

Three-Dimensional Imaging of Submicrometer Colloidal Particles in Concentrated Suspensions Using Confocal Scanning Laser Microscopy

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Confocal scanning laser fluorescence microscopy (CSLM) has been used as a novel and versatile tool to study the interparticle structure of colloidal particles in the bulk of concentrated suspensions. Micrographs showing individual particles were made of a "colloidal glass", a "colloidal crystal", and a thermotropic reversible gel. The imaged particles consisted of newly developed monodisperse, fluorescent silica spheres. The fluorophore fluorescein isothiocyanate was chemically incorporated deep inside the 1-octadecanol-coated particles. Such model spheres in combination with the improved resolution and optical sectioning of CSLM enable three-dimensional imaging and other measurements that are very difficult with other microscopic techniques or indirect methods like scattering.

Introduction

Many interesting colloidal phases can be observed in concentrated dispersions,¹⁻³ like "colloidal fluids"⁴⁻⁵ with short-range dynamic positional order, "colloidal glasses"⁶ with an essentially frozen-in fluid structure, "colloidal crystals"⁷ with long-range positional correlations, and thermotropic reversible gels.^{5,8} Mostly, the structure and dynamics of these phases have been studied using scattering techniques. Direct observations of individual colloidal particles have, however, also been made.

The use of microscopy to study colloids dates back to R. Brown (1773-1858), who correctly hypothesized about the origin of the irregular, "Brownian", movements of pollen particles. Perrin viewed colloids with a microscope to determine Avogadro's number.⁹ In 1957 a microscopic observation of a colloidal crystal consisting of spherical insect virus particles was reported.¹⁰ Nowadays, microscopy is used to study colloidal crystals consisting of charged, synthetic latex spheres in water.¹¹⁻¹³ The main disadvantages of conventional microscopy are the limited ability to observe more than only a few layers of particles under the cover glass, and the difficulties encountered in the determination of a truly three-dimensional structure.¹¹

With confocal scanning laser fluorescence microscopy (CSLM) an exceptionally short depth of field is combined

with an increased resolution. These improvements are realized by imaging only one picture point at a time. The object is illuminated point-by-point by the image of a point source (a pinhole) focused on the surface or inside the sample, whereas the (con)focused fluorescent light is imaged on a point photodetector.^{14,15} With excitation light of 488 nm a width of the imaging point transverse to the optical axis of 0.2 μm has been obtained. Along the optical axis a resolution of 0.65 μm is possible.¹⁶ With the setup used in this letter the scanning is performed by moving the laser spot over a stationary sample, and it takes about 1 s to image a section of 20 \times 20 μm containing 512 \times 512 pixels. Together with digital image processing, the optical sectioning of a sample makes three-dimensional analysis straightforward.¹⁷ Although the resolution of electron microscopy is much higher, and has proven very useful in revealing the structure of gem opals¹⁸ (also consisting of regularly arranged silica spheres of colloidal size), the requirements of high vacuum make it unsuitable to study dispersions in a liquid.

Experimental Section

The CSLM graphs were made with a MRC 500 confocal laser scanning microscope from BioRad mounted on a Zeiss Axioplan. The z-axis drive from BioRad was modified to drive the course focus of the stage. The objective used was a 40 \times oil N.A. 1.3 lens; dye excitation took place at 488 nm. The cuvettes used were flat glass capillaries (width 2 mm, thickness 200 μm , Vitro Dynamics, Inc., Rockaway, NJ).

Transmission electron micrographs were made with a Philips CM10 electron microscope. The full characterization of the particles with static and dynamic light scattering and fluorescence spectroscopy is described elsewhere.¹⁹

The detailed synthesis of the fluorescent silica spheres studied in this letter is also described in ref 19. However, as a typical example the synthesis of the particles shown in Figures 2 and 3 is briefly described: The fluorophore fluorescein isothiocyanate (isomer I, Sigma, FITC) was covalently attached to the silane

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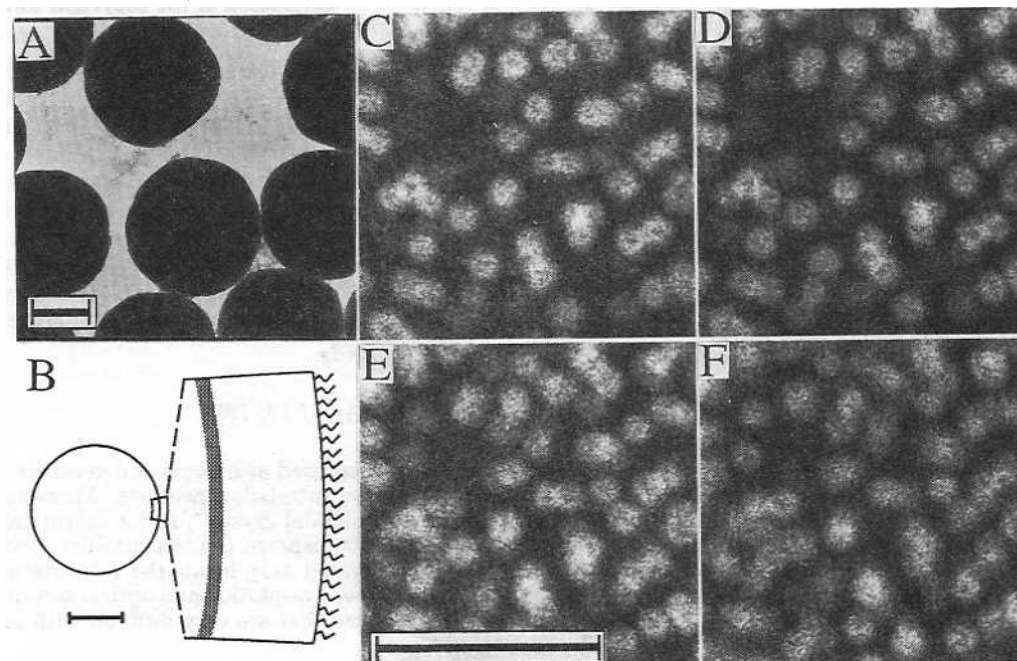


Figure 1. 1-Octadecanol-coated silica spheres in the confocal scanning laser micrographs (C–F) and in the transmission electron micrograph (A). The radius of the spheres is 596 nm with a relative standard deviation in the size of 5%. The fluorescent molecules are only present in a few nanometer thick layer 20 nm deep under the surface of the sphere (B; shaded region is fluorescent). The fraction of the dispersion volume occupied by all the particles was initially 30% in cyclohexane. After sedimentation, the volume fraction was close to random close-packed (64%).⁶ Together with the fluorescent spheres, the same number of particles without any dye and with approximately the same radius were present. The micrographs (C–F) were made after settling of the particles through sedimentation into a glassy state (see also Figure 2C) 50 μm under the surface of the cuvette. Subsequent sections (C–F) were evenly spaced by 0.6 μm in the direction along the optical axis (scale bars in A and B, 500 nm; in E, 5 μm).

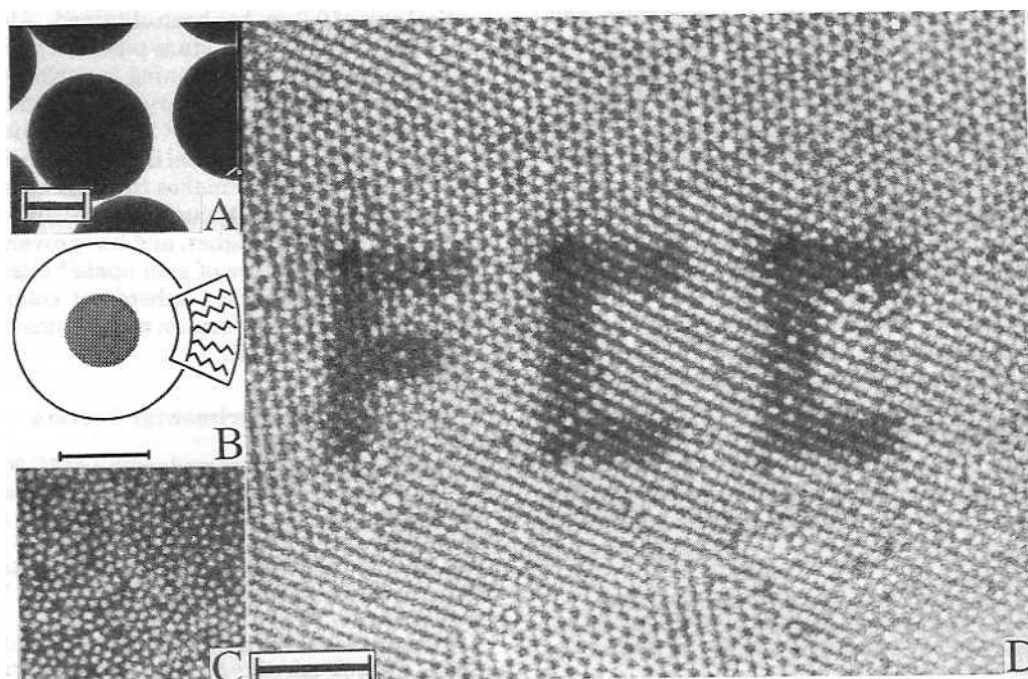


Figure 2. A polycrystalline section 30 μm under the surface of a capillary containing 1-octadecanol-coated silica spheres in chloroform of initial volume fraction 20% (D). The dye molecules are contained in a core of radius 212 nm (B; shaded area contains the fluorescence). The total particle radius was 505 nm with a relative standard deviation of 1.9% (A). The interparticle distance in the crystal phase in chloroform was 1.5 μm , indicating a particle charge (see text). The interparticle distance in the glassy state in cyclohexane (C; contrary to Figure 1 all spheres are fluorescent), obtained as described in Figure 1, was as expected: 1 μm . The photobleached pattern was created by a 100-fold illumination of the darkened area with the imaging beam (scale bars in A and B, 500 nm; in CSLM graphs C and D, 10 μm).

coupling agent 3-(aminopropyl)triethoxysilane (APS, Janssen) by stirring for 24 h a solution of 10.6 mg of FITC (2.73 μmol) and 11.4 mg of APS (52 μmol) in 5 mL of ethanol (Nedalco). The reaction product between APS and FITC was incorporated into a silica core by addition of the APS–FITC–ethanol to the following mixture in 75 mL of ethanol: 8.5 mL of ammonia (24%, Merck) and 3.3 mL of tetraethoxysilane (TES, 14.8 mmol). The

fluorescent silica core particles were grown larger by subsequent addition of TES after removal of unreacted dye by centrifugations, and finally, the spheres were coated with 1-octadecanol as described in ref 20.

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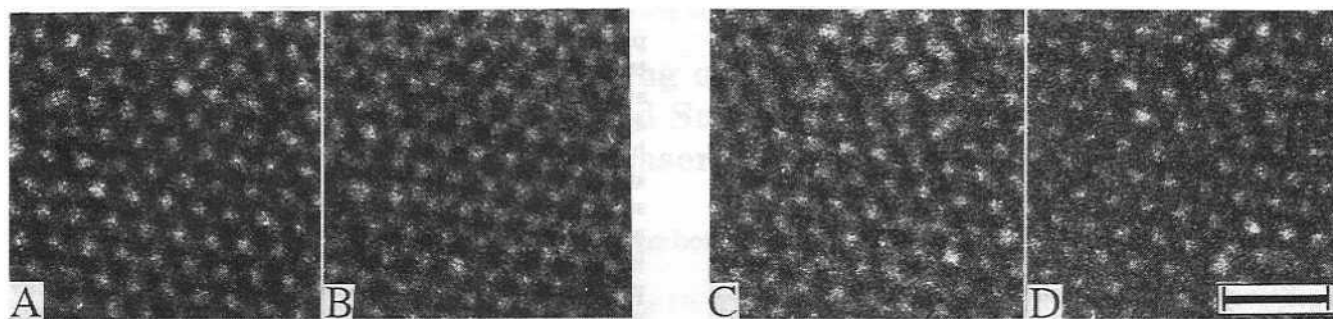


Figure 3. Same as in Figure 2, but now with several sections (planes parallel with the glass surface) shown starting 30 μm under the surface with a separation distance of 1.4 μm . Section A shows a hexagonal layer parallel to the glass surface. The subsequent layers (B-D) demonstrate the stacking order of a hexagonal close-packed structure, HCP: ABAB (scale bar 5 μm).

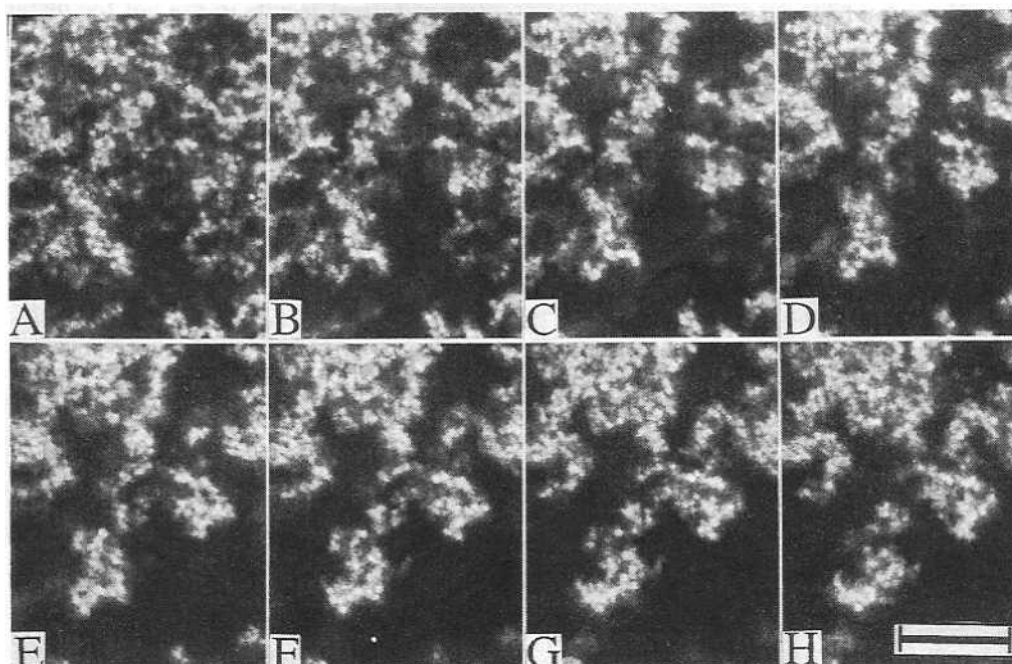


Figure 4. Silica spheres of outer radius 386 nm and polydispersity in size 2.3% (fluorescent core 204 nm), analogous to the particles shown in Figure 2, but now dispersed in hexadecane at 20 $^{\circ}\text{C}$. The gelation temperature for this system at a volume fraction of 15% is approximately 25 $^{\circ}\text{C}$. At 30 μm under the glass surface scans were made with a separation of 1 μm . The order of the sections is from A to G (scale bar 10 μm).

Results and Discussion

Using fluorescent particles, the features of CSLM can be fully exploited to investigate colloidal systems. Silica spheres coated with 1-octadecanol can be dispersed in many apolar solvents with refractive indices close to that of the particles (1.45²⁰). In this way, the scattering of spheres with radii of 0.5 μm can be markedly reduced, so that even concentrated dispersions are not very turbid. Recently, a synthetic route was developed by us to incorporate fluorescent molecules covalently inside silica spheres.¹⁹ Such spheres were used to measure diffusion coefficients with fluorescence recovery after photobleaching.²¹ The place where the fluorescent molecules (fluorescein isothiocyanate) are located inside the particle core can be controlled. In Figure 1, particles are depicted that have a thin fluorescent layer close under their surface. The spheres were mixed with almost identical nonfluorescent spheres to make them more visible as separate particles. In Figure 2, particles are depicted that have a small fluorescent region near the center. These spheres are suitable for discriminating separate particles in a concentrated suspension.

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In Figures 2D and 3 silica particles in chloroform are shown in a crystalline state. In Figure 2D the letters FCC, which are initials of the name of our group, were obtained by localized photobleaching where a high intensity of light destroys the fluorescence irreversibly. The darkened region thus created was used for positioning different optical sections as illustrated in Figure 3. For the colloidal crystals this was necessary, because on top of the Brownian movements around the equilibrium positions, a slow collective movement of whole crystal planes was often observed.

In chloroform crystallization occurs rapidly (minutes), and a large interparticle spacing is found in the crystal phase: 1.5 μm . From this spacing a volume fraction of 36% can be calculated. This is a low close-packed volume fraction compared to that of a hard sphere system and is a strong indication that the particles are charged. The finding of an electrical charge on the spheres is remarkable given the low dielectric constant of chloroform and the hydrophobic character of the particle surface. The crystal structure found for several crystallites (Figure 2D) was hexagonal close-packed (Figure 3). However, with the relatively small number of layers that have so far been investigated, stacking faults of the hexagonal layers cannot

be excluded. Random stacking of the hexagonal close-packed layers, as reported for hard spheres,²² seems unlikely.

Contrary to the crystallization in chloroform, in cyclohexane only a glassy structure formed upon sedimentation of the spheres (Figure 2C). Here the interparticle distance equals the diameter of the particles, as expected.

In hexadecane (*n*-C16) 1-octadecanol-stabilized silica spheres are known to form a volume-filling gel phase at lower temperatures.⁵ Optical sections made inside such a gel are shown in Figure 4. After the sample was heated to 30 °C, which is above the gel temperature, a homogeneous dispersion gradually reappeared, in which Brownian displacements of the individual particles could be observed between successive images.

Conclusions

The examples shown in this letter demonstrate the potentials of CLSM in the study of a variety of colloidal

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phenomena. In a direct way and deep inside a concentrated dispersion—far away from wall effects—particle structures can be observed. Digital image processing enables a more quantitative analysis than is presented here, whereas use of a video system makes a real time study of the dynamics possible as well. The use of model spheres with the fluorophores buried deep inside the silica assures that the particle interaction potential is not influenced by the labeling and that the interaction potential still can be varied independently, e.g., by changing the particle surface coating. Presently, we are studying the observed crystal structures in chloroform in more detail and are also working with mixtures of particles containing two different dyes. We expect to obtain more (quantitative) results in the not too distant future.

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