

Developing a Non-Immunogenic and Biocompatible Polymeric Self-Assembly By Using RAFT Methodology for Therapeutics Application

Amit Kumar^{1,a}, Ashutosh Anand^{1,a}, Rishi Kant Singh¹, Praveen Kumar Verma¹, Surya Pratap Singh¹, Sandeep Kumar¹ and Arbind Acharya^{1*}

¹Cancer Immunology Lab, Department of Zoology, Institute of Science, Banaras Hindu University, Varanasi-221005, (UP), India.
kumaramitsoni.87@gmail.com, ashutoshanan@gmail.com, acharya@bhu.ac.in*

Abstract: Poly N-isopropylacrylamide (PNIPAM) is a precise thermoresponsive polymer, which is synthesized by reversible addition-fragmentation chain transfer (RAFT) polymerization method from monomer N-isopropylacrylamide (NIPAM) by a chain transfer agent (CTA) i.e. 4-cyano-4 ((dodecyl-sulphanylthiocarbonyl) sulphanyl)pentanoic acid. The polymerization of NIPAM follows pseudo-first-order kinetics and the linear evolution of the molar mass was obtained with the polydispersity index (PDI) 1.21 - 1.23 in dioxane. The synthesized RAFT polymer was fully characterized by Fourier-transform infrared (FTIR) spectroscopy, nuclear magnetic resonance (NMR) spectroscopy, and gel permeation chromatography (GPC) techniques. The ¹H NMR spectrum study confirmed the presence of chain-end functional groups on homopolymer. PNIPAM undergoes a reversible phase transition at low critical solution temperature (LCST) was about 32-33°C in aqueous solution. The cytotoxicity of PNIPAM was evaluated by Trypan blue exclusion and MTT assay against various normal cells such as macrophages, splenocytes, and thymocytes and found that the biocompatibility dose of PNIPAM is 200 mg/mL.

Index Terms: Low critical solution temperature, PNIPAM, Poly Dispersity Index (PDI), RAFT, Thermoresponsive polymer.

I. INTRODUCTION

Poly(N-isopropylacrylamide) (PNIPAM) is one of the most widely used temperature-sensitive polymers, which have been extensively studied in grafting on many kinds of surfaces due to its unique properties, such as its lower critical solution temperature (LCST) and the anti-fouling properties in normal

room temperature (Ebara et al 2007; Curti et al., 2005). PNIPAM undergoes a reversible phase transition state at low critical solution temperature (LCST) which is around 32-33°C in water (Gil and Hudson, 2004). At temperature above the LCST, intramolecular hydrogen bonding between (-CONH) groups in PNIPAM is dominant which results in the aqueous solution of PNIPAM is cloudy in appearance (phase separation occurred) and thus polymer becomes hydrophobic. In contrast, at temperatures below the LCST, the intermolecular hydrogen bonding between PNIPAM chains and water molecules is dominant, and therefore PNIPAM is hydrophilic (a homogeneous solution). Recently the properties of PNIPAM in aqueous media have attracted attention because of its scientific interest and applications to several biomedical fields (Schild et al., 1991; Crowther and Vincent, 1998; Hirotsu, 1988). PNIPAM is constantly copolymerized with other monomers and/or conjugate with other several biological molecules through weak mechanic properties, due to this nature of the polymer, it has been used in several therapeutic applications (Kim et al., 2001; Kondo et al., 1994; v et al., 1997; Yamato et al., 2000).

For the synthesis of polymers possessing a controlled molecular mass, structure, composition and low PDI within a desired range, there are several reversible-deactivation radical polymerization methods which are found to be accessible and dynamic, viz. reversible addition-fragmentation chain transfer (RAFT) (Moad et al., 2008; Mayadunne et al., 1999; Moad et al., 2005; Moad et al., 2000) polymerization, nitroxide mediated polymerization (NMP) (Hawker et al., 2001; Nicolas et al., 2013; Zamfir et al., 2013) and atom transfer radical

^a Authors have contributed equally to this work.

polymerization (ATRP) (Matyjaszewski, 2012; Boyer et al., 2011). Among these, the RAFT method of polymerization was found to be more applicable due to its nature of compatibility (Moad et al., 2008; Singh et al., 2015) with the monomers containing a larger range of functional groups such as an acid, amine, amide (or lactam), etc. Principles governing the reversible deactivation radical polymerization reaction include temporal termination or reversible deactivation, which arose from rapid equilibration reactions between the monomeric radicals and latent species (Thomas et al., 2012; Giz et al., 2001). Various types of polymers possessing a controlled molecular weight and low dispersity have been synthesized to date, which has a wide range of applications in the field of biotechnology, biomedical/ pharmaceutical technologies (Mori and Endo, 2012). RAFT methodology has become more practical due to the research work has been done by scientists in the polymerization area for different types of monomers. From the literature survey, it is not straightforward to control the polymerization of less active monomer because of propagating radicals of non-conjugated monomers which are highly reactive and have an affinity to undergo various side reactions during polymerization (Liang et al., 2014). Although *N*-isopropyl acrylamide has been polymerized successfully through RAFT methods, there are many conventional free radical polymerization (FRP) methods that are available today for synthesizing several controlled polymer. The popularity of PNIPAM in biomedical areas increases due to low toxicity, good solubility in aqueous as well as organic phases and biocompatibilities (Zelikin et al., 2016).

Given the above discussion, our present study reports the synthesis of PNIPAM with reversible-deactivation radical polymerization, in which 4-cyano-4 ((dodecyl-sulphanylthiocarbonyl) sulphanylpentanoic acid was used as a RAFT agent with controlled molecular weight and optimum PDI value. PDI is the ratio of weight-average molar mass (M_w) to number average molar mass (M_n) i.e. M_w/M_n . If the M_w/M_n is larger, then the molecular weight of the polymer will be broader. The polymerization of *N*-isopropylacrylamide (NIPAM) by the RAFT method follows pseudo-first-order kinetics with a PDI value of ≤ 1.21 . The cytotoxicity and biocompatibility of synthesized PNIPAM were tested on different normal healthy cells such as thymocyte, splenocyte and macrophage cells (Penit et al., 1986; Franken et al., 2015). Considering the above characteristics, our synthesized thermoresponsive polymer may be treated as a promising delivery vehicle for hydrophilic molecules (e.g. drugs). From the literature study, it can be conferred that the polymeric micelles may have the ability to carry several hydrophilic drugs as well as controlled release of the highly water-soluble drug at the target sites. Apart from this advantage, the synthesized polymer is found to be non-immunogenic, biodegradable and biocompatible.

II. EXPERIMENTAL SECTION

A. Materials

4-cyano-4 ((dodecyl-sulphanylthiocarbonyl) sulphanylpentanoic acid) (ECT) chain transfer agent was purchased from Sigma (Sigma-Aldrich, HPLC grade, 99.9%). 2,2'-Azobisisobutyronitrile (AIBN, Sigma, 98%) and *N*-isopropylacrylamide (Aldrich, 97%) was recrystallized from methanol and hexane, respectively. Dry solvent 1,4-dioxane (Aldrich), acetone (Sigma-Aldrich, HPLC grade, 99.9%), *n*-pentane (EMD), sodium chloride (EMD), hydrochloric acid (1N, Macron), anhydrous sodium sulfate (EMD), sodium carbonate (EMD), sodium bicarbonate (EMD), anhydrous dimethyl sulfoxide (DMSO) (Sigma), phosphate-buffered saline (PBS) (Sigma), phosphate-buffered saline tween 20 (PBST) (Sigma), SureBlue TMB 1-Component Microwell Peroxidase Substrate (KPL), SuperBlockR PBS (Thermo Scientific), GelCode Blue Safe Protein Stain (Thermo Scientific), high sensitivity Streptavidin- HRP (Thermo Scientific), $CDCl_3$ (Cambridge Isotope, 99% D) were used as received, unless otherwise noted. RPMI 1640 culture medium was purchased from HiMedia, Mumbai, India and Fetal Bovine Serum (FBS) were obtained from Invitrogen, Grand Island, NY, USA. MTT (3-(4, 5-dimethylthiazol 2-yl) -2,5-diphenyltetrazolium bromide) and All other solvents have been purified by a conventional procedure. All other reagents were obtained from Sigma-Aldrich and used without further purification unless otherwise noted.

B. Method

The reaction parameters have been calculated by using the following formulae (Moad et al., 2006):

$$\text{Conversion (NMR)} = \frac{A(\text{polymer})}{A(\text{polymer}) + A(\text{monomer})} \dots (1)$$

For the kinetic study, the conversion of monomer was carried out by 1H NMR spectral analysis of crude sample taken at a different time gap. The calculation of monomer conversion was done by comparing the peak of the monomer against the peak area of the repeating unit of the polymer. The theoretical number average molar mass (M_n) of the polymer can be calculated with the help of Eq. 1 and 2.

$$M_n(\text{NMR}) = M(\text{RAFT}) + M(\text{NIPAM}) \times \frac{NIPAM_0}{RAFT_0} \times \text{Conversion(NMR)} \dots (2)$$

Where $A_{(\text{monomer})}$ and $A_{(\text{polymer})}$ are the peak area of monomer and polymer of the same protons of the crude sample. $M_{(\text{NIPAM})}$ and $M_{(\text{RAFT})}$ are the molecular mass of monomer and RAFT agent and $NIPAM_0$ and $RAFT_0$ are the initial concentration of monomer and RAFT agent, respectively.

1) Synthesis of poly(*N*-isopropyl acrylamide) macro chain transfer agent (PNIPAMmacroCTA)

RAFT polymerization of NIPAM was done in 1,4 dioxane under a nitrogen atmosphere at 60°C. Inhibitor-free crystal of NIPAM (1.135 g, 10.0 mmol), CTA (20 mg, 0.05 mmol), a magnet stir and drysolvent 1-4 Dioxane (15 mL), was added to a three-neck round-bottom flask fitted with a rubber septum under continuous purging of nitrogen gas for 35 min, then initiator 2,2'-azobisisobutyronitrile (0.4 mg, 2.25 µmol) was added to the round-bottom flask. Further, the solution was deoxygenated by three freeze-pump thaw cycles under a nitrogen atmosphere. The molar ratio of monomer to chain transfer agent to initiator was 200:1:0.05. Furthermore, the reaction mixture was placed in a preheated paraffin oil bath at 60 °C. An aliquot of the reaction mixture was taken out from the flask time to time and was analyzed through ¹H NMR for calculation of % conversion and gel permeation chromatography (GPC) was conducted for determination of molecular weight determination & PDI. After 12 h, the reaction was stopped by rapid cooling with liquid nitrogen, when the reaction mixture was cooled, diluted with 1-4 dioxane and the resulting solution was precipitated by adding a drop wise adequate amount of n-pentane with vigorous stirring to obtain the precipitate of the polymer. This precipitate was redissolved in a small amount of 1, 4dioxane/acetone and precipitated again. This process was repeated four (x4) times to get pure poly (*N*-isopropylacrylamide), dried at 48°C under vacuum for 48 h, pure poly(*N*-isopropylacrylamide) was obtained with the conversion of 80.0%. GPC: Mn = 16700 g/mol with Mw/Mn =1.21.

III. CHARACTERIZATION

A. FT-IR Spectroscopy and FT-NMR

Fourier transform infrared (FT-IR) spectra were recorded by making pellets of synthesized polymer in KBr/neat using Varian Excalibur 3100 spectrometer (Palo Alto, CA). ¹H NMR spectra were recorded on a JEOL AL500.159 MHz NMR at 25°C in CDCl₃ and chemical shifts were reported in part per million (ppm) from internal reference tetramethylsilane (TMS). The NMR spectra were plotted by using Delta™ 5.0.5 NMR Data Processing Software provided by the JEOL, Ltd.

B. Gel permeation chromatography (GPC)

Absolute molecular weights and molecular weight distributions were determined via Gel permeation chromatography (GPC) which was performed on a Younglin ACME 9000 in THFat 40°C with a flow rate of 1.0 mL/min on two polystyrene gel columns. (PL gel 5 µm 10E 4Å columns (300 ×7.5 mm²)) connected in series to Younglin ACME 9000 Gradient Pump and Younglin ACME 9000 RI detector. The columns were calibrated against the PMMA standard samples (Polymer Lab, PMMA Calibration Kit, S M2-10).

IV. ANIMAL MODEL

Pathogen-free inbred populations of H-2d strain of *Mus musculus* (BALB/c) of either sex (male or female) and 2-3 months of age were used. All animals were kept in conventional cages on sterilized bedding and given food and water ad libitum. All animals were inspected daily for survival and maintained with the utmost care, under the guidelines of Animal Ethical Committee, Banaras Hindu University, Varanasi.

V. CYTOTOXICITY MEASUREMENT

A. Primary Cell Culture and synthesized polymer exposure

Mice colonies in suitable ambience were maintained in the animal house of Zoology Department, BHU as per the guidelines of Animals Ethical Committee, Banaras Hindu University. Normal mice of 8-12 week were euthanized through cervical dislocation; different types of cells were isolated from mice for the experimental purpose. After injecting Phosphate Buffer Saline (PBS) into the peritoneal cavity of mice, normal cells (like macrophage, splenocytes, and thymocytes) were harvested and washed with PBS. Isolated cells were adhered on Petri-dish and supplemented with Fetal Bovine Serum (FBS), after incubation, non-adherent cells were washed. The adherent cells which are macrophages was collected. Thymus and spleen tissues were removed after dissection of normal mice after that single-cell suspension was prepared by using homogenizer and debris was removed by passing the cells through a strainer (70 micron). Collected cells were washed with chilled PBS. All the isolated cells (macrophage, thymocytes, and splenocytes) were incubated with synthesized polymer and cultured in RPMI-1640 medium supplemented with 10% FBS, streptomycin 100 U/mL, and penicillin 100 µg/mL in 5% CO₂ at 37°C. For treatment conditions, macrophage, splenocytes, and thymocytes cells were treated with different concentrations of synthetic polymer (25, 50, 100, 200, 400, 800 and 1000 mg/ml) for cytotoxic activity by using different parameters (Tomar et al., 2018).

B. Trypan Blue Exclusion Assay

To examine the level of cytotoxicity, Trypan blue exclusion assay was carried out to examine the cytotoxic effect of synthesized polymer. Trypan blue dye is commonly used for staining the dead cells in a population of cells suspension to determine the cytotoxicity. Live cells having intact cell membranes, permit the uptake of some compounds that pass through the membrane while it excludes the trypan blue dye. The cytotoxicity of the synthesized polymer was analyzed by using Trypan blue exclusion dye. After isolation of 1 × 10⁶ viable normal cells (macrophages thymocytes, and splenocytes) were cultured in culture media (RPMI 1640 and 10% PBS) containing different concentrations of (25, 50, 100, 200, 400, 800 and 1000 µg/ml) polymer at 37 °C for 6 hours duration. After incubation, cells were collected and an equal amount of trypan blue dye was added. Thereafter, dead and live cells were counted by

hemocytometer under an inverted light microscope and the result was expressed as a percentage of live cells (Strober, 2001). The percentage of live cells was calculated using the following formula:

$$(\% \text{ of Live Cells}) = \frac{\text{Number of Live cells}}{\text{Total Number of cells}} \times 100$$

C. MTT assay

MTT is colorimetric assay in comparison to trypan blue because it is very easy to use, very safe, has high reproducibility and is widely used in determining the cell viability. Besides membrane integrity, cell viability together with its proliferation can also be monitored by using MTT assay. For the assessment of cytotoxic behavior of synthesized polymer (NIPAM) on different types of normal cells (thymocytes, splenocytes, and macrophages) was further evaluated for 24 hours by using the MTT assay. To perform the colorimetric assay, different types of normal cells were seeded into 96-well round-bottom culture plates at 4×10^4 cells/200 μ l per well in triplicate. The medium was removed after 24 h of incubation and fresh complete culture media containing different concentrations of polymers (25 to 1000 μ g/ml) were added. Thereafter, 10 μ l of MTT (3-(4, 5-dimethylthiazole-2-yl)-2, 5 diphenyltetrazolium bromide) solution was added to each well of the culture plate. Formazan crystals were observed after incubation with MTT in culture plates at 37°C for 4 h due to reaction with succinate dehydrogenase enzyme present in live cells. Further, 100 μ l DMSO was added to each well to dissolve the formazan crystals. The absorptions were read at 540 nm in ELISA reader and results were recorded as percentage absorbance relative to untreated control cells (Kumar et al., 2016). The viability of cells was calculated with the help of following formula:

$$(\% \text{ Cell Viability}) = \frac{\text{Mean OD}(\text{sample})}{\text{Mean OD}(\text{Control})} \times 100$$

D. Statistical Analysis

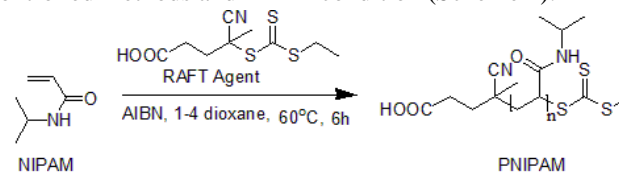
Each value represents the mean \pm standard error of the mean (SE_M) of three independent experiments in each group except for an *in vivo* stimulation experiment where two independent experiments were conducted. Data were analyzed by using a two-tailed student's t-test on statistical software package Sigma Plot, version 10.0. Differences were considered statistically significant at $p < 0.05$.

VI. RESULT AND DISCUSSION

A. RAFT polymerization of NIPAM

NIPAM monomer was first polymerized through RAFT polymerization using 4-cyano-4 ((dodecyl-

sulphonylthiocarbonyl) sulphonylpentanoic acid) as CTA under controlled methods and RDRP condition (Scheme 1).

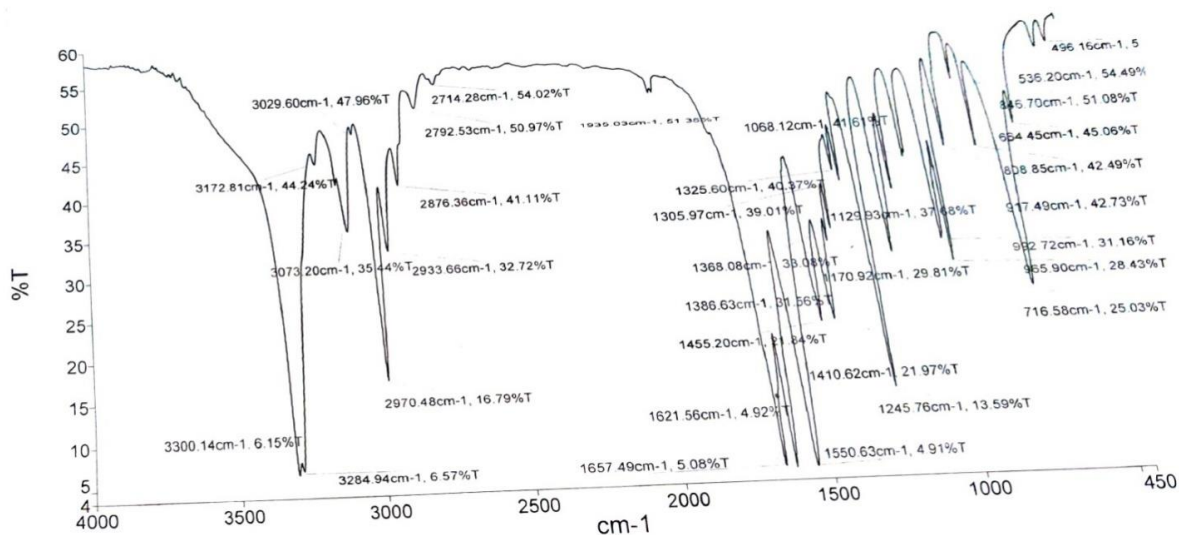


Scheme 1. Synthetic pathway of PNIPAM by using 4-cyano-4 ((dodecyl-sulphonylthiocarbonyl) sulphonylpentanoic acid) as RAFT agent in 1, 4dioxane at 60 °C RAFT polymerization method

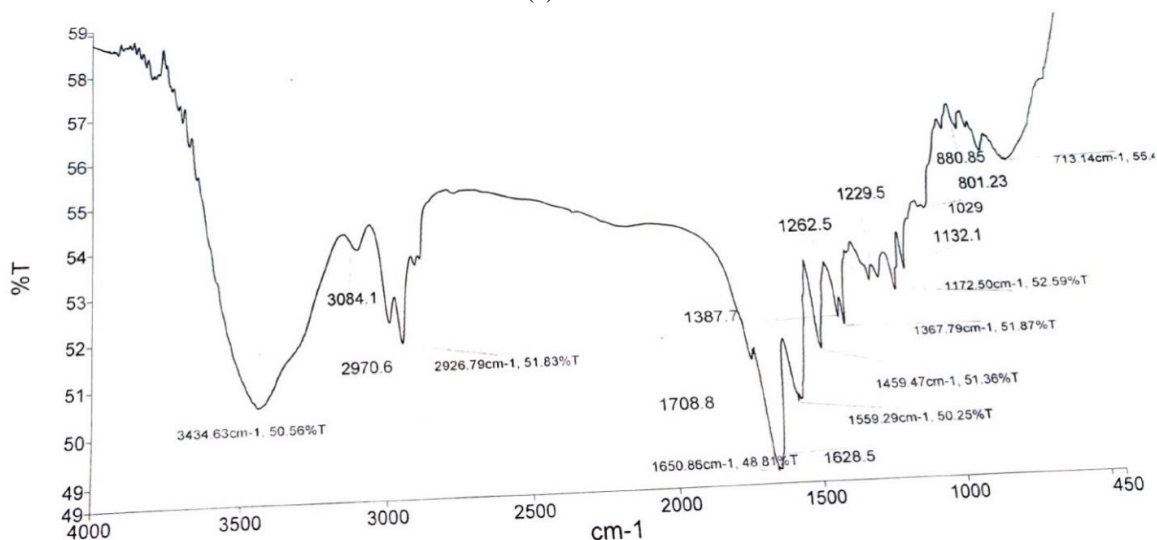
The polymerization of *N*-isopropyl especially NIPAM is very tough in a controlled manner using other reversible-deactivation radical polymerization techniques such as ATRP, NMP. The CTA i.e. 4-cyano-4 ((dodecyl-sulphonylthiocarbonyl) sulphonylpentanoic acid) has provided good control on polymerization of the NIPAM monomer. The RAFT polymerization achieved in the presence of AIBN initiator in dioxane with the (RAFT/AIBN) ratio = 2 and (NIPAM)/(RAFT) ratio = 200 at 60 °C. In ongoing polymerization, polymer samples were taken out and quenched at regular time intervals for ¹H NMR and GPC analyses for studying structure, conversion and molecular weight of PNIPAM.

The FTIR spectra of NIPAM monomer and PNIPAM (KBr/ neat, cm⁻¹) are illustrated in Fig 1. An important peak was obtained in the IR spectrum of NIPAM which is due to C=C stretching at around 1621 cm⁻¹ which disappears upon polymerization. Its disappearance is not that evident as upon polymerization a new broad peak appears around 1638 cm⁻¹ due to amide carbonyl group stretching. The main characteristic peaks of the polymer were found at around 1459, 1559, and 1628 cm⁻¹ in the spectrum of PNIPAM which are attributed to -CH₃ (bend.), C-N (stretch.), and C=O (stretch.), respectively (Katsumoto et al., 2002).

The successful RAFT polymerization of NIPAM was further confirmed by ¹H-NMR spectroscopy (Fig.2), which shows the assignment of proton signals according to the polymer structure shown in the inset of the figure (Sun et al., 2013; Schonhoff et al., 2002). A broad signal peak is obtained at 6.391 (e) ppm, which show the presence of proton of -NH group. Signals peaks at δ 3.9-4.06 (d), 3.7 (b), 2.0-2.16 (a), 1.5 (c) ppm are attributed to the CH group (CH close to C=O), CH group (CH close to -NH), CH₃ group and CH₂ group respectively, presented in the polymer chain. Also, there are absence of alkene proton at the range of 5-7 ppm in NMR spectrum which confers that the formation of PNIPAM. All the above frequencies confirm the successful polymerization of NIPAM into PNIPAM *via* RAFT methodology.



(a) NIPAM



(b) PNIPAM

Fig 1. FT-IR spectra of NIAPM (a) and PNIPAM (b)

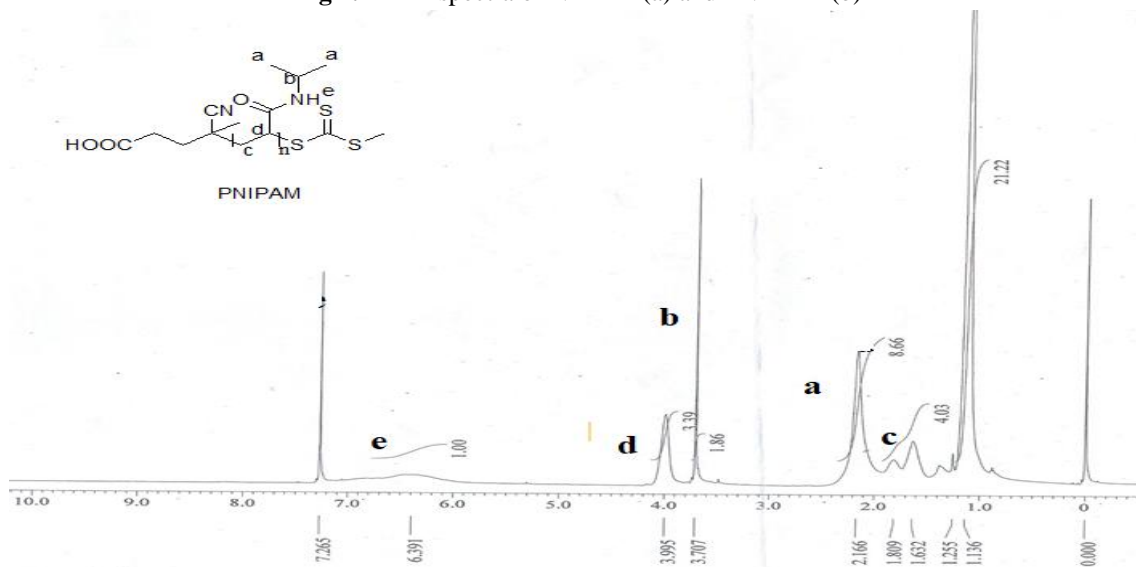


Fig 2. ¹H NMR spectrum of PNIPAM in CDCl₃

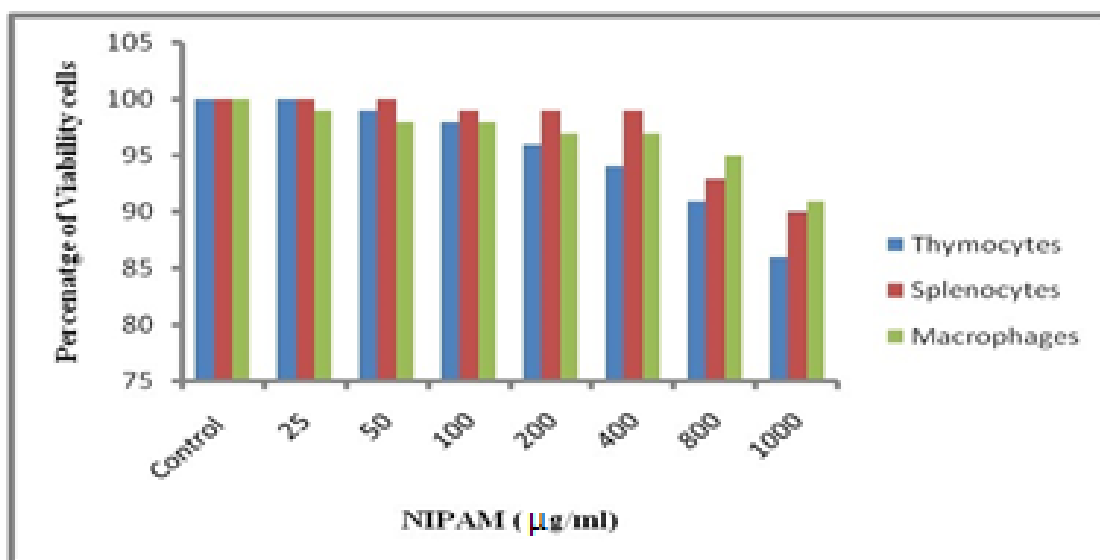


Fig. 3. Trypan blue exclusion assay. Normal cells were treated with different concentrations of synthesized polymer (25, 50, 100, 200, 400, 800, and 1000 µg/mL) and incubated in a CO₂ incubator for 6 h.

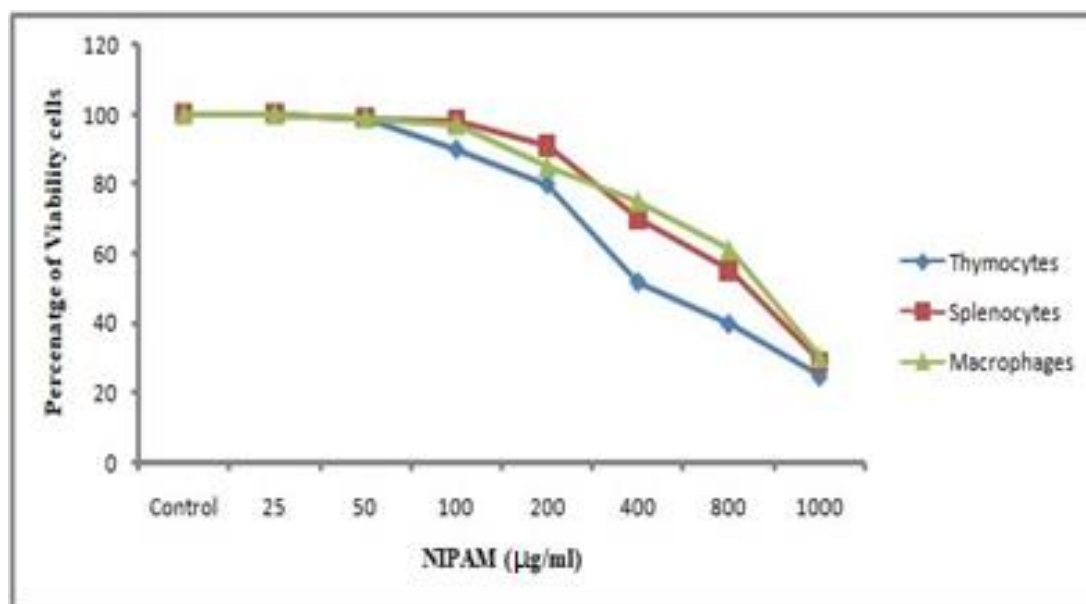


Fig 4. The cytotoxic effect of the polymer (PNIPAM) on thymocytes, splenocytes, and macrophages after 24 h of incubation. Cytotoxicity was determined by MTT assay and results are expressed as a percent viable cell concerning control.

B. Non-toxic, biocompatible behavior of polymer

1) Trypan Blue Method

To examine the non-toxic, biocompatible properties of synthesized Raft polymer by using a trypan blue assay. Normal cells (thymocytes, splenocytes, and macrophage) are seeded and treated with different concentrations of newly synthesized polymer (25, 50, 100, 200, 400, 800, and 1000 µg/ mL) for 6 h. The result observed that nontoxicity was up to 800 µg/mL treatment of polymer (Fig. 3).

Our result showed that the polymer up to 200µg/mL concentration treatment showed negligible toxicity on thymocytes, splenocytes, and macrophage cells. Further, we found that, after 200µg/mL to 400µg/mL concentration of treatment, toxicity for thymocytes is 5% while for the splenocytes and macrophage cells, almost 100% viability was observed. After 400 µg/mL to 800 µg/mL concentration of biocompatible polymer, the thymocytes showed toxicity 7%, while for the other two types of cell the toxicity was less than 5%. Further study confirmed that a higher concentration of

polymer treatment (1000 µg/mL) showed a reduction in cell viability of around 15% for thymocytes, while for splenocytes and macrophages showed toxicity 10% and 8%, respectively.

2) MTT Colorimetric assay Method

Cytotoxicity after 24 h incubation of PNIPAM was measured to access the biocompatibility effect on thymocytes, splenocytes and macrophage cells. The polymer used in this study did not show any cytotoxic effect on normal cells. In a brief description, in our case, we studied after 24-hour exposure of cells with the developed thermoresponsive polymer. The result after 24 hours unfolded level of toxicity for polymer, up to 50 µg/mL concentration of polymer, all three types of above cells showed 100% cell viability. Further, after 50 µg/mL to 100 µg/mL concentration of polymer showed the 20% toxicity for thymocytes, while for splenocytes and macrophages approx 100% cell viability was observed.

Furthermore, beyond 100 µg/mL concentration, the polymer showed toxicity for all three types of cell viz. for thymocytes, splenocytes and macrophages toxicity was 40%, 15%, and 25%, respectively. Besides, the half-maximum inhibitory concentration (IC50) value of PNIPAM against different types of normal cells was evaluated and was found to be >400 ~200, and >400 µg/mL for macrophages, thymocytes, and splenocytes respectively, Table I. The MTT results showed the measure of cytotoxic effect of polymer on various kinds of cells that can be used in a drug delivery system safely. The percentage of cell viability as compared to the concentration of PNIPAM is presented in Fig. 4. The result shows that the PNIPAM did not induce a significant cytotoxic effect even at a higher concentration of the used synthetic polymer. These findings suggest that synthesized polymer has biocompatibility in nature.

Table I: IC50 values of synthetic polymer against macrophages, thymocytes, and splenocytes after incubation of 24h.

| Type of Cells | Macrophages | Thymocytes | Splenocytes |
|--|-------------|------------|-------------|
| Incubation time (24 h) IC50 (µg/mL) | >400 | ~200 | >400 |

CONCLUSION

The thermal sensitive polymer (PNIPAM) has been effectively synthesized by the RAFT method using new 4-cyano-4 ((dodecyl-sulphanylthiocarbonyl) sulphanylpentanoic acid) acting as RAFT agent in the presence of a small quantity of a prevalent radical initiator AIBN and monomer. The conversion and reaction time decides the controlled molecular mass of the PNIPAM. The presence of the end group in the polymer chain

was confirmed by ¹H-NMR. The cytotoxic study confirmed that the treatment of a higher concentration of polymer shows no cytotoxic effect on various normal cells. Thus our findings justify the use of this Raft polymer safely in biomedical applications. In the future, we will design nano-scaled PNIPAM particles containing a carboxylic group at the terminal end by introducing an acrylic acid monomer. These carboxylic groups were applied to conjugate with the amino group of the specific antibody which has been used in selectively binding of targeted cell surface for biomedical application.

ACKNOWLEDGMENT

The authors are grateful for the financial support received from UGC, New Delhi, Govt. India in the form of Junior Research Fellowship (UGC-JRF) to Amit Kumar and DST-SERB (P-07/705) fellowship to Ashutosh Anand. Authors are thankful to Prof. B. Ray and Central facility of Chemistry Department for providing help to access the GPC, IR and NMR.

REFERENCES

- Boyer, C., Soeriyadi, A. H., Zetterlund, P. B., & Whittaker, M. R. (2011). Synthesis of complex multiblock copolymers via a simple iterative Cu (0)-mediated radical polymerization approach. *Macromolecules*, 44(20), 8028-8033.
- Chen, G., Ito, Y., & Imanishi, Y. (1997). Regulation of growth and adhesion of cultured cells by insulin conjugated with thermoresponsive polymers. *Biotechnology and bioengineering*, 53(3), 339-344.
- Crowther, H. M., & Vincent, B. (1998). Swelling behavior of poly-N-isopropylacrylamide microgel particles in alcoholic solutions. *Colloid and Polymer Science*, 276(1), 46-51.
- Curti, P. S., de Moura, M. R., Veiga, W., Radovanovic, E., Rubira, A. F., & Muniz, E. C. (2005). Characterization of PNIPAAm photografted on PET and PS surfaces. *Applied surface science*, 245(1-4), 223-233. (3) Gil, E. S.; Hudson, S. M. *Prog. Polym. Sci.* 2004, 29, 1173-1222.
- Ebara, M., Hoffman, J. M., Stayton, P. S., & Hoffman, A. S. (2007). Surface modification of microfluidic channels by UV-mediated graft polymerization of non-fouling and 'smart' polymers. *Radiation Physics and Chemistry*, 76(8-9), 1409-1413.
- Franken, L., Klein, M., Spasova, M., Elsukova, A., Wiedwald, U., Welz, M., & Kurts, C. (2015). Splenic red pulp macrophages are intrinsically superparamagnetic and contaminate magnetic cell isolates. *Scientific reports*, 5, 12940.
- Gil, E. S., & Hudson, S. M. (2004). Stimuli-responsive polymers and their bioconjugates. *Progress in polymer science*, 29(12), 1173-1222.
- Giz, A., Catalgil-Giz, H., Alb, A., Brousseau, J. L., & Reed, W. F. (2001). Kinetics and mechanisms of acrylamide

- polymerization from absolute, online monitoring of polymerization reaction. *Macromolecules*, 34(5), 1180-1191.
- Hawker, C. J., Bosman, A. W., & Harth, E. (2001). New polymer synthesis by nitroxide mediated living radical polymerizations. *Chemical reviews*, 101(12), 3661-3688.
- Hirotsu, S. (1988). Critical points of the volume phase transition in N-isopropylacrylamide gels. *The Journal of chemical physics*, 88(1), 427-431.
- Katsumoto, Y., Tanaka, T., Sato, H., & Ozaki, Y. (2002). Conformational change of poly (N-isopropylacrylamide) during the coil– globule transition investigated by attenuated total.
- Kim, S. Y., Kanamori, T., & Shinbo, T. (2002). Preparation of thermal-responsive poly (propylene) membranes grafted with n-isopropylacrylamide by plasma-induced polymerization and their water permeation. *Journal of applied polymer science*, 84(6), 1168-1177.
- Kondo, A., Kaneko, T., & Higashitani, K. (1994). Development and application of thermo-sensitive immunomicrospheres for antibody purification. *Biotechnology and bioengineering*, 44(1), 1-6.
- Kumar, S., Gautam, P. K., Tomar, M. S., & Acharya, A. (2016). CD28-mediated T cell response is upregulated by exogenous application of autologous Hsp70–peptide complex in a tumor-bearing host. *Immunologic research*, 64(1), 313-323.
- Liang, X., Kozlovskaya, V., Cox, C. P., Wang, Y., Saeed, M., & Kharlampieva, E. (2014). Synthesis and self-assembly of thermosensitive double-hydrophilic poly (N-vinylcaprolactam)-b-poly (N-vinyl-2-pyrrolidone) diblock copolymers. *Journal of Polymer Science Part A: Polymer Chemistry*, 52(19), 2725-2737.
- Matyjaszewski, K. (2012). Atom transfer radical polymerization (ATRP): current status and future perspectives. *Macromolecules*, 45(10), 4015-4039.
- Mayadunne, R. T., Rizzardo, E., Chiefari, J., Chong, Y. K., Moad, G., & Thang, S. H. (1999). Living radical polymerization with reversible addition– fragmentation chain transfer (RAFT polymerization) using dithiocarbamates as chain transfer agents. *Macromolecules*, 32(21), 6977-6980.
- Moad, G., Chiefari, J., Chong, Y. K., Krstina, J., Mayadunne, R. T. A., Postma, A., ... & Thang, S. H. (2000). Living free radical polymerization with reversible addition– fragmentation chain transfer (the life of RAFT). *Polymer International*, 49(9), 993-1001.
- Moad, G., Chong, Y. K., Postma, A., Rizzardo, E., & Thang, S. H. (2005). Advances in RAFT polymerization: the synthesis of polymers with defined end-groups. *Polymer*, 46(19), 8458-8468.
- Moad, G., Rizzardo, E., & Thang, S. H. (2006). Living radical polymerization by the RAFT process—a first update. *Australian Journal of Chemistry*, 59(10), 669-692.
- Moad, G., Rizzardo, E., & Thang, S. H. (2008). Toward living radical polymerization. *Accounts of chemical research*, 41(9), 1133-1142.
- Mori, H., & Endo, T. (2012). Amino-Acid-Based Block Copolymers by RAFT Polymerization. *Macromolecular rapid communications*, 33(13), 1090-1107.
- Nicolas, J., Guillaneuf, Y., Lefay, C., Bertin, D., Gimes, D., & Charleux, B. (2013). Nitroxide-mediated polymerization. *Progress in Polymer Science*, 38(1), 63-235.
- Penit, C., & Papiernik, M. (1986). Regulation of thymocyte proliferation and survival by deoxynucleosides. Deoxycytidine produced by thymic accessory cells protects thymocytes from deoxyguanosine toxicity and stimulates their spontaneous proliferation. *European journal of immunology*, 16(3), 257-263.
- Schild, H. G., Muthukumar, M., & Tirrell, D. A. (1991). Cononsolvency in mixed aqueous solutions of poly (N-isopropylacrylamide). *Macromolecules*, 24(4), 948-952.
- Schönhoff, M., Larsson, A., Welzel, P. B., & Kuckling, D. (2002). Thermoreversible polymers adsorbed to colloidal silica: a 1H NMR and DSC study of the phase transition in confined geometry. *The Journal of Physical Chemistry B*, 106(32), 7800-7808.
- Singh, P., Srivastava, A., & Kumar, R. (2015). Synthesis and characterization of nano micelles of poly (N-acrylamidohexanoic acid)-b-poly (N-vinylcaprolactam) via RAFT process: Solubilizing and releasing of hydrophobic molecules. *Polymer*, 57, 51-61.
- Strober, W. (2001). Trypan blue test of cell viability. *Curr. Protoc. Immunol*, 21.
- St. Thomas, C., Maldonado-Textle, H., Rockenbauer, A., Korecz, L., Nagy, N., & Guerrero-Santos, R. (2012). Synthesis of NMP/RAFT inifers and preparation of block copolymers. *Journal of Polymer Science Part A: Polymer Chemistry*, 50(14), 2944-2956.
- Sun, S., Wu, P., Zhang, W., Zhang, W., & Zhu, X. (2013). Effect of structural constraint on dynamic self-assembly behavior of PNIPAM-based nonlinear multihydrophilic block copolymers. *Soft Matter*, 9(6), 1807-1816.
- Tomar, M. S., Kumar, S., Kumar, S., Gautam, P. K., Singh, R. K., Verma, P. K., & Acharya, A. (2018). NK cell effector functions regulation by modulating nTreg cell population during progressive growth of Dalton's lymphoma in mice. *Immunological investigations*, 47(1), 40-56.
- Yamato, M., Konno, C., Kushida, A., Hirose, M., Utsumi, M., Kikuchi, A., & Okano, T. (2000). Release of adsorbed fibronectin from temperature-responsive culture surfaces requires cellular activity. *Biomaterials*, 21(10), 981-986.
- Zamfir, M., Rodriguez-Emmenegger, C., Bauer, S., Barner, L., Rosenhahn, A., & Barner-Kowollik, C. (2013). Controlled growth of protein resistant PHEMA brushes via S-RAFT

polymerization. *Journal of Materials Chemistry B*, 1(44), 6027-6034.

Zelikin, A. N., Ehrhardt, C., & Healy, A. M. (2016). Materials and methods for delivery of biological drugs. *Nature chemistry*, 8(11), 997.reflection/infrared spectroscopy and density functional theory calculation. *The Journal of Physical Chemistry A*, 106(14), 3429-3435.
