Cell-assisted assembly of colloidal crystallites

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Many cells ingest foreign particles through a process known as phagocytosis. It now turns out that some cell types organize phagocytosed microparticles into crystalline arrays. Much like the classic crystallization of colloidal particles in a thermal bath, crystallization within the cell is driven by the spatial confinement of mutually repelling particles, in this case by the cell membrane. Cytoskeleton-driven motions exert a randomizing force, similar to but stronger than thermal forces; these motions anneal defects and purify the colloidal crystals within the cells. Bidisperse mixtures of microspheres phase separate within the cell, with the larger particles crystallizing around the nucleus and the smaller particles crystallizing around the perimeter of the large particle array. Mitochondria also participate in this kind of size segregation, which appears to be driven by membrane tension and curvature minimization. Beyond the curiosity of the phenomenon itself, cell-assisted colloidal assembly may prove useful as a new tool to study a variety of biophysical processes including cytoskeletal rearrangements, organelle–membrane interactions, the in vivo mechanics of microtubules, the cooperativity of molecular motors and intracellular traffic jams on cytoskeletal filaments.

Introduction

Cells orchestrate forces to organize organelles within themselves during cell division, intracellular transport, and other processes. During cell crawling, internally created forces propel the entire cell forward. In order to unravel the chemo-mechanical origins of these forces, as well as their influence on the cell, a multitude of approaches have been developed to measure either forces exerted by the cell (see ref. 1–4, and references therein) or the local viscoelasticity of cells. Several of the cited approaches follow the dynamics of single particles or organelles inside the cell to determine cellular viscoelasticity or localized cell forces. A more recent approach analyzes the entire distribution of organelles within cells. The theory combines assumptions about intracellular transport mechanisms with known details of the cell’s internal structural environment, and makes predictions that are in agreement with observed distributions of mitochondria, endosomes, melanosomes and peroxisomes in various cell types. This new approach is useful because it indirectly validates presumed transport mechanisms and it helps identify the predominant mechanisms. It also addresses how multiple processes such as actin and microtubular transport are coupled and how this coupling impacts organelle patterns within the cell.

In a similar vein, this article addresses the surprising spatial distribution of a large number of phagosomes (phagocytosed particles) after their ingestion by a fibroblast cell. We report for the first time that fibroblast cells actively assemble phagocytosed, micron-sized particles into extended, hexagonally close packed arrays in two or three dimensions (Fig. 1). Each array constitutes a small colloidal crystal within a cell; such particle arrays will be referred to as crystallites throughout this paper. The phenomenon is robust and reproducible for a range of particles 0.75–6.0 μm in diameter. Further, when supplied with particles of bimodal size distribution, fibroblast cells segregate the two sizes such that the larger particles crystallize around the cell nucleus while the

Fig. 1 Scanning electron micrograph (SEM) of a fibroblast cell with phagocytosed 6 μm polystyrene particles organized into a hexagonal array (accelerating voltage 10 kV). The cell membrane visibly extends beyond the area filled with particles. Cell-assisted crystallite assembly occurs for polysterene microspheres ranging from 750 nm to 6 μm in diameter.
smaller particles do so around the rim of the large particle cluster. Despite the rather unnatural conditions presented to the cell, the observation of these unique spatial distributions sheds light on relevant biological processes and on new aspects of the physical environment within the cell. We suggest that similar studies of the dynamics and distribution of multiple particles could shed insight on plasma membrane–organellar coupling, cytoskeletal regulation, cytoskeletal traffic jamming, stiffness of biopolymers in cells, and cellular stress fields during adhesion and crawling.

Experimental

Cells

Rat embryonic fibroblast wild type cultures (REF 52) and 3T3 mouse fibroblasts, and MDCK kidney cells were grown in Dulbecco’s minimal essential medium (DMEM) containing 10% fetal bovine serum (FBS), 1% l-glutamine and 1% penicillin–streptomycin in a humidified incubator with 5% CO₂. Rat chondrocytes (RCJ-P) were grown in alpha-MEM medium containing 15% FBS and 2% l-glutamine. All cell cultures were harvested with 2.5% trypsin–EDTA. In preparation for phagocytosis assays, cells were cultured on round glass coverslips (30 mm) attached to the bottoms of 50 mm petri dishes with vacuum grease. Live cell imaging was performed in glass bottomed petri dishes purchased from MatTek Corporation, USA which were coated with covalently bound fibronectin to minimize cell motility.

Microspheres and microsphere coating

All microspheres used in these assays were made of polystyrene and purchased from Polysciences, Inc. Phagocytosis, retrograde motion, and crystallite formation reliably occurred for many different particle surface chemistries, including carboxylate, sulfate and amine groups, as well as for beads coated with non-specifically absorbed BSA, fibronectin, poly-L-lysine, or covalently bound immunoglobulin G (IgG).

IgG coating of microspheres

Bovine serum albumin (BSA) was covalently coupled to the carboxylate microspheres after activating the surface with EDC (1-ethyl-3-(3-dimethylaminopropyl))- and NHS (N-hydroxysuccinimide) from Sigma-Aldrich, followed by incubation with affinity purified rabbit anti-BSA IgG (Sigma-Aldrich) for 1 hour at 24 °C.

Fibronectin coating of glass slides

Glass-bottomed petri dishes or glass slides were cleaned with ‘piranha’ solution (H₂O₂ and concentrated H₂SO₄ at 1 : 1) for 30 minutes followed by incubation in aminosilane solution for 30 minutes. The glass was then rinsed with a mixture of methanol, water, aminosilane and acetic acid (1 : 0.05 : 0.01 : 0.006) and dried for 10 minutes in an oven at 60 °C. The activated surfaces were then incubated for 30 minutes with fibronectin diluted in PBS (10 μg ml⁻¹). A final washing step with PBS was performed to remove any non-bound fibronectin. Fibronectin coating mildly suppressed cell motility, thus enabling the tracking of the retrograde motion and the formation of colloidal crystals during live video microscopy.

Depolymerization experiments of actin and microtubules

The role of the cytoskeleton in the formation of the phagosome crystallites was studied by depolymerising the actin cytoskeleton with 2 μM of cytochalasin D or depolymerising microtubules with 2 μM nocodazole. The cytoskeletal-altering drugs were added for 1 hour before the sample was washed and fresh medium was added. After the replacement of the medium, the depolymerised actin or microtubules could repolymerize. Movies of the experiments were made by taking an image every 20 seconds (3 fps).

Immunofluorescent staining and imaging

To prepare the cells for staining, they were permeabilized in 0.1% Triton X-100 (Sigma-Aldrich) in 3% glutaraldehyde for 2 minutes, followed by washing with PBS and incubation for 15 minutes in 3% glutaraldehyde. Non-specific binding was blocked by incubation with 1% BSA (Sigma-Aldrich ) in PBS for 30 minutes. Actin was stained using 0.02% Rhodamine–phalloidin (Sigma-Aldrich). Microtubule staining was performed using 0.001% monocolonal α-tubulin (Invitrogen, GmbH) for 60 minutes followed by 0.01% Alexa Fluor 488 for 30 minutes. The cell membrane was stained using 0.02% Vybrant DiO (Invitrogen, GmbH) on living cells for 30 minutes, which were then fixed for imaging. Mitochondria were stained in living cells using 300 nM Mitotracker (Molecular Probes, Inc.) for 30 minutes.

Fluorescence imaging was performed on a confocal laser scanning microscope (LSM 5, Carl Zeiss, GmbH) equipped with a 50 mW 405 nm diode laser and a 75 mW 532 nm diode-pumped solid state laser. Vertical stacks of horizontal images were made by taking an image every 20 seconds (3 fps). Samples were prepared for scanning electron microscopy by critical point drying and sputtering with a thin layer of gold. Images were made with a Leo 1530 field emission scanning electron microscope (Carl Zeiss, GmbH) using an accelerating voltage of either 3 or 10 kV (as labelled in the Figures).

Live video microscopy

Extended observation of crystallite formation in fibroblast cells was performed on a Delta Vision real time restoration imaging system microscope (Applied Precision, LLC.) using bright field, phase contrast, and fluorescence microscopy. Throughout the measurements, the cells were maintained at 37 °C in a 5% CO₂ atmosphere. A programmable stage made it possible to visit multiple sites repeatedly, increasing the probability of finding a cell uptaking and transporting particles. Images were taken every 30 seconds.
Formation of colloidal crystallites in cells

The cells were seeded in glass-bottomed petri dishes and allowed to adhere/spread for 3 hours. Then the microspheres were added to the Petri dish at varying concentrations to control the speed and degree of particle uptake. Depending on the relative concentration of cells to microspheres and the particle size and surface covering, the crystallites form over a period of 1–5 days.

Analysis of maximal microsphere coverage in first crystallite layer

Cells densely packed with microspheres that had already begun to form a second layer on the colloidal crystallite were stained with the membrane dye DiO and imaged with confocal microscopy. A Matlab routine was used to measure the surface area of 26 cells and the area occupied by the first layer of microspheres.

Results

Phagocytosis and retrograde motion

Phagocytosis, or the uptake of large objects (>0.5 μm) by cells is a common process undertaken by specialized cells such as macrophages and neutrophils. Critical for the management of infectious agents and senescent cells, phagocytosis plays a role in development, tissue remodelling, the immune response and inflammation. Macrophages and neutrophils are classified as professional phagocytes because of their dedicated phagocytic receptors and quick uptake kinetics. However, most cells have some phagocytic capacity. For example, fibroblasts employ phagocytosis for tissue remodelling while thyroid and bladder epithelial cells phagocytose erythrocytes in vivo.

During phagocytosis, the interaction of specific receptors on the cell surface with an object external to the cell initiates actin polymerization which drives the gross perturbation of the cell’s outer cell (plasma) membrane around the particle. Once encapsulated by the membrane, the membrane buds off and the particle is swept into the cell via an actin (and possibly a myosin motor) based mechanism. The internalized membrane-coated particle, or phagosome, is then transported towards the nucleus for further digestion via a process called retrograde motion. In professional phagocytes and fibroblasts, transport towards the nucleus is believed to be the result of active transport by molecular motors along cytoskeletal filaments; although in some cases, especially in non-professional phagocytes, the transport may be driven by cortical actin flow. Studies on macrophages indicate that the type of active cytoskeletal transport may depend on the size of the object, where 3 μm particles are affiliated with actin-based motor transport while 0.9–1.0 μm objects are associated with microtubule-dynein motor transport.

Our experiments demonstrate that rat embryonic fibroblast (REF 52) cells readily ingest colloidal particles between the size of 500 nm and 6 μm. Live video microscopy reveals that phagocytosis occurs near the cell edge, consistent with scanning electron microscope (SEM) images. An ingested particle is then actively transported through the cytoplasm from their point of entry to the perinuclear region, the area around the cell nucleus. SEM (Fig. 1, Fig. 2, inset) and confocal microscopy (images not shown) also verified that the particles are located inside of the cell rather than on the cell surface where directed motion may also take place due to lipid membrane flow or a transmembrane interaction with a submembranous cortex of moving actin. The particle track in Fig. 2 represents a typical retrograde motion event for a 4.5 μm diameter particle in a REF 52 fibroblast. 4.5 μm diameter beads are measured to have an average velocity of about 1.5 μm per minute during a typical transit period.

The fibroblasts in these experiments phagocytose an extraordinary number of colloidal particles relative to their total cell volume when fed with a medium that contains the particles at high density. The phagosomes’ subsequent retrograde motion within the cell crowds the particles around the cell nucleus. As the number of internalized particles increases, the packed phagosomes begin to appear as a colloidal crystal with hexagonal order. The degree of crystalline regularity increases with time, reaching a final nearly static state after several days to a week as the cells mature and reach confluence (i.e., the cells cover the adhesion area on the surface, leaving little room for cell motion and suppressing cell division). Fig. 3a–d and the corresponding online movie (movie 1 in the ESI) show the initial stages of particle organization. Fig. 3c,d demonstrate that the particles can rearrange significantly due to cell motions in just tens of minutes when the cell is only partially filled.

Fibroblasts assemble colloidal crystallites. Cell-assisted colloidal crystallization is robust and easily reproducible. In REF 52 fibroblasts, we show that particles with diameters up to at least 6 μm and with a minimum diameter of 750 nm form into crystallites. The first observations of colloidal crystallization in cells were made by daily observation of the cell culture extended over a week. These observations indicated a slow build-up of quasi hexagonally-packed particles around

Fig. 2 Typical trajectory of phagocytosed 4.5 μm particle (phagosome) versus time. Inset: SEM image of particle inside of cell illustrates severe membrane deformation near the cell edge which is ~200 nm thick (accelerating voltage 10 kV).
the nucleus. In the early stages, a pearl necklace of beads decorates the nucleus. It then appears to grow outward, into a crystallite. The dynamics captured during live video microscopy experiments verify this observation (Fig. 3 and movie 1 in the ESI). Fig. 4–5 give typical examples of cellular crystallites for polystyrene particles with diameters of 0.75, 2, 3 and 6 μm. Approximately $4.5 \times 10^7$ beads diluted in PBS at a ratio 1 : 25 were added to each sample. Images were taken 3–4 days later. During that time, two-dimensional crystallites are formed around the cell nucleus with relatively good hexagonal order. The degree of order decreases for particle sizes of 1 μm and less, with occasional exceptions like the beautiful three-dimensional array of 0.75 μm beads in the SEM image in Fig. 5. Some of the cells shown in Fig. 4a,c have begun to form a second colloidal layer which appears out of focus here.

Similar results were reproduced in 3T3 mouse fibroblasts and rat chondrocytes (RCJ-P) cells, while they failed to form in MDCK kidney cells. The structures in 3T3 fibroblasts were less extensive but similar to those in REF 52 fibroblasts. The RCJ-P cells also produced crystalline structures, but they were more disordered and smaller than those of the 3T3 and REF 52 fibroblasts. The difference appears to result from the different shape of the RCJ-P cells which appeared less flat and extended than the fibroblasts, allowing the first layer of beads to inhabit a quasi-3D space. The MDCK cells, whose total size is smaller than the fibroblasts and chondrocytes, uptook on average only a few particles before the cells stopped phagocytosis.

Phagocytosis and colloidal organization occurs in most or all the REF 52 fibroblasts in a typical sample, as shown in the confluent cell monolayers imaged in Fig. 4b,c. In general, the extension of the crystallites depends on cell size while the number of defects is related to the cell shape. In these images, the cells are small and seeded at a high density, which results in smaller crystallites with more defects. Larger cells present extended areas without the structural curvature that arises at the nucleus and the cell periphery, permitting extended defect-free crystalline domains to form. Selective separation of larger fibroblast cells via centrifugation produced cells capable of growing extended crystallites as shown in Fig. 4a and the second supplemental movie. In most cases, the beads are located adjacent to the nucleus where the large holes in the crystallites are cell nuclei.

The time scale of the crystallite formation, assuming equal initial concentrations, depends on the size of the beads. Two micron beads form well-ordered crystallites most efficiently, while the process is slower for both larger and smaller beads. For uncoated 2 μm polystyrene spheres, adding $4.5 \times 10^7$ beads to approximately $3 \times 10^4$ cells plated on a 50 mm diameter area, the process of full crystallite assembly requires 2–3 days. Increasing or decreasing the particle concentration shortens or lengthens the time scale, although very high bead concentrations can kill the cells.

For particles sizes of 500 nm, crystalline order is never achieved (Fig. S1 in the ESI). Instead, the clumping of the 500 nm particles into large sacs distributed around the nucleus often occurs (Fig. S1 in the ESI). It is unclear if the clumping results from the fusion of single bead phagosomes, which has been reported previously in macrophages, or through the initial simultaneous uptake of multiple beads which for example, can occur in amoeba cells. Such clumps were also occasionally seen in regions between the lamellopodia and the perinuclear area (Fig. S1 in the ESI). We attempted to minimize clumping by adding low concentrations of particles several times over a few days. However, the 500 nm particles never become well-organized. The lack of crystallization could result from different transport mechanisms for smaller particles. This is possible if the particles were endocytosed rather than phagocytosed, and therefore follow the endocytic pathway (500 nm is the approximate particle size where both mechanisms can occur). More likely, the lack of crystallization could result from the decreased membrane–particle confinement (see the Discussion section). Particles of 200 and 300 nm were also found to be ingested by the cells but consistent with the 500 nm particles, failed to form crystalline structures.

**Cell crystallites form 3D pyramidal structures**

The area of phagosome occupation never extends to the outer edge of the cell, as shown in Fig. 6 by immunofluorescent...
staining of the plasma membrane. This remains true even when very high initial particle concentrations are used or when additional particles are sequentially added to the cells over time. Instead, at extremely high particle concentrations, a second crystalline layer begins to form when the first layer nears completion. The three-dimensional structures can be extensive in large cells as shown in Fig. 5, forming multiple layers with hexagonal close packing. Each additional layer is smaller than the one below it so that the layered crystallite appears like a truncated pyramid.

By saturating the fibroblasts until the second crystallite layer begins to form, we measured the maximum ratio of the area occupied by the phagosomes versus the cell's total area for 26 fibroblasts to be $36.8 \pm 13.3\%$. What limits the extent of the particle organization to the perinuclear area and drives the particles to then travel above (or in some cases below) the first layer is not understood, although it may be related to membrane–particle interactions in the thinner sections of the cell and the presence of membrane curvature in the perinuclear region (see the Discussion section).

Size segregation of binary mixtures of phagosomes. When presented with a bidisperse solution of two particle sizes, fibroblasts predictably phagocytose both species and transport

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**Fig. 4** (a) 2 μm diameter sulfated polystyrene particles organized into extended crystalline arrays with several domains inside of REF52 fibroblast cells. Each island of particles represents part of a different cell which is not visible due to the microscope’s focus on the particles. The circular gaps in the crystalline arrays are the cell nuclei. (b) 3 μm carboxylated polystyrene particles organize into crystallites within numerous cells despite the microspheres' slight irregularities. (c) 6 μm sulfated polystyrene particles fill every cell in the field of view where they are hexagonally close packed. Out of focus sections in the cells represent the formation of a second crystalline layer.
This may be due to the relative size of the particle diameter and the cell dimensional structures while the two dimensional arrays lack order. The particle size is 750 nm. 750 nm and 1 μm particles tend to form well-ordered crystallites when there are enough particles to create three dimensional structures while the two dimensional arrays lack order. This may be due to the relative size of the particle diameter and the cell height in the perinuclear region.

Fig. 5 SEM image of a multi-layered three-dimensional colloidal crystallite inside of a large fibroblast cell (accelerating voltage 3 kV). The particle size is 750 nm. 750 nm and 1 μm particles tend to form well-ordered crystallites when there are enough particles to create three dimensional structures while the two dimensional arrays lack order. This may be due to the relative size of the particle diameter and the cell height in the perinuclear region.

Fig. 6 Membrane staining of fibroblast cells filled with 3 μm microspheres. The staining was performed after a second crystalline layer had begun to form to ensure that the first layer was maximally full. A confocal image of (a) the bottom of the cell compared with (b) the top of the cell shows that the cell surface area is much larger than the microsphere-filled area. The first crystallite layer covers an average of 37% of the cell’s surface area (average of 26 cells). The particle size is 750 nm. 750 nm and 1 μm particles tend to form well-ordered crystallites when there are enough particles to create three dimensional structures while the two dimensional arrays lack order. This may be due to the relative size of the particle diameter and the cell height in the perinuclear region.

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them to the perinuclear area. More unexpectedly, after several days the larger particles appear closer to the nucleus and separated from the smaller ones. This size-dependent spatial segregation within the cells occurs for multiple combinations of bead sizes, as shown in Fig. 7. Occasionally, individual small particles appear between the larger particles but the larger particles are always clustered around the nucleus.

In the next section it is shown that the normal halo-like distribution of the mitochondria around the nucleus is disrupted by the presence of the packed microspheres within the cells. The majority of the mitochondria, which are approximately 0.5–1 μm sized organelles responsible for cellular energy (ATP) production, become marginalized on the outside of the crystalline structure of larger beads (Fig. 9). It has also been observed that other small cellular objects also size-segregate with respect to externally-added microspheres. These unidentified organelles, like the mitochondria, are smaller than the phagosomes. They too build up around the phagosome crystallite, and in the case of bidisperse phagosomes, become the third and smallest species at the edge of the crystal. However, they do not appear to crystallize.

Multiple biological mechanisms could explain this outcome. For example, slower kinetics of the uptake of smaller particles, or the differential speed/force of retrograde motion for different particle sizes could help the larger particles reach the nucleus first. To eliminate the first possibility, we added only the smaller particles to the cell culture and allowed them to organize into medium sized crystallites over 36 hours. The larger particles were then added to the cell culture. However, in a few days time, the end result was identical to the earlier results: the larger particles phase separate from the smaller particles and gather around the nucleus.

We then tested the hypothesis that size segregation arises from differences in the transport force of large versus small beads along the cytoskeleton. For example, if the number of working molecular motors on a bead increases with its size, the force on large beads might be higher than that exerted on small beads, allowing the large beads to travel to penetrate through the smaller beads. To eliminate this possibility, we compared the average velocity of 3 and 4.5 μm beads during retrograde motion in average sized fibroblasts. Surprisingly, the velocity has a simple inverse dependence on the particle radius as expected in a Newtonian fluid. Assuming that the applied force is correlated with the average velocity, 3 μm particles must experience a similar or larger force than the 4.5 μm particles. Thus, the theory of differential transport forces is also eliminated. Another possible sorting mechanism, arising from membrane–microsphere coupling, is addressed in the Discussion section.

Impact of colloidal crystallite on cell function. Time lapse video microscopy and immunofluorescent staining were used to study the crystallite’s impact on the cell function and organization. Despite the large volume occupied by the beads, the cells continue to divide and proliferate, although at a slightly slower rate. During cell division, the colloidal particles are, on average, equally distributed between the mother and the daughter cell, as are many organelles. Cell division temporarily destroys the crystallite structure because the cell releases its attachments to the surface and assumes a spherical shape. However, after division the two resultant cells respread on the surface and two new crystallites are quickly reformed within tens of minutes. The flow of the cytosol during cell spreading and cytoskeletal rearrangement appears to play an important role in organizing the particles. This phase of cell spreading is also a very active time for additional particle take-up through phagocytosis because the two processes of cell spreading/adhesion and phagocytosis are closely related.

The actin distribution is similar to that in control cells. Confocal microscopy of fluorescently stained actin reveals that actin stress fibers are located below and above the beads, as shown in Fig. 8. On the other hand, the microtubules, which always extend from the centrosome located near the nucleus, are dramatically altered. A comparison of the microtubular distribution in a normal cell (see Fig. S2 in the ESI†) with those in a cell containing 4.5 μm beads shows that the normally dense, radially-extended microtubules are less dense and snake
outwards through the beads (Fig. S3 in the ESI†). The beads’ size and their crystallization regulate the microtubular network’s mesh size and orientation. If microtubules are indeed the key transport system of retrograde motion, the microtubules may become structured this way during retrograde motion and the consequent organization of the microspheres. Another interesting possibility is that the microtubules, which in normal fibroblast cells grow and shrink every 10 minutes,44 maintain this so-called dynamic instability and grow through the crystallite, bending as they encounter the beads as obstacles. In either case, the required bending around the beads is interesting given the stiff nature and long persistence length (on the order of millimeters) of microtubules.45 Using even larger beads may allow for the exploration of the mechanical stiffness and the buckling of microtubules within the cell.46–48 Experiments might also be related to recent work which predicts that the persistence length of the microtubules increases with longer contour lengths.49

The distribution of microtubules will greatly impact the intracellular transport system of the cell. Microtubules are the main highway along which multiple organelles, including mitochondria, secretory and endocytotic vesicles and mRNA are distributed throughout the cell. In the cells with colloidal crystallites, the spatial distribution of mitochondria is significantly altered (Fig. 9). In bead-free cells, the mitochondria extend in a halo of slightly decreasing concentration outward from the cell nucleus (Fig. S4 in the ESI†). In bead-filled cells, the mitochondria cluster around the edge of the crystallite and fill any large gaps within it. Confocal microscopy reveals that few mitochondria are found throughout or above the crystal. The localization of the mitochondria to the outside of the microspheres may reflect that more ATP consumption takes place in that area, since mitochondria can become concentrated in regions with high energy needs. They may also become segregated to the outside of the microspheres because it is difficult to transport them through the beads. It is unclear...
how or why they are excluded from the perinuclear region during crystallite formation, although their segregation may result from the same mechanism responsible for the size separation of bidisperse particles in cells.

**Dynamics of crystallite formation**

The formation and evolution of the crystalline structures was visualized with time-lapse video microscopy to understand the mechanisms responsible for phagosome crystallization. The continual uptake of particles combined with their retrograde motion towards the nucleus gives rise to an imperfect hexagonal close packing of the particles. Movies 1 and 2 in the ESI† show two stages of the formation. The first movie, as discussed earlier, shows the early formation of the crystallite due to the retrograde motion of 4.5 µm particles in a normal-sized fibroblast cell. The second movie shows the late stage formation of the outer parts of a crystallite in a much larger cell.

Differently from the typical cell in movie 1 (and Fig. 3), the giant fibroblast cell in movie 2 gathers particles along lines with the same directionality as the actin stress fibers in a such cell. These stress fibers tend to lie tangential to the cell nucleus, so that the particle motion, presumably along the stress fiber, is nearly perpendicular to a classic retrograde motion trajectory. Bead motion occurs in both directions such that the particles move with respect to one another until they collide and become bunched together. Such bidirectional motion may reflect contraction of the actin stress fiber by myosin II, which would slide the beads affiliated with different filaments in the stress fiber closer towards one another.

Once clustered together, the beads appear as a snake of particles. Such snakes are then coherently transported towards the nucleus, perpendicular to the stress fiber. The transport occurs both towards and away from the nucleus, although it occurs more frequently towards the nucleus. The observation of bidirectional motion thus excludes cortical actin flow as a driving mechanism. Recent studies show that mixed actin and microtubular transport is possible in some cells. However, the simultaneous and coordinated transport of a group of particles along parallel microtubules is unlikely statistically. The snake of particles might also be fused together, allowing for the possibility that a single bead on the snake could be attached to and pulled along a microtubule. In that case, the snake should experience a torque that forces it to rotate slightly in the cytosol, but this does not occur. Furthermore, the particles do not always remain neighbors after their transport to the crystallite. Similarly, the probability that a group of particles is thermally-activated to simultaneously hop from one stress fiber to a next is small. It is also improbable that a large, fused snake of particles has enough thermal energy to hop to a neighboring stress fiber. The one remaining explanation is that the smooth motion of a snake of particles towards or away from the nucleus is mediated by the gross motions of the underlying actin stress fiber.

This process further builds up well-ordered domains by clustering the snakes of particles at the edge of the crystallite. Thereafter, the particles continue to occasionally rearrange, with individual particles hopping into vacant holes. Sometimes, the hopping even displaces another particle. Over
time, the overall order of the crystallite improves further. Thermal energy is not sufficient to drive these rearrangements, given the tight packing of the colloidal spheres in the relatively viscous and highly confined cytosol. The hopping must be driven by local forces arising from the cell’s structural rearrangements driven by the cytoskeleton. This is consistent with reports that the cytoskeleton exerts forces on the 100s of picoNewtons to the nanoNewton scale. Thus, it appears that intracellular motions produce a kind of background noise that helps anneal defects in the crystallites, analogous to temperature annealing defects in crystals of atoms or colloidal particles.

The role of the cytoskeleton in crystal formation was determined by depolymerising the cytoskeletal filaments. The actin filaments were partially depolymerised using 2 μM cytochalasin D for 1 hour. This led to the partial withdrawal of the cell from the surface and a slight cell rounding because the actin cytoskeleton is largely responsible for the cell’s shape and anchorage to the adhesion surface. These changes disrupt or even destroy the crystallites if the cytochalasin is strong enough. Similar structural rearrangements and loss of order were witnessed during the exposure of phagosome crystallites to 2 μM nocodazole, a microtubule depolymerising drug. After the partial disruption of the crystallites by 60 minutes of exposure to the cytoskeletal altering drugs, the medium was exchanged. Movies 3 and 4 in the ESI show the regrowth of the actin cytoskeleton and the microtubules, respectively, and its dramatic impact on the reorganization and recrystallization of the crystallites (3 frames per second, 121 and 192 minutes, respectively). These experiments show that both types of cytoskeletal filaments can strongly influence the distribution and packing of the phagosomes.

To study the evolution of the crystallite after its formation, we sequentially added two sets of 2 μm particles to the cells with a time delay. First, a set of non-fluorescent particles were allowed to form an array that filled about two thirds of the area within the cell occupied by the first crystallite layer. Then, after washing the sample, a second group of fluorescently-labelled particles was added. This allowed for the visualization of the evolution of the crystallite, as well as for information about the collision of particles as they encounter the crystal’s edge. It was found the fluorescent particles stopped at the edge of the crystal. There, a ring of the second particle type builds up (Fig. 10a–c). At longer times, however, the second type of particle begins to penetrate or diffuse into the crystallite, presumably from the constant jittering and structural rearrangements of the cell (Fig. 10d–e). If the two-dimensional crystallite grows relatively large compared to the cell’s surface area, the newly added particles sometimes transit above or below the crystallite (forcing other particles upwards) while others continue to build up at the crystallite edge. When the cell is filled with densely packed phagosomes, cell motions can also force particles in the middle of the crystallite to pop upward into another plane.

Thus, these experiments show that the constant shuffling and motion of the cell adds an additional energy component to the formation and evolution of crystallites.
anneal defects in the crystallite. Indeed, cells may try to optimize the order of the phagosomes into a hexagonally-packed crystallite to minimize the energetic and material costs of stable mechanical attachment to the surface. Further experiments comparing the statistical dynamics of phagosomes in cells containing a sparse number of phagosomes versus the dynamics of phagosomes in cells with many particles, might help support or eliminate this theory.

**Discussion**

This paper reports previously unknown phenomena related to the transport and organization of monodisperse and bidisperse microparticles in cells. Spherical particles that are phagocytosed into fibroblast cells in large numbers become ordered into hexagonally close packed arrays. The phenomenon is robust and reproducible for particle diameters ranging from 750 nm up to at least 6 μm particles. For particles with 2 μm diameters up to at least 6 μm diameters, the crystalline arrays are two or even three dimensional when sufficient numbers of particles occupy the interior of the cell. The order of two dimensional crystallites is reduced for 750 nm and 1 μm phagosomes, but at very high particle concentrations, a well-ordered three dimensional crystal forms within the cells. Particles with diameters below 750 nm do not exhibit crystalline order at any stage, even when particle clustering is suppressed. Lastly, bidisperse mixtures of particles spatially segregate within the fibroblasts by size, with larger particles located closer to the nucleus and the smaller particles surrounding the periphery of the larger beads. This section presents a simple explanation for the observed crystallization and size-segregation of phagosomes within fibroblasts.

The fibroblast cell presents a highly confined volume to a phagosome, shaped approximately like a fried egg. The area near the cell perimeter is about 200 nm thick, much thinner than the microparticles in this study. The membrane is significantly deformed as a phagocytosed bead is pulled through this region during retrograde motion (Fig. 2, inset). In the perinuclear area, where the cell is thick enough to accommodate the nucleus, phagosomes and other organelles have more space in which to move. The average nuclear height of REF 52 fibroblasts is 5.1 ± 1.1 μm, as measured for 16 cells with DAPI stained nuclei visualized with confocal microscopy. Both membrane–phagosome interactions and the non-Newtonian, heterogeneous nature of the cytosol play an important role in the crystallite assembly.

Classic colloidal crystallization of mutually-repulsive microspheres in a thermal bath occurs when the microspheres’ density is above some critical concentration. The mechanism for phagosome crystallization is similar to classic colloidal crystallization. In fibroblasts, the spatial confinement by the cell membrane is the driving force of the crystallization. Bound by the membrane and pumped into the perinuclear area by retrograde motion, the particles become concentrated to the point of crystallization. Much like purely thermal forces in a classic colloidal crystal, the cytoskeleton-mediated intracellular motions help anneal defects and purify the phagosome crystal. This cytoskeletal noise dominates thermal effects in the highly viscous and heterogeneous cell interior.

This simple explanation is supported by experiments involving changes of cell shape. For example, fibroblast cells, whose shape is more extended and flattened than that of RCJ-P cells, form more ordered crystallites than the RCJ-P cells. Furthermore, altering a cell’s shape by disruption of cell adhesion and/or the cytoskeleton, disturbs or destroys the crystalline structures. This is witnessed experiments using cytoskeletal depolymerizing drugs, the cell adhesion severing enzyme trypsin (data not shown), as well as during cell division. In all three cases, the cell partially or fully rounds up, increasing the three-dimensional space accessible to the phagosomes. Inversely, the flattening out of cells through cell adhesion and cell extension improves the crystalline order. The removal of the depolymerising cytoskeletal drugs results in the re-adhesion and spreading of the cell on the surface, again creating a thin extended volume which drives the reconstruction of the crystallite. Recrystallization is also seen in the daughter cells as they spread after cell division, or in cells reseeded on the surface after trypsinization.

Phagosome crystallization occurs only for certain particle sizes and particle densities. The relative size of the particle diameter versus the local cell height determines the accessible volume and thus the local particle concentration. In regions where particle diameter is greater than the average local cell thickness, the particles are confined to two dimensions and crystallization occurs. Particles equal or greater than 2 μm crystallize at any position within the cell. However, in thicker regions of the cell, smaller particles retain their ability to move in three dimensions. As a result, 750 nm and 1 μm particles form crystallites only when the concentration becomes so large, that a three dimensional structure can be formed. Experiments show that 500 nm particles never crystallize. These observations are consistent with the premise that the cell boundary confines particles and forces crystallization. For 500 nm particles, the confinement remains insufficient to reach high enough particle concentrations, while for 750 nm and 1 μm particles, confinement becomes sufficient if the particles become crowded enough to fill the three dimensional volume. Similar effects might be possible for organelles, if large enough concentrations were reached.

The wedge-like boundary of a cell in the perinuclear region significantly impacts the final distribution of polydisperse microparticles, as seen when bi- and tridisperse particle distributions phase separate within a cell. The cell’s boundary is an elastic sheet produced by the protein coupling of the plasma membrane and the actin cortex. Deformations of the composite membrane caused by noise-driven particles in regions of spatially varying height give rise to elastic restoring forces that drive the particles towards regions with a larger height. Since larger particles deform the membrane more than smaller particles, the membrane’s elastic restoring force will be larger during encounters with large particles. This size-dependent source is the driving force of size segregation. As experiments show, regardless of the initial particle distribution, large particles are driven to more voluminous regions where the membrane is not deformed such as the area around the cell nucleus. Smaller particles can coexist there, or if there is a sufficient number of large particles, the smaller particles will be driven to the area outside of the large particle domain. A
similar effect may be responsible for the limitation to the extent of the crystallites within the cell which was measured to be $36.8 \pm 13.3\%$ for 3 \textmu m beads. If so, it might be possible to measure a difference in the average area of occupation for various bead sizes. If these explanations are correct, phagosome–membrane coupling has implications for the distribution of organelles throughout the cell.

Further analysis of crystallite formation within cells could reveal additional details about the relevant aspects of the physical environment within cells. It may also lead to new tools for the study of cytoskeletal rearrangements, cytoskeletal forces and stress, and of organelle–membrane interactions. Studies of the relative transport of multiple particles might yield further insights into cooperativity of molecular motors, intracellular traffic jams on cytoskeletal filaments, and rearrangements and dynamics of the cytoskeleton. Studies of the more static, extended crystallites could shed light on the in vivo mechanical properties of microtubules, on the dynamics of microtubules and on the intracellular transport networks of cells.

Conclusions

Cell-assisted assembly of colloidal crystallites has been reported for the first time. These studies reveal that the crystallites are formed by the combined processes of phagocytotic internalization, retrograde motion, and cytoskeletal manipulation of colloidal particles in a highly confined space. Cellular colloidal crystallites form inside of fibroblasts from both uncoated and functionalized polystyrene particles ranging from 750 nm up to at least 6 \textmu m in diameter. Once the first crystalline layer covers approximately 37\% of the cell area, three dimensional crystallites with hexagonal close packing grow when the cells are provided enough particles. Particle sizes of 2 \textmu m or larger result in improved crystalline order, although smaller particles can form well-ordered three-dimensional structures.

Simple biophysical explanations of these observations are introduced. It is proposed that phagosome crystallization is driven by cellular confinement and that the crystallites are purified by the constant rattling of the colloidal particles by intracellular motions. These manipulations occur during cell spreading, cell motion, and structural rearrangements of the cytoskeleton. Further studies might demonstrate that the cell actively rearranges the particles to minimize their strain on the cell and maximize the stability of the cell’s attachment to the surface. This would be an interesting example of internal mechanosensing and response.

Observation of the size separation and spatial segregation of different sized particles reflect the importance of membrane curvature and phagosome–membrane coupling within the cell. Size separation of particles is driven by a membrane–cortex restoring force arising from the cost of bending energy. The observation of a size dependent particle distribution around the nucleus may have important consequences for the cell, especially if it arises for objects less than 750 nm, a typical size for organelles. Our studies show that mitochondria are excluded from the perinuclear region when 4.5 \textmu m particles are present; however they do not crystallize.

The clustering of large numbers of micrometer-sized particles within a cell offers new opportunities to explore physical and biological aspects of the cellular interior. Phagosome crystallization is proof of the cramped environment of the cell, while particle size segregation demonstrates that the lipid bilayer–cortex composite membrane can influence transport inside of the cell. Observing multiple phagosomes during cell adhesion, crawling, or during the regrowth of the cytoskeleton during repolymerization experiments could be a new way to track the cytoskeleton and perhaps even to map the forces it exerts. Other interesting territory lies in experiments that study the simultaneous active transport of multiple phagosomes. Growing research in experiments that address the cooperativity of molecular motors, intracellular traffic jams on cytoskeletal filaments, and rearrangements and dynamics of the cytoskeleton might also be facilitated by this approach.

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43 In preparation for separate publication.