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Alginic Aldehyde-Gelatin Nanogel as Sustainable Drug Delivery System of Azithromycin: Development, Characterization and In Vitro Evaluation

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Abstract **-** The present study deals with the encapsulation of Azithromycin in alginic aldehyde and gelatin nanogel as an intestinal site-specific and controlled therapeutic delivery system against gram-negative intracellular Salmonella Typhi, which is synthesized through inverse miniemulsion technique in three different drug to polymer ratios, and exploration the physicochemical properties and antibacterial activity of the system. The characterizations of the azithromycin-loaded nanogel were done by DLS and FT-IR. The hydrodynamic diameter of the azithromycin-loaded nanogel is 118±4 nm with negative zeta potential (-34.2±1.5 mV). We observed absolute transition from the crystalline form of the azithromycin to amorphous state while was loaded to the AA-Gel nanogel by FT-IR, and the FT-IR measurements demonstrate that there are not any detectable chemical and intramolecular interactions between AZM and nanogel network. The encapsulation efficiency of the loaded nanogels were influenced by the ratio of drug to polymer, which the highest value (86.3±2.3%) was demonstrated for the 1:3 formulation. In vitro azithromycin release profile from AA-Gel nanogels presents an initial burst release over a period of 4 hours that is followed by controlled release until the 24 hours. Nanogels showed significant bacteriostatic activities in comparison to free azithromycin. Our results indicate proper physicochemical, in vitro and ex vivo features of the azithromycin nanogel, which can be a worthy candidate for oral administration against Salmonella Typhi.

Keywords: Nanogel; azithromycin; Salmonella Typhi; oral drug delivery; alginic-aldehyde; gelatin

1. Introduction

Common infectious diseases as new and ancient threats of world health could be controlled effectively by utilization of antibiotics or antiretroviral drugs, but recently, their function as intracellular chemotherapy agents could be limited due to antibiotic resistance of bacteria, compliance issues, and physicochemical limitations of drugs such as low bioavailability, short half – life or variable absorption [1,2]. To overcome these challenges, the nanoparticulate systems are designed as improved delivery platforms to infected sites for loading antibiotics [2].

The objective of this work is to develop, optimize and evaluate the physicochemical properties of the alginic aldehyde - gelatin nanogel (AA-GEL nanogel) system for sustainable and controlled delivering of azithromycin (AZM) as a poorly-water soluble antibiotic agent against Salmonella enterica serovar Typhi (S. Typhi). AZM is an azalide antimicrobial agent, which is the derivative of erythromycin, contains a methyl-substituted nitrogen in the lactone ring. Its bioavailability is 37% for a single oral dose of 500 mg, which is so low and related to its poorly water-solubility property and existence of some gastrointestinal (GI) tract responses such as diarrhea [3,4].

The nanostructured drug is synthesized by means of the modified inverse miniemulsion technique, to enhance therapeutic agent's bioavailability and improve antibacterial activity of that which leads to reduction of side effects along with maximum therapeutic efficiency against S. Typhi as a gram-negative bacterium, the causative agent of typhoid fever, the globally life-threating disease, and according to reports; one of the effective therapeutic agents to remedy of multidrug-resistant typhoid fever (MDRTF) is Azithromycin [5].

2. Materials and Methods

2.1. Materials

Sodium alginate (medium viscosity grade, viscosity of 2% solution: 2000 cps at RT), gelatin (type A, gel strength=300), sodium tetra borate 99% (borax), Span 20, Tween 80, Durapore PVDF Millipore membrane filter (0.22 µm) were purchased from Sigma Aldrich, and Phosphate buffered saline from Thermo Fisher. Sodium metaperiodate, acetone, Millex-HV syringe filter (0.45 µm) were obtained from Merck. Dialysis tubing (3.5 kDa MWCO) was procured from Spectramlabs. Azithromycin dihydrate powder and enteric-coated capsules were obtained as a gift from DEVA pharmaceutical company, and Lonza Pharma & Biotech respectively. All other chemicals used were of HPLC and analytical reagent-grade.

2.2. Preparation of azithromycin-loaded alginic aldehyde-gelatin nanogel

Nanogel matrix was synthesized through inverse miniemulsion technique [6] at the different ratios of drug to polymer (1:1, 1:2 and 1:3). Initially, sodium alginate was oxidized by sodium metaperiodate [7, 8]. The 5% (w/v) alginic aldehyde and 5% (w/v) gelatin solutions were prepared by dissolving AA and gelatin respectively in borax 0.1 M and ultrapure water. The mixture of 250 µl of AA and 250 µl of gelatin was drop-wise added to the organic phase that consisted of dissolved 0.2 gr Span 20 (2%, wt% with respect to organic phase) and 25 mg AZM powder in chloroform (10ml) under sonication over a period of 5 min. To attain the powder form of nanogel for evaluation of physicochemical and antibacterial properties, the precipitation of produced nanogel emulsion was done by dropwise addition into acetone (50 ml) while stirring on magnetic stirrer, and the precipitate was separated by centrifugation (9500 rpm, 30 min) and washed thrice with distilled water, and then dried under vacuum.

2.3. Physicochemical characterization of nanogels

The particle size, polydispersity index (PDI), and surface charge of AZM loaded nanogel formulations were measured by dynamic light scattering (DLS) (Malvern Zetasizer Nano ZS, UK) equipped with a He-Ne laser (633 nm) at 25 °C. The reported values are the average of five series of measurements and each measurement were carried out three times with nine sub-runs.

2.4. Determination of encapsulation efficiency

Encapsulation efficiency of azithromycin loaded AA-gel nanogel was measured by utilization of HPLC method (Shimadzu, Prominence-i LC2030C) that equipped with a diode array detector and a reverse-phase C18 column (250 × 4.6 mm, pore size 5 µm). The mobile phase was consisted of dipotassium hydrogen phosphate 10 mM (pH 9.0)-absolute methanol (15:85, v/v); flow rate of 1.5 ml/min was adopted. The detection wavelength of azithromycin was 210 nm. Each experimental process was repeated in triplicate. The drug encapsulation efficiency (EE) was calculated as below, Eq (1):

$$(\text{EE }\%) = \frac{\text{Actual drug content in nanogel}}{\text{Initial drug content in nanogel}} \times 100 \quad (1)$$

2.5. In vitro release evaluation

To evaluate the in vitro dissolution behaviour of azithromycin, free AZM and AZM loaded nanogels (each containing 10 mg AZM) were manually filled into the enteric-coated capsules and was incubated at 37 ± 0.5 °C in USP rotating basket apparatus I (Pharma test, PTWS-820 MA, Germany) with dissolution medium volume 900 ml, 0.1 N HCl pH 1.2 and 0.1 M phosphate buffer pH 6.8 separately containing 0.1% w/v Tween 80 [9], at continuous rotational speed 50 rpm [10]. At specified time intervals aliquot (3 ml) of release medium was withdrawn and an equivalent volume (3 ml) of fresh dissolution media was replenished, and the samples assayed by HPLC at 210 nm. The cumulative percentage of azithromycin release was calculated from the following equation (2) and the results are representative of triplicate measurements:

$$\text{Accumulative release percentage (\%)} = \frac{\text{Amount of AZM in the aliquot}}{\text{Total amount of AZM}} \times 100 \quad (2)$$

2.6. FT-IR analysis

Fourier-transform infrared spectroscopy of sodium alginate, alginate aldehyde, gelatin, azithromycin, AA-Gel nanogel and AZM-loaded AA-Gel nanogel were recorded by usage an FT-IR spectrometer (Perkin Elmer Spectrum 100 FT-IR Spectrometer, PerkinElmer Inc., Waltham, MA, USA) in the scanning range of 4000-800 cm^{-1} with a spectral resolution of 4 cm^{-1}.

2.7. In vitro antibacterial activity analysis

Bacterial growth inhibitory properties of azithromycin and AZM/AA-Gel nanogels against pathogenic gram-negative S. Typhi (ATCC-19214) were determined using microtitre broth dilution method. The stock bacterium culture was grown in LB agar medium; and to elevate the exponential growth of culture, single colony was transferred to separate LB medium flask and incubated at 37 °C with shaking at 180 rpm. Then bacterial liquid cultures were diluted in PBS to inoculum concentration of (4–6 ×105) CFU/ml based on optical density at 600 nm [11]. The diluted AZM and AZM-loaded suspensions in decreased concentration ranges were inoculated with S. Typhi in log-phase to yield a final concentration (4–6 ×105 CFU/ml) and after incubation for 24 h at 37 °C the bacterial growth were inspected at intervals of 1 hour. Furthermore, in order to investigate the antibacterial effect of AA-Gel nanogel themselves, a suspension of blank nanogel in similar concentration to the one of AZM-loaded nanogels suspension was prepared. Experiments were carried out as three individual replicates.

3. Results and Discussions

3.1. Physicochemical characterization of nanogels

The particle size, polydispersity index, and zeta potential of azithromycin-loaded nanogels are shown in (Table 1). By referring to the obtained results of different weight ratio of alginic aldehyde and gelatin; by increasing the amount of AA, the size of the droplets were decreased, which is related to the high availability of aldehyde groups in the medium for promoted cross-linking process. The zeta potential measurement reveals the negative charges that is necessary for the extended colloidal system stability, due to the existence of carboxylic acid moieties of alginic aldehyde, so increasing the volume of AA causes the rise of negative zeta potential value [12].

Table 1: Characteristics of azithromycin loaded nanogels ($\bar{x}\pm s$, n=3).

Formulation	Aqueous phase (µl)		Span 20 (wt %)	Azithromycin (mg)	Chloroform (ml)	Average particle size	PDI	Zeta potential (mV)
	Alginic aldehyde	Gelatin						
F1	350	150	0.2	25	10	110±3	0.128±0.041	-35.1±0.2
F2	250	250	0.2	25	10	118±4	0.139±0.009	-34.2±0.5
F3	150	350	0.2	25	10	130±2	0.201±0.025	-30.9±0.3

3.2. Encapsulation efficiency

To evaluate the drug encapsulation efficiency, and in vitro release behaviour, the formulation of F2 was chosen (Table 1) and the nanogels with different ratio of drug to polymer matrix (1:1, 1:2 and 1:3) were prepared and characterized by DLS method (Table 2). It can be concluded from the obtained data, the drug to polymer ratio could influence the encapsulation efficiency (%) in a way that the low drug:polymer ratio (1:3) leads to the highest E.E (%) (86.3±2.3) along with reduction of

average particle size and zeta potential. That could be due to the higher viscosity of AA that leads to stronger crosslinking matrix, which would decrease the diffusion coefficient of AZM.

Table 2: Particle size, PDI, zeta potential and encapsulation efficiency of different drug to polymer ratios. ($\bar{x}$±s, n=3)

Formulation	Polymer (mg)	Drug (mg)	Particle size (nm)	PDI	Zeta potential (mV)	Encapsulation Efficiency (%)
F2a	25	25	118±4	0.139±0.009	-34.2±0.5	64.7±2.1
F2b	25	12.5	116.3±2.8	0.152±0.012	-39.8±1.2	74.9±4
F2c	25	8.3	106.9±3.6	0.187±0.007	-44.6±2.8	86.3±2.3

3.3. Dissolution study

The release profile (Fig.1) results demonstrate the required time for 70 % release of azithromycin for the different drug:polymer ratios (1:1, 1:2 and 1:3) and intact AZM are as follows: 161.4, 210, 328.7, and 23.3 min respectively; therefore, the synthesized nanogels provide the slower and controlled release and decreasing the dissolution rate. It must be considered that a burst release over a period of 4 hour has been occurred in the initial phase (burst azithromycin release of 91.62%, 72.95% and 56.95% for drug:polymer ratios 1:1, 1:2 and 1:3 respectively) and that is followed by a controlled release until 24 hr. The reason for a burst release is related to the nature of synthesized AA-gel nanogels, which the protonation of carboxylic acid functional groups on the nanogel network happens in the neutral pH of intestinal tract environment, and forward large osmosis, leading the nanogels initiate to swell significantly and this phenomenon conducts to the rapid drug diffusion from the superficial layers of nanogels, while the drug release from the core of the network is slowly and provide the controlled release behaviour.

Utilization of a drug delivery system with an initial burst release can be an excellent choice for carrying the antibiotics because this property has potential to eradicate the intruding bacteria before their proliferation, besides the slow release in the plateau phase averts the growth of the survived bacteria from the initial burst release [13,14].

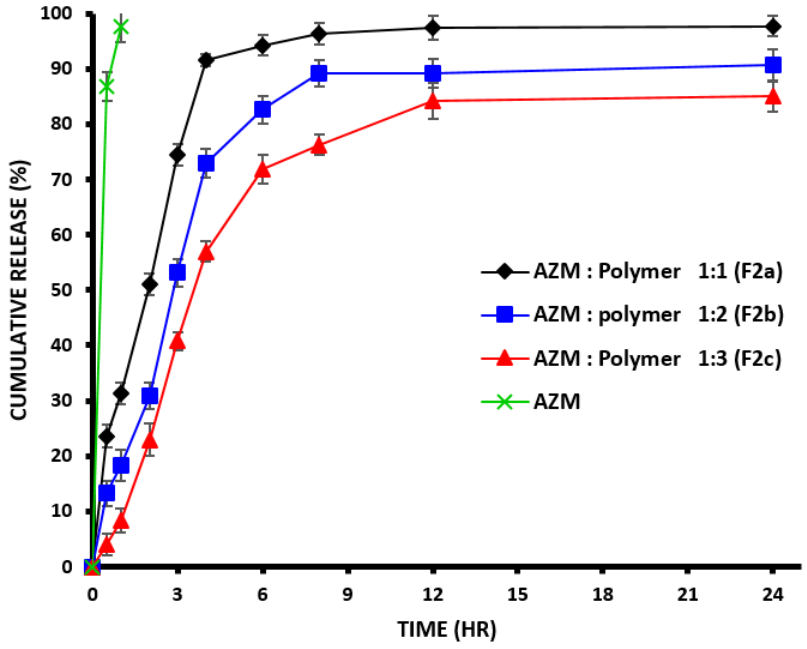

Fig 1: Release profile of azithromycin and AZM-loaded nanogels.

3.4. FT-IR characterization

By taking advantage of Fourier-Transform infrared spectroscopy (FT-IR) method the cross-link between alginic aldehyde and gelatin through Schiff's base reaction, besides the loading of azithromycin into the nanogel network are investigated that are shown in (Fig.2).

In spectrum of bare nanogel, the peaks of 1612.4 cm^{-1} and 1546.3 cm^{-1} (C═N stretching) show the formation of Schiff's base between aldehyde and amino groups that the broad band of 1612.4 cm^{-1} as a result of Schiff's base is overlapped by 1631.9 cm^{-1} band of amide I corresponding to gelatin and the band of 1525.2 cm^{-1} (amide II) is shifted to 1546.3 cm^{-1} in nanogel network [15]. In the spectrum of azithromycin, intense peaks at the range of 1049.2-1377 cm^{-1} (ether C-O stretching), the peak at 1720.1 cm^{-1} (ester C═O stretching), peaks at the scale of 2784.9-2971.5 cm^{-1} (aliphatic C-H stretching), besides the bands at 3489.8 cm^{-1} and 3612.3 cm^{-1} due to the existing of O-H stretching in this dihydrate drug, have been observed [16]. The characteristic spectra of AZM-loaded nanogel demonstrates that there are not any chemical and intermolecular interactions between AZM and nanogel network due to that the peaks corresponding to O-H stretching in drug were substituted by a broad peak (3659.4 cm^{-1}) that could be explained by transition of azithromycin from crystalline to amorphous form after loading to the nanogel and the other mentioned peaks of azithromycin remain intact meanwhile preparation process [16-17].

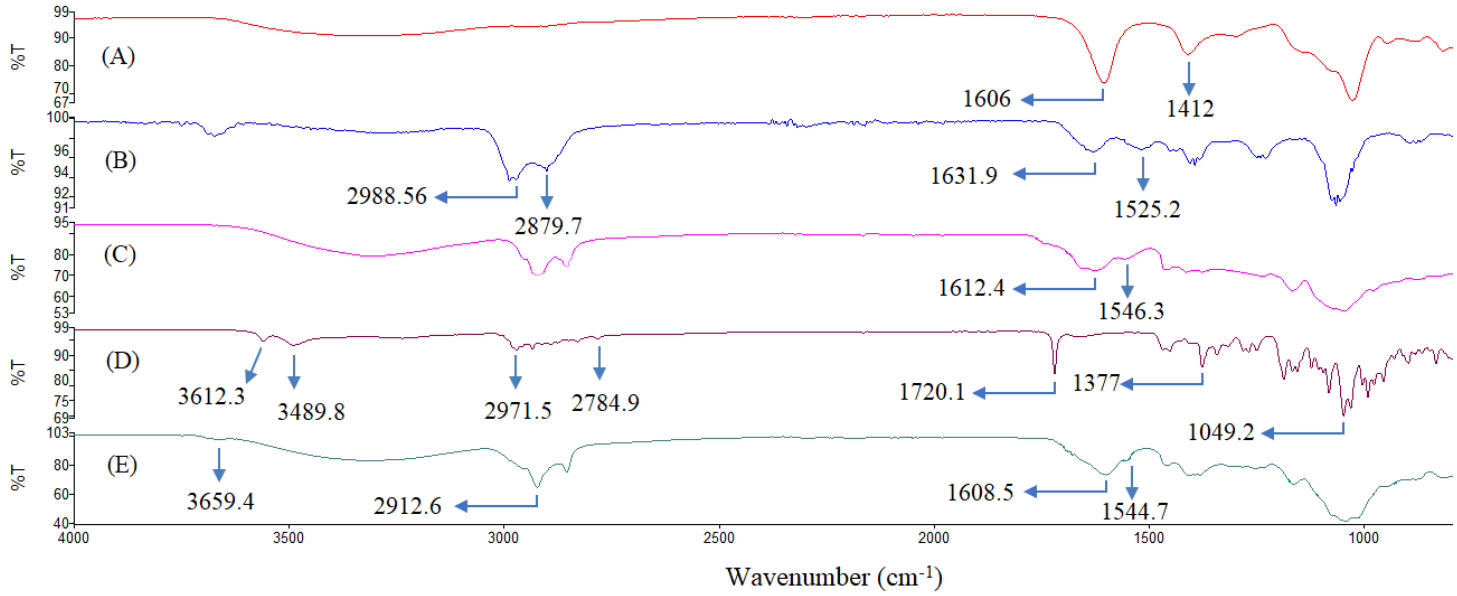

Fig 2: FT-IR spectra of alginic aldehyde (A), gelatin (B), bare nanogel (C), azithromycin (D) and AZM-loaded nanogel (E).

3.5. In vitro antibacterial activity analysis

The minimum inhibitory concentrations (MIC) against S. Typhi of AZM/AA-Gel nanogels at different drug to polymer ratios were shown in Fig (2) that demonstrate the azithromycin loaded nanogels are more effective in comparison to free drug and it is noteworthy that the amount of polymer in the nanogel matrix did not affect the antibacterial activity of the delivery system. The higher antibacterial efficacy of AZM loaded nanogels may have resulted mainly from higher diffusion of the nanogels through the bacteria cells; consequently, the consumed dose of this antibiotic against S. Typhi and its side effects could be decreased.

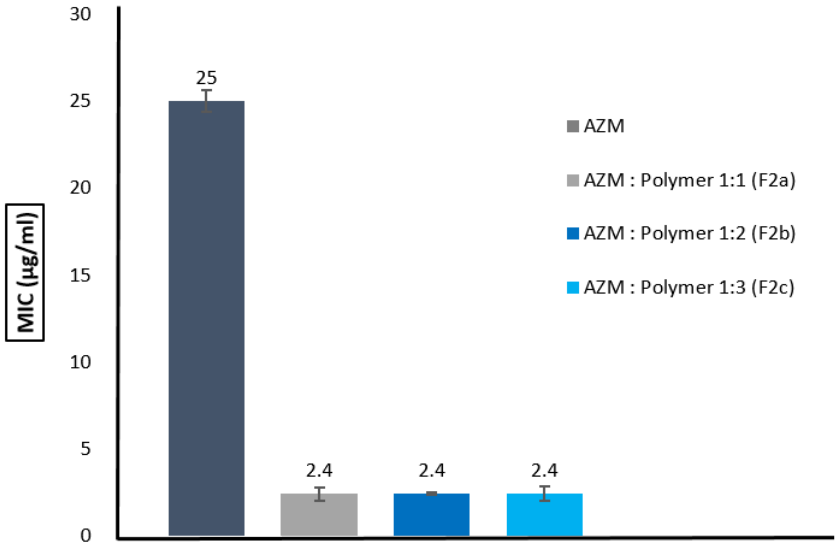

Fig 2: MICs of intact azithromycin (AZM), and azithromycin loaded nanogels with different drug:polymer ratios (($\bar{x}\pm s$, n=3).

4. Conclusion

In this study, the AA-gel nanogels were successfully developed and optimized through inverse miniemulsion technique, as a sustainable and controllable delivery vehicle for azithromycin through the oral administration rout, which own negative zeta potential with high encapsulation efficiency and high antibacterial activity against gram-negative intracellular Salmonella Typhi. Consequently, the developed nano-carrier for delivery of azithromycin can enhance the bioavailability, dissolution rate along with the reduction of side effects. By referring to the obtained results, the nanogel formulation of the azithromycin has a potential to use clinically for more efficiently therapy, in comparison to the conventional formulations of this pharmaceutical agent. In future, the in vivo studies can be done to investigate the antimicrobial efficacy of azithromycin in a nanostructured form.

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