Review Article

Chitosan Microspheres in Novel Drug Delivery Systems

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Mitra and Dey: Chitosan Microspheres Drug Delivery Systems

The main aim in the drug therapy of any disease is to attain the desired therapeutic concentration of the drug in plasma or at the site of action and maintain it for the entire duration of treatment. A drug on being used in conventional dosage forms leads to unavoidable fluctuations in the drug concentration leading to under medication or overmedication and increased frequency of dose administration as well as poor patient compliance. To minimize drug degradation and loss, to prevent harmful side effects and to increase drug bioavailability various drug delivery and drug targeting systems are currently under development. Handling the treatment of severe disease conditions has necessitated the development of innovative ideas to modify drug delivery techniques. Drug targeting means delivery of the drug-loaded system to the site of interest. Drug carrier systems include polymers, micelles, microcapsules, liposomes and lipoproteins to name some. Different polymer carriers exert different effects on drug delivery. Synthetic polymers are usually non-biocompatible, non-biodegradable and expensive. Natural polymers such as chitin and chitosan are devoid of such problems. Chitosan comes from the deacetylation of chitin, a natural biopolymer originating from crustacean shells. Chitosan is a biocompatible, biodegradable, and nontoxic natural polymer with excellent film-forming ability. Being of cationic character, chitosan is able to react with polyanions giving rise to polyelectrolyte complexes. Hence chitosan has become a promising natural polymer for the preparation of microspheres/nanospheres and microcapsules. The techniques employed to microencapsulate with chitosan include ionotropic gelation, spray drying, emulsion phase separation, simple and complex coacervation. This review focuses on the preparation, characterization of chitosan microspheres and their role in novel drug delivery systems.

Key words: Biopolymer, bio-compatible, coacervation, chitosan, drug targeting, ionotropic gelation, microcapsules, phase separation

Drug delivery focuses on maximizing bioavailability at specific places in the body and over a period of time. Nanomedical approaches to drug delivery concentrate on the development of nanostructure devices like the microcapsules or nanospheres to improve the bioavailability of the drug and target it to the specific site of interest[1]. In this present era, science and technology are giving their maximum stress on the development of sustained-release pharmaceuticals. This matter continued to be the focus of a great deal of attention in both industrial and academic laboratories. There currently exist numerous products in the market formulated for both oral and injectable routes of administration that claim sustained or controlled drug delivery. The strength of drug delivery system is its ability to alter the pharmacokinetics and bio-distribution of the drugs[2]. Nanotechnology appears to possess the potential to improve drug delivery and drug targeting leading to increased efficacy and reduced toxicity, which would result not only in a great benefit to patients but also to pharmaceutical and drug delivery companies by creating new market opportunities[3,4].

Drug delivery some times is aimed at crossing specific barriers such as the blood brain barrier, in order to increase the drug concentration at the site of action to improve effectiveness; or to find alternative and acceptable route of delivery for protein drugs that cannot be delivered through gastro-intestinal tract due to degradation[4]. A novel drug delivery system (NDDS) is a system that offers multiple drug delivery solutions such as: Oral Drug Delivery Systems and Materials, Parenteral and Implant Drug Delivery Systems, Pulmonary and Nasal Drug Delivery, Transmucosal Drug Delivery, Transdermal and Topical Drug Delivery, Delivery of Proteins and Peptides, Drug Delivery Pipelines, Drug Delivery Deals. Use of lipid or polymer based nanoparticles have shown improved pharmacological and therapeutic actions and have overall benefits in the novel drug delivery systems.

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In the design of controlled release dosage formulations choice of polymer is of vital importance since it acts as drug carrier[5]. Chitosan is a polysaccharide comprising copolymers of glucosamine and N-acetyl glucosamine. Being biodegradable and biocompatible, chitosan has been used in the formulation of particulate drug delivery systems to achieve controlled drug delivery[6]. Chitosan has been widely investigated as a drug carrier for many possible routes of administration as chitosan has favorable biological properties, such as non-toxicity, biocompatibility, biodegradability, and antibacterial characteristics[6,7]. The physical and chemical properties of Chitosan, such as inter- and intra-molecular hydrogen bonding and the cationic charge in acidic medium, makes this polymer more attractive for the development of conventional and novel pharmaceutical product. Chitosan can serve a number of purposes including as a coating agent, gel former, controlled-release matrix, with desirable properties, such as mucoadhesion and permeation enhancement to improve oral bioavailability of drug. Chitosan is a good candidate for site specific drug delivery[7]. Chitosan microspheres can be prepared by a number of techniques as discussed below.

METHODS OF CHITOSAN MICROSPHERES PREPARATION

Chitosan microspheres/nanoparticles are used to provide controlled release of many drugs and to improve the bioavailability of degradable substances such as proteins, as well as to improve the uptake of hydrophilic substances across the epithelial layers. Chitosan microspheres can be prepared by reacting chitosan with controlled amounts of multivalent anion resulting in cross-linking between chitosan molecules. This cross-linking can be achieved in acidic, neutral, or basic environments depending upon the methods applied. Chitosan microspheres/nanoparticles can be prepared by various methods such as cross-linking with anions, precipitation, complex-coacervation, modified emulsification and ionotropic gelation, precipitation-chemical cross-linking, glutaraldehyde cross-linking, thermal cross-linking etc[7,8]. Selection of any of the methods depends upon factors such as particle size requirement, thermal and chemical stability of the active drug molecule, reproducibility of the release kinetic profiles, stability of the final product and residual toxicity associated with the final product. Different methods used in the preparation of chitosan micro/nanoparticles and the characterization of those particles along with their role in the drug delivery system will be discussed in this review. However, selection of any particular method for preparing chitosan microspheres depends upon the nature of the active drug molecule that is to be encapsulated as well as the type of the delivery device[8].

Emulsion cross linking:

This method utilizes the reactive functional amine group of chitosan to cross-link with aldehyde groups of the cross-linking agent. In this method, water-in-oil (w/o) emulsion is prepared by emulsifying the aqueous solution of chitosan in the oil phase. Aqueous droplets are stabilized using a suitable surfactant. The stable emulsion, thus formed, is cross-linked by using an appropriate cross-linking agent such as glutaraldehyde to harden the droplets. Microspheres are filtered and washed repeatedly with n-hexane followed by alcohol and then dried. By this method, particle size of the microspheres can be controlled. The size of final product depends upon the extent of cross-linking agent used while hardening as well as the speed of stirring during the formation of emulsion. Thus chemical cross-linking method for preparation of chitosan microspheres involves emulsification followed by cross-linking with a suitable cross-linking agent (e.g. glutaraldehyde). The degree of stirring (i.e. time and speed of stirring during emulsification) determines the size of dispersed droplets. By varying any one or both of these parameters, the size of droplets can be changed to obtain the product (i.e. chitosan microspheres) in the desired size range. The emulsion cross-linking method suffers from few drawbacks since it is a tedious procedure and use of harsh cross-linking agents that can enter into chemical reactions with the active drug molecule of interest. Moreover, complete removal of the un-reacted cross-linking agent is difficult in this process. Cross-linking agents mostly used in this methods are glutaraldehyde, sulphuric acid, and heat treatment. Further preparation and performance of microspheres are affected by concentration of chitosan and the stabilizing solution. Emulsion cross-linking method has been used to prepare chitosan microspheres to encapsulate diclofenac sodium, phenobarbitone, nifedipine, theophyllin, griseofulvin, pentazocine, progesterone and a number of drugs of choice.

Thanoo *et al*.[9] prepared the chitosan microspheres by emulsion technique where cross-linking of chitosan solution in paraffin oil as an external medium with glutaraldehyde was done using dioctyl sulfosuccinate as the stabilizing agent. Addition of stabilizing agent during particle formation produced microspheres with spherical geometry and smooth surfaces. Encapsulation efficiencies up to 80% were achieved for theophylline, aspirin or griseofulvin. A 2.5% (w/v) chitosan solution in aqueous acetic acid was prepared. This dispersed phase was added to continuous phase (125 ml) consisting of light liquid paraffin and heavy liquid paraffin in the ratio of 1:1 containing 0.5% (w/v) span 85 to form a water in oil (w/o) emulsion. Stirring speed was maintained at 2000 rpm. A drop-by-drop solution of a measured quantity (2.5 ml each) of aqueous glutaraldehyde (25% v/v) was added at 15, 30, 45, and 60 min. Stirring was continued for 2.5 h to obtain microspheres, which were separated by filtration under vacuum and washed, first with petroleum ether (60-80°) and then with distilled water to remove the adhered liquid paraffin and glutaraldehyde, respectively. The volume of glutaraldehyde was varied to affect the cross-linking density. The microspheres were then finally dried in a vacuum desiccator.

Shiraishi *et al*.[10] reported the interaction of chitosan with tripolyphosphate (TPP). The microspheres were formed by dropping the bubble-free dispersion of chitosan through a disposable syringe (10 ml) onto a gently agitated (magnetic stirrer) 5% or 10% w/v TPP solution. The chitosan microspheres were separated after 2 h by filtration and rinsed with distilled water; then they were air dried.

Denkbas *et al*.[11] used the mixture of mineral oil/petroleum ether in the ratio of 60/40 (v/v) as the external medium to prepare chitosan microspheres using glutaraldehyde as a cross-linking agent and Tween-80 as an emulsifier. Smaller microspheres with narrow distributions were produced when chitosan/solvent ratio and drug/chitosan ratio were lower. 5-fluorouracil microspheres prepared by this method were loaded up to a concentration of 10.4 mg/g of chitosan. One hundred milliliters of paraffin oil was placed in a 250-ml plastic beaker. One milliliter of Tween 80 was mixed with the oil in the beaker by stirring. To this, 3 ml of chitosan solution of different concentrations (0.8%, 1.4%, 2% w/v), prepared by dissolving chitosan in 5% acetic acid, was added drop-wise using a 22-gauge hypodermic syringe. This addition was accompanied with stirring of paraffin oil at different speeds (2000 rpm, 3000 rpm, and 4000 rpm) with the help of a high-speed stirrer with propellers. Stirring was continued for 5 min after the complete addition of chitosan solution into oil. Later 0.25 ml of glutaraldehyde was added to the mixture with continuous stirring at the same speed. Stirring was continued at the same speed for next 5 min, and then stirring speed was reduced to 500 rpm. Glutaraldehyde (0.25 ml) was added twice to the mixture, once after 1 h and then after 2 h, respectively, with continuous stirring. Stirring was stopped after 1 h of the final addition of glutaraldehyde. Suspension of chitosan microspheres in paraffin oil thus obtained was allowed to stand for 1 h to let the microspheres settle down under gravity. Clear supernatant was decanted and microspheres obtained as residue were washed 4 times with solvent ether. After the final wash, microspheres were allowed to dry in air.

Jameela *et al*.[12] prepared smooth, highly spherical, cross-linked chitosan microspheres in the size range of 45–300µm for the controlled release of progesterone. An aqueous acetic acid dispersion of chitosan containing progesterone was emulsified in the dispersion medium consisting of liquid paraffin and petroleum ether stabilized by using sorbitan sesquioleate and the droplets were hardened by glutaraldehyde cross-linking. Extent of cross-linking showed a significant influence on drug release characteristics. Highly cross-linked microspheres released only about 35% of steroid in 40 days compared to 70% release from the lightly cross-linked microspheres. Evaluation of *in-vivo* bioavailability by intramuscular injection in rabbits showed that a plasma concentration of 1 to 2 ng/ml was maintained up to 5 months without showing any high burst release effect. The data suggests the usefulness of cross-linked chitosan microspheres as potential carriers for long-term delivery of steroids.

Al-Helw *et al*.[13] used Glutaraldehyde extracted in toluene as a cross-linking agent to prepare chitosan microspheres encapsulated with phenobarbitone. Uniform and spherical microspheres with loading efficiency up to 57.2% were produced. Loading efficiency was dependent upon the preparation conditions. Parameters affecting the preparation and performance of microspheres are molecular

weight and concentration of chitosan as well as concentration of the stabilizing agent. Particle size of the microspheres varied in the range 274–450 μm. Release rates of phenobarbitone from different formulations of microspheres showed high initial release (burst effect) of the drug and about 20–30% of the drug was released in the first hour. Release was faster from the small size microspheres, i.e., almost 75–95% of the drug was released within 3 h depending upon the molecular weight of chitosan.

Thermal cross linking method:

Orienti *et al*.[14] prepared the microspheres by using Citric acid, as a cross-linking agent. Citric acid was added to 30 ml of an aqueous acetic acid solution of chitosan (2.5% w/v) maintaining a constant molar ratio between chitosan and citric acid (6.90×10^{-3} mol chitosan: 1 mol citric acid). The chitosan cross-linker solution was cooled to 0° and then added to 25 ml of corn oil previously maintained at 0°, with stirring for 2 min. This emulsion was then added to 175 ml of corn oil maintained at 120°, and cross-linking was performed in a glass beaker under vigorous stirring (1000 rpm) for 40 min. The microspheres obtained were filtered and then washed with diethyl ether, dried, and sieved.

Ionotropic gelation method:

Owing to the tedious procedure and use of harsh cross-linking agents in emulsion cross-linking methods, use of complexation between oppositely charged macromolecules to prepare chitosan microspheres has attracted much attention because the process is very simple and mild. In addition, reversible physical cross-linking by electrostatic interaction, instead of chemical cross-linking, has been applied to avoid the possible toxicity of reagents and other undesirable effects. Tripolyphosphate (TPP) is a polyanion, which can interact with the cationic chitosan by electrostatic forces. In the ionic gelation method, chitosan is dissolved in aqueous acidic solution to obtain the cation of chitosan. This solution is then added drop-wise under constant stirring to polyanionic TPP solution. Due to the complexation between oppositely charged species, chitosan undergoes ionic gelation and precipitates to form spherical particles. However, TPP/chitosan micro-particles formed have poor mechanical strength thus, limiting their usage in drug delivery. Xu and Du[15] have studied different formulations of chitosan nanoparticles produced by the ionic gelation of TPP and CS. TEM indicated their diameter ranging between 20 and 200 nm with spherical shape. FTIR confirmed tripolyphosphoric groups of TPP linked with ammonium groups of chitosan in the nanoparticles. Factors that affect the delivery have been studied using bovine serum albumin (BSA) as a model protein. The factors include molecular weight and deacetylation degree of chitosan, concentrations of chitosan and BSA, as well as the presence of polyethylene glycol (PEG) in the encapsulation medium. With the increasing molecular weight of chitosan from 10 to 210 kDa, BSA encapsulation efficiency was enhanced nearly twice. The total release of BSA in phosphate buffered saline pH 7.4 in 8 days was reduced from 73.9 to 17.6%. Increasing deacetylation degree from 75.5 to 92% promoted the encapsulation efficiency with a decrease in release rate. Encapsulation efficiency decreased greatly with the increase in the initial concentration of BSA and chitosan. Higher loading capacity of BSA enhanced the BSA release from nanoparticles. However, adding PEG hindered the BSA encapsulation and increased the release rate.

A modified ionotropic gelation method is reported which makes use of a high voltage electrostatic field to prepare protein-loaded chitosan microspheres using bovine serum albumin (BSA) as a model protein[16]. Microspheres prepared by this technique exhibited good sphericity and dispersity when the mixture of sodium tripolyphosphate (TPP) and ethanol was applied as coagulation solution. Higher encapsulation efficiency (>90%) was achieved for the weight ratio of BSA to chitosan below 5%. 35% of BSA was released from the microspheres cured in 3% coagulation solution, and more than 50% of BSA was released from the microspheres cured in 1% coagulation solution at pH 8.8. However, only 15% of BSA was released from the microspheres cured in 1% coagulation solution at pH 4. The results suggested that ionotropic gelation method combined with a high voltage electrostatic field will be an effective method for fabricating chitosan microspheres for sustained delivery of protein. It is also reported that chitosan microspheres prepared by tripolyphosphate cross-linking and emulsification ionotropic gelation can be used as an excellent mucoadhesive delivery system.

Insulin-loaded chitosan nanoparticles have been prepared by mixing insulin with TPP solution and

then adding this to chitosan solution under constant stirring. Two types of chitosan in the form of hydrochloride salt (SeacureR 210 Cl and ProtasanR 110 Cl), varying in their molecular weight and degree of deacetylation, were utilized for nanoparticle preparation. For both types of chitosan, TPP concentration was adjusted to get a chitosan/TPP ratio of 6:1. Chitosan nanoparticles thus obtained were in the size range of 300–400 nm with a positive surface charge ranging from +54 to +25 mV. Using this method, insulin loading was modulated reaching the values up to 55%. Efficiency of the method was dependent upon the deacetylation of chitosan, since it involves the gelation of protonated amino groups of chitosan.

Pan *et al*.[17] prepared the insulin-loaded chitosan nanoparticles by ionotropic gelation of chitosan with TPP anions. Particle size distribution and zeta potential were determined by photon correlation spectroscopy. The ability of chitosan nanoparticles to enhance the intestinal absorption of insulin and the relative pharmacological bioavailability of insulin was investigated by monitoring the plasma glucose level of alloxan-induced diabetic rats after the oral administration of various doses of insulin-loaded chitosan nanoparticles. The positively charged, stable chitosan nanoparticles showed particle size in the range of 250-400 nm. Insulin association was up to 80%. *In vitro* release experiments indicated initial burst effect, which is pH-sensitive. Chitosan nanoparticles enhanced the intestinal absorption of insulin to a greater extent than the aqueous solution of chitosan *in vivo*. After administration of 21 iu/kg insulin in the chitosan nanoparticles, hypoglycemia was prolonged over 15 h. The average pharmacological bioavailability relative to subcutaneous injection (SC) of insulin solution was up to 14.9%.

Coacervation/precipitation method:

This method utilizes the physicochemical property of chitosan. Since it is insoluble in alkaline pH medium, but precipitates/coacervates when it comes in contact with alkaline solution. Particles are produced by blowing chitosan solution into an alkali solution like sodium hydroxide, NaOH-methanol or ethanediamine using a compressed air nozzle to form coacervate droplets. Further particles were separated by filtration/centrifugation followed by successive washing with hot and cold water. Varying compressed air pressure or spray-nozzle diameter controlled the size of the particles and then using a cross-linking agent to harden particles can control the drug release.

Preparations of chitosan–DNA nanoparticles using the complex coacervation technique have been reported. Important parameters such as concentrations of DNA, chitosan, sodium sulfate, temperature, pH of the buffer and molecular weights of chitosan and DNA have been investigated. At the amino to phosphate group ratio between 3 and 8 and chitosan concentration of 100 μg/ml the particle size was optimized to 100–250 nm with a narrow distribution. Surface charge of these particles was slightly positive with a zeta potential of 112 to 118 mV at pH lower than 6.0, and became nearly neutral at pH 7.2. The chitosan–DNA nanoparticles could partially protect the encapsulated plasmid DNA from nuclease degradation.

In another technique, sodium sulfate solution was added drop-wise to an aqueous acidic solution of chitosan containing a surfactant subjected to stirring and ultrasonication for 30 min[18]. Microspheres prepared were purified by centrifugation and re-suspended in demineralized water. Particles were cross-linked with glutaraldehyde. Particles produced by this method have better acid stability than observed by other methods.

Chitosan microspheres loaded with recombinant human interleukin-2 (rIL-2) have been prepared by dropping of rIL-2 with sodium sulfate solution in acidic chitosan solution. When protein and sodium sulfate solutions were added to chitosan solution and during the precipitation of chitosan, the protein was incorporated into microspheres. This method is devoid of cross-linking agent[19].

Emulsion-droplet coalescence method:

The novel emulsion-droplet coalescence method was developed by Tokumitsu *et al*.[20] which utilizes the principles of both emulsion cross-linking and precipitation. However, in this method, instead of cross-linking the stable droplets, precipitation is induced by allowing coalescence of chitosan droplets with NaOH droplets. First, a stable emulsion containing aqueous solution of chitosan along with drug is produced in liquid paraffin oil and then, another stable emulsion containing chitosan aqueous solution of NaOH is produced in the same manner. When both emulsions are mixed under high-speed

stirring, droplets of each emulsion would collide at random and coalesce, thereby precipitating chitosan droplets to give small size particles. Gadopentetic acid-loaded chitosan nanoparticles have been prepared by this method for gadolinium neutron-capture therapy. Since gadopentetic acid is a bivalent anionic compound, it interacts electrostatically with the amino groups of chitosan, which would not have occurred if a cross-linking agent is used that blocks the free amino groups of chitosan. Particles produced using 100% deacetylated chitosan had the mean particle size of 452 nm with 45% drug loading. Nanoparticles were obtained within the emulsion-droplet. Thus, it was possible to achieve higher gadopentetic acid loading by using the emulsion-droplet coalescence method compared to the simple emulsion cross-linking method.

Spray drying technique:

In this method, chitosan is first dissolved in aqueous acetic acid solution, drug is then dissolved or dispersed in the solution and then, a suitable cross-linking agent is added. This solution or dispersion is then atomized in a stream of hot air. Atomization leads to the formation of small droplets, from which solvent evaporates instantaneously leading to the formation of free flowing particles. Various process parameters are to be controlled to get the desired size of particles. Particle size depends upon the size of nozzle, spray flow rate, atomization pressure, inlet air temperature and extent of cross-linking.

He *et al*.[21] prepared both un-crosslinked and cross linked chitosan microparticles by spray-drying method for the delivery of cimetidine, famotidine and nizatidine. Conti *et al*.[22] produced Cetylpyridinium chloride, an antiinfective agent chitosan microspheres by spray-drying technique exposing the spray-dried particles to vapors containing cross-linking agents. Extent of cross-linking was controlled by the time of exposure to cross-linking agent.

Ganza-Gonzalez *et al*.[23] have demonstrated spray-drying technique as fast, simple and reliable to obtain microspheres. Microspheres were prepared by spray drying of aqueous chitosan dispersions containing metoclopramide hydrochloride using different amounts of formaldehyde as a cross-linker. Microspheres released the drug for more than 8 h, independent of the pH of the medium.

Lorenzo-Lamosa *et al*.[24] prepared the micro-encapsulated chitosan microspheres for colonic delivery of sodium diclofenac. Sodium diclofenac was entrapped into chitosan microcores by spray-drying and then, micro-encapsulated into Eudragit R L-100 and Eudragit R S-100 using an oil-in-oil solvent evaporation method. By spray-drying, chitosan microspheres of 1.8–2.9 μm sizes were prepared and efficiently microencapsulated into Eudragit R microspheres ranging in size between 152 and 223 μm to form the multi-reservoir system. Numbers of variables such as type and concentration of chitosan, the core/coat ratio and the type of enteric polymer have been investigated to optimize the microspheres properties.

Huang *et al*.[25] prepared chitosan microspheres by the spray-drying method using type-A gelatin and ethylene oxide–propylene oxide block copolymer as modifiers. Surface morphology and surface charges of the prepared microspheres were investigated using SEM and microelectrophoresis. Shape, size and surface morphology of the microspheres were significantly influenced by the concentration of gelatin. Betamethasone disodium phosphate-loaded microspheres demonstrated a good drug stability (less 1% hydrolysis product), high entrapment efficiency (95%) and positive surface charge (37.5 mV).

In another study vitamin D_2, also called ergocalciferol, was efficiently encapsulated into chitosan microspheres prepared by spray-drying method. The microencapsulated product was coated with ethyl cellulose. The sustained release property of VD2 microspheres was used for the treatment of prostatic diseases. Spray-drying method was also used to prepare ampicillin-loaded methylpyrrolidone chitosan microspheres by taking different drug-to-polymer weight ratios.

Reverse micellar method:

In this method, the surfactant is dissolved in an organic solvent to prepare reverse micelles. Reverse micelles are thermodynamically stable liquid mixtures of water, oil and surfactant. To this, aqueous solutions of chitosan and drug are added with constant vortexing to avoid any turbidity. The aqueous phase is regulated in such a way so as to keep the entire mixture in an optically transparent microemulsion phase. Additional amount of water may be added to obtain nanoparticles of larger size. To this transparent solution, a cross-linking agent is added with constant

stirring, and cross-linking is achieved by stirring overnight. The maximum amount of drug that can be dissolved in reverse micelles varies from drug to drug and has to be determined by gradually increasing the amount of drug until the clear microemulsion is transformed into a translucent solution. The organic solvent is then evaporated to obtain the transparent dry mass. The material is dispersed in water and then adding a suitable salt precipitates the surfactant out. The mixture is then subjected to centrifugation. The supernatant solution is decanted, which contains the drug-loaded nanoparticles. The aqueous dispersion is immediately dialyzed through dialysis membrane for about 1 h and the liquid is lyophilized to dry powder.

Mitra *et al*.[26] have encapsulated doxorubicin–dextran conjugate in chitosan nanoparticles prepared by reverse micellar method. The surfactant sodium bis(2-ethylhexyl)sulfosuccinat was dissolved in n-hexane. To 40 ml of surfactant solution (0.03 M), 100 µl of 0.1% chitosan solution in acetic acid, 200 µl doxorubicin–dextran conjugate (6.6 mg/ml), 10 µl liquor ammonia and 10 µl of 0.01% glutaraldehyde solution were added with continuous stirring at room temperature. This procedure produced chitosan nanoparticles encapsulating doxorubicin–dextran conjugate. Solvent was removed by rotary evaporator and the dry mass was re-suspended in 5 ml of pH 7.4 Tris–HCl buffer by sonication. To this, 1 ml of 30% $CaCl_2$ solution was added drop-wise to precipitate the surfactant as calcium salt of diethylhexyl sulfosuccinate. The precipitate was pelleted by centrifugation at 5,000 rpm for 30 min at 48º. The pellet was discarded and the supernatant containing nanoparticles was centrifuged at 60,000 rpm for 2 h to pellet the nanoparticles. The pellet was dispersed in 5 ml of pH 7.4 Tris–HCl buffer.

Sieving method:

Agnihotri and Aminabhavi[27] have developed a simple, yet novel method to produce chitosan microparticles containing the drug clozapine. In this method, a suitable quantity of chitosan was dissolved in 4% acetic acid solution to form a thick jelly mass that was cross-linked by adding glutaraldehyde. The non-sticky cross-linked mass was passed through a sieve with a suitable mesh size to get micro particles. The micro particles were washed with 0.1N NaOH solutions to remove the un-reacted excess glutaraldehyde and dried overnight in an oven at 40°. Clozapine was incorporated into chitosan before cross-linking with entrapment efficiency up to 98.9%. This method is devoid of tedious procedures, and can be scaled up easily. However microparticles produced were irregular in shape, with the average particle sizes in the range 543–698 µm. The *in vitro* release was extended up to 12 h, while the *in vivo* studies indicated a slow release of clozapine.

CHARACTERIZATION OF CHITOSAN MICROSPHERES

The chitosan beads prepared by the different techniques discussed above will be characterized for their morphology like size, size distribution and shape analysis using optical microscopy, SEM and particle size analysis. Swelling kinetics can be studied in different physiological conditions. Once the technique is well standardized, drug is to be incorporated into chitosan as a function of concentration at different pH. Drug loading in micro/nanoparticulate systems can be done by two methods, i.e., during the preparation of particles (incorporation) or after the formation of particles (incubation). In these systems, drug is physically embedded into the matrix or adsorbed onto the surface. Various methods of loading have been developed to improve the efficiency of loading, which largely depends upon the method of preparation as well as physicochemical properties of the drug. Maximum drug loading can be achieved by incorporating the drug during the formation of particles, but it may get affected by process parameters such as method of preparation, presence of additives, etc. Both water-soluble and water-insoluble drugs can be loaded into chitosan-based particulate systems. Water-soluble drugs are mixed with chitosan solution to form a homogeneous mixture, and then, particles can be produced by any of the methods discussed before. Water-insoluble drugs and drugs that can precipitate in acidic pH solutions can be loaded after the formation of particles by soaking the preformed particles with the saturated solution of drug. Water-insoluble drugs can also be loaded using the multiple emulsion technique. In this method, drug is dissolved in a suitable solvent and then emulsified in chitosan solution to form an oil-in-water (o/w) type emulsion. Sometimes, drug can be dispersed into chitosan solution by using a surfactant to get the suspension. Thus, prepared o/w emulsion or suspension can be further emulsified into liquid paraffin to get the oil-water-oil (o/w/o) multiple

emulsions. The resulting droplets can be hardened by using a suitable cross-linking agent.

Morphological study of microspheres:
Photomicrographs of the unloaded chitosan microspheres can be obtained using a digital optical microscope. Microspheres were characterized in terms of sphericity and clumping of microspheres, as observed from the photomicrograph.

Determination of mean particle size and particle size distribution:
Particle size analysis of unloaded and drug-loaded chitosan microspheres can be performed by optical microscopy using a compound microscope[28]. A small amount of dry microspheres was suspended in purified water (10 ml). The suspension was ultrasonicated for 5 seconds. A small drop of suspension thus obtained was placed on a clean glass slide. The slide containing chitosan microspheres was mounted on the stage of the microscope and Ferret's diameter of at least 300 particles was measured using a calibrated ocular micrometer.

Determination of percentage drug entrapment:
Efficiency of drug entrapment for each batch can be calculated in terms of percentage drug entrapment (PDE) as per the following formula: PDE=(Practical drug loading/theoretical drug loading)×100.

Theoretical drug loading was determined by calculation assuming that the entire drug present in the chitosan solution used gets entrapped in microspheres and no loss occurs at any stage of preparation of microspheres.

Determination of Practical drug loading can be done by taking a weighed quantity of chitosan microspheres (approximately 25 mg) in a 25-ml volumetric flask. Sufficient quantity of methanol is to be added to make the volume 25 ml. After shaking the suspension vigorously it was left for 24 h at room temperature with intermittent shaking. Supernatant was collected by centrifugation and drug content in supernatant was determined by UV spectrophotometer at suitable wavelength.

Determination of uniformity index:
Uniformity index can be determined by the formula- $UI=D_w/D_n$, where D_w and D_n are weight average diameter and number average diameter, respectively, and can be calculated as follows: $D_w=\sum Ni\ Di^4/\sum Ni\ Di^3$, $D_n=\sum Ni\ Di/\sum Ni$, where Ni is the number of particles with *Di* diameter. As per Shukla *et al*.[29] values of UI ranging from 1.0 to 1.1 and 1.1 to 1.2 indicate monodisperse and nearly monodispersed particles.

Scanning electron microscopy:
A monolayer of dry microspheres was mounted on an aluminium slab using double-sided carbon tape. The sample was coated with a 10 nm thick gold film using a sputter coater. Coated samples were examined using an electron acceleration voltage of 20 KeV. Size distribution and average particle diameter can be determined analyzing 5 to 10 images.

DRUG RELEASE KINETICS OF CHITOSAN MICROSPHERES

Drug release from chitosan-based particulate systems depends upon the extent of cross–linking, morphology, size and density of the particulate system, physicochemical properties of the drug as well as the presence of adjuvant. *In vitro* release also depends upon pH, polarity and presence of enzymes in the dissolution media[30]. The release of drug from chitosan particulate systems involves three different mechanisms: (a) drug release from the particle surface (b) diffusion of drug molecules through the swollen rubbery matrix and (c) release of drug due to the erosion of polymer. In case of release from the surface, adsorbed drug instantaneously dissolves when it comes in contact with the release medium. Drug entrapped in the surface layer of particles also follows this mechanism. Such type of drug release leads to burst effect. He *et al*.[21] observed that cimetidine-loaded chitosan microspheres have shown burst effect in the early stages of dissolution. Most of the drugs were released within few minutes when microparticles were prepared by spray drying technique. Increasing the cross-linking density can prevent the burst release.

Drug release by diffusion involves three steps. First, penetration of water into particulate system, which causes swelling of the matrix; secondly, the conversion of glassy polymer into rubbery matrix and the third step is the diffusion of drug from the swollen rubbery matrix. Hence, the release is slow initially and later, it becomes fast. This type of release is more prominent in case of hydrogels.

Kweon and Kang[31] prepared the chitosan–poly vinyl alcohol (PVA) matrix to study the release of prednisolone under various conditions. Relationship between the amount of drug release and square root of time was linear indicating the diffusion-controlled release. Drug release was controlled by the extent of PVA grafting, heat treatment or cross-link density, but it was less affected by the pH when compared to plain chitosan. Ganza-Gonzalez *et al*.[23] analyzed the drug release data using Higuchi equation which was used to describe the release of a solute from a flat surface, and the good fit obtained suggests that the release rate depends upon the rate of diffusion through the cross-linked matrix.

Agnihotri and Aminabhavi[27] analyzed the dynamic swelling data of chitosan microparticles using to predict drug release from the water uptake data of the microparticles cross-linked with glutaraldehyde. It was observed that as the cross-linking increases, swelling of chitosan microparticles decreases.

Jameela *et al*.[12] have obtained a good correlation fit for the cumulative drug released vs. square root of time, demonstrating that the drug release from the microsphere matrix is diffusion-controlled and obeys Higuchi equation[32]. It was demonstrated that the rate of release depends upon the size of microspheres. Orienti *et al*.[14] studied the correlation between matrix erosion and release kinetics of indomethacin-loaded chitosan microspheres. Release kinetics was correlated with the concentration of chitosan in the microsphere and pH of the release medium. At high concentrations of chitosan and at pH 7.4, deviations from Fick's law to zero order kinetics have been observed. Nam and Park have demonostrated the *in vitro* release test of drug loaded chitosan microspheres[30].

Weighed quantities of drug loaded microspheres were dispersed in 30 ml of normal saline phosphate buffer (pH 7.4) in a conical flask, mouth of the flask plugged with cotton. The system was kept in an incubator at 37°. Three milliliters of the dispersion medium was drawn after definite time intervals and was replaced with 3 ml of dissolution media. The drawn sample was filtered using Whatman filter paper (grade 2, Whatman, Kent, UK). The residue was returned to the suspension. The clear filtrate was subjected to UV spectrophotometry (after dilution, to prepare definite concentrations) for determination of drug content.

APPLICATIONS

Chitosan microspheres have several applications in novel drug delivery systems. Some of which are mentioned below.

GI-delivery-systems:
Floating systems have a density lower than the density of the gastric juice. Thus, the gastric residence time and hence the bioavailability of drugs that are absorbed in the upper part of the GI-tract will be improved. Both chitosan granules and chitosan-laminated preparations could be helpful in developing drug delivery systems that will reduce the effect of gastrointestinal transit time. Floating hollow microcapsules of melatonin produced have an interesting gastro retentive controlled-release delivery system for drugs.

Colon and intestinal drug delivery:
Since, chitosan is degraded by the microflora that are available in the colon it was found to be promising for colon-specific drug delivery. Chitosan esters, such as chitosan succinate and chitosan phthalate have been used successfully as potential matrices for the colon-specific oral delivery of diclofenac sodium. Systems for colon delivery containing paracetamol, mesalazine, and insulin have been studied and give satisfactory results. Sustained intestinal delivery of drugs, such as 5-fluorouracil (choice for colon carcinomas) and insulin (for diabetes mellitus) seems to be a feasible alternative to injection therapy. A formulation was developed that could bypass the acidity of the stomach and release the loaded drug for long periods into the intestine by using the bioadhesiveness of polyacrylic acid, alginate, and chitosan tract[33-35].

Opthalmic drug delivery:
Chitosan is found to be a unique material for designing ocular drug delivery vehicles. Due to its elasticity ophthalmic chitosan gels have shown excellent adhesion to mucin, which coats the conjunctiva and the corneal surface of the eye.

Chitosan based colloidal systems were found to work as transmucosal drug carriers, facilitating the transport of drugs to the inner eye (chitosan-coated colloidal system containing indomethacin) or their accumulation into the corneal/conjunctival epithelia (chitosan nanoparticulate containing cyclosporin). The microparticulate drug carrier (microspheres) seems a

promising means of topical administration of acyclovir to the eye[36].

Oral, buccal and sublingual drug delivery:

Being a muco/bioadhesive polymer, chitosan is considered a good candidate for oral cavity drug delivery[23]. Chitosan is a biologically safe polymer and prolongs the adhesion time of oral gels and drug release from them. Chitosan also inhibits the adhesion of *Candida albicans* to human buccal cells and has antifungal activity. Chitosan containing quick-hardening paste was developed as a bone substitute for dental purpose. The use of this paste will minimize the inflammation in gums. Chitosan gel and chitosan film containing chlorhexidine gluconate for local delivery were developed. A monolayer and multilayered film of chitosan PLGA containing ipriflavone were showed to prolong drug release for 20 days *in vitro*[37].

Buccal tablets based on chitosan micropsheres containing chlorhexidine diacetate showed a prolonged release of the drug in the buccal cavity. The loading of chlorhexidine into chitosan was to improve the antimicrobial activity of the drug. Bioadhesive tablets of nicotine containing 0% to 50% w/w glycol chitosan give acceptable adhesion. Mucoadhesion developed by a chitosan hydrogel appears to be suitable for prolonging the residence time of the drug and improving the therapeutic effect.

The buccal bilayered devices (bilaminated film, bilayered tablets) using a mixture of drugs and chitosan, with and without anionic cross-linking polymers demonstrated that these devices show promising potential for use in controlled delivery of drugs to the oral cavity.

Nasal and transdermal drug delivery:

Various chitosan salts (chitosan lactate, chitosan aspartate, chitosan glutamate, and chitosan hydrochloride) showed nasal sustained release of vincomycin hydrochloride. Bioadhesive chitosan microspheres of pentazocine for intranasal systemic delivery showed improved bioavailability with sustained and controlled blood level profiles in comparison to intravenous or oral administration. Diphtheriatoxoid (DT) associated to chitosan microparticles results in systemic protection and local immune response against DT, and enhances significant IgG production after nasal administration. Different types of nasal vaccine systems include cholera toxin, microspheres, nanoparticles, liposomes, attenuated virus and cells, and outer membrane proteins (proteosomes). Nasal formulations induced significant serum IgG responses similar to that induced by a parenteral administration of the vaccine[3].

Chitosan-alginate poly electrolyte complex (PEC) has been prepared as *in situ* in beads and microspheres for potential applications in transdermal controlled release systems, and wound dressings[22]. Chitosan gel was used as the drug-reservoir. The drug-release profiles showed that drug delivery is completely controlled by the devices. The rate of drug release was found to be dependent on the type of membrane used. A combination of chitosan membrane and chitosan hydrogel containing lidocaine HCl, a local anesthetic, is a good transdermal system for controlled drug delivery. Chitosan gel beads containing the antiinflammatory drug prednisolone showed sustained release of drug with reduced inflammation and resulted in improved therapeutic efficacy[38].

Vaginal drug delivery:

Chitosan vaginal tablet containing metronidazole, acriflavine, and other drugs gave adequate release, therapeutic action, and good adhesion properties. By introducing thiol groups, the mucoadhesive properties of the polymer were strongly improved, and this resulted in an increased residence time of the drug in the vaginal mucosa tissue ensuring good controlled drug release in the treatment of mycotic infections[3].

Vaccine delivery system:

Various chitosan-antigen nasal vaccine have been developed whose responses were found to be same or superior when compared with the parenteral administration of the vaccine. Bovine serum albumin and diptheria toxoid loaded chitosan microspheres showed prolonged drug release action *in vivo*[3].

Gene delivery system:

Many hereditary disorders, multi genetic diseases can be treated by genetic delivery. Gene delivery systems include viral vectors, cationic liposomes, polycation complexes, and microencapsulated systems. Chitosan has been used as a carrier of DNA for gene delivery applications[39,40]. Also, chitosan could be a useful oral gene carrier because of its adhesive and transport properties in the GI tract. MacLaughlin *et al*.[40] showed that plasmid DNA containing cytomegalo virus promoter sequence and a luciferase reporter

gene could be delivered *in vivo* by chitosan and depolymerized chitosan oligomers to express a luciferase gene in the intestinal tract.

CONCLUSION

Chitosan, a natural biopolymer of crustacean origin exhibiting a wide variety of physicochemical and biological properties has numerous applications in the fields of agriculture, textile, nutritional enhancement and food processing, waste water management, cosmetics, drug delivery and many other medical and pharmaceutical applications. Being characterized by biocompatibility, non toxicity, lack of allergenicity, biodegradability chitosan is really an attractive biopolymer for delivering a wide variety of drugs in a controlled/sustained manner and can be successfully targeted for site specific drug delivery. Problems asociated with dose dumping, burst out effect, unavoidable fluctuations in drug concentrations (mostly associated with conventional dosage form) can be eliminated/reduced by use of such biopolymers resulting in enhanced efficacy and lesser incidences of adverse effects associated with the drugs.

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