RESEARCH ARTICLE

Physicochemical Characteristics of Fe_3O_4 Magnetic Nanocomposites Based on Poly(N-isopropylacrylamide) for Anti-cancer Drug Delivery

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Abstract

Background: Hydrogels are a class of polymers that can absorb water or biological fluids and swell to several times their dry volume, dependent on changes in the external environment. In recent years, hydrogels and hydrogel nanocomposites have found a variety of biomedical applications, including drug delivery and cancer treatment. The incorporation of nanoparticulates into a hydrogel matrix can result in unique material characteristics such as enhanced mechanical properties, swelling response, and capability of remote controlled actuation. Materials and Methods: In this work, synthesis of hydrogel nanocomposites containing magnetic nanoparticles are studied. At first, magnetic nanoparticles (Fe_3O_4) with an average size 10 nm were prepared. At second approach, thermo and pH-sensitive poly (N-isopropylacrylamide -co-methacrylic acid-co-vinyl pyrrolidone) (NIPAAm-MAA-VP) were prepared. Swelling behavior of co-polymer was studied in buffer solutions with different pH values (pH=5.8, pH=7.4) at 37˚C. Magnetic iron oxide nanoparticles (Fe_3O_4) and doxorubicin were incorporated into copolymer and drug loading was studied. The release of drug, carried out at different pH and temperatures. Finally, chemical composition, magnetic properties and morphology of doxorubicin-loaded magnetic hydrogel nanocomposites were analyzed by FT- IR, vibrating sample magnetometry (VSM), scanning electron microscopy (SEM). Results: The results indicated that drug loading efficiency was increased by increasing the drug ratio to polymer. Doxorubicin was released more at 40˚C and in acidic pH compared to that 37˚C and basic pH. Conclusions: This study suggested that the poly (NIPAAm-MAA-VP) magnetic hydrogel nanocomposite could be an effective carrier for targeting drug delivery systems of anti-cancer drugs due to its temperature sensitive properties.

Keywords: Magnetic nanocomposite - poly (N-isopropylacrylamide) - doxorubicin - targeted drug delivery

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Introduction

Hydrogel nanocomposites can be obtained by incorporating different types of nanoparticles, such as metallic, clay, and carbon nanotubes, into a hydrogel matrix. Hydrogel nanocomposites have unique properties such as improved mechanical strength, stimuli responsive behavior, biological interactions, optical properties, and ability of remote actuation (Gattas-Asfura et al., 2003; Frimpong et al., 2007;Thomas et al., 2008). For example, the high water content and elasticity of hydrogels typically leads to inferior mechanical performance and limits their applications.

The incorporation of nanoparticulate material like hydroxyapatite has improved the mechanical properties and made the nanocomposites attractive materials for a broader range of tissue engineering applications (Xu et al., 2007). In cases where the nanoparticle could induce an undesired biological response, the hydrogel can prevent the direct interaction of the nanoparticle with the biological system. Additionally, hydrogels can be made responsive to external stimuli by incorporation of stimuli-specific nanoparticulates. In order to impart remote controlled (RC) capabilities, a variety of approaches have been employed including the incorporation of magnetic nanoparticles (Satarkar and Hilt, 2008) gold nanoparticles (Shiotani et al., 2007), or carbon nanotubes (Miyako et al., 2008).

Magnetic nanoparticles are well known for their ability to generate heat when exposed to alternating magnetic fields (Wang et al., 2007). Hydrogel nanocomposites can

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be roughly divided into three categories based on their size: bulk nanocomposites, particle nanocomposites, and core-shell nanocomposites (Figure1). The bulk hydrogel nanocomposites are predominantly synthesized in the form of thin films. The nanocomposites in particle form can range from tens of nanometers to microns in diameter and can contain several to many nanoparticle fillers. The core-shell nanocomposites, on the other hand, consist of a single nanoparticle core or small agglomerate of nanoparticles surrounded by a hydrogel shell (Xu et al., 2004; Chen et al., 2005; Muller-Schulte and Schmiz-Rode, 2006; Frimpong et al., 2007; Budhlall et al., 2008). The field of controlled drug delivery continues to be one of the key areas of current research in pharmaceutics and medicine. Due to unique and tailorable properties, hydrogels have been extensively studied as materials for drug delivery applications (Peppas et al., 2000; Qiu and Park, 2001; Hoare and Kohane, 2006). For example, the hydrophilicity and porosity of hydrogel network can be controlled by choice of the monomer(s) and the crosslinker. The manipulation of network design allows for the development of drug delivery systems with a desired release profile for a given drug (Lin and Metters, 2006). The incorporation of nano- and micro-scale materials in the hydrogel matrix allows additional control on the network properties (e.g., potential for RC actuation).The various types of hydrogel nanocomposites have been developed in the past few years, and their swelling and drug release properties have been extensively studied (Kost and Longer et al, 2001). Hydrogel nanocomposites with magnetic nanoparticles have been demonstrated as potential candidates for pulsatile drug delivery and soft actuator applications. The aim of this research was to assess the merits of poly (NIPAAm-MAA-VP) magnetic nanocomposite as anticancer drug carrier. For this purpose, poly (NIPAAm-MAA-VP)-Fe_3O_4 Nanocomposite was synthesized by incorporation of superparamagnetic Fe_3O_4 nanoparticles in temperature sensitive poly (NIPAAm-MAA-VP) hydrogel.

Doxorubicin was encapsulated within this system with the floating and swelling method. Poly (NIPAAm-MAA-VP) magnetic nanocomposite loaded with Dox was characterized in terms of size and in-vitro release of doxorubicin (Valizadeh et al., 2012; Akbarzadeh et al., 2012a; 2012b; 2012c; 2012d).

Materials and Methods

Ferric chloride hexahydrate ($FeCl_3.6H_2O$), ferrous chloride tetrahydrate ($FeCl_2.4H_2O$) and ammonium hydroxide (25wt%) were purchased from Fluka (Buchs, Switzerland). N-iso propylacrylamide (NIPAAm), N-vinyl- 2-pyrrolydone, Methacrylic acid (MAA), Benzoyl proxide (B.P.O), 1,4- dioxan, Methylene-bis-acrylamide (BIS) were purchased from Merck. Doxorubicin hydrochloride (DOX.HCl) was purchased from Sigma-Aldrich. X-ray diffraction, Rigaku D/MAX-2400 x-ray diffractometer with Ni-filtered Cu K_α radiation, and scanning electron microscopy (SEM) measurements were conducted using LEO 1430VP. The drug-loading capacity and release behavior were determined using an ultraviolet visible 2550 spectrometer (Shimadzu, Tokyo, Japan). Infrared spectra were recorded in real-time with a Perkin Elmer series FT-IR. The magnetic property was measured on a vibrating sample magnetometer (Meghnatis Daghigh Kavir, Iran) at room temperature. ^{1}H NMR spectra was recorded in realtime with a Brucker DRX 300 spectrometer operating at 300.13 mHz.

Synthesis of Fe_3O_4 nanoparticles

Superparamagnetic magnetite NPs were prepared via improved chemical coprecipitation method (Dresco et al., 1999). According to this method, 3.1736 g of $FeCl_2.4H_2O$ (0.016 mol) and 7.5684 g of $FeCl_3.6H_2O$ (0.028 mol) were dissolved in 320 mL of deionized water, such that $Fe^{2+}/Fe^{3}+=1/1.75$. The mixed solution was stirred under N_2 at 80˚C for 1h. Then, 40ml of $NH_3{\cdot}H_2O$ was injected into the mixture rapidly, stirred under N_2 for another 1h and then cooled to room temperature. The precipitated particles were washed five times with hot water and separated by magnetic decantation (Figure 2). Finally, magnetic NPs were dried under vacuum at 70˚C:

$$2FeCl_3+FeCl_2+4H_2O+8NH_3\rightarrow Fe_3O_4+8NH_4Cl$$

Preparation of (NIPAAm-MAA-VP) co-polymer

A copolymer of NIPAAM-VP-MAA was synthesized through free radical mechanism. Water soluble monomers, NIPAAM, VP and MAA, were used in 75.7:9.5:14.8 molar ratios and the polymer were cross-linked with MBA. Using a standard experimental protocol, 4.25g NIPAAM, 0.55 ml VP and 0.172 ml MAA (also freshly distilled) were mixed in 20 ml of 1,4- dioxin. To cross-link the polymer chain, 300 ml of MBA (0.05 g/ml) was added in the aqueous solution of monomers. The dissolved oxygen was removed by passing nitrogen gas for 30 min. To initiate the polymerization, B.P.O (0.087g) was added to mixture. The polymerization was fulfilled at 25°C for 24 h in nitrogen atmosphere. The polymerization was carried out under vacuum. The copolymer was precipitated

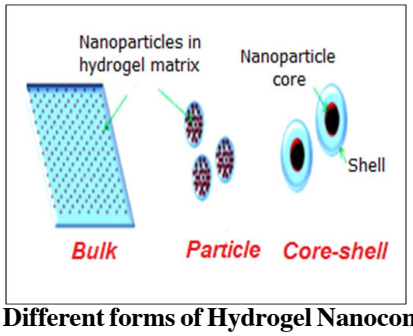

Figure 1. Different forms of Hydrogel Nanocomposites

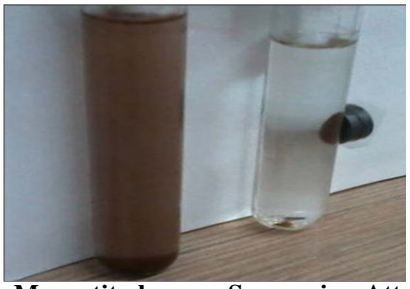

Figure 2. Magnetite-hexane Suspension Attached to a Magnet

in ice-cold n-hexane. The yield of copolymer was 75%. The synthesis process of NIPAAm-MAA-VP copolymer is shown in schem1.

LCST measurement of co-polymer

Optical transmittance of the co-polymer solution (14g/li) at various temperatures (25-50°C with 1°C intervals) was measured at 500 nm with a UV-Vis spectrophotometer UV-Vis spectrophotometer. Poly N-isopropylacrylamide (PNIPAAm) is a well- known temperature- sensitive polymer. When the temperature is higher than the LCST, PNIPAAm aqueous solution is cloudy (phase separation occurred). In contrast, when the temperature is lower than the LCST, PNIPAAm is soluble in water.

Characterization of swelling behavior

Swelling behavior of poly (NIPAAm- MAA-VP) were performed in buffer solutions with different pH values (5.8, 7.4) at 37 ºC to characterize the effect of pH on swelling behavior. For the swelling measurements, vacuum dried hydrogels were placed in to a Petri dish, the Petri dish was then filled with solutions of desired pH (5.8, 7.4) at 37°C. Swollen gels removed from desired solution after 5 minute and were dried superficially with filter paper and then weighted .The percentage that the gels swelled was calculated using the formula:

$$Swelling\ (\%)=(W_t/W_0)\times 100$$

Where W_t is the weight of the gel at predetermined time and W_0 is the dry mass of the gel

Doxorubicin-loaded on magnetic hydrogel nanocomposite

Doxorubicin- loaded poly (NIPAAm-MAA-VP)-Fe_3O_4 nanoparticle were prepared using the floating and swelling method. Doxorubicin is floating in NIPAAm-MAA-VP-Fe_3O_4 nanoparticles when the temperature is lower than the LCST and then temperature increased to higher than the LCST, so Doxorubicin incorporated into magnetic hydrogel nanocomposite. Loading of Dox is carried out in two drug different molar ratio to polymer (1 to 10 and 1 to 5).

Loading of doxorubicin with 1 to 10 molar ratio of drug to polymer

500 mg of co-polymer, 10 mg of Fe_3O_4 nanoparticles and 50 mg of doxorubicin dispersed in 5 ml of buffer solution (pH=7.4). The solution was stirred at 25ºC (lower than the LCST) for 2 days and then temperature increased to higher than the LCST, so doxorubicin incorporated into magnetic hydrogel nanocomposite. This value was then compared with the total amount of DOX to determine the DOX loading efficiency of the nanoparticles. The amount of non- entrapped doxorubicin in aqueous phase was determined by UV-Vis (λ= 483nm) spectrometer. This procedure permits analysis of doxorubicin solutions with removal of most interfering substance. The amount of doxorubicin entrapped efficiency within nanoparticles was calculated by the difference between the total amount used to prepare nanoparticles and the amount of doxorubicin present in the aqueous phase.

Loading efficiency was calculated according to the following formula:

Loading efficiency % = [(amount of loaded drug in mg (amount of added drug in mg)] × 100%

In vitro drug release kinetics study

To study drug release, two different sets of experiments were performed. They include two different temperatures (40 and 37ºC) and two different pHs (5.8 and 7.4). In each drug release experiment, 2.0 mg of the drug carrier bonded with smart polymer was sealed in a 20 ml of Na_2HPO_4-KH_2PO_4 buffer solution with pH of 5.8 or 7.4. The test tube with the closer was placed in an incubater maintained at 40ºC (>LCST), 37ºC (<LCST).The release medium (~2 ml) was withdrawn at predetermined time intervals (1, 3, 5, 8, 12, 24, 36, 48, 60, 80, 100, 130, 160, 190 and 400 h) and the amount of the free doxorubicin (W free DOX) in the buffer solution was quantified using Lambert–Beer law. After each measurement, the withdrawn medium was returned back to the system. Since the measurement time was very short while the drug release predetermined time interval was significantly large, the influence of the returned medium on drug release during the measurement time is expected to be insignificant.

Characterization

The IR spectra were recorded by a Fourier transform infrared spectrophotometer (FT-IR) and the sample and KBr were pressed to form a tablet. The magnetization curves of samples were measured with a vibrating sample magnetometry (VSM) at room temperature. Power X- ray diffraction was used to investigate the crystal structure of the magnetic nanoparticles. The size and shape of the nanoparticles were determined by scaning electron microscope (SEM), the sample was dispersed in ethanol and spread a small drop onto a 400 mesh copper grid.

Results

FT-IR spectroscopy of Fe_3O_4 and NIPAAm-MAA-VP co-polymer

FT-IR spectroscopy was used to show the structure of Fe_3O_4 and NIPAAm-MAA-VP copolymer. From the infrared spectra shown in figure 3a, the absorption peaks at 580 cm^{-1} belonged to the stretching vibration mode of Fe-O bonds in Fe_3O_4. Figure 3b, show strong peaks in the range of 800-1000 cm^{-1} corresponding to the stretching mode of vinyl double bonds, which disappeared in the spectrum of polymer, indicating that polymerization has taken place. Absorbance of amide carbonyl groups in PNIPAAm occurs at 1650 cm^{-1} and bending frequency of amide N-H appears at 1550 cm^{-1}. An intense and broad peak in 3300-3400 cm^{-1} illustrates formation of hydrogel polymer due to the water of hydration attached to the polymer, which gives rise to broad spectrum. The C-H stretching vibration of the polymer backbone is manifested through a strong peak at 2928 cm^{-1}.

X-ray diffraction pattern of Fe_3O_4 nanoparticles

Figure 4 shows the x-ray diffraction pattern for pure Fe_3O_4. It is apparent that the diffraction pattern for our

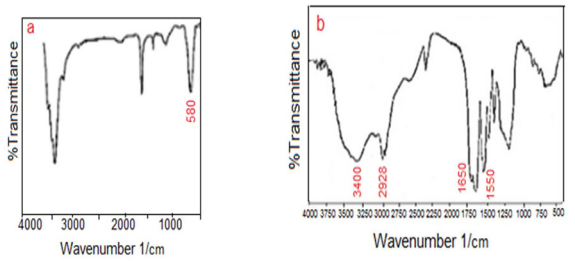

Figure 3. Fourier Transform Infrared Spectra. (a) Pure Fe_3O_4 Nanoparticles and (b) NIPAAm-MAA-VP Copolymer

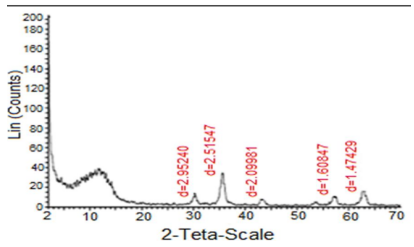

Figure 4. X-ray Diffraction Patterns of pure Fe_3O_4 Nanoparticles

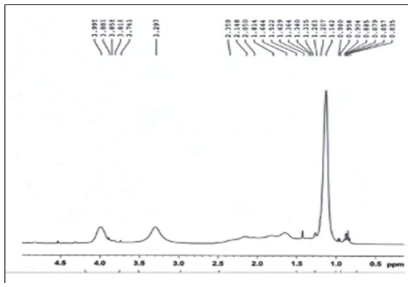

Figure 5. ^{1}H Nuclear magnetic resonance spectrum of poly (NIPAAm-MAA-VP)

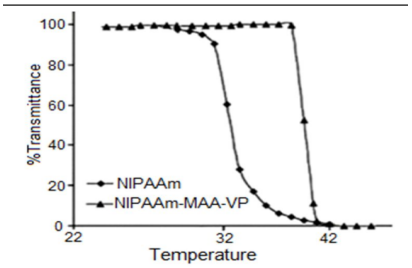

Figure 6. LCST of NIPAAm and NIPAAm-MAA-VP Co-polymer

Fe_3O_4 nanoparticles is close to the standard pattern for crystalline magnetite. The characteristic diffraction peaks are marked, respectively, by their indices (220), (311), (400), (422), (511), and (440), which could be well indexed to the inverse cubic spinel structure of Fe_3O_4 (JCPDS card 85-1436). The average crystallite size D was about 15 nm and obtained from the Sherrer equation $D=K\lambda/(\beta \cos \theta)$, where K is the constant, λ is the x- ray wavelength, and β is the peak width of half maximum.

^{1}H-NMR spectrum of NIPAAm-MAA-VP co-polymer

The basic chemical structure of NIPAAm-MAA-VP copolymer is confirmed by ^{1}H-NMR spectra that were recorded in real- time with a Brucker DRX 300 spectrometer operating at 400 mHz. In figure 5, NIPAAm signal appeared in δ=3.9 ppm, which represents N-CH-$(CH_3)2$, and δ=1.6 ppm related to (CH-CH), and δ= 1.1 ppm related to CH-(CH3)2. The peaks of vinyl pyrrolidone appeared at δ= 3.3 ppm, which related to (CH_2-CH) and δ=2.9 ppm relates to (CH-N-).

LCST of co-polymer

To determine the temperature at which the phase transition occurs in the nanoparticles, UV- Vis spectrophotometer was used. As shown in figure 6, the LCST of N-isopropylacrylamid was 32°C. Also, the phase transition of NIPAAm-MAA-VP co-polymer occurs sharply at 39°C. In addition to the LCST measurements, the phase transition of the nanoparticles can easily be seen when the solution goes from clear to cloudy at each specific LCST.

Swelling behavior of co-polymer

Figure 7, represents the effect of pH on the degree of swelling at 37°C. With increasing pH of solution, the swelling ratios of gel show a continuous decrease. The results obtained show that swelling behavior of hydrogel depending on pH (Table 1).

Drug-loading efficiency

In this study, the effect of the drug/polymer ratio on the drug loading efficiency was investigated. In figure 8 the drug/ polymer ratio of 1/10 showed excellent drug loading efficiency (69.5%) without formation of large aggregates and precipitated products (Table 2).

Magnetism test

The magnetic properties of the nanoparticles were analyzed by vibrating sample magnetometry at room temperature. Figure 9 shows the hysteresis loops of the samples. The saturation magnetization was found to be 18 emu/g for doxorubicin-loaded NIPAAm-MAA-VP magnetic nanocomposite, less than for the pure Fe_3O_4 nanoparticles (65emu/g). This difference suggests that a large amount of polymer encapsulated the Fe_3O_4 nanoparticles and doxorubicin. With the large saturation magnetization, the doxorubicin- loaded NIPAAm-MAA-VP magnetic nanoparticles could be separated from the reaction medium rapidly and easily in a magnetic field. In addition, there was no hysteresis in the magnetization, with both remanence and coercivity being zero, suggesting

Table 1. Conditions Used for Preparation of P (NIPAAm-MAA-VP)

Sample	Mole ratio NIPAAm-MAA:VP	BPO percent	Polymerization efficiency (%)	LCST (°C)
P (NIPAAm-MAA-VP)$_1$	5:10:58	0.01	78%	42±0.2
P (NIPAAm-MAA-VP)$_2$	5:35:60	0.01	65.5%	34±0.5
P (NIPAAm-MAA-VP)$_3$	25:35:40	0.01	54.8%	32±0.8

Table 2. Physical Characteristics of Synthesized Polymers

Polymer type	Encaosulation efficiency (%)	Particle size (nm)
P (NIPAAm-MAA-VP)$_1$	68.5±10	116
P (NIPAAm-MAA-VP)$_2$	59.6±8.5	285
P (NIPAAm-MAA-VP)$_3$	55.5±7	350

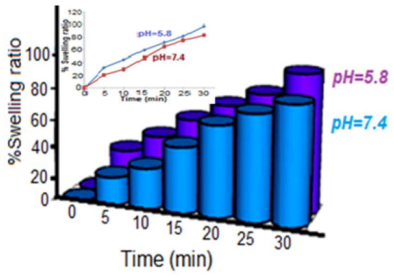

Figure 7. Swelling% in Different pHs at 37ºC

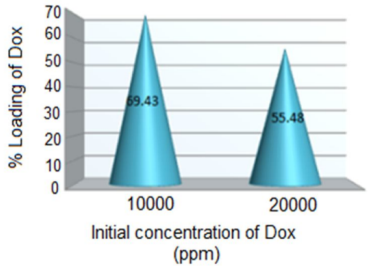

Figure 8. %Loading at Different Concentration of Doxorubicin

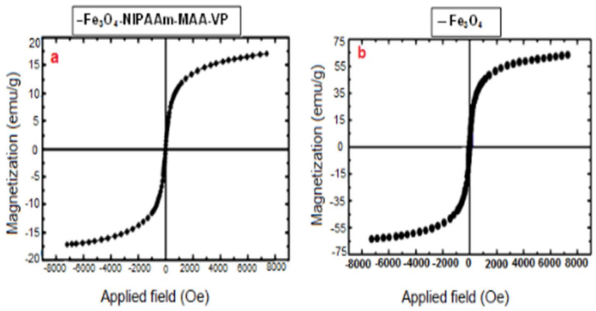

Figure 9. Vibrating Sample Magnetometry (VSM). A) NIPAAm-MAA-VP-Fe_3O_4 and **B)** pure Fe_3O_4

that these magnetic nanoparticles are superparamagnetic. When the external magnetic field was removed, the magnetic nanoparticles could be well dispersed by gentle shaking. These magnetic properties are critical for application in the biomedical and bioengineering.

Size and morphology of nanoparticles (SEM)

The SEM micrographs of pure Fe_3O_4 nanoparticles and doxorubicin loaded on poly (NIPAAm-MAA-VP)-Fe_3O_4 nanoparticles are shown in figure 10. Observing the photograph (10a), nanoparticles were aggregated seriously, which was due to the nanosize of the Fe_3O_4, and they were about 25 nm, according to the result of XRD. At figure 10b, the size of particles was changed to be 60-100 nm.

Release kinetics (Drug release)

The release behavior of the nanoparticles was studied for 16 days (400 h) in PBS (0.1 M, pH=7.4, t=37ºC) and (pH=5.8, t=40°C). The percentage of cumulative release of DOX at 40°C was significantly higher than at 37°C.

Figure 11a shows the release profile of Dox loaded NIPAAm-MAA-VP-Fe_3O_4 nanoparticles in pH=7.4, T=37ºC and figure 11b shows the release profile in pH=5.8, T=40ºC. In two cases Release percentage of Dox in 69.43 drug loading percentage is more than 55.48

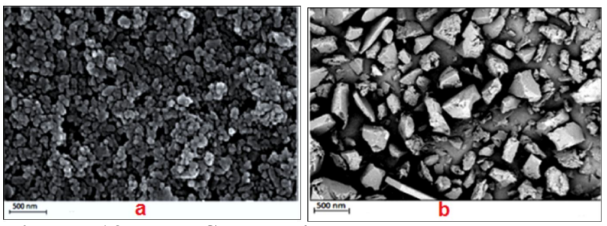

Figure 10. The SEM Micrographs. a) Pure Fe_3O_4 Nanoparticles and **b)** Dox loaded on poly (NIPAAm-MAA-VP)- Fe3O4 nanoparticles

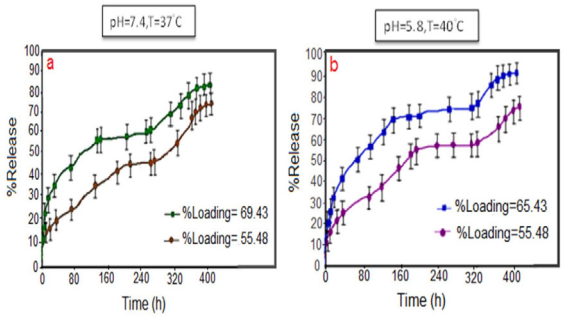

Figure 11. *In vitro* Release Profiles of DOX Over 400 Hours between Poly (NIPAAm)

drug loading percentage. At first hours burst release was occurred and 29% of the encapsulated Dox was released and after 80 hours at 69.43 loading percentage, 44% of Dox was released at 37ºC whereas at 40ºC approximately 50% was released. It is generally assumed that a drug is released by several process such as diffusion through the polymer matrix, release by polymer degradation and solubilization and diffusion through microchannels that exist in the polymer matrix or are formed by erosion. Poly (NIPAAm-MAA-VP) exhibit reverse thermal and pH-dependent gelation properties. Hydrolysis of amid groups in this co-polymer will cause the swelling to increase with time as hydrolysis proceeds. The gel becomes increasingly pH-sensitive as hydrolysis proceeds. We can consider that the drug is released from the Fe_3O_4-NIPAAm-MAA-VP nanoparticles through the swelling mechanism in vitro. The swelling of the particles increased in acidic buffered solutions due to the protonation of amine groups.

Discussion

We have synthesized a smart magnetic hydrogel nanocomposite containing NIPAAm, MAA, VP and Fe3O4 nanoparticles. For this porpose superparamagnetic iron oxide nanoparticles were prepared using an improved chemical co-precipitation method and then incorporated with doxorubicin (model cancer drug) into NIPAAm-MAA-VP co-polymer. Saturation magnetization was found to be 18 emu/g. Our results indicated that the aqueous solution of poly (NIPAAm-MAA-VP) exhibited a noticeable thermo-responsive behavior. The phase transition temperature (LCST) of NIPAAm-MAA-VP co-polymer (39ºC) was higher than the normal PNIPAAm (32ºC). Swelling experiments were performed in buffer solutions with different pH to establish the behavior of the hydrogel was depended on pH. The obtained results show that as the pH increased, swelling ratio % of

NIPAAm-MAA-VP decreased. In pH=5.8, hydrogel is water soluble but in pH=7.4 slightly less water soluble or water insoluble. So this co-polymer will be used as stimuli sensitive drug carrier. Then we use Dox to investigate Dox loading and release pattern. Drug loading efficiency in 1/10 polymer to drug is more than the 1/5 and was found 69.43%. Furthermore, the drug release studies indicated that Dox released from these nanoparticles were in response to changes in temperature with the highest percentage of release occurring at 40ºC, Higher and faster doxorubicin release was observed for 69.43 loading percentage than for 55.48 loading percentage at 40ºC and pH=5.8. The release rate decreased with the increase of pH values. The pH value of the tumor was 5.0-6.0, which was lower than the pH value of the normal tissue, so the DOX could be released at the tumor. Therefore this nanocomposite can be used for controlled drug delivery due to their temperature sensitive properties.

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