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ABSTRACT

Phagocytosis by macrophages represents a fundamental process essential for both immunity and tissue homeostasis. The size of targets to be eliminated ranges from small particles as bacteria to large objects as cancerous or senescent cells. Most of our current quantitative knowledge on phagocytosis is based on the use of solid polymer microparticles as model targets that are well adapted to the study of phagocytosis mechanisms that do not involve any lateral mobility of the ligands, despite the relevance of this parameter in the immunological context. Herein we designed monodisperse, IgG-coated emulsion droplets that are efficiently and specifically internalized by macrophages through *in-vitro* FcγR-mediated phagocytosis. We show that, contrary to solid polymeric beads, droplet uptake is efficient even for low IgG densities, and is accompagnied by the clustering of the opsonins in the zone of contact with the macrophage during the adhesion step. Beyond the sole interest in the design of the material, our results suggest that lateral mobility of proteins at the interface of a target greatly enhances the phagocytic uptake.

1. Introduction

Phagocytosis is a process that consists in the ability of a cell to internalize objects larger than 0.5 microns. Whereas unicellular organisms use phagocytosis to capture and eat preys, in multicellular organisms it represents a fundamental part of innate immunity, organ homeostasis and tissue remodeling. Innate immunity relies on a specialized subset of cells, the professional phagocytes, which patrol the organism, identify, ingest and eliminate pathogens. Among them, macrophages are versatile cells residing in tissues that are able to scavenge worn-out cells and participate to the activation of the adaptive immune response [1]. Phagocytosis by macrophages is triggered by the binding of the target to specific receptors, present at the surface of the phagocyte. Several receptors have been identified so far, each involving different signaling pathways and ingestion mechanisms [2]. In the case of Fcγ receptors-mediated phagocytosis [3], antigens present at the surface of the target are bound by specific soluble immunoglobulins (IgGs). Fc regions of those IgGs are actively recognized [4] by the Fcγ receptors (FcγR) from the phagocyte surface, which form clusters and trigger the internalization. Engulfment then occurs by an actin-driven membrane extension and closure [5] of a phagocytic cup around the foreign body to create a specific degradative compartment: the phagosome [6].

Most of our current quantitative knowledge on phagocytosis is based on the use of various model particles such as heat- or chemically inactivated bacteria or yeast [7] and polymer microparticles [8]. The versatility and reproducibility of the latter has allowed monitoring the influence of parameters such as size and surface chemistry [8–13], shape [14,15] and mechanical properties [16] of the target on the mechanism of phagocytosis. It was observed that the uptake of polymer particles is the most efficient for 2-3 microns-large targets, increases with the density of IgGs attached to the surface [8–12,14,17,18] and depends on the local curvature of the target in contact with the cell [14,15].

Antigens present on the surface of endogenous targets, such as erythrocytes [19], cancerous and apoptotic cells [20,21] exhibit a lateral mobility [22,23] that can't be mimicked by adhesive proteins adsorbed on the solid surface of common polymeric targets [24]. However, in the immunological context, phenomena as for example the formation of the immune synapse [25,26], or the fabrication of artificial antigen presenting cells [27], require the ability of antigens or receptors to be laterally mobile at the interface of the target. There is hence a need of novel particulate materials allowing the free diffusion of the adhesive molecules bound to their surface.

Oil-in-water emulsions have been already used since half a century as colloidal drug carriers for various therapeutic applications [28]. Versatile in terms of volume and surface composition, they can be fabricated with a narrow size distribution ranging from a few tenth of nanometers to several hundreds of microns [29]. Emulsions have also been used in a biophysical context as deformable objects to measure forces existing in living embryonic tissues [30] and those generated by growing actin networks *in-vitro* [31]. Carefully functionalized with biologically-relevant adhesive molecules, they are able to interact with cells in a specific manner [32] and can be used as model particles for cell adhesion modeling [33–35]. In addition to giving access to a controlled range of biomolecules densities at the surface, emulsion droplets have a liquid interface allowing proteins bound to it to be laterally mobile [34–36], as in our case the IgGs. However, to our knowledge there is no report of any observations at the scale of a single droplet interacting with a phagocyte, nor obvious elements about the possible influence of the nature of the interface (liquid *vs*. solid) on the uptake, despite its biophysical [37] and biological relevance [4,22,23].

We thus propose to use IgG-functionalized oil-in-water emulsion droplets for phagocytosis studies as probes able to mimick the lateral mobility of antigens present on the surface of cellular targets. Herein we decribe a fabrication route of monodisperse, IgG-coated

emulsion droplets made from soybean oil and biotinylated phospholipids. We show that IgGs-coated liquid emulsion droplets are efficiently and specifically internalized through FcγR-mediated phagocytosis *in-vitro*. During the recognition by macrophages, we show that IgGs are driven in the contact zone and colocalize with an increase of the local concentration of FcγRs, while polymerized F-actin is visible during the extension and closure of the phagocytic cup. By comparing the phagocytosis efficiency of droplets and polystyrene particles in similar conditions of IgG coating, we suggest that the lateral mobility of the IgGs at the interface of a target enhances its ability to be internalized.

2. Materials and methods

2.1. Biotinylation and opsonization of the emulsion droplets

The lipid-containing oil was obtained by dilution of DSPE-PEG(2000)-Biotin phospholipids (Avanti Lipids, Alabama, USA) in soybean oil at concentrations ranging from 0.015 to 0.15 $mg.mL^{-1}$ (30 min sonication followed by evaporation of the chloroform from the oil). This oil, cooled to room temperature, was dispersed and emulsified by hand in an aqueous continuous phase containing 15 %w/w of Poloxamer 188 block polymer surfactant (CRODA, East Yorkshire, UK) and 1 %w/w sodium alginate (Sigma-Aldrich, St. Louis, MO, USA) at a final oil fraction equal to 75%. The rough emulsion was sheared in a Couette cell apparatus at a controlled shear rate of 5000 s^{-1} following the method developed by Mason *et al.* [38] to narrow the droplet size distribution to 7 ± 2 μm. For storage and handling purposes the emulsion were diluted to an oil fraction of 60 %w/w with 1 %w/w of poloxamer 188 in the continuous phase and stored at 12°C in a Peltier-cooled cabinet for several weeks. Size distribution of the emulsion droplets was measured by microscopy and image analysis. Coupling of IgGs to biotins present on the surface of the droplets was obtained after a 30 min incubation of the droplets in 0.003 – 0.3 $mg.mL^{-1}$ (2.10^{-8} – 2.10^{-6} $mol.L^{-1}$ with a molecular

weight of 150 kDa) fluorescent antibiotin IgGs solutions (Alexa Fluor 488-conjugated IgG fraction monoclonal mouse anti-biotin (Jackson Immunoresearch, West Grove, PA, USA) at room temperature in phosphate buffer (PB, pH=7.2, 20 mM, 0.2 %w/w Tween 20). The droplets were rinsed twice in the same buffer and finally suspended in (DMEM, Life Technologies, Carlsbad, CA, USA) containing high glucose, no glutamine and no phenol red directly prior to use in cell assays.

2.2. Opsonization of the polystyrene beads by direct adsorption of IgGs

Polystyrene beads (6 µm diameter, Polysciences, Warrington, PA, USA) were functionalized by direct adsorption of rabbit anti-goat IgGs FITC conjugate (Sigma-Aldrich) at concentrations ranging from 0.4 to 1 $mg.mL^{-1}$ for one hour at room temperature.

2.3. Opsonization of the polystyrene beads using biotinylated BSA and anti-biotin IgGs

Polystyrene beads (6 µm diameter, Polysciences, Warrington, PA, USA) were incubated with biotin-conjugated BSA (Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 5 $mg.mL^{-1}$ for 1 hour in a PBS buffer. Beads were then washed and incubated for 30 min at 37°C in a fluorescent antibiotin IgG solution (Alexa Fluor 488-conjugated mouse anti-biotin, Jackson Immunoresearch, West Grove, PA, USA) at concentration of 2.10^{-1} $mg.mL^{-1}$ in PBS.

2.4. Characterization of opsonization degree of the particles

Liquid and solid particles were characterized with a BD Accuri C6 cytometer (BD Biosciences, New Jersey, USA) according to a method we developed in the past [36] for quasi-monodisperse emulsions. In brief, the fluorescence intensity of the particles is proportional to the amount of fluorescent proteins on their surface and can be converted in a

total number of fluorophores per particle using a commercial quantification kit (Quantum™ Alexa Fluor® 488 MESF beads and Quantum™ FITC-5 MESF Premix, Bangs Laboratories, Fishers, IN, USA). The number of IgGs per droplet is then estimated by dividing the number of fluorophores per droplet by the average number of dyes per IgG, which ranges from 5-8 for Alexa 488 and 3-4 for FITC according to the manufacturer. Using a value of 5 Alexa 488 dyes per IgG (or 3 FITC), the number of IgGs ranges from 10^3 to 10^5 per droplet.

2.5. Cell culture

Lifeact-mCherry RAW 264.7 murine macrophages [39] were obtained from Pierre Jurdic (IGFL, ENS Lyon) and used as model macrophages. The cells were cultured at 37°C under a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (Life Technologies), 1.0 $g.L^{-1}$ D-glucose, 1.1 $g.L^{-1}$ sodium pyruvate (Life Technologies) and 1% penicillin-streptomycin (Life Technologies). The expression of the Lifeact-mCherry was maintained through the intermittent addition of 4 $\mu g.mL^{-1}$ puromycine (Life Technologies) to the culture medium. Confluent monolayers of cells were resuspended after trypsinization and plated in 6 wells cell culture plates lined with glass coverslips (20x20mm, VWR, Radnor, PA, USA) 24 hours prior to phagocytosis studies. Experiments were performed with cell densities close to 10^6 cells per glass coverslip that correspond to *ca.* 60% confluence.

2.6. Experimental setup of the phagocytosis assay.

The custom-built chamber consisted of two glass coverslips assembled with 100 μm thick double-sided tape (3M) to form a 20 x 20 mm experimental chamber. The coverslips with the adherent cells were first washed with DMEM without FBS to eliminate dead cells. Lipid droplets or polystyrene beads suspended at an initial concentration of two drops per cell in

DMEM without FBS were injected in the observation chambers and incubated at 37°C under 5% CO_2 for up to 120 min in the presence of the macrophages. For each time point, the solution in the chambers was replaced by a fixation solution for 20 min (4 %w/w paraformaldehyde in DPBS-1X, Sigma-Aldrich). Once fixed, the cells were washed again with DPBS-1X, and a mixture of Atto 555-phalloidin and DAPI (Sigma-Aldrich, St. Louis, USA) was added to each chamber for 30 min to respectively make polymerized actin filaments and cell nuclei fluorescent for further observation. The chambers were rinsed 3 times with DPBS-1X and observed under the microscope. Unless stated, all the experiments were run in triplicate.

2.7. Microscopy

Brightfield and fluorescent images of cells with attached and internalized droplets were acquired on a Zeiss Axio Observer Z1 microscope (Oberkochen, Germany) equipped with a Clara E CCD camera (Andor Scientific, Belfast, UK) and controlled by the μManager software [40]. Confocal microscopy observations were performed on a Zeiss LSM 710 microscope. Observations were performed in DMEM without FBS at 37°C for live-cell imaging.

2.8. Quantification of phagocytosis

Phagocytosis efficiency was characterized by manually measuring, over *ca*. 20 fields of observation, the percentage of cells having internalized from 0 to a maximum of 5 droplets on a subpopulation of around 200 randomly chosen macrophages per condition. The percentage of internalizing cells (%IC) after a given incubation time and the phagocytic index (PI) were calculated. PI is defined as the weighted arithmetic mean of the total number of internalized particles per cell. The data related to the phagocytic index are reported in the

Supporting Materials. The evolution of %IC as a function of the IgG density on the droplets is fitted by a Hill equation [41] :

$$\%\mathrm{IC} = \frac{\%\mathrm{IC}_{Max}}{1 + \left({\mathrm{IgG}_{50}}/{(\mathrm{IgG\ per\ droplet})}\right)^{n}}$$

where ($\%IC_{Max}$, IgG_{50}, n) are the adjustable parameters. $\%IC_{Max}$ corresponds to the efficacy, *i.e*. the saturation value of the curve, IgG_{50} to the potency, *i.e*. the number of IgGs per droplet necessary to reach 50% of the saturation value and *n* to the Hill slope.

2.9. Immunolocalization

After a 15 min incubation with the droplets, cells were fixed and further incubated for 30 min with NH_4Cl 50 mM and 30 min with 1% BSA (Sigma-Aldrich), at room temperature. For immunodetection of FcγRs, primary antibody anti CD16/CD32 was incubated overnight at 4°C at a concentration of 1 $\mu g.mL^{-1}$ (Rat anti-mouse CD16/CD32 mAb 2.4G2, BD Pharmingen™ Biosciences, New Jersey, USA). After washing, samples were incubated for 1 hour at room temperature with secondary antibody at a concentration of 5 $\mu g.mL^{-1}$ (Goat anti-rat Alexa Fluor® 647, Life Technologies, Carlsbad, CA, USA), DAPI and Atto 555-phalloidin (Sigma-Aldrich). After extensive washing, coverslips were mounted in Fluoroshield (Sigma-Aldrich) and imaged on a Zeiss LSM 710 confocal microscope.

Diode laser 405 nm was used to excite DAPI, argon laser 488 nm for Alexa Fluor® 488, helium laser 543 nm for Atto 555 and 633 nm for Alexa Fluor® 647. Emission was detected between 430 and 540 nm for DAPI, 485–540 nm for Alexa Fluor 488, 550–580 nm for Atto 555, and 640-790 for Alexa Fluor® 647. Acquisition was made in channel-separated mode with a line averaging of 8.

2.10. Statistical analysis

Distribution of data was assessed by d'Agostino & Pearson omnibus normality test. All data followed a non Gaussian distribution. Unless stated, statistical significance was evaluated by Wilcoxon signed rank test or ANOVA and Dunn's multiple-comparison test using Prism software. $P < 0.01$ was considered significant: *** indicates $p < 0.001$ and ** indicates $p < 0.01$.

3. RESULTS

3.1 Design of opsonized lipid droplets

A series of IgG-coated lipid droplets, with an average diameter of 7 ± 2 µm, were produced (**Figure S1A**) to study their internalization by macrophages *in-vitro*. Soybean oil was chosen for the lipidic core of the droplets as it gives stable and biocompatible emulsions that are commonly used both in pharmaceutical formulations and for biophysical studies [32,33,42,43]. Quasi-monodispersity was achieved by using the method developed by Mason *et al.* [38]. To induce FcγR-mediated phagocytosis, the surface of the droplets exposes the Fc domain of IgGs. To ensure functional opsonisation of the droplets, fluorescent mouse anti-biotin IgGs were conjugated to biotins present at the surface of the droplets. This functionalization was obtained by first incorporating in the soybean oil phospholipids with a poly(ethylene)glycol spacer linked to a biotin (**Figure 1A)**. The amphiphilic nature of the modified lipids makes them gather at the interface of the droplets (**Figure 1B)**, allowing biotins to be further conjugated. The IgGs were chosen fluorescent to allow their visualization and the quantification of the opsonization on the surface of the droplets (**Figure 1C)** by flow cytometry (see **Supplementary Information**). Presence of biotinylated lipids at the interface is a necessary condition to have an attachment of IgGs on the surface of the droplets as direct adsorption of the IgGs alone is not efficient (**Figure S1B**). In our conditions, the number of IgGs per droplet varies from 10^3 to 5.10^5, which corresponds to densities

ranging from a very dilute to an almost close-packed monolayer (*ca.* 10^2 nm^2 per IgG [44]) of opsonins at the interface. Most of the experiments are done with droplets coated with a intermediate amount of 3.10^4 IgGs per droplet that corresponds to a physiological density of *ca.* 2.10^2 IgGs per μm^2 [45].

3.2 Phagocytosis of the lipid droplets

RAW 264.7 murine macrophages were incubated with lipid droplets in custom-built observations chambers made from two glass coverslips and double-sided tape spacers (**Figure 2A**). Droplets are injected in the chambers at initial concentrations of 2 droplets per cell and then, chambers are turned upside-down during incubation to let the buoyancy force make the droplets encounter the cells. Incubation time was varied from 5 min to 2 hours. Brightfield microscopy reveals that macrophages successfully engulf the droplets (**Figure 2B)** and that during the internalization by phagocytosis, the cell creates an actin-rich phagocytic cup by extending its membrane around the droplet (**Figure 2C)**. To quantify the phagocytosis process, we measure the distributions of internalizing cells as a function of the number of internalized droplets at different exposure times. After 45 minutes, the system reaches a saturation where 50% of cells are able to internalize (**Figure 3A)** between 1 and 5 droplets (**Figure 3B)**, with a majority of macrophages engulfing from 1 to 3 droplets**.** This plateau correspond to about 50% of the droplets initially added being internalized. **Figure 3A** shows that the phagocytosis assay has a characteristic time of *ca.* 15 min.

Once the phagocytic process starts, an IgG-coated droplet is fully internalized in approximately 5 minutes (**Figure 3C)**, which is in accordance with what has been measured so far in the literature with polymer particles in the same size range [14].

3.3 Specificity and IgG concentration-dependent phagocytosis

To assess the specificity of the droplets uptake towards the presence of IgGs on their surface we have compared the phagocytic efficiency of droplets with different amounts of IgGs: bare droplets made from pure soybean oil, biotinylated droplets made from soybean oil in which the biotinylated lipids have been dissolved, and opsonized droplets with anti-biotin IgGs on their surface. While bare and biotinylated droplets are almost inert towards phagocytosis, IgGs-coated droplets are efficiently internalized by the macrophages (**Figure 4A** and **Figure S2C**). The excellent level of control of opsonization density on the lipid droplets, in addition to the specificity of their phagocytosis, is used to measure the dose-response relationship of the phagocytosis by macrophages with the number of IgGs on the surface of droplets. **Figure 4B** and **Figure S3A** show that the percentage of internalizing cells (or the phagocytic index) increases with the number of IgGs and reach a plateau above *ca.* 10^4 IgGs per droplet . This value corresponds to a surface density of roughly 1% of a close-packed IgGs monolayer. In addition, phagocytosis efficiency values reported in **Figure 4B** and **Figure S3A** follow a Hill equation from which we extract a potency value of *ca.* $IgG_{50} = 2000$ IgGs per droplet necessary to reach 50% of the maximal phagocytosis efficiency. At the single cell level, **Figure S3D** also shows that the proportion of macrophages internalizing more than one droplet increases with the amount of IgGs on the surface.

3.4 Nature of the interface and IgGs clustering in the zone of contact

To qualitatively evaluate the influence of the nature of the material on the ability of a target to be internalized, we have compared the phagocytosis of IgGs-coated droplets to IgGs-coated solid polystyrene beads in the same size range (6 microns). As polystyrene beads are denser than water, the beads were allowed to settle down by gravity on the coverslips plated with cells, as shown on the **Figure S5**. Two opsonin densities within the plateau region of the

dose-internalization curve (**Figure 4B)** were investigated. **Figure 5A** shows that polystyrene particles coated with a low density of IgGs are poorly internalized, whereas at higher opsonization densities, their uptake efficiency is comparable to the opsonized droplets value within the plateau region. As expected, the spatial distribution of the IgGs on the surface of the polystyrene beads remains homogeneous, no matter the IgG density. Conversely**,** droplets show a constant uptake efficiency over the range of IgG densities (**Figure 5B**) that have been considered. **Figure 5B** also shows that at a low IgG density, the spatial distribution of the IgGs is heterogeneous and exhibits a strong clustering of the opsonins in the zone of contact with the cells. At higher opsonization densities, however, no clustering is observed with hardly any effect on the phagocytosis efficiency as compared to the low IgG condition**.** Immunolocalization experiments show that when an IgG-coated droplet (3.10^4 IgGs per particle) adhere to the macrophage, the IgG clusters colocalize with an increase of the local concentration of FcγRs (**Figure 5C**). At this stage, no nascent phagocytic cup is visible and the actin cortex is not yet modified. These clusters form within a few minutes of contact between a droplet and a macrophage and exhibit a highly dynamic behavior during the recognition phase (**Figure 5D)**.

4. DISCUSSION

A wide range of liquid emulsified systems have been industrially developped these last decades with commercial applications in drug and oxygen delivery, in topical formulations or as dietary substitutes [28,47]. Most of the characterization studies in which such materials are involved rely on macroscopic measurements made at the level of an organ or the whole organism with the aim to improve the stability and monitor the fate and the effect of emulsions on the body [47]. In the context of *in-vitro* phagocytosis studies, emulsions coated by generic biomolecules (LPS, albumin, *etc*…) have enabled to monitor the effect of physico-chemical parameters such as temperature, surface composition or the presence of various drugs and chemical species in the medium [46,48–51] on the phagocytic process. Although experimental procedures and sizes of objects used here are different, the timescales of the uptake reported on the **Figure 3** are in accordance with former kinetics measurements reported in the literature related to the uptake of opsonized droplets [48,50] and polystyrene beads [13,14].

Specificity of the uptake towards the presence of opsonins is a crucial requirement when dealing with the conception of new materials relevant for phagocytosis studies and targeting. **Figure 4A** shows that whereas IgG-caoted droplets are efficiently internalized, bare and biotinylated droplets, from which IgGs are absent from their surface, are not uptaken by macrophages. This absence of internalization of non-opsonized droplets agrees with results reported in the past [46] for non-opsonized emulsions fabricated with similar ingredients and in the absence of FBS in the medium in which the phagocytic assays were performed. As particles bearing a strong positive or negative surface charge are known to be readily internalized in a non-specific manner [8], we measured the ζ-potential of the different lipid droplets (**Figure S4**) to assess its variation upon opsonization. ζ-potential of the droplets is slightly negative, close to -20 mV, and does not depend on the functionalization of the

droplet surface. Hence this parameter cannot account for the observed differences in terms of efficiencies between bare, biotinylated and IgG-coated droplets. Thus the strong phagocytosis efficiency of IgGs-coated droplets is a consequence of the sole presence of the IgGs on their surface.

The specific internalization of IgG-coated droplets allows measuring the phagocytic efficiency of emulsion droplets over a two orders of magnitude dynamics of IgG densities. Uptake of the droplets increases with the amount of IgGs on the surface and reaches a maximum for IgG densities above *ca.* 2.10^4 IgGs per droplet (**Figure 4B** and **Figure S3A**). This value corresponds to roughly 1% of a full monolayer of IgGs if we consider an occupation area of 120 nm^2 per IgG at the saturation of the interface [44].

Recently, Gallo *et al.* [19] studied the influence of IgG density at the surface of red blood cells (sRBC) on their phagocytosis by primary macrophages. RBCs were opsonized by IgGs in density ranges that are similar to those measured in our experiments. The analysis of the potency values of IgGs-coated droplets and opsonized sRBC shows that the uptake of droplets is one order of magnitude more sensitive to the IgG density than for sRBCs. Indeed, as few as 3.10^3 IgGs are sufficient to reach 50% of the saturation value for opsonized droplets, whereas this value is close from 3.10^4 IgGs in the case of RBCs. If we hypothesize that the two kinds of macrophages have comparable behaviors, we may conclude that IgG-coated droplets could constitute a valuable alternative material to model targets such as red blood cells for *in-vitro* studies.

Within the range of opsonins densities where the droplet uptake is maximal, our experiments reveal noticeable differences in terms of phagocytic efficiencies between liquid emulsion droplets and solid polystyrene particles. Whereas the phagocytic efficiency is the same for low and high opsonization densities, **Figure 5A** shows that polystyrene beads need to have their surface almost fully saturated with IgGs to be at the maximum of internalization.

This last observation is in accordance with previous studies performed with opsonized particles similar in size to the ones used in our experiments [4,9,18].

For low IgG densities, the different internalization efficiency between the beads and the droplets could be a consequence of a difference in terms of mechanical properties of the targets. Indeed, in the case of polyacrylamide beads fabricated with various reticulation degrees, it has been shown that rigid particles are more efficiently uptaken than deformable ones [16]. Our experiments are not following this tendency, since rigid polystyrene beads are less efficiently uptaken than deformable liquid droplets. This parameter is hence unable to explain the discrepancy between the internalization efficiency of opsonized beads and droplets.

Beads and droplets differ in terms of mechanical properties, but also in terms of the nature of their interface, with strong implications on the functionalization protocols: whereas IgGs present at the interface of the droplets are specifically bound to biotinylated phospholipids dissolved in the oil, those bound the the beads are directly adsorbed on the polystyrene surface. The functionality of an antibody deposited on a surface by adsorption can decrease either by a wrong orientation of the Fc region towards the FcγRs of the cells, or by its partial denaturation on the surface [52,53]. The discrepancy between the beads and the droplets uptake at low IgG density could hence be a consequence of the coating strategy used for the beads on the functionality of the IgGs bound to them. In comparison to the measurement of the phagocytic efficiency of the IgG-adsorbed beads, we measured the internalization efficiency of polystyrene beads coated with an alternate functionalization route that avoids the contact of the IgGs with the solid surface. In this latter case, anti-biotin mouse IgGs are specifically bound to a primary and saturated layer of biotinylated BSA adsorbed on the beads, hence diminishing the possibility of the IgGs to be denaturated and possibly improving their orientation. The comparaison of the phagocytic efficiency of beads

prepared following this protocol and beads opsonized by direct adsorption show that the functionalization strategy of the particles has no effect on the internalization efficiency (**Figure 5A** and **Figure S6**). The hypothesis of a bias in the measurements related to the chemical nature of the surfaces and its potential effect on the IgG functionality can be ruled out.

Microscopic observations show drastic visual differences about the behavior of the IgGs present on the surface of the beads or the droplets during the adhesion step. The IgG distribution remains homogeneous around the polystyrene beads no matter the IgG density, as a consequence of the solid nature of the surface that forbids the lateral diffusion of the opsonins. Conversely, IgGs present on the surface of the droplets cluster in the zone of contact with the cell for low IgG densities whereas no clustering is visible for high IgG densities (**Figure 5A**). Clustering of proteins bound to the interface of emulsion droplets has been reported in various experiments involving the adhesion of droplets to functional substrates [32–34]. It has been shown that when attached on a liquid interface through mobile linkers as phospholipids, adhesion molecules could migrate from the outer region of the droplets to the region of contact and form dense clusters. The clustering can be driven by non-specific interactions solely, which create an energetically more favorable environment within the zone of contact, without the need of the formation of ligand-receptor complexes, nor cytoskeleton remodeling [33]. A rough estimate of the IgGs density in the cluster shown on **Figure 5B** indicates that the opsonins are almost close-packed in the region of contact with the cell. Indeed, a random close-packed region containing all the 3.10^4 IgGs initially present on the droplet would occupy an area of 5 μm^2 for a molecular area of *ca*. 120 nm^2 per IgG [44], thus constituting a lower limit of the cluster size that is consistent with the cluster dimensions. Immunolocalization experiments on the **Figure 5C** show that IgGs colocalize with an increase of the local concentration of FcγRs on the cell surface. For the high IgG

density condition, the surface of the droplets is almost saturated with opsonins, which makes the local variations in IgG surface densities barely observable by and explains why no cluster is visible in this case.

According to Zhang *et al.* for the case of polystyrene beads as large as the emulsion droplets of our study, the density of IgGs present on the surface of the particles controls the efficiency of the internalization, but has no influence on the kinetics of the cup closure [18]. We understand these results as characteristic from an all-or-none ingestion mechanism similar to the phagocytic *trigger* model [54], and activated above a certain density of IgG-FcγR complexes in region of contact between the bead and the cell. According to this scheme, IgG-coated droplets have a competitive advantage over solid beads since sole passive diffusion on the liquid interface can help gather all the IgGs present on the droplet, increase the local concentration of opsonins in the zone of contact, increase the expression of receptors, cluster IgG-FcγR complexes and finally cross the threshold that activates the uptake, as sketched on the **Figure 5E**.

5. CONCLUSION

In conclusion, we have designed and characterized new synthetic targets for phagocytosis studies, IgGs-coated emulsion droplets, that are efficiently and specifically uptaken by macrophages. From the material point of view, droplets need one order of magnitude less opsonins on their surface as compared to polystyrene beads to reach the maximal internalization efficiency, which can be a valuable advantage if we think in term of particle design for *e.g.* pharmaceutical applications. Microscopic analyses have shown that the adhesion of droplets to macrophages is accompagnied by the formation of IgG clusters in the region of contact, which increase the local effective concentration of opsonins and finally

triggers phagocytosis. To rationalize the characterization of the engineered droplets, we consider looking in a near future at the macrophage response cascade occurring following the internalization and the phagosome maturation, by assaying first cytokine secretion, respiratory burst and reactive oxygen species production, and second the fate of the droplets on a long-term timescale.

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FIGURES AND CAPTIONS

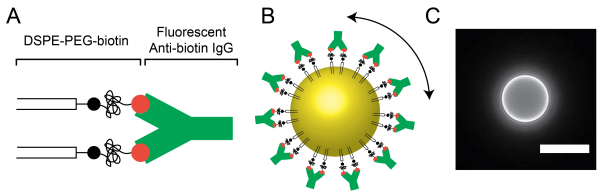

Figure 1: (A) Schematic view of the immunoglobulin-functionalized phospholipids. (B) Schematic view of the interface of the lipid droplets (C) Epifluorescence image of a droplet bearing 2.10^4 fluorescent IgGs on its surface. Scale bar : 10 μm.

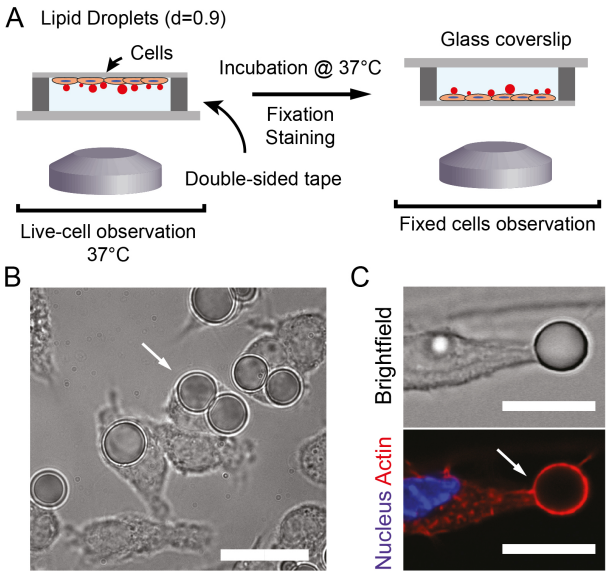

Figure 2: (A) Schematic view of the experiment. (B) Representative brightfield microscopy image of a phagocytic assay. (C) Brightfield and fluorescence images of the phagocytosis of an IgG-coated droplet after fixation and staining with DAPI and Atto 555-phalloidin. Scale bar: 10 μm.

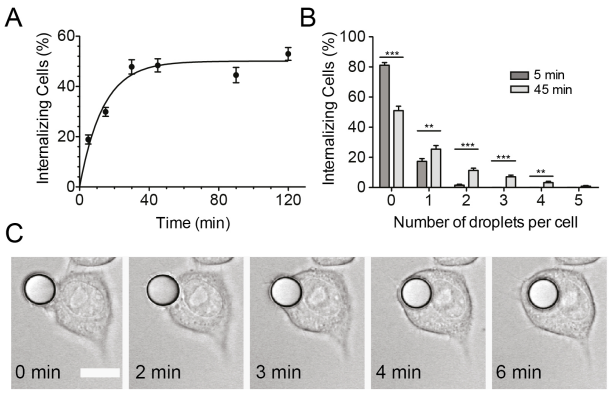

Figure 3: (A) Time evolution of the percentage of internalizing cells after incubation with IgG-coated lipid droplets (3.10^4 IgGs per droplet). (B) Evolution (after 5 and 45 min incubation) of the percentage of internalizing cells with n (from 0 to 5) IgG-coated droplets (3.10^4 IgGs per droplet). (C) Time-lapse observation of macrophages ingesting opsonized lipid droplets. Scale bar: 10 μm. *** indicates $p < 0.001$, ** indicates $p < 0.01$. Error bars represent the SEM of three independent experiments.

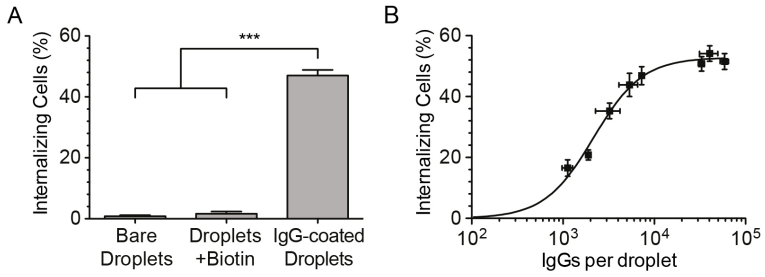

Figure 4: (A) Dependence of the percentage of internalizing cells upon surface functionalization of the droplets: fbare droplets, biotinylated droplets (0.03 $mg.mL^{-1}$ DSPE-PEG-Biotin) and IgG-coated droplets (2.10^4 IgGs per droplet) after 45 min of incubation with n=100. (B) Influence of the number of IgGs per droplet on the percentage of internalizing cells (after 45 minutes of incubation). The curve is fitted by a Hill equation: $\%IC_{Max}$ = 52 ± 2; IgG_{50} = 2150 ± 200 IgGs per droplet; n = 1.62 ± 0.25. Each point represents the mean of two independent experiments and error bars correspond to SEM.

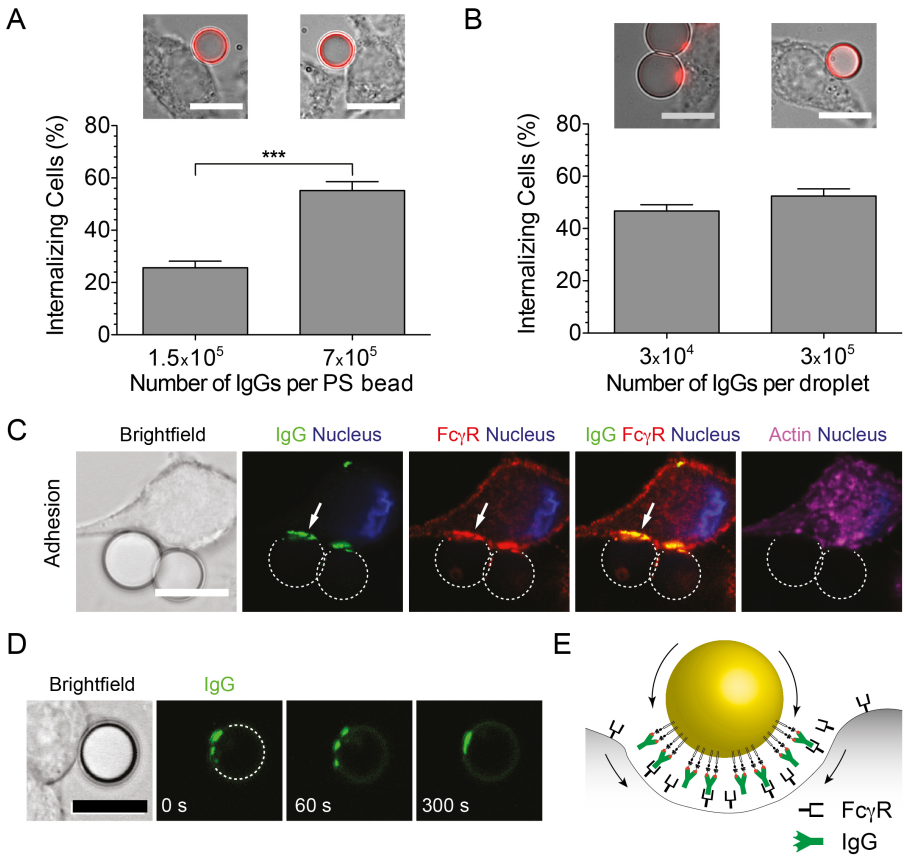

Figure 5: Internalization of polystyrene solid beads (A) *vs.* liquid droplets (B) for two different IgG densities per particle (45 min of incubation time). Pictures above each bar chart are representative brightfield and epifluorescence overlays of beads and droplets adhering to the macrophages for each experimental condition. The IgGs are shown in red to enhance the readability of the images. *** indicates $p < 0.001$. Error bars represent the SEM of three independent experiments. Scale bar: 10 μm. (C) Fluorescence microscopy images of immunolocalisation during the adhesion step: IgGs (green, Alexa-488), Fcγ receptors (red, Alexa-647), actin network (magenta, Atto-555), nucleus (blue, DAPI). Scale bar: 10 μm. The dashed lines indicate the position of the droplets. (D) Time-lapse images of a droplet engaged in a phagocytic event during the adhesion step: IgGs (green, Alexa-488). The dashed line

indicate the position of the droplets. Scale bar: 10 μm. (E) Schematic view of clustering process of IgGs and FcγRs in the zone of contact between the droplet and the cell.

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