DERIVATIZED SILICA SPHERES AS IMMUNOSPECIFIC MARKERS FOR HIGH RESOLUTION LABELING IN ELECTRON MICROSCOPY

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ABSTRACT

For high resolution labeling of influenza virus cell surface antigens on HeLa cells, an immunospecific marker is used with silica sphere cores of 13-14 nm average diameter. These markers are formed using commercially available silica sphere sols. Two other size ranges are available, 7-8 nm and 22-25 nm. The steps for chemical derivatization are described in detail. Amino and aldehyde functions are covalently introduced onto the sphere surface. Sols of these derivatized silica spheres (DSS) are physicochemically stable and therefore usable for years. Coupling of IgG to DSS followed by permeation chromatography on controlled pore glass results in size-defined immunospecific silica sphere markers (DSS-markers). Saturation labeling of cell surface antigens on HeLa cells on cover slips is obtained with the final sphere concentration of $10^8$ DSS-marker/cm$^3$ within 20 min. With usual protective conditions, the marker stability and labeling ability are preserved for months. The visibility and the fine structure of the DSS-marker on cell surfaces are shown by using transmission electron microscopy (TEM) with stereo replicas and ultrathin sections.

KEY WORDS  cell surface antigens  - immunospecific markers  - high resolution labeling  - derivatized silica spheres  - defined silica sphere markers

It has become the practice in electron microscopy to visualize cell surface components by labeling with markers (13, 22). Because of the resolution limits when using the conventional scanning electron microscope (SEM), such markers have to be larger than 20-30 nm. This imposes limitations upon the localization of the binding sites. More recent advances in replica techniques (16) have made it possible to use the transmission electron microscope (TEM) for three-dimensional analysis of cell topography, and therefore much smaller markers can be employed due to higher available resolution.

A recent addition to the field of markers for electron microscopy has been surface derivatized silica spheres (DSS) (17). These small markers, which have high density and are easily recognized in the SEM, appear to have promise for high resolution labeling of cell surfaces. The markers
consist of dense, amorphous silica spheres of perfect roundness to which IgG ligands are covalently attached. Since the silica spheres are similar in size to the attached immunoglobulin molecules, the size of the whole silica-sphere globulin complex is the computed dimension of these markers; thus we are regarding the silica spheres as "cores" of the marker.

The silica core consists of pure, amorphous SiO$_2$ which is grown into spherical form from nascent low molecular weight silicic acid solution. The silica core carries a negative surface charge, stabilized by positive counter ions in the aqueous suspension medium. A range of suspensions with different sphere sizes is commercially available. Other sizes can be synthesized in the laboratory. This will be described in a further paper. All the silica cores used in this study are based upon commercially available suspensions.

In this paper we wish to report methods for the derivatization of immunospecific markers from silica cores and to demonstrate the use of these surface-derivatized silica spheres as immunospecific markers for high resolution labeling of influenza virus antigens in the TEM.

MATERIALS AND METHODS

Silica Spheres

The following types of silica spheres were used: (a) 7-8 nm average diameter, 30 weight percent solution, pH 9.9, trade name: Ludox SM 30; (b) 13-14 nm average diameter, 40 weight percent solution, pH 9.7, trade name: Ludox HS 40; and (c) 22-25 nm average diameter, 49 weight percent solution, pH 8.9, trade name: Ludox TM. Suspensions (a), (b), and (c) were obtained from E. I. du Pont de Nemours & Co., Inc. (Wilmington, Del.).

Chemical Reagents

The following chemical reagents were used: y-amino-propyltriethoxy silane (silane coupling agent A 1100, Union Carbide Corp., New York); glutaraldehyde, 25% (cat. no. 23114, Serva Feinbiochemica, D-6900 Heidelberg, Federal Republic of Germany); polyethylene glycol, 20,000 mol wt was obtained from Union Carbide Corp. or from the vendors of the column accessories or the controlled pore glass (see below). All other chemicals were analytical grade (E. Merck, D-6100 Darmstadt 1, Federal Republic of Germany).

Dialysis

Visking-dialysis hoses were 20 mm circumference (cat. no. 44104, Serva, Feinbiochemica).

Ultrasonication

For sonication a Sonifier B-12 (Branson Sonic Power Co., Danbury, Conn.) was used.

Chromatography

Chromatography on controlled pore glass (CPG) was done by described standard procedures (5-7). Ready-packed columns and accessories were obtained from H. Hözel (D-8250 Dorfen, Federal Republic of Germany). For purification and fractionation of IgG derivatized 13-14 nm silica spheres, we used glyceryl-CPG with 51.9 nm or 25.5 nm pore size and 120-200 mesh packing (Electro-nucleonics, Inc., Fairfield, N. J.). Buffer used was 0.15 M NaCl and 10 M Tris adjusted to pH 8.75 with HCl.

Biological System

HeLa cells (flow cat. no. 0-042) were used, infected with influenza A$_v$ virus, strain PR$_6$ (American Type Culture Collection, Rockville, Md.). Anti A$_v$PR$_6$ antiserum was induced in rabbits by immunization with virus, produced in embryonated duck eggs, and anti-rabbit-IgG antiserum was produced in sheep. IgG fractions were isolated as further described (17). For indirect immunofluorescence technique, commercial anti-rabbit-IgG-FITC (Sevac, Prague, Czechoslovakia) was used.

Labeling Technique

Indirect labeling procedure was done at 37°C using HeLa cell cover slip cultures after incubation with A$_v$PR$_6$ virus for 18 h with or without prefixation with formaldehyde (17, 19). First, the cover slip cultures were overlaid with 0.2 ml anti-influenza serum. After 15 min, they were washed for 15 min in a flow-through cell and overlaid with 0.2 ml of anti-rabbit-IgG-DSS (10$^4$ DSS/cm$^2$). After 20 min of labeling, they were again washed for 15 min.

EM Preparation

Fixation, dehydration, and critical point drying of the cells were done according to the exchange method (18, 17). Stereo replicas were produced of the dried cells in two steps, as described in detail elsewhere (16). First, Pt-C shadow-casting at 20°C and an angle of 30° was done in a vacuum evaporator (EPA 100, Leybold-Heraeus, D-5000 Köln 51, Federal Republic of Germany) with a thickness of 1.5 nm in direction of the evaporation. Then a 10 nm supporting film was deposited by glow discharge, using a Hummer sputter coater (Technics Inc. Alexandria, Va.) in an argon-benzene atmosphere. The replica was then removed and cleaned for a short time with hydrofluoric acid and then with sodium
hypochlorite solution. ERL-embedding (20) of cells and ultrathin sectioning with diamond knives were done as previously described (19).

Electron Microscopy

For electron microscopy, a Siemens AG Elmiskop I with anti-contamination device was used at 80 kV.

Principle of Marker Preparation

The commercially available silica sphere solutions are stabilized with sodium counter ions. The spheres carry a negatively charged silanol (Si-OH) surface hydroxyl which is identical to the silanol groups present on silica glass surfaces. Much of the coupling chemistry used in this paper has been derived from experiences gained in surface-derivatizing of CPG, which also has a surface which consists primarily of silanol groups. The first step of converting the inorganic silica surface into a surface of organic groups which can be further derivatized by the usual technique of organic chemistry consists of reacting the silica with a bifunctional silane coupling agent. Because of the versatility of amino groups for further derivatization, the reaction of silica with γ-aminopropyltriethoxysilane has become particularly popular in the derivatization of CPG. We also use this reaction in the first step of derivatizing the silica cores. It should, however, be mentioned that the sphere suspensions are colloidal systems. The spheres are kept in suspension by electric forces, and interference with these forces causes instability and coagulation. All chemical steps of derivatization have to be chosen under consideration of these facts, and such experimental parameters as pH and concentration are critical to the stability of the colloid.

Derivatization of silica spheres of 13-14 nm nominal average diameter is described. An electron micrograph of the spheres is shown in Fig. 1. The commercial suspension (Ludox HS 40) contains 40 weight percent silica and has a density of 1.294 g/cm³. Assuming a sphere density of 2.18 g/cm³, one can calculate that 1 cm³ of the solution contains $1.84 \times 10^{17}$ spheres which have a surface area of 105.5 m².

The first derivatization steps (Fig. 2, IA and IB) consisted of converting the sphere surface from silanol to amino functionality by covalently grafting to it propylamino groups (IA). Such derivatizations had previously been performed on CPG by reaction with γ-aminopropyltriethoxysilane. From this experience, it was known that the resulting amino-functional glasses had a real derivatization yield of 8-10 μmol of covalently bound amine/m² of glass surface. This figure was used to calculate the ratio of reactants, since it was desirable to obtain exhaustive surface coverage without the presence of an excess of γ-aminopropyltriethoxysilane in the solution. Excess of the silane causes coagulation and also interferes with the later-described steps.

The addition of the amino-silane reverses the surface charge of the glass, causing instantaneous gelling of the solution. Prompt dispersion by shaking of the gel in acid (Fig. 2, IB), however, leads again to a stable sol of approx. pH 1. Sols of amino-functional silica spheres made 3 yr ago are still stable.

In the next steps (Fig. 2, II A and II B), an excess of

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3 W. Haller. 1965. See footnote on page 362 of reference 9.
glutaraldehyde is added to the strongly acidic sol (II A) and the sol is dialyzed against alkaline buffer (II B) until it has a modestly acid pH (~6.0), and the unbound glutaraldehyde is removed. Subsequently, the pH is suddenly changed to alkaline (pH 9.5) by addition of ethanolamine. Since the silica spheres themselves have amino groups are eliminated with a 10-fold excess of Tris or sodium hydroxide. Under vigorous Vortex shaking, equal 4-cm$^3$ vol of the dialyzed silica sphere suspension and pH 11.5 buffer are mixed, resulting in a pH close to 9. Vigorous mixing is important to avoid aggregation while shifting the pH. The silica sphere suspension is now dialyzed overnight at 4°C against 10 mM sodium tetra-borate buffer. It can be calculated that the particle density is now near 1.7 $\times$ 10$^{15}$/cm$^3$.

**PHASE III (LIGAND COUPLING):** To bind protein to activated silica spheres, the above solution is diluted 10-fold in 10 mM sodium tetra-borate buffer. 1 cm$^3$ of the dilution is flushed quickly, under vigorous Vortex shaking, into 1 cm$^3$ of protein solution with 10-20 mg of protein per cm$^3$. The ratio of 0.85 to 1.7 $\times$ 10$^6$ activated silica spheres per 10-20 mg of protein is optimal, otherwise clumping or multiple binding of several markers to one protein molecule will occur.

**Procedure**

**PHASE I (AMINIZATION):** In a 250-cm$^3$ bottle, 50 cm$^3$ of Ludox HS 40 (representing 5,276 m$^2$ of silica surface) is mixed with 50 cm$^3$ of water. 50 cm$^3$ of $\gamma$-aminopropyltriethoxysilane representing 0.0425 mol is added. The bottle is shaken until gelling is complete. 25 cm$^3$ of 3 N HCl is added and the bottle is vigorously shaken until most of the gel has redissolved. Traces of undispersed gel are removed by filtration through cotton wool. It can be calculated that the resulting sol has 6.8 $\times$ 10$^6$ particles per cm$^3$ if all gel was redispersed.

The CPG chromatography of the reaction mixture produced precise size-defined fractions of markers which greatly improved their usability in the described study.

**FIGURE 2** Schematic representation of the derivatization steps leading to defined silica sphere markers.
use of 20 mg of IgG/1.7 × 10^{19} activated spheres is equivalent to ~50 IgG molecules per sphere. The mixture of protein and silica has a turbid, opalescent appearance. It is sonicated for 30 s (50 watts) and then incubated at room temperature under shaking for 6–15 h. 5–15 IgG are found to be bound to each sphere group, 1 cm^3 of 2 M Tris (or ethanolamine) solution is added. The mixture is incubated under agitation, for another 2 h, then it is sonicated again for 30 s (50 watts).

The reaction mixture was chromatographed on CPG columns with glyceryl surface functionality (glyceryl-CPG). In addition, the CPG was pretreated with an aqueous 1% (wt/vol) solution of polyethylene glycol (PEG) of 20,000 mol wt. The column was washed with water and buffer after the PEG treatment. It is quite likely that the use of either the glyceryl-CPG or the PEG treatment alone may have been sufficient to prevent adsorption. To effectively separate the marker from unreacted protein, nonspecific adsorption of the proteins to the markers is prevented by a sample volume of 2 M KCl injected into the column followed by the sample. On a 0.8 × 100 cm column, up to 5 ml of reaction mixture can be fractionated. Since the peak positions are highly reproducible, routine separation can be done without a monitor. Dilution factor under this condition is not more than two. The final particle concentration is ~10^{14}/cm^3.

Marker solutions have a slight opalescence. They can be stored for several weeks. Precipitations occurred if procedures were faulty. Partially precipitated marker solutions can be reclaimed by ultrasonication and subsequent chromatography on CPG columns.

RESULTS

Labeling with Defined Silica Sphere Markers

Direct and indirect labeling of cell surfaces is possible with this marker. Indirect labeling is more practical, because it requires only a single anti-IgG marker, which binds to different antisera as long as they come from the same animal species. In this work, we describe indirect labeling with sheep-anti-rabbit-IgG markers which attach to infected cells, treated with rabbit anti-influenza serum and direct labeling with rabbit-anti-influenza-IgG markers.

For the production of sheep-anti-rabbit-IgG markers or of the rabbit anti-influenza-IgG markers, we coupled 1 ml of aldehyde functional silica spheres (5 × 10^{19} spheres/cm^3) with 1.5 cm^3 purified sheep-anti-rabbit-IgG (10 mg protein/cm^3) or with rabbit-anti-influenza (AₐPRₐ) IgG. In both cases, the markers were size fractionated and purified by CPG chromatography.

When using small markers, it is important to calculate the number of markers which are required to saturate the available cell-binding sites. The influenza (AₚPRₚ)-infected HeLa cells were grown as cover slip cultures, whereby the cells touched each other with their extensions, without forming a dense coherent tissue. Assuming that the cells are flat and that all of them have been infected so that the area of antigen-carrying cell surface is identical to the cover slip area (8 × 50 mm), 2 × 10^{14} markers of 40 nm diameter are required. An amount of 0.2 ml of marker suspensions represents therefore a 100-fold excess of markers above the calculated maximum saturation required. Such surplus of markers is needed because only a fraction of the IgG is antigen specific, and, due to chemical procedures, parts of the antibodies may be inactivated. Indirect labeling with IgG-fluorescein isothiocyanate (FITC) and fluorescence observation in the light microscope indicated infection of ~2/3 of the cells. Prefixation with formaldehyde produces a uniform distribution of the surface antigen over the entire cell surface. The same applies for DSS labeling, where a complete surface coverage with the marker is observed (Fig. 3). The markers appear densely packed and there is good contrast between the marked surfaces of infected labeled cells and the nonmarked surfaces of neighboring noninfected cells. One observes dense coverage with spheres while the noninfected cells do not show nonspecific label. In Fig. 4 at greater magnification (× 125,000), one observes a clear demarcation between labeled and unlabeled surfaces. This high magnification reveals substructure of the markers themselves which is caused by the elongated IgG molecules protruding from the smooth sphere core. In spite of the marker’s structured surface, it is easy to distinguish it from the more fine-grained surface of the nonlabeled cells. Other details of the nonmarked cell surface, such as budded structures, can easily be recognized as being not labeled. No labeling was detectable on the labeling controls (not shown in the figures), either on noninfected or infected HeLa cells treated with anti-rabbit IgG markers alone, or on noninfected HeLa cells treated with anti-influenza markers.

Because of the recognizable characteristic structure of the markers, they can also be used for single receptor labeling on the cell surface. This is demonstrated on infected HeLa cells that have not been prefixed. When first treated with anti-influenza serum, IgG cross-linking results in a dislocation and capping of surface antigens of cells...
FIGURE 3 Stereo replica of HeLa cells in TEM. Cells infected with influenza virus, prestabilized, and indirectly labeled with defined silica sphere marker. The infected cell shows complete surface coverage with the markers (LC, labeled cell), whereas noninfected cell (ULC, unlabeled cell) is not labeled. Arrow indicates the area of Fig. 4. Bar, 2.5 μm. × 16,500.

which have not been fixed (19); however, some uncapped antigens remain. After labeling with DSS, Fig. 5 shows the uniformly distributed marked areas of equal size which represent the remaining antigens. Neighboring noninfected cells do not show this pattern. In high magnification (Fig. 6, × 70,000), one can see that the markings consist of aggregates up to six markers which delineate regions of bound IgG.

Ultrathin sections of infected, marked cells also reveal the substructure of the markers. Prefixed infected HeLa cells, directly labeled with anti-influenza markers, show a uniform distribution of surface antigens. At high magnification (× 300,000), one can see the silica cores of the markers, surrounded by bound IgG molecules (Fig. 7). Under favorable sectioning conditions, the binding sites of the markers at the cell surface are visible.

DISCUSSION
The labeling capacity of, and the ability to visualize, the new defined immunosilica sphere markers are a result of their particular design. Their uni-
Figure 4  Detail from Fig. 3. Boundary between labeled (LC) and unlabeled (ULC) cells is clearly visible. Substructure of markers can be seen at this high magnification but can be distinguished from unlabeled cell surface by its uniformity. Budded structures (BS, arrowheads) are not labeled. Bar, 0.2 μm, × 125,000.

Formity and the good contrast of a silica core result in good visibility in high resolution SEM (17) as well as in TEM. The silica spheres are among the smallest known markers. The smallest sizes of high polymer markers, such as polymethacrylate (500 nm), polystyrene (230 nm), and copolymer latexes (30 nm), are far above the resolution of stereo replication methods (4, 12, 14). Small biological macromolecules such as hemocyanin (35 x 50 nm) and spherical viruses (30 nm) are of a similar range (15, 21). Only ferritin particles (12 nm) and gold granules (16 nm) compare (10, 11) with the silica spheres (13-14 nm) used in this study. The last-mentioned markers have high uniformity and contrast similar to those of silica spheres, but seem to have a tendency for coagulation and unspecific labeling. The chemical composition of the silica spheres has certain advantages. The core of the silica sphere is pure SiO₂ and the surface of the sphere has closely spaced silanol groups which can be exhaustively derivatized. This leads to a dense assembly of desired active groups without interference from the original surface of the core. Aside from the aminopropylglutaraldehyde coupling reaction which is described in this paper, other coupling techniques could be used like those used with CPG derivatizations described in the past years (8). The similarity of the silanol hydroxyl and the cellulose hydroxyl furthermore allows the application of procedures originally pioneered for the affinity chromatography on cellulose substrates (2, 3). To obtain a
FIGURE 5  Influenza virus-infected HeLa cells, indirectly labeled with markers without prestabilization. Small patches of markers locate antigen on infected cell (LC). Noninfected cell (ULC) is free of patches. Bar, 1 μm. × 35,000.

FIGURE 6  Cells of the same preparation as in Fig. 5. Groups of markers are distributed over the cell surface (triangles). Immunoglobulin on marker core is discernible by its substructure. Bar, 0.5 μm. × 70,000.
FIGURE 7  Thin section of influenza-infected HeLa cells which were directly labeled with DSS after prestabilization. Markers are distributed on the surface of the cell. Note the fine structures of DSS marker (triangles), consisting of the silica core and attached IgG. Bar, 100 nm. x 300,000.

wider variety of marker sizes, one can modify the core size by appropriate variations in the preparation of the silica sol, and one can also change the total marker size by varying the ratio of cores to ligands and/or the ligand sizes. In any case, one will obtain a statistical distribution of sizes from which a desired size fraction can be obtained by permeation chromatography on CPG. CPG chromatography selects by the largest dimension of a rigid particle and this dimension is very close to the size seen in EM if the particles are nearly symmetrical. Due to the uneven distribution of ligands on the core surface (Fig. 7), the size of the markers in EM will be more uniform if the dimension of ligands is much smaller than that of the cores. This could be obtained by using silica spheres coated either with Fab fragments of immunospecific IgG or with staphylococcal protein A. The latter will be specific against the Fc portion of IgG and therefore suitable for indirect labeling techniques (1). The concentration of spheres in the available sols is high, and even after dilution and purification steps the sphere concentration exceeds any possible requirements for exhaustive marking, a prerequisite for any quantitative assay. Another possible utilization of the DSS markers is their use for selective attachment to particles or membrane vesicles in mixtures, which can subsequently be fractionated by centrifugation, due to increased specific weight imparted by the attached silica spheres.

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