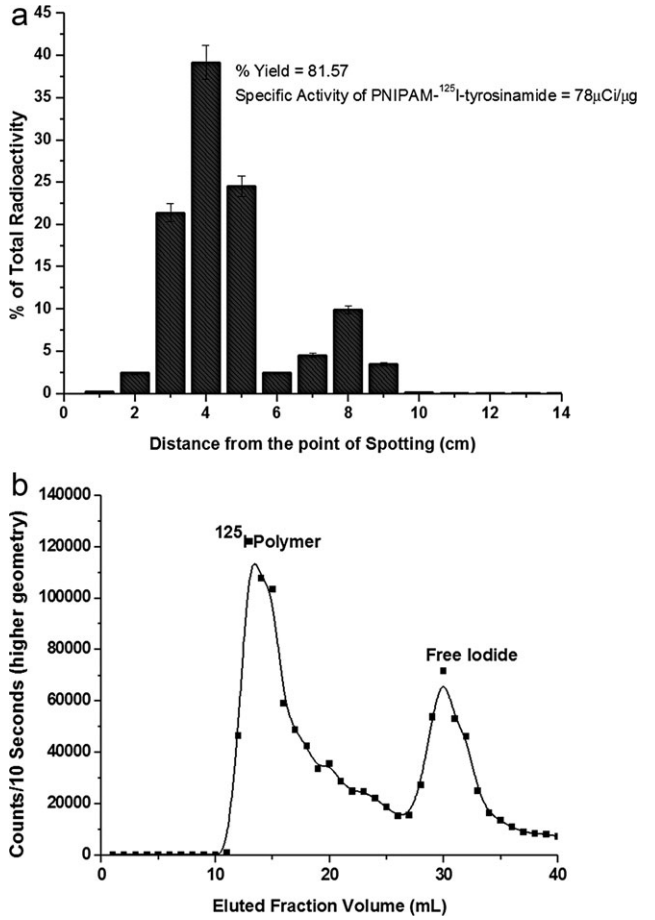

Figure 7. (a) Paper electrophoresis of reaction mixture PNIPAM-^{125}I-tyrosinamide polymer. (b) Elution pattern of PNIPAM-^{125}I-tyrosinamide polymer as a function of radioactivity.

the second peak. The specific activity of the radioiodinated polymer was calculated to 78−80 μCi/μg. On gel filtration through Sephadex G-25 using 0.05*M* phosphate buffer, pH–7.4, the purified radiolabeled polymer is separated from the unreacted iodide. A typical elution profile is shown in Figure 7(b). The first peak observed in the elution pattern indicates the pure PNIPAM-^{125}I-tyrosinamide, which can be subsequently pooled together and lyophilised till further use. The purification procedure based on gel-filtration thus standardized was highly reproducible, which could effectively separate the unreacted free iodide from the pure PNIPAM-^{125}I-tyrosinamide.

Biodistribution Studies of PNIPAM-^{125}I-Tyrosinamide Polymer

The percentage uptake of the injected radioactivity [% injected dose (i.d.)] for the various organs at different time points is given in Figure 8(a). It can be seen that the major clearance of radioactivity was through the kidneys and to some extent through hepatobiliary route. Less than 5% of i.d. was observed in thyroid at 5d p.i., indicating insignificant *in vivo* deiodination of the radiolabeled polymer, particularly when compared with the results obtained for radioactive iodide (Na^{125}I) injection (control mice), described later. Nearly 30% of the total activity associated with the radiolabeled polymer (injected dose) was retained in the tumor at 2 h p.i., which was due to the thermoresponsive property of the polymer. However, the % radioactivity in the tumor was observed to decrease with time. This could arise from the diffusion of unassociated PNIPAM from the site of injection to the aqueous cell fluid due to the concentration gradient or due to enzymatic degradation of polymer−protein conjugate. No other organs showed retention of radioactivity throughout the study.

Mice injected with only Na^{125}I (aqueous solution) intratumorally under identical experimental conditions were used as a control group. In this group of mice, accumulation of activity in the thyroid was much higher (∼ 28−30% of i.d.) in comparison with the PNIPAM-^{125}I-tyrosinamide treated animals at 1d p.i. The comparison of the biodistribution in thyroid and tumor mass of radioiodinated polymer and unlabeled Na^{125}I is shown in Figure 8(b). This figure showed that at 1d p.i., control mice

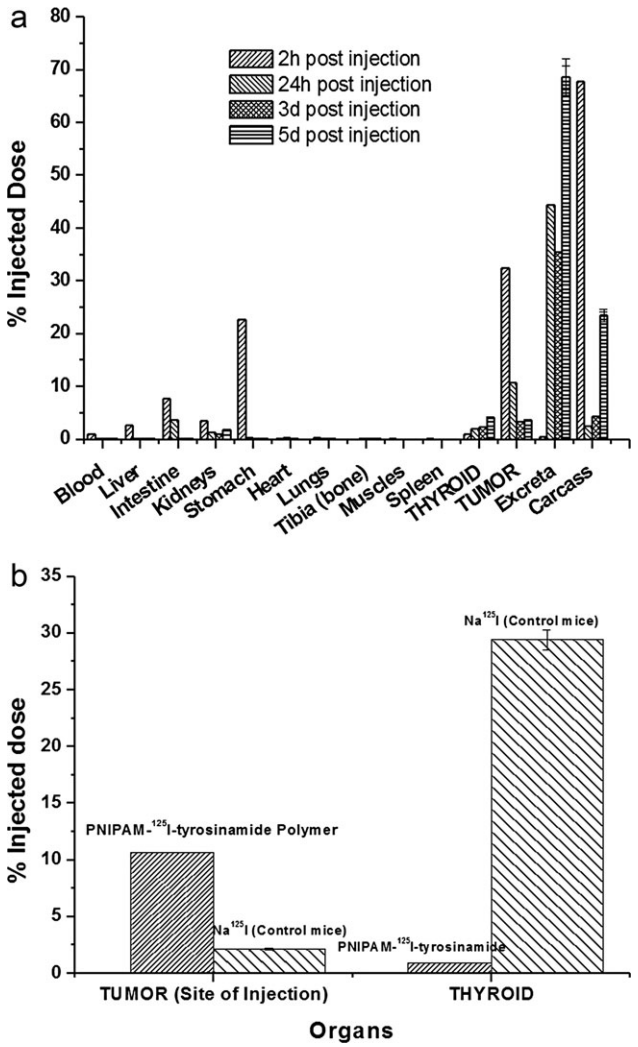

Figure 8. (a) Biodistribution of PNIPAM-^{125}I-PNIPAM polymer in mice at various organs. (b) Comparison of Biodistribution of PNIPAM-^{125}I-polymer and Na^{125}I at 24 h post injection in tumor (site of injection) and thyroid.

showed high radioactivity uptake in thyroid which is very low in the thyroid of animals injected with radioiodinated polymer mice (experimental mice), thereby supporting the findings of no significant *in vivo* deiodination of the polymer. Based on this, it may be inferred that the excretion of the radioiodinated PNIPAM polymer from the tumor observed at 1d p.i. might not be in the form of free iodide, but in the form of radioiodinated polymer diffusing from the tumor site. Such diffusion process is enhanced in low molecular weight polymers and could be controlled by using polymer particles of narrow polydispersity and improved aggregation characteristics which will be taken up in future studies. Literature showed the retention of polymers was enhanced by using organic solvents which might prove to be harmful to the animals. Our studies reflected the higher retention of the polymer in the tumor without using any harmful organic solvents in the experimental mice and thus the superiority of its potentiality of making it a promising radiopharmaceutical for solid tumors.

CONCLUSIONS

In this study, the radiolabeling and purification of an in-house synthesized thermoresponsive polymer and its usefulness in localized radiotherapy is demonstrated. The use of ^{125}I in this study can be replaced by ^{131}I for therapeutic application. The higher retention of the radiolabeled polymer observed at the tumor site when injected intratumorally clearly indicates the potential of this agent for use in localized tumor radiotherapy. The amount of free iodide leaching out of the tumor after probable enzymatic degradation of the radioiodinated polymer is very small. However, even this small leached activity leading to unsolicited exposure to the thyroid could be well taken care of by way of blocking the organ with "Lugols Iodine" solution when treating the solid tumor with radioiodinated polymer. The thermoresponsive polymeric particles described by us for direct loco-regional administration is an attractive approach, as this could particularly result in high therapeutic radiation dose at the tumor site. Such treatment modality could be a better choice for treatment of solid approachable tumors, for complete regression or even shrinking of the tumor, without the high systemic exposure or adverse side effects. Efforts towards increasing the retention property of this radioiodinated polymer would be our future scope of work.

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