Rapid, Simple, and Sensitive Immunoagglutination Assay with SiO₂ Particles and Quartz Crystal Microbalance for Quantifying Schistosoma japonicum Antibodies

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Background: The resurgence of the parasitic disease schistosomiasis calls for more efficient diagnostic tests. We developed a rapid, simple, portable, and sensitive immunoagglutination assay that uses SiO₂ particles and quartz crystal microbalance (QCM) for quantifying Schistosoma japonicum (Sj) antibodies (SjAb).

Methods: As replacements for traditional latex microspheres, we prepared submicrometer-sized silica particles derivatized with Sj antigens to specifically agglutinate in the presence of SjAb targets, and we used the QCM monitor to measure the resulting frequency shifts. We optimized the assay medium by adding poly(ethylene glycol) (PEG) as a response accelerator of immunoagglutination. To minimize or eliminate any nonspecific agglutination or adsorption interferences, we conducted appropriate sealing procedures separately for silica particles and the QCM probe.

Results: The measured frequency changes were linearly related to the SjAb concentrations in infected rabbit serum. The PEG-assisted immunoagglutination system is quantitatively sensitive to SjAb concentrations ranging from −0.70 to 32.31 mg/L, with a detection limit of −0.46 mg/L. The obtained linear regression equation was: y = 43.61 x + 80.44 (r = 0.9872). Several serum specimens were evaluated by the developed QCM immunoassay in comparison with the ELISA, validating the feasibility of practical applications.

Conclusions: This novel immunoagglutination-based QCM detection format is rapid, simple to use, and more portable than conventional diagnostic immunoassays, thus offering a promising alternative tool that can be used for point-of-care clinical diagnosis of schistosomiasis, particularly in epidemic situations.
In recent years, the rapid development of materials with many improved properties for bioanalytical applications (3,10) has led to a resurgence of interest in the development of particle-enhanced immunoassays. Nano-meter- or submicrometer-sized particles may be suitable for designing innovative agglutination-based diagnostic tests (3,10–13). For example, Costanzo et al. used biotin-modified SiO2 nanoparticles for the agglutination-based analysis of avidin with a complex, dynamic light-scattering instrument, enabling investigation of aggregation rates for various-sized nanoparticle mixtures (14). Because silica (SiO2) particles have higher density (~2 g/mL) (3,10,15), greater surface-to-volume ratio, and more rugged surfaces than do polymeric latex beads, small SiO2 particles may facilitate larger anchoring of immunoreactive entities. Moreover, SiO2 particles with hydroxyls on their surfaces are more hydrophilic and less susceptible to physical deformation than polymeric latex microspheres and thus are less likely to produce hydrophobic interactions, a potential cause for aggregation or instability. In addition, chemically functionalized SiO2 particles may provide a sol-gel matrix for firm and efficient binding of Sj antigens (SjAg) from adult worms or eggs, which are labile, large-sized, and otherwise difficult to immobilize. In the present work, we used submicrometer-sized SiO2 particles, sensitized with SjAg, as replacements for latex microspheres in a quantitative LPEIA alternative method for measuring Sj antibodies (SjAb) in infected rabbit serum (IRS) and human serum specimens.

**Materials and Methods**

**APPARATUS**
The transmission electron microscope (TEM; Hitachi-H800) was from Hitachi, Ltd. Copper grids (No. 50–230) were provided by Scientific Instruments Ltd. of the Chinese Academy of Science (Beijing, China). The quartz crystal analyzer was obtained from SEIKOEI&G Co. Ltd., and QCMs (9 MHz, gold electrodes), which we operated at their fundamental frequencies, were from Chenxing Radio Equipment. One side of the QCM was sealed with an O-ring of silicone rubber. The ultrasonic rinse implement (B2205-T) was from Binengxin Ultrasonic, Ltd. The platform-type centrifuge was from Pingfan Instruments, Ltd. A laboratory-made detection cell was used for the agglutination-based immunoassay, in which an optimized assay buffer solution was gently agitated with a magnetic stirrer. The experimental temperature was controlled at 25 °C with a thermostat obtained from Chongqing Experimental Equipment (Model CSS 501).

**REAGENTS**

3-(Aminopropyl) trimethoxysilane was purchased from Sigma-Aldrich; tetraethyl orthosilicate (TEOS) from Wulan Chemicals; ammonia, ethanol, methanol, and poly(ethylene glycol) (PEG, M₆,000) from Tiantai Fine Chemistry and Engineering Company; and glutaraldehyde (GLU) from Wuhan Organic Chemistry Reagent Company. SjAg (4.2 g/L, M, 32,000) were obtained from soluble homogenate of adult Sj worms through an elaborated purification procedure as described in (16). Sj antibodies ([SjAb] 2.1 g/L) were obtained by purifying the immunoglobulins of IRS. Normal rabbit serum (NRS), IRS, and human serum specimens were kindly provided by the Institute of Schistosomiasis, Xiangya School of Medicine, Central South University (Hunan, China). Bovine serum albumin (BSA) was purchased from Shanghai Shensuo Biological Products. Phosphate buffered saline (PBS) solutions of various pH were prepared with 0.01 mol/L Na₂HPO₄ and 0.01 mol/L KH₂PO₄. The Piranha reagent was a mixture of H₂O₂ and H₂SO₄ at a volume ratio of 3:7. All other reagents were of analytical reagent grade. Doubly distilled water was used throughout the experiments.

**PREPARATION AND IMMUNOSENSITIZATION OF SILICA PARTICLES**

*Synthesis and surface functionalization of SiO₂ particles.* We prepared SiO₂ particles with the tetraethyl orthosilicate hydrolyzation procedure with ammonia as the catalyzer and PEG as the surfactant, according to the method reported elsewhere (17). The submicrometer-sized silica particles were ultrasonically washed in methanol and water, each for 10 min, and were sequentially centrifuged at ~1200 rpm to eliminate any unreacted chemical substances.

A portion of the dried SiO₂ particles (~1.0 g) was ultrasonically resuspended in the mixed solution of methanol (10 mL) and 3-(Aminopropyl) trimethoxysilane (0.50 mL) and stirred overnight at room temperature so as to complete the silanized reaction. Then the mixture was isolated by centrifuge and washed for several times with methanol and PBS solution (pH 7.0). The resulting amine-functionalized SiO₂ particles were stored in PBS solution (pH 7.0, 4.0 mL) containing 1.5% PEG at 4 °C.
Preparation of SjAg-derivatized SiO2 particles. The prepared amine-functionalized SiO2 particle suspension (10 mL) was ultrasonically resuspended in 2.5% GLU (5.0 mL), stirred for 3 h at room temperature to achieve the aldehyde-activated surface, and then washed twice with water. The resulting silica particles were suspended in the PBS solution (pH 7.0, 4.0 mL), followed by the addition of a portion of SjAg solution (4.2 g/L, 1.0 mL) with continual stirring for 3 h at room temperature. After being washed twice with water, the obtained SjAg-derivatized SiO2 particles were resuspended in PBS solution (pH 7.0, 4.0 mL). We then introduced an appropriate BSA-NRS reagent consisting of 10 g/L BSA and NRS at a 1:1 volume ratio into the silica suspension, which was then stirred for 1.5 h to block the unreacted aldehyde and any nonspecific adsorption sites on the silica surfaces. Furthermore, we isolated the SjAg-derivatized silica suspension centrifuge and washed it twice with PBS solution (pH 7.0). Finally, the resulting immunosensitized SiO2 particles were stored in PBS solution (pH 7.0, 4.0 mL) containing 1.5% PEG at 4 °C. This preparation may remain stable with no considerable change in immunoactivity for ~3 months, but the silica particle suspensions should be ultrasonically resuspended before use.

TEM MEASUREMENTS
We dropped 15 μL of the immunoagglutination assay solution with SjAg-derivatized SiO2 particles of ~1.76 × 10¹⁵ beads/mL in the presence of 21.0 mg/L SjAb onto a copper grid. Another portion of SjAg-sensitized silica particle suspension without added SjAb was processed in parallel as the control test. Each of the grids was then drained on tissue paper and air-dried. We then carried out separate high-resolution TEM measurements (images shown in Fig. 1).

IMMUNOAGGLUTINATION ASSAY PROCEDURE
Before analysis, the clean QCM probe was incubated with a portion of BSA solution (10 g/L, 20 μL) for 1 h at 37 °C to block any protein-nonspecific adsorption sites on the crystal. The BSA-modified QCM probe was then inserted into the detection cell with 3.0 mL of the optimized assay buffer solution (pH 7.0, containing 1.5 g/L PEG and 40 mmol/L NaCl). A schematic diagram of the total QCM system setup is shown in Fig. 2. Under gentle stirring and thermostatic control, 80 μL SjAg-derivatized SiO2 particle suspension was introduced with the microinjector into the assay solution. After stabilization of the resonance frequency (shift <~1.0 Hz/min) within several minutes, 25 μL of each serum sample to be analyzed was separately injected into the detection cell. The induced frequency shifts were monitored in real time by the quartz crystal analyzer, and the data were displayed and recorded by the linking computer. To avoid possible errors from different additions of analytes and any nonspecific background adsorption, we recorded the frequency responses to the analytes from 30 s after analyte addition until equilibrium was reached. For all experiments, unless otherwise indicated, the recorded frequency responses were the mean (SD) frequency changes of triplicate measurements.

At the completion of each detection assay, the assay buffer solution and the silica immunoagglutinated entities were discharged from the detection cell, which we then rinsed twice with PBS. Moreover, QCM probes used repeatedly for ~3–5 immunoassay runs were treated with Piranha reagent for ~5 min to peel off any proteins from the crystal, then rinsed twice with water and air-dried. The regenerated QCM probes were remodified with BSA for the next assay cycle.

Results and Discussion
IMMUNOAGGLUTINATION QCM ASSAY WITH SILICA PARTICLES
QCM devices are highly sensitive to changes of the surface mass loading of adsorbates and the interfacial properties relating to some nonmass response factors of the test solution, such as the density (ρ), viscosity (η), and conductivity (18–22). We used submicrometer-sized SiO2 particles as replacements for traditional latex micro-
spheres to label SjAg for a new QCM immunoagglutination assay. The specific agglutination of SjAg-derivatized SiO2 particles in the presence of SjAb targets induced a primarily linear change of $(\rho \eta)^{1/2}$ leading to proportional frequency shifts of the crystal, which can be derived from the pioneering theory model (equation) for a QCM in contact with liquid (21–22). The measured frequency changes should be linearly related to SjAb concentrations in samples to be quantified. The silica immunoagglutination events in the presence and absence of SjAb were comparably characterized by the high-resolution TEM (Fig. 1). Before the immunoagglutination, silica particles in the suspension are mostly spherical, uniform, and monodispersed, with an estimated mean diameter of ~280 nm (Fig.1B). When the immunoagglutination occurred in the presence of SjAb, the TEM image fields were dominated by large micrometer-sized aggregations (Fig. 1B), which may be the source of the changes in the solution parameters (i.e., $\rho$ and $\eta$) that induced the frequency shifts of the QCM probe.

The submicrometer-sized silica particles used in these experiments have properties that may make them more suitable for immunoagglutination assays than are common polymer latex (i.e., polystyrene) microspheres. These special properties include larger surface-to-volume ratio, higher density, more hydrophilic surface, and higher colloidal stability. Moreover, silica particles derivatized with amine groups by silanization might provide an efficient sol-gel matrix for increased binding of SjAg through entrapment and GLU-mediated covalence interactions. Therefore, a silica particle-enhanced immunoagglutination assay with the highly sensitive QCM measurements might be expected.

**DOSAGE OF IMMUNOSENSITIZED SILICA PARTICLES**

We used SjAg-derivatized silica suspensions with various particle concentrations to investigate the effects of immunosensitized SiO2 particle dosage on silica immunoagglutination assays for SjAb serum samples (Fig. 3). The

![Fig. 2. Schematic diagram of the total QCM system setup for the immunoagglutination-based detection of SjAb. QCM, quartz crystal microbalance.](image)

![Fig. 3. The dosage-dependence of the silica immunoagglutination on the SjAg-derivatized SiO2 particles ($C_{SjAg-SiO2}$), where the frequency changes ($\Delta f$) were obtained by immunoagglutination assays for 21.0 mg/L SjAb serum in the optimized assay medium with SjAg-derivatized silica suspensions of various concentrations.](image)
frequency responses obtained for the immunoagglutination assays peaked at $\sim 1.76 \times 10^{15}$ beads/mL of the silica particles (Fig. 3). Particle concentrations that are too high or too low may lead to a serious imbalance of antigen-antibody ratios and thus decrease formation of lattice-like immunocomplexes (23), resulting in very little change $(\pm n)^{1/2}$ of the assay solution or, conversely, steric hindrance of silica agglutination that is too high, as manifested by frequency responses.

OPTIMIZATION OF IMMUNOAGGLUTINATION ASSAY MEDIUM COMPOSITION
Because composition of the assay buffer may play a vital role in controlling the physicochemical processes of immunological recognition and agglutination (13, 24–25), we sought to optimize the composition of the reaction medium for the immunoagglutination assays within required ranges of pH, ionic strength, and surfactants.

We investigated the effects of pH values on the frequency responses of silica immunoagglutination (see Fig. 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol52/issue11). The biggest frequency response was obtained with the assay solution at pH 7.0. Such a neutral condition might optimize the recognition of SjAb by SjAg-sensitized silica particles and subsequently the agglutination by appropriate electrostatic forces, because the immunoagglutination interaction process might involve pH-dependent charged protein species. Moreover, it was found that the frequency responses tend to slightly increase when pH $< 2.0$, presumably because of the nonspecific acid agglutination of silica particles.

We investigated the optimum ionic strength for the silica immunoagglutination assays by adding various concentrations of NaCl to the assay buffer solution (see Fig. 2 in the online Data Supplement). We observed that the frequency responses to immunoagglutination increased with the increase in NaCl concentrations, up to 40 mmol/L. When the concentrations of added salt were increased to an ionic strength $> 40$ mmol/L, a gradual decrease in the frequency response occurred. Moreover, suitable increases in NaCl concentrations experimentally increased the immunoagglutination response rates (data not shown), suggesting that salt in the test solution might in some way promote the immunorecognition/agglutination of SiO$_2$ particles. However, excessive ionic strength in the test solution ($> 40$ mmol/L NaCl, the optimum ionic strength in test solution) may hamper silica immunoagglutination, inducing nonspecific precipitation of labile substances in the samples.

PEG as a polyelectrolyte surfactant has been commonly used as the accelerator or precipitant of immunoagglutination for various homogeneous immunoassays. We studied the promotion effects of PEG on the silica immunoagglutination by adding PEG to the assay buffer solution and compared frequency responses between the immunoagglutination assays with and without PEG (see Fig 3 in the online Data Supplement). Use of PEG could obviously speed the immunoagglutination to equilibrium and amplify the frequency response signal, shifting from $\sim 332$ Hz to $\sim 565$ Hz, possibly because the steric exclusion contributed by PEG could accelerate silica immunoagglutination in the presence of the complementary targets (13, 25).

DEPRESSION OF NONSPECIFIC SILICA IMMUNOAGGLUTINATION
Nonspecific silica agglutination adversely affects analytical performances of the immunoagglutination QCM assays through the nonspecific surface adsorption and self-aggregation of silica particles. By use of BSA-NRS reagent to treat SjAg-derivatized SiO$_2$ particles, we blocked blank sites on silica particle surfaces that might conduct nonspecific adsorptions. We observed a difference in frequency change of $\sim 100$ Hz for SjAg-derivatized silica particles treated with BSA-NRS compared with the untreated particles (see Fig. 4). The silica particles were disposed of or stored in PBS solution containing 1.5% PEG, in which PEG chains may be entangled on the anchored silica particle surface, forming steric and electrostatic barriers to the self-aggregation of these particles, even at high ionic strength, as observed elsewhere for the polymer microspheres (26–27). Additionally, premodification of the QCM probe with BSA could greatly reduce or eliminate adventitious nonspecific adsorption on the crystals of proteins or molecules from complex sample backgrounds.

CHARACTERISTICS OF FREQUENCY RESPONSE FOR IMMUNOAGGLUTINATION
We investigated characteristics of time-dependent frequency response for silica immunoagglutinations under
optimized assay conditions, in which the immunoaggregation processes were monitored in real time by the QCM system (Fig. 4). The observed frequency shift trends were quite different for IRS vs NRS as the control test. The response values obtained for the former (Fig. 4B, ∼567 Hz) were 3-fold greater than that for the latter (Fig. 4A, ∼121 Hz), indicating that the frequency response to the nonspecific agglutination of NRS (the blank or noise signal) may be insignificant. These observations may demonstrate that the silica immunoagglutination is attributable only to specific SjAg-SjAb interactions. The total time for the specific silica immunoagglutination process was ∼35 min, which may vary with the target SjAb concentrations in the samples.

**QUALITATIVE ANALYSIS**

We investigated the quantitative analysis capabilities of the developed immunoassay under optimized conditions with a series of serum samples with various SjAb concentrations. The calibration curve describing the relationship between the frequency changes (ΔF) of immunoagglutination and SjAb concentrations is shown in Fig. 5. The ΔF values were highly linear for SjAb concentrations of ∼0.70–32.31 mg/L. The detection limit was estimated to be ∼0.46 mg/L according to the 3 SD rule, defined as the concentration at the mean 3 SD of several determinations (here, 6 determinations) of the blank or zero calibration (28). The response signals may saturate at >32.31 mg/L SjAb, presumably because of the limited amount of SjAg available on the surfaces of silica particles; moreover, a value of 21.0 mg/L SjAb serum was assayed 5 times. The obtained mean (SD) frequency change was 563 (57) Hz, with a relative SD of 10.3%, reflecting good analytical reproducibility of the proposed technique.

**SPECIMEN EVALUATION**

The developed QCM immunoagglutination system was applied to evaluate several unpurified IRS specimens with various degrees of Sj infection, denoted by the numbers of Sj cercarias (parasitic larvae) and days of infection. The results of frequency shifts were recorded in real time (see Fig 4 in the online Data Supplement). We observed that frequency responses increased with an increase in the degree of Sj infection of the samples. For example, IRS specimens infected by 1000 Sj cercarias for 31, 45, and 54 days corresponded to mean (SD) frequency changes of 312 (34) Hz, 449 (38) Hz, and 506 (42) Hz, respectively. Meanwhile, the frequency changes obtained for the IRS samples infected for 54 days with varying Sj cercarias numbers of 1000, 2000, and 3500 were 506 (42) Hz, 622 (59) Hz, and 815 (67) Hz, respectively. Accordingly, the developed immunoagglutination assay can easily define the Sj infection degree of IRS specimens. Of note, positive samples from patients may be identified by frequency response values >3 times that of the blank in the control test.

To further investigate the feasibility of the developed QCM immunoassay for practical applications, we analyzed 8 human serum specimens obtained from patients living in endemic schistosomiasis areas, and compared our SjAb titer results with those obtained by ELISA performed at the Institute of Schistosomiasis, Central South University (Fig. 6). By comparing the results, we obtained a regression equation of y = 1.009 x + 61.68, with a correlation coefficient of 0.9827. Moreover, the statistically significant difference estimated by the common F-test showed no significant difference (P > 0.05) between the results of the 2 methods. In particular, as can be seen from Fig. 6, they showed consistent results in identifying specimens with very low or zero SjAb titers.
These results indicate that the QCM immunoassay should be comparable to ELISA in terms of analytical sensitivity and specificity, providing an alternative tool in rapid clinical diagnosis, particularly for epidemic screening of schistosomiasis.

**Conclusion**

We have successfully extended LAT technology to develop a silica particle-enhanced immunoagglutination assay for quantifying SjAb through sensitive QCM measurements. The proposed assay enables quantitative detection of SjAb concentration as low as ~0.46 mg/L. Specimens with different degrees of Sj infection can be easily identified with no sample pretreatment. Our results for human serum specimens demonstrate that the detection sensitivity and specificity of the QCM immunoassay are comparable to those of ELISA. In addition, our portable system offers advantages over the conventional method in that the analytical operations are more rapid, easier to perform, and less expensive. The new QCM method can be tailored as a bedside or field-applicable tool for rapid clinical diagnosis and screening of schistosomiasis in epidemic situations, and can be further integrated and miniaturized as a commercial chip. Moreover, this novel technology might be extended to other immunoagglutination tests for wider diagnostic applications.

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**References**