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A novel nanocomposite containing graphene oxide and ABC triblock copolymer for methotrexate delivery

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Abstract

Background and objective: Graphene oxide has been extensively used in theranostics due to its drastic properties, biocompatibility, and chemical stability. Graphene has a large surface area and provides enough space for loading of anticancer drugs. In our study, a novel thermo-and pH-responsive graphene-containing nanocomposite was synthesized for methotrexate (MTX) delivery into cancer cells.

Materials and methods: Triblock copolymer of poly[(2-hydroxyethylmethacrylate)-*b*-(*N*-isopropyl-acrylamide)-*b*-(dimethylaminoethyl methacrylate)] abbreviated as poly(HEMA-*b*-NIPAM-*b*-DMAEMA) was prepared by reversible addition fragmentation chain-transfer (RAFT) polymerization. The triblock copolymer was attached onto the surface of graphene oxide nanoparticles via carboxylic groups of graphene oxide. Structure of poly(HEMA-*b*-NIPAM-*b*-DMAEMA) was studied by Fourier transform infrared (FT-IR) spectroscopy and Proton nuclear magnetic resonance (^{1}HNMR). Morphology of the nanocomposite was studied by field emission scanning electron microscope (FESEM) and its thermo-responsive behavior was investigated by lower critical solution temperature (LCST), dynamic light scattering (DLS), and thermogravimetric analysis (TGA). Polydispersity index (PDI) was evaluated by gel permeation chromatography. pH-responsive behavior of the nanocomposite was also studied by evaluation of MTX release from the structure at pH 5.4 and 7.4 in the laboratory.

Results and conclusion: Graphene oxide/poly(HEMA-*b*-NIPAM-*b*-DMAEMA) has a sheet-like structure with average thickness of 55.6 nm. The triblock chains successfully covered graphene oxide. Characterization of poly(HEMA-*b*-NIPAM-*b*-DMAEMA) resulted in M_n = 26875 g, M_W = 33862 g, and PDI = 1.26. Encapsulation efficiency of the structure was 91% for MTX. Release rate of MTX from the graphene nanocomposite was pH-dependent. In a buffer solution, release rate of 31.2% was achieved at pH 7.4 and temperature of 37 °C after 150 h. In comparison, release rate of 52.4% was calculated for pH 5.4 after 150 h at the same temperature. Therefore, the synthesized graphene nanocomposite is an appropriate candidate as a carrier of anticancer drugs in treatment of cancer cells.

Keywords: Cancer therapy, graphene nanocomposite, methotrexate, pH-responsive, thermo-responsive

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1. Introduction

Nanomedicine is an interesting filed in the world due to its multi-functionality and targetability as well as its potential to overcome multi-drug resistance and also entrap the poorly soluble drugs [1,2]. In this regard, graphene and its derivatives such as graphene oxide (GO) are of the most interested candidates because of their specific physicochemical and biological characteristics [3-5]

In GO, carbon atoms bond together in a honeycomb lattice, in which oxygen-containing functional groups are attached to the backbone to form a two-dimensional structure [6,7]. Graphite oxidation is the common way of GO synthesis. Excessive oxidation results in oxygen-containing functional groups on the structure. Graphene has large surface area that provides desired space for loading of anticancer drugs. In addition, GO is well-loaded with anticancer drugs classified as aromatic medicines [8-10].

Presence of oxygen-containing functional groups in the structure is desirable for delivery of anticancer drugs because of their active involvement in chemical reactions [11,12]. The basal planar structure of GO and its high surface area with sp^2 domain result in its high loading capacity, biocompatibility, and solubility. Simple chemical or physisorption conjugation can be used in synthesis of multimodal GO by conjugation of proteins, polymers, and biomolecules to GO structure [13]. GO has been studied for delivery of several drugs because it has a large hydrophobic area able to load hydrophobic drugs via non-covalent adsorption driven by π-π stacking and interaction with aromatic compounds [14-16]. Drug loading in GO is occurred at low temperatures, which allows loading of temperature sensitive formula [17].

Poly(N-isopropylacrylamide) (PNIPAM) has been used as a thermo-responsive copolymer [18-21]. It has a cloud point (or lower critical solution temperature; LCST) of 32 °C [22-25]. PNIPAM copolymers are interested in pharmaceutical applications. Local hyperthermia that is important in nanomaterials target cancer cells after release of their loaded drug could be achieved by PNIPAM copolymers [26-28].

In this study, our objective was development of a simple way for synthesis of stimuli-responsive magnetic nanocomposite able to diagnose and treat cancer cells simultaneously. Copolymer of poly(2-hydroxyethylmethacrylate-*b*-N-isopropylacrylamide-*b*-N,N-dimethylaminoethylmethacrylate) abbreviated as poly(HEMA-*b*-NIPAM-*b*-DMAEMA) was synthesized by reversible addition fragmentation chain-transfer (RAFT) polymerization. Synthesized GO was attached to poly(HEMA-*b*-NIPAM-*b*-DMAEMA) via covalent bond. Characteristics of the graphene nanocomposite were studied in the laboratory. Release kinetic of methotrexate from the structure was also studied under *in vitro* trial.

2. Materials and methods

2.1. Materials

RAFT agent of 4-cyano-4-[(phenylcarbothioyl) sulfanyl] pentanoic acid was synthesized in the laboratory [30]. 2-hydroxyethyl methacrylate (HEMA, 98%), N,N-dimethylaminoethyl methacrylate (DMAEMA, 97%), N-isopropyl-acrylamide (NIPAM, 97%), graphite, sodium nitrate, potassium permanganate, and sulfuric acid were purchased from Merck, Germany. Initiator of 2,2-azobisisobutyronitrile (AIBN) was obtained from Fluka company (Switzerland).

2.2. Synthesis of poly(HEMA) and poly (HEMA-*b*-NIPAM)

Poly(HEMA), as one of macro-RAFT agents, was synthesized according to Scheme 1 [31]. Poly(HEMA-*b*-NIPAM) was further synthesized by using poly(HEMA) as macro-RAFT agent and NIPAM monomer [32].

2.3. Synthesis of poly(HEMA-*b*-NIPAM-*b*-DMAEMA) triblock copolymer

Macro-RAFT agent of P(HEMA-*b*-NIPAM) (1.8 g, 0.1 mmol), AIBN (2.5 mg, 0.015 mmol), DMAEMA monomer (2 g, 12.7 mmol), and dimethylformamide (DMF) (15 ml) were added to a reactor. The mixture was degassed and refluxed at 75 °C for 30 h. Then, the reactants were precipitated in cold diethyl ether (100 ml).

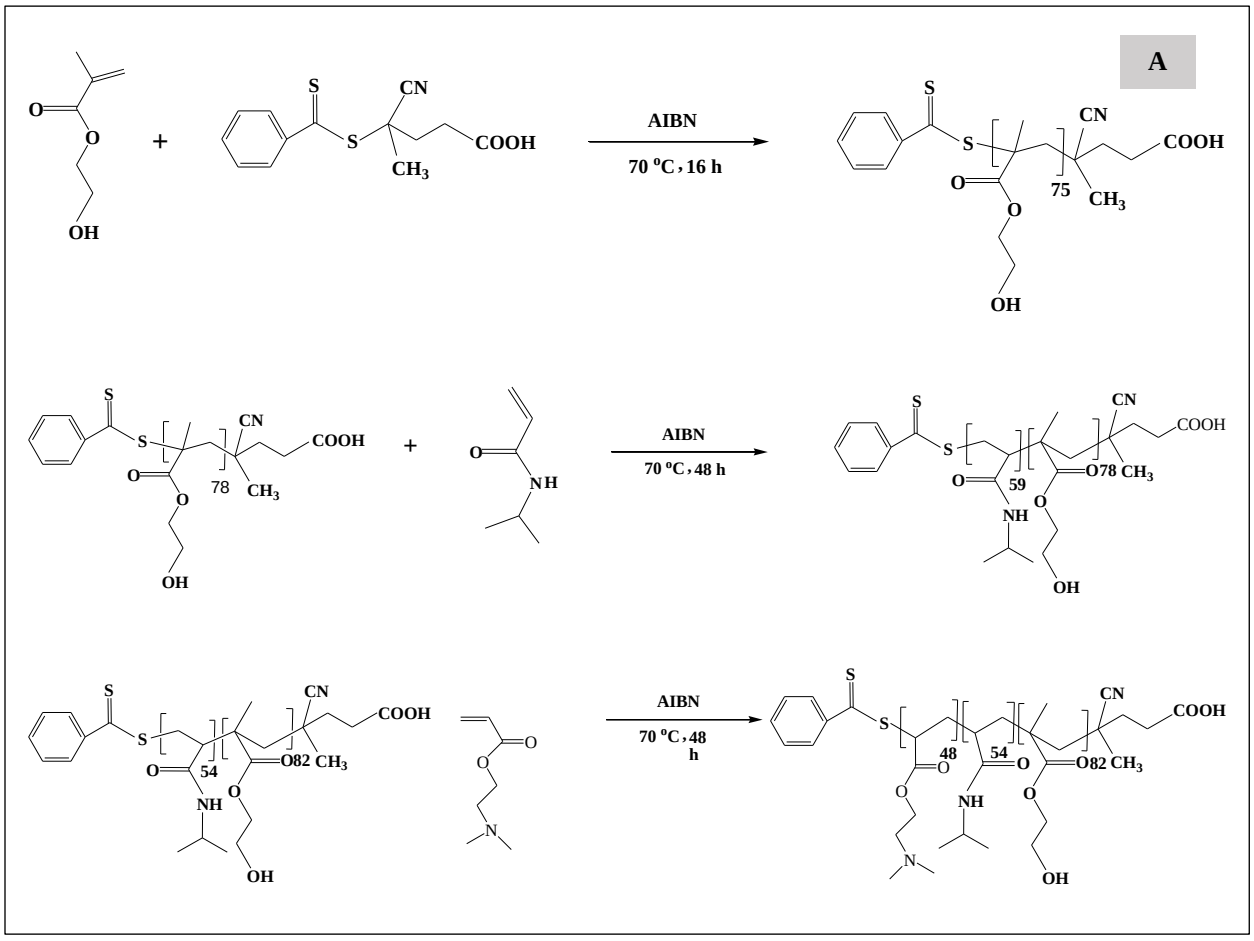
The synthesized triblock copolymer was finally filtrated and dried under vacuum at room temperature (Scheme 1).

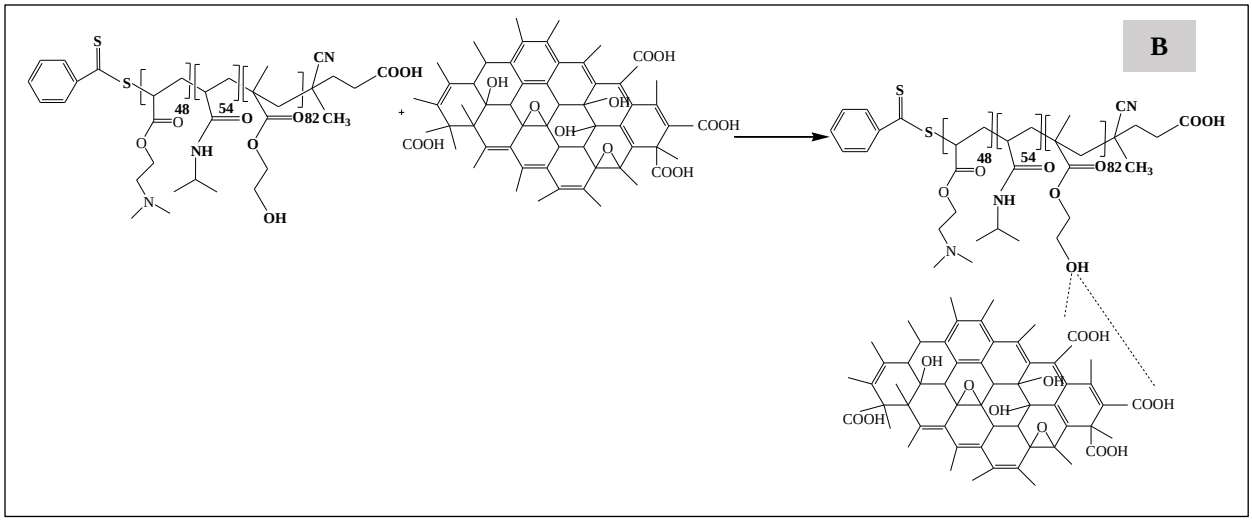
2.4. Synthesis of GO

GO was prepared by oxidation of graphite powder. Sodium nitrate (1.5 g), sulfuric acid (135 ml), and graphite powder (4 g) were added to a 250-ml flask and stirred for 50 min at 25 °C. Then, potassium permanganate (14 g) was slowly added to the flask and the mixture was stirred at 35 °C for 8 h. In the next step, the content was diluted by 700 ml deionized water followed by addition of 35 ml H_2O_2 to reduce unreacted potassium permanganate. The final mixture was centrifuged (7000 rpm, 20 min), and the precipitate was washed with 0.1 M hydrochloric acid and distilled water to reach pH ~ 7. At the end, GO was dried at 65 °C under vacuum.

2.5. Synthesis of graphene/poly(HEMA-*b*-NIPAM-*b*-DMAEMA) nanocomposite

500 mg poly(HEMA-*b*-NIPAM-*b*-DMAEMA) copolymer and 30 ml GO solution (300 mg GO dissolved in 10 ml DMF) were mixed. Then, the mixture was heated at 60 °C for 3.5 h under stirring. GO/poly(HEMA-*b*-NIPAM-*b*-DMAEMA) dispersion was centrifuged at 6000 rpm for 10 min and the precipitate was dried under vacuum at room temperature for 24 h (Scheme 1).





Scheme 1- Synthesis of A) poly(HEMA-*b*-NIPAM-*b*-DMAEMA) copolymer, and B) GO/poly(HEMA-*b*-NIPAM-*b*-DMAEMA) nanocomposite

2.6. Preparation of MTX-loaded GO/poly (HEMA-*b*-NIPAM-*b*-DMAEMA) nanocomposite

Drug-loaded nanocomposite was prepared by membrane dialysis method. For this, 200 mg nanocomposite was dispersed in 5 ml dimethyl sulfoxide and treated by ultrasound waves at 1.5 Hz frequency at 25°C. Then, 20 mg MTX was added to the solution and stirred for 45 h in darkness. Final solution was moved into a membrane bag and dialyzed against 250 ml deionized water for 2 days. Then, MTX-loaded nanocomposite was centrifuged at 6000 rpm for 15 min to be separated. UV-Vis absorbance of MTX-loaded nanocomposite was measured at 290 nm to ensure of encapsulation.

2.7. Characterization

Size exclusion analysis was carried out by gel permeation chromatography (GPC) (Waters 1515, USA) equipped with isocratic pump (Breeze 1515) and manual injector (7725). Fourier transform infrared (FT-IR) spectrophotometer (Shimadzu 8101M, Kyoto, Japan) at wavenumber ranges of 4000 to 400 cm^{-1} was used for investigation of chemical interactions. Proton nuclear magnetic resonance (^{1}HNMR) spectra were obtained at 400 mHz at 25 °C by Bruker spectrometer (Ettlingen, Germany). For this, the samples were prepared in deuterated dimethyl sulfoxide solvent (DMSO-d6). UV-Vis spectra were obtained by Shimadzu 1650 PC UV-Vis spectrophotometer (Kyoto, Japan). Size of nanocomposites was measured by laser-scattering technique (Zetasizer Nano ZS90, Malvern, UK) at 25, 35, and 45 °C. Field emission scanning electron microscope (FESEM) (model 1430 VP, UK) was used for determination of the nanocomposites' morphology. For this, the samples were spread on a SEM stub and coated with gold before analysis.

3. Results and discussion

3.1. Characterization of poly(HEMA-*b*-NIPAM-*b*-DMAEMA) copolymer

FT-IR and ^{1}HNMR spectra of poly(HEMA-*b*-NIPAM-*b*-DMAEMA) are shown in Figures 1 and 2. Main bands in FT-IR spectra are vibration of stretching C–O at 1392 cm^{-1}, stretching carbonyl at 1734 cm^{-1}, bending C–H at 1471 cm^{-1}, stretching C–O–C at 1177 cm^{-1}, and stretching hydroxyl at 3502 cm^{-1} [31,32].

Synthesis of poly(HEMA-*b*-NIPAM-*b*-DMAEMA) was approved by chemical shifts at 2.93, 3.22, and 4.1 ppm, which were related to N–$(CH_3)_2$ protons, N-CH_2, and O-CH_2 [33] of poly(DMAEMA) segments. All the chemical shifts are addressed in ^{1}HNMR spectra of the triblock copolymer (Figure 2).

Gel permeation chromatogram of poly(HEMA-*b*-NIPAM-*b*-DMAEMA) is shown in Figure 3. Number average molecular weight (M_n)= 26875,

average molecular weight (M_W) = 33862, and polydispersity index (PDI) = 1.26 were achieved by GPC, and M_n = 22944 was achieved by ^{1}HNMR for poly(HEMA-*b*-NIPAM-*b*-DMAEMA). The triblock copolymer synthesized by RAFT polymerization had relatively low PDI, which shows the good performance of RAFT technique in this regard.

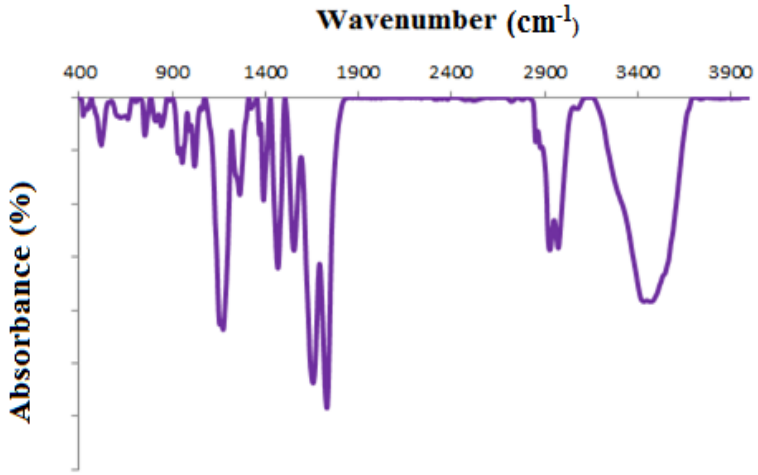


Figure 1- FT-IR spectra of poly(HEMA-b-NIPAM-b-DMAEMA) triblock copolymer; FT-IR spectra of PHEMA and PNIPAM were presented in our previous studies [31,32].

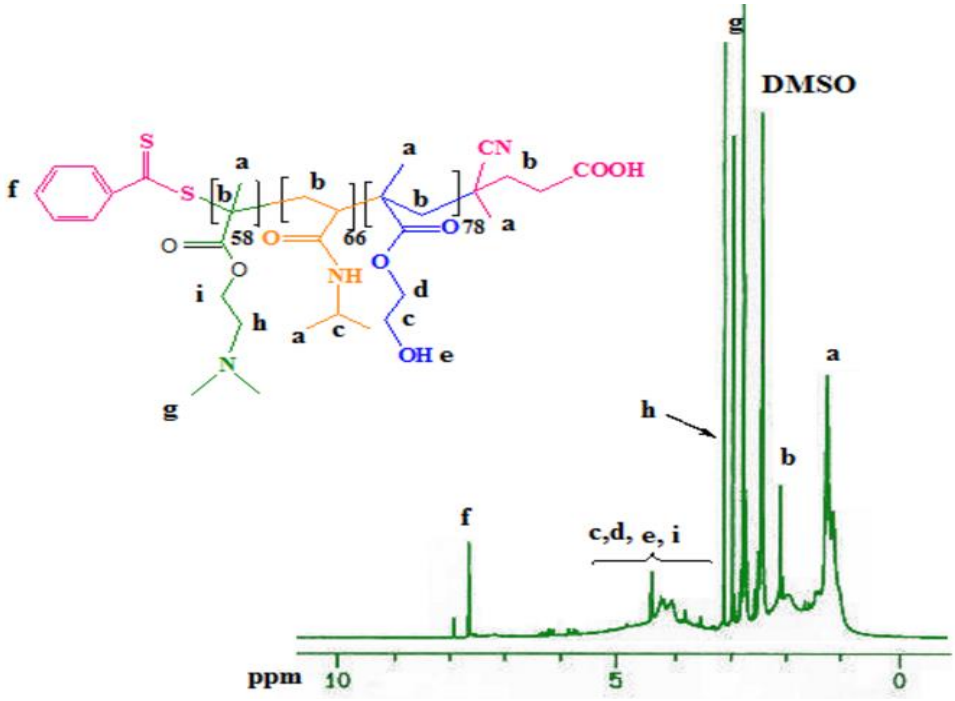


Figure 2- ^{1}HNMR spectra of poly(HEMA-*b*-NIPAM-*b*-DMAEMA) triblock copolymer; ^{1}HNMR spectra of PHEMA and PNIPAM were presented in our previous studies [31,32].

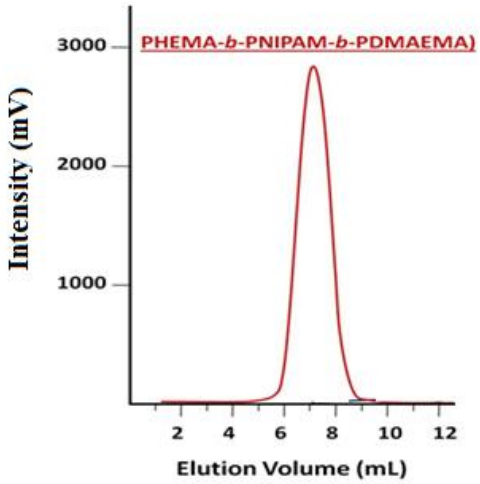
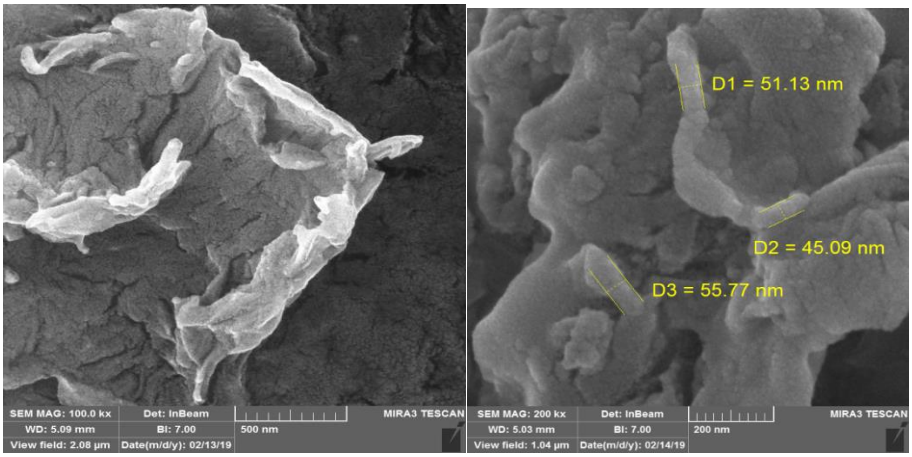


Figure 3- Gel permeation chromatogram of poly(HEMA-*b*-NIPAM-*b*-DMAEMA) triblock copolymer

3.2. Characterization of nanocomposite

FESEM images of GO and GO/poly(HEMA-*b*-NIPAM-*b*-DMAEMA) nanocomposite are presented in Figure 4. The synthesized GO had sheet-like structure in 25 nm thickness with smooth surface containing small wrinkles. GO/poly(HEMA-*b*-NIPAM-*b*-DMAEMA) nanocomposite had thickness of 50.66 nm and also wrinkled surface. According to the figure, poly(HEMA-*b*-NIPAM-*b*-DMAEMA) chains successfully covered GO nano-sheets.



(A) **(B)**

Figure 4- Field emission scanning electron microscope images of A) synthesized GO, and B) GO/poly(HEMA-*b*-NIPAM-*b*-DMAEMA) nanocomposite

Poly(N-isopropylacrylamide) (PNIPAM) is a reversible temperature-responsive polymer, which has a low critical solution temperature (LCST) of 32 °C [26]. It is well-known that PNIPAM-containing copolymers show tunable phase transition behavior that is interested in biomaterials' application [33]. LCST behavior of aqueous solution of the copolymers was studied by UV-Vis transmission spectrum. According to Figure 5, LCST of 36-38 ℃ was observed for GO/poly(HEMA-*b*-NIPAM-*b*-DMAEMA) nanocomposite.

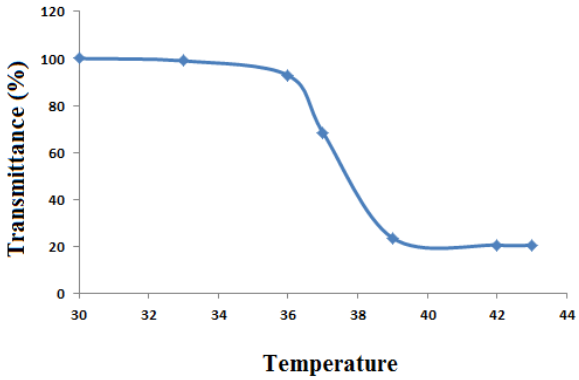


Figure 5- Low critical solution temperature of GO/poly(HEMA-*b*-NIPAM-*b*-DMAEMA) nanocomposite

Thermo-sensitivity of GO/poly(HEMA-*b*-NIPAM-*b*-DMAEMA) nanocomposite was evaluated by dynamic light scattering (DLS). Particle size of the nanocomposite was also analyzed by DLS at three different temperatures. Average thickness of GO/poly(HEMA-*b*-NIPAM-*b*-DMAEMA) nanocomposite at 25, 35, and 42 ℃ was 95, 135, and 110 nm, respectively (Figure 6). Importantly, particles size decreased to 66 nm at temperature above LCST (42 °C) due to collapse of PNIPAM chains and shrinkage of the nanocomposite network [34,35].

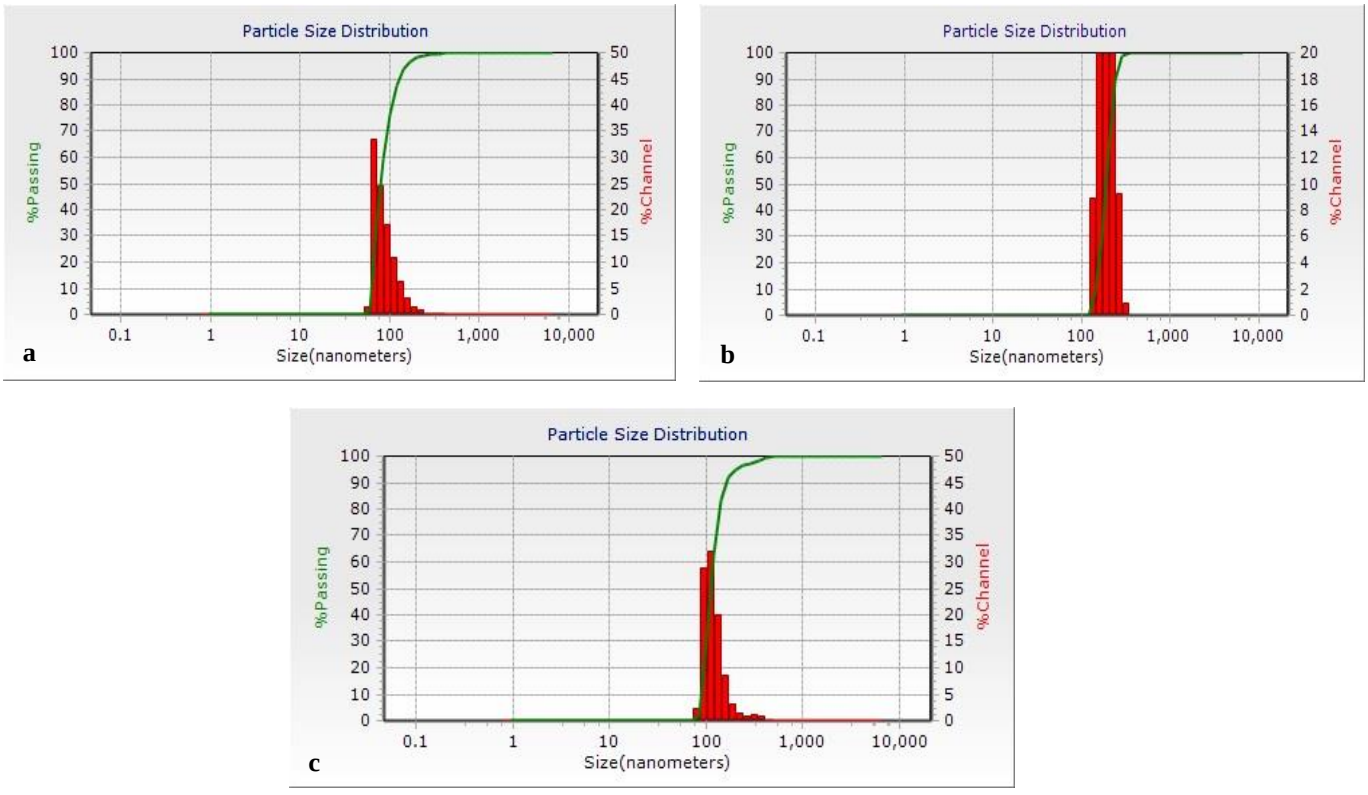


Figure 6- Dynamic light scattering of GO/poly(HEMA-*b*-NIPAM-*b*-DMAEMA) nanocomposite at a) 25 ºC, b) 35 ºC, and c) 42 ºC

Thermal stability of GO and GO/poly(HEMA-*b*-NIPAM-*b*-DMAEMA) nanocomposite was investigated by thermogravimetric analysis (Figure 7). Below 100 °C, 22.1% weight loss was calculated for GO due to evaporation of intercalated water molecules. Consequently, a two-step weight loss was observed. The first was 36.4% in the range of 110–250 °C due to release of CO_2, CO, and steam from the structure, and the second was 15.14% in the range of 250-350 °C due to degradation of stable oxygens. In comparison, weight loss of the nanocomposite was about 6.3% below 140 °C due to evaporation of intercalated water molecules. Then, weight loss continued up to 37.18% of initial weight between 140-150 °C (Figure 7) which was related to release of CO_2 and steam from GO. The nanocomposite degraded in the range of 150–500 °C. Final weight loss of 21% was calculated for GO/poly(HEMA-*b*-NIPAM-*b*-DMAEMA) nanocomposite. Thermogravimetry behavior of the nanocomposite confirmed the attachment of GO nano-sheet to P(HEMA-*b*-NIPAM-*b*-DMAEMA) copolymer.

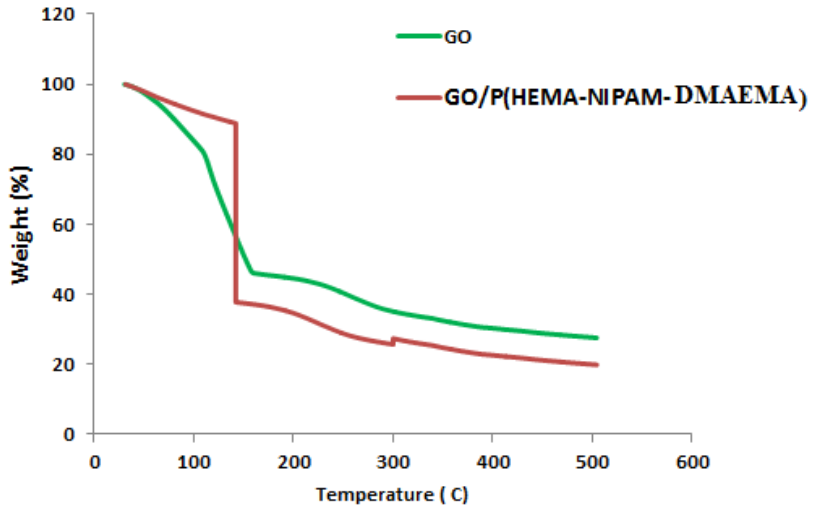


Figure 7- Thermo-gravimetry curve of GO and GO/poly(HEMA-*b*-NIPAM-*b*-DMAEMA) nanocomposite

3.3. MTX release from nanocomposite *in vitro*

GO-containing nanocomposites have been commonly used in drug delivery because of their ability to target specific sites and appropriate size [36]. Our synthesized GO/poly(HEMA-*b*-NIPAM-*b*-DMAEMA) nanocomposite had homogenous structure and there was no sign of aggregation. MTX was loaded in the nanocomposite by electrostatic interactions between carboxylate groups of MTX and protonated amine groups of the nanocomposite at pH 7.4. Release behavior of MTX from GO/poly (HEMA-*b*-NIPAM-*b*-DMAEMA) was studied in a buffer solution at 37 °C. Interestingly, release rate was low (31.2%) at pH 7.4 after 150 h, while 52.4% of MTX released from the nanocomposite at pH 5.4 after 150 h (Figure 8). Indeed, amine groups of the nanocomposite and MTX are protonated under acidic condition. It further weakens π–π stacking followed by release of MTX from the structure. On the other hand, hydrogen bonds are dissociated at lower pH which leads to higher release of MTX in the system. Such behavior is interesting in cancer therapy because pH of cancer cells is lower than normal cells. Therefore, the synthesized GO nanocomposite can be considered as a candidate in cancer chemotherapy due to its high loading capacity and pH-dependent release behavior.

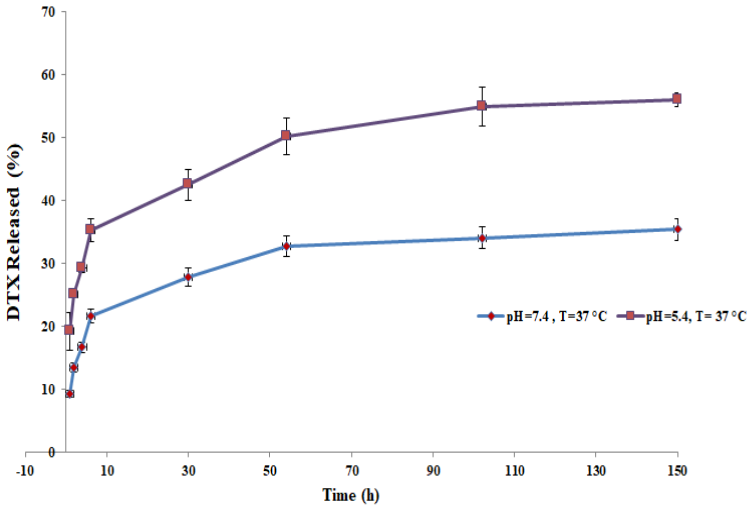


Figure 8- *In vitro* release of MTX from GO/poly(HEMA-*b*-NIPAM-*b*-DMAEMA) nanocomposite at 37 °C

4. Conclusion

Graphene, sp2 hybridized carbon framework with one atom thickness, has manifold applications in electronics and fabrication of sensors, composites, and catalysts. In recent decades, it has been interested in biomedicine. A thermo- and pH-responsive graphene nano-composite was developed to study its behavior for cancer therapy. Poly(HEMA-*b*-NIPAM-*b*-DMAEMA) triblock copolymer was prepared by RAFT polymerization technique. GO was also synthesized by graphite oxidation. The triblock copolymer was attached to GO through interaction of its carboxyl groups with hydroxyl groups of GO. PDI of 1.26 was achieved for poly(HEMA-*b*-NIPAM-*b*-DMAEMA) after RA-FT technique that it is a living free-radical polymerization. MTX was successfully loaded to the nanocomposite with encapsulation effici-ency of 91%. Release rate of MTX from GO/poly(HEMA-*b*-NIPAM-*b*-DMAEMA) nanocomposite was relatively low at pH 7.4, while the release rate increased at pH 5.4. The synthesized nanocomposite can be studied further as a carrier of chemotherapy drugs in treatment of cancer cells which have lower pH than normal cells.

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