

A SURFACE PLASMON RESONANCE BIOSENSOR FOR REAL-TIME IMMUNOLOGIC DETECTION OF *ESCHERICHIA COLI* O157:H7

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1. INTRODUCTION

Testing of foods for the presence of pathogenic organisms requires methods different than those employed in the clinical laboratory. In foods there could be very low levels of pathogenic bacteria and the organisms are often in a poor physiological state because of exposure to stressful conditions in the food environment or to stresses encountered during food processing and storage. The organism must be identified in the presence of a large population of competing flora. Therefore, a period of enrichment culturing of the sample in liquid growth medium is usually required in order to allow the organism to recover from injury and to allow for selective growth of low numbers of the target bacteria to detectable levels. Traditional methods for detection and identification of pathogenic bacteria in foods and other samples have relied on the use of specific microbiological media to isolate and enumerate viable bacterial cells followed by a series of biochemical and serological tests for confirmation.

In recent years, methods for detection of food-borne pathogens have evolved from traditional culture techniques to more rapid and specific immunological assays involving use of specific antibodies and genetic methods based on nucleic acid probes or the polymerase chain reaction. Many of these methods, however, are still laborious, require at least 24 to 48 hours to obtain final results, and may require a significant amount of training to perform. Biosensors hold considerable promise and potential for on-line quality and safety monitoring in the food industry; however, biosensor technology is still in its infancy. Recently, various types of biosensors have been developed which allow direct measurement of chemical species in biological samples¹. However, reports on the use of biosensors for detection of food-borne pathogens are lacking.

2. MATERIALS AND METHODS

2.1. Bacteria

The bacteria used in this study were the following: *E. coli* O157:H7 B1409 and *E. coli* O157:H7 933 (Centers for Disease Control and Prevention, Atlanta, GA), *E. coli* O157:H7 ATCC 43890, *Salmonella typhimurium* ATCC 14028 (American Type Culture Collection, Rockville, MD), *E. coli* O157:H7 93–569 (Food Safety and Inspection Service, Beltsville, MD), *E. coli* O103:H2 87.1368 (*E. coli* Reference Center, University Park, PA), and *Yersinia enterocolitica* GER O:3 (Food and Drug Administration, Seattle, WA). Bacteria were grown in brain heart infusion broth at 37°C for 16 h with aeration. One milliliter aliquots of the cultures were then centrifuged at $16,000 \times g$ for 2 min and the cell pellet was suspended in 1 ml of HBS buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% BIAcore® surfactant P20 [Biacore, Inc., Piscataway, NJ]).

2.2. Reagents and Instrumentation

These studies were performed on the BIAcore® instrument (Biacore, Inc.) equipped with BIAlogue command software. Monoclonal antibody 8–9H (IgG2a subtype) reactive against *E. coli* O157:H7 was kindly provided by Dr. Rebecca Durham at Organon Teknika, Rockville, MD. Unlabeled goat anti-*Escherichia coli* O157:H7 affinity-purified polyclonal antibody and goat anti-*Salmonella* (CSA-1) were purchased from Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD. Other materials included: Protein A (purified from *Staphylococcus aureus*; Sigma, St. Louis, MO), purified recombinant Protein G (Pierce, Rockford, IL), sensor chip CM5, and the amine coupling kit containing *N*-ethyl-*N'*-(dimethylaminopropyl)-carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS) and ethanolamine (Biacore, Inc.)¹⁴. Additional chemicals used were of reagent grade.

2.3. Immobilization

Ligands were immobilized onto flow cells of sensor chip CM5 by the amine coupling method¹⁴. Briefly, with this coupling method, the carboxymethylated dextran layer on the sensor surface is activated with an EDC/NHS mixture to produce NHS-esters which can react with ligands containing primary amino groups. After immobilization of the ligand, unreacted NHS-esters are deactivated following injection of a large excess of ethanolamine hydrochloride. Noncovalently bound ligand is then washed from the surface with 100 mM HCl. Ligands bound to the sensor chip flow cells included: Protein A (300 µg/ml in 10 mM NaOAc, pH 4.5), Protein G (150 µg/ml in 10 mM NaOAc, pH 4.5), polyclonal affinity-purified antibody (100 µg/ml in 10 mM NaOAc, pH 4.5), and monoclonal antibody 8–9H (ascites, 100 µg/ml in 10 mM NaOAc, pH 4.5). HBS, pH 7.4, was used as the “running” (wash) buffer between injections.

2.4. Detection of Bacteria

Following immobilization of Protein A or Protein G on the sensor chip surface, either monoclonal or polyclonal antibodies against *E. coli* O157:H7 were applied at concentrations of 200 µg/ml (diluted in HBS, pH 5.0 or 7.4) or 50 µg/ml (diluted in HBS), respectively, at a flow rate of 3 µl/min for a total of a 10-min injection. The bacterial

suspension (in HBS, pH 5.0 or 7.4) was then injected across the surface also at a flow rate of 3 $\mu\text{l}/\text{min}$ for a total of a 10-min injection and allowed to interact with the bound antibodies. Between injections, the surface was washed with HBS (pH 5.0 or 7.4) to remove unbound material. The sensor chip surface was regenerated with two 1-min pulses of 6 M guanidine-HCl, pH 1.0.

The bacterial suspensions were also injected over surfaces with immobilized monoclonal or polyclonal *E. coli* O157:H7 antibodies. This was followed by injection of either monoclonal or polyclonal antibodies at concentrations of 200 $\mu\text{g}/\text{ml}$ or 50 $\mu\text{g}/\text{ml}$, respectively. Between injections, the surface was washed with HBS to remove unbound material. The bacterial suspensions were applied for 10 min and the second antibody was also injected for 10 min at a flow rate of 3 $\mu\text{l}/\text{min}$.

To determine the limit of detection, 10-fold serial dilutions, prepared in HBS, of *E. coli* O157:H7 cultures were injected over a monoclonal antibody surface. Following a wash with HBS, polyclonal antibody diluted in HBS to a concentration of 50 $\mu\text{g}/\text{ml}$ was injected for 10 min at a flow rate of 3 $\mu\text{l}/\text{min}$.

To visualize bacteria bound to antibody on the sensor chip surface, various levels of *E. coli* O157:H7 were injected over a surface to which monoclonal antibody was bound. The surface was washed with HBS. The sensor chip was removed and the surface was then examined by optical microscopy for the presence of bacteria.

2.5. Inhibition Assay

E. coli O157:H7 cultures were resuspended and serially diluted in HBS (pH 5.0) to ca 10^4 to 10^{10} colony forming units (CFU)/ml. Aliquots of the bacterial suspension (100 μl) and 200 μl of polyclonal *E. coli* O157:H7 antibody (diluted to 5, 25, and 50 $\mu\text{g}/\text{ml}$ in HBS, pH 5.0) were combined in 1.5 ml polypropylene tubes and reacted with shaking on an IKA-Vibrax-VXR (Janke & Kunkel IKA Werk, Staufen, Germany) at a motor setting of ca 900 for 30 min at room temperature. Following reaction, the bacteria with associated antibodies were pelleted by centrifugation at $16,000 \times g$ for 5 min and 200 μl of the supernatant was removed for concentration analysis with the BIAcore[®]. The supernatant, which contained unbound antibody, was flowed across a Protein G-immobilized CM5 sensor chip for 10 min at a flow rate of 3 $\mu\text{l}/\text{min}$. Removal of bound antibody to regenerate the Protein G sensor surface was effected through two 5-min injections of 0.1 M HCl flowed across the sensor chip at a rate of 3 $\mu\text{l}/\text{min}$.

3. RESULTS AND DISCUSSION

In the present study, various test systems were used to monitor binding of whole bacterial cells to the BIAcore[®] sensor surface in efforts to develop a real-time assay for detection of *E. coli* O157:H7. Binding of *E. coli* O157:H7 B1409 at concentrations of ca 5×10^9 CFU/ml to monoclonal antibody 8-9H immobilized on the sensor surface generated a response signal of 633 RU (Fig. 1). A 10-min pulse of a *S. typhimurium* suspension (ca 1×10^9 CFU/ml) did not generate a notable response (3 RU). Injection of *Y. enterocolitica* or *E. coli* O103:H2 also produced only negligible RUs (data not shown). These results indicate that the antibody was specific against *E. coli* O157 and had insignificant cross-reactivity with other bacteria tested. Also, bacteria did not bind non-specifically to the dextran matrix. Injection of whole bacteria through the instrument's liquid handling system did not cause disturbances in the flow system.

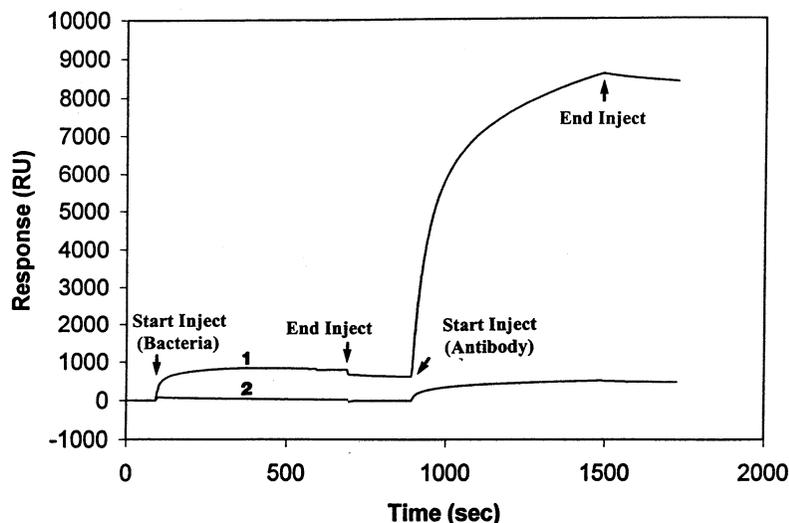


Figure 1. Overlay plots of sensorgrams showing the interaction of monoclonal antibody 8-9H (ligand) with *E. coli* O157:H7 (1) and *S. typhimurium* (2) followed by injection of polyclonal antibody at a concentration of 50 $\mu\text{g/ml}$. The bacteria were injected at a concentration of ca 5×10^9 CFU/ml.

In order to enhance the signal obtained with binding of *E. coli* O157:H7 and to confirm the specificity of the interaction, injection of bacteria was followed by a 10-min pulse of polyclonal antibody. A signal of 7779 RU was obtained with surfaces to which *E. coli* O157:H7 bound (Fig. 1). Since *S. typhimurium* did not bind to the monoclonal antibody, only a minimal response with polyclonal antibody reactive against *E. coli* O157:H7 was obtained. Other ligand-second antibody combinations were tested including polyclonal antibody as ligand followed by polyclonal antibody and polyclonal antibody followed by monoclonal antibody. With every combination, signal was enhanced reproducibly with injection of the second antibody. Generally, the combination of monoclonal antibody as the ligand followed by polyclonal antibody produced the best response with *E. coli* O157:H7. Three other *E. coli* O157:H7 strains (933, 93-569 and ATCC 43890) were tested and similar results were obtained as with strain B1409, while *E. coli* O103:H2 did not generate a notable signal (data not shown). To further confirm the specificity of the assay for *E. coli* O157:H7, antibody against *Salmonella* species was injected as the second antibody following a 10-min pulse of the bacterial suspensions over a monoclonal antibody 8-9H surface. No enhanced response was obtained with *E. coli* O157:H7 and since no remarkable binding of *S. typhimurium*, *Y. enterocolitica* or *E. coli* O103:H2 occurred with immobilized monoclonal antibody, a response signal was not generated with injection of antibody reactive against *Salmonella*.

The detection limit of the assay was ca 5×10^7 CFU/ml injecting a total of 30 μl (3 $\mu\text{l}/\text{min}$ for 10 min, therefore, ca 1.7×10^6 CFU injected). Generation of significant binding responses following injection of *Staphylococcus aureus* over surfaces with immobilized human IgG (through binding of Protein A molecules on the cell membrane to IgG) and binding of erythrocytes to immobilized concanavalin required injections of 5×10^{12} CFU/ml of *S. aureus* and 4×10^9 erythrocytes/ml¹³. RU's obtained with injection of *E. coli* O157:H7 suspensions were rather low, therefore, to determine the level of bacteria bound to the sensor surface, the sensor chip was examined by optical microscopy. Bacteria were

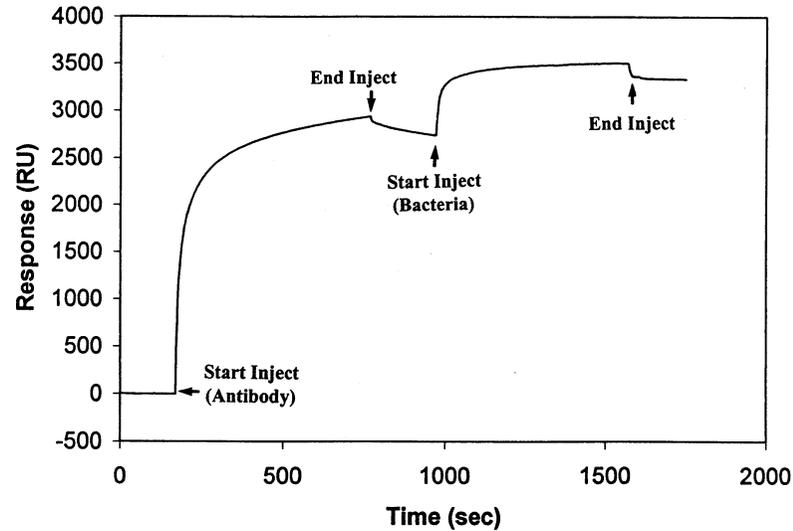


Figure 2. Sensorgram showing the interaction of Protein G (ligand) with polyclonal antibody applied at a concentration of 200 µg/ml followed by injection of *E. coli* O157:H7 at a concentration of ca 5×10^9 CFU/ml.

visible and evenly distributed over the entire flow cell of the sensor chip when injected at a concentration of 2×10^9 CFU/ml generating a signal of 403 RU. When injected at a level of 1×10^{10} CFU/ml, higher levels of bacteria bound to the surface and a signal of 876 RU was obtained (data not shown).

Several factors should be taken into consideration when monitoring interactions of whole cells using the BIAcore®. The instrument generates a response with changes in the refractive index of the solution close to the gold film on the sensor surface. The effective penetration depth of the evanescent wave which arises under conditions of surface plasmon resonance is 0.3 µm. Therefore, refractive index changes occurring at distances less than 0.3 µm from the surface will cause a signal change. The bacteria probably do not penetrate the dextran layer (0.1 µm thick) which coats the gold surface. Thus, when bacteria bind to ligands bound to the dextran surface, only a small portion of the bacterial cell which is close to the sensor surface generates a response signal. Furthermore, the BIAcore® detection system measures an average SPR angle over an area of approximately 0.25 mm² of the sensor surface. Since bacterial cells are large and do not evenly cover an area measured, the response signal is decreased.

When antibodies are coupled to the sensor surface, it is probable that they are bound in different orientations, some of which may not allow binding of antigen to the antibody binding site. To ensure that antibody was bound in the proper orientation, a second assay format was designed. Protein A or Protein G was immobilized onto the sensor surface and used to capture the F_c portions of the monoclonal or polyclonal antibodies which in turn captured the bacteria through the binding sites. When the assay was performed using HBS at pH 7.4 and a Protein G sensor surface, injection of polyclonal antibody at a concentration of 200 µg/ml generated an RU of approximately 2752 (Fig. 2), and similar RU values were obtained in each analysis. The signal obtained following interaction of the *E. coli* O157:H7 (5×10^9 CFU/ml) with the protein G-bound antibody was 588 RU. Injection of *S. typhimurium*, *Y. enterocolitica*, or *E. coli* O103:H2 did not produce notable RUs (data

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Table 1. Interactions of immobilized Protein A and Protein G with monoclonal and polyclonal antibodies against *E. coli* O157:H7 and of bound antibody with *E. coli* O157:H7^{a,b}

Ligand	Antibody ^c	Resonance units generated with interactions			
		Using HBS at pH 5		Using HBS at pH 7.4	
		Antibody	<i>E. coli</i> O157:H7	Antibody	<i>E. coli</i> O157:H7
Protein A	Polyclonal	388	180	372	77
Protein G	Polyclonal	3174	310	2752	588
Protein A	Monoclonal	1712	220	1400	326
Protein G	Monoclonal	4240	613	1500	284

^aProtein A or Protein G were immobilized on the sensor surfaces. Either monoclonal or polyclonal antibodies were passed over the Protein A or G surfaces. Subsequently interactions of suspensions of *E. coli* O157:H7 with bound antibodies were measured. Results are from one experiment and similar results were obtained with repeated trials.

^bBacterial suspensions were prepared in HBS pH 5 or pH 7.4 at a concentration of 5×10^9 CFU/ml.

^cMonoclonal antibody 8-9H and polyclonal antibody were diluted in HBS at pH 5 or 7.4 at a concentration of 200 μ g/ml.

not shown). Interestingly, this assay format did not appear to enhance the capture of *E. coli* O157:H7 or to enhance the signal generated compared to use of the assay format in which antibody was directly immobilized onto the sensor surface (Fig. 1).

Both monoclonal and polyclonal antibodies bound at higher levels to Protein A and Protein G, in particular to Protein G, when they were prepared and tested in HBS, pH 5, than when tested in HBS, pH 7.4 (Table 1). However, the level of bacteria which subsequently bound to either monoclonal or polyclonal antibody was not influenced by the pH of HBS. This is in agreement with Medina and Palumbo¹¹ who found that optimum binding of sheep IgG to Protein G occurred using HBS, pH 5. They found that binding of the sheep IgG to Protein G was 7-fold higher than to Protein A as has also been reported by others¹⁵. The binding capacities of Protein A and G with antibodies derived from different animal species can vary. Under the binding conditions used in the present study, both monoclonal (from mouse) and polyclonal (from goat) antibodies bound at higher levels to Protein G, than to Protein A, especially at pH 5. According to Newman et al.¹⁵, the binding capacities of mouse IgG, subtype 2a, to Protein A and G and of goat IgG to Protein A and G are similar. However, binding is influenced by reaction conditions, particularly pH.

In addition to the direct binding of bacteria to sensor chips, an indirect approach was also employed for the detection of bacteria. This latter method entailed the initial reaction of anti-*E. coli* O157:H7 antibodies with viable *E. coli* O157:H7 followed by removal of unbound or "free" antibody and detection of the free antibody with a protein G-immobilized sensor chip. Prior to performing the indirect assay, a saturation curve for the protein G-immobilized sensor chip was established by flowing varying concentrations of anti-*E. coli* O157:H7 antibody across the surface. Antibody concentrations of ca 50 μ g/ml were determined to nearly saturate the Protein G immobilized on the sensor chip (data not shown). Subsequently, antibody concentrations no greater than 50 μ g/ml were used in the indirect assay for the detection of *E. coli* O157:H7. Results of the indirect assay, displayed in Figure 3 (inset), indicate that initial antibody concentrations ranging from 5 to 50 μ g/ml resulted in a detection limit of between 10^6 to 10^8 *E. coli* O157:H7 CFU/ml in a total assay time of less than 1 h per sample. A detection limit of ca 10^6 – 10^7 CFU/ml was obtained when using an initial antibody concentration of 5 μ g/ml which is somewhat lower than 5×10^7 CFU/ml obtained using the antibody sandwich assay described above. Conversion of the data for the indirect inhibition assay to percent inhibition and replotting the results (displayed in Fig. 3) facilitated visualization of the limits of detection corresponding to

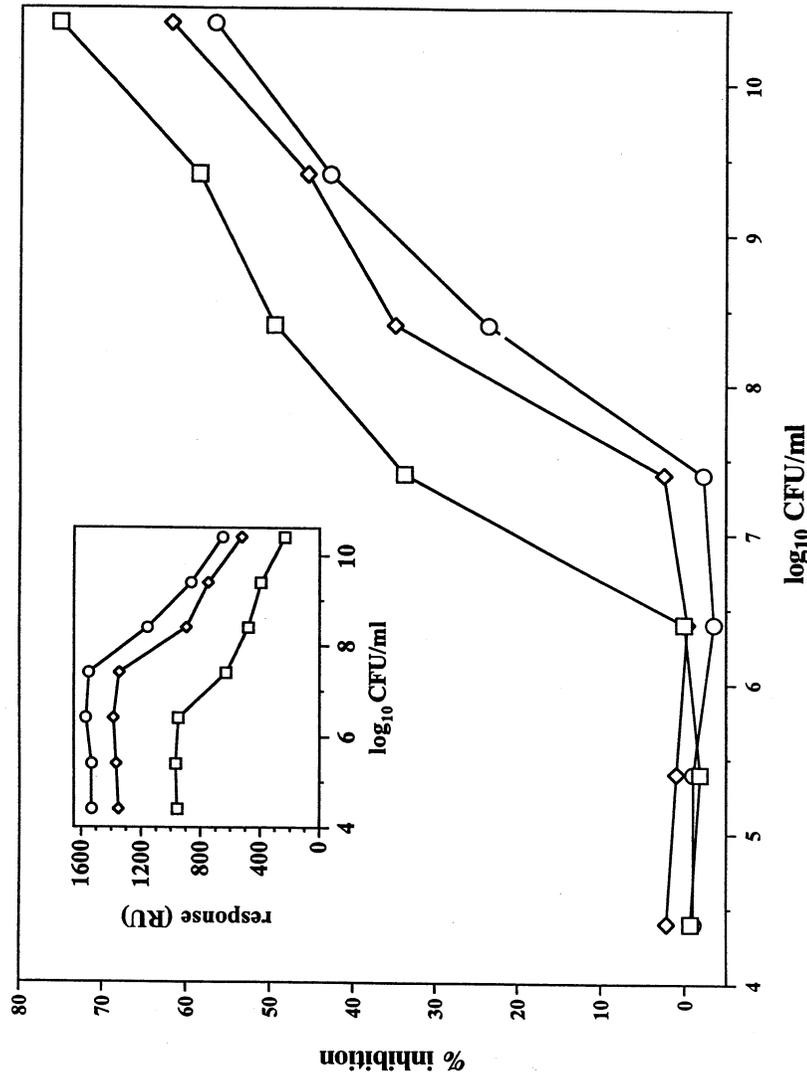


Figure 3. Indirect detection of whole *E. coli* O157:H7 cells using an inhibition assay format. *E. coli* O157:H7 cells were serially diluted in HBS (pH 5.0), aliquots (100 μ l) of the diluted cells were reacted with 200 μ l of anti-*E. coli* O157:H7 antibody (diluted in HBS, pH 5.0, to 5 μ g/ml [squares], 25 μ g/ml [diamonds], and 50 μ g/ml [circles]) for 30 min. Antibody not associated with the bacteria was separated through centrifugation and then flowed over a Protein G surface, thus effecting detection of the 'free' antibody concentration via surface plasmon resonance. (The inset displays the response (in RU) for the varying concentrations of bacteria that were tested).

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the varying initial antibody concentrations. Antibody concentrations of less than 5 µg/ml did not markedly improve the limit of detection (data not shown).

Antibody and Protein A and G surfaces were readily "regenerated." During regeneration, only layers of antibody and/or antigen are removed, leaving the covalently immobilized ligand undisturbed. Therefore, many separate analyses can be performed on a single flow cell of a sensor chip. In the present study, the sensor chip surface could be employed for at least 50 different analyses.

Sensitivity for detecting whole cells was somewhat inferior (10^6 – 10^7 CFU/ml) to that of standard ELISA-based assays. With improved BIAcore® system instruments which are currently becoming available (BIAcore® 1000, BIAcore® 2000) and new sensor chip designs, sensitivity should also be enhanced considerably. It is envisioned that the biosensor can be employed for rapid identification of bacterial colonies recovered from selective medium if appropriate antibodies are used. For examining interactions of small analytes, the lower limit of detection of the BIAcore® system which was used in this study is approximately 10 pg analyte/mm² (16). Reports have shown that the BIAcore® can be employed for monitoring levels of antibodies, hormones, veterinary drugs and antibiotics in foods and other materials even using crude samples^{5, 13, 15}. Medina et al.¹² monitored interactions of *E. coli* O157:H7 antibody with immobilized whole bacteria thus demonstrating the usefulness of the BIAcore® for studying interactions of cells/cell membranes with molecular analytes.

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