
Preparation and In vitro Evaluation of Isoniazid-Containing Dex-HEMA-Co-PNIPAAm Nanogels

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Abstract

In this work, Dex-HEMA-Co-PNIPAAm nanogels containing Isoniazid antibiotic were made. Characteristic features of nanogels were studied by Fourier transform infrared spectroscopy (FTIR), dynamic light scattering (DLS) and scanning electron microscopy (SEM). Drug loading capacity and entrapment efficiency were determined. In vitro drug release amount was estimated at room and body temperature. Biocompatibility of gels was investigated through cytotoxicity assay. Finally antimicrobial properties of synthesized gels were studied. It was shown from the experimental data that the nanogels size after drug loading increased about 1-2%. %Isoniazid loading and %entrapment efficiency were in the range of 15-22% and 37-48% respectively. After 10 days of degradation ca. 80% at 25°C and ca. 90% at 37°C of the nanogel structures were destructed. No significant toxic product produced while degradation and all nanogels depicted good biocompatibility. No antimicrobial features observed through the test condition against gram negative E Coli.

Keywords: nanogel, Isoniazid, Dex-HEMA-PNIPAAm, MTT, Antibacterial.

1 Introduction

In recent years, with the development of pharmacy many efforts have been done to improve the drugs performance and reduce the drugs side effects. Due to low drug absorption, fast metabolism and rapid elimination of drug from body, drug concentration dose not stay steady (Haddadi-Asl, 2011). To solve these types of problems introducing new kinds of drug delivery systems using drug carriers seems necessary.

Drug delivery includes any formulation and method capable of drugs molecules convey in to the body. Such systems are prepared in different kinds, like micro/nano particles, micro/nano capsules, gels and hydrogels, liposomes, micelles and dendrimers (Patel Kundan, 2012, .Oreilly, 2013. Hang, 2010., Mohammadpour, 2012., Michelle, 2013). In addition to drug transportation, drug delivery will enhance drug release, distribution and bioavailability.

Lots of materials can be used as drug carriers such as metals, ceramics, biomaterials, composites and polymers. Among these, in accordance with cheapness, light weight, high chemical resistance and different physical, chemical and mechanical properties polymers can be suitable and worthy candidate as a pharmaceuticals carrier. Polymers can be used in the forms of synthetic, natural or either a combination of both types (Gemma, ***. Zhe, 2013. Franssen, 1999, Huang, 2004).

Biodegradable polymers which mostly have ester, amide and ether functional group in their structure can break down to pieces. If they are utilized as drug carrier they can release drugs as they are degrading. As the polymer is degrading it converted to the small parts that can be excreted from the body or can be used for microorganism in biological process (Aguilar, 2013, Qiu, 2009, Dijk-Wolthuis, 1997,).

Polysaccharides are a group of natural polymers that can be digested enzymatically. In Dextran, a polysaccharide made of glucose molecules, the linear and main chain consists of α -1, 6 glycosidic linkages between glucose molecules,

and branches begin from α -1, 3 links. Dextran hydrolyze or dextranase degrade the dextran by endohydrolysis of α -1, 6 glycosidic linkages.

Hydrogels, 3D hydrophilic networks, may be building even by physical or chemical cross links. High amount of water can be absorbed by gels and duo to this feature gels are like body tissue. Hence, gels show good biocompatibility when using as drug carriers. Stimuli sensitive hydrogels can control the time of drug release by phase changes and transient when facing stimuli. Stimuli can be physical like temperature, light or electrical and magnetic fields, chemical like pH and finally biological (Franssen, 1999, Dijk-Wolthuis, 1997, Huang, 2004, Lui, 2011).

Temperature or thermo sensitive gels are among the most investigated drug delivery systems, in which swelling/deswelling occur as the environment temperature changes. In this way drug will release in an on/off manner (Lowe, 2005, Georgiou, 2011).

Poly N-isopropylacrylamide (PNIPAAm) and its copolymers are widely used in thermo sensitive carrier design. This polymer shows negative behavior, which means that they show lower critical solution temperature. In the simplest way at low temperature the drug will be released and on high temperatures the system will be off (Perju, 2011, Kurecic, 2012, Huang,2004) .

We have synthesized and characterized a novel drug delivery system previously (Jafari, 2015). Our system was consisting of dextran biodegradable natural polymer functionalized by adding hydroxyl ethyl Meta Acrylate (HEMA) to add hydrolysable group which was then copolymerized by PNIPAA to make thermosensitive nanogels. Swelling and LCST temperature of the gels were determined carefully. In this work Isoniazid as a model drug was loaded to the nanogels and drug loading parameters were evaluated. Thereafter dug release, degradation, biocompatibility and antimicrobial features were studied.

2 Experimental methods

2.1 Materials

Dextran was obtained from Fluka. N-Isopropylacrylamide (NIPAAm) and 2-Hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone (Irga cure 2959) and MTT color were purchased from Aldrich. 2-Hydroxyethyl methacrylate, 1, 1-Carbonyldiimidazole, 4-(Dimethylamino)pyridine (DMPA), anhydrous Magnesium sulphate (MgSO₄), N, N'-Methylenediacrylamide (MBA) and mono and dibasic sodium hydrogen phosphate were available from Merck. Isoniazid, dialysis tube (12000 Dalton) and L929 cell line were provided by Iran Pasture institute. Triton X-100 and Dimethyl sulfoxide were obtained from scharlau. Hydroquinone monomethyl ether, lecithin and HCl were provided from Riddle, Pasargad novin and Chem.-Lab, respectively. Tetra hydro- furan (THF), ethyl acetate and chloroform were provided by Dae-Jung, South Korea.

2.2 Preparation of Isoniazid loaded nanogel

Isoniazid loaded nanogels were prepared exactly in the same procedure as nanogels were synthesized previously. In a few words, freeze dried Dex-HEMA, obtained by coupling the hydroxyl group of HEMA to dextran as described by Dijk- Wolthuis, NIPAAm monomer, photo initiator, cross linking agent and Isoniazid with various amounts as given in table 1 were mixed and dissolved in PBS (pH=7). Afterward, these dispersions were added to the thin soy lecithin film, which was gained after vacuum evaporation of lecithin-chloroform solution. Resulting dispersions were mixed completely and were left motionless overnight. Finally, prepared dispersions were extruded 20 times, through a syringe coupled to 450 nm filters to make nano sized polymerization reactors. Subsequently to avoid polymerization outside the formed liposomes the dispersions were diluted and then after, radical polymerization of samples commenced by 30 minutes UV irradiation. The end products were achieved after liposomes washed out using triton x-100.

2.3 Characterization of Isoniazid loaded nanogel

The structures of Isoniazid loaded nanogels, from 400-4000 cm⁻¹, were investigated by means of Fourier transform infrared (ATR-FTIR, Bruker). Each spectrum was resulted from 32 times scan and no preparation was exerted to the nanogels solution prior to study.

The particles size and size changes due to drug loading, after suspending the nanogels in distilled water with viscosity of 0.89 CP, were evaluated by dynamic light scattering method (DLS, Brookhaven Instrument Corporation, Brookhaven, Georgia, US) at 657nm.

The surface morphology of nanogels and Isoniazid loaded nanogels were monitored using scanning electron microscopy (SEM, Seron Technologies Inc., AIS 2100). Samples were coated by gold before scan to make their surface conductive.

Table 1: Composition and ingredients amount in feed

Sample No.	1	2	3	4	5
Dex-HEMA to NIPAAm monomer ratio	0.5	1	2	2	0.5
Cross linking agent (gr)	0.01	0.02	0.01	0.03	0.03
Photo initiator (gr)	0.01	0.01	0.01	0.01	0.01
Isoniazid (gr)	2	2	2	2	2

2.4 Loading capacity and efficiency evaluation

To study the nanogel yield (NY), Isoniazid loading capacity (LC) and encapsulation efficiency (EE), Isoniazid loaded nanogel suspensions were centrifuged at room temperature for 20 minutes at 10000 rpm. The precipitate that was consisting of Isoniazid loaded nanogels were collected for additional procedure such as liposome lyses and frees drying. The supernatants containing free Isoniazid were gathered and investigated by UV-VIS spectrophotometer at the wavelength of 263 nm to find out free Isoniazid concentration.

The nanogel yield, Isoniazid loading percent and entrapment efficiency of nanogels were computed as follow:

$$\% NY = \frac{\text{nanogels weight} \times 100}{\text{polymer weight} + \text{initial isoniazid amount}}$$

$$\% LC \left(\frac{\mu g}{ml} \right) = \frac{(initial\ isoniazid\ amount - free\ isoniazid\ amount) \times 100}{nanogels\ weight}$$

$$\% EE \left(\frac{\mu g}{ml} \right) = \frac{(initial\ isoniazid\ amount - free\ isoniazid\ amount) \times 100}{initial\ isoniazid\ amount}$$

2.5 In vitro release studies

The in vitro drug release from Dex-HEMA-Co-PNIPAAm nanogels was tested by dialysis method. Particular mass of lyophilized Isoniazid loaded gels were weighted and subsequently suspended in 3 ml of PBS (pH = 7.4) and poured in individuals dialysis bag. Afterwards each dialysis bag was placed in separate glass beaker filled with 15 ml of PBS (pH = 7.4), incubated at 37 °C, while was stirred. At different time points, the absorptions of PBS solution, out of dialysis tube, were measure by UV-VIS spectroscopy (LKB Biochrom 4050 ultrospec II UV-VIS spectrophotometer) at the wavelength of 263 nm to find out the released Isoniazid concentration.

2.6 Hydrolytic degradation studies

5 mg of dispersed nanogels in PBS buffer solution were poured in polyethylene tubes and then the tubes were incubated at 37°C for 14 days. At different time intervals, sample were collected and probed by DLS to evaluate the nanogels diameter changes which represent the degradation of the nanogels.

2.7 Biocompatibility studies

The biocompatibility studies were performed by cytotoxicity and cellular viability using MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide) assay. The cytotoxicity of Isoniazid loaded five different series of nanogels were determined facing L929 cells. A 96- well was used in order to seeding cells. Each well was then filled with 100 µL of DMEM medium with 10 % PBS and was maintained at the temperature of 37 °C in CO2 atmosphere. One day in the rear of plating, 50,100,200, 500 µg ml⁻¹ of Isoniazid loaded nanogels were added to the wells. After 24, 48, 72 hr the medium was removed, washed and then 50 µL of 5 µg ml⁻¹ MTT solution in PBS

was added to each well and the plate was kept in 37 °C for 3-4 hr. the medium was removed and 150 µL of DMSO containing formazan salt was added to each well and read on microplate reader. Tests were performed in triplicate. The absorbance of samples was observed at 570 nm, which is sign of cell growth and proliferation.

2.8 Antimicrobial studies

The antimicrobial specification of isoniazid loaded nanogels were assest against E Coli (ATCC 25912, PTCC 1399), gram negative organism, by help of zone of inhibition test. 1.5* 10⁸ CFU ml⁻¹ of E Coli was prepares and cultivated on LB Agar medium. The suspension of 200 mg ml⁻¹ of samples were made in distilled water . disks were soaked in sample suspentions and then were placed on plated. Plates were incubated at 37°C for 72 hr.

3 Results and discussion

3.1 Isoniazid loaded nanogels characterization

Figure 1 illustrat the isoniazid loaded nanogels spectra. Peaks relating to water (OH~1630, H-O-H~3300) are vividly distinguishable. Peaks belong to Free NH₂ (~1220), NH bend (~1650), pyridine group (~ 1440) of isoniazid could been recognized, too.

The size, size increase of drug loaded nanogels to empty nanogels and PDI mesured by DLS are given in table 2 .

SEM images have demonstrate the morphology and size of nanogels. The nanogel were almost spherical and had an smoot surface, and size range about 400nm which is given in table 2 with more detailes. From the camparison of size data catched by both method it is notable that possibly because of freez dried particle cogulating to each other bigger size have been gotten.

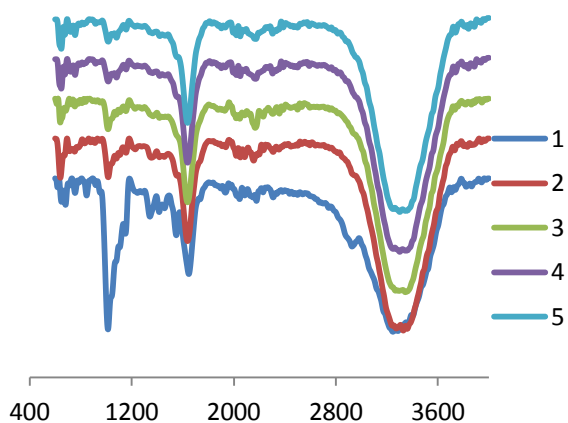


Figure 1. Isoniazid loaded nanogels FTIR spectra

Table 2: Size and size distribution obtained by DLS and SEM comparison

Sample name	Isoniazid loaded nanogel Size (nm)- DLS	Size increase after drug loading (%) -DLS	PDI	Isoniazid loaded nanogel Size (nm)- SEM	Size increase after drug loading (%) -SEM
1	339.9	1.46	0.255	424.2	2.38
2	349.8	0.75	0.263	565	2.52
3	354	1.51	0.269	406.6	0.95
4	329	0.86	0.238	352.5	1.48
5	373.5	1.55	0.268	439.3	1.99

For instance figure 2 represents DLS and SEM images of sample 2 with and without Isoniazid.

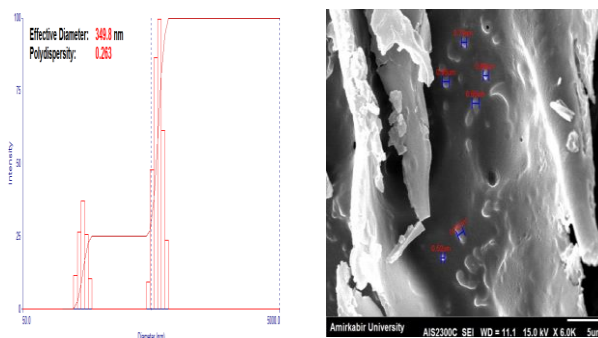
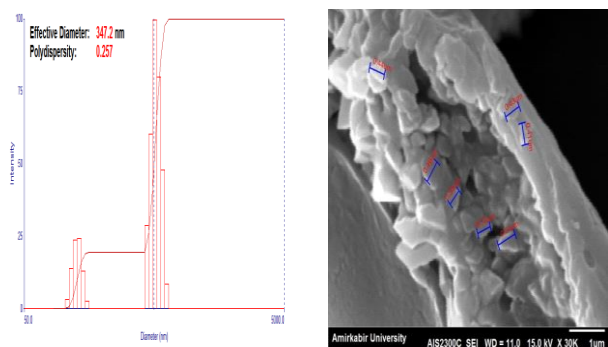


Figure 2. (top row) Isoniazid free nanogel size, (bottom row) Isoniazid loaded nanogels size measure by DLS and SEM respectively

3. 2 Loading capacity, encapsulation efficiency and nanogels yield

The amounts of Isoniazid which loaded in nanogels were measured by subtracting the free Isoniazid value from initial Isoniazid content. Nanogels yield, Isoniazid loading and entrapment efficiency were calculating according to formulas (1) to (3) are reported in table 3.

As it is obvious, the less the cross linking agent was used the more drug loading and entrapment efficiency percent were resulted.

Table 3: Characteristic of Isoniazid loaded nanogels

Sample name	% nanogels yield	% Isoniazid loading	% entrapment efficiency
1	73.42	21.77	48.11
2	72.96	18.32	40.31
3	72.92	20.86	45.78
4	72.51	20.90	37.81
5	71.85	15.29	38.18

3. 3 In vitro Isoniazid release study

while a part of synthesized nanogels consisted of PNIPAAm, a thermosensitive polymer, whole nanosized carrier found the thermoresponding behavior in which the gel swell and deswell according to temperature shifts and the net mesh size could change automatically. Hence the release behavior in both temperatures limits, below and upper the LCST temperature, were studied to evaluate the differences in release mechanism. The results of Isoniazid release during 10 days from gels at 25°C and 37°C is depicted in figure 3.

As the results show at 37° C and at the temperature upper LCST which is ca. 32.5° C, in the physiological conditions nanogels shrink and the portion of their trapped content were released suddenly which is observable as a burst release in figure 3. After about 1 hr that all 5 series of freeze dried Isoniazid loaded nanogels reached the media temperature they deswelled and 69.88, 80.72, 88.52, 79.38 and 67.82 % of their content including Isoniazid released respectively. Then the release regime in all gels followed the same way slowly by the degradation mechanism and finally after 10 days the drug release percent touched the 82.61, 90.38, 96.80, 89.73 and 79.95% respectively. The sample 2 having the mix combination of the highest ratio of Dex-HEMA to PNIPAAm, having the most amount of hydrolysable ester bond in its structure and cross linking agent of 0.01 gr which was the less value showed the most amount of release among other samples. Next ranks of Isoniazid release belong to samples 3 and 4 respectively. The effect of Dex-HEMA to PNIPAAm ratio that results in better degradation were more serious than the cross linking agent. Last places were occupied by sample 1 and 5 by the previous analysis.

At 25°C that is bellow LCST temperature the release behavior was different. At this temperature nanogels swelled and the release mechanism was controlled by diffusion. The ultimate values for Isoniazid release after 10 days were 80.46, 80.08, 79, 77.83 and 77.97 for all the 5 series. As the results represent the values are almost similar and no tangible differences were filled, but even in this case the impact of cross linking agent can be seen. The more the cross linking agent observed the less swelling and consequently less release attached.

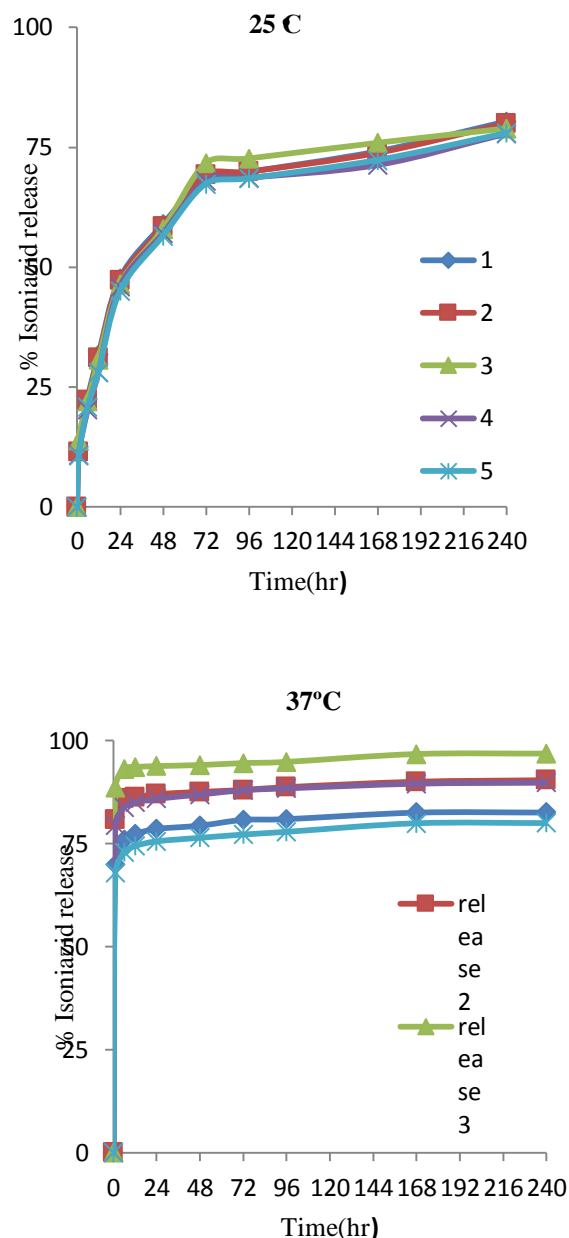


Figure. 3. Drug release% from nanogels at 25 and 37°C

3. 4 Isoniazid loaded nanogels degradation

To study the hydrolytic degradation, Isoniazid loaded nanogels of different composition incubated in physiological condition. At very beginning times an increase in gels size could be observed which sign of freeze dried gel swelling was. The swelling was not considerable because the testes were holding on upper LCST temperature in which nanogels were in their shrinking form. Then after the gels sizes have been decreased slowly within 2

weeks. Figure 4 shows the percent of particle size decrease as a function of degradation time.

As results depict, size decrease due to hydrolytic degradation occurred most in sample 4, 3, 2, 1 and 5 respectively that can be related to the quantity of hydrolysable group which is found in Dex- HEMA part of the nanogels.

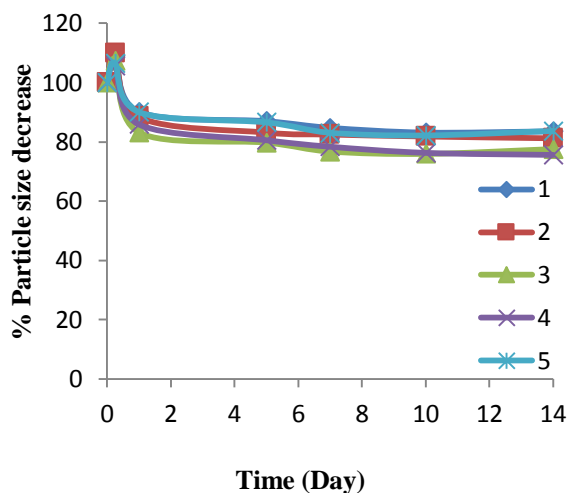


Figure 4. Particle size decrease % due to hydrolytic degradation

3.5 In vitro cytotoxicity assay

The cytotoxicity of Isoniazid loaded nanogels against L929 cells was evaluated and outcomes are represented in figure 5.

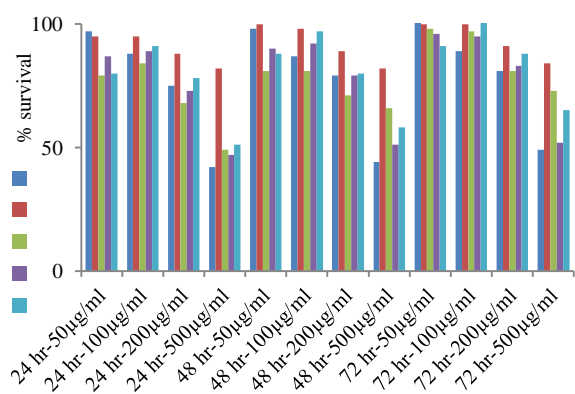


Figure 5. Cell viability (proliferation) during 24, 48, 72hr at concentration: 50, 100, 200, 500 µg/ml

At the same concentration of Isoniazid loaded nanogels, the more the time was the higher cell survival observed. On the other hand, at similar

time intervals, as the concentration raised, the cell viability fallen.

3.6 In vitro antimicrobial assay

The antimicrobial behavior of nanogels tested against gram negative E Coli. Unfortunately, not any traces of inhibition zone were detected in all 5 series of samples. According to Gerald Tritz work inhibitory nature of E coli by Isoniazid depends on the initial cell concentration, Isoniazid concentration, and chemical composition of the medium, hence these parameters could be the origin of losing antimicrobial feature of the Isoniazid loaded nanogels.

4. Conclusion

In this work, Isoniazid loaded biodegradable and thermosensitive nanogels were prepared through UV polymerization method. By incorporating PNIPAAm in the nanogels structure the thermosensitivity characteristic was given to the gels which affected the degradation and drug release from the gels in physiological condition. Hydrolytically degradable specification also donated to nanogels because of Dex-HEMA portion. The degradation product were nontoxic but had not any antimicrobial features. Totally these nanogels seem to be a good candidate in controlled drug delivery.

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