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Development and Characterization of Mitoxantrone-Loaded Glutaraldehyde Crosslinked Sodium Alginate Nanoparticles for the Delivery of Anticancer Drugs

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Authors' contributions

This work was carried out in collaboration between both authors. Author MWA designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author MAGR managed the analyses of the study and literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

Every year millions of new cases of various types of cancers are diagnosed, leading to an alarming rate of fatalities. Mitoxantrone is an anthracenedione antineoplastic agent which is used in the treatment of various types of cancer, mostly acute myeloid leukemia and prostate cancer. In spite of its therapeutic applications, it possesses numerous limitations and side effects including specific targeting and systemic toxicity. Sodium alginate is a biodegradable, mucoadhesive and biocompatible polymer commonly used in drug delivery applications. Glutaraldehyde is a saturated dialdehyde and is used as a polymer cross linker. In this study, mitoxantrone-loaded glutaraldehyde-sodium alginate nanoparticles were developed by ionic gelation method and characterized (determination of particle size, drug entrapment efficiency, drug release and its kinetics) for the delivery of anticancer drugs. The nanoparticles mean particle size was found to be within the acceptable range. The entrapment efficiency was also on the higher side with sustained drug release. The findings of this study reveal promising potential of delivery system and project the way forward for further *in vitro* and *in vivo* investigations.

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1. INTRODUCTION

Cancer is a deadly set of diseases which involves uncontrolled growth of cells with the possibility of invading or spreading across the body [1,2]. Commonly observed symptoms comprise a lump, alterations in bowel movements, abnormal loss of weight, chronic cough and abnormal bleeding [3]. Though these are the common symptoms of cancer, nonetheless they can also be due to other ailments [3]. A plethora of cancers are known to affect humans [2].

Mitoxantrone is an anticancer drug [4,5] which shows strong near infrared absorption in the range of 600–700 nm. It has been extensively used to treat breast and prostate cancer as it is clinically approved and has a broad spectrum of anticancer activity [6]. In children suffering from acute lymphoblastic leukemia (ALL) relapse, mitoxantrone has been found to improve the survival rate [7]. A prednisone-mitoxantrone combination is approved as a second line of treatment against hormone-refractory prostate cancer. Nevertheless, the therapeutic efficacy of Mitoxantrone is significantly inadequate because of its poor systemic toxicity and specific targeting. Furthermore, chemotherapeutics also triggers adjacent normal cells to discharge some growth factors or cytokines which stimulate tumor cells growth eventually resulting in drug resistance [8]. This chemotherapy resistance [9,10] additionally leads to the over-expression of multidrug resistance gene (P-gp) and support the efflux of drug to reduce the drug concentration in cells, inadequate to reach the lethal dose to kill cancer cells. These unwanted side effects of mitoxantrone can possibly be addressed by using nanoparticles. These nanoparticles can efficiently load and carry the drug, target it to the delivery site, and enhance drug accumulation, in addition to reducing the drug's side effects [11]. Past two decades have seen the discovery of a range of biodegradable polymers showing anticipated controlled, sustained, and selective targeting effect while simultaneously keeping the concentration of drug inside the therapeutic window.

Sodium alginate is a polymer renowned for its mucoadhesive, biocompatible and biodegradable characteristics [12]. Its source is marine brown algae and is a sodium salt of alginic acid. It comprises β-D-mannuronic and α-L-glucuronic acids, as well as two uronic acids. Glucuronic acids interchange sodium ion and react with calcium ions and α-L-glucuronic acid groups, therefore linking together by divalent cations. A gel network if formed due to dimerization of alginate chains. Key characteristics of sodium alginate owing to which it is extensively used in several areas include its unique cross-linked structure and its ionic gelatin potential [13]. Out of numerous known protein cross-linking agents, glutaraldehyde possesses widest applications in several fields including pharmaceutical sciences.

The goal of this study was to design and develop a nanoparticulate formulation for the efficient delivery of mitoxantrone that can enhance drug delivery while reducing systemic toxicity and enhancing specific targeting.

2. EXPERIMENTAL

2.1 Materials

Mitoxantrone hydrochloride (ATCO Laboratories Pakistan), Sodium tripolyphosphate, Glacial acetic acid, Sodium hydroxide, Monobasic potassium phosphate, glutaraldehyde, chitosan, sodium alginate, Dialysis membrane with molecular weight cut off of 12,000-14,000 dalton (Carl Roth, Germany).

2.2 Mitoxantrone Nanoparticles Pre paration

To investigate the concentration-dependent interaction between glutaraldehyde-alginate from pre-gel, aggregation and precipitation areas owing to the formation of alginate ion-glutaraldehyde bond, a nephelometer was used. To a 20 ml (0.1 % w/v) sodium alginate solution, 10 ml glutaraldehyde was added dropwise considering the turbidity changes. For each glutaraldehyde addition, the nephelometer cell was continuously stirred, followed by system and solution equilibration for 10 min and measurement of turbidity (Nephelometric Turbidity Units, NTU) [7]. Glutaraldehyde and sodium alginate were used at different ratio combinations to yield different formulations named as MIT-4, MIT-5, MIT-6 MIT-7, and MIT-8. All mitoxantrone nanoparticulate formulations were prepared in triplicates viz MIT-4 (i, ii, iii), MIT-5 (i, ii, iii), MIT-6 (i, ii, iii), MIT-7 (i, ii, iii), and MIT-8 (i, ii, iii). Various concentrations (ranging

from 0.4-1 mg/ml) of sodium alginate aqueous solutions were prepared in distilled water by stirring at 700 rpm for 30 min while maintaining a temperature of 37 °C. To alginate solution, mitoxantrone was added and allowed to stir for 25 min to facilitate complete mixing. 18 and 36 mM glutaraldehyde solutions were prepared in 100 ml deionized water, respectively. Glutaraldehyde solution was injected dropwise into sodium alginate solution under the influence of stirring at 1500 rpm for 60 min, followed by the purification of resultant nanoparticles using ultracentrifugation technique.

A series of glutaraldehyde-sodium alginate ratios were used in the formulation of chitosan-alginate nanoparticles. The reason behind aqueous glutaraldehyde addition was the identification of optimum glutaraldehyde-sodium alginate mass ratio that could formulate nanoparticles with maximum particle concentration, small mean particle size, and low polydispersity (PDI).

2.3 Mitoxantrone Conjugates Preparation

Different molar ratios of glutaraldehyde-sodium alginate were used to prepare various formulations namely MIT-4, MIT-5, MIT-6, MIT -7 and MIT-8. Sodium alginate aqueous solutions (ranging between 0.4-1mg/ml) were prepared followed by the addition of mitoxantrone and 25 min stirring to facilitate thorough mixing.

2.3.1 Influence of glutaraldehyde-sodium alginate mass ratio on nanoparticulate particle size

Chitosan–alginate nanoparticles were formulated using a set of glutaraldehyde-sodium alginate ratios. The reason behind aqueous glutaraldehyde addition was the identification of optimum glutaraldehyde-sodium alginate mass ratio that could formulate nanoparticles with maximum particle concentration, small mean particle size, and low PDI. The mass of sodium alginate was kept uniform to identify glutaraldehyde-sodium alginate mass ratio which varied to prepare chitosan alginate nanoparticles [14].

2.3.2 Determination of particle size by zetasizer

The average particle size was recorded employing dynamic light scattering (DLS) technique using a particle size analyzer (Nicomp® N3000). Nano-dispersion was suitably diluted in order to record the measurements of average particle size and PDI [15].

In a volumetric flask, mitoxantrone (10 mg) was added to phosphate buffered saline (25 ml, maintained at pH 7.4), followed by magnetic stirring for sufficient time to facilitate maximum drug dissolution. Afterwards, the solution was diluted with phosphate buffered saline to 100 ml yielding a final drug concentration of 0.01 mg/ml.

2.3.4 Drug entrapment efficiency determination

The encapsulated drug percentage was determined by the separation of nanoparticles. Ultracentrifugation technique was used for the separation of nanoparticles and the operating conditions were 15000 rpm for 60 min, while the temperature was maintained at 4 ºC. The nanoparticles were isolated from the supernatant, followed by a 15 min sonication thus yielding a uniform solution. The entrapped mitoxantrone percentage was determined using spectrophotometric technique, two absorption bands at 660 and 610 nm, and a shoulder at about 570 nm. Mitoxantrone percentage entrapment efficiency (EE) was calculated as in Eq 1 [16].

$$EE = \{(T - S)/T\}100 \quad (1)$$

where T is the total nanoparticulate mitoxantrone and S is the amount of free mitoxantrone in the supernatant.

2.4 Investigation of *In Vitro* Drug Release

Dialysis method was used to study the *in vitro* release profile of mitoxantrone from nanoparticles. Release medium used in the study was phosphate buffered saline (PBS) (maintained at pH 7.4). 100 ml nano-suspension of the drug-loaded nanoparticles was centrifuged, the supernatant was discarded, followed by the suspension of remaining portion into buffer (5 ml). The dialysis tubing was first rinsed using distilled water and the suspended nano-formulation was added, the tubing was sealed followed by placement into buffer (100 ml) in a beaker under the influence of continuous stirring at 100 rpm while maintaining the temperature at 37 ± 1 °C. After every 60 min, 5 ml sample was withdrawn and replenished by equal aliquot of fresh buffer. The experiment was performed in triplicates [15].

2.4.1 Analysis of drug release kinetics

The drug release kinetics of all formulations were studied. The highest values of regression coefficient were determined using DDSolver

software to study in detail the drug release pattern and identifying the best fit model (zero order, first order, korsmeyer-peppas, hixon-crowell, highuchi) [17].

2.4.2 Korsmeyer-peppas model application

To better understand the kinetics of drug release from a polymeric system Korsmeyer-Peppas derived an equation. The pattern of drug release from hydrophilic matrix can be easily understood using this model. The graphs were plotted between log cumulative drug release (%) versus log time as described below in Eq 2.

$$Ktn = Mt/M\alpha \quad (2)$$

Where K is a constant which includes the characteristics of the drug and macromolecular polymeric system, n is a kinetic constant associated with the drug release mechanism and Mt/Mα is the quantity of drug release at time t. The value of n smaller than or equal to 0.43 shows that the release pattern is following fickian diffusion, instead if n's value is in the range of 0.43-0.89 then the pattern of drug release is non-fickian, if n = 0.89 for case II the release is zero order or relaxational and in case if the value of n > 0.89 then it is super case II release [18].

2.5 Statistical Analysis

The data are expressed as mean ± standard deviation (SD). SPSS version 18 was used to perform the statistical analysis. The formulations were compared using one–way analysis of variance (one-way ANOVA). The values of $p < 0.05$ were considered as statistically significant.

3. RESULTS

3.1 Analysis of Particle Size

The mean particle sizes of various formulations of mitoxantrone-loaded sodium alginate nanoparticles were found to be in the range of 320 - 467 nm. The readings were recorded on nicomp particle size analyzer (Table 2).

3.2 Entrapment Efficiency (%)of Mitoxantrone-Loaded Nanoparticles

The entrapment efficiencies (%) of mitoxantrone-loaded nanoparticles were found to be in the range of 62-81 % (Table 3).

Percentage entrapment efficiencies of mitoxantrone-loaded nanoparticulate formulations were determined by the measurement of absorbance at 610nm. The mitoxantrone formulation MIT-8 exhibited the maximum entrapment efficiency of 81%. The polymer concentration was found to be directly proportional to the absorbance. A direct correlation was also found between entrapment efficiency and the concentration of dispersing agent.

3.3 *In vitro* Drug Release Profile Of Mitoxantrone

The release study of mitoxantrone from the nanoparticles (in phosphate buffer and pH maintained at 7.4) was carried out up to 32h. Polymer concentration and the rate of drug release from nanoparticles were found to be inversely proportional. The drug release profiles of three formulations including MIT-4, MIT-5 and MIT-6 were studied, and a line graph of cumulative drug release versus time was plotted (Fig. 1). The rate of mitoxantrone release from MIT-4 was found to be fastest and was discovered to drop gradually for MIT-5 and MIT-6.

The findings of mitoxantrone release profile from nanoparticles endorsed the correlation between

Table 1. Mitoxantrone nanoparticles concentration

Formulation Name	Drug Concentration (mg/ml)	Alginate Concentration (mg/ml)	Glutaraldehyde Concentration (mM)
MIT-4	0.1	0.4	36
MIT-5	0.1	0.5	36
MIT-6	0.1	0.6	36
MIT -7	0.1	0.8	18
MIT-8	0.1	1.0	18

18 and 36 mM aqueous glutaraldehyde solutions were prepared and injected dropwise into sodium alginate solution and kept stirring at a speed of 1500 rpm for 60 min. The isolation of nanoparticles was done using ultracentrifugation technique

Table 2. Mean particle size and polydispersity index of formulations

Formulation Name	Mean Particle Size (nm)	Polydispersity Index
MIT-4	320	0.435
MIT-5	442	0.241
MIT-6	467	0.352
MIT -7	2554	1
MIT-8	4521	1

Table 3. Entrapment efficiency (%) of mitoxantrone-loaded nanoparticles

Formulation Name	Mean Absorbance at 610 nm (n=3)	Entrapment efficiency (%)
MIT-4	0.522	62
MIT-5	0.576	66
MIT-6	0.634	70
MIT -7	0.685	76
MIT-8	0.701	81

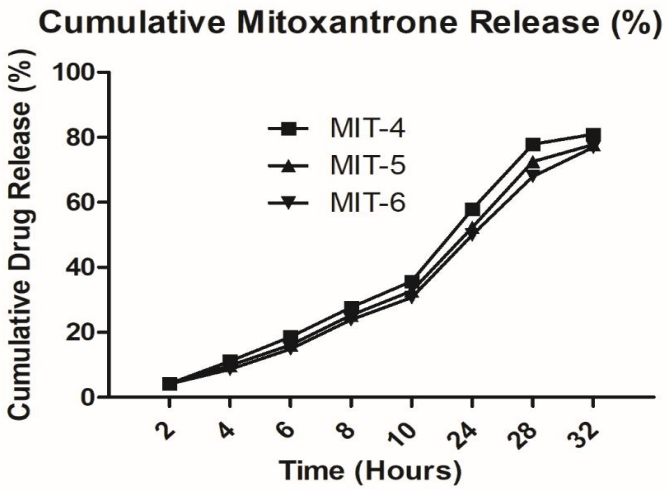

Fig. 1. *In vitro* drug release profile of mitoxantrone

Table 4. Drug release kinetics models

Kinetic model		Formulation		
		MIT-4	MIT-5	MIT-6
Zero	R^2	0.6720	0.4169	0.5231
	K_0	4.022	4.173	4.124
First	R^2	0.8693	0.9156	0.90
	K_1	0.126	0.178	0.115
Higuchi	R^2	0.825	0.8865	0.8832
	K_{HC}	19.02	19.31	19.147
Korsmeyer-Peppas	R^2	0.943	0.9555	0.943
	K_P	27.98	26.14	26.57
	n	0.345	0.378	0.382
Hixon-Crowell	R^2	0.864	0.879	0.863

polymer concentration and the rate of drug release. An increase in the concentration of polymer was found to decrease the drug release from nanoparticles and vice versa. However, in case of entrapment efficiencies (%) and polymer concentration, a direct correlation was found.

3.4 Drug Release Kinetics

Using DDSolver software, the data of all formulations obtained from *in vitro* release experiment were fit into Korsmeyer-Peppas model. Higher regression coefficient values showed that the mitoxantrone release followed Korsmeyer-Peppas model. The formulation MIT-1 showed the value of n <0.43, whereas the values of n for MIT-2 and MIT-3 were found to be 0.43 to 0.45, respectively.

4. DISCUSSION

Coacervation technique was used in the preparation of mitoxantrone-loaded nanoparticles for anticancer drug delivery. The *in vitro* experiment revealed that mitoxantrone was released from the nanocarrier in a sustained manner. Sodium alginate is a biodegradable hydrophilic polymer and is commonly used in the preparation of polymeric nanoparticles. Owing to its ability to react with cations and ionic gelation characteristic, it has been extensively reported in the fabrication of microparticle preparations [12].

Here, mitoxantrone-loaded nanoparticles prepared by using ionic gelation technique exhibited some exciting advantages over some of previously reported studies of mitoxantrone loading. An interesting finding of this study was the relatively higher entrapment efficiency of the drug, secondly a direct correlation between entrapment efficiency and polymer concentration. The formulations MIT-4 - MIT-7 exhibited relatively lower entrapment efficiencies in comparison to others. This phenomenon might be attributed to the difference in stirring speed or pH during adding of cross-linker to the polymer solution [14].

The rate of mitoxantrone release was found to be dependent on the polymer concentration. An increase in polymer concentration resulted in the decrease of mitoxantrone release from nanoparticles. This can possibly be attributed to the augmented concentration buildup due to crosslinking between the cross linker and polymer. This results in the retardation of drug release. At high polymer and low cross linker concentration, the particles were found to be out of range with highest polydispersity index (1). At a low alginate and high glutaraldehyde concentration, the mean particle size was found to be decreased. The findings of formulation MIT-1 revealed that the drug release followed Fickian diffusion. The release of mitoxantrone from formulations MIT-2 and MIT-3 followed the anomalous transport kinetics with a combination of super case II transport and two diffusion mechanisms. This sort of finding was also formerly reported by Marcato et al. [19].

5. CONCLUSION

Mitoxantrone-loaded sustained release nanoparticles were successfully prepared using ionic gelation method. The nanoparticles exhibited higher entrapment efficiency of mitoxantrone (around 70%). The nanoparticles exhibited exciting physicochemical characteristics and drug entrapment efficiency and are promising candidates for further *in vitro* and *in vivo* studies, thus indicating their potential for chemotherapeutic application.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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