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ScienceDirect
International Journal of Pharmaceutics 323 (2006) 146–152
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Preparation, characterization and *in vitro* drug release studies of novel polymeric nanoparticles

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Received 24 February 2006; received in revised form 18 May 2006; accepted 24 May 2006
Available online 7 July 2006

Abstract

Polymeric nanoparticles of AADG cross-linked with MBA encapsulating water soluble macromolecules such as FITC-Dextran have been prepared in the reverse micellar system. The particles obtained were of >85 nm in diameter which were highly monodisperse. An optically clear solution was obtained on redispersing these nanoparticles in aqueous buffer. Size and morphology of the particles remains the same on re-dispersing the lyophilized powder of these nanoparticles in aqueous buffer. The size dependency of the particles on the monomer and surfactant concentration was observed. The average size of the nanoparticles as obtained from DLS studies ranges from 74 to 114 nm in case 0.06 M AOT and 62–104 nm in case of 0.1 M AOT concentration. FITC-Dextran was entrapped into nanoparticles with high efficiency (>70%). The pH dependent release of the entrapped molecules from these nanoparticles was also studied. At pH 5.0 solution, ~43% of FITC-Dx was released and at pH 7.4 it was about 70%.

Keywords: Nanoparticles; Polymeric; FITC-Dextran; Monodisperse; Surfactant

1. Introduction

With the advent of genomic era, new drug targets have been identified, because of which drug delivery (including both controlled release and drug targeting) has become popular both in academia and industry. The rapid progress in biotechnology has led to the development of a large number of bioactive molecules and vaccines based on polysaccharides, peptides, proteins and oligonucleotides. For site specific delivery of these bioactive molecules, a specific carrier is required which can deliver these agents in a controlled manner. To develop such carrier systems have been a major challenging task before the pharmaceutical industry from the last few decades. In search of an ideal system, over the years, a number of carrier systems have been proposed for controlled delivery of these biomolecules/drugs (Schacht, 1987; Kopecek and Ducan, 1987; Seymour, 1992; Ravi Kumar, 2000). Of the various systems proposed, biodegradable polymers have emerged as potential candidates for the development of carriers for targeting drugs to specific sites in the body (Tomlin, 1986; Davis and Illum, 1988b; Davis et al., 1993; Allerman et al., 1993; Stolnik et al., 1995). These polymeric materials are usually biocompatible, non-antigenic and highly hydrophilic in nature. Hydrophilic drugs can easily be incorporated into these polymers. The preparation of nanoparticles from these biodegradable polymers has been a major area of interest, as these can carry water soluble drugs, including proteins and nucleic acids in the naked forms or in the form of pro-drugs, thereby rendering protection against the various degrading enzymes present in the body fluid. Enormous amount of efforts have been made to prepare nanoparticles for the targeted and controlled delivery of drug molecules to specific sites.

Most of the preparation methodologies produce nanoparticles of diameter > 100 nm with high polydispersity (Allerman et al., 1993; Davis and Illum, 1988a,b; Curt, 1989; Davis, 1997). This size of nanoparticles is quite large as compared to the histology of the endothelial barrier, whose fenestration is around 50–60 nm in diameter or in the case of vasculature in solid tumor sinusoids which are less than 100 nm in diameter (Mayerson

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doi:10.1016/j.ijpharm.2006.05.065

et al., 1959). Nanoparticles, below 100 nm, with hydrophilic surface are better site specific delivery agents particularly for solid tumors (Davis and Illum, 1988a,b; Curt, 1989). The body's defense mechanism comprising of reticuloendothelial system (RES), mainly the Kupffer cells in the liver engulfs the polymeric nanoparticles with hydrophobic surface leading to rapid clearance from the vasculature system. Surface modifications, using amphiphilic polymers like polyethylene glycol (PEG) or surfactant molecules such as poloxamers and poloxamines, of nanoparticles significantly increases the blood circulation time but could not escape the RES uptake (Kataoka et al., 1993; Mao et al., 2005; Ziady et al., 2003; Li et al., 2003; Kwon and Okano, 1996). Due to this reason, the delivery of biomolecules to sites other than RES presents an important challenge. From these studies, it can be inferred that nanoparticles should have a diameter less than 100 nm with hydrophilic surface to escape from RES clearance and to have longer bioavailability.

Delivering bioactive agents employing biodegradable delivery systems is highly desirable, as it discards the need for a surgical procedure to remove the delivery system. Moreover, controlled release of bioactive agents reduces the frequent administration by maintaining the therapeutic levels of the drug. With the objective of developing a nanoparticulate system having hydrophilic core as well as surface, glucosamine based nanoparticles have been prepared. Earlier studies have proposed that the conjugation of bioactive molecules, like enzymes with synthetic polymers containing sugar residues have been found to impart stability under harsh conditions (Kim and Park, 1993). Glucosamine is an amino monosaccharide found in chitin, glycoproteins and glycosaminoglycans such as hyaluronic acid and heparan sulfate. Glucosamine have been found to play pivotal role in the promotion and maintenance of the structure and function of cartilage in the joints of the body thereby relieving osteoarthritis pain. Glucosamine hydrochloride reacts with acryloyl chloride to give a glucose containing vinyl monomer AADG. Free radical co-polymerization of AADG with MBA with two redox initiators in the aqueous nanoreactors results in the formation of nanoparticles.

In this study, a process for preparation of cross-linked acrylamido-2-deoxy-glucose (AADG) nanoparticles of average diameter 85 nm and below, using the aqueous core of reverse micellar droplets as nanoreactors has been described. The cross-linking was realized by using *N*,*N*′-methylene bis-acrylamide (MBA) as a cross-linking agent. Since, the reverse micellar droplets are highly monodisperse and can be varied; the nanoparticles prepared, using this medium, are nearly monodisperse with narrow size distribution. These nanoparticles were characterized using DLS, and TEM. The *in vitro* release of FITC-Dextran (FITC-Dx) entrapped into nanoparticles was also investigated at three different pH.

2. Experimental

2.1. Materials

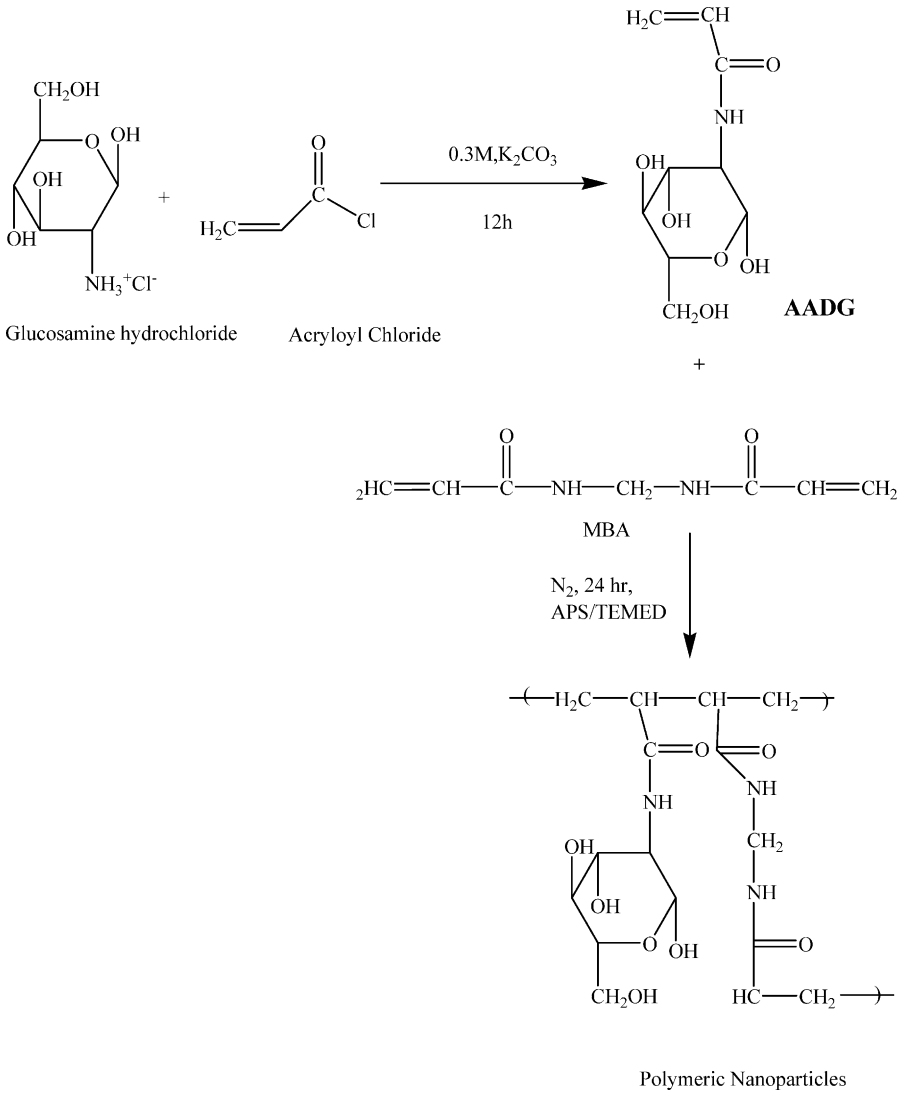
Aerosol OT, i.e., AOT (sodium bis-2-ethylhexylsulfosuccinate) (purity > 99%), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), ammonium persulfate (APS), *N*,*N*-methylene bis-acrylamide (MBA), glucosamine hydrochloride and Fluorescein isothiocynate-dextran (FITC-Dx) molecular weight approximately 20,000 Da. were products of Sigma, USA and were used directly without further purification. Acryloyl chloride from Aldrich, USA. *n*-Hexane (99%), sodium monohydrogen phosphate, and dihydrogen phosphate, and ferrous ammonium sulfate (FAS), potassium carbonate were procured from SRL (India) and are of high purity grade. Double-distilled water was used for all the work.

2.2. Preparation of acrylamido-2-deoxy-glucose (AADG) monomer

Acrylamido-2-deoxy-glucose (AADG) monomer was synthesized following a reported procedure (Kim and Park, 1993). In a round bottom flask, glucosamine hydrochloride (21.56 g) and sodium nitrite (0.2 g) were dissolved in potassium carbonate solution (0.3 M, 50 ml) and resulting solution was cooled in an ice-salt bath. Acryloyl chloride (16.20 ml) was added drop-wise from a dropping funnel under vigorous stirring over a period of 1 h. After complete addition, the reaction mixture was further stirred at room temperature for 12 h (Scheme 1). Then, the reaction mixture was added in a rapidly stirred dry ethanol. The solid was removed and to the filtrate was added diethyl ether. The resulting solution was kept for crystallization at 0 °C for 24 h. The crystalline material, separated from the solvent, was dried to obtain AADG in 79% yield.

2.3. Preparation of FITC-Dx entrapped *N*-acryloylglucosamine nanoparticles

Preparation of *N*-acryloylglucosamine nanoparticles was carried out by microemulsion polymerization method in AOT reverse micelles. The surfactant, sodium bis-2-ethylhexyl sulfosuccinate (AOT) (0.1 M) in *n*-hexane (40 ml), was taken in a two-necked round bottom flask. To this, *N*-acryloylglucosamine monomer (23.31 mg/ml, 280 μl), methylenebisacrylamide (MBA) (49 mg/ml, 50 μl), 11.2% *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) (20 μl), 1% ferrous ammonium sulphate (20 μl), 20% ammonium persulphate (20 μl) and 3.2% (w/v) of FITC-Dx (50 μl) were added with vigorous stirring. Additional water (280 μl) was added to adjust the required water to surfactant ratio, $W_0 = 8$. The homogeneous and transparent solution was further stirred for 24 h at 25 °C under nitrogen to complete the polymerization (Scheme 1). The amount of solubilized water is usually expressed as a molar ratio of water to surfactant, or $W_0 = ([H_2O]/[AOT])$. The ratio W_0 represents the number of water molecules added per molecule of surfactant (AOT) present in the solution and it has been shown to be a representative of the size of aqueous droplets. For $W_0 < 10$, water is spontaneously solubilized by the micelles giving micellar solutions and for $W_0 > 10$, larger aggregates with distinct aqueous core known as water-in-oil microemulsion is formed. After completion of polymerization reaction, the work up was carried out by two methods.



Scheme 1. Preparation of AADG monomer and AADG nanoparticles.

Method 1: The organic solvent was completely evaporated off on rotary evaporator. The residue was re-suspended in water (10 ml) and 30% calcium chloride solution (w/v) (15 ml) was added drop-wise with continuous stirring to precipitate the surfactant as calcium salt of bis-(2-ethylhexyl)sulphosuccinate, [Ca(DEHSS)$_2$]. The solution was centrifuged (10,000 rpm) for 30 min and the supernatant containing the nanoparticles was separated from [Ca(DEHSS)$_2$]. The cake of [Ca(DEHSS)$_2$] containing some nanoparticles adsorbed on it, was re-dissolved in *n*-hexane (5 ml) and washed with 1 ml of water. Aqueous layer was collected and added to the original supernatant. This aqueous solution was dialyzed for 2 h using dialysis membrane (12 kDa cut off) to get rid of unreacted monomer and other unwanted small polymer chains. The aqueous phase containing nanoparticles was lyophilized to obtain nanoparticles in powder form (yield: 81%).

Method 2: After the polymerization reaction, the organic solvent was evaporated off to half of its volume on rotary evaporator. Then, excess methanol was added to precipitate out the polymeric nanoparticles from solution. The precipitate of nanoparticles was washed with acetone (3× 5 ml) and then air-dried (yield: 87%).

AADG nanoparticles with two different surfactant concentration, i.e. 0.06 and 0.1 M AOT and different monomer concentration from 0.04 to 0.12 M were also prepared employing above methodology.

2.4. Characterization of nanoparticles

2.4.1. FT-IR studies

IR spectra of *N*-acryloylglucosamine, and cross-linked *N*-acryloylglucosamine nanoparticles were taken in KBr pellets using a Perkin-Elmer Fourier transform infrared (FT-IR) spectrophotometer (Spectrum BX series) with the following scan parameters: scan range 4400–400 cm^{-1}: number of scan 16: resolution 4.0 cm^{-1}: interval 1.0 cm^{-1}: units *%T*.

2.4.2. Size determination

2.4.2.1. Dynamic light scattering (DLS). All the dynamic light scattering (DLS) measurements for the determination of the

average hydrodynamic radii of the nanoparticles were carried out using Photon Correlation spectrometer, PHOTOCOR FC fitted with argon ion laser operated at 632.8 nm as the light source with digital correlator. All the measurements were carried out at fixed angle, i.e. 90° to the incident light and data were collected over a period of 3 min. The average particle sizes reported in the study were obtained by the method of cumulants. A weighed amount of lyophilized nanoparticles (2 mg) was suspended in double distilled water (1.0 ml) and sonicated for 3 min using a probe sonicator prior to measurements.

2.4.2.2. Transmission electron microscopy (TEM). Lyophilized powder (2 mg) of nanoparticles was dispersed by sonication in double distilled water (1 ml) to obtain a clear solution, which was later used for preparing samples for TEM. The sample solution (3 μl) was put on a formvar (polyvinyl formal) coated copper grid and air-dried. A drop of 0.5% (w/v) solution of formvar was placed on the water (previously degassed) surface. A thin film was formed on the water surface, onto which several clean copper grids were placed, with matty surface downwards. After 2–3 s, the grids along with the film were lifted off by a piece of filter paper with forceps. TEM pictures were taken on a JEOL JEM 2000 Ex 200 Model electron microscope. Prior to visualization of samples, a blank grid without sample was also scanned.

2.5. Determination of loading in the nanoparticles

The lyophilized nanoparticles (2 mg), prepared using above methodology, were dispersed in 10 ml double distilled water. The solution was then divided into 20 aliquots of 500 μl each and one of the aliquots was filtered through a Millipore Centricon YM-100 (100-kDa cut off) membrane filter. The nanoparticles were retained while the free FITC-Dx passed through the filter. The amount of FITC-Dx present in the filtrate was determined spectrophotometrically using a Perkin-Elmer Lambda Bio 20 UV/VIS spectrophotometer at a wavelength of 494 nm. The entrapment efficiency (*E*%) was calculated from the total concentration of the added amount of FITC-Dx present in the system ($[\text{FITC-Dx}]_T$) and that in the filtrate ($[\text{FITC-Dx}]_f$) using the equation

$$E\% = \frac{[\text{FITC-Dx}]_T - [\text{FITC-Dx}]_f}{[\text{FITC-Dx}]_T} \times 100.$$

2.6. In vitro release kinetic studies

A known amount of lyophilized nanoparticles (2.1 mg) encapsulating FITC-Dx was dispersed in 10 ml buffer, pH 7.4, and the solution was divided in 20 micro tubes (500 μl each). The tubes were kept in a thermostated water bath set at a temperature of 25 °C. At predetermined intervals of time, the solutions were filtered through a Millipore filter as mentioned above to separate free FITC-Dx from the loaded nanoparticles. The concentration of free FITC-Dx in the filtrate was determined spectrophotometrically by adding 100 μl of filtrate in 3 ml of buffer solution (pH 7.4) and measuring the absorbance at a wavelength of 494 nm. The percentage of FITC-Dx released was determined from the equation

$$\text{release}\,(\%) = \frac{[\text{FITC-Dx}]_{ft}}{[\text{FITC- Dx}]_T} \times 100,$$

where $[\text{FITC-Dx}]_{ft}$ is the concentration of FITC-Dx in the filtrate at time *t* and $[\text{FITC-Dx}]_T$ is the total amount of FITC-Dx entrapped in the nanoparticles.

Similarly, the release kinetics of FITC-Dx entrapped nanoparticles was studied at pH 5.0 and 8.0 at 25 °C and also at 37 °C.

3. Results and discussion

3.1. Infrared studies of the polymeric nanoparticles

Cross-linked AADG nanoparticles were prepared by the polymerization of monomers (AADG) using MBA as cross-linking agent and ammonium persulfate (APS) as initiator. The nanoparticles thus formed in this present study were characterized by FT-IR spectroscopy. As evident from the IR data, strong peak at 992 cm^{-1} corresponding to the vinyl C–H out of plane bending disappeared from the spectrum of the nanoparticles and also the peak at 3066 cm^{-1} for terminal vinyl C–H stretching vibration disappeared in the spectrum of the nanoparticles, indicating that the polymerization reaction had taken place. The C–H stretching vibration of the polymer backbone in the nanoparticles is shown by a strong peak at 2969 cm^{-1} (Fig. 1).

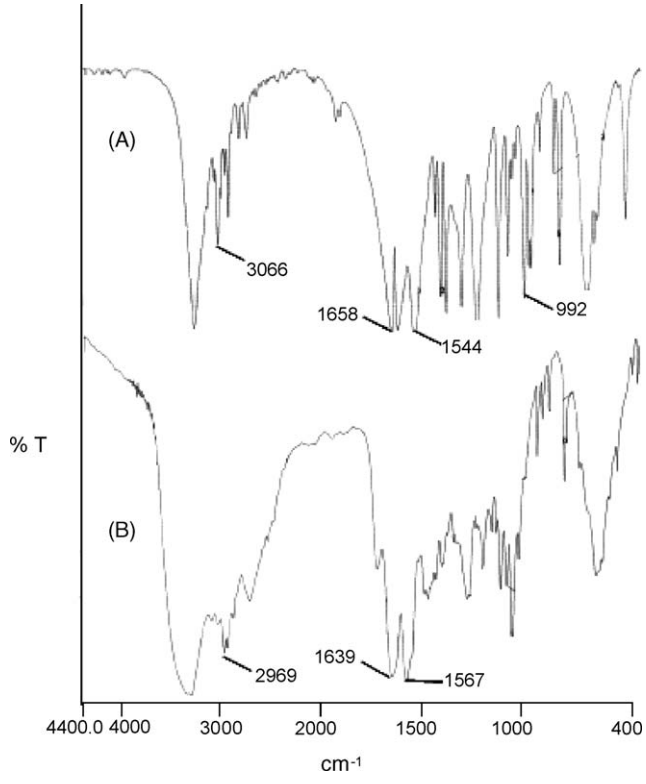


Fig. 1. FT-IR spectra of (A) AADG monomer and (B) cross-linked AADG nanoparticles.

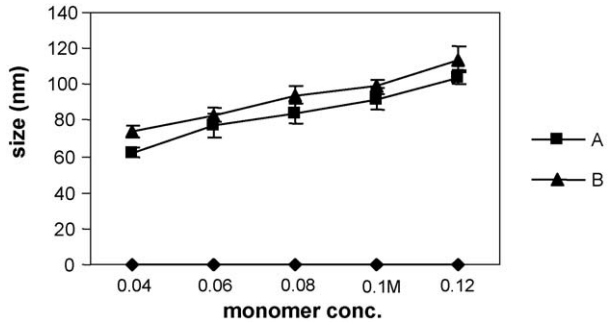


Fig. 2. Size distribution pattern of AADG nanoparticles at different surfactant concentrations (A) 0.1 M AOT and (B) 0.06 M AOT concentration. The nanoparticles were prepared in three different batches, standard error values shown.

3.2. Effect of monomer concentration on nanoparticles size

A plot of the size of the AADG nanoparticles versus the concentration of the monomer (AADG) used is shown in Fig. 2. With the increase in the concentration of the monomer (0.04–0.12 M), the size of the nanoparticles thus formed has increased from 62 to 104 nm (hydrodynamic diameter) when the reaction carried out in 0.1 M AOT solution (Fig. 2A). A similar pattern was observed when 0.06 M AOT solution was used for polymerization reaction, i.e. the size increased from 74 to 114 nm (Fig. 2B).

3.3. Effect of surfactant concentration on nanoparticles size

Nanoparticles were prepared at two different surfactant concentrations, i.e. 0.1 and 0.06 M AOT. As expected the size of the nanoparticles formed in 0.06 M AOT was found to be larger than that of 0.1 M. The size distribution at the two different surfactant concentration with various monomer concentrations is shown in Fig. 2.

3.4. Size and morphology of the AADG nanoparticles

The sizes and surface morphology of AADG nanoparticles entrapping FITC-Dx were determined employing DLS and TEM. The nanoparticles (0.1 M monomer) entrapping FITC-Dx were found to be around 104 nm diameter as determined by DLS (Fig. 3). The same particles exhibit diameter of about 80–85 nm in the TEM pictures (Fig. 4). It is well established fact that DLS measures the hydrodynamic radii by dispersing particles in aqueous phase or solvents whereas TEM measures the size of dried samples loaded onto to copper grids. We believe that the hydration and swelling of the particles in aqueous buffer or in the aqueous cores of reverse micellar droplets may be the possible reason for observing larger size by DLS measurements as compared to TEM. The concentration of monomer is one of the major factors governing the size of the nanoparticles in the synthesized polymer. With increase in the monomer concentration more molecules are likely to react with the cross-linking molecules to form larger polymeric networks which in this case appear as nanoparticles.

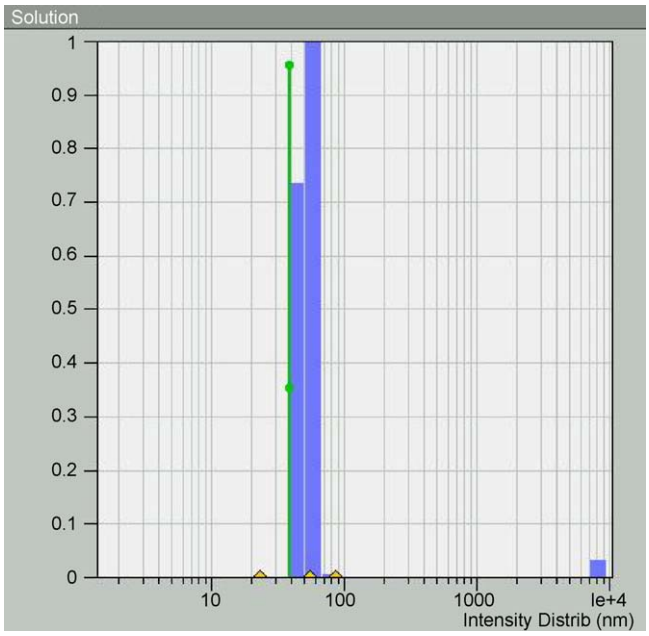


Fig. 3. Representative dynamic light scattering spectrum of AADG nanoparticles in double distilled water. Average size of nanoparticles is 104 nm.

3.5. Entrapment efficiency

The entrapment efficiency (*E*%) of the AADG nanoparticles entrapping FITC-Dx was found to be about 71% when 3.2% (w/w) of FITC-Dx was added to the polymeric material. The

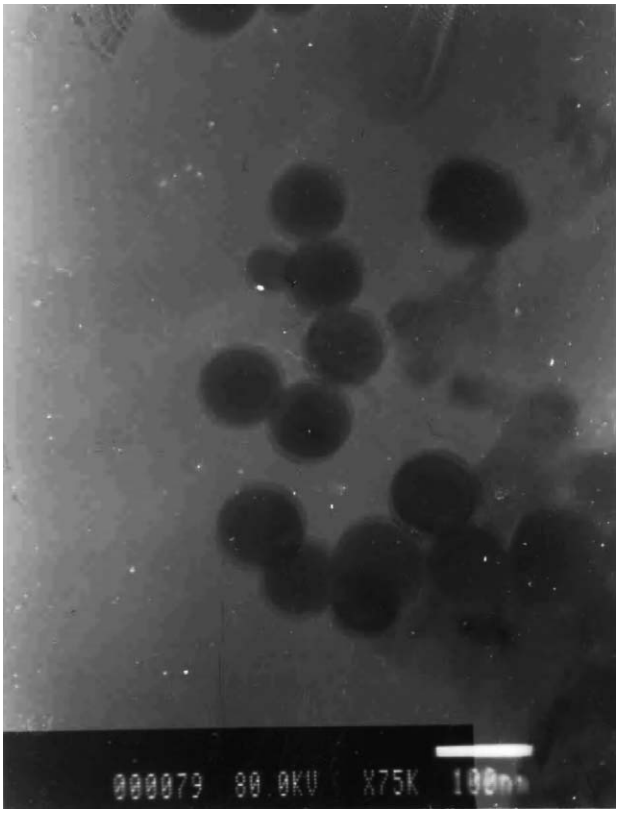


Fig. 4. Transmission electron microscope image of AADG nanoparticles. Average size of nanoparticles is 85 nm.

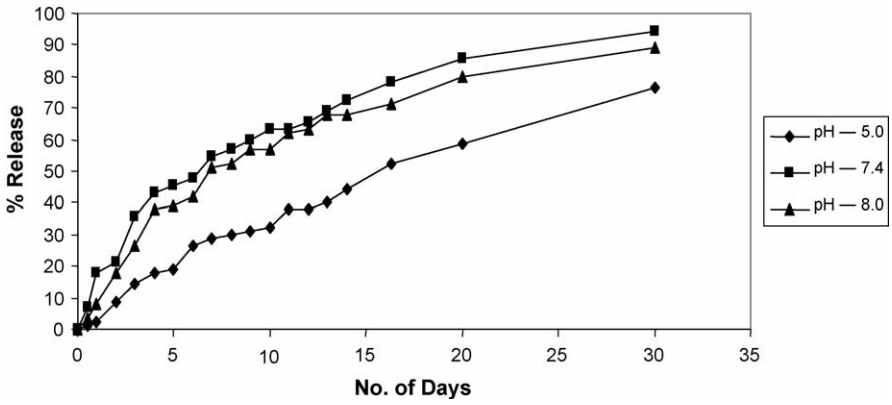


Fig. 5. Release kinetics of FITC-Dx from AADG nanoparticles (1.2%, w/w MBA) dispersed in aqueous buffers of different pH (indicated against each curve). FITC-Dx loading = 3.2% (w/w) of polymeric material.

E%, however, remains practically constant at about 70% within the broad range of AADG nanoparticles prepared in the present investigation.

3.6. In vitro release kinetic studies

Since the major objective behind the synthesis of these nanoparticles was to prepare particles that are stable under different physiological pH. So, to determine the stability FITC-Dx entrapped nanoparticles were suspended in different aqueous buffer and FITC-Dx thus released measured spectrophotometrically. The cumulative percentage of FITC-Dx released from nanoparticles at different time intervals has been shown in Fig. 5. The *in vitro* release from the nanoparticles was monitored for few days but it never reached 100%. FITC-Dx is a large molecule (molecular weight 20 kDa), therefore, simple diffusional release of the molecule from the polymer matrix of the nanoparticles is difficult unless the polymer is swelled or eroded in an aqueous medium. AADG cross-linked with MBA has a structure in which polymeric erosion through cleavage may take place only through the hydrolysis of the amide bond of the cross-linking agent with consequent generation of a loose polymeric network. However, Torchilin et al. indicated that the rate of cleavage of amide bonds in MBA cross-linked polymer is extremely low, particularly when the networks are prepared with more than 1% (w/w) MBA (Torchilin et al., 1977). Therefore, it is certain that the release of FITC-Dx from the AADG nanoparticles precedes polymer degradation. The plausible route of drug release, under these circumstances, is, therefore, believed to be due to a diffusion-controlled process from the highly swollen gel nanoparticles. To understand this we have determined the profile of FITC-Dx release from the AADG nanoparticles at different pH (5.0, 7.4 and 8.0) and temperature (25 and 37 °C). The *in vitro* release of entrapped FITC-Dx from AADG nanoparticles at pH 5.0, 7.4 and 8.0 determined at 25° C has been shown in Fig. 5. It is evident from Fig. 5 that the release rate is slower both in higher and in lower pH and it attains a maximum at pH around 7.4. While after 14 days, ∼43% of FITC-Dx is released in pH 5.0 solution, this is increased to about 70% during the same period in pH 7.4. This behavior clearly indicates that AADG nanoparticles may undergo swelling in aqueous dispersion and the swelling is maximal in physiological pH. No significant difference in the *in vitro* release profile of entrapped FITC-Dx from AADG nanoparticles was observed at the two temperatures, i.e. 25 and 37 °C studied.

4. Conclusions

A novel methodology for preparing controlled size and size distribution employing reverse micellar system of polymeric AADG nanoparticles have been optimized. This strategy leads to the preparation of nanoparticles of average diameter 85 nm (TEM) and below encapsulating water-soluble polysaccharide. The particles, thus prepared, were lyophilized and re-dispersed in aqueous buffer without having any change in their size and surface morphology. The nanoparticles size with respect to the monomer and surfactant concentration was also studied. The average size of the nanoparticles as determined by the DLS measurements was from 74 to 114 nm in case 0.06 M AOT and 62–104 nm in case of 0.1 M AOT concentration. The FITC-Dextran incorporation efficiency was quite high (>70%) into nanoparticles. The pH dependent release of the entrapped molecules from these nanoparticles revealed about 70% release at pH 7.4. These particles are quite stable in aqueous dispersion and can be further modulated to be used as efficient drug delivery agent.

Acknowledgements

Authors gratefully acknowledge the help rendered by Dr. N.C. Mehra (University Science Instrumentation Center, University of Delhi) for TEM studies. SN, RK and PC are grateful to CSIR, India for financial assistance.

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