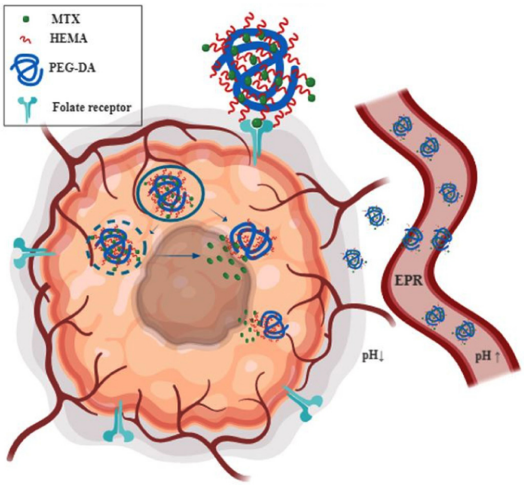
Assessment of pH Responsive Delivery of Methotrexate Based on PHEMA-st-PEG-DA Nanohydrogels

Javad Farzanfar, Fatemeh Farjadian*, Amir Roointan, Soliman Mohammadi-Samani*, and Lobat Tayebi

Macromol. Res., ***29***, *54 (2021)*

The principal purpose of this project is to synthesize a biocompatible nanohydrogel (NH) consisting of poly-(hydroxyethyl)methacrylate (HEMA) with poly(ethyleneglycol) diacrylate (PEG-DA) as cross-linker by using reversible addition-fragmentation chain-transfer (RAFT) method, with the potential to carry methotrexate (MTX) into breast cancer cell lines (MCF-7). The pH-sensitive delivery for MTX using the synthesized final formula can be considered as an innovation in this design. Due to enhanced permeability and retention (EPR) effect the NH could accumulate in tumor site and release MTX through pH changes.



Macromolecular Research Article

DOI 10.1007/s13233-021-9007-6 www.springer.com/13233 pISSN 1598-5032 eISSN 2092-7673

Assessment of pH Responsive Delivery of Methotrexate Based on PHEMA-st-PEG-DA Nanohydrogels

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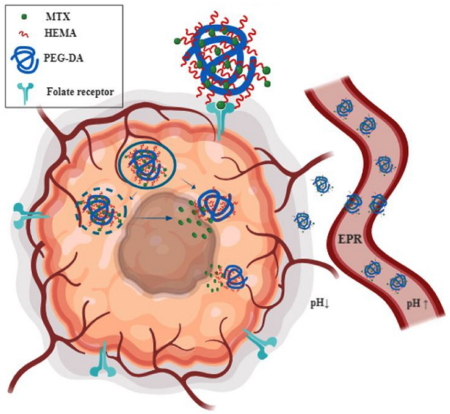
Received August 3, 2020 / Revised November 17, 2020 / Accepted November 22, 2020

Abstract: Nanohydrogels (NHs) are novel and attractive carriers for various anti-cancer factors delivery. The objective of present study is development of a safe NH for pH responsive delivery of methotrexate (MTX). Herein, poly (hydroxyethyl methacrylate) is utilized as the main structure, which is cross-linked with poly(ethyleneglycol) diacrylate (PEG-DA) through reversible addition fragmentation chain transfer polymerization technique. After synthesis, the developed structure is characterized using different methods, including ^{1}H NMR, FT-IR, size exclusion chromatography, transmission electron microscopy and dynamic light scattering. The results confirm successful synthesis of the NH with acceptable yield and nano scale mean size of 194 nm. Methotrexate is conjugated with the aforementioned structure through pH responsive esteric bond. The efficiency of the prepared NH in loading and release of the anti-cancer drug, methotrexate, is tested. The developed NH shows great potential in methotrexate loading, as well as a faster release rate of methotrexate in acidic pH. The results of *in vitro* toxicity assessment on MCF-7 as a breast cancer cell line reveal an improved cytotoxicity induction by the methotrexate loaded particles when compared with the free MTX molecules. The suitable size (<200 nm), great potential in loading and release of the methotrexate and cytotoxicity induction in cancer cells are the reliable features of NH as an ideal anti-cancer vehicle.



Keywords: RAFT synthesis, nanohydrogel, PHEMA, PEG-DA, methotrexate, drug delivery, cancer.

1. Introduction

Naoparticles have found distinct utilization as therapeutic agents' carriers in modern medicine.[1-3] Polymeric nanoparticles (PNPs) are colloidal nanoparticles with a wide range of sizes (10-1000 nm).[4,5] Nanohydrogels (NHs) are a special type of polymeric nanomaterials that have recently gained interest due to their specific features.[6] NHs can absorb water over their weight, while remaining in the environment as insoluble.[7] These polymeric systems can be developed in nano-scale sizes. Their high sorption content and, importantly, their biocompatibility plus all the beneficial features of nanomaterials, such as high circulation half-life and easy penetration into tissues make them suitable carriers in pharmacy and medicine.[8] The hydrophilic nature and high water absorption capacity of hydrogels have extended their applicability in different fields.[9] Due to such features, along with versatility in concentration, different monomers and cross-linker have enabled scientists to design various types of hydrogels suitable for different tasks in biomedicine, engineering and agriculture.[10,1] So far, various NH formulations have been developed for *in vivo* delivery of different drugs.[11-13] Their biocompatibility, high loading capacity, stability and similarity to biopolymers, along with all the benefits of nanomaterials (like passive targeting to solid tumors), allow them to be great candidates for anti-cancer drug delivery.[14]

One of the simplest, reliable and diverse methods of synthesis for the production of polymeric nanoparticles, such as NHs, among the controlled free radical polymerization (CRP) methods is the reversible addition fragmentation chain transfer (RAFT) polymerization. The versatility of this method in synthesizing different co-polymers with a defined structure is the main reason of its popularity in this context. There is no need to use hazardous catalysts, like organometallic compounds; in addition, producing polymers with a narrow poly dispersity indices (PDIs) is another benefit of RAFT techniques.[15]

In the case of drug carriers, numerous NHs have been developed using monomers. Hydroxyethyl methacrylate (HEMA) is an outstanding monomer for applying hydrophilicity to surfaces

Acknowledgments: The supports of present work from Shiraz University of Medical Sciences - Iran (Grant No. 95-01-36-11579) and Iran National Science Foundation (Grant No. 97007686) is highly acknowledged. The authors wish to thank Mr. H. Argasi at the Research Consultation Center (RCC) of Shiraz University of Medical Sciences for his invaluable assistance in editing this manuscript.

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and preparation of NHs.[16] This hydrophilic monomer, with a swelling behavior, is an ideal candidate for synthesizing biocompatible drug delivery vehicles.[17] Good swelling behavior and acceptable mechanical properties of HEMA-based polymers are usually controlled by bi-functional crosslinking, like poly ethyleneglycol diacrylates (PEG-DA). These cross-linking agents affect and maintain the mechanical strength of the whole structure.[18,19]

Methotrexate (MTX), as one of the most common anti-cancer agents, is applied to remedy various tumors, like lung, bladder and breast cancers. MTX is an analog of folic acid (FA) containing a pteridine ring, a p-aminobenzoic acid residue and a glutamic acid residue. The over-expressed folate receptors on various types of cancer cells can be good attractions for pteridine rings of MTX molecules. Therefore, MTX entrance into cancer cells is similar to FA transport pathway. The mechanism of MTX is to stop the formation of tetrahydrofolate, therefore stopping synthesis of purines and pyrimidines, which are building blocks of DNA and RNA molecules. Specifically, the MTX molecules inhibit synthesis of DNA and RNA molecules through inhibition of dihydrofolate reductase (DHFR) enzyme. Various types of nanocarriers such as dendrimers, carbon nanotubes, micelles, hydrogels, magnetic nanoparticles and gold nanoparticles have been developed to deliver MTX into cancerous cells.[20]

One of the major cancers between women is breast cancer. Usual treatments for breast cancer have encountered a major challenge due to the emergence of drug-resistant cancer cells. Here, methotrexate resistance is very well documented by some research. Accordingly, there is a genuine need to develop alternative strategies to overcome this problem. Drug delivery systems could be a great platform to improve therapeutic efficacy of anti-cancer drugs.[21,20,22-26]

Previously, Farjadian *et al*. reported the synthesis of hydroxylated magnetite nanoparticles (NPs) and presented it as potent system for pH sensitive delivery of MTX. The hydroxyl groups of this NP have great avidity for carboxylate groups of methotrexate molecules.[22] In other efforts by this team, smart pH-responsive NHs of poly(HEMA-co-N,N'-dimethylaminoethylmethacrylamide),[27] temperature and pH responsive delivery system based on poly (N-isopropylacrylamide-co-acrylamide[28] and lysine modified poly(vinylcaprolactam) have successfully applied for doxorubicine delivery.[29]

The principal purpose of this project is to synthesize a biocompatible NH consisting of poly-HEMA with PEG-DA as cross-linker by using RAFT method, with the potential to carry MTX into breast cancer cell lines (MCF-7). The use of an effective and reliable cross-linker such as PEG-DA as well as pH-sensitive delivery for MTX using the synthesized final formula can be considered as an innovation in this design. The *in vitro* anticancer efficacy of the as prepared NH with and without MTX was evaluated in MCF-7 cell line.

2. Experimental

2.1. Instruments

In the present study, to check the synthesis and assess the size of the developed nanohydrogels, several characterization methods, including ^{1}H nuclear magnetic resonance (^{1}H NMR, Bruker 300 MHz), Fourier-transform infrared spectroscopy (FT-IR, Vertex 70), size exclusion chromatography (SEC , TSK gel G3000 SWXL 300 mm * 7.8 mm, 5 μm), transmission electron microscopy (TEM, Philips, CM10) and dynamic light scattering (DLS, NANO-flex® 180°) were utilized. The zeta potential of the polymerized NH was checked by Nanotrac wave MN402 (USA). The drug release profile and cytotoxicity assay were determined *via* a 96-well ELISA plate reader (USA, Stat Fax 2100 Microplate Reader). All examinations were performed in triplicate, and for explaining values was used from the mean ± standard deviation.

2.2. Materials

Azo-bis-isobutyronitrile (AIBN), HEMA, PEG-DA, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), 4-dimethylaminopyridine (DMAP), and 4-Cyano-4-[(dodecylsulfanylthiocarbonyl)sulfanyl]pentanoicacid were prepared from Sigma-Aldrich, Germany. Initially, the recrystallization of AIBN performed by using methanol, and the purification of both the PEG-DA and HEMA performed by using a silica column. Afterward, a combination of 30% water and 70% ethanol solution was prepared and used as the main reaction solvent. Before that, the main solvent was degassed by pure N_2 purging for 30 min. Human breast cancer cells (MCF-7) were supplied from Pasteur Institute Cell bank (Iran).

2.3. Synthesis of PHEMA-st-PEG-DA

Based on the previous RAFT method,[27,30] 0.09 mmol of AIBN, 0.225 mmol of RAFT agent (4-Cyano-4-[(dodecylsulfanyl thiocarbonyl)sulfanyl]pentanoicacid), 6.55 mmol, of purified HEMA and 0.20 mmol, of purified PEG-DA with ratios of 0.4, 1 and 30 (HEMA: PEG-DA 97%:3%), respectively, and 10 mL of the basic solvent were poured into a 20 mL vacuum tube. After 15 min nitrogen bubbling and successive stages of freezing using liquid N_2, thawing by degassing pump and degassing with N_2 gas, the tube was immersed in an oil bath at 80 °C for 48 h. Finally, PHEMA-st-PEG-DA co-polymer was washed four times and purified by cold n-hexane.[27]

2.4. Characterization of the nanohydrogel

The structure of NH was verified via ^{1}HNMR and FT-IR. The size and morphology of the particles were checked via DLS and TEM. The NH particles were dispersed in PBS (pH 7.4), using a magnetic stirrer for about 1 h before characterization by TEM and DLS. The molecular weight was obtained via SEC analysis. In SEC procedure, PBS with pH 6.5 and flow rate of 0.5 mL/min for one hour) was used as the running buffer. The zeta potential of the particles in 5 mM PBS (pH 7.4) was measured by Nanotrac Wave MN402 instrument.

2.5. Preparation of methotrexate-loaded nanohydrogel particles

The conjugation of MTX with NH was carried out via the esterification reaction amongst the -OH groups of HEMA and the -COOH

group of MTX, similar to our previous report.[22] At first, 0.05 mmol EDC), as carboxyl group activator, was added to 920 µL aqueous solution of 0.05 mmol MTX in the presence of DMAP (0.005 mmol). Then, the admixture was stirred for 60 min, followed by addition of 8 mg of NH to the mixture. The tube was shaken at temperature 40 °C for a full day, and the admixture was then centrifuged, washed with acetone/deionized water and dried at temperature 25 °C. The amount of MTX was determined by hydrolysis of PHEMA-st-PEG-DA/MTX in acidic aqueous medium (pH of 1.2) during 48 h at 40 °C through measurement via a UV-Vis spectrophotometer at wavelength 306 nm.[22]

2.6. Assessment of MTX release profile of PHEMA-st-PEG-DA/MTX

To assess the MTX release profile, MTX-NH solutions (100 µg/mL) in two various pH values of 7.4 and .5), two sets of tubes were placed on a shaker incubator and were shaken slowly at temperature 37 °C at 450 rpm for times of 0.5, 1, 2, 4, 8, 12, 24, 48, 72 and 96 h. The released amounts of the MTX into the medium were measured via UV-vis spectrophotometer at wavelength 306 nm, and then the cumulative drug release amount (%) was calculated using the Eq. 1.[22]

$$\text{Accumulative drug release percentage} = \frac{\text{released amount of drug}}{\text{total amount of drug}} \times 100 \quad (1)$$

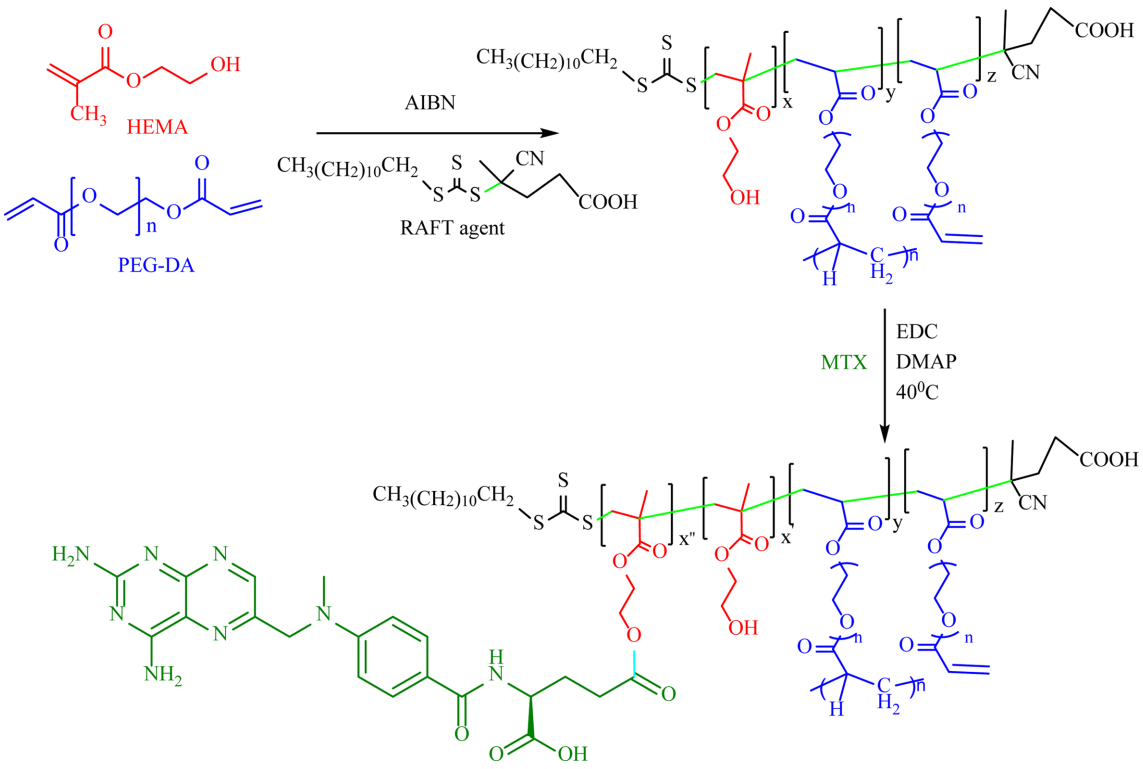
2.7. *In vitro* sample's cytotoxicity

Cell culture procedures performed by using RPMI 1640 from Sigma-Aldrich with 10% inactivated FBS from Merck, streptomycin (100 mg/mL) and penicillin (100 µL/mL). Trypsin/EDTA 0.25% from Shellmax/China was used for cell passaging procedure. 3-(4,5-Dimethyl-1,3-thiazol-2-yl)2,5-diphenyl-2H-tetrazol-3-ium bromide (MTT, 5 mg/mL from Sigma-Aldrich) assay was utilized for the cytotoxicity evaluations. MCF-7 cells were cultivated in 96 well plates and incubated for 24 h (temperature 37 °C, 5% CO_2) (7000 cells per well). After 80% confluency, cells were treated with different concentrations of NH-MTX (0, 2, 5, 10, 20, 50 and 100 µg/mL) and equivalent concentrations of free MTX for 24 h were performed, in a quadruplet manner. After finishing incubation, each well medium was removed and then, 20 µL/well of (5 mg/mL) MTT in phosphate-buffered saline (PBS) solution was added. The 96 well plates were incubated for 4h at temperature 37 °C in a darkroom. Subsequently, dimethyl sulfoxide (100 µL) was added to wells, and after gentle shaking for 15 min, the absorbance of each well was obtained using ELISA plate reader (570 nm). Percentage of cell viability was calculated by Eq. (2). The similar procedure was performed by using only NH at various concentrations, 2/ 5/ 10/ 20/ 50 and 100 µg/mL to test its possible efficacies on MCF-7 after two days of incubation.[27]

$$\%\ \text{Cell viability} = \frac{\text{Abs (test)}}{\text{Abs (Control)}} \times 100 \quad (2)$$

2.8. Statistical analysis

The data were analyzed in Graph Pad Prism (V 7.04) and presented as mean ± standard error of the mean (S.E.M). The significance of data was calculated using a two-tailed t-test for independent samples.



Scheme 1. Schematic presentation of two step synthesis pathway of PHEMA-st-PEGDA and MTX conjugated structure.

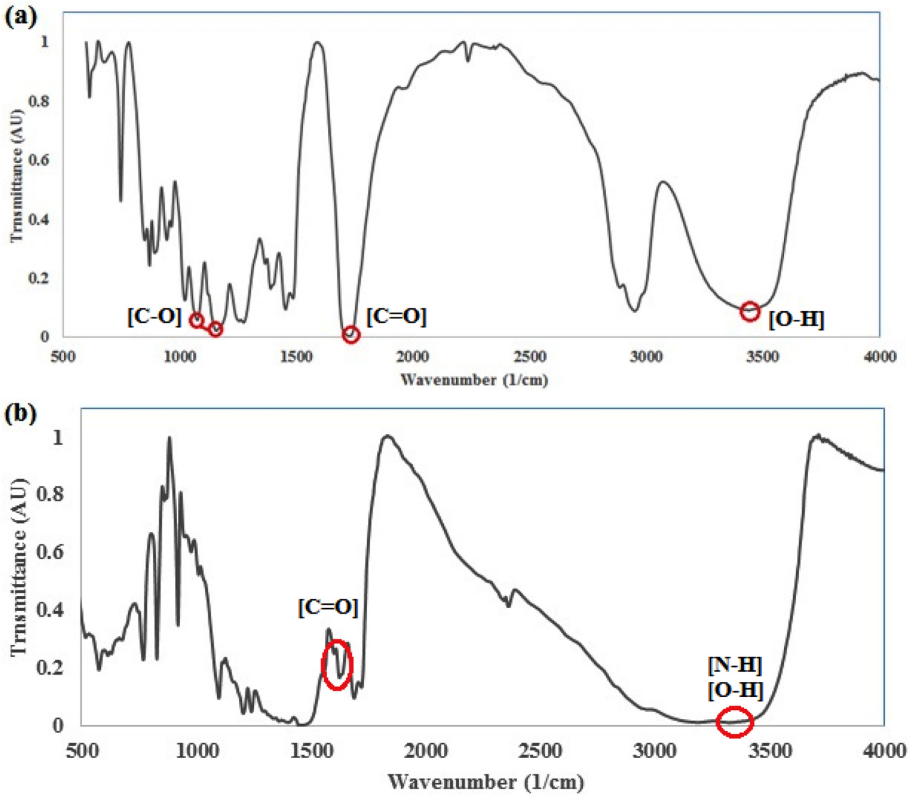


Figure 1. The FT-IR spectrum of the synthesized PHEMA-st-PEGDA (a) and NH-MTX (b).

3. Results and discussion

3.1. Synthesis and characterization

The NH synthesis was successfully performed by RAFT technique (Scheme 1). In the RAFT procedure, different ethanol and water mixtures were applied as the reaction solvent. The results show that the reaction solvent composition can play a main role in the polymer synthesis yield. Due to the effect of various ratios of ethanol and water on yield of synthesis (data not shown here), at first different ratios of ethanol and water are used to obtain the optimum reaction solvent for maximum synthesis yield of the polymer. The optimized and the finest solvent mixture for synthesizing the NH is determined at 70% ethanol and 30% water. The synthesized NH using this mixture reveals better solubility and dispersion behavior in comparison with the synthesized NHs that use other solvents, or ethanol and water in other ratios. Advantages of RAFT polymerization method include RAFT applicability in different temperatures, high functional group tolerance and possessing a great control on molecular weight (MW) and structure of polymers.[31]

NH synthesis, using HEMA, PEG-DA monomers and RAFT synthesized method was successfully accomplished and proven by observing the FT-IR and ^{1}H NMR results.

FT-IR spectroscopy was performed to show the presence of functional group in synthesized PHEMA-st-PEGDA structure (Figure 1). The FT-IR peaks show the accurate place of the functional groups, including C-O groups of PEG-DA (1076-1157 cm^{-1}), O-H groups of HEMA (~3439 cm^{-1}) and C=O groups in all monomers (~1726 cm^{-1}) in the spectrum.[27,32]

^{1}HNMR results also reveal the peaks that represent the protons of each monomer group and confirm the results of FT-IR (Figure 2). In the ^{1}HNMR spectrum, the peaks around the 3.3 and 3.63 ppm show the HEMA functional groups, while the peaks in 3.09, 3.24 and 4.21 ppm show the protons of PEG-DA functional groups. The methyl and methylene groups related protons of the RAFT agent can be seen in 0.5 to 2.1 ppm. Peaks around 5.41 and 5.79 ppm are ascribed to the unreacted functional vinyl groups of PEG-DA.[27,33]

After integration of the peak areas, the synthesized NH composition is estimated. The NH composition 97.15% PHEMA and 2.85% PEG-DA, respectively. The average molecular weight of the synthesized NH is estimated to be 3957 Da. The calculated MW of MTX-NH by SEC experiment is about 6000 Da, which is in compromise with the estimated MW from the ^{1}HNMR results.

The size of synthesized NH and NH-MTX was measured using DLS and TEM imaging. The obtained results showed the average sizes of 194 nm and 191.4 nm for the dispersed NH and NH-MTX in phosphate buffer (pH: 7.4), respectively (Figure 3). Particles with zeta potential of +7.3 mV possess a positive charge. The PDI/poly dispersity index of the NH particles is 1.2. These data were in reasonable range for the selected synthesis method.[27]

Based on the results of DLS and TEM microscopy, the NH particles have a size smaller than 200 nm. According to the literature, when administered intravenously, particles with size lower than 200 nm have longer blood circulation. This can improve their accumulation in solid tumors via what is known as "enhanced permeation and retention" effects (EPR). EPR is a special spec-

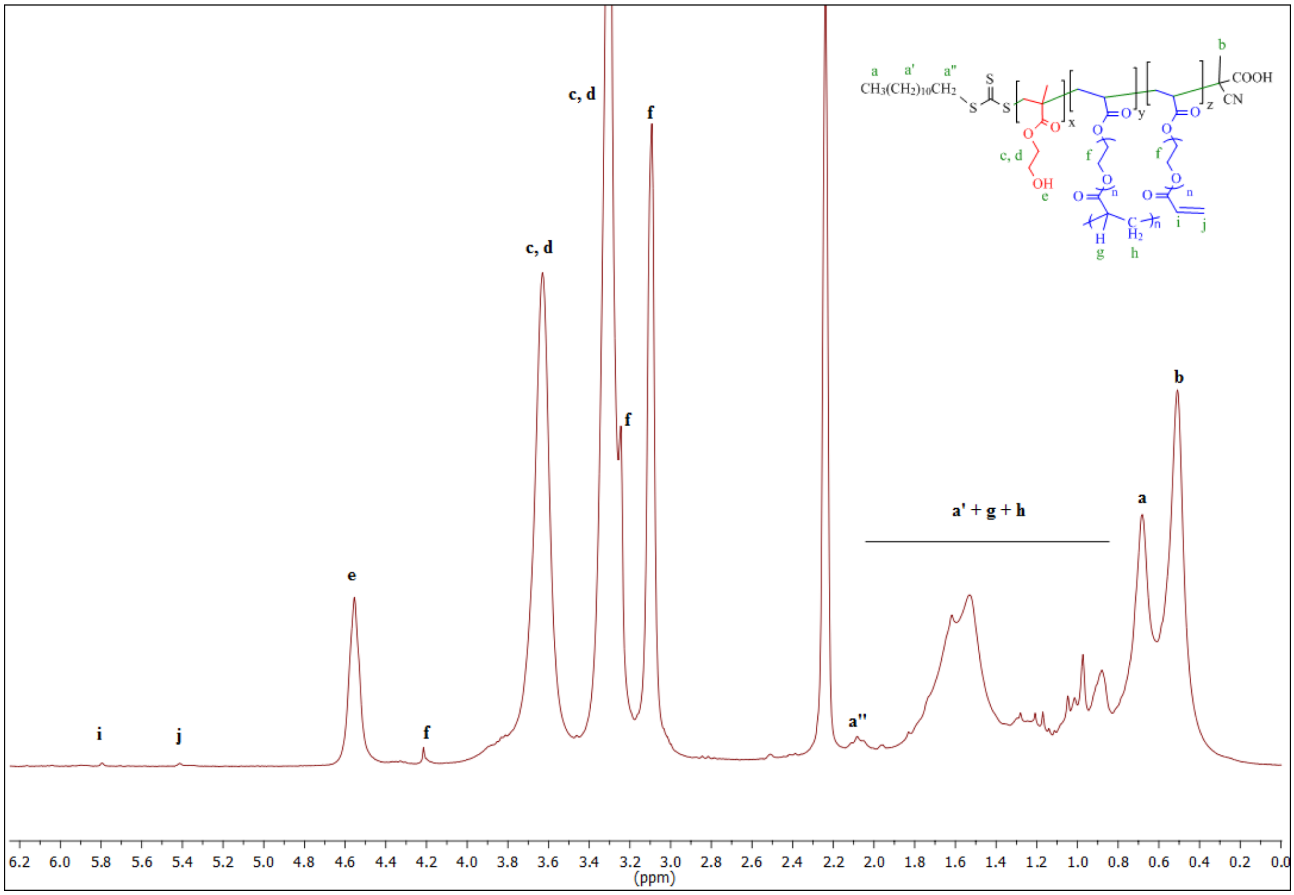


Figure 2. ^{1}HNMR spectrum of the synthesized PHEMA-st-PEGDA in dimethyl sulfoxide-d6. The letters indicate the specified atoms in the NH. (a, a′, a′′ and b) represent the CH_3, CH_2s, CH_2 and CH_3 groups of the RAFT agent (CTA), (c and d) represent CH_2, (e) represents the hydroxyl groups of PHEMA, (f) represents the CH_2 groups of PEG-DA, (g and h) represent the reacted functional vinyl groups of PEG-DA and (i and j) represent the unreacted functional vinyl groups of PEG-DA.

ification of solid tumors and, according to the literature, particles with size <400 nm can easily penetrate and remain in tumor tissues.[34] Other research also shows that the particle influence depth into the solid tumor tissues is linked to their sizes, where the smaller particles have higher penetration rate. On the other hand, based on other reports, the blood clearance rate of such particles (< 200 nm) is lower than larger particles.[35] Overall, having a suitable size (194 nm) and having the benefits of EPR effect, the developed NH could exhibit a better efficiency in passive transfer of different anticancer factors into solid tumor tissues.[35,36]

3.2. MTX conjugation and release study

Herein, MTX is conjugated with the available PHEMA hydroxyl groups on the NH structure (Scheme 1). The conjugation process is carried out through the formation of ester bonds between PHEMA-st-PEG-DA hydroxyl groups and carboxylic acid groups of MTX molecules.

To confirm the conjugation processes, the resulted conjugated molecules (NH-MTX) are analyzed using FT-IR. The FT-IR peaks showed the accurate place of the functional groups of MTX, including N-H, O-H (~3400 cm^{-1}), and C=O groups (~1640-1610 cm^{-1}) in the spectrum (Figure 1). Moreover, to check the loading process, a complete breakage of the ester bonds between MTX and PHEMA groups in NH is done in an acidic medium, and then the quantity of the hydrolyzed MTX molecules is analyzed using UV-vis spectroscopy.[22] In this regard, the calculated loading amount is 1.43 mmol MTX/g NH, equal to 65%.

The drug release profile of the prepared NH-MTX is evaluated in two various pH values 5.5 and 7.4 at 37 °C (Figure 4). During 24 h incubation time, the NH-MTX reveals an exponential growth in MTX released into the medium. Interestingly, the release pattern is more desirable in the pH of 5.5. This could be due to higher hydrolysis of the ester bonds between the MTX molecules and hydroxyl groups of HEMA at a more acidic pH.[22] The overall MTX release over time was not more than 41% in the normal pH (pH: 7.4) within 96 h. However, the released amounts of MTX in the acidic pH (pH: 5.5) was 67% after 96 h of incubation. The release mechanism of MTX from the conjugated delivery system was assessed according to curve fitting to zero order, first order and Higuchi model.[37] Due to the esteric nature of the synthesized delivery system best results were obtained to first order model with r^2 about 0.991 for pH 7.4 and 0.972 for pH 5.5 indicating a pH responsive system for delivery of MTX.

These observations show the potential of synthesized NH in carrying and releasing anti-cancer agents in higher quantity in solid tumor microenvironment, where the pH is acidic (pH: 5.5-6).[38,39]

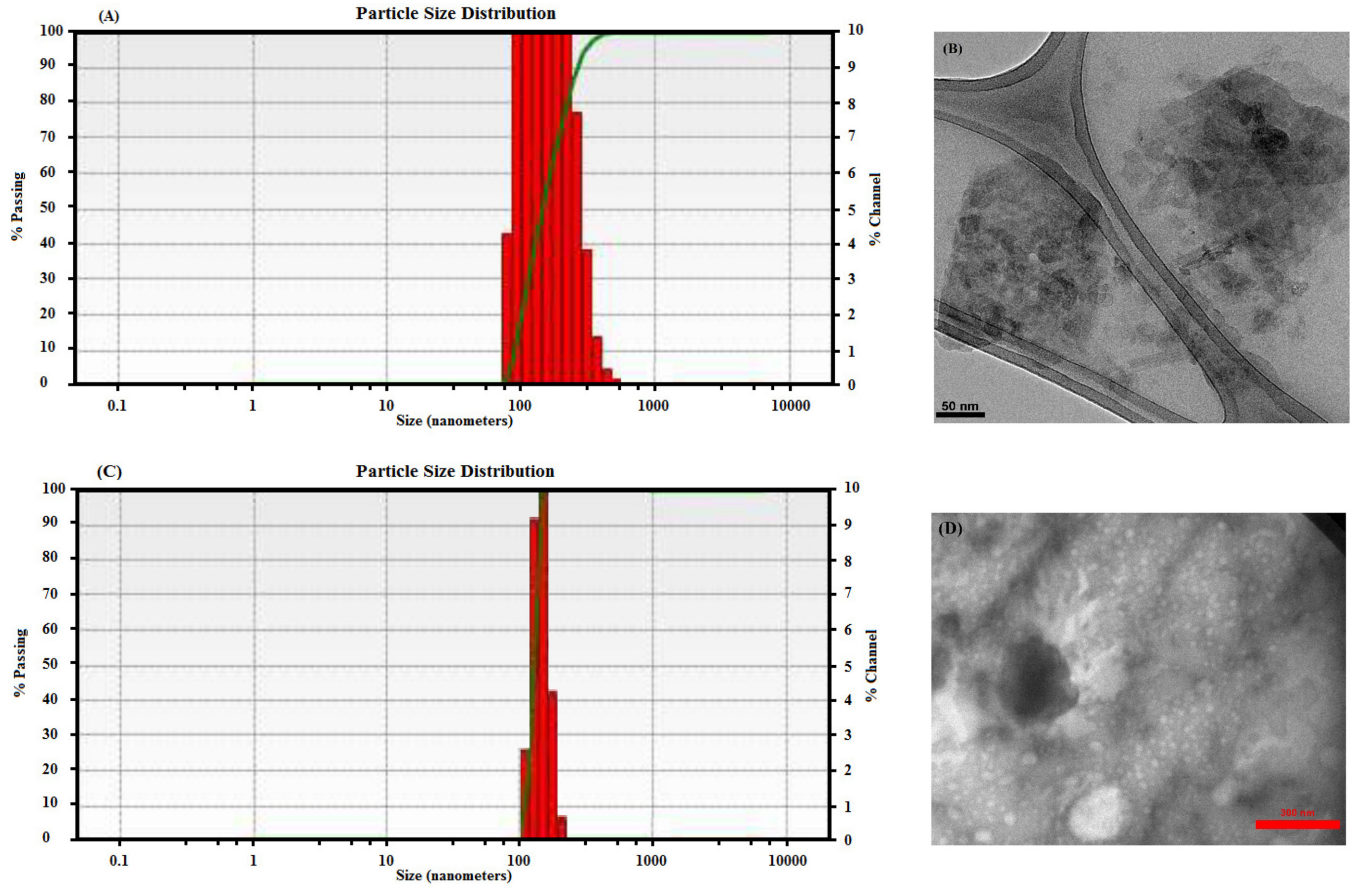


Figure 3. DLS and TEM results of PHEMA-st-PEGDA (A and B) and NH-MTX (C and D) at pH 7.4.

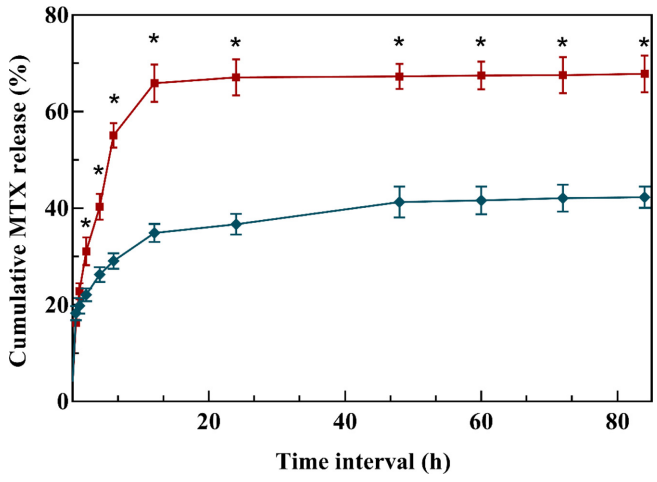


Figure 4. Cumulative release study of methotrexate from NH-MTX particles based on time and pH alterations. Square and diamond symbols stand for pH 5.5 and pH 7.4, respectively. Values are presented as mean ± S.E., (n = 3, *p<0.01).

3.3. *In vitro* cellular cytotoxicity studies

In this section, the ability of NH-MTX complexes in inducing cytotoxicity in MCF-7 is assessed. Here, MCF-7 cells are treated with different MTX-free concentrations, and with the same concentrations of the MTX-NH (equivalent drug concentrations in the NH). MCF-7 cell is also treated with NH to evaluate the cytotoxicity of the NH by itself. Figure 5 shows the results of MTT assay after 48 h incubation (Figure 5). The obtained results reveal a dose-dependent cytotoxicity in the cells treated with MTX alone, also the treated cells with NH-MTX conjugates. In a comparative view, the NH-MTX conjugates result in a higher cell cytotoxicity when compared with the MTX alone, especially in higher concentrations. The NH-MTX in certain concentrations of 20, 50 and 100 μg/mL that correspond to 13, 32.5 and 65 μg/mL of MTX, respectively, reveal a considerable cell cytotoxicity after 48 h of incubation (Figure 5(A)). On the other hand, there was no significant toxicity in the treated cells with the NH alone. Such observations reveal the safety of the developed NH, where the highest concentration of the NH had no significant cytotoxic effect, even after 48 h of incubation (Figure 5(B)). The IC_{50} amounts for MTX alone and prepared NH-MTX are obtained as 8.34 μg/mL and 6.5 μg/mL, respectively. Compared to the MTX, the lower IC_{50} values of NH-MTX show the enhanced effectiveness of the prepared conjugates in killing the cancerous cells.

The higher cytotoxicity of the NH-MTX complexes could attributed to their different cellular entrance pathways.[23] MTX molecules are well-known substrates of multidrug resistance protein 1 (MDR1), extremely represented on the cell surface of most cancer cells. Based on reports, it is assumed that the MTX molecules in conjugated complexes (NH-MTX) have better chance of cellular entrance and cellular retention in the cytosol of cancer cells, when compared to the bare MTX molecules. For instance, in one experiment performed by Kohler *et al.*,[40] it is shown that

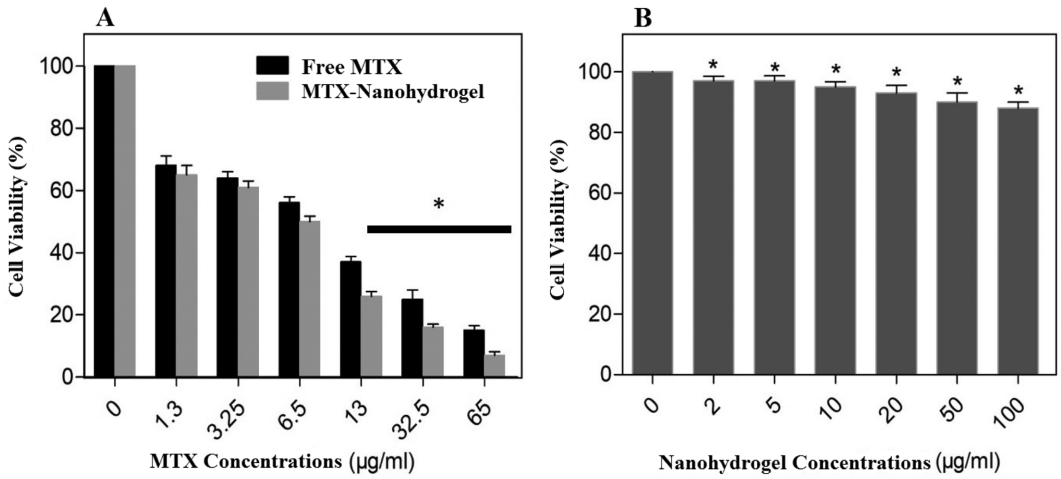


Figure 5. The results of cytotoxicity (MTT) assay against MCF-7 cells. (A): Cytotoxicity results by using different concentrations of free MTX, MTX-NH and (B) NH particles for 48 h. No cellular morphological changes and no significant toxicity by NH, indicating the safety of NH, as well as the cellular toxicity of MTX (not the NH) on the cancer cells.

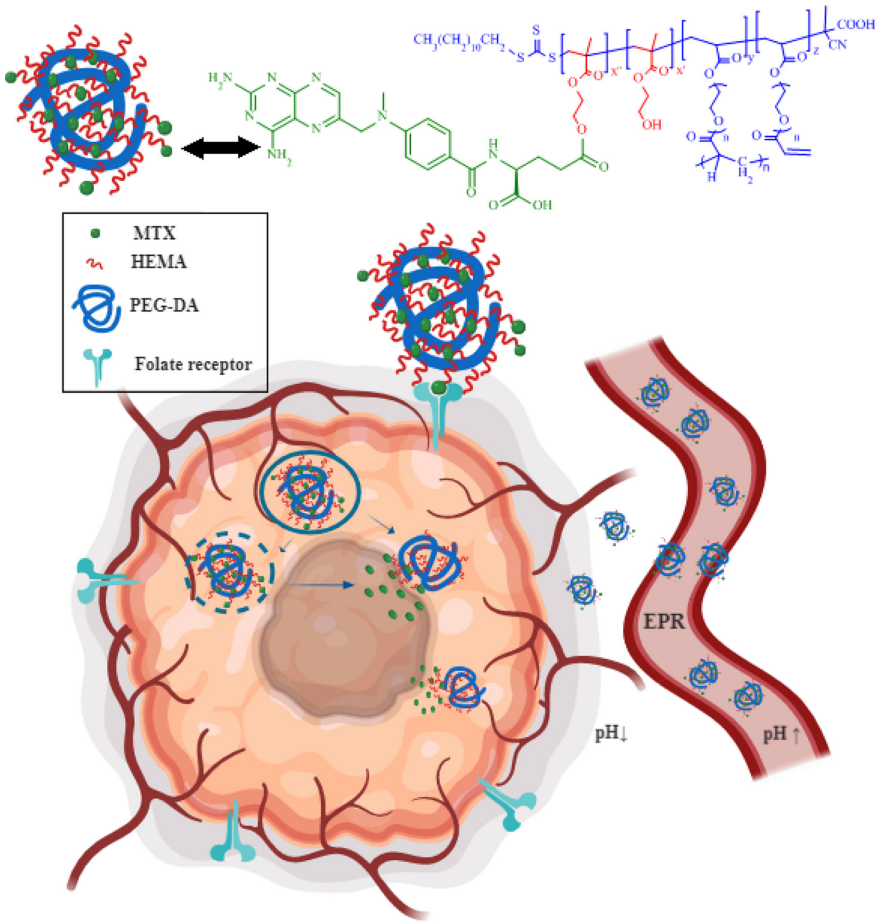


Figure 6. Schematic illustration of enhanced permeation and retention (EPR) effect in solid tumors. The developed NH particles (<200 nm), could be a great candidate for anti-cancer drug delivery into tumor cells.

the nanoparticles carrying MTX molecules could internalize into cells and accumulate into lysosomal compartments in higher amounts than bare MTX molecules. Due to the acidic environment of the lysosomes, MTX release will occur at faster rates; hence, higher cytotoxicity is possible. The higher chance of cellular entrance in the cancer cells' cytosol is due to MTX similarity with transport pathway of FA, also since MDR1 protein cannot identify MTX-NH. Hence, cellular retention rate is higher together with the benefits of EPR effect, resulting from the suitable size of NH particles, leading to higher cytotoxicity of the NH-MTX complexes compared to the bare MTX molecules (Figure 6).

4. Conclusions

In summary, a nanohydrogel containing inert and safe molecules of PHEMA and PEG-DA was synthesized via RAFT methodology. The characterization results confirm the successful RAFT methodology in synthesizing this anti-cancer drug delivery model. The novelty of this experiment is the application of PEG-DA as a cross linker in combination with pHEMA in a hydrogel based system, and application of this developed NH in delivering MTX into the MCF-7 cancer cells. The developed NH reveals good water dispersion, as well as a great efficiency in conjugation and also release of MTX molecules into the cancer cells.

The morphology of the developed NH are fully tested by DLS and TEM techniques. With the suitable size of 194 nm and MW of about 3957 Da, we introduce this NH structure as an effective vehicle for systemic delivery of anti-cancer agents. In previous experiments, we used features like pH or magnet for a smart drug delivery. But, in this structure, we took the benefits of NH sizes (below than 200 nm), and enhanced permeation and retention effect (EPR) in tumor area. Both of the mentioned items finally cause a longer circulation of NPs and their more accumulation in the tumor area. However, more evaluations are necessary to confirm the safety and efficiency of the developed NH in delivering MTX or other anti-cancer agents. In case of MTX delivery, using the developed NH, high availability of HEMA hydroxyl groups made NH a suitable platform for an exquisite conjugation with MTX through ester bond formation. The MTX release pattern reveals a time- and pH-dependent release behavior of the prepared NH-MTX conjugates. *In vitro* cellular cytotoxicity shows higher anti-cancer activity of the NH-MTX conjugates in comparison with the free MTX molecules. Our recommendation is to conduct different safety assessments, such as checking the NH-MTX cellular entrance and the *in vivo* efficiency of the developed NH in delivering MTX drug molecules.

References

(1) F. Farjadian, A. Ghasemi, O. Gohari, A. Roointan, M. Karimi, M. R. Hamblin, *Nanomedicine*, **14**, 93 (2018).
(2) F. Farjadian, M. Moghoofei, S. Mirkiani, A. Ghasemi, N. Rabiee, S. Hadifar, A. Beyzavi, M. Karimi, and M. R. Hamblin, *Biotechnol. Adv.*, **36**, 968 (2018).
(3) F. Farjadian, A. Roointan, S. Mohammadi-Samani, and M. Hosseini, *Chem. Eng. J.*, **359**, 684 (2018).
(4) M. Hosseini, F. Farjadian, and A. S. H. Makhlouf, in *Industrial Applications for Intelligent Polymers and Coatings*, Springer, 2016, pp 1-26.
(5) A. Roointan, S. Kianpour, F. Memari, M. Gandomani, S. M. Gheibi Hayat, and S. Mohammadi-Samani, *J. Polym. Mater. Polym. Biomater.*, **67**, 1028 (2018).
(6) O. Erol, A. Pantula, W. Liu, and D. H. Gracias, *Adv. Mater. Technol.*, **4**, 1900043 (2019).
(7) M. Akram and R. Hussain, in *Nanocellulose and Nanohydrogel Matrices: Biotechnological and Biomedical Applications*, 2017, pp 297-330.
(8) P. K. Bolla, V. A. Rodriguez, R. S. Kalhapure, and J. Renukuntla, *J. Drug Deliv. Sci. Technol.*, **46**, 416 (2018).
(9) B. Massoumi, Z. Mozaffari, M. Jaymand, *Int. J. Biol. Macromol.*, **117**, 418 (2018).
(10) M. Rizwan, R. Yahya, A. Hassan, M. Yar, A. Azzahari, V. Selvanathan, F. Sonsudin, and C. Abouloula, *Polymers*, **9**, 137 (2017).
(11) J. Li and D. J. Mooney, *Nature Reviews Materials* **1**, 1 (2016).
(12) S. S. Mishra and A. K. Sharma, *Pharma Innovation J.*, **7**, 182 (2018).
(13) H. Gangadharappa, S. M. C. Prasad, R. P. Singh, *J. Drug Deliv. Sci. Technol.*, **41**, 488 (2017).
(14) W.-F. Lai and Z.-D. He, *J. Control. Release*, **243**, 269 (2016).
(15) G. Moad, *Polym. Chem.*, **8**, 177 (2017).
(16) F. Farjadian, S. Schwark, and M. Ulbricht, *Polym. Chem.*, **6**, 1584 (2015).
(17) C. N. Kotanen, D. R. Janagam, R. Idziak, L. Rhym, R. Sullivan, A. M. Wilson, T. L. Lowe, and A. Guiseppi-Elie, *Europ. Polym. J.*, **72**, 438 (2015).
(18) A. Bhat, B. Smith, C.-Z. Dinu, and A. Guiseppi-Elie, *Mater. Sci. Eng. C*, **98**, 89 (2019).
(19) S. Gatti, A. Agostini, R. Ferrari, and D. Moscatelli, *Polymers*, **9**, 389 (2017).
(20) G. Choi, T.-H. Kim, J.-M. Oh, and J.-H. Choy, *Coord. Chem. Rev.*, **359**, 32 (2018).
(21) S. Davaran, H. Fazeli, A. Ghamkhari, F. Rahimi, O. Molavi, M. Anzabi, and R. Salehi, *J. Biomater. Sci., Polym. Ed.*, **29**, 1265 (2018).
(22) F. Farjadian, S. Ghasemi, and S. Mohammadi-Samani, *Int. J. Pharm.* **504**, 110 (2016).
(23) C. de Oliveira, S. Büttenbender, W. Prado, A. Beckenkamp, A. Asbahr, A. Buffon, S. Guterres, and A. Pohlmann, *Nanomaterials*, **8**, 24 (2018).
(24) N. Silva, A. Riveros, N. Yutronic, E. Lang, B. Chornik, S. Guerrero, J. Samitier, P. Jara, and M. Kogan, *Nanomaterials*, **8**, 985 (2018).
(25) J. Zhao, M. Zhao, C. Yu, X. Zhang, J. Liu, X. Cheng, R. J. Lee, F. Sun, L. Teng, and Y. Li, *Int. J. Nanomedicine*, **12**, 6735 (2017).
(26) J. Xie, Z. Fan, Y. Li, Y. Zhang, F. Yu, G. Su, L. Xie, and Z. Hou, *Int. J. Nanomedicine*, **13**, 1381 (2018).
(27) A. Roointan, J. Farzanfar, S. Mohammadi-Samani, A. Behzad-Behbahani, and F. Farjadian, *Int. J. Pharm.*, **552**, 301 (2018).
(28) F. Farjadian, S. Ghasemi, Z. Andami, and B. Tamami, *Iranian Polym. J.*, **29**, 197 (2020).
(29) F. Farjadian, S. Rezaeifard, M. Naeimi, S. Ghasemi, S. Mohammadi-Samani, M. E. Welland, and L. Tayebi, *Int. J. Nanomedicine*, **14**, 6901 (2019).
(30) S. Ghasemi and Z. A. Harandi, *RSC Adv.*, **8**, 14570 (2018).
(31) S. B. Perrier, *Macromolecules,* **50**, 7433 (2017).
(32) J. Farzanfar and A. R. Rezvani, *Res. Chem. Int.,* **41**, 8975 (2015).
(33) Q. Xu, A. Sigen, Y. Gao, L. Guo, J. Creagh-Flynn, D. Zhou, U. Greiser, Y. Dong, F. Wang, and H. Tai, *Acta Biomater.*, **75**, 63 (2018).
(34) D. Rosenblum, N. Joshi, W. Tao, J. M. Karp, and D. Peer, *Nat. Commun.*, **9**, 1410 (2018).
(35) J. Kydd, R. Jadia, P. Velpurisiva, A. Gad, S. Paliwal, and P. Rai, *Pharmaceutics*, **9**, 46 (2017).
(36) S. K. Golombek, J.-N. May, B. Theek, L. Appold, N. Drude, F. Kiessling, and T. Lammers, *Adv. Drug Deliv. Rev.*, **130**, 17 (2018)
(37) S. Mohammadi-Samani, S. Zojaji, and E. Entezar-Almahdi, *J. Drug Deliv. Sci. Technol.*, **47**, 427 (2018).
(38) J. Chen, X. Yang, L. Huang, H. Lai, C. Gan, and X. Luo, *Drug Deliv.*, **25**, 1932 (2018).
(39) C. Schwall and I. Banerjee, *Materials*, **2**, 577 (2009).
(40) N. Kohler, C. Sun, J. Wang, and M. Zhang, *Langmuir*, **21**, 8858 (2005).

