Development of a New Drug Carrier Made from Alginate

M. RAJANARIVONY, C. VAUTHIER, G. COUARRAZE, F. PUSIEUX, AND P. COUVREUR

Received May 15, 1992, from Laboratoire de Physico-chimie, Biopharmacie, Pharmacootechnie, URA CNRS 1218, Université de Paris XI, Faculté de Pharmacie, 92296 Chatenay-Malabry, France. Accepted for publication December 23, 1992.

Abstract A new approach for the preparation of nanoparticles is presented. The method is based on control of the gelification phenomenon of alginate by calcium ions, and it leads to small particles of a wide range of very well-defined sizes (250–850 nm) depending on the alginate concentration. The particles are formed in a sodium alginate solution by addition of calcium chloride and then poly-L-lysine. The concentrations of sodium alginate and of calcium chloride were lower than those required for gel formation and corresponded to the formation of a pregel state. The size of the particles formed is greatly dependent on the order of addition of calcium and poly-L-lysine to the sodium alginate solution. This phenomenon can be attributed to the difference in the nature of the interactions between calcium and alginate and between poly-L-lysine and alginate. Furthermore, the data indicate that the formation of the particles probably occurs during the addition of the first component to the sodium alginate solution. Evaluation of the drug-loading capacity was done with doxorubicin as a drug model. The results indicate that alginate nanoparticles are interesting carriers because the drug-loading capacity could be >50 mg of doxorubicin per 100 mg of alginate.

Most of the colloidal particles used as drug carriers are rapidly taken up from the blood flow by the mononuclear phagocytes system (MPS), especially by the Kupffer cells in the liver. This phenomenon is of great interest for targeting a drug to organs of the MPS. In fact, spectacular results were recently reported with doxorubicin-loaded nanoparticles to treat in vivo experimental liver metastasis induced in mice. Interesting results were also reported with ampicillin, an antibiotic that is generally inefficient towards intracellular infections. On the contrary, rapid capture by the MPS becomes a drawback to gain access to other target sites in the body, to keep the drug carrier in the blood flow for a prolonged time, or to control the release of a drug in this compartment.

To avoid the rapid clearance of colloidal particles from the blood flow by the MPS, some authors have modified the surface properties of nanoparticles or liposomes to render them more hydrophilic. This approach consists of the adsorption of hydrophilic copolymers onto nanoparticle surfaces or the incorporation of sialic acid-rich gangliosides or polyethyleneglycol fatty acid derivatives into liposomes. As a consequence of those modifications, liposomes as well as nonbiodegradable polymeric nanoparticles are generally less recognized by the MPS and present a prolonged life time in the blood flow. However, the tissue distribution profile of biodegradable polyalkylcyanoacrylate nanoparticles remained unchanged even after they were coated with hydrophilic copolymers. This could be due to the fact that the stability of the copolymer adsorption layer onto the surface of biodegradable nanoparticles is still undefined, and desorption can occur when such particles are introduced into biological fluids. Thus, desorption of adsorbed hydrophilic copolymers can be the reason for the rapid clearance observed with biodegradable nanoparticles. With liposomes, a prolonged blood half-life was shown with radiolabeled material. The stability of liposomes after introduction into the body being still under discussion, the integrity of the radiolabeled phospholipidic vesicles has not yet been demonstrated. In light of these problems, this paper proposes a new concept to obtain “stealth” colloidal particles, directly prepared from a hydrophilic polymer, to overcome the problem of desorption of the coating layer. This concept has been developed with sodium alginate as a hydrophilic polymer model (sodium alginate was previously described as having satisfactory hemocompatibility). In fact, repeated systemic administration of alginate solutions did not cause any adverse immunoglobulin G (IgG) and IgM humoral response. Also, alginites were not observed to accumulate in any of the major organs, and some evidence for the polymeric degradation was even described. The preparation of alginate nanoparticles described here is based both on gel properties of this polysaccharide and on its ability to form stable polyelectrolyte complexes in the presence of polypeptides. A mechanism is proposed for the formation of these nanoparticles that is quite different from the one proposed by Davison et al. that consists of water-soluble nonstochiometric polyelectrolyte complex structures made from poly(methacrylic acid) and quaternized poly(vinyl pyridine). In fact, those complexes were only obtained through a self-assembly principle based on structural complementarity. In this paper, the evaluation of the drug-loading capacity has also been examined with doxorubicin, which was chosen as a drug model because, according to in vitro release profiles, it was reported by Bhakoo et al. that interactions may exist between alginate gels and doxorubicin.

Experimental Section

Chemicals—Sodium alginate (75 000–100 000), extracted from Macrocystis pyrifera, was purchased from Sigma Chemical (St. Louis, MO); it contained 61% mannanuronic acid and 39% guluronic acid. Poly-L-lysine, HBr (1000–4000) was a commercial sample provided by Sigma. Calcium chloride was purchased from ProLabo as the hydrated crystalline form. All those chemicals were used without further purification. Aqueous solutions were prepared with distilled water.

Conditions for Nanoparticles Formation—Alginate nanoparticles were obtained by inducing the gelification of a sodium alginate solution with calcium chloride. Poly-L-lysine was then added to make a polyelectrolyte complex. The scheme for nanoparticle preparation is shown in Figure 1. Various volumes (0.5–5 mL) of a calcium chloride solution (18 mM) (Ca_{\text{Initial Conc.}}) were added to 5 to 9.5 mL of a sodium alginate solution containing various concentrations of alginate (Alg). The suspension was 6.8. However, the tissue distribution profile of biodegradable polyalkylcyanoacrylate nanoparticles remained unchanged even after they were coated with hydrophilic copolymers. This could be due to the fact that the stability of the copolymer adsorption layer onto the surface of biodegradable nanoparticles is still undefined, and desorption can occur when such particles are introduced into biological fluids. Thus, desorption of adsorbed hydrophilic copolymers can be the reason for the rapid clearance observed with biodegradable nanoparticles. With liposomes, a prolonged blood half-life was shown with radiolabeled material. The stability of liposomes after introduction into the body being still under discussion, the integrity of the radiolabeled phospholipidic vesicles has not yet been demonstrated. In light of these problems, this paper proposes a new concept to obtain “stealth” colloidal particles, directly prepared from a hydrophilic polymer, to overcome the problem of desorption of the coating layer. This concept has been developed with sodium alginate as a hydrophilic polymer model (sodium alginate was previously described as having satisfactory hemocompatibility). In fact, repeated systemic administration of alginate solutions did not cause any adverse immunoglobulin G (IgG) and IgM humoral response. Also, alginites were not observed to accumulate in any of the major organs, and some evidence for the polymeric degradation was even described. The preparation of alginate nanoparticles described here is based both on gel properties of this polysaccharide and on its ability to form stable polyelectrolyte complexes in the presence of polypeptides. A mechanism is proposed for the formation of these nanoparticles that is quite different from the one proposed by Davison et al. that consists of water-soluble nonstochiometric polyelectrolyte complex structures made from poly(methacrylic acid) and quaternized poly(vinyl pyridine). In fact, those complexes were only obtained through a self-assembly principle based on structural complementarity. In this paper, the evaluation of the drug-loading capacity has also been examined with doxorubicin, which was chosen as a drug model because, according to in vitro release profiles, it was reported by Bhakoo et al. that interactions may exist between alginate gels and doxorubicin.
Figure 1—Nanoparticle preparation process.

of doxorubicin. Then, 2 mL of poly-L-lysine aqueous solution (0.05%) were added to the system, giving a final concentration of alginate (Alg_{alg. sys. Conc.}) equal to 0.06%. The final concentration of doxorubicin (Dox_{final sys. Conc.}) ranged from 0.05 to 0.4 mg/mL. The suspension of alginate nanoparticles loaded with doxorubicin was then stirred for 30 min and kept at room temperature overnight. The same procedure was followed for preparing nanoparticles without drug.

Characterization of the Alginate Systems—Rheological Measurements—The rheological behavior of the alginate solutions and suspensions was determined with a cone and plate rheometer CLS 100 (CARRI-MED, U.K.) at 25 °C. The samples were placed between the plate and the cone to measure the shear stress as a function of the shear rate. The geometric characteristics of the cone-plate were as follows: diameter, 6 cm; angle, 2°/2.

Viscometric Measurements—Viscometric measurements were also performed with an Ubbelohde capillary viscometer on the alginate systems displaying a Newtonian behavior at 25 °C. From those experiments, reduced viscosity (η_red) was determined. This parameter was calculated from eq 1:

\[ \eta_{\text{red}} = \frac{\eta - \eta_0}{\eta_0 C} \]  

In eq 1, \( \eta_0 \) is the viscosity of the solvent, \( \eta \) is the value of the viscosity obtained from the viscometer, and \( C \) is the concentration of alginate in the dispersion. The following systems were studied: 1 or 0.12% (wt/vol) sodium alginate solutions (Alg_{alg. sys. Conc.}) gelled with calcium chloride solutions to a final calcium concentration (Ca_{alg. sys. Conc.}) ranging from 0.9 to 2.7 mM. Results are expressed as percentages of the viscosity measured for a pure alginate solution at the same concentration (0.5 and 0.06%, wt/vol, Alg_{alg. sys. Conc.}). Then, different dilutions of those preparations with distilled water (1/10, 1/5, 3/10, 2/5, 3/5, 4/5) were also used for viscometric measurements.

Size of Alginate Nanoparticles—The apparent hydrodynamic diameter of the nanoparticles was determined without dilution by quasi-elastic light scattering with a Nanosizer N4 MD (Coultertronics, Margency, France).

Evaluation of Doxorubicin Loading Capacity of the Nanoparticles—Doxorubicin-loaded nanoparticles were separated from the aqueous suspension medium by ultracentrifugation at 35 000 rpm for 1 h (Beckman L7-55-Rotor 70Ti-USA). The amount of free drug remaining unfixed to the nanoparticles (Cs) was measured in the clear supernatant by HPLC with a column packed with nucleosil C18 (Novenpak, Waters, St. Quentin-en-Yvelines, France). The mobile phase consisted of methanol-acetate buffer (0.01 M) and concentrated acetic acid (60:39:1). Spectrofluorimetric detection (247–550 nm) was used (Millipore, Waters 470).

The drug loading capacity onto nanoparticles (L) was calculated from eq 2:

\[ L (\%w/w) = \frac{Dox_{\text{final sys. Conc.}} - Cs}{Alg_{\text{final sys. Conc.}}} \times 100 \]  

In eq 2, Dox_{final sys. Conc.} is the total concentration of doxorubicin in the suspension (bound + free), Cs is the concentration of doxorubicin in the supernatant (free), and Alg_{final sys. Conc.} is the polymer concentration (0.5 mg/mL).

The association capacity of the drug (R) with respect to initial drug concentration was expressed as a percentage:

\[ R (\%) = \frac{Dox_{\text{final sys. Conc.}} - Cs}{Dox_{\text{final sys. Conc.}}} \times 100 \]  

Results and Discussion

Conditions for Nanoparticle Formation—Preliminary Experiments—Gelification of alginate was induced in our experiments by the addition of calcium. Under these conditions, gel formation resulted from the complexation by calcium of the oligopolyglyuronic sequences.18 To find conditions for nanoparticles formation, systems that were able to produce gel aggregates or microgels were identified depending on both the rate of calcium addition and the concentration of the calcium chloride solution (Ca_{alg. sys. Conc.}).12 Preliminary assays have shown that the addition of an 18 mM calcium chloride solution to sodium alginate at a concentration of 1%, wt/vol (Alg_{alg. sys. Conc.}) induced the formation of a homogeneous gel at the limit between the formation of a homogeneous gel and of gel aggregates. The use of higher concentrations of calcium chloride (Ca_{alg. sys. Conc.}) led to the formation of both gel aggregates and microgels, whereas lower Ca_{alg. sys. Conc.} induced continuous gels. Thus, a concentration of 18 mM calcium chloride (Ca_{alg. sys. Conc.}) was chosen to prepare all the systems used in this study.
Systems Containing a High Sodium Alginate Concentration (Alg<sub>0.5% wt/vol</sub>, 0.5%, wt/vol)—The macroscopic aspects of the systems obtained by adding various amounts of calcium chloride (Ca<sub>initial Conc.</sub>, 18 mM) to a 1% (wt/vol) alginate solution (Alg<sub>Initial Conc.</sub>) are reported in Table I. The formation of a system containing a true solution was observed with calcium chloride concentration of 2.7 mM (Ca<sub>alg, sys. conc.</sub>) or below. Above this concentration, a continuous gel was observed (G). As shown in Figure 2, systems (S) prepared with concentrations of 0.9, 1.8, or 2.7 mM calcium chloride (Ca<sub>alg, sys. conc.</sub>) showed a linear relationship between the shear stress and the shear rate, corresponding to Newtonian flow behavior. In contrast, the systems with a gel state (G) showed a shear thinning behavior. These results indicated that sol-gel transition occurred clearly for a concentration of calcium chloride (Ca<sub>alg, sys. conc.</sub>) around 3.6 mM. Viscometric measurements were then carried out on the different alginate solutions with Newtonian flow behavior (alginate systems noted S) to determine the reduced viscosity. The curves obtained (Figure 3) were compared with a curve drawn under the same conditions for a pure sodium alginate solution at the same concentration (0.5%, wt/vol). All the curves showed a typical pattern for polyelectrolytes with the appearance of a strong electrosorptive effect ("increase of viscosity due to the adsorption of a counter ions layer") for high dilution (final concentration of alginate in the dilution, <0.2%, wt/vol).<sup>17</sup> This behavior was very reproducible and was observed with measurements by both cone-plate and capillary techniques. The system containing 2.7 mM calcium chloride (Ca<sub>alg, sys. conc.</sub>) presented reduced viscosity (Figure 3) and viscosity (Figure 4) higher than the systems with a lower calcium chloride concentration (Ca<sub>alg, sys. conc.</sub>). These results indicate that the system containing 2.7 mM calcium ions (Ca<sub>alg, sys. conc.</sub>) was located in the proximity of the sol-gel transition. Surprisingly, systems containing 0.9 and 1.8 mM calcium chloride (Ca<sub>alg, sys. conc.</sub>) showed lower viscosity than the pure sodium alginate solution (Figure 4). This suggested that calcium divalent cation induced a rearrangement of the alginate molecules, allowing the formation of microdomains with high local concentrations of alginate instead of an infinite network of polymer. Formation of those microdomains, described elsewhere as a "pregel" state,<sup>18</sup> could be responsible for the observed reduced viscosity decrease of the polymer solution near the sol-gel transition. Indeed, the dispersion medium of the aggregates had a lower viscosity than the macromolecular solution.<sup>18</sup>

Systems Containing a Low Sodium Alginate Concentration (Alg<sub>0.06% wt/vol</sub>, 0.06%, wt/vol)—With the sodium alginate solution at a lower concentration (Alg<sub>initial Conc.</sub>, 0.12%, wt/vol), macroscopic gel aggregates or microgels were formed instead of a continuous gel when calcium chloride was added at concentrations (Ca<sub>alg, sys. conc.</sub>) >3.6 mM (Table I). Below this concentration, homogeneous solutions (S) were obtained. Viscosity measurements carried out on homogeneous solutions (S) of low alginate concentration showed a similar behavior to that observed for the systems with high alginate concentration. Only with a calcium concentration of 2.7 mM (Ca<sub>alg, sys. conc.</sub>) was a difference noted. Viscosity increased with high alginate concentration solutions, yet tended to a plateau for low alginate concentration solutions (Figure 4).
This is probably due to the fact that in the case of low alginate concentration solutions, the transition occurred from a solution to microgels instead of gels with high alginate concentration solutions. The addition of poly-L-lysine to the different systems led to the formation of visible polymer aggregates for all calcium chloride concentrations (Ca_{Alg, Sys, Conc.}) except for the lower concentration (0.9 mM) for which a slightly bluish coloration appeared (Tyndall effect) after the addition of poly-L-lysine. With this preparation, quasielastic light scattering measurements showed the presence of nanoparticles characterized by a mean diameter of 280 nm, with a rather narrow size distribution (Figure 5). Thus, the formation of nanoparticles was observed for very dilute solutions of sodium alginate and for a calcium chloride concentration (Ca_{Alg, Sys, Conc.}) lower than the one that was required to induce microgel formation. Moreover, the calcium chloride concentration (Ca_{Alg, Sys, Conc.}) that was able to produce nanoparticles was shown to form a pregel state with higher alginate concentrations. Viscometric data suggested that a pregel state also existed for the systems containing low alginate concentration.

The formation of aggregates was probably due to the proximity of the sol–gel transition. As a matter of fact, Mutin has shown that the internal concentration of polymer within the microdomain forming pregels decreased when the systems approached the sol–gel transition. Thus, aggregates of alginate molecules in the pregel state could become less compact than those obtained for lower concentration of calcium and could lead to macroscopic aggregates after addition of poly-L-lysine. Another reason to explain the appearance of aggregates instead of nanoparticles in those systems could be that the electrical charge of the aggregates in the pregel state was different than that of the system containing 0.9 mM calcium chloride (Ca_{Alg, Sys, Conc.}) that is able to induce the formation of nanoparticles. In fact, the formed nanoparticles probably resulted from ionic interactions between negatively charged polyelectrolytes and calcium and between positively and negatively charged polyelectrolytes. On the other hand, the concentration of calcium producing aggregates was shown to be higher than that allowing nanoparticle formation. Thus, the net electrical charge of the complex between alginate and calcium and the number of sites of interactions should not be the same in the two cases. As a consequence, with 1.8 or 2.7 mM calcium, poly-L-lysine could not interact in the same way with the “alginate–calcium” complex as it did with 0.9 mM calcium concentration. Therefore, aggregates were formed.

**Factors Influencing Nanoparticle Formation**—To understand the process by which alginate nanoparticles were formed, factors such as alginate concentration (Alg_{Initial, Conc.}) and influence of the procedure of preparation were studied. As shown in Figure 6, the size of the nanoparticles depended greatly on the sodium alginate concentration (Alg_{Final, Sys, Conc.}) sizes increased proportionally with the concentration of sodium alginate (Alg_{Final, Sys, Conc.}).

The experimental procedure for nanoparticle preparation was also found to be a factor influencing the size of the nanoparticles. As a matter of fact, the order in which calcium chloride or poly-L-lysine was added to the sodium alginate solution was very important. Indeed, as shown in Figure 7, the nanoparticles were characterized by a small size (280 nm) when calcium chloride was added first to the sodium alginate solution. In contrast, when poly-L-lysine was added first, before calcium chloride, alginate nanoparticles were bigger (850 nm). The formation of alginate nanoparticles was thus strongly influenced by the nature of the first compound (either calcium chloride or poly-L-lysine) added to the alginate solution. This observation indicated that the mechanism of nanoparticle formation was probably different when poly-L-lysine was first added to the sodium alginate solution instead of calcium chloride. As a matter of fact, it seems that the formation of nanoparticles resulted mainly from the interaction of alginate with the first added compound and that the formation was less dependent on the addition of the second component. Indeed, to reverse the order of addition of sodium alginate with one of the two other compounds (either calcium or poly-L-lysine) did not affect the final size obtained. This could also be related to the formation of a pregel state. The size differences between the two types of particles could be explained by the structure of the complexes that was formed either between calcium and alginate or between poly-L-lysine and alginate. Within the calcium–alginate complex, the interaction between the calcium ions and the alginate polymer occurred at the level of the oligopolyglyuronic sequences. Furthermore, calcium ions induced a parallel packing of the oligopolyglyuronic sequences to give egg-box structures. This type of interaction should lead to the formation of very compact domains in the alginate molecules. Thus, the respective concentrations of alginate and calcium allowing the appearance of a pregel state led to the formation of very small aggregates of alginate molecules. The addition of poly-L-lysine allowed only strengthening of this system to obtain small and well-defined particles.

On the contrary, interaction between peptides, such as poly-L-lysine, and alginate was described to take place on mannuronic residues. These structures are found in oligopolyglyuronic sequences and in random mannuronic-guluronic sequences. In this situation, the formation of the complex should not induce the appearance of very well-organized and close packed domains as it did with calcium ions. In fact, parallel arrangement of oligopolyglyuronic sequences and
formation of compact egg-box structures should not be possible if poly-L-lysine was added first because, in that case, the alginate molecules were most probably setting by the interaction with poly-L-lysine. This could explain that the size of the obtained particles was bigger than when calcium chloride was added first. These results indicate that the gelification step of the alginate molecules by calcium was a critical factor for the preparation of well-defined nanoparticles.

Nanoparticle Doxorubicin-Loading Capacity—Evaluation of the ability of alginate nanoparticles to carry a drug was performed with doxorubicin as a model drug. The association capacity and the loading capacity were determined as a function of the initial amount of drug. Results (Figure 8) show that almost 100% (97–99%) of doxorubicin initially dissolved in the alginate solution was associated with the nanoparticles, whatever the initial concentration of doxorubicin was. However, the drug loading capacity of doxorubicin onto nanoparticles increased linearly with the concentration of the drug (Figure 8). For the lowest concentration (0.05 mg/mL, $\text{Dox}_{\text{Final Sys. Conc.}}$), the loading capacity was 9 mg of doxorubicin/100 mg of alginate, whereas for the more concentrated suspension (0.4 mg/mL, $\text{Dox}_{\text{Final Sys. Conc.}}$), it was much higher (79 mg of doxorubicin/100 mg of alginate). The size of the nanoparticles increased in the presence of doxorubicin (Table II). The main limitation was due to the appearance of aggregates when the loading capacity became higher than 59 mg of doxorubicin/100 mg of alginate. The important drug loading most likely resulted from a great affinity of doxorubicin for alginate polymer. As a matter of fact, the amino group of doxorubicin is possibly involved with the uronic acid residues of alginate. Indeed, Bhakoo et al.\(^1\) have already hypothesized that an interaction between doxorubicin and alginate was responsible for the different release patterns observed with high guluronic alginate and high mannuronic alginate content; there was a greater degree of release of doxorubicin from high guluronic gels compared with high mannuronic gels. Moreover, it could be assumed that if doxorubicin was added to alginate before calcium has induced the gelling process, the interaction between doxorubicin and alginate led to a steric hindrance for a conformational arrangement of the macromolecule in favor of the formation of the “egg-box” structure with calcium, leading to bigger particles. Such a behavior was also noted when poly-L-lysine was added before calcium to the sodium alginate solution.

Conclusions

This paper proposed a new type of nanoparticles made from alginate and prepared by controlling the gelification of alginate with divalent cations, such as calcium. Evaluation of the loading capacity was done with doxorubicin, which has an important drug loading capacity that was never obtained with other nanoparticles.$^{11-12}$ Hemocompatibility of alginate as well as some evidence for in vivo degradation and elimination$^{11}$ suggest that it would be a good candidate for drug targeting purposes. In addition, the hydrophilicity of most polysaccharide compounds could result in reducing the opsonization of alginate nanoparticles. Further experiments are needed to study the ability of alginate nanoparticles to avoid the MPS uptake.

### References and Notes


**Acknowledgments**

This work was supported by the GDR G 0965, Centre National de la Recherche Scientifique (CNRS), France.