Expert Opinion

informa healthcare

Alginate-based sustained release drug delivery systems for tuberculosis

Zahoor Ahmad & GK Khuller[†]
[†]*Postgraduate Institute of Medical Education & Research, Department of Biochemistry, 160 012, Chandigarh, India*

Drug delivery systems have wide biomedical applications owing to their distinct therapeutic advantages, such as controlled release of drugs over prolonged periods, protection against premature drug degradation, reduction in drug toxicity and drug–drug interactions. All these factors are important considerations in the treatment of chronic infectious diseases such as tuberculosis. In tuberculosis, patient non-compliance is a vexing problem which is responsible not only for treatment failure, but also for the emergence of multi-drug resistant cases. Alginate, a natural polymer, has attracted researchers owing to its ease of availability, compatibility with hydrophobic as well as hydrophilic molecules, biodegradability under physiological conditions, lack of toxicity and the ability to confer sustained release potential. It is not therefore surprising that the controlled release phenomenon of this polymer has been documented for a vast array of drugs. In particular, the ability of alginate to co-encapsulate multiple antitubercular drugs and offer a controlled release profile is likely to have a major impact in enhancing patient compliance for better management of tuberculosis.

Keywords: alginate, alginate microspheres, alginate nanoparticles, antitubercular drugs, chitosan, drug delivery system, liposomes, microparticles, poly (dl-lactide-co-glycolide), solid lipid nanoparticles, tuberculosis

Expert Opin. Drug Deliv. (2008) 5(12):1323-1334

1. Introduction

1.1 Tuberculosis

Tuberculosis (TB) is an infectious disease, caused by *Mycobacterium tuberculosis*, that usually affects the lungs (pulmonary TB) and can also affect the central nervous system, lymphatic system, circulatory system, genito-urinary system, bones and joints [1,2]. Over one-third of the world's population now has the TB bacterium in their bodies and new infections are emerging at a faster rate [2]. Although tuberculosis predominantly affects the developing world, a rising number of people in the developed world contract tuberculosis owing to their compromised immune systems due to immunosuppressive drugs, HIV/AIDS, or by infections with multi-drug resistant or extensively resistant strains of *M. tuberculosis* [3,4].

The control of TB revolves around prevention – vaccination and a cure – chemotherapy [5]. Although vaccination is the most desirable means of preventing this infection, the vaccine currently available for TB, that is Bacillus-Calmette-Guerin (BCG), suffers from several demerits such as variable efficacy in different populations, short-term immunity and limited success against pulmonary infection, which accounts for most of the disease burden [3]. However, efforts are being made to improve BCG or to develop new vaccines. In this regard carriers such as chitosan/alginate, etc have been employed with some degree of success [6,7]. BCG incorporated in alginate microspheres demonstrated an almost ninefold increase in viable bacilli

10.1517/17425240802600662 © 2008 Informa UK Ltd ISSN 1742-5247

in simulated gastric fluid (SGF) after 1.5 h, in comparison to free BCG, thereby demonstrating the potential of encapsulated BCG for oral vaccination [8]. In another study, alginate-encapsulated BCG proved (reduced more colony forming units (CFU)) to be more protective in mice even after oral administration as compared to an un-encapsulated counterpart that was administered either orally or subcutaneously [9]. Significant improvements in both humoral and cell-mediated immune responses have been observed upon the encapsulation of subunit tuberculosis vaccine antigen Ag85B-ESAT-6 in liposomes [10]. Nevertheless, it is apparent that several decades will elapse before a candidate TB vaccine could take over from BCG and this is precisely the reason why an improvement in the current TB chemotherapy should be the immediate short-term goal [11].

1.2 Drug delivery

It is appreciated globally that in order to combat various diseases in a logical manner, the development of novel delivery systems for pre-existing drugs is as important as new drug identification [12]. An ideal drug delivery system is designed to alter the pharmacokinetics and biodistribution of the drug, or to function as a drug reservoir that controls the rate and/or site of drug release [13]. Drug delivery systems improve the bioavailability of drugs by entrapment of the drugs within a suitable carrier such as liposomes and micro/nanoparticles of natural or synthetic polymers [12]. These systems are mainly aimed at the improvement of already existing drugs with established therapeutic profiles.

The underlying concept behind drug delivery systems is to get the maximum therapeutic benefit while removing or minimizing the side effects of the drugs [14]. Sustained release drug delivery occurs when a polymer, whether natural or synthetic, is judiciously combined with a drug or other active agent in such a way that the active agent is released from the material in a predesigned manner [14]. The release of the active agent may be constant over a prolonged period which may be cyclic, or it may be triggered by the environment or other external events. In any case, the purpose behind controlling the drug delivery is to achieve more effective therapies while eliminating the potential for both under and overdosing. Other advantages of using controlled delivery systems are the maintenance of drug levels within a desired range, the need for fewer administrations, optimal use of the drug in question and increased patient compliance [15].

The goal of all drug delivery systems is to deploy medications intact to specifically targeted parts of the body through a medium that can control drug bioavailability by means of either a physiological or chemical trigger [12]. To achieve this goal, researchers are turning to advances in the worlds of micro and nanotechnology [14]. During the past decade, polymeric microspheres, polymer micelles and hydrogel-type materials have been shown to be effective in enhancing drug targeting specificity, lowering systemic drug toxicity, lowering dosing frequency and providing protection of pharmaceuticals against biochemical degradation, hence improving treatment success rates [12,14,15]. In addition, several other experimental drug delivery systems look promising, including those composed of biodegradable polymers, dendrimers (so-called star polymers), electroactive polymers and modified C-60 fullerenes (also known as 'buckyballs'). Several drug delivery systems have received regulatory approval and these are listed in **Table 1**.

In a drug delivery system, delivery is the only variable parameter, while the other three – drug, destination and disease – cannot be changed. Hence, when a drug formulation is designed in such a way that the rate and/or place of drug release are altered, the formulation is called a modified release system. Those modified release systems that provide slow and controlled release of the drug are called sustained release drug delivery systems. Sustained release is generally achieved by means of encapsulation of drugs in carriers. In this review we discuss the potential of alginate as a sustained release carrier for the delivery of antitubercular drugs (ATD).

1.3 The need for antitubercular drug delivery systems

The therapy of tuberculosis is challenging because *M. tuberculosis* is not susceptible to many classes of antibacterial agents, it grows slowly and bears strong potential to develop resistance [16]. Hence, most of the drugs used for treating infections with other bacteria have failed to act on it. The antibiotic treatment of TB was initiated by the discovery of streptomycin (SM), followed by the identification of its activity against *M. tuberculosis* [17]. In spite of the availability of effective chemotherapy and BCG vaccine, TB remains a leading infectious killer worldwide [18].

The current therapy for drug-susceptible TB uses daily oral administration of antituberculosis drugs, that is isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA) and ethambutol (EMB) for a period of 6 – 9 months or more [16]. However, the administration of these drugs alone (without encapsulation in a drug delivery system) suffers from certain pitfalls: the drugs often reach the target sites at low concentrations due to premature and rapid degradation, dilution (drugs are diffused through the circulatory medium and hence become diluted), reduced cellular permeability of drugs, drug–drug interactions, hepatotoxicity, nephrotoxicity, nausea, vomiting and fever [17,19]. Most patients are therefore either unwilling or unable to adhere to such daily dosing, which results in treatment failure and the emergence of drug resistance [20]. The ongoing DOTS (Directly Observed Treatment, Short course) program has not been completely successful in solving the problem of patient non-compliance.

To assure the success of treatment, a number of basic requirements must be met: i) antibiotics must be given in combination to prevent the selection of resistant mutants; ii) antibiotics must be given for a long period of time, at least 6 months, to prevent relapse after treatment is stopped; and iii) patient as well as clinician compliance must be monitored to ensure proper administration and intake of

Table 1. Examples of drug delivery systems that have received regulatory approval.

Drug or therapeutic agent (trade name), manufacturer(s)	Disease
Liposomal amphotericin B (AmBisome), Gilead, USA; Fujisawa, USA	Fungal infections, leishmaniasis
Liposomal amphotericin B (Fungisome), Lifecare, India	Fungal infections, leishmaniasis
Goserelin Acetate (Zoladex), Zeneca, UK	Prostate cancer
PEG-adenosine deaminase (Adagen), Enzon, USA	Severe combined immunodeficiency disease
Styrene maleic acid and neocarzinostatin copolymer in Ethiodol (SMANCS/Lipiodol, Zinostatin stimalamer), Yamanouchi, JPN	Hepatocellular carcinoma
Stealth (PEG-stabilized) liposomal doxorubicin (Doxil/Caelyx), ALZA, USA; Schering-Plough, USA	Kaposi's sarcoma, refractory ovarian cancer, refractory breast cancer
Liposomal cytosine arabinoside (DepoCyt), SkyePharma, USA	Lymphomatous meningitis, neoplastic meningitis
Denileukin diftitox or interleukin 2-diptheria toxin fusion protein (ONTAK), Seragen, USA	Cutaneous T-cell lymphoma
Liposomal doxorubicin (Myocet), Elan, Ireland	Metastatic breast cancer in combination with cyclo-phosphamide
Gemtuzumab ozogamicin or anti-CD33–linked calicheamicin (Mylotarg), Wyeth-Ayerst, USA	CD33_ relapsed acute myeloid leukemia
PEG-interferon-2b (PEG-Intron), Enzon, USA; Schering-Plough, USA	Hepatitis C
PEG–granulocyte colony stimulating factor or pegfilgrastim (Neulasta), Amgen, USA	Reduction of febrile neutropenia associated with chemotherapy
90Y-ibritumomab tiuxetan or 90Y anti-CD20 (Zevalin), IDEC, USA	Relapsed or refractory non-Hodgkin's lymphoma
131I-tositumomab (anti-CD20) (Bexxar), Corixa, GlaxoSmithKline, UK	CD20_ relapsed non-Hodgkin's lymphoma

antibiotics. These requirements are often difficult to meet, especially in developing countries and in the context of HIV epidemics. The vast majority of the TB burden is in developing countries, which is the main reason why only 23% of prevalent active cases receive appropriate antituberculosis treatment [21]. The results are therefore such that even after the availability of such a strong therapeutic bullet, 9×10^6 new cases of active disease occur each year [21].

The research efforts to improve chemotherapy of TB include: i) derivatize existing ATDs into more potent compounds; ii) screening of compounds active against replicating as well as latent bacilli; iii) identification of novel drug targets and designing appropriate inhibitors; and iv) targeting host–pathogen-related processes essential for survival in human disease. While these strategies are indeed worthwhile to go ahead with, the challenges are: i) the involvement of intense research efforts; ii) the difficulty in targeting latent bacilli; and iii) uncertainty with respect to toxicity and drug resistance [22]. Considering the fact that for several decades now no new ATDs have been developed, despite global efforts, it would be prudent and rational to develop sustained release drug formulations (using ATDs already in current clinical practice) in order to reduce the dosing frequency. Therapies based on sustained release drug delivery systems could be administered more intermittently (i.e., once weekly or even less frequently) without sacrificing therapeutic outcome. This will reduce the burden of supervising drug administration and make treatment more widely available, as well as more acceptable.

2. Types of carriers

A carrier is that part of a drug delivery system that holds the active moiety, that is the drug, by encapsulation/adsorption, etc, and helps in its delivery and pre-designed release pattern. In addition to different delivery system designs, carriers also provide the flexibility for selecting the route of delivery. There are number of carriers used in drug delivery systems that are broadly classified into synthetic and natural [15]. The synthetic carriers that are used as drug delivery vehicles include poly(D,L-lactide-co-glycolide) (PLG), polylactic acid (PLA), polyglycolic acid (PGA), polyanhydrides, polymethyl acrylates, carbomers, etc, while lipids (liposomes and solid lipid nanoparticles), alginic acid, chitosan, gelatin, dextrins, etc, are examples of natural carriers [14,15,23,24]. The present focus is on the natural carrier alginate.

2.1 Alginate

Alginic acid is a natural copolymer of guluronic acid and mannuronic acid (**Figure 1**) and was first reported by the British chemist EC Stanford at the end of 19th century. This polymer is obtained by the treatment of brown seaweed *(Phaeophyceae*, mainly *Laminaria)* (where they play structural role) with alkali, followed with mineral acids. Alginate is the most abundant marine biopolymer and the second most abundant biopolymer (next to cellulose) in the world. Alginate can be obtained economically in an ultra-pure form and may be prepared in neutral/charged

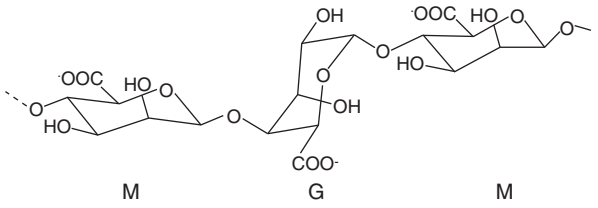

Figure 1. Structure of alginate.
Reticulation reaction
$2Na(Alginate) + Ca^{2+} \rightarrow Ca(Alginate)2 + 2Na^{+}$.
G: (alpha)-L-guluronic acid unit; M: (beta)-D-mannuronic acid unit.

forms which make it compatible with a broad range of substances [25,26].

Alginate represents a family of unbranched polysaccharides composed of the epimers β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues, that can be arranged either in homopolymeric blocks of each type (MM, GG) or in heteropolymeric blocks (MG) (**Figure 1**). The alginate monomer composition is of major concern in drug delivery as it governs the drug release property [26,27]. High 'M' content alginates are suitable for thickening applications, whereas those with high 'G' content are best for gelation. 'Designer' alginates are currently being developed, involving the 5-epimerization of β- (1→4) linked 'M' residues to α- (1→4) linked 'G' residues in algal alginates using bacterial epimerases.

The main property of alginates is their ability to form gel-like structures in aqueous solutions with the help of divalent counterions, for example Ca^{2+}. These cations specifically bind to carboxylic groups of guluronate in GG sequences, leading to a particular structure three-dimensional network often called an 'egg-box model'. The gelation process is very fast, and gel network and homogeneity depends on the cation concentration. With an excess of Ca^{2+}, a modified 'egg-box' with multiple alginate chains in the gelling zone may be formed with different physicochemical properties [28]. The homogenous hydrogels can only be obtained by the controlled addition of cations. The M/G ratio is an important consideration in the process – as the ratio decreases, the requirement of Ca^{2+} for crosslinking increases. Gels bearing alginate with a high 'G' content exhibit high porosity, low shrinkage during gelation and do not swell after drying [29]. Hence, such alginates ensure the formation of more rigid gels which are less prone to erosion and the drug release is slow. When the 'M' content increases, the gels become softer, more elastic, shrink, porosity reduces and such gels swell after drying. Alginic acid possesses unique features because of its ability to form two types of gels depending upon the pH of the surrounding medium. At low pH, hydration of alginic acid leads to the formation of a high viscosity 'acid gel' due to intermolecular binding; this property is being exploited for cell immobilization/encapsulation studies. Ionotropic hydrogels are formed with divalent/multivalent cations, except the Mg^{2+} form. Based on its ability to form two types of gels that have a wide range of physicochemical properties, alginate is suitable for granule formation, lyophilization and direct compression into tablets. In addition, alginate is non-toxic, stable at room temperature and sterilization may be performed by filtration or specific heat treatment [25,30]. With the discovery of alginate lyases, either mannuronate or guluronate lyases, it is now possible to produce novel alginate polymers for diverse applications [31].

2.2 Alginate-based drug delivery

Alginate has wide industrial applications due to its gelling and stabilizing properties [32] and in the pharmaceutical industry it is in clinical use for the supportive treatment for reflux esophagitis. It has diverse applications as a binding and disintegrating agent in tablets, a suspending and thickening agent in water miscible gels, lotions and creams and as a stabilizer for emulsions [31,32]. Several attributes make alginate an ideal drug delivery vehicle (**Table 2**). Hence, it is not surprising that alginate has been used as a carrier for the controlled release of numerous molecules, for example insulin, indometacin, sodium diclofenac, nicardipine, dicoumarol, gentamicin, vitamin C, ketoconazole, amoxicillin and ATDs [33-44]. There are two broad types of alginate-based drug delivery systems: the membrane system and the matrix system. In the membrane reservoir system, the drug release from the inner reservoir core is controlled by the polymeric encapsulating membrane having a specific permeability. As the thickness of the coat/membrane increases, the release rate decreases. Moreover, the co-encapsulation of certain non-polar substances may further reduce the release rates. This property was advantageously used in the encapsulation and controlled release of indometacin, where the sudden release of the drug is highly undesirable because it is well known that indometacin is irritant to the gastrointestinal mucosa [34]. In contrast, in the matrix system or more specifically the 'swelling–dissolution–erosion system', the drug molecules are dispersed in a rate controlling polymer matrix. The matrix swellings, as well as dissolution/erosion occurring concomitantly at the matrix periphery ,are the factors that modulate drug release [43]. Broadly speaking, factors related to the development of a particular formulation, as well as factors encountered once the formulation is inside a living system, can influence alginate-based drug delivery systems to a great extent (**Table 3**).

2.3 Alginate microspheres as antitubercular drugs carriers

The method used entails the encapsulation of ATDs during the cation-induced gelation of alginate, followed by recovery of microspheres by filtration. On drying, the microspheres showed that mean particle size was between 90 – 100 μm [41]. The entrapment of INH, RIF and PZA was found to be

Table 2. Alginate: the polymer of choice in drug delivery.

A natural polymer
Large-scale production in an economic fashion
Compatible with a wide range of substances
Simple encapsulation procedure for the majority of drug(s)
Mucoadhesive
Biodegradable
On-toxic
Formulation of different delivery systems
Sustained drug release
Enhanced bioavailability of drugs
Applications in biotechnology

Table 3. Factors influencing drug encapsulation and release from alginate-based systems.

pH of the surrounding medium
The relative proportion of G and M residues
Molecular weight and viscosity of alginate
Drug–polymer ratio
Ionic nature of the drug
Nature and amount of cross-linker
Gelling time
Variation in particle size 47
Addition of regulatory molecules

25 – 35%, 40 – 70% and 33 – 43%, respectively. The release of INH, RIF and PZA from drug-loaded alginate microparticles was studied in simulated intestinal fluid (SIF) and simulated gastric medium (SGM). All the drugs showed sustained release in SIF and SGM over a period of 25 days [41]. *In vivo* drug disposition studies were carried out in guinea pigs by oral administration of a single dose of alginate microparticles containing individual or a combination of drugs and a sustained release was observed in the plasma for 3 – 4 days. Peak plasma concentration (C_{max}), T_{max}, elimination half-life ($t_{½}e$) and $AUC_{0-\infty}$ of alginate drugs were all significantly higher than those of free drugs. The encapsulation of drugs in alginate microparticles resulted in up to a ninefold increase in relative bioavailability compared with free drugs [41]. When the tissue disposition of drugs was examined, sustained release of INH, RIF and PZA was detected above the minimum inhibitory concentration (MIC) in all the organs up to 7 days. Based on tissue distribution, free and alginate-encapsulated drugs were administered daily and weekly respectively for 8 weeks. Both these regimens resulted in no detectable bacilli in spleen and lungs as compared to untreated controls, which exhibited 5.1 and 5.0 log units of *M. tuberculosis* in lungs and spleen respectively [41]. A new form of alginate microspheres called alginate blends–INH microspheres has been evaluated recently for INH delivery in rabbits [43]. This formulation has an encapsulation efficiency of 89% w/w with an *in vitro* sustained release potential up to 36 h. Bio-distribution studies showed accumulation of the formulation in liver, intestine, lungs and kidneys, indicating a hepatobiliary and renal route of excretion.

2.4 Alginate–chitosan microspheres

In order to further improve the drug encapsulation and pharmacokinetics of the formulation, a few critical modifications were introduced, such as maintaining a constant drug : polymer ratio, the addition of chitosan as a stabilizer and the reduction of the gelling time. This resulted in a reduction in microsphere size (65 – 75 µm in diameter), encapsulating approximately 85% of the initial amount of RIF and 70% of INH/PZA [45]. The microspheres encapsulating ATDs at therapeutic doses were first evaluated in mice by the oral route. The plasma profile, organ distribution and key pharmacokinetic parameters including bioavailability (with respect to oral free drugs) were calculated. It was observed that all the drugs could be detected in the plasma for 7 days and in the organs (lung, liver, spleen) for 9 days with a striking improvement in the pharmacokinetic parameters [45]. In particular, the relative bioavailability was increased by 29 – 39-fold for all three drugs. The study was repeated in other animal species to rule out the possibility of interspecies variation, but similar results were obtained in rats, guinea pigs and rabbits. Moreover, it was also possible to obtain sustained release behavior even after a reduction in drug dose. Indeed, drugs could be detected in the plasma for 5 days and in the organs for 7 days following a single oral dose of alginate encapsulated ATDs at half the therapeutic dose [45]. This formulation was non-toxic even at a very high dose tested in mice as opposed to an equivalent dose of free drugs, which proved to be lethal. It was observed that 150 times the therapeutic dose of drug-loaded microspheres did not produce any adverse effects, whereas the same dose of free drugs resulted in 100% mortality within 24 h [45].

Once the pharmacokinetics and safety of the microspheres was established, the chemotherapeutic efficacy studies were carried out in *M. tuberculosis*-infected guinea pigs, because TB progression in guinea pigs largely resembles human pathology. As predicted by the pharmacokinetic data, a schedule of microsphere administration at every 10 days (therapeutic dose) as well as every 7 days (half therapeutic dose) was followed. Free drugs administered daily (as per the conventional chemotherapy) served as one of the control groups. Five doses of microspheres (therapeutic dose) adminis-tered every 10 days or seven doses of microspheres (half therapeutic dose) administered every 7 days, were as efficacious as 46 doses of free drugs [45]. This study clearly demonstrates the feasibility of reducing the dosing frequency and the dosage.

2.5 Alginate nanoparticles as drug carriers

In order to take advantage of both alginate and nanotechnology, alginate nanoparticles encapsulating ATDs were developed and evaluated. This development has also addressed a big question of particulate delivery systems. Particulate systems (liposomes, microparticles and nanoparticles) used as drug carriers are rapidly taken up from the blood by the mononuclear phagocytes (MPS), especially by the Kupffer cells in the liver [28]. To avoid this problem surface properties of nanoparticles and liposomes have been modified to render them more hydrophilic. This approach consists of the adsorption of hydrophilic co-polymers on the nanoparticle surface or the incorporation of sialic acid-rich gangliosides or polyethylene glycol fatty acid derivatives into liposomes [28]. However, the tissue distribution profile of nanoparticles remained unchanged, even after they were coated with hydrophilic co-polymers. In addition, the integrity of the liposomes (modified by the above approach) has not yet been demonstrated under *in vivo* conditions. In light of these problems, in recent years 'stealth' colloidal particles (nanoparticles) have been directly developed from hydrophilic polymers to overcome the problem of MPS uptake. This concept has been developed with sodium alginate (a hydrophilic polymer) [28]. These nanoparticles have been shown to encapsulate both hydrophilic as well as hydrophobic drugs. Thus, alginate nanoparticles were used as an effective drug delivery system for antitubercular drugs.

The development of alginate-based drug delivery systems makes use of the ability of this polymer to undergo gelation in the presence of divalent cations. However, a critical adjustment in the relative concentrations of alginate and the cation results in a pregel state, that is alginate nanoparticles that can be harvested by high-speed centrifugation [46,28]. This principle was initially exemplified taking doxorubicin as a model drug and the same technique was employed for encapsulating ATDs [47]. The original method of nanoparticle production was modified at two steps: the substitution of chitosan in place of poly-L-lysine and a reduction in the polymer : drug ratio [47]. Chitosan was substituted in place of poly-L-lysine owing to its non-toxicity, bioadhesiveness and lower cost [46]. The size of alginate nanoparticles developed (235 nm) favors their use as oral drug delivery vehicles, as particles less than 400 nm are known to be taken intact from the intestine [46,47]. Further, it has been demonstrated that alginate nanoparticles can cross the intestinal barrier and be distributed to various organs. The drug encapsulation efficiency ranged from 70 – 90% [47,48]. The efficiency of drug encapsulation/loading depends on the type of drug, the resultant drug–polymer interactions and solvent–drug interaction. The higher drug loading observed in alginate nanoparticles in this study can be attributed to a higher drug : polymer ratio. Further alginate due to high gel porosity is reported to have higher drug loading capacity [31,28].

2.5.1 *Studies with orally administered ATD-loaded alginate nanoparticles*

Following a single oral administration of drug-loaded alginate nanoparticles to mice, all the four ATDs (EMB, RIF and INH/PZA) in plasma were observed up to 7, 9 and 11 days respectively [47,28]. These results depicted a definite improvement in $AUC_{0-\alpha}$ and relative bioavailability of ATDs encapsulated in alginate nanoparticle over free drugs or drugs encapsulated in other carriers. The pharmacodynamic parameters C_{max}/MIC, $AUC_{0-\alpha}$ /MIC and T_MIC achieved significantly higher values in case of encapsulated drugs than reported to be required for the substantial killing of *M. tuberculosis* [47-48]. Thus, results of pharmacodynamic parameters further supported the use of alginate nanoparticles as an ideal carrier for antitubercular drugs. The sustained release of encapsulated drugs can be explained on the basis that both alginate and chitosan possess bioadhesive characteristics, which is probably responsible for the prolonged adhesion of the formulation to the intestinal mucosa, thereby increasing the time period available for its absorption. Chitosan itself is known to modulate the intestinal tight junctions, thereby augmenting the paracellular transport process. The prolonged plasma stay of drugs encapsulated in alginate nanoparticles might be attributed to their longer half-life (nanoparticles) coupled with sustained release potential in general and to the affinity of alginate to amino groups in the case of INH and PZA in particular. These factors collectively contribute towards enhancing the pharmacokinetics and oral bioavailability of ATDs when administered as alginate nanoparticles.

Upon oral administration of ATD-loaded alginate nanoparticles to guinea pigs, the plasma profile of all four frontline ATDs was significantly better than un-encapsulated counterparts; the improvements were even better than those observed in mice (**Figure 2**) [49]. This can be attributed to slower metabolic rates in guinea pigs than mice owing to lesser surface area. All the pharmacokinetic as well as pharmacodynamic parameters of encapsulated drugs were better than their free counterparts ($p < 0.001$) [49].

Upon oral administration of alginate nanoparticles to mice/guinea pigs, all the ATDs were present at or above MIC in the organs (lungs, liver and spleen) up to day 15 [47-49]. This formed the basis of the chemotherapeutic schedule wherein the alginate formulation was administered to TB infected mice or guinea pigs on every 15th day through the oral route, as against free antitubercular drugs daily. It was observed that only two doses of this formulation, administered every 15 days in mice, were equi-efficient to 30 doses of oral free drugs administered daily in achieving undetectable CFU [47-49]. The efficacy of the alginate nanoparticle formulation in animals harboring higher bacillary loads of 6 – 7 log CFU that are commonly found in human TB lesions was also evaluated. A bacillary load of ~ 7.2 log CFU in lungs/spleen at day 15 post-challenge confirmed the establishment of infection. Six weeks of chemotherapy, that is either three doses of alginate formulation or 45 doses of

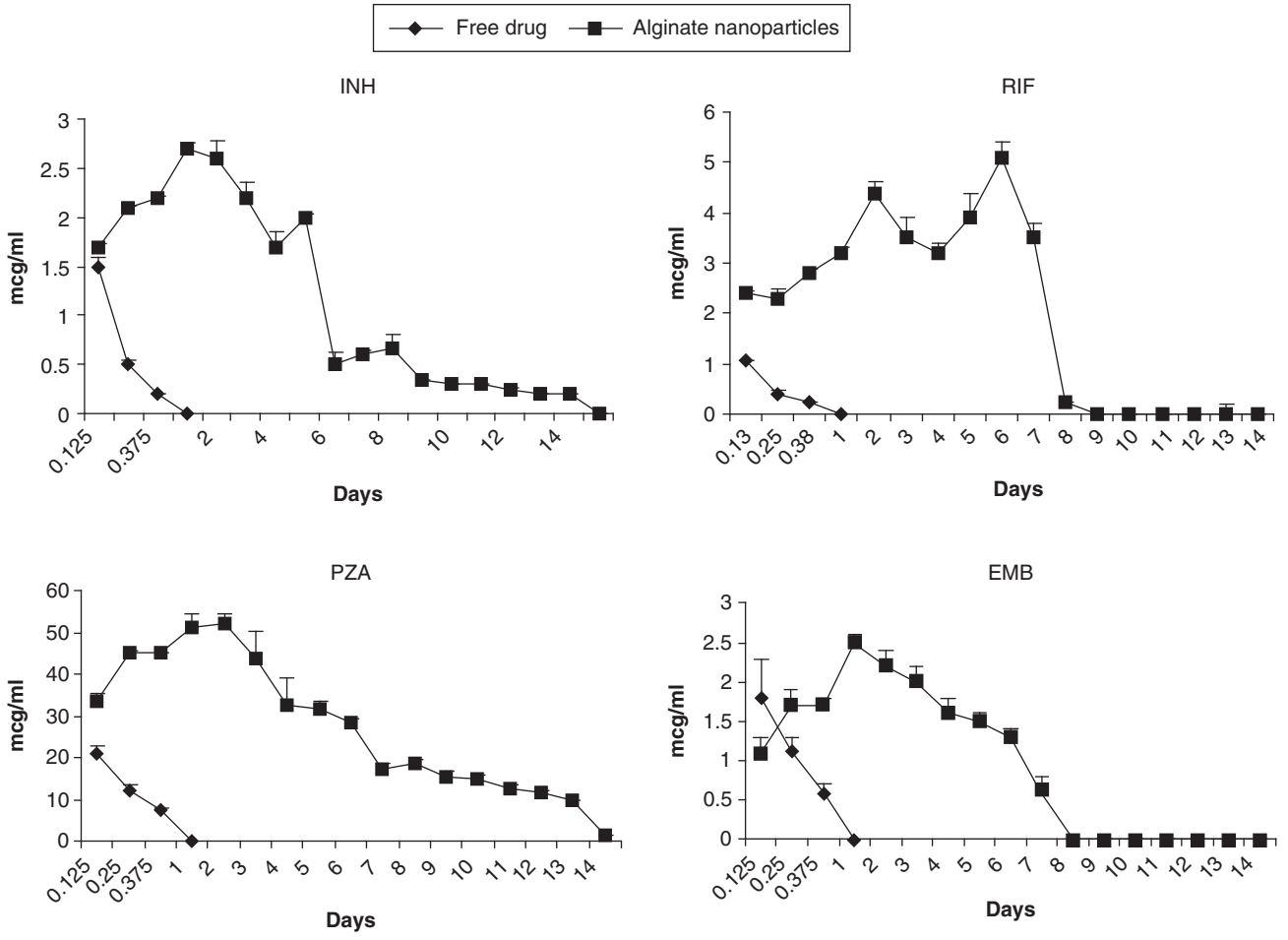

Figure 2. Plasma drug profile following a single oral dose of alginate–nanoparticle encapsulated ATDs to guinea pigs.
Values are mean ± SD, $n = 6$.

free drugs, resulted in undetectable CFU levels in the lungs and spleen of infected animals (**Figure 3**). Hence, fortnightly alginate nanoparticle-based ATD delivery will be equi-efficient to daily conventional drugs against bacterial loads that are normally found in human TB lesions.

The formulation was also evaluated against a guinea pig model of tuberculosis and it was observed that either two doses of four drugs or three doses of three drug formulations, administered every 15 days, were equi-efficient to 30 (four drugs) or 45 (three drugs) doses of oral free drugs administered daily in achieving undetectable CFU [49].

2.5.2 *Studies with inhalable ATD-loaded alginate nanoparticles*

Alginate nanoparticles were also administered via the aerosol route to guinea pigs, and INH, RIF and PZA were detected in all the tissues above MIC until 15 days [50]. These observations clearly demonstrate that alginate nanoparticles encapsulating ATDs when administered through the aerosol route not only increased the bioavailability of the drug in the lung, but also at any other site in the body. Further, it was observed that only three nebulized doses of this formulation, administered every 15 days, were equi-efficient to 45 doses of oral free drugs administered daily in achieving undetectable CFU [50].

A comparative account of alginate-based drug delivery systems that have been used for the delivery of ATDs is presented in **Table 4**.

2.5.3 *Nanoparticles as novel drug delivery systems for azole antifungals*

During recent years azole antifungal drugs have been suggested to have antitubercular activity [51]. In fact, these drugs have been proven to bear strong potential against *in vitro* and *ex vivo M. tuberculosis* [52]. More recently, *in vivo* studies have demonstrated that econazole can reduce the CFU load by 90% in the lungs and spleen of mice infected with *M. tuberculosis* and the chemotherapeutic potential of econazole was equal to RIF [53]. Further, the results of this study showed that econazole could replace RIF, INH or both in

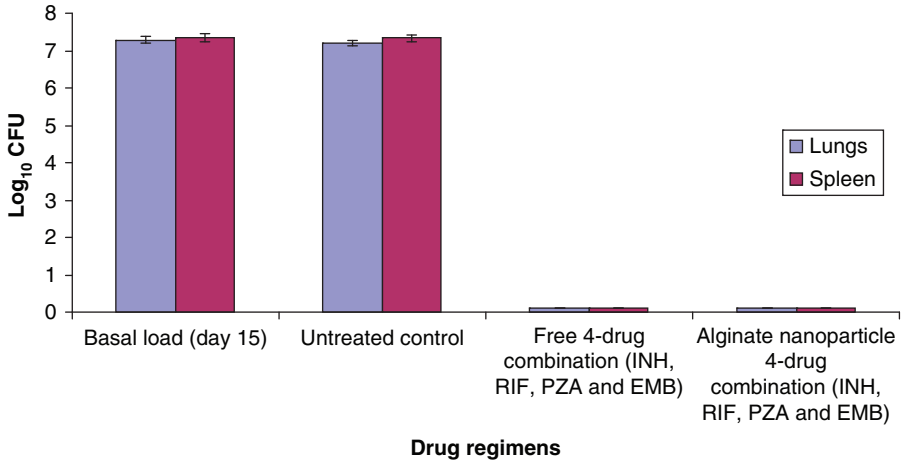

Figure 3. Chemotherapeutic efficacy of orally administered ATD-loaded alginate nanoparticles in mice. Free drugs were administered daily (seven doses/week) for 45 days and nanoparticle drugs were administered fortnightly (total three doses/45 days). Basal load is the $\log_{10}$ CFU load found at day 15 after infection (start of treatment). A value of 0.1 in each bar indicates no detectable bacilli.
Values are mean ± SD of six animals.

Table 4. Comparison of alginate-based drug delivery systems employed for ATDs.

Drug delivery system [ref.]	Drug encapsulation efficiency	Route of administration	Plasma drug stay with a single dose (days)	Increase in relative bioavailability (fold)	Chemotherapeutic schedule (days)
Comment on property	The higher the value, the better the system	The more routes, the better the feasibility of administration	The longer the duration, the more benefit	The higher the value, the more benefit	The higher the value, the more benefit
Alginate microspheres [41]	INH = 25 – 35% RIF = 40 – 70% PZA = 33 – 43% EMB = N.D.	Oral	INH = 4 RIF = 4 PZA = 4.5 EMB = N.D.	INH = 3.7 RIF = 9.3 PZA = 1.5 EMB = N.D.	7
Alginate–chitosan microspheres [45]	INH = 65 – 75% RIF = 81 – 85% PZA =65 – 76% EMB = N.D.	Oral	INH = 7 RIF = 7 PZA = 7 EMB = N.D.	INH = 18.4 RIF = 17.6 PZA = 19.1 EMB = N.D.	10
Alginate nanoparticles [47,48]	INH = 70 – 90% RIF = 80 – 90% PZA = 70 – 90% EMB = 88 – 95%	Oral and aerosol	INH = 11 – 14 RIF = 9 PZA = 11 – 14 EMB = 7	INH = 32.25 RIF = 76.85 PZA = 198.87 EMB = 290.24	15

N.D.: Not done.

tuberculosis chemotherapy [53]. Azole drugs have strong antimycobacterial potential against latent *M. tuberculosis* under *in vitro* conditions and these drugs prevent the formation of drug-induced latency [54]. Their antimycobacterial activity against MDR strains of *M. tuberculosis* has also been demonstrated recently. In fact the MIC_{90} and $MBC_{>99.99}$ of econazole were found to be in the ranges of 0.120 – 0.125 µg/ml and 0.125 – 0.150 µg/ml respectively against the strains bearing resistance patterns that include resistance to INH and RIF but sensitive to all other drugs; resistance to INH, RIF, EMB and streptomycin but sensitive to ofloxacin; and resistance to INH, RIF and ofloxacin (with or with out resistance to EMB and streptomycin) [55]. Thus, these strains represent the most common multi-drug resistant (MDR) strains that have emerged globally and are posing a threat to treatment [55].

Although azole drugs have been shown to have strong antimycobacterial potential against latent/persistent MDR and active murine tuberculosis, their bioavailability is poor via the oral route. Therefore, studies were designed to improve the oral bioavailability of two clinically important antifungal drugs – clotrimazole and econazole. Each drug was encapsulated in either PLG-NP or alginate nanoparticles.

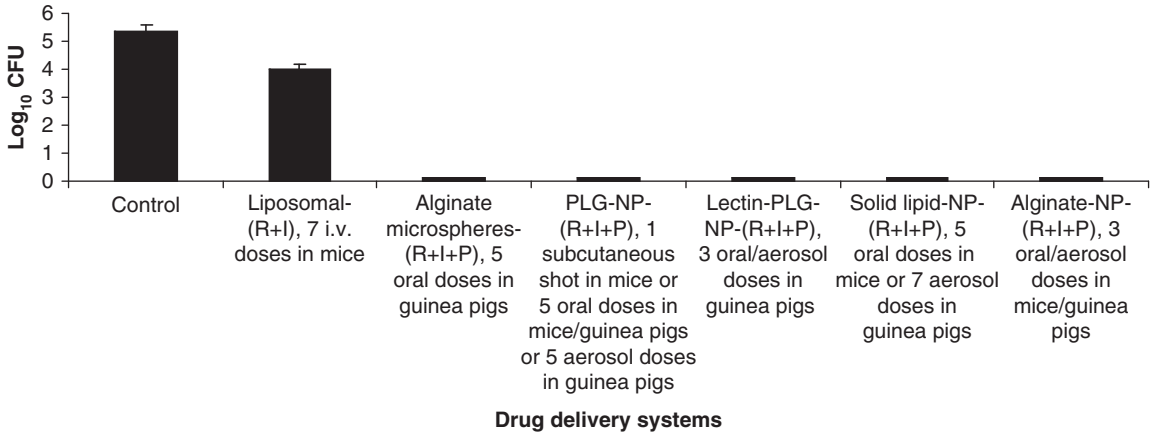

Figure 4. Therapeutic efficacy of various controlled release antitubercular drug delivery systems. Each bar indicates the mean log CFU in the lungs after the respective treatment. A value of 0.1 indicates no detectable CFU.
R: Rifampicin; I: Isoniazid; P: Pyrazinamide; E: Ethambutol; MP: Microparticles; NP: Nanoparticles; i.v: Intravenous; SLN: Solid lipid nanoparticles.

Drug encapsulation efficiency was better (> 90%) for the alginate formulation compared with the PLG formulation (nearly 50%) [56]. The biodistribution/pharmacokinetic data suggested that there was a controlled drug release for 5 – 6 days with each of the formulations (**Figure 4**) as compared with un-encapsulated drugs which were cleared within 3 – 4 h of oral/i.v. administration. There was also a striking improvement in the relative and absolute bioavailability of each drug [56]. Further, drugs were detected in the tissues (lungs, liver, spleen) for up to 6 – 8 days in the case of nanoparticles, whereas free drugs were cleared by 12 h. These results emphasize the power of nanotechnology to make the oral administration of azole antifungal drugs a reality [56].

Chemotherapeutic studies using alginate nanoparticle-encapsulated or freeeconazole (with or without ATDs) against murine tuberculosis have been carried out. Eight doses of econazole administered weekly resulted in approximately 90% clearance of bacilli from the lungs and spleen [57]. The administration of four drug-loaded alginate nanoparticles to *M. tuberculosis*-infected mice resulted in undetectable mycobacterial CFU in lung and spleen homogenates (undiluted/1 in 10 diluted) as compared to ~ 4 log CFU in lungs and spleens (**Table 5**) in the untreated control group [57]. These results clearly demonstrate that econazole can replace INH and RIF in chemotherapy of tuberculosis in free and in encapsulated forms.

2.5.4 Alginate nanoparticle-based therapy and relapse rates

Conventional ATDs can be administered daily or intermittently, although the latter is associated with post-treatment relapse. Hence it is of great interest to evaluate intermittent therapy based on drug delivery technology for relapse cases. This aspect was investigated in mice, employing dexamethasone as an immunosuppressant to allow post-treatment relapse. It was observed that two doses of ATD-loaded alginate nanoparticles administered fortnightly, or three doses of ATD-loaded PLG-NP administered every tenth day, or 30 doses of free drugs administered daily resulted in undetectable mycobacterial CFU as compared to ~ 4 log CFU in lungs and spleen of untreated control group. Further, similar observations were seen in animals killed even after six weeks of immunosuppression (the time period given to allow reactivation of persistent/latent bacilli if any) (unpublished observations). These observations were in contrast to untreated controls, where 5.73 log CFU load was seen in lungs and spleen after immunosuppression (unpublished observations). These results clearly demonstrate that nanoparticle-based intermittent therapy leads to a total clearance of bacilli rather than persisters.

3. Expert opinion and conclusion

Out of the four components of a drug delivery system – drug, destination, disease and delivery – the delivery is the only component that can be variable, or adjusted according to conditions. If the design of a drug formulation is made in such way to alter its natural disposition by effecting its rate and/or place of drug release, the formulation is called a modified release system. Several such systems have been used for the delivery of antitubercular drugs using both synthetic and natural carriers. A comparison of therapeutic efficacy of these sustained release antitubercular drug delivery systems has been presented in **Figure 4**. As is clear from the Figure, alginate represents one of the most promising systems in the form of nanoparticles. There are a number of factors that affect drug loading and release from alginate-based systems. These include the pH of the surrounding medium, the composition of alginate, viscosity of alginate, drug : polymer ratio, ionic nature of the encapsulated drug, the crosslinker, gelling/curing time, variation in microsphere size, presence of substances which modulate the properties of alginate. In general the mechanical

Table 5. Chemotherapeutic efficacy of azoles alone or in combination with ATDs in free or alginate nanoparticle encapsulated form against murine tuberculosis (ref. [57]).

Groups	Log_{10} CFU			
	4 weeks		6 weeks	
	Lung	Spleen	Lung	Spleen
INH, PZA, EMB and RIF	< 1.0	< 1.0	< 1.0	< 1.0
Econazole, INH, EMB and PZA	< 1.0	< 1.0	< 1.0	< 1.0
Econazole, RIF, EMB and PZA	< 1.0	< 1.0	< 1.0	< 1.0
Econazole, EMB and PZA	2.3 ± 0.03‡	2.32 ± 0.05‡	< 1.0	< 1.0
Encapsulated INH, PZA, EMB and RIF	< 1.0	< 1.0	< 1.0	< 1.0
Encapsulated econazole, INH, EMB and PZA	< 1.0	< 1.0	< 1.0	< 1.0
Encapsulated econazole, RIF, EMB and PZA	< 1.0	< 1.0	< 1.0	< 1.0
Encapsulated econazole, EMB and PZA	2.3 ± 0.06‡	2.33 ± 0.03‡	< 1.0	< 1.0
Untreated controls	4.02 ± 0.03	4.1 ± 0.04	4.71 ± 0.04	4.73 ± 0.03

Values are mean ± SD of six to eight animals; ‡$p < 0.001$ as compared to untreated controls. < 1.0 indicates no detectable bacilli.
EMB: Ethambutol; INH: Isoniazid; PZA: Pyrazinamide; RIF: Rifampicin.

stability and the fast drug release of Ca^{2+}-alginate based systems can be improved by modifying the microsphere surface using polycations (chitosan/poly-L-lysine), or by applying layer by layer strategies. The main advantages of the proposed coating strategies are the reduced use of toxic chemicals and the ease of preparation. Many efforts have been made to develop procedures for the preparation of alginate-based drug systems for the delivery of antitubercular drugs with small dimensions and narrow size distributions. This literature evaluation may convince readers that this field can be considered mature and that new publications can only add information on the use of this type of drug carriers with slight innovation.

Although pharmacokinetic improvements of antitubercular drugs have been well documented by the application of drug delivery technology, in TB it is generally believed that the tubercle lesions/cavities represent pathological barriers for drug–bug contacts. Hence, it is important to design studies to address the issue of drug penetration to these cavities after the administration of encapsulated drugs. Such studies can be designed with ease in animal models like guinea pigs or rabbits, where formation of cavitory lesions has been reported. The issue can also be addressed using hollow fiber mouse models of tuberculosis and *in vitro* pharmacokinetic and pharmacodynamic systems, as both these models have some kind of microenvironmental similarities with natural tubercle lesions.

Antitubercular drug delivery systems have been extensively studied in mouse as well as in guinea pig models. Further studies should be conducted to address some important issues in this area, which include evaluating these systems against aerosol models of infection, especially against high bacterial burden, evaluating them at human equipotent drug doses, and performing actual six-month studies miming both phases of conventional TB therapy and assessment of post-treatment relapse.

In conclusion, drug delivery systems hold great promise in order to improve patients' compliance with tuberculosis chemotherapy. Although all the drug delivery systems have definite advantages, the alginate nanoparticle-based ATD delivery system certainly holds several unique attributes to the extent that it seems to have the potential to turn the tide against tuberculosis.

Acknowledgements

The work carried out in the author's laboratory was supported by grants from the Department of Science and Technology (DST), New Delhi and the Indian Council of Medical Research (ICMR), New Delhi.

Declaration of interest

The authors state no conflicts of interests and have received no payment in the preparation of this manuscript.

Bibliography

1. Dye C, Watt CJ, Bleed DM, et al. Evolution of tuberculosis control and prospects for reducing tuberculosis incidence, prevalence, and deaths globally. JAMA 2005;293:2767-75
2. Williams BG, Granich R, Chauhan LS, et al. The impact of HIV/AIDS on the control of tuberculosis in Indi. Proc Natl Acad Sci USA 2005;102:9619-24
3. Fine PEM. Variation in protection by BCG: implications of and for heterologous immunity. Lancet 1995;346:1339-45
4. Chaisson RE, Martinson NA. 'Tuberculosis in Africa – combating an HIV-driven crisis' N Engl J Med 2008;358:1089-92
5. Xing Z. The Hunt for New Tuberculosis Vaccines: Anti-TB Immunity and Rational Design of Vaccines. Curr Pharm Des 2001;7:1015-37
6. Kirby DJ, Rosenkrands I, Agger EM, et al. PLGA microspheres for the delivery of a novel subunit TB vaccine. J Drug Target 2008;16:282-93
7. Zhu BD, Qie YQ, Wang JL, et al. Chitosan microspheres enhance the immunogenicity of an A985B-based fusion protein containing multiple T-cell epitopes of Mycobacterium tuberculosis. Eur J Pharm Biopharm 2007;66:318-26
8. Dobakhti F, Rahimi F, Dehpour AR, et al. Stabilizing effects of calcium alginate microspheres on Mycobac-terium bovis BCG intended for oral vaccination. J Microencapsul 2006;23:844-54
9. Ajdary S, Dobakhti F, Taghikhani M, et al. Oral administration of BCG encapsulated in alginate microspheres induces strong Th1 response in BALB/c mice. Vaccine 2007;25:4595-601
10. Kirby DJ, Rosenkrands I, Agger EM, et al. Liposomes act as stronger sub-unit vaccine adjuvants when compared to microspheres. J Drug Target 2008;16:543-54
11. du Toit LC, Pillay V, Danckwerts MP. Tuberculosis chemotherapy: current drug delivery approaches. Respir Res 2006;7:118
12. Jain KK. Delivery systems – an overview. Methods Mol Biol 2008;437:1-50
13. Amar HO, Khalil RM. Preparation and evaluation of sustained release solid dispersions of drugs with Eudragit polymers. Drug Dev Ind Pharm 1997; 23:1043-54
14. Bala I, Hariharan S, Kumar MN. PLGA nanoparticles in drug delivery: the state of the art. Crit Rev Ther Drug Carrier Syst 2004;21(5):387-422
15. Pandey R, Khuller GK. Polymer based drug delivery systems for mycobacterial infections. Curr Drug Del 2004;1:195-201
16. Nuermberger E, Grosset J. Pharmacokinetic and pharmacodynamic issues in the treatment of mycobacterial infections. Eur J Clin Microbiol Infect Dis 2004;23:243-55
17. Schatz A, Bugie B, Waksman S. Streptomycin, a substance exhibiting antibiotic activity against gram-positive and gram-negative bacteria. Proc Soc Exp Biol Med 1944;55:65-9
18. World Health Organization. Global tuberculosis control: surveillance, planning financing. WHO/HTM/TB/2005. WHO Report 2005; Geneva, Switzerland
19. Chopra P, Meena LS, Singh Y. New drug targets for Mycobacterium. tuberculosis. J Med Res 2003;117:1-9
20. Christine MJ, Pandey R, Sharma S, et al. Oral therapy using nanoparticle encapsulated antituberculosis drugs in guinea pigs infected with Mycobacterium. Tuberculosis. Antimicrob Agents Chemother 2005;49:4335-8
21. Giovanni DP, Bonora S. Which agents should we use for the treatment of multidrug-resistant Mycobacterium tuberculosis? J Antimicrob Chemother 2004;54:593-602
22. Zhang Y. The magic bullets and tuberculosis drug targets. Annu Rev Pharmacol Toxicol 2005;45: 529-64
23. Falk R, Randolph TW, Meyer JD, et al. Controlled release of ionic compounds from poly (L-lactide) microspheres produced by precipitation with a compressed antisolvent. J Contr Rel 1997:441:77-85
24. You JO, Park SB, Park HY, et al. Preparation of regular sized Ca-alginate microspheres using membrane emulsification method. J Microencapsul 2001;18:521-32
25. Smidsrod O, Draget KI. Chemistry and physical properties of alginates. Carbohydr Eur 1996;14:6-13
26. Skjak-Braek G, Espevik T. Application of alginate gels in biotechnology and biomedicine. Carbohydr Eur 1996;14:19-25
27. Takka S, Acarturk F. Calcium alginate microparticles for oral administration: I. effect of sodium alginate type on drug release and drug entrapment efficiency. J Microencapsul 1999;16:275-90
28. Rajaonarivony M, Vauthier C, Couarraze G, et al. Development of a new drug carrier made from alginate. J Pharm Sci 1993;82:912-17
29. Ostberg T, Vesterhus L, Graffner C. Calcium alginate matrices for oral multiple unit administration: II. Effect of process and formulation factors on matrix properties. Int J Pharm 1993;97:183-93
30. Tonnesen HH, Karlsen J. Alginate in drug delivery systems. Drug Dev Ind Pharm 2002;28:621-30
31. Wong TY, Preston LA, Schiller NL. Alginate lyase: review of major sources and enzyme characteristics, structure–function analysis, biological roles, and applications. Annu Rev Microbiol 2000;54:289-340
32. Matricardi P, Meo CD, Coviello T, Alhaique F. Recent advances and perspectives on coated alginate microspheres for modified drug delivery. Expert Opin Drug Deliv 2008;5(4):417-25
33. Raj NK, Sharma CP. Oral insulin – a perspective. J Biomater Appl 2003;17:183-96
34. Joseph I, Venkataram S. Indomethacin sustained release from alginate–gelatin or pectin-gelatin coacervates. Int J Pharm 1995;126:161-8
35. Gonzalez-Rodriguez ML, Holgado MA, et al. Alginate–chitosan particulate systems for sodium diclofenac release. Int J Pharm 2002;232:225-34
36. Chickering III DE, Jacob JS, Desai TA, et al. Bioadhesive microspheres: III. An in vivo transit and bioavailability study of drug loaded alginate and poly (fumaric-co-sebacic anhydride) microspheres. J Control Release 1997;48:35-46
37. Iannuccelli V, Coppi G, Cameroni R. Biodegradable intraoperative system for bone infection treatment. I. The drug/polymer interaction. Int J Pharm 1996;143:195-201
38. Cui JH, Goh JS, Park SY, et al. Preparation and physical characterization of alginate microparticles using air atomization method. Drug Dev Ind Pharm 2001;27:309-19
39. Whitehead L, Collett JH, Fell JT. Amoxycillin release from a floating dosage form based on alginates. Int J Pharm 2000;210:45-49

40. Lucinda-Silva RM, Evangelista RC. Microspheres of alginate–chitosan containing isoniazid. J Microencapsul 2003;20:145-52

41. Ain Q, Sharma S, Khuller GK, et al. Alginate based oral drug delivery system for tuberculosis: pharmacokinetics and therapeutic effects. J Antimicrob Chemother 2003;51:931-8

42. Ciofani G, Raffa V, Menciassi A, et al. A drug delivery system based on alginate microspheres: mass-transport test and in vitro validation. Biomed Microdevices 2007;9(3):395-403

43. Samad A, Sultana Y, Khar RK, et al. Radiolabeling and evaluation of alginate blend-isoniazid microspheres by Tc-99m for the treatment of tuberculosis in rabbit model. J Drug Target 2008;16:509-15

44. Takka S, Ocak OH, Acarturk F. Formulation and investigation of nicardiine HCl-alginate gel beads with factorial design-based studies. Eur J Pharm Sci 1998;6:241-6

45. Pandey R, Khuller GK. Chemotherapeutic potential of alginate chitosan microspheres as antitubercular drug carriers. J Anitimicrob Chemother 2004;53:635-40

46. Berrada M, Serreqi A, Dabbarh F, et al. A novel non-toxic camptothecin formulation for cancer chemotherapy. Biomaterials 2005;26:2115-20

47. Ahmad Z, Pandey R, Sharma S, et al. Pharmacokinetic and pharmacodynamic behaviour of antitubercular drugs encapsulated in alginate nanoparticles at two doses. Int J Antimicrob Agents 2006;27:420-27

48. Ahmad Z, Pandey R, Sharma S, et al. Alginate nanoparticles as antitubercular drug carriers: formulation development, pharmacokinetics and therapeutic potential. Indian J Chest Dis Allied Sci 2006;48:171-6

49. Ahmad Z, Pandey R, Sharma S, et al. Evaluation of antitubercular drug loaded alginate nanoparticles against experimental tuberculosis. Nanoscience 2005;1:81-5

50. Ahmad Z, Sharma S, Khuller GK. Inhalable Alginate Nanoparticles as Antitubercular Drug Carriers against Experimental Tuberculosis. Int J Antimicrob Agents 2005;26:298-303

51. Guardiola-Diaz HM, Foster LA, Mushrush D, et al. Azole–antifungal binding to a novel cytochrome P450 from Mycobacterium. tuberculosis. Biochem Pharmacol 2001;61:1463-70

52. Ahmad Z, Sharma S, Khuller GK. In vitro and ex vivo antimycobacterial potential of azole drugs against M. tuberculosis. $H_{37}R_V$. FEMS Microbiol Lett 2005;251:19-22

53. Ahmad Z, Sharma S, Khuller GK. Azole antifungals as novel chemotherapeutic agents against murine tuberculosis. FEMS Microbiol Lett 2006;261:181-86

54. Ahmad Z, Sharma S, Khuller GK. The potential of azole antifungals against latent/persistent tuberculosis. FEMS Microbiol Lett 2006;258:200-3

55. Ahmad Z, Sharma S, Khuller GK, et al. In vitro antimycobacterial potential of Econazole against multidrug resistant strains of Mycobacterium. tuberculosis. Int J Antimicrob Agents 2006;28(6):543-4

56. Pandey R, Ahmad Z, Sharma S, et al. Nano-encapsulation of azole antifungals: potential applications to improve oral drug bioavailability. Int J Pharm 2005;301:268-76

57. Ahmad Z, Sharma S, Khuller GK. Chemotherapeutic evaluation of alginate nanoparticle-encapsulated azole antifungal and antitubercular drugs against murine tuberculosis. Nanomedicine 2007;3:239-43.

Affiliation

Zahoor Ahmad[2] & GK Khuller[†1]
[†]Author for correspondence
[1]Professor
Postgraduate Institute of Medical Education & Research,
Department of Biochemistry,
160 012, Chandigarh, India
Tel: +91 981 420 4024;
Fax: +91 0172 2744 401, 2745 078;
E-mail: gkkhuller@yahoo.co.in
[2]Johns Hopkins University School of Medicine,
Center for Tuberculosis Research,
Department of Medicine,
Baltimore, Maryland, USA