Interaction of Bacterial Endotoxine (Lipopolysaccharide) with Latex Particles: Application to Latex Agglutination Immunoassays

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The latex agglutination immunoassay technique uses polymer colloids as carriers for antibodies or antigens to enhance the immunological reaction. In this work, the interaction of a lipopolysaccharide (LPS) of Brucella Melitensis with two conventional latexes has been studied. Some experiments on the physical adsorption of the LPS onto these polystyrene beads have been performed and several complexes with different coverage degrees were obtained by modifying the incubation conditions. Regarding the application in the development of diagnostic test systems, it is advisable to study the latex–LPS complexes from an electrokinetic and colloidal stability point of view. The complexes were electrokinetically characterized by measuring the electrophoretic mobility under different redispersion conditions. The colloidal stability was determined by simple turbidity measurements. Experimental and theoretical data have been employed to study the molecular disposition of the LPS in the latex particle surface to compare with the outer membrane of bacterial cells. Latex complexes covered by different LPS amounts showed high colloidal stability and adequate immunoreactivity that remains for a long time period.

Key Words: polystyrene latex; LPS; electrokinetic characterization; particle-enhanced immunoassays.

I. INTRODUCTION

The interaction between biomolecules and latexes has considerable importance in the development of particle-enhanced immunoassays. The use of synthetic polymer colloids for serological diagnosis is normally possible by coupling of ligands, antigens, or antibodies to the surface of particle latex. Several procedures for physical and covalent coupling of proteic ligands have been previously described (1–5). However, the antigenic characteristics for a great number of microorganisms do not reside in protein molecules, making very interesting the study of the interaction between non-proteic ligands and latex particles.

Lipopolysaccharide (LPS), a major constituent of the outer membrane of gram-negative bacteria, is the main antigen of these bacterial cells (6). Interaction of LPS with latex particles is of great interest since it allows the development of rapid serological immunodiagnostic tests (7, 8). In these works, however, colloidal properties of the latex complexes obtained are not studied, when it is very important for their practical application.

The adsorption of a ligand is a complex process in which several physico-chemical factors are involved. The amount and way in which the ligand is adsorbed depends on the nature of the ligand, the characteristics of the solid surface, and the solution conditions. The LPS molecule, with two well-defined parts (hydrophobic and hydrophilic), may interact with latex particles to render surface complexes similar to those of the bacterial outer membranes.

In this work, we have used two latexes with different particle sizes to study the coupling of Brucella Melitensis LPS. Adsorption experiments with different solution conditions of pH and ionic strength have been performed to obtain different LPS–latex complexes. These biointerfaces were characterized from an electrokinetic point of view to study the change in the electrical state of the particle covered by LPS as well as to obtain information about the possible configuration of this molecule on the surface.

Another important characteristic of these complexes is their colloidal stability that prevents particle aggregation in the absence of specific antibodies. We have studied the conditions in which these complexes are collooidally stable. This property will be useful to check if these LPS–latex systems can be used for immunological applications. In this way, the presence of specific Brucella antibodies in rabbit and human sera was proved by the immunoagglutination of the complex particles in different serum dilutions.

II. MATERIALS AND METHODS

All chemicals used in this study were of analytical grade and were used without further purification. Water used in all experiments was doubly distilled and deionized with a Milli-Q water purification system (Milipore).

Two polystyrene latexes were employed in this study: a sulfonate latex obtained by a “shot-growth” emulsion polymerization technique, using sodium styrene sulfonate as the comonomer (latex A) (9), with an average particle diameter of 195 ± 10 nm and a surface charge density of −4.2 ± 0.6 μC/cm², and a sulfate sample obtained by a surfactant-free emulsion.
polymerization technique (latex B) (10), with an average particle diameter of 602 ± 12 nm and a surface charge density of −4.1 ± 0.3 μC/cm². The particle size of the latex samples was obtained by transmission electron microscopy (TEM) and automatic image analysis with Bool2k software (AQ systems, Spain). Surface charge densities were determined by conductimetric and potentiometric titrations, verifying the presence of strong acid groups on the particle surface.

*Brucella Melitensis* (16 M) crude S-Lypopolysaccharide (LPS) was obtained by methanol precipitation of the phenol phase of a water–phenol extraction (11). The structure of the LPS molecule is shown in Fig. 1 (12). LPS concentration in

![Diagram of LPS molecule](image)

**FIG. 1.** Structure of the LPS molecule. O-chain and core (hydrophilic zone); Lipid A (hydrophobic zone).
solution was determined using a phenol–sulfuric acid total carbohydrate determination (13).

Physical adsorption experiments were performed for 2 h at room temperature by adding 0.4 m² of latex surface from polymer suspensions in several buffers with different conditions of pH and ionic strength, each containing different amounts of LPS. The sensitized particles were centrifuged at 20,000 g for 30 min and the supernatants filtered using a Nucleopore polycarbonate filter before the LPS concentration was measured. After two centrifugation cleaning steps, LPS–latex complexes were resuspended in the appropriate buffer.

The buffers used were acetate at pH 4 and 5, phosphate at pH 6 and 7, and borate at pH 8 and 9, with a final ionic strength of 2 mM. Higher ionic strength values were reached with NaCl.

The electrophoretic mobility of the latex particles, with and without LPS on their surface, was measured with a Zeta-Master (Malvern Instruments, U.K.) by calculating the average of six measurements at the stationary level in a cylindrical cell.

The colloidal stability has been expressed in terms of the critical coagulation concentration (CCC). We have estimated this parameter by using a static method. Latex particles were mixed with NaCl solutions at the adequate concentration and kept at room temperature for 24 h. CCC values were then determined by measuring the average aggregate size with a photon correlation spectroscopy (PCS) technique (4700 system, Malvern Instruments, U.K.). Due to the strong acid character of the surface groups for both latexes, CCC values should be independent of pH. The particle size of the complexes was determined with PCS as well.

The immunoreactivity of particles sensitized with different amounts of LPS was measured using a test slide. On the slide, 20 μl of reagent, previously treated with glycine saline solution containing 1 mg ml⁻¹ of bovine serum albumin (GBS-BSA), were mixed with an equal volume of different dilutions of sheep and human serum samples in the same glycine buffer. The slide was rotated for 8 min and aggregation was ascertained visually. Serum samples were previously tested with a commercial ELISA test (Vircell S.L., Spain) to distinguish positive (presence of specific antibodies to Brucella) and negative (absence of these specific antibodies) serum samples.

III. RESULTS AND DISCUSSION

A. Adsorption Experiments

LPS adsorption experiments onto both latexes were carried out at different conditions of pH and ionic strength. The LPS of Brucella shows anionic character with two well-defined parts. In Fig. 1 we can distinguish a hydrophobic zone, the Lipid A, and a hydrophilic zone, the Chain O, responsible for its antigenic character. Both parts are joined by the core, a negatively charged oligosaccharide. In comparison with other gram-negative bacteria, the amount of negative charged groups in the core and lipid A is lower (12).

Figure 2 shows the maximum adsorption of LPS vs pH onto A and B latexes at low ionic strength. Similar adsorption behavior was observed when higher ionic strengths were tested (50 and 100 mM). The adsorbed amounts of this molecule seem to be practically independent of pH, and only when this parameter is lower than 5 did the adsorption increase slightly, maybe due to some protonation of the LPS acidic groups, leading to a reduction in the electrostatic repulsion between LPS molecules and the negative latex surface.

Carmona-Ribeiro and Herrintong (14) propose a mechanism in which the lipid molecules are bound to the surface of anionic latexes by means of a hydrophobic interaction between the hydrophobic tails of the lipid and the polystyrene surface, obtaining a lipidic monolayer around the particles and avoiding lipid desorption. An entropic increase that favors the adsorption process is caused by the dehydration of the hydrophobic zones of the latex and LPS molecules. The hydrophobic interaction should then be assumed as the main force on this process. Upon studying the adsorption of surfactants onto latex particles with the same sign of charge, some other authors obtained a similar conclusion (15, 16). In this way, the adsorbed LPS molecules would present an orientation similar to that on the outer membrane of the bacterial cell. This situation will be checked later in the electrokinetic characterization. Table 1 shows the adsorbed amounts when the medium is saline phosphate buffer (PBS) with an ionic strength of 170 mM. We did not observe differences in the adsorption when the complexes were obtained at low ionic strength, but surprisingly they showed very poor immunological behavior. At low ionic strength electrostatic interactions are more effective, and then we can expect a stronger repulsion between the negative groups of the LPS molecule and the latex surface that, probably, may promote a different orientation of this molecule onto the surface.
TABLE 1
Adsorption Isotherm Values in PBS at pH 7

<table>
<thead>
<tr>
<th>Initial [LPS] (mg of LPS/m² of latex)</th>
<th>Adsorbed amount (mg of LPS/m² of Latex A)</th>
<th>Adsorbed amount (mg of LPS/m² of Latex B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.31</td>
<td>0.64</td>
</tr>
<tr>
<td>2</td>
<td>0.56</td>
<td>0.93</td>
</tr>
<tr>
<td>3</td>
<td>1.27</td>
<td>1.62</td>
</tr>
<tr>
<td>4</td>
<td>1.58</td>
<td>1.84</td>
</tr>
<tr>
<td>5</td>
<td>2.25</td>
<td>2.05</td>
</tr>
<tr>
<td>6</td>
<td>1.94</td>
<td>1.78</td>
</tr>
</tbody>
</table>

B. Electrokinetic Characterization and Colloidal Stability

Electrophoretic mobility data of the bare latexes and the LPS–latex complexes when they are redispersed at different pHs are shown in Figs. 3 and 4. The mobility of the sensitized particles is influenced by the presence of LPS molecules at acid pH, where the electric charge of the LPS molecules are diminished as a consequence of the protonation of their acid groups. For the complexes with the higher amount of LPS adsorbed, around 2 mg/m², the surface charge of the particles is completely screened and the mobility even changes to positive values. In this situation, the particle seems to be fully covered by LPS. As the adsorbed amount decreases, we found an increase in the negative value of the mobility. At neutral and basic pH, however, the negative electrophoretic mobility increases and the values for all complexes approach those of the bare latexes, as a consequence of the increase in the net charge of the LPS molecules. This situation was found for both latexes and is indicative of their colloidal stability.

Table 2 shows the CCC values for bare latexes and several complexes at different pHs. As was to be expected, complexes with a low adsorbed amount present lower CCC values at pH 5 than uncovered latex particles. Nevertheless, their stabilities increase with the adsorbed amount, reaching values similar or higher than those from bare latex. As can be seen in Figs. 3 and 4, at pH 5 the electrophoretic mobility of complexes with the maximum adsorbed amount is considerably reduced with respect to bare latexes. This situation could be indicative of an additional contribution, steric or hydration forces, to the electrostatic interaction in the colloidal stability of these complexes (17–20). Hechemy et al. (7), working with latex particles coated with LPS from Escherichia coli, obtained a similar result. Spontaneous aggregation of the coated particles occurred when 50% of the surface was covered by the ligand, but complexes became stabilized when the ligand covered the whole surface. This situation, usually found also in protein–latex systems

<table>
<thead>
<tr>
<th>Sample</th>
<th>Adsorbed amount (mg of LPS/m² of latex)</th>
<th>pH 5 (μm/m²)</th>
<th>pH 7 (μm/m²)</th>
<th>pH 9 (μm/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Latex A</td>
<td>0</td>
<td>150 ± 10</td>
<td>200 ± 10</td>
<td>150 ± 10</td>
</tr>
<tr>
<td></td>
<td>1.27</td>
<td>60 ± 10</td>
<td>100 ± 10</td>
<td>200 ± 10</td>
</tr>
<tr>
<td></td>
<td>2.25</td>
<td>200 ± 10</td>
<td>500 ± 50</td>
<td>1000 ± 50</td>
</tr>
<tr>
<td>Latex B</td>
<td>0</td>
<td>80 ± 10</td>
<td>80 ± 10</td>
<td>80 ± 10</td>
</tr>
<tr>
<td></td>
<td>0.93</td>
<td>20 ± 10</td>
<td>60 ± 10</td>
<td>100 ± 10</td>
</tr>
<tr>
<td></td>
<td>1.62</td>
<td>50 ± 10</td>
<td>120 ± 10</td>
<td>350 ± 50</td>
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<tr>
<td></td>
<td>2.05</td>
<td>80 ± 10</td>
<td>200 ± 10</td>
<td>500 ± 50</td>
</tr>
</tbody>
</table>
(21, 22), is attributed to the bridging between ligand molecules bound to the particles and the free surface of another latex particle.

To obtain additional information about the interaction between LPS and latex particles and the orientation of these molecules on the surface, we have applied some theoretical studies on the electrokinetic behavior of colloidal particles with a structured surface to our system. Ohshima and co-workers (23, 24) developed an approximate formula for the electrophoretic mobility of particles with a surface charge layer, assuming that the surface charges are distributed uniformly throughout the surface layer of finite thickness and that the electrolyte ions can penetrate the layer. This equation gives the electrophoretic mobility for structured solid–liquid interfaces as a function of three parameters:

$$\mu = -\frac{\epsilon_{el}KT}{v\eta} \left[ \ln \left( \frac{N}{2vn} \right) - \left( \left( \frac{2vn}{N} \right)^2 + 1 \right)^{1/2} \frac{2vn}{N} \right]$$

$$ \times \left( \frac{1}{2} \right) \left[ 1 + \left( \frac{N}{2vn} \right)^{2/4} \tanh \lambda d - 1 \right]$$

$$ - \frac{Ne}{\eta\lambda^2} \left( 1 - \frac{1}{\cosh \lambda d} \right).$$

$N$ is the density of charged groups in the ligand layer; $\lambda$ gives information about the frictional force that the ligand layer exerts on the surrounding liquid; and $d$ is the thickness of this surface layer liquid. The theoretical and experimental mobility data at different pHs for the complex of latex A with 2.25 mg of LPS/m$^2$ are shown in Fig. 5. The best fit between experiments and theory was obtained when $\lambda$ approximated 0.3 nm$^{-1}$ and $N$ increased its value from 0.016 M at pH 5 to 0.022 M at pH 7. The variation of this parameter is related to the increase in the net charge of the LPS molecules when the pH changes from acid to neutral values. The third parameter, $d$, is the most important in our study, and the value obtained by the fit is 7 nm. This result is in consonance with the thickness value experimentally obtained by means of PCS. Using this technique we have estimated in 6 ± 1 nm the thickness of the adsorbed layer, comparing the diameter of particles with and without LPS. These two results are in agreement with the length of the LPS molecule that, having taken into account the chain O structure and the length of the hydrocarbon chains of the lipid A, could be estimated between 5 and 10 nm (12). The good correlation between these results could verify the idea previously expounded in which the surface of the LPS–latex complexes present a disposition similar to that observed in the outer membrane of bacterial cells. Thus, the molecular orientation of LPS would be optimum for the immunological reaction with specific antibodies.

![Figure 5](image-url)  
**FIG. 5.** Theoretical and experimental electrophoretic mobility of a LPS–Latex A complex (2.25 mg of LPS/m$^2$). Experimental mobility for the following: – • bare latex; – □, LPS–Latex A complex at pH 5; ○, LPS–Latex complex at pH 7.

**C. Immunoreactivity**

The last step of this work is to check the immunoreactivity of some colloidal complexes under physiological conditions.

To suppress nonspecific interactions between complexes and serum proteins, the nonoccupied parts of the latex particles have to be blocked with a second ligand. The most commonly used is a protein, the bovine serum albumin (BSA) (25). In this way, we have made a previous treatment of the complexes with a solution of BSA at pH 5 (15 mg m$^{-2}$ added) to obtain more specific reactives. After this process, the complexes are suspended in saline buffer glycine with BSA (GBS-BSA) and pH between 7 and 8, which is normally used to stabilize these systems (26).

Figure 6 shows the agglutination intensity of several complexes of both latexes with different amounts of LPS as a function of the dilution serum. These complexes were previously tested against different dilutions of other serum samples without specific antibodies (negatives) and no aggregation was observed, verifying the specific behavior of our systems. As can be seen in this figure, none of the complexes present aggregation in GBS-BSA, which is indicative of their colloidal stability in the absence of agglutinant. The experimental curves so obtained show the typical bell shape of the immuno-precipitin reaction (27). Such a response can be explained considering that an antibody molecule acts as a bridge to coagulate two sensitized particles. Before reaching the maximum, it is easy to understand why the immunological response increases as the antibody concentration increases. At higher antibody concentrations the system seems to lose reactivity. This may be due to the saturation of the attached LPS antigens with solution antibodies, and thus not favorable for the bridging process.
or other forces) is low but nonnegligible. Much care must be

due to other repulsive interactions different from the electrostatics. Under appropriate conditions, the LPS covered-latex has been shown to be a good system to detect specific antibodies against Brucella specific antibodies in sheep and human serum samples. A surpris-

ging high colloidal stability is observed for these complexes, suggesting the existence of other repulsive interactions different from the electrostatics. Under appropriate conditions, the

FIG. 6. Immunoactivity of several complexes LPS–latex: ○, 1.27 mg of LPS/m² of Latex A; □, 2.25 mg of LPS/m² of Latex A; ●, 1.62 mg of LPS/m² of Latex B; ■, 2.05 mg of LPS/m² of Latex B.

The results obtained for each latex system, differing in particle size, are clearly different in two aspects: the sensitivity, related to the agglutination intensity, and the response as a function of the amount of LPS at the surface. It can be observed in Fig. 6 that the smaller particles, latex A, give rise to the highest agglutination when intermediate coverage is employed, but it drops down dramatically for complexes with higher coverage. With a bigger particle, latex B, the immunoresponse is practically the same for the two studied coverages (medium and high). To under-

stand these results, we have to consider the possible interactions between covered particles. As was shown before when studying the electrokinetic behavior of these complexes, some kind of interaction apart from the electrostatic repulsion is overstabilizing these particles so as to provoke the high values of CCC found for complexes with high coverage, ascribing this effect to steric or hydration forces. It is then plausible to suppose that the same forces, which do not allow a close approach of the particles, may hamper the immunological interaction of the antigen-covered particle with the free specific antibody as well as the later reaction with another covered particle. This way, according to Table 2, it can be observed that LPS–Latex A complexes with the highest coverage do not show good immunoreactivity but high colloidal stability. On the other hand, the same latex with lower coverage and lower CCC shows a considerable increase in immunoreactivity. In the case of the other latex system, that of a bigger size (latex B), at the same time the differences in colloidal stability between complexes with different coverages are not so high, the immunoreactivity is quite similar as well.

Therefore, to develop antigen–latex systems for immunoagglutination assays, it is convenient to obtain systems where repulsion between particles (mediated by electrostatic interaction or other forces) is low but nonnegligible. Much care must be taken to ensure that adequate colloidal stability is attained to avoid nonspecific aggregation in the absence of the correspon-
dent antibody. To achieve this objective, an optimization strategy must be designed including ligand coverage of the particles, pH, and ionic strength of the reaction medium, which constitute the critical variables.

The immunoresponse of these complexes remained without appreciable variations for a long time period (6 months at least), verifying the stable bond between the LPS molecules and the surface latex particles.

Particle size is another important point in relation to the sensi-
tivity of the covered latex particles since aggregates of bigger particles will grow faster, but smaller ones will diffuse easier. As can be seen in Fig. 6, the best immunoresponse is achieved for the smallest particle at intermediate coverage. If the immunodetection system is going to be adapted to light-scattering techniques, it is desirable to use small particles (ca. 200 nm) (28). In this case, a wider concentration range and better linearity could be obtained before saturation of the signal. Future work involves this adaptation of the system to automatic techniques.

IV. CONCLUSIONS

We have obtained several LPS–latex complexes in which a hydrophobic bond seems to be the main interaction that allows LPS molecules to remain strongly joined to the surface latex particles. The surface characterization of the LPS–latex particles indicates a disposition of the LPS molecules on the surface similar to that observed in the outer membrane of a bacterial cell. Our system remains colloidal stable at neutral or basic pH in physiological serum conditions (150 mM), which allows, using this system, detection of the presence of Brucella specific antibodies in sheep and human serum samples. A surpris-

ingly high colloidal stability is observed for these complexes, suggesting the existence of other repulsive interactions differ-

ent from the electrostatics. Under appropriate conditions, the LPS covered-latex has been shown to be a good system to detect specific antibodies against Brucella in mammal serum samples.

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