Self-Gelling Primaquine−Gum Arabic Conjugate: An Injectable Controlled Delivery System for Primaquine

K. K. Nishi and A. Jayakrishnan*

Polymer Chemistry Division, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Satelmond Palace Campus, Trivandrum, Kerala 695 012, India

Received June 28, 2006; Revised Manuscript Received October 16, 2006

Primaquine, an 8-aminoquinoline, forms a cross-linked gel with periodate-oxidized gum arabic rapidly by simply mixing the drug with the oxidized polysaccharide due to Schiff's base formation between the two amino groups of primaquine and the aldehyde groups in the oxidized polysaccharide. The speed of gelation is determined by the degree of oxidation of polysaccharide, its quantity, and the drug payload. Estimation of the cross-linking density of the gels showed that the higher is the degree of oxidation of gum arabic, the higher is the cross-linking density. In vitro release of primaquine into phosphate buffered saline (PBS) at 37 °C demonstrated that the extent of release depended on the cross-linking density and drug payload. Repeated extraction using PBS soon after gel formation showed that not all of the primaquine was conjugated to the polysaccharide and the release seen in vitro was mostly from the unconjugated drug especially from matrices with higher cross-linking density. The gels were found to degrade in PBS, the kinetics of degradation being dependent on the cross-linking density. Cytotoxicity evaluation using MTT assay against L_{929} mouse fibroblasts showed that oxidized gum arabic having a degree of oxidation of 50% was only very mildly cytotoxic at a concentration of 0.025 g/mL. An injectable, biodegradable drug depot with controlled release of primaquine over several days or weeks would be advantageous for long-term delivery of this drug against malaria or leishmaniasis, and the present study shows that a primaquine−polymer conjugate that can be formed in situ could be an interesting possibility.

1. Introduction

"Polymer therapeutics" is an emerging and promising area of research in the field of drug delivery and includes polymeric drugs, polymer−drug conjugates, polymer−protein conjugates, polymeric micelles to which the drug is covalently bound, and multicomponent polyplexes for DNA delivery.[1−3] Polymer-based therapeutic agents are designed to increase plasma half-life, targetability, controlled degradation of polymer−drug bonds, and improve cellular uptake.[4−6] In polymer−drug conjugates, drug conjugation is usually achieved by linking the active pharmaceutical agent using pendent groups along the polymer backbone chain or by conjugation to the end groups in the polymer chain. While the former method allows many drug molecules to be conjugated to the polymeric carrier, the latter method allows only a maximum of one or two molecules to be linked. The latter method has been preferred to link peptide and protein-drugs to poly(ethylene glycol), a technique now well known as PEGylation with many therapeutic advantages.[7]

Polymer−drug conjugates retained in the body for a longer period of time can serve as a depot enabling controlled release of the drug. The stability of the linkage between drug and the polymer determines the fate of release. Generally, polymeric prodrugs intended for controlled release are designed to release the drug by the enzymatic or nonenzymatic hydrolysis of the drug−polymer bond.[3]

Polymeric prodrugs are also being increasingly investigated to improve the solubility of many drugs and reduce their toxicity. Thus, Domb et al.[8−10] and Charvalos et al.[11] have recently demonstrated that the anti-fungal drug amphotericin-B, a polyene antibiotic with negligible aqueous solubility, can be conjugated to oxidized polysaccharides or poly(vinyl pyrrolidone) to improve its solubility, reduce toxicity, and at the same time maintain its high anti-fungal activity. The concept of drug conjugation to polymeric carriers has recently been exploited to transform even an inactive drug into an active drug. Donmurado et al.[12,13] have recently shown that norfloxacin, an antibiotic that is inactive against mycobacteria in its native form, can be transformed into an active drug by conjugating it to mannose-bearing dextran.

Malaria causes death for 1.5−2.7 million of the 500 million people infected annually.[14] Primaquine, an 8-aminoquinoline, is the only currently available drug for therapy to prevent relapse of the disease. Considerable confusion surrounds the dose regimen of primaquine to prevent relapse; recent recommendations consist of a regimen of 30 mg daily for 14 days or 0.5 mg/kg body weight.[15] Primaquine is also used for anti-leishmanial therapy.[16] The clinical use of primaquine, however, poses problems due to its toxicity.[17] To improve the therapeutic efficacy of the drug and diminish its toxicity, various approaches have been examined. These include linking the drug to a carrier protein such as albumin,[18] linking peptide derivatives of the drug onto biodegradable polyacryl starch microspheres,[19] and encapsulation in polycyanoacrylate and polylactide nanoparticles,[20,21] in erythrocytes,[22] and in liposomes.[23,24]

In a recent communication, we showed that primaquine-conjugated gum arabic microspheres of <2 μm size suitable for macrophage uptake could be prepared from periodate-oxidized gum arabic and primaquine by heat denaturation.[25] During the course of this investigation, it was observed that both the aliphatic and the hindered aromatic amino groups of primaquine entered into Schiff's reaction with the aldehyde functions, leading to cross-linking of the polysaccharide. We

* Corresponding author. Tel.: +91-471-2340801. Fax: +91-471-2341814. E-mail: dr_jayakrishnan@yahoo.co.in.

10.1021/bm060612x CCC: $37.00
Published on Web 11/29/2006

therefore suggested that this phenomenon provided an opportunity for constructing an injectable controlled delivery system for primaquine. A curative single dose treatment of malaria in a single injection would be a great advantage as opposed to multiple, long-term treatment regimens for preventing relapse of this disease. Although there are not many studies on this, liposomal primaquine has been investigated to this end by the intravenous route.[23,26]

An intramuscularly or intraperitoneally injected biodegradable drug depot with controlled release of primaquine over several days or weeks would be an exciting possibility for long-term delivery of this drug against malaria or leishmaniasis. To this end, we believe that a simple injectable system composed of the polymer−drug conjugate would be worth investigating. Here, we show that a sustained release of primaquine lasting several days could be obtained from the primaquine−gum arabic conjugate, which can be prepared by simply mixing the drug with the oxidized polysaccharide, and the extent of release could be manipulated by varying the degree of oxidation of gum arabic and the drug payload.

2. Materials and Methods

2.1. Materials. Gum arabic (from acacia tree) of approximate molecular weight 250 000 (Product No. G-9752), primaquine phosphate, sodium *m*-periodate, 2,4-dinitrophenylhydrazine, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT), and Dulbecco's Minimum Essential Medium (DMEM) were purchased from Sigma Chemical Co., St. Louis, MO. Dialysis tubing (Spectra/Por, MWCO 6000−8000) was from Spectrum Laboratories Inc., CA. All other reagents such as boric acid, Borax, disodium hydrogen phosphate, monosodium hydrogen phosphate, sodium chloride, starch, sodium thiosulphate, etc., were of analytical grade and were procured locally. L_{929} mouse fibroblast cells for cytotoxicity evaluation were subcultured from stock culture obtained from National Center for Cell Sciences, Pune, India. Borate buffer of pH 11 was prepared by dissolving 6.18 g of boric acid and 9.54 g of Borax in 1 L of distilled water, and the pH was adjusted to 11 by the addition of sodium hydroxide. Phosphate buffered saline (PBS, pH 7.4, 0.1 M) was prepared by dissolving 17.97 g of disodium hydrogen phosphate, 5.73 g of monosodium hydrogen phosphate, and 9 g of sodium chloride in 1 L of distilled water.

2.2. Methods. *2.2.1. Oxidation of Gum Arabic and Its Characterization.* Gum arabic was oxidized using varying quantities of sodium *m*-periodate as reported before to obtain 80%, 50%, or 20% oxidized polysaccharide.[25] The aldehyde content was estimated using the hydroxylamine hydrochloride method[27]and by the 2,4-dinitrophenylhydrazine method.[28] Thermal stability of the samples was analyzed by thermogravimetry using a simultaneous TGA-DTA instrument (model SDT 2960 TA Instruments Inc., New Castle, DE). Experiments were carried out in N_2 atmosphere at a heating rate of 10 °C/min. Mass change of the sample was recorded continuously as a function of a combination of temperature with time.

2.2.2. Preparation of Primaquine Cross-Linked Gum Arabic Aldehyde. Gum arabic−primaquine gel was prepared as follows. One mL of a 10% solution of oxidized gum arabic having different degrees of oxidation (80%, 50%, 20%) prepared in borate buffer (pH 11) was introduced into a glass vial of 15 mL capacity. Into this, a 10% aqueous solution of primaquine phosphate was added to obtain drug payloads of 20, 30, 40, and 50 wt % with respect to gum arabic. The solutions were mixed well using a vortex mixer for 5 min. The color of the mixture turned from light yellow to dark red on Schiff's base formation and cross-linking. The vials were kept at room temperature for 30 min when a primaquine cross-linked gum arabic gel was obtained.

2.2.3. Characterization of the Gel. 2.2.3.1. Scanning Electron Microscopy (SEM). Surface morphology of the primaquine cross-linked gum arabic hydrogels was examined using SEM. A piece of lyophilized gel was placed on a double-sided tape, sputter coated with gold, and examined using the microscope (Hitachi, model S-2400, Japan). For examining the internal structure, the gel was sectioned using a razor blade, coated with gold, and examined.

2.2.3.2. Swelling Characteristics. Gels were prepared with oxidized gum arabic having different degrees of oxidation (20%, 50%, and 80%) with 20 wt % primaquine payload as before ($n = 3$). PBS (5 mL) was introduced into each vial, and the gels were allowed to swell at 37 °C in the incubator for 24 h till they attained equilibrium swelling. The PBS was aspirated using a Pasteur pipet from each vial, and the gels were gently blotted using tissue paper and weighed in an analytical balance. Degree of swelling (Q) was defined as the reciprocal of the volume fraction of the polymer in the hydrogel (υ_2) and was calculated using the equation,

$$Q = \nu_2^{-1} = [(1/\rho_p)[(Q_m/\rho_s) + (1/\rho_p)]^{-1}]^{-1}$$

where ρ_p is the polymer density (0.262 g/cm^3), ρ_s is the density of water (0.9971 g/cm^3 at 25 °C), and Q_m is the swelling ratio, defined as the mass ratio of absorbed water and the dried gel.[29]

2.2.3.3. Cytotoxicity. Cytotoxicity of gum arabic and oxidized gum arabic having a degree of oxidation of 50% was quantitatively assessed by MTT assay.[30] L_{929} cells were cultured in multi-well tissue culture plates, and when monolayer was attained, culture medium was removed, rinsed with PBS, and 100 μL each of gum arabic and oxidized gum arabic (50% oxidized) at concentrations of 0.1, 0.05, and 0.025 g/mL of the medium were added to different pre-labeled wells containing cells. Cells with medium alone served as control. Culture medium (100 μL) was used as reagent blank. After the plates were incubated for 24 h at 37 °C in 5% carbon dioxide atmosphere, the medium was removed, and 200 μL of MTT working solution was introduced into each well and incubated at 95% humidified atmosphere at 37 °C for 8 h. After the reagent solution was removed and the material was rinsed with PBS, 200 μL of isopropanol was added to each well and incubated for 20 min at 37 °C in a shaker incubator (Labline Instruments, Melrose Park, USA). The absorbance of the resulting solution in each well was recorded immediately at 570 nm using an automated micro plate reader (Bio-Tek Instruments, VT). Results were expressed as O.D. after blank (i.e., medium only) subtraction. Reported values are the mean of three replicates.

2.2.3.4. In Vitro Degradation. The gels were prepared using 80%, 50%, and 20% oxidized gum arabic with 20 wt % primaquine payload in vials having 15 mL capacity as before. Free primaquine was extracted using phosphate buffer, and the initial weights of the gels were determined after lyophilization and drying. Multiple samples of the gels ($n = 3$ for each time interval) were then incubated with 5 mL of PBS at 37 °C. At definite intervals of time (24 h and then every week up to 6 weeks), PBS was aspirated from the vials, rinsed once with distilled water, and the samples were frozen and lyophilized to dryness and their weights were noted. The extent of degradation of the gels was determined from their change in weights at different times of incubation.

2.2.4. In Vitro Drug Release. Drug release from gels prepared with gum arabic having different degree of oxidation (80%, 50%, and 20%) and having different primaquine payloads (20, 30, 40, and 50 wt % with respect to gum arabic) was examined in PBS at 37 °C. Gels were prepared in vials having 20 mL capacity as before, and 10 mL of PBS was introduced into these vials and they were rocked in a reciprocating bath shaker (model SW-22, Jualbo Labortechnik, Seelbach, Germany) at 100 strokes/min. Aliquots of 0.5 mL were withdrawn at different time intervals, filtered through a 0.2 μm filter, and the drug released was measured spectrophotometrically at 355 nm. A constant volume of buffer was maintained in each vial by adding fresh buffer.

2.2.5. Statistical Analysis. Statistical analysis of data was performed by one-way analysis of variance (ANOVA), assuming a confidence level of 95% ($p < 0.05$) for statistical significance. All data were expressed as mean ± standard deviation (S.D.).

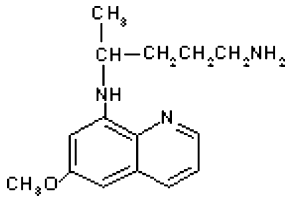

Figure 1. Structure of primaquine.

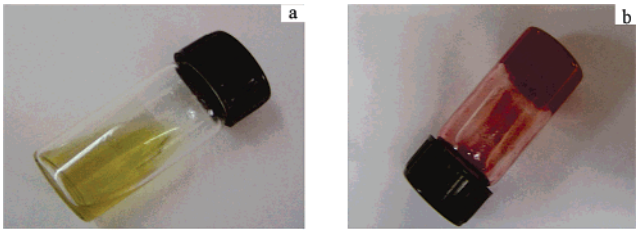

Figure 2. Solution of 50% oxidized gum arabic and primaquine before gelation (a) and after gelation (b).

3. Results and Discussion

Gum arabic is a natural polysaccharide obtained from the exudates of acacia tree with the major component being arabinogalactan (90%) and is extensively used in food, pharma, and cosmetic industries.[31] It is reported to be fermented and metabolized in the caecum and the colon.[32,33] Periodate oxidation of polysaccharides offers a convenient route to synthesize polymer−drug conjugates especially with drugs possessing aliphatic amino functions via imino bonds in a simple manner.[8−10] The Schiff's base formation between the amine and the aldehyde group is a rapid reaction under the appropriate conditions.[9]

Hydrogels are a three-dimensional network of hydrophilic polymers.[34,35] These networks are able to retain a large quantity of water within their structure without dissolving and are extensively investigated as drug delivery vehicles. Various cross-linking agents have been designed to prepare hydrogels from natural as well as synthetic polymers.[35] Primaquine contains an aliphatic primary amino group as well as a hindered aromatic amino group of unequal reactivity (Figure 1) and therefore is an interesting candidate for such an approach in the preparation of a hydrogel-type polymer−drug conjugate where the drug itself is the cross-linking agent.

When primaquine was mixed with an aqueous solution of oxidized gum arabic, it was seen that the solution slowly turned into an insoluble gel on standing (Figure 2). The change in viscosity of the system with time was followed at 37 °C using 10 mL samples with a Brookfield viscometer (model DV-II+, Brookfield Laboratories, MA), a small sample adapter, and spindle S-31 at 50 rev/min, and the time when viscosity rose to infinity was taken as the gelation time.[25] With 20 wt % primaquine payload, the gelation times were 17 min for 80% oxidized gum arabic, 26 min for 50% oxidized gum arabic, and 32 min for 20% oxidized gum arabic. The fast gelation seen with 80% oxidized gum arabic is believed to be due to the large number of aldehyde groups present in it, whereas 20% oxidized gum arabic took a longer time to form the gel because it contained less number of aldehyde groups. The gelation time with 50% oxidized gum arabic was in between. The ratio of aldehyde to amine in the system composed of 80% oxidized gum arabic and 20 wt % primaquine is calculated to be 10.6, whereas for the system composed of 50% and 20% oxidized gum arabic with 20 wt % primaquine the values were 6.6 and 2.7, respectively. Thus, the more oxidized is the polysaccharide, the greater is the availability of aldehyde functions for the cross-linking to take place, which is reflected in the decrease in the gelation time with increase in the degree of oxidation. However, when gelation was followed using a small sample according to the procedure of Mo et al.[36] by stirring 1 mL of solution using a magnetic stir bar at 37 °C and noting the time for the stir bar to stop, considerably reduced gelling times were obtained. With 20 wt % primaquine, the gelling times for 80%, 50%, and 20% oxidized gum arabic were 1 min 35 s, 2 min 8 s, and 4 min 17 s, respectively. Thus, gelation times appropriate for the injected material to stay at the intended site could be obtained depending on the amount of the material introduced. It is also possible to visually examine the system for its transition from sol to gel and then inject the material before complete gelation occurs. Whether all of the primary and secondary amino groups present in primaquine enter into reaction with the available aldehyde is not certain due to the difference in the reactivity of the primary and the secondary amino functions; the fact that gelation did take place leading to an insoluble product points to the fact that both amino groups are entering into reaction with the aldehyde present on the polysaccharide.

The most important parameters that define the structure and properties of swollen hydrogels are the polymer volume fraction in the swollen state and cross-linking density. Highly cross-linked gels have a tighter structure and will swell less as compared to the hydrogels with lesser cross-linked structure.[29] The polymer volume fraction in the swollen gel is a measure of the amount of fluid that a hydrogel can incorporate into its structure. It also gives an idea about the interaction between polymer chains. Hydrogels prepared from gum arabic of different degree of oxidation with 20 wt % primaquine payload were allowed to swell in PBS for 24 h at 37 °C, and the weight of the swollen gel was noted. Swelling ratio (Q_m) was calculated from the ratio of weight of PBS uptake to the weight of dried gel. Degree of swelling (Q) is the reciprocal of volume fraction of the polymer (v_2) in the hydrogel, which is a measure of interaction between polymer chains.[29] Typical values of Q for highly swollen gels range from 5 to 100 and sometimes even 1000. For moderately swollen gels, Q value varies from 1.5 to 5. Primaquine−gum arabic aldehyde hydrogels have a Q value in the range 2−5, showing that they are moderately swollen (Table 1). Cross-linking density v_e was calculated from the Flory−Rehner equation,

$$v_e = -[\ln(1 - v_2) + v_2 + \chi_1 v_2^2][V_1(v_2^{1/3} - 2v_2/f)]^{-1}$$

where χ_1 is the Flory−Huggins interaction parameter, f is the cross-linking functionality, V_1 is the molar volume of water (18.062 cm^3/mol), and v_2 is the volume fraction of polymer in the hydrogel when it reaches the equilibrium swollen state. In general, for a polymer to be soluble in water at a particular temperature, χ_1 must be lower than 0.5; the value of χ_1 was assumed to be 0.35 for gum arabic aldehyde, as it has been reported for a similar interaction involving other polymeric aldehydes.[37] In the primaquine−gum arabic aldehyde system, because both amino groups of primaquine participate in the cross-linking reaction involving the aldehyde groups on the oxidized polysaccharide, the cross-linking functionality f was taken as four. Assumptions made on these calculations are that the hydrogels are neutral, swelling is isotropic, and polymer chains have Gaussian distribution.

Cross-linking density was highest for gels prepared from 80% oxidized gum arabic, and this decreased with decrease in the degree of oxidation. Cross-linking depends on the number of aldehyde groups available to take part in the reaction, and because the 80% oxidized gum arabic contains more aldehyde

Table 1. Cross-Linking Parameters of 20 wt % Primaquine Cross-Linked Gum Arabic Gels

degree of oxidation of gum arabic (%)	swelling ratio (Q_m)	polymer volume fraction (ν_2)	degree of swelling (Q)	cross-linking density ($\nu_e \times 10^5$)
80	8.46 ± 0.47	0.4885	2.05 ± 0.23	716.7 ± 0.002
50	9.31 ± 0.77	0.2898	3.45 ± 0.35	224.4 ± 0.008
20	9.36 ± 0.87	0.1068	4.61 ± 0.29	105.6 ± 0.001

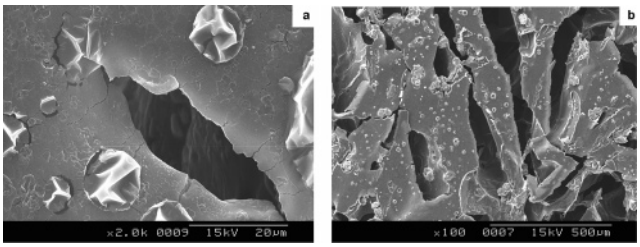

Figure 3. SEM of gum arabic−primaquine gel. (a) Surface and (b) internal structure.

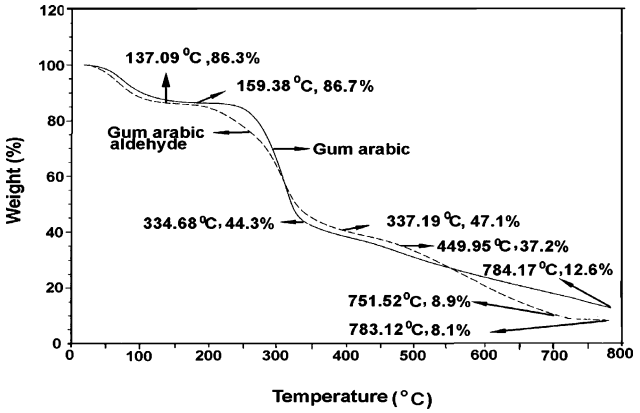

Figure 4. Thermogram of gum arabic (−) and gum arabic aldehyde (- - -).

groups, a higher cross-linking density seen was in accordance with the expectation.

Morphology of the gel surface was studied using SEM (Figure 3). The gel had a rather smooth surface with occasional pits and cracks. The internal structure looked less solid in nature with a number of perforations. These results, however, should be interpreted with caution because it was not the wet gel that was examined in the microscope, but the lyophilized gel that underwent considerable shrinking and fluting during the lyophilization process. Nevertheless, these pictures give a rough idea about the morphology and internal structure of these novel materials.

The thermal stability of gum arabic and oxidized gum arabic aldehyde was analyzed by thermogravimetry. Gum arabic aldehyde was somewhat less stable as compared to gum arabic. Gum arabic started to degrade at 159.38 °C and lost about 13% weight at this stage, and the second stage of decomposition was at 334.68 °C losing about 55% of the weight. A linear decrease in weight was observed thereafter. Gum arabic aldehyde started to degrade at a lower temperature; the first stage of decomposition was observed at 137.09 °C and lost about the same weight as gum arabic, and the second stage was at 337.19 °C losing about 53% of the weight. As in the case of gum arabic, a linear weight loss was observed subsequently (Figure 4). Thus, the thermal stability of the material has not undergone drastic change due to oxidation. The slightly reduced thermal stability of the oxidized product could be attributed to the opening of the rings

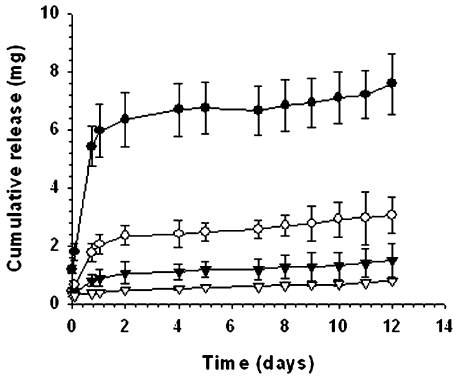

Figure 5. In vitro primaquine release from 80% oxidized gel. Release from gels having 50% (●), 40% (○), 30% (▼), and 20% (▽) drug payload.

by periodate oxidation and altering the conformation of the polymer by imparting free rotation to the β-glycosidic linkages.

In vitro drug release was examined from gels prepared from gum arabic having different degrees of oxidation (80%, 50%, and 20%) with different primaquine payloads (20, 30, 40, and 50 wt %). The drug release pattern obtained varied depending on the cross-linking and swelling characteristics of different gels. Drug release was examined up to 12 days. The release of the drug was slow from all preparations, although it increased with time. As expected, the release from the gel having the highest payload was more rapid as compared to release from gels having lower drug payloads. The maximum amounts released were 15%, 8%, 5%, and 4% of primaquine from gels formed from 80% oxidized gum arabic with 50%, 40%, 30%, and 20% primaquine payload (Figure 5). A significant difference ($p < 0.05$) was observed in the amount of primaquine released from gel with the highest drug payload and other gels. Primaquine released from gels with the lowest payloads of 30 and 20 wt % was not significantly different ($p > 0.05$). With 50% drug loading, although the 80% oxidized material will have about 4 times the aldehyde functions as compared to the amino functions from primaquine, some of the primaquine molecules may not be covalently bonded to the polysaccharide due to the high viscosity of the system, and the release of about 15% that is seen in vitro could be mostly from the unconjugated primaquine. When the drug payload is reduced, the cumulative release was reduced drastically especially from formulations containing 20 and 30 wt % primaquine. In the case of 30 and 20 wt % loading, the ratio of aldehyde to amine is of the order of 6.9 and 10.4, respectively. This infers that the extent of conjugation and cross-linking in these specimens would be high and the free primaquine available for immediate release is low.

The extent of primaquine released from 50% oxidized gum arabic matrix was 11%, 4.8%, 4.4%, and 4.3% for 50%, 40%, 30%, and 20% primaquine payload, respectively (Figure 6). A significant difference ($p < 0.05$) was observed in the amount of primaquine released from gel with the highest payload and other gels. However, there was no significant difference in the amount of primaquine released from gels with 40%, 30%, and

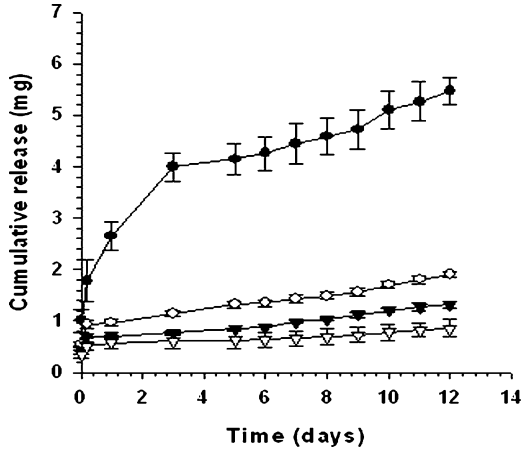

Figure 6. In vitro drug release from 50% oxidized gel. Release from gels having 50% (●), 40% (▽), 30% (▼), and 20% (○) drug payload.

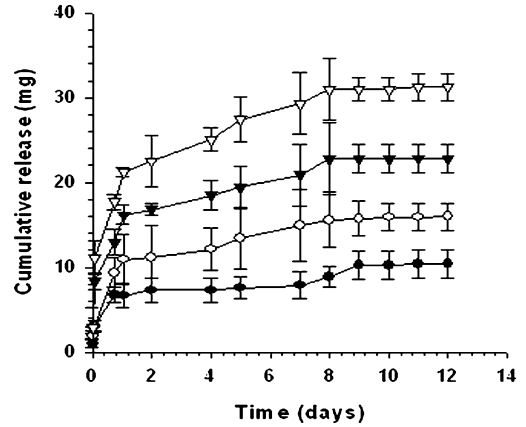

Figure 7. In vitro drug release from 20% oxidized gel. Release from gels having 50% (▽), 40% (▼), 30% (○), and 20% (●) drug payload.

20% primaquine payload. As in the case of 80% oxidized gum arabic with the highest payload of primaquine, the amount of free drug present in the matrix is believed to be high with high drug payload.

Gels prepared from 20% oxidized gum arabic matrix also demonstrated an apparent payload-dependent release pattern. The extent of release observed for 12 days was 63%, 57%, 53%, and 51% from gels having 50%, 40%, 30%, and 20% primaquine payload (Figure 7). Statistical analysis, however, showed that there was significant difference ($p < 0.05$) between the amounts of primaquine released from gels with different payloads.

Thus, a faster release of primaquine was seen from the gels prepared from 20% oxidized gum arabic. Various factors contribute to this; 20% oxidized gum arabic has the lowest number of aldehyde groups as compared to the other two matrices, and the cross-linking density is least with this matrix (Table 1). The ratio of aldehyde to amine in the system is about 1.06, 1.32, 1.76, and 2.7 for drug payloads of 50, 40, 30, and 20 wt %, respectively. Therefore, the extent of drug conjugation and cross-linking is believed to be less with this matrix. Earlier studies on release of drugs from gel matrices have shown that cross-linked structure of polymer membranes exerts a screening effect on drug diffusion even in the swollen systems;[38] this screening effect will be more for 80% oxidized and 50% oxidized matrices, thus slowing down the rate of drug diffusion.

To confirm whether the primaquine released into the medium is the free primaquine, an extraction study was conducted to

Table 2. Free Primaquine Extracted from Microspheres and Cumulative Release in Vitro

degree of oxidation (%)	primaquine payload (wt %)	primaquine released on extraction (%)	primaquine released in vitro (%)
20	20	42.9 ± 5.8	51.8 ± 6.5
20	30	40.3 ± 5.0	53.3 ± 1.6
20	40	52.5 ± 11.4	57.1 ± 1.7
20	50	55.2 ± 2.6	62.5 ± 1.6
50	20	5.4 ± 0.2	4.3 ± 0.2
50	30	8.1 ± 1.9	4.4 ± 0.1
50	40	10.6 ± 5.3	4.8 ± 0.1
50	50	11.4 ± 2.2	10.9 ± 0.5
80	20	3.9 ± 0.2	4 ± 0.5
80	30	4.78 ± 1.2	5 ± 0.6
80	40	5.6 ± 1.2	8 ± 0.6
80	50	4.6 ± 1.0	15.1 ± 2.1

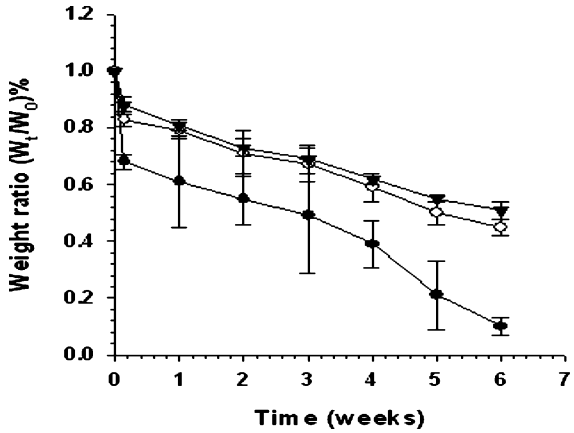

Figure 8. Degradation profiles of 20% (●), 50% (○), and 80% (▼) oxidized gel having a primaquine payload of 20%.

substantiate the results obtained from in vitro drug release. Gels were prepared, and after 30 min they were extracted with 5 mL of PBS five times. These extracts were collected, and the extracted primaquine was estimated. It was found that in every preparation, there was free primaquine that could be extracted easily (Table 2). In the case of the system composed of 20% oxidized gum arabic, about 40−50% of the drug could be extracted. This amount is reflected in the in vitro release profiles. When the degree of oxidation is increased, decreasing amounts of primaquine only could be extracted in accordance with the in vitro release profiles seen from these systems. Thus, primaquine that is physically encapsulated in the matrix is getting released faster into the medium, and the remaining primaquine bound by Schiff's linkage to the polysaccharide is expected to be released slowly by hydrolysis and polymer degradation.

Degradation of the gels was studied in PBS at 37 °C. Degradation of the matrix under physiological conditions would be a desirable feature of implantable drug delivery systems. The degradation was found to be faster for gel prepared from 20% oxidized gum arabic, whereas gels prepared from 50% and 80% oxidized material degraded more slowly (Figure 8). Statistical analysis revealed that weight loss from different gels was significantly different ($p < 0.05$). Gels prepared from 20% oxidized gum arabic was least cross-linked and therefore undergo a faster degradation, while gels prepared from 80% and 50% oxidized gum arabic are more cross-linked and therefore undergo a slow degradation. These results are in accordance with the release and extraction experiments. In the case of 20% oxidized gum arabic, the amount of primaquine

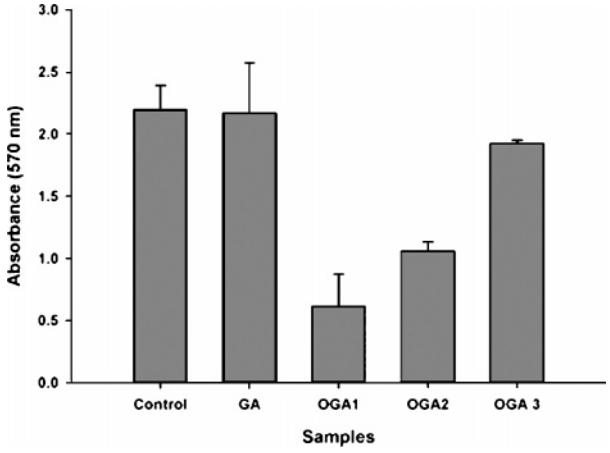

Figure 9. MTT reduction by mouse fibroblast cells challenged with gum arabic (GA) at 0.1 g/mL and 50% oxidized gum arabic (OGA1−0.1 g/mL, OGA2−0.05 g/mL, OGA3−0.025 g/mL) in comparison with control (cells with medium alone) for 24 h.

extracted by PBS was less than the amount released into the medium at all drug payloads over a period of 12 days (Figure 7 and Table 2). The amount in excess of the extractable could therefore be primaquine released as a result of hydrolysis and polymer degradation. In the case of 50% and 80% oxidized gum arabic, the extent of degradation is about 20% in 10 days and the in vitro release profile of primaquine from these gels does not reflect any significant amount released by hydrolysis and degradation. The amount extracted into PBS and the amount released in vitro were roughly the same.

Partially oxidized polysaccharides are known to degrade in the physiological medium as opposed to their unoxidized counterparts.[39,40] The backbone and the side chains of gum arabic consist of 1,3-linked β-D-galactopyranosyl units,[31] and opening up of these rings by periodate is also expected to hasten the degradation of oxidized gum arabic. The Schiff's linkages formed between the primary amino groups and the aldehyde groups are known to be susceptible to hydrolysis. The two amino groups of primaquine are not equally reactive, although both are capable of Schiff's reaction. However, it is known that when there is an aryl group on the nitrogen, the resultant adducts are known to be more stable.[41] The relative hydrolytic stability of primaquine−gum arabic conjugates is reflected in the release profile of primaquine, because most of the primaquine released into the medium is the unconjugated drug as evidenced by the extraction experiments. The resistance to degradation by the conjugates prepared from highly oxidized gum arabic as opposed to conjugate prepared from less oxidized gum arabic could also be explained on the basis of their high cross-linking density. However, about 40% weight loss is seen for these highly cross-linked conjugates in about 6 weeks (Figure 8). Therefore, one would anticipate release of primaquine when the matrix undergoes continuous biodegradation over a long period of time.

The cytotoxicity of gum arabic and 50% oxidized gum arabic was assessed using MTT assay. While gum arabic did not show any sign of cytotoxicity at 0.1 g/mL, in the case of oxidized gum arabic, slight cytotoxicity was observed at a concentration of 0.025 g/mL (Figure 9). Furthermore, when the aldehyde groups on the oxidized gum arabic react with the drug, the slight cytotoxicity that is due to the presence of adehyde groups should diminish considerably. Detailed cytotoxicity studies on dextran aldehyde reported recently have shown a cytotoxic effect on murine RAW 264.7 cells at a concentration of 130 μg/mL, whereas the widely used protein cross-linker glutaraldehyde was cytotoxic at less than 0.15 μg/mL.[10] Thus, as compared to oxidized dextran, oxidized gum arabic appears to be much less cytotoxic at concentrations orders of magnitude higher than oxidized dextran.

Considerable confusion exists regarding the dose regimen of primaquine for anti-malarial therapy. In India, the National Malarial Eradication Programme recommends a dose of 15 mg for 5 days in the case of adults, while the dose for children in the age group of 1−14 ranges from 2.5 to 10 mg.[42] Even though the systems reported here are not optimized to deliver ideal therapeutic concentrations, it can be seen from the release profiles that different delivery rates are possible. It is difficult to predict how these systems would behave in terms of delivery profiles when implanted intramuscularly or intraperitoneally because the in vivo release profiles could be quite different from what is seen in vitro. Hypothetically, a gel composed of 0.8 g of 20% oxidized gum arabic and 0.4 g of primaquine should, in principle, deliver 240 mg (60%) of the drug in 8 days. Whether dose-dumping would result with such a high payload in vivo can only be evaluated by in vivo studies.

4. Conclusions

An in situ forming, injectable polymer−drug conjugate from oxidized gum arabic and primaquine could be easily fabricated by simply mixing the drug with the oxidized polysaccharide. Because both the aliphatic and the hindered aromatic amino groups of primaquine enter into Schiff's base formation, the resulting conjugate is a cross-linked gel. With the same drug payload, the cross-linking density of the resulting gel increases with increase in the degree of oxidation of the polysaccharide, resulting in different release profiles of primaquine, thereby providing an opportunity to control the release profile of the drug. The drug−polymer conjugate was found to degrade in physiological media, and the kinetics of degradation depended again on the cross-linking density of the gel network. Prolonged release of primaquine is a possibility concurrent with the polymer degradation. Oxidized gum arabic was not found to be cytotoxic at concentrations orders of magnitude higher than oxidized dextran. Drugs that can conjugate to a polymer spontaneously and form insoluble cross-linked gel networks could possibly find application as injectable delivery depots for controlled and sustained delivery.

Acknowledgment. A.J. dedicates this paper to Professor Eugene P. Goldberg, University of Florida, Gainesville, on the occasion of his 78th birthday. K.K.N. thanks the University Grants Commission, New Delhi, for a Senior Research Fellowship. Thanks are also due to the Director, SCTIMST, for permission to publish this work.

References and Notes

(1) Duncan, R. The dawning era of polymer therapeutics. *Nat. Rev. Drug Discovery* **2003**, *2*, 347−360.
(2) Brocchini, S.; Duncan, R. Pendent drugs, release from polymers. In *Encyclopedia of Controlled Drug Delivery*; Mathiovitz, E., Ed.; Wiley: New York, 2000; Vol. 2, pp 786−815.
(3) D'Souza, A. J. M.; Topp, E. M. Mini review. Release from polymeric prodrugs: Linkages and their degradation. *J. Pharm. Sci.* **2004**, *93*, 1962−1979.
(4) Maeda, H., Kataoka, K., Kabanov, A., Okano, T., Eds. *Polymer Drugs in the Clinical Stage: Advantages and Prospects*; Springer: New York, 2003.
(5) Ouchi, T.; Ohya, Y. Macromolecular prodrugs. *Prog. Polym. Sci.* **1999**, *20*, 211−257.
(6) Veronese, F. M.; Morpurgo, M. Bioconjugation in pharmaceutical chemistry. *Farmaco* **1999**, *54*, 497−516.

(7) Roberts, M. J.; Bentley, M. D.; Harris, J. M. Chemistry for peptide and protein PEGylation. *Adv. Drug Delivery Rev.* **2002**, *54*, 459−476.
(8) Flak, R.; Domb, A. J.; Polacheck, I. A novel injectable water-soluble amphotericin B-arabinogalactan conjugate. *Antimicrob. Agents Chemother.* **1999**, *43*, 1975−1981.
(9) Ehrenfreund-Kleinman, T.; Azzam, T.; Falk, R.; Polacheck, I.; Golenser, J.; Domb, A. J. Synthesis and characterization of novel water soluble amphotericin B-arabinogalactan conjugates. *Biomaterials* **2002**, *23*, 1327−1335.
(10) Sokolsky-Papkov, M.; Domb, A. J.; Golenser, J. Impact of aldehyde content on amphotericin-B-dextran imine conjugate toxicity. *Biomacromolecules* **2006**, *7*, 1529−1535.
(11) Charvalos, E.; Tzatzarakis, M. N.; Bambeke, F. V.; Tulkens, P. M.; Tsatsakis, A. M.; Tzanakakis, G. N.; Mingeot-Leclercq, M.-P. Water-soluble amphotericin-B-polyvinylpyrrolidone complexes with maintained antifungal activity against *Candida* spp and *Aspergillus* spp and reduced haemolytic and cytotoxic effects. *J. Antimicrob. Chemother.* **2006**, *57*, 236−244.
(12) Coessens, V.; Schacht, E. H.; Domurado, D. Synthesis and in vitro stability of macromolecular prodrugs of norfloxacin. *J. Controlled Release* **1997**, *47*, 283−291.
(13) Roseeuw, E.; Coessens, V.; Balazuc, A.-M.; Lagranderied, M.; Chavarot, P.; Pessina, A.; Neri, M. G.; Schacht, E.; Marchal, G.; Domurado, D. Synthesis, degradation, and antimicrobial properties of targeted macromolecular prodrugs of norfloxacin. *Antimicrob. Agents Chemother.* **2003**, *47*, 3435−3441.
(14) World Health Organization (WHO) Fact Sheet No. 94. Geneva, WHO, 1996.
(15) Baird, J. K.; Hoffman, S. L. Primaquine therapy for malaria. *Clin. Infect. Dis.* **2004**, *39*, 1336−1345.
(16) Heurtault, B.; Legrand, P.; Mosqueira, V.; Devissaguet, J. P.; Barratt, G.; Bories, C. The antileishmanial properties of surface-modified, primaquine-loaded nanocapsules tested against intramacrophagic Leishmania donovani amastigotes in vitro. *Ann. Trop. Med. Parasitol.* **2001**, *95*, 529−533.
(17) White, N. J. The treatment of malaria. *New Eng. J. Med.* **1996**, *335*, 800−806.
(18) Hofsteenage, J.; Capuano, A.; Altszuler, R.; Moore, S. Carrier-linked primaquine in the chemotherapy of malaria. *J. Med. Chem.* **1986**, *29*, 1765−1769.
(19) Borissova, R.; Lammek, B.; Stjarnkvist, P.; Sjoholm, I. Biodegradable microspheres 16. Synthesis of primaquine-peptide spacers for lysosomal release from starch microspheres. *J. Pharm. Sci.* **1995**, *84*, 249−255.
(20) Gasper, R.; Opperdoes, F. R.; Preat, V.; Roland, M. Drug targeting with polyalkylcyanoacrylate nanoparticles: in vitro activity of primaquine-loaded nanoparticles against intracellular Leishmania donovani. *Ann. Trop. Med. Parasitol.* **1992**, *86*, 41−49.
(21) Rodrigues, J. M., Jr.; Croft, S. L.; Fessi, H.; Bories, C.; Devissaguet, J. P. The activity and ultrastructural localization of primaquine-loaded poly(D,L-lactide) nanoparticles in Leishmania donovani infected mice. *Trop. Med. Parasitol.* **1994**, *45*, 223−228.
(22) Talwar, N.; Jain, N. K. Erythrocyte-based delivery system of primaquine: in vitro characterization. *J. Microencapsulation* **1992**, *9*, 357−364.
(23) Pirson, P.; Steiger, R.; Trouet, A. The disposition of free and liposomally antimalarial primaquine in mice. *Biochem. Pharmacol.* **1982**, *31*, 3501−3507.
(24) Stensrud, G.; Sande, S. A.; Kristensen, S.; Smistad, G. Formulation and characterization of primaquine loaded liposomes prepared by pH gradient using experimental design. *Int. J. Pharm.* **2000**, *198*, 213−228.
(25) Nishi, K. K.; Jayakrishnan, A. Preparation and in vitro evaluation of primaquine conjugated gum arabic microspheres. *Biomacromolecules* **2004**, *5*, 1489−1495.
(26) Pirson, P.; Steiger, R.; Trouet, A.; Gillet, J.; Herman, F. Primaquine liposomes in the chemotherapy of murine malaria. *Ann. Trop. Med. Parsitol.* **1980**, *74*, 383−391.
(27) Zhao, H.; Heindel, N. D. Determination of degree of substitution of formyl groups in polyaldehyde dextran by the hydroxyl amine hydrochloride method. *Pharm. Res.* **1991**, *8*, 400−402.
(28) Vogel, A. I. *Practical Organic Chemistry*, 3rd ed.; ELBS: London, 1956; p 1060.
(29) Peppas, N. A.; Bures, P.; Leobandung, W.; Ichikawa, H. Hydrogels in pharmaceutical applications. *Eur. J. Pharm. Biopharm.* **2000**, *50*, 27−46.
(30) Ciapetti, G.; Cenni, E.; Pratelli, L.; Pizzoferrato, A. In vitro evaluation of cell/biomaterial interaction by MTT assay. *Biomaterials* **1993**, *14*, 359−64.
(31) Verbeken, D.; Dierckx, S.; Dewettinck, K. Mini-Review: Exudate gums: occurance, production, and applications. *Appl. Microbiol. Biotechnol.* **2003**, *63*, 10−21.
(32) Ross, A. H.; Eastwood, M. A.; Brydon, W. G.; Busuttil, A.; McKay, L. F. A study of the effects of dietary gum arabic in the rat. *Br. J. Nutr.* **1984**, *51*, 47−56.
(33) Ross, A. H.; Eastwood, M. A.; Brydon, W. G.; Anderson, J. R.; Anderson, D. M. A study of the effects of dietary gum arabic in humans. *Am. J. Clin. Nutr.* **1983**, *37*, 368−375.
(34) Hoffman, A. S. Hydrogels for biomedical applications. *Adv. Drug Delivery Rev.* **2002**, *43*, 3−13.
(35) Hennink, W. E.; van Nostrum, C. F. Novel crosslinking methods to design hydrogels. *Adv. Drug Delivery Rev.* **2002**, *54*, 13−56.
(36) Mo, X.; Iwata, H.; Matsuda, S.; Ikada, Y. Soft tissue adhesive compound of modified gelatin and polysaccharides. *J. Biomater. Sci., Polym. Ed.* **2000**, *11*, 341−51.
(37) Lee, K. Y.; Bouhadir, K. H.; Mooney, D. J. Degradation behavior of covalently cross-linked poly(aldehyde guluronate) hydrogels. *Macromolecules* **2000**, *33*, 97−101.
(38) Langer, R. S.; Peppas, N. A. Present and future applications of biomaterials in controlled drug delivery systems. *Biomaterials* **1981**, *2*, 201−214.
(39) Boontheekul, T.; Kong, H.-J.; Mooney, D. J. Controlling alginate gel degradation utilizing partial oxidation and bimodal molecular weight distribution. *Biomaterials* **2005**, *26*, 2455−2465.
(40) Singh, M.; Ray, A. R.; Vasudevan, P. Biodegradation studies on periodate oxidized cellulose. *Biomaterials* **1982**, *3*, 16−20.
(41) March, J. *Advanced Organic Chemistry*; Wiley: New York, 1992; pp 896−97.
(42) http://www.malariasite.com/malaria/primaquine.htm (URL last accessed on September 25, 2006).

BM060612X