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In Situ Assembly of Colloidal Particles into Miniaturized Biosensors

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We show how to create arrays of biosensors by in situ assembly of colloidal particles onto micropatterned electrodes. Latex microspheres from suspension are collected via dielectrophoresis in the micrometer-sized gaps between planar electrodes. The assembled particulate patches are fixed by changing the colloidal interactions to induce coagulation. Immuno-active sites on the latex surfaces bind the target molecules. A direct electric conductivity readout is accomplished after secondary tagging with colloidal gold and its enhancement by silver nucleation. The method holds promise for creating disposable on-chip arrays of highly sensitive miniature sensors for specific proteins, DNA fragments, or other biomolecules.

Introduction

Recent research in the assembly and fabrication of micrometer- and nanometer-sized colloidal structures[1−8] has shown promise for the creation of novel materials and devices.[9−15] Of particular interest are devices in which electronic microchips are created out of, and interface with, fluid-borne colloidal and biological systems. New directions in this research have been provided by lithographic technologies for the fabrication of micropatterned substrates.[8,10−14] Besides providing a well-defined organized template for colloidal assembly, lithographically patterned substrates also allow electrical manipulation of colloidal systems and living cells on a micron scale.[16−19] As we show here, similar techniques allow for the creation of minia-

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(1) Fendler, J. H. *Chem. Mater.* **1996**, *8*, 1616.
(2) Ahlers, M.; Müller, W.; Reichert, A.; Ringsdorf, H.; Venzmer, J. *Angew. Chem., Int. Ed. Engl.* **1990**, *29*, 1269.
(3) (a) Denkov, N. D.; Velev, O. D.; Kralchevsky, P. A.; Ivanov, I. B.; Yoshimura, H.; Nagayama, K. *Nature* **1993**, *361*, 26. (b) Nagayama, K. *Colloids Surfaces A* **1996**, *109*, 363.
(4) (a) Chiruvolu, S.; Walker, S.; Israelachvili, J.; Schmitt, F.-J.; Leckband, D.; Zasadzinsky, J. A. *Science* **1994**, *264*, 1753. (b) Velev, O. D. *Adv. Biophys.* **1997**, *34*, 139. (c) Walker, S. A.; Kennedy, M. T.; Zasadzinski, J. A. *Nature* **1997**, *387*, 61.
(5) Velev, O. D.; Furusawa, K.; Nagayama, K. *Langmuir* **1996**, *12*, 2374.
(6) Trau, M.; Saville, D. A.; Aksay, I. A. *Science* **1996**, *272*, 706.
(7) Tsukruk, V. V. *Prog. Polym. Sci.* **1997**, *22*, 247.
(8) (a) Dinsmore, A. D.; Crocker, J. C.; Yodh, A. G. *Curr. Opin. Colloid Interface Sci.* **1998**, *3*, 5. (b) Huck, W. T. S.; Tien, J.; Whitesides, G. M. *J. Am. Chem. Soc.* **1998**, *120*, 8267.
(9) (a) Velev, O. D.; Jede, T. A.; Lobo, R. F.; Lenhoff, A. M. *Nature* **1997**, *389*, 447. (b) Yang, P. D.; Deng, T.; Zhao, D. Y.; Feng, P. Y.; Pine, D.; Chmelka, B. F.; Whitesides, G. M.; Stucky, G. D. *Science* **1998**, *282*, 2244.
(10) Groves, J. T.; Ulman, N.; Boxer, S. G. *Science* **1997**, *275*, 651.
(11) van Blaaderen, A.; Ruel, R.; Wiltzius, P. *Nature* **1997**, *385*, 321.
(12) (a) Xia, Y.; Whitesides, G. M. *Langmuir* **1997**, *13*, 2059. (b) Xia, Y. N.; Whitesides, G. M. *Angew. Chem., Int. Ed. Engl.* **1998**, *37*, 551.
(13) Burmeister, F.; Schäfle, C.; Keilhofer, B.; Bechinger, C.; Boneberg, J.; Leiderer, P. *Adv. Mater.* **1998**, *10*, 495.
(14) Braun, E.; Eichen, Y.; Sivan, U.; Ben-Yoseph, G. *Nature* **1998**, *391*, 775.
(15) (a) Decher, G.; Eckle, M.; Schmitt, J.; Struth, B. *Curr. Opin. Colloid Interface Sci.* **1998**, *3*, 32. (b) Musick, M. D.; Keating, C. D.; Keefe, M. H.; Natan, M. J. *Chem. Mater.* **1997**, *9*, 1499. (c) Freeman, R. G.; Grabar, K. C.; Allison, K. J.; Bright, R. M.; Davis, J. A.; Guthrie, A. P.; Hommer, M. B.; Jackson, M. A.; Smith, P. C.; Walter, D. G.; Natan, M. J. *Science* **1995**, *267*, 1629. (d) Asher, A.; Holtz, J.; Weissman, J.; Pan, G. *MRS Bull.* **1998**, *23*, 44.
(16) Pethig, R.; Huang, Y. H.; Wang, X.-B.; Burt, J. P. H. *J. Phys. D: Appl. Phys.* **1992**, *24*, 881.

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turized biosensors by assembly of colloidal particles onto microchip substrates.

Immunological tests are usually performed in practice by means of latex agglutination assays that are simple and versatile tools for immunodetection.[20−22] However, agglutination tests have a number of disadvantages, including the need for a relatively large amount of sample, ambiguous optical detection, and unsuitability for miniaturization and simple electronic readout. Electronic immunosensors to date are typically based on surface plasmon resonance, total internal reflection, or piezoelectric oscillators (e.g., refs 23−26), but they tend to be complex to align and use, require a relatively large surface for sample adsorption, and may not be suitable for disposable assays.[23,25,27] Even when miniaturization is possible via, e.g., ink-jet or screen-printing techniques,[26] the precise deposition and readout of microscopic bioactive elements is difficult.

In this paper, we describe a method in which microscopic sensor patches are assembled in situ from the same latex particles used in traditional agglutination assays. The glass substrates for our sensors carry photolithographically fabricated gold patterns that form addressable electrodes of micron size with small gaps between them. To generate the active area of the sensors, the particles, suspended in a low-electrolyte aqueous medium, are collected in the gaps between the electrodes under the action of a nonuniform alternating electric field, an effect known as dielectrophoresis.[28] The mere collection of the microspheres is, however, not enough to produce sensor patches, as the latexes are stabilized by either electrostatic or steric repulsion,[29] and so disassemble when the field is turned off. The key step in particle immobilization into sensor patches is to decrease the repulsive interactions to a degree where the van der Waals and hydrophobic attractive forces[29] coagulate the particles. Particles that are stabilized by electrostatic repulsion from charged groups can be coagulated by slow adsorption of an oppositely charged surfactant on the surface. Particles stabilized by nonionic surfactant are easily bound together by washing away the adsorbed steric protective layer. The assembly procedure can be repeated many times by exchanging the particle suspension and addressing different gaps so that different sensors can be assembled on the same "chip".

After the sensor patches are assembled, they are washed in saline containing serum protein and nonionic surfactant. The protein and surfactant in the medium restore the steric protective coat on the already assembled particles, block subsequent nonspecific adsorption, and allow only immunospecific binding events to take place and be discriminated. The sensor patches on the chips are then incubated with medium that may carry a target immune molecule, which in our model experiments was human immunoglobulin (IgG). The biospecific agent attached to the latex particles in this case is protein A, which binds to the Fc portion of IgG molecules. The schematics of the main stages involved in the preparation and read-out of the biosensors are presented in Figure 1.

If IgG molecules are present, the latexes are coated with an IgG layer. The detection of the test results is carried out by first tagging the IgG molecules with colloidal gold[30,31] conjugated to a secondary antibody, in this case, antihuman IgG. This yields a layer of strongly and specifically attached colloidal gold particles around the latex microspheres. Before the test results are read, the gold particles are enlarged and fused together by a metastable solution of silver salts, which via nucleation deposits a silver layer on top of the gold (this is called "silver enhancement"[31]). If the test results are positive, the electrodes become effectively short-circuited. The direct readout is then carried out by simply measuring the resistance between the electrodes.

Experimental Section

Substrates. The pattern from an e-beam-generated mask was photolithographically transferred onto 24 × 24 × 1 mm glass slides. Layers of 20 Å of Ti and 1500 Å of Au were deposited in the etched photoresist by vacuum evaporation to form the electrode patterns. The free glass surface was cleaned and hydrophilized by 15 s treatment in 10% HF. Before each experiment, the substrates were cleaned by immersion for 30 min in a strong oxidizer (Nochromix), followed by abundant washing with deionized water.

Setup. The "chips" were mounted on the stage of an Olympus BH2 microscope, allowing observation in both transmitted bright-field and fluorescent illumination. The source of the AC current was a Beckmann FG3A signal generator. Each of the four addressable electrodes was connected to the AC source through a 3 kΩ resistor and a switch that grounds the electrode when disconnected from the generator. The shape and the amplitude of the signal were monitored by an oscilloscope and Keithley 179A multimeter. The resistance of the electrode pairs was measured by a Radio Shack 22-168A multimeter. After the experiments some of the chips were covered by a sputtered gold layer and examined on a JEOL 840 scanning electron microscope (SEM) at an accelerating voltage of 15−20 KV.

Further experimental details are provided in the Supporting Information.

Results and Discussion

The glass substrates carried four pairs of 30 μm wide gold electrodes, each connected to contact pads near the plate periphery and having a gap of 7−15 μm in the middle. A low-magnification optical image of the active sensor area is shown in Figure 2A. Before the experiments, a small flow-though glass chamber of volume ≈5 μL with adjacent "corrals" for liquid insertion and removal was assembled on top of the electrode area, the substrates were fixed on the stage of a fluorescence microscope, and electric leads were attached near the edges.

Two types of Protein A covered latexes were used in the assembly of the IgG sensitive elements, namely, pure

(17) (a) Fuhr, G.; Müller, T.; Schnelle, Th.; Hagedorn, R.; Voigt, A.; Fiedler, S. *Naturwissencschaften* **1994**, *81*, 528. (b) Müller, T.; Gerardino, A.; Schnelle, Th.; Shirley, S. G.; Bordoni, F.; De Gasperis, G.; Leoni, R.; Fuhr, G. *J. Phys. D: Appl. Phys.* **1996**, *29*, 340.
(18) Pethig, R. *Crit. Rev. Biotechnol.* **1996**, *16*, 331.
(19) Fuhr, G.; Shirley, S. G. *Top. Curr. Chem.* **1998**, *194*, 83.
(20) Bangs, L. B.; Meza, M. B. *IVD Technol.* **1995**, *17*, 20.
(21) Bangs, L. B. In *Applying Latex Based Technology in Diagnostics Biotechnology*; Seaman, G. V. F., Pollock, C. L., Eds.; Health and Sciences Comm.: Washington, DC, 1990; pp 79−102.
(22) Bangs, L. B. In *Liquid and Surface-Borne Particle Measurement Handbook*; Knapp, J. Z., Barber, T. A., Lieberman, A., Eds.; Marcel Dekker: New York, 1996; Chapter 15.
(23) Morgan, C. L.; Newmann, D. J.; Price, C. P. *Clin. Chem.* **1996**, *42*, 193.
(24) Kress-Rogers, E., Ed. *Handbook of Biosensors and Electronic Noses*; CRC Press: Boca Raton, FL, 1997.
(25) Collings, A. F.; Caruso, F. *Rep. Prog. Phys.* **1997**, *60*, 1397.
(26) Lambrechts, M.; Sansen, W. *Biosensors: Microelectrochemical Devices*; IOP Pub.: Philadelphia, 1992; pp 98−155.
(27) Cornell, B. A.; Braach-Maksvytis, V. L. B.; King, L. G.; Osman, P. D. J.; Raguse, B.; Wieczorek, L.; Pace, R. J. *Nature* **1997**, *387*, 580.
(28) Pohl, H. A. *Dielectrophoresis*; Cambridge University Press: Cambridge, 1978.
(29) Israelachvili, J. N. *Intermolecular and Surface Forces*; Academic Press: London, 1992.
(30) Hayat, M. A., Ed. *Colloidal Gold: Principles, Methods, and Applications*; Academic Press: San Diego, 1989.
(31) Hayat, M. A., Ed. *Immunogold−Silver Staining: Principles, Methods and Applications*; CRC Press: Boca Raton, FL, 1995.

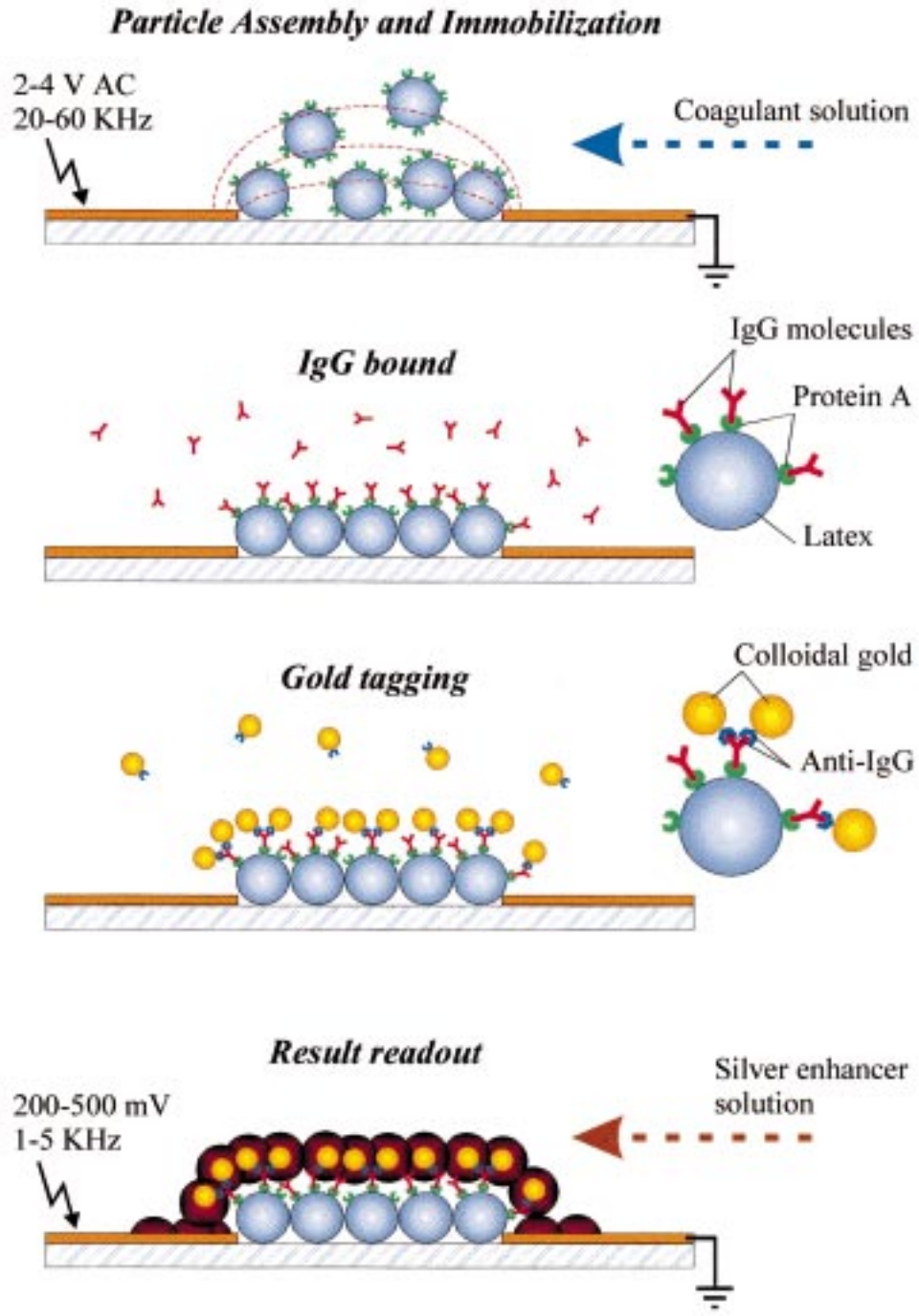

Figure 1. Schematics of the main stages of sensor assembly and functioning. The procedure is illustrated by an immunoglobulin test.

polystyrene beads and beads with magnetic material encapsulated in the polymer shell. Although working elements could be made from either type, the magnetic beads were preferable because of their easier cleaning, washing, and stronger dielectrophoretic responsiveness. Uncoated fluorescent beads were used as model particles for negative control experiments. The AC fields can cause both attraction or repulsion of the beads from the electrode gap, and the optimal dielectrophoretic conditions depend on the type of the latex used.[16,17]AC (3 V) square wave fields of frequency 20−60 MHz proved universally applicable to our systems.

A microphotograph sequence of experimentally observed events is shown in Figure 2. First, the alternating field is applied to the leftmost pair of electrodes and a bridge of the paramagnetic Protein A covered latexes is collected (the bridge is somewhat uneven because the particles used were partially aggregated). When proteins are covalently coupled to latex microspheres, some of the charged groups on the surface are used to create chemical bonds, which compromises the stability of the particles. For this reason, the microspheres are stabilized with an adsorption layer of the nonionic surfactant Tween 20, which can be desorbed by washing with water, incurring particle aggregation.[20] Thus the Protein A covered latexes collected in the bridge were assembled within 3−10 min by a few successive washes with deionized water. Once the first patch is assembled (Figure 2B), the AC field to that pair of electrodes is turned off, and the electrode area is incubated with a suspension of nonfunctionalized sulfate yellow-

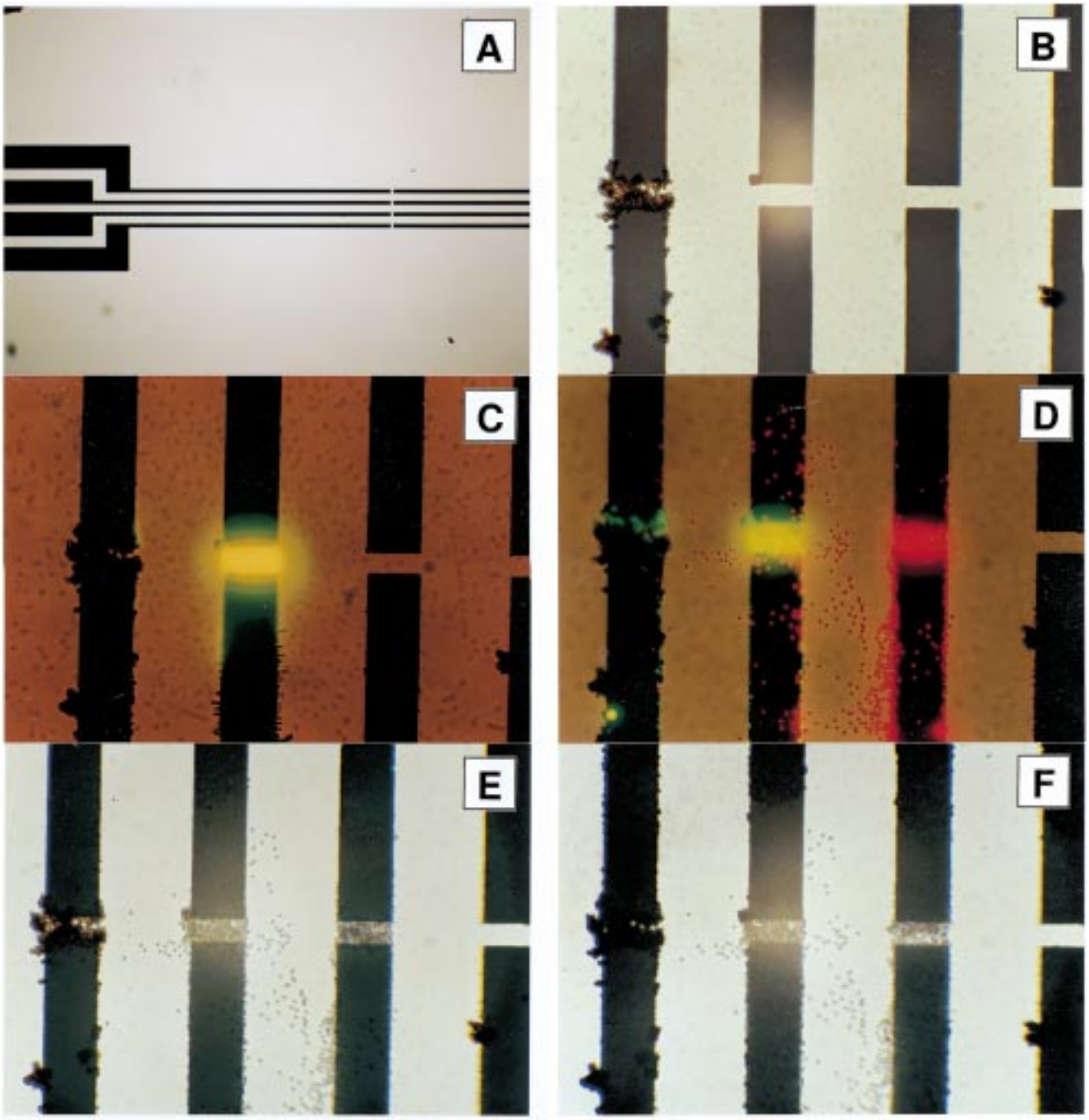

Figure 2. Microphotographs of the sensor area during an experimental run. The scale in the frames is given by the width of the electrodes (30 μm). (A) Low-magnification view of the active area with the four pairs of addressable electrodes. (B) High-magnification view of the electrode gaps after magnetic protein A-functionalized particles have been collected by an AC field at the leftmost electrode pair and coagulated; (C) A second pair of electrodes is energized, and nonfunctionalized sulfate yellow-green fluorescent latex beads are collected there to form a "negative control" sensor. Microphotograph in combined fluorescent and bright-field illumination; (D) A third model sensor from fluorescent red carboxylate latex is assembled between the third pair of electrodes. (E) Bright-field photograph of the three sensor patches before the immunospecific experiment. (F) Picture after the "chip" has been treated with human IgG, tagged with anti-human IgG gold and silver stained. The left electrode has become short-circuited by the metal layer (black) deposited around the functionalized particles. Note the low background on the other two latex patches.

green fluorescent latexes. When the second pair of electrodes is energized, these particles are collected in the second gap (Figure 2C). Sulfate and carboxylic latexes have high negative surface charges that cannot be removed by washing. They were coagulated with a saturated solution of the cationic surfactant dimethyldipalmitylammonium bromide (DMDPB). DMDPB, which is very slightly soluble in water, adsorbs almost irreversibly on the mostly hydrophobic surface of the latex beads and causes particle coagulation in 20−30 s. A bridge of yet another type of fluorescent latex (nonfunctionalized carboxylic beads) is assembled by the same procedure between the third pair of electrodes (Figure 2D,E). Thus the method can create arrays of *different* addressable sensors of micron dimensions.

The sensor area is then washed with phosphate-buffered saline containing 0.3 wt % bovine serum albumin and 0.1 wt % Tween 20 and is incubated for 15 min with 50 μg/mL solution of human IgG in the same medium. Only the leftmost particles in Figure 2E carry Protein A and are

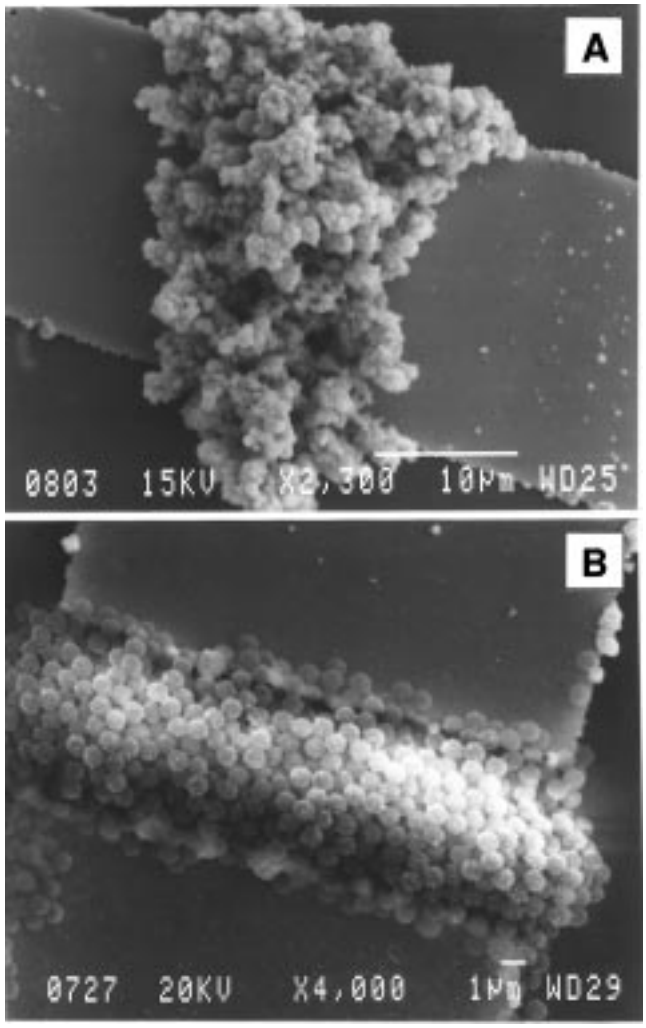

Figure 3. SEM micrographs of gold-tagged and silver-enhanced latex bridges between electrodes in IgG-specific experiments. (A) Protein A-functionalized latexes heavily coated by deposited metal that short-circuits the electrodes. (B) Nonfunctionalized particles in the negative control patches only marginally tagged by deposited metal. The bridge remains nonconductive.

able to bind specifically the IgG molecules from solution. The electrode area is then thoroughly washed with saline and incubated with a diluted suspension of 5 nm colloidal gold conjugated to goat antihuman IgG. The colloidal gold can be specifically attached only to the leftmost IgG sensor. The other two latex patches in this experiment serve as negative control sensors, which prove that a response is not triggered by nonspecific attachment of IgG or gold to the latex surfaces. After 45 min, the excess colloidal gold is washed away with water and a silver layer is deposited onto the gold particles with an electron microscopy enhancing reagent for 10 min. The excess silver reagent is removed by extensive washing with distilled water.

Microscopic observations after the silver enhancement show that the latex particles in the leftmost bridge are heavily darkened. This is caused by the high number of specifically attached gold particles that have triggered silver deposition (Figure 2F; note that the other two particulate bridges lack silver deposition, which validates the specificity of the gold attachment to the leftmost sensor). Scanning electron microscope observations of the particulate bridges demonstrate that only the functionalized microspheres are coated by particulate metal shells (Figure 3). Though the exact mechanisms of particle merging and conductance require further investigation, these metal shells obviously cause electrode short-circuiting.

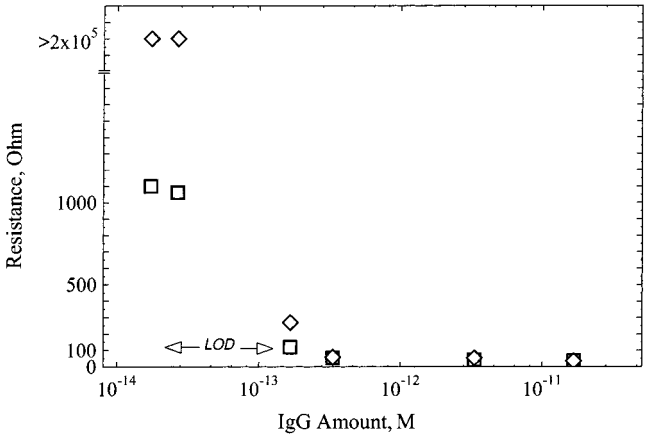

Figure 4. Sensor resistance as a function of the IgG amount. The squares and diamonds at each concentration show the results of two different experimental runs. The two points above the scale break denote an open circuit (resistance higher than the multimeter range).

All positive sensors (15 micropatches total) had a resistance of 50−70 Ω in the wet state and 40−50 Ω after drying. The resistance of the electrode pairs in the parallel negative controls was above 10^3 Ω (6 micropatches). Additional negative control experiments were carried out first by omitting the gold tagging. No signal was observed, proving that only the gold particles induce silver deposition onto the functionalized latexes. Negative results were also obtained after presaturating the gold suspension with excess IgG. This shows that the tagging of the latexes with gold in a positive test was a result of the specific IgG/anti-IgG binding.

A cycle of experiments at lower concentrations of IgG allowed an estimate of the limit of detection (LOD) of the sensors. The response of the sensor in these experiments is shown in Figure 4. The resistances were measured after drying the patches and the amounts shown were calculated for the 10 μL samples loaded on the chip. The resistance of the bridges was constantly low with decreasing IgG concentration down to 1.7×10^{-13} M and then jumped up to become ambiguous and erratic (resistances $\approx 10^3$ Ω or no short-circuiting) for an IgG amount of about 2×10^{-14} M or less. Thus, we take the LOD of our model sensors to be in the range of 2×10^{-13}-2×10^{-14} M, which is comparable to the better IgG agglutination assays and immunosensors.[32] Note that the above estimate is conservatively based on the amount of sample used. The quantity of IgG that had been actually adsorbed on the latex patch and that triggered the sensor response is estimated to be in the range of 10^{-18}−10^{-19} M and can be reduced by further miniaturization.

To demonstrate that the method is transferable to other types of latex particles and other biospecific interactions we conducted experiments with the biotin−streptavidin interaction pair (with the biotin attached to the latex and the streptavidin to the gold). The data of the positive and the negative tests were similar to those discussed above, although the silver deposition on the latex bridges was somewhat lower and the final resistance was higher than that in the IgG experiments. This lower degree of coverage for the biotin−streptavidin pair could be explained by the lack of multiplication of the binding sites or the lower

(32) Scheller, F.; Schubert, F. *Biosensors*; Elsevier: Amsterdam, 1992; pp 275−287.

activity of the sterically inhibited biotin groups on the latex interface.

In general, the biosensor appears applicable as a substitute to most of the more than 100 qualitative latex agglutination tests available today for detection of specific diseases, physiological conditions, and pathogens (summaries of such tests can be found, e.g., in refs 21 and 33). The chips are not reusable but could be made cheaply. Unlike the common optical and piezoelectric biosensors, which respond to adsorbed mass, and are therefore prone to errors caused by nonspecific adsorption, our method includes secondary immunolabeling with gold and thus may be less susceptible to nonspecific protein adsorption. However the high miniaturization of the sensor area (now on the order of the size of blood cells), means that cells, bacteria, and particles need to be removed from the investigated liquid.

As seen in Figure 4, the sensor response is close to that of a threshold (yes or no) and thus is not applicable to quantitative concentration assays. This stems from the mode of sensor operation wherein the electrodes are short-circuited above a certain density of the adsorbed and silver-enhanced gold particles. One possibility for future exploration is the reading of the high frequency impedance of the gold-covered latexes without the silver-enhancement stage. This signal is likely to be concentration-dependent. A number of other interesting possibilities for the future improvement and optimization of the method exist, such as using easily assembled latexes with customized interactions, increasing the speed and sensitivity by generating an electro-osmotic flux over the sensor area, and micromachining a microscopic sample chamber.

(33) Bangs, L. B. *Review of Commercial Latex Diagnostic Tests: The Latex Course Lecture 1* Doc. # 40; Bangs Labs., Carmel, IN, 1996.

Concluding Remarks

The exploratory results presented have shown that a method of interfacing colloidal assemblies with electronic circuits could be used to create functional devices with sensitivities comparable to those of clinical assays. As the sensors are assembled from commercially available particles, the principle is universally applicable to most of the latex agglutination tests in use today and can be extended to assays for DNA fragments and other genetic markers. Because the microscopic active elements are formed from very few particles, their theoretical sensitivity is higher than that of the agglutination assays and in principle approaches the lowest imaginable limit of a few tens or hundreds of molecules. The in situ colloidal assembly method may eventually help create miniature "on chip" electronic devices that simultaneously measure many different parameters.

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Supporting Information Available: List of materials, schematics of the patterned substrates, and of the experimental arrangement. This material is available free of charge via the Internet at http://pubs.acs.org.

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