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Abstract

The ability of microorganisms to survive under extreme conditions is closely related to the physicochemical properties of their wall. In the ubiquitous protozoan parasite *Toxoplasma gondii*, the oocyst stage possesses a bilayered wall that protects the dormant but potentially infective parasites from harsh environmental conditions until their ingestion by the host. None of the common disinfectants are effective in killing the parasite, since the oocyst wall acts as a primary barrier to physical and chemical attacks. Here, we address the structure and chemistry of the wall of the *T. gondii* oocyst by combining wall surface treatments, fluorescence imaging, electron microscopy and measurements of its mechanical characteristics by using Atomic Force Microscopy (AFM). Elasticity and indentation measurements indicated that the oocyst wall resembles common plastic materials, based on the Young moduli, *E*, evaluated by AFM. Our study demonstrates that the inner layer is as robust as the bilayered wall itself. Besides wall mechanics, our results suggest important differences regarding the non specific adhesive properties of each layer. All together, these findings suggest a key biological role for the oocyst wall mechanics in maintaining the integrity of the *T. gondii* oocysts in the environment or after exposure to disinfectants, and therefore their potential infectivity to humans and animals.

Introduction

Resistance to physical and chemical degradation is essential for the perpetuation of the life cycle of environmentally exposed microbial pathogens. In the coccidian parasite *Toxoplasma gondii*, this function is served by the oocyst, the only stage of the parasite structurally equipped to survive in harsh environments (1). Oocyst-related infections in humans and other warm-blooded animals worldwide have been increasingly reported as more prevalent and severe than previously thought (2–6). Infections lead to possible deleterious ocular and neurological complications, and even death (7). In this context, a global effort has emerged to decipher the basic structures (8, 9) and biological processes of the oocyst (10–13) that allow the parasite to survive different environmental conditions and disinfectants (14–18).

The oocyst is the result of sexual multiplication of *T. gondii* in the intestinal epithelium of cats (19–21). A few days post-infection, unsporulated (uninfective) spheroid oocysts (10 x 12 µm) are excreted in cat feces and become rapidly infective following aerobic sporulation (22). Sporulation results in different subpopulations of maturing oocysts during the first few days (22): unsporulated (NS), 'sporoblast-staged' (SB) and fully sporulated (SP) oocysts (11, 22). SP oocysts are ovoid, measure 11 x 13 µm in size and have two sporocysts (6 x 8 µm), each containing four infective banana-shaped sporozoites (2 x 6-8 µm) (*SI Appendix*, Fig. S1) (23, 24).

Toxoplasma gondii oocysts are highly resistant to environmental influences and this resistance to various physical and chemical stressors, including disinfectants such as UV, ozone, and chlorine-based products, is attributed to the oocyst wall (4, 25, 26). In contrast, oocysts are rapidly inactivated following exposure to temperatures above 60°C

for few minutes (27). The oocyst wall is bilayered with the outer layer being thinner than the inner layer (24). The layers are not tightly bound to each other since the outer layer can be stripped off easily using sodium hypochlorite (10, 12, 22, and the present study). The oocyst wall is made of more than 90% proteins with several structural cysteine- and tyrosine-rich proteins having been identified in the outer layer only (13) or in whole oocyst wall fractions (10, 12). How these different proteins are processed and/or packed to form the oocyst wall is still not clear (8). Current models support a strong contribution of protein-tyrosine cross-linking in the formation and hardening of the oocyst wall in *Toxoplasma*-related coccidia (8, 12, 26, 28) and results in the development of its typical blue autofluorescence (AF) under UV excitation (10, 26, 28, 29) (*SI Appendix*, Fig. S1B). This complex polymeric organization also suggests an important robustness of the *T. gondii* oocyst wall in terms of mechanics (8, 26). Thus, measuring mechanical properties of the *T. gondii* oocyst wall appears to be relevant to addressing the respective roles of structure and chemistry of each layer of the oocyst wall in the overall resistance of the oocyst to various physical and chemical agents (8).

Atomic Force Microscopy (AFM) is a rather new technique in biology that fits perfectly for this purpose (30). The AFM provides valuable information regarding the surface topography and/or allows force measurements in physiological media, with fixed or unfixed samples, from proteins to cells (31–33). AFM uses a finger-like tip, at the extremity of a very soft cantilever (30). This tip can be used to: (i) delineate the surface (imaging mode, (34–36)), (ii) indent the objects surface by pressing on them allowing measurement of their mechanical properties (force mode, mechanics, (37–40)) or (iii) probe the adhesion of surface molecules when decorated with suitable haptens and

pulling the lever off the surface until all built bridges are broken. This allows direct quantifying of the force that these bridges can sustain (force mode, adhesion). For modes (ii) and (iii), the AFM cantilever is held at a given x,y above the sample (a surface or a cell) and ramped in z-direction. Measuring cantilever deflection as a function of piezo position produces force-extension curves (FC) (30).

Using the AFM tip as a microindentor and using the part of the FC where the tip is pressed on the surface, one can gain information regarding the local elastic properties as measured as a Young's modulus, *E*, using a Hertz model for elastic indentation. *E* moduli measured for different eukaryotic cell types vary greatly from cell type to cell type and usually ranges from 1 kPa to several 100 kPa (37, 38). Using the part of the FC where the tip is retracted from the surface, adhesion force measurements can be performed and have been employed in cell biology, from single molecules measurements to cell/cell measurements (31, 41–47). The sensitivity of force determination is usually limited by the thermal noise of the system and the properties of the chosen cantilever (30).

Here, we investigate using AFM the mechanics of the wall of *T. gondii* oocysts submitted to physical (heat inactivation) and chemical (bleach exposure) treatments separate or in combination in order to evaluate the contribution of each layer in the overall mechanics of the oocyst wall. Our results present a simple way to gently but firmly immobilize oocysts on surfaces to image them using fluorescence microscopy or test their mechanics using AFM under moderate forces. Our findings may be correlated to structural modifications of the oocyst wall and suggest a key biological role of the wall mechanics in maintaining the integrity of the *T. gondii* oocysts in the environment or exposed to disinfectants, therefore affecting potential infectivity to humans and animals.

Results

Microscopic Characteristics of *T. gondii* Oocysts Following Sporulation in Water. In order to evaluate the basic mechanics of the *T. gondii* oocyst wall, we limited the use of chemicals to avoid any modification of the wall structure due to handling or storage conditions. For this, oocysts were sporulated in water rather than in 2% aqueous sulfuric acid solution which is commonly used for oocyst sporulation and subsequent storage (11, 22, 48). After a 5-day sporulation process, the oocyst suspension contained 18.6 ± 2.7 % of NS, 18.3 ± 6.4 % of SB and 63.0 ± 5.9 % of SP oocysts (*SI Appendix*, Fig. S1). All these different oocyst subpopulations exhibited the same typical autofluorescence (AF) under UV excitation (*SI Appendix*, Fig. S1B). Careful observation, before and during AFM experiments, was a key step to the exact categorization of the objects.

Characterization of the Bleach Effects on *T. gondii* Walls by Electron Microscopy. Electron microscopy confirmed that control (H_2O-conserved) oocysts retained their typical double-layered wall (observed thickness ~50 nm, Fig. 1A,B). In contrast, the outer layer (observed thickness ~18-20 nm) was absent in bleach-treated oocysts with only the inner layer (observed thickness ~30 nm) remaining with, in certain cases, slight remnants of the outer layer persisting (Fig. 1C). The oocyst wall thicknesses that were observed here are consistent with but in the lower end of the reported values of such structures reported in literature (up to 100 nm for the bilayered wall) (24). It is interesting to note that the bilayered structure of the *T. gondii* sporocyst wall was maintained following bleach treatment of the oocyst wall (Fig. 1D,E).

Characterization of *T. gondii* Walls by Fluorescence Microscopy. The properties of the wall of NS, SB and SP oocysts were first assessed microscopically by analyzing their reactivity to the monoclonal antibody (mAb) 4B6, which is specific to the inner layer of the oocyst wall (49). In order to induce structural modifications of the bilayered oocyst wall, the parasites were treated by bleach to remove the outer layer and/or heated at 80°C. In contrast to bleach treatment, heating oocysts at 80°C efficiently kills the sporozoites, however, the effects on the wall structure remain largely unknown. Oocysts exposed only to water during their maturation and storage served as controls. The percentages of oocysts at different maturing stages exposing partially or totally their inner wall following these different surface treatments are shown in Fig. 2. Corresponding immuno-fluorescence and AF representative images are shown in *SI Appendix*, Fig. S2.

Between 21.2 and 25.6% of H_2O-exposed oocysts were labeled with antibody mAb 4B6 (Fig. 2). A mixture of unstained to totally mAb 4B6-stained oocysts were observed irrespective of the oocyst developmental stage (*SI Appendix*, Fig. S2A). Fluorescent staining appeared randomly distributed at the oocyst surface, and ranged from almost continuous staining of the entire surface (*SI Appendix*, Fig. S2A, case 1) to very discrete patches (*SI Appendix*, Fig. S2A, case 2). In these conditions, 4B6 staining of the inner wall layer appeared to result from the infiltration of the antibody through cracks in the oocyst wall rather than the exposure of the outer aspect of the inner layer of the oocyst wall. This hypothesis was further supported by the fact that ~25-30% of H_2O-exposed oocysts were permeable to FITC in solution, again indicating possible openings in the

oocyst wall (*SI Appendix*, Fig. S3). Irrespective of their 4B6 pattern, SB and SP oocysts were more autofluorescent than NS oocysts (*SI Appendix*, Fig. S2A and Fig. S4).

Heating oocysts at 80°C for 10 min led to a significant reduction of the percentage of 4B6-positive oocysts at all maturation stages (0.6-3.1%) (Fig. 2). The few positive oocysts observed were very faintly stained in localized areas of the oocyst wall (*SI Appendix*, Fig. S2B). In these experimental conditions, heating did not appear to alter significantly the microscopic structure or internal content of NS, SB and SP oocysts or the AF of the oocyst wall (*SI Appendix*, Fig. S2B compared to Fig. S2A).

In contrast, there was a significant increase in mAb 4B6 staining of oocysts bleached with 3% bleach solution for 30 min (final mAb 4B6-labelled oocysts from 48.3 to 80.7% depending on the maturing stage) compared to control or heated oocysts (Fig. 2). In these experimental conditions, the antibody stained, at least partially, the inner layer of NS, SB and SP oocyst wall usually with a strong intensity (*SI Appendix*, Fig. S2C) indicating that modifications of the wall structure increase the access of mAb 4B6 to the outer aspect of the inner layer of the wall, but not its infiltration through it since only ~25-30% of bleach-exposed oocysts were again permeable to FITC due to possible fractures in their wall (*SI Appendix*, Fig. S3). We also observed that several bleach-treated oocysts displayed a reduced AF pattern of the oocyst wall compared to the control oocysts (*SI Appendix*, Fig. S4, suggesting possible differences in the biochemical content of the oocyst wall between untreated and bleach-treated oocysts.

When bleached oocysts were subsequently heated at 80°C, the percentages of mAb 4B6-positive parasites were similar to that observed with bleaching alone (40.1-74.3%) (Fig. 2). Microscopically, these oocysts had similar 4B6 and AF patterns compared to

bleached-only oocysts, irrespective of the stage of maturation (*SI Appendix*, Fig. S2D compared to Fig. S2C). However, a few mAb 4B6-positive oocysts still had a very discrete wall AF (*SI Appendix*, Fig. S2D, case 5).

Attachment of the Oocysts for AFM Experiments. The mechanical properties of the wall of H_2O-stored, bleach- and/or heat-treated oocysts at different maturing stages were then evaluated using AFM. For this, oocysts were first allowed to adhere onto Poly-L-Lysine (PLL)-coated glass microscopic slides prior to being approached by the AFM tip (*SI Appendix*, Fig. S5A,B,C). We verified that the coating procedure on glass did not change significantly the initial observed proportions of the different subpopulations of the oocysts irrespective of their pretreatment (*SI Appendix*, Fig. S5B). This coating procedure was suitable for firmly attaching the oocysts onto the glass surface thus allowing repeated contacts between the AFM tip and each oocyst at a preset contact force of 1 nN. The fine positioning of the AFM tip on top of the substructures (i.e. sporocysts) does not largely affect the measurements (*SI Appendix*, Fig. S5D,E).

Elastic Properties of the Oocyst Wall. Following our immobilization protocol, repeated force curves were obtained for each adhered object (Fig. 3A,B). We observed that the indentation depth was <50 nm and typically ~ 20 nm (Fig. 3C,D) irrespective of the maturing oocyst stages or their pretreatment (*SI Appendix*, Table S1). This median indentation is of the same order of magnitude as the most outer layer of the oocyst wall (20 nm) and smaller than the thickness of the most inner layer (30 nm) as measured in EM micrographs. Following this, (i) in absence of bleach treatment, we concluded that

we measured the mechanics of, if not the outer layer alone, the bilayered structure of the wall, and (ii) following bleach treatment, we were able to access the mechanical properties solely of the inner layer of the wall.

Young moduli, *E*, obtained for NS, SB and SP H_2O-stored oocysts were typically in the 10^6-10^7 Pa range and were not significantly different from each other (Fig. 3E,F). Those elevated *E* values are similar to the ones reported for artificial polymeric capsules of comparable thicknesses (50). The median *E* moduli showed no significant variation between the different maturing oocyst stages irrespective of the oocyst pretreatment (*SI Appendix*, Tables S2 and S4). Force relaxation experiments during contact of the tip on the oocyst and the superposition of trace and retrace parts of the force curves indicated that no viscous behavior could be identified in this range of stimulations (Fig. 3A,B). In addition, repetitions of contacts lead to rather unvarying indentation depths, with no apparent tendency of any plastic deformation of the oocyst wall for the investigated forces (Fig. 3C).

Pressing the oocysts at higher forces (30 or 120 nN) did not appear to strongly modify the mechanics of the oocyst wall (*SI Appendix*, Fig. S5F,G). However, in these conditions, we observed that oocysts were accidently removed from the slide because of the higher pressing force, making the AFM tip into a golf club and the oocyst the ball.

Adhesive Properties of the Oocyst Wall. In addition to indentation and elasticity measurements, the non-specific adhesive properties of the oocyst wall were examined. Surface adhesion (Fig. 4) was measured as the force required to detach the AFM tip from the surface of the oocyst after indentation at 1 nN, with a fixed pulling speed of 1 μm/s.

We observed that at least 50% of the force curves showed some adhesion (Fig. 4A vs. Fig 4B). The proportion of oocysts showing a detectable adhesion was observed to depend on the developmental stage and on the treatment of the oocyst surface. Temperature alone lowered the proportion of oocysts with adhesive surface properties while application of bleach increased it (Fig. 4C and D). Upon repeated pulling on the same object, adhesive forces did not show any marked tendency (Fig. 4E) indicating that materials coming from the wall did not pollute the tip. We observed that the strength of adhesion (i.e. the force required to fully detach the tip from the oocyst) was rather low (<100 pN) for control and temperature-treated oocysts but was significantly stronger when bleach was applied as a first treatment (Fig. 4F and *SI Appendix*, Table S3) suggesting important bleach-induced modifications of the wall. In conclusion, bleach treatment increases both the proportion of oocysts showing detectable adhesion and the overall strength of the adhesion while high temperature treatment alone did not affect adhesion force but reduces the proportion of oocysts showing significant adhesion.

Discussion

The *T. gondii* oocyst is a superstructure that protects the dormant but potentially infective sporozoites from many extreme conditions that would be deleterious for survival (24). Facing the external environment, the oocyst wall acts as a primary barrier to physical and chemical attacks as long as its complex polymeric organization is perfectly maintained (8, 12, 26). Different, but complementary, approaches have been applied to investigate the structure and molecular basis of the oocyst wall resilience, such as electronic microscopy (9, 12, 24, 51) and proteomics studies (10, 12). However, oocysts are

technically difficult to process for electron microscopy examination because of the impervious nature of the walls and proteomics usually require large numbers of highly purified oocysts, which are difficult to obtain since oocysts cannot be generated in vitro. Here, we addressed the structure and chemistry of each oocyst wall layer by measuring their respective mechanical properties by combining wall treatments, fluorescence and electron microscopic observations, and AFM techniques on immobilized parasites.

In coccidian parasites such as *T. gondii*, the oocyst wall results from the particular arrangement of structural proteins through a sclerotization process involving both quinone tanning and protein dityrosine crosslinking and dehydration (12, 26). This process probably takes place very early in the development of the oocyst wall, from the NS stage before it leaves the host (21), and is thought to lead to hardened structures, which excludes water-soluble molecules in order to form a complex polymeric covering capable of resisting extended physical and chemical-induced disorganization. Interestingly, it has been recently claimed that the inner wall layer of *Eimeria tenella* oocysts possesses discrete pores of 5-250 nm (9). However, such structures in the *T. gondii* oocyst wall were not observed in the present or previous studies (26).

The oocyst wall layers in *T. gondii* are assumed to differ in their thickness and molecular content, the inner one being thicker, less electron dense and more resistant to chemical degradation than the outer one (8). Using fluorescence imaging and electron microscopy combined with different treatments, we provide new insights on the structure and chemistry of the wall of *T. gondii* oocysts. Specific immuno-staining of the inner wall layer of H_2O-exposed oocysts was infrequent and appeared to result from the infiltration of the antibody through focal openings of the oocyst wall rather than exposure of the

external surface of the inner layer. After heating, antibody staining was significantly decreased in control oocysts while it had little, if no, effect on bleach-treated oocysts. This would suggest that heating is not denaturing the antigen recognized by the antibody and probably results from heat-induced reticulation of poly-protein structures of the outer layer reducing penetration of the antibody. In contrast, bleach treatment clearly affected both the structure and chemistry of the oocyst wall, by removing the outer layer as seen in our EM micrographs and significantly affecting the oocyst wall AF. Consequently, most of the 4B6 staining observed in bleached oocysts could be linked to the exposure of the external surface of the inner layer of the oocyst wall.

Then, we investigated the mechanical properties of the double-layered oocyst wall, and then those specific to the inner layer after removing the outer layer by treatment with bleach. We observed high *E* moduli comparable to polymeric shells (50) with neither viscous nor plastic behaviors. The *E* moduli were not significantly different between the different maturing stages and treatments involving temperature and bleach (alone or in combination). Considering the small indentation and high *E* modulus, our results strongly support that the global stiffness of the bilayered oocyst wall is in the same order as that of the inner layer alone. Interestingly, we showed that the oocyst wall stiffness did not vary significantly following parasite incubation at 80°C, which was quite unexpected since heat-induced stresses usually result in increasing stiffness of polymeric multilayer microcapsules due to an increased shell thickness or reticulation (52). The conservative hypothesis suggests that each wall layer retains its basic mechanical properties by maintaining to large extend its molecular architecture, even at this temperature.

Besides mechanical measurements, AFM has also permitted examination of the non-specific adhesive properties of each layer of the wall. It was observed that the proportion of oocyst showing adhesive properties was lower as was the strength of the adhesion (i.e. overall force needed to fully detach the tip from the oocyst) in parasites retaining their typical double-layered wall structure, whereas there was a higher proportion of oocysts with adhesive properties and stronger adhesion was recorded in oocysts exposing solely the inner wall layer (*SI Appendix*, Fig. S6). This contrasting behavior might indicate the existence of important differences regarding the biochemical nature of the molecules and/or their arrangement from one wall layer to the other resulting from oocyst treatments modulating the structure of the wall. We speculate an increasing number of residual polypeptidic chains due to removal of (most of, if not all) the most outer layer of the wall (*SI Appendix*, Fig. S6). Such differences have also been hypothesized by others (10), based on proteomics analyses of purified wall fractions of *T. gondii* oocysts exposed or not to bleach. It is not yet clear whether these different adhesive properties play a role in the oocyst fate. Recent studies have shown that the negative charges covering the surface of the oocyst wall prevent in most cases any aggregation of the parasites with other particles, thus allowing the parasites to disperse freely in fresh water (8). Further investigations, in particular on molecule-specific adhesion and the effects of digestive enzymes found in the host's gut, are required to extend our study and refine the biological significance of the adhesive properties of the oocyst wall.

From a methodological point of view, this study proposes a simple but efficient way to immobilize hardened biological microparticles such as *T. gondii* oocysts on glass slides for investigating the biophysical properties of their multilayer wall by using AFM and

fluorescence microscopy techniques. It opens the possibility to extend such studies to immobilized *T. gondii* sporocysts and to other environmentally-resistant parasitic pathogens such as *Cryptosporidium* and *Giardia* (53–56).

In conclusion, our study demonstrates that the overall rigidity of the bilayered *T. gondii* oocyst wall is as high as common plastic materials and that the inner layer is as robust as the bilayered wall itself. These findings strongly suggest that the mechanical characteristics of the *T. gondii* oocyst wall sustain the survival of the enclosed sporozoites facing physical and chemical attacks outside the host. In particular, our results suggest that chlorine-based products used as surface disinfectants or for treating drinking water are ineffective for efficiently killing *T. gondii* oocysts because these compounds are not able to permeabilize or disrupt the oocyst wall. However, it is clear that these properties have to be circumvented following oocyst ingestion by the host, in order to safely deliver the sporozoites near the enterocytes. As chemicals are ineffective in breaking the oocyst wall, a supplementary physical stimulus (still to be determined) seems to be required to prime oocyst-related infections in humans and animals.

Materials and Methods

Oocyst Purification and Sporulation. Oocysts of the genotype II TgNmBr1strain of *T. gondii* (57) were harvested from feces of cat 6-8 days after feeding infected mouse tissues to a *T. gondii* free cat (1), then purified by flotation, and allowed to sporulate at RT for 5 days. More details can be found in SI. Oocyst suspension was stored in distilled water at 4°C until used within 3 months. More details can be found in *SI Appendix*.

Chemical and Physical Treatment of the Oocysts

Bleach Treatment. H_2O-stored oocysts were washed three times in PBS at 5000g 5 min and then incubated with 1 mL of bleach solution containing 3% sodium hypochlorite (Fouque Chimie Service, Marseille, France) in PBS for 30 min at 4°C. The oocysts were then washed three times in PBS to remove bleach prior to be immobilized on coverslips for AFM experiments.

Heat Inactivation. H_2O-stored or bleach-treated oocysts were washed three times in PBS at 5000 g for 5 min, resuspended in 500 µL PBS and then placed in a dry block heater for 10 min at 80°C to allow their inactivation prior to AFM experiments.

Electron Microscopy. Samples of water maintained and bleached oocysts were mechanically ruptured prior to fixation in 2.5% glutaraldehyde in 0.1M phosphate buffer and processed for routine electron microscopy. In summary, the samples were post-fixed in osmium tetroxide, dehydrated in ethanol, treated with propylene oxide and embedded in Spurr's epoxy resin. Thin sections were cut and stained with uranyl acetate and lead citrate prior to examination in a Jeol 1200EX electron microscope.

Immunofluorescence Assay (IFA). The effects of bleach and heat treatments on the integrity of the oocyst wall were evaluated by IFA combined with the autofluorescent signal (AF). We labeled oocysts in suspension using a monoclonal antibody (IgM mAb 4B6), which was previously shown to react mainly with the inner layer of the oocyst wall (49, 58). More details can be found in *SI Appendix*.

FITC Infiltration Assay. The permeability of the wall of oocysts exposed or not to bleach treatment was assessed by incubating oocysts with FITC at 0.5 mg/mL in PBS for 1 hr at room temperature. Oocysts were subsequently washed four times in PBS by gentle centrifugations at 5000 g for 5 min and examined for fluorescence of FITC bound to internal proteins (*SI Appendix*, Fig. S3).

Measurements of the Oocyst Wall Autofluorescence Intensity. The effects of bleach treatment on the autofluorescence pattern of the oocyst wall were evaluated on AF gray scale images as described in *SI Appendix*, Fig. S4.

AFM Experiments

Oocyst Immobilization on Glass Coverslides. Clean glass coverslides were coated with poly-L-lysine (PLL) after activation using a residual air-based plasma After rinsing and mounting on an observation chamber, a diluted suspension of untreated, heat-inactivated, or bleach-treated oocysts was seeded onto the PLL treated zone and let to settle for 45min to 1 hr at RT before removal of non adherent objects. We observed that this procedure did not grossly affect the different sub-populations ratios as compared to the original suspension (*SI Appendix*, Fig. S5B). More details can be found in *SI Appendix*.

AFM Measurements. A Nanowizard I (JPK Instruments, used in closed loop mode) sitting on an Axiovert 200 (Zeiss) equipped with 10x and 40x lenses (with an optional 1.6x lens) was used to measure the oocyst mechanics. The system was sitting on an active damping table (Halcyonics) to suppress mechanical noise. Blunt AFM levers (MLCT, Veeco, nominal spring constant 10 pN/nm) were used and calibrated in situ. The spring

387constant (∼ 15-18 pN/nm) was determined in situ using a built-in thermal calibration
388method, far from the glass surface to avoid any hydrodynamical bias due to the coupling
389with the substrate (59).
390Using bright field, a given oocyst was chosen and a calibrated AFM cantilever was
391positioned on top of it (*SI Appendix*, Fig. S5C,D). We checked that the measurements
392were not affected by the fine positioning of the tip on top of the structures (e.g. over the
393two substructures of SP oocysts and between them, so only the mid position was used to
394quantify the structure's mechanics (*SI Appendix*, Fig. S5D,E).
395At least 10 force curves, with a preset contact force of 1 nN, a preset contact time of 0
396sec, pressing/pulling speeds of 1 μm/sec and an acquisition frequency of 1024 Hz, were
397acquired per oocyst (Fig. 3A,B). Each force curve was evaluated by eye and processed on
398a PC using the built-in JPK IP software (JPK Instruments), resulting in 3 to more than 15
399data points extracted for each structure. We first observed that the maximal indentation
400depth at 1 nN was in average ~ 20 nm (Fig. 3C,D), that is less than the thickness of the
401bilayered wall structure, so the model was accurate enough to allow us to extract the
402Young modulus of the structures, *E*, one per valid force curve. To quantify the
403mechanics, we used as a first approximation a Hertz model for contact to fit the pressing
404part of the force curves assuming a pyramidal tip of $\alpha=21°$ half angle (calculated from
405manufacturer data) and incompressibility of the material ($\nu=0.5$) (37) (Fig. 3A,B). Since
406the indentation was <50 nm, we also tried the Hertz model for a spherical indentor of 25-
40750 nm radius and found little differences in the quality of the fit and subsequently
408calculated *E* moduli.
409For adhesion measurements, we retrieved from the return/pulling part of the force curves

the maximum detachment force (Fig. 4B) and frequency of adhesion events (i.e. the ratio between the number of force curves having adhesion divided by the total number of force curves taken into account) (Fig 4C,D). We then calculated median adhesion forces and plotted the entire force distribution as whisker plots. We did not observe any tendency of adhesion force versus upon repetitions of tests (Fig. 4E).

Experiments lasted maximum 2 hr at RT before changing the sample and the cantilever with occasional supplementations of water to counteract the evaporation.

Data Processing and Statistical Analysis

The distributions of the pooled data being non gaussian, median values with data points and/or whisker plots were then plotted as a function of the oocyst stage and treatment using Prism 5.0 and 6.0 (GraphPad). The data sets were compared using non-parametric tests such as Kruskal-Wallis.

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Author contributions

A.D. and P.-H.P designed research; A.D., J.P.D., D.J.P.F. and P.-H.P. performed research; A.D., J.P.D., D.J.P.F., P.B., N.A. and P.-H.P. contributed reagents/materials/analysis tools; A.D., J.P.D., D.J.P.F. and P.-H.P analyzed the data, and A.D., J.P.D., D.J.P.F. and P.-H.P wrote the paper.

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584**Figure Legends**

Figure 1: Ultrastructure of the *T. gondii* oocyst wall. (A) Low magnification image of a control (water maintained) oocyst showing the ruptured oocyst wall (OW) and the remnants of a sporocyst (Sp). Bar is 1 µm. (B) Detail of the oocyst wall from a control oocyst showing the thinner outer layer (O) and the thicker inner layer (I). Bar is 100 nm. (C) Detail of the oocyst wall from a bleached oocyst showing the inner layer (I) with loss of the outer layer except for a few remnants (arrows). Bar is 100 nm. (D) Detail of the sporocyst wall from a control oocyst showing the outer (O) and inner (I) layers of the wall. Bar is 100 nm. (E) Detail of the sporocyst wall from a bleached oocyst showing the retention of both the outer (O) and inner (I) layers for the wall. Bar is 100 nm.

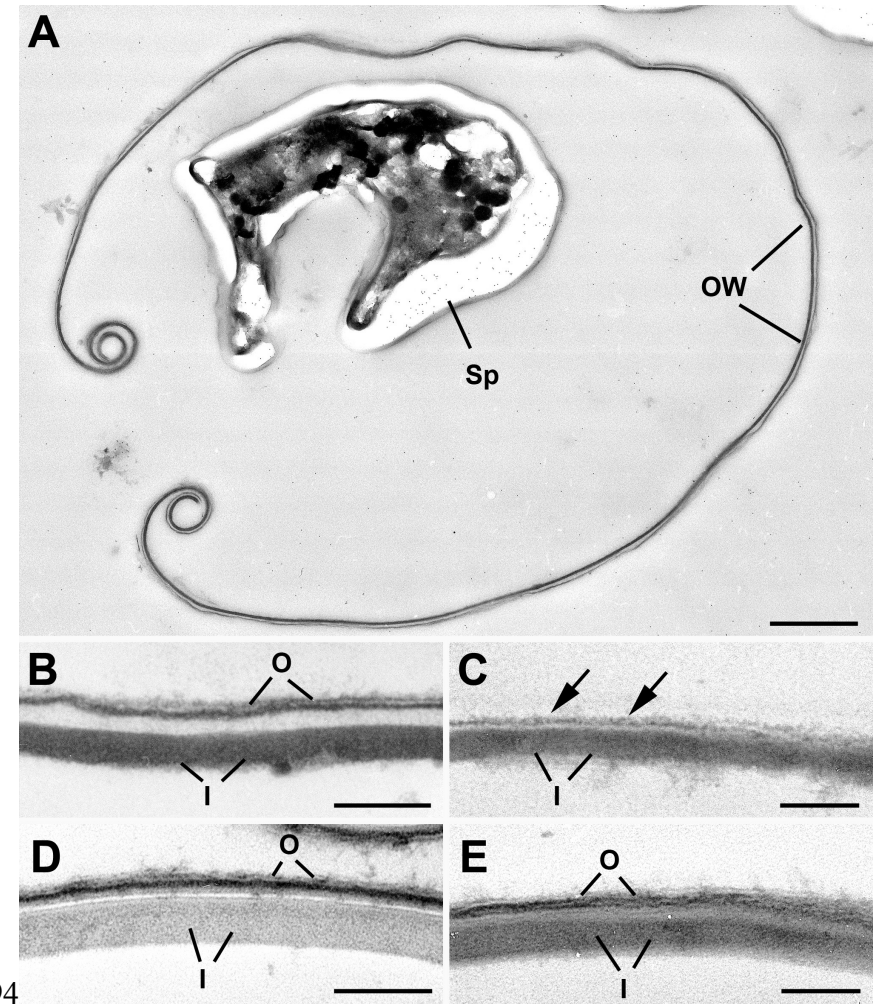

Figure 2: Fluorescence labeling of the inner wall of *T. gondii* oocysts submitted to different surface treatments. Non sporulated (NS), sporoblast-staged (SB) and sporulated (SP) oocysts were bleached and/or heated prior to be allowed to react with 4B6 antibody specific to their inner wall (49, 58). The percentages of labeled parasites in each treatment condition are presented. No significant differences were observed between the stages of oocyst maturation for any given treatment. However there were significant differences among treatments, with control H_2O oocysts differing from heated, bleached, and bleached then heated oocysts (p values <0.001-0.05) and heated oocysts differing from bleached, and bleached then heated oocysts ($p<0.001$). No statistical difference was noted between bleached oocysts and bleached then heated oocysts at any maturing stage. The corresponding typical fluorescence images are presented on *SI Appendix*, Fig. S2.

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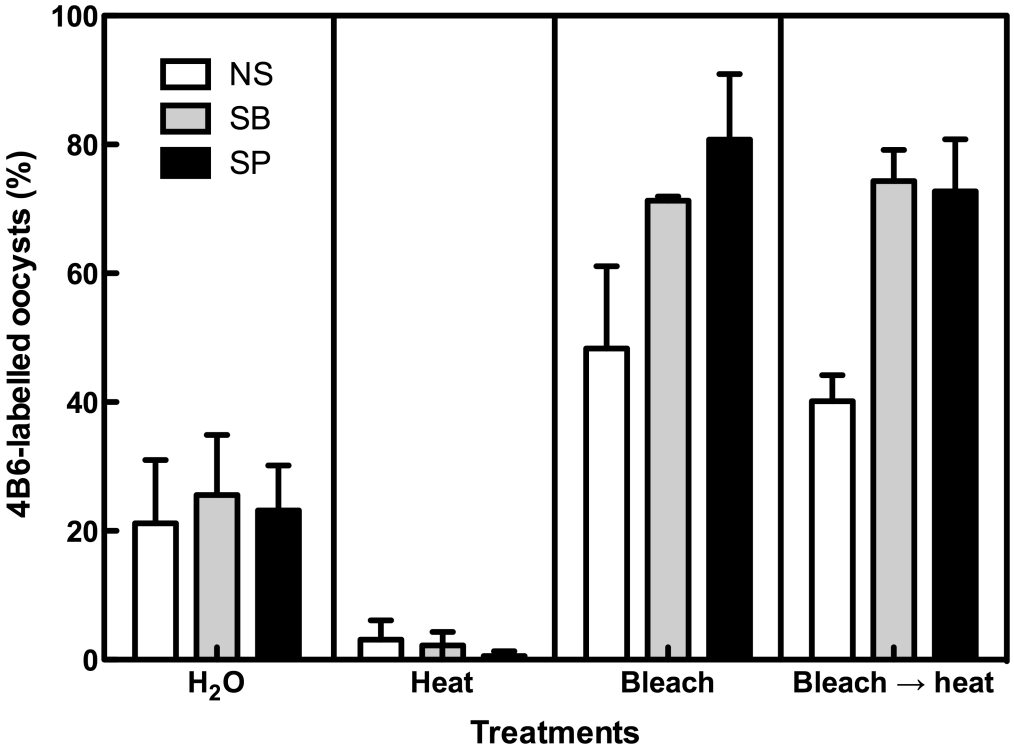

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Figure 3: Measuring mechanical properties of *T. gondii* oocysts. (A) Typical force curve (Force vs. Tip-sample separation) used for quantifying the Young modulus (note that pressing and pulling curves are almost superimposed). The dotted line is the point of contact. (B) Zoom on the contact region of the curves presented in A, showing the superimposition of pressing and pulling curves together with the Hertz fit described in the text (dotted line), and *d* the maximal indentation. The superimposition of pressing and pulling curves shows that little if no dissipation is occurring in the material when indenting, ruling out any viscoelastic behavior under the conditions of our experiments. (C) Example of maximal indentation for untreated oocysts of different subtypes, showing no tendency upon repeated indentation. Note that 100 nm was used as the maximum of the scale as it is the upper bound of the thickness of the oocyst wall found in literature. (D) Maximal indentation under a force of 1 nN as a function of oocyst subtype and treatment. (E) Repeated measured values of Young modulus of 3 untreated oocysts of various subtypes, showing that for a given object no tendency can be observed upon repeated indentation. Note that the value of 10^4 Pa used as the lower limit of the scale corresponds to the values recorded for hard eukaryotic cells as found in literature. (F) Young modulus as a function of oocyst subtype and treatment. In D and F, each point is the median value obtained for a single oocyst upon successive indentations. The line is the median of the subsequent distribution. No significant differences are observed between the conditions neither for indentation (D and *SI Appendix*, Tables S1, S4) nor for Young modulus (F and *SI Appendix*, Tables S2, S4).

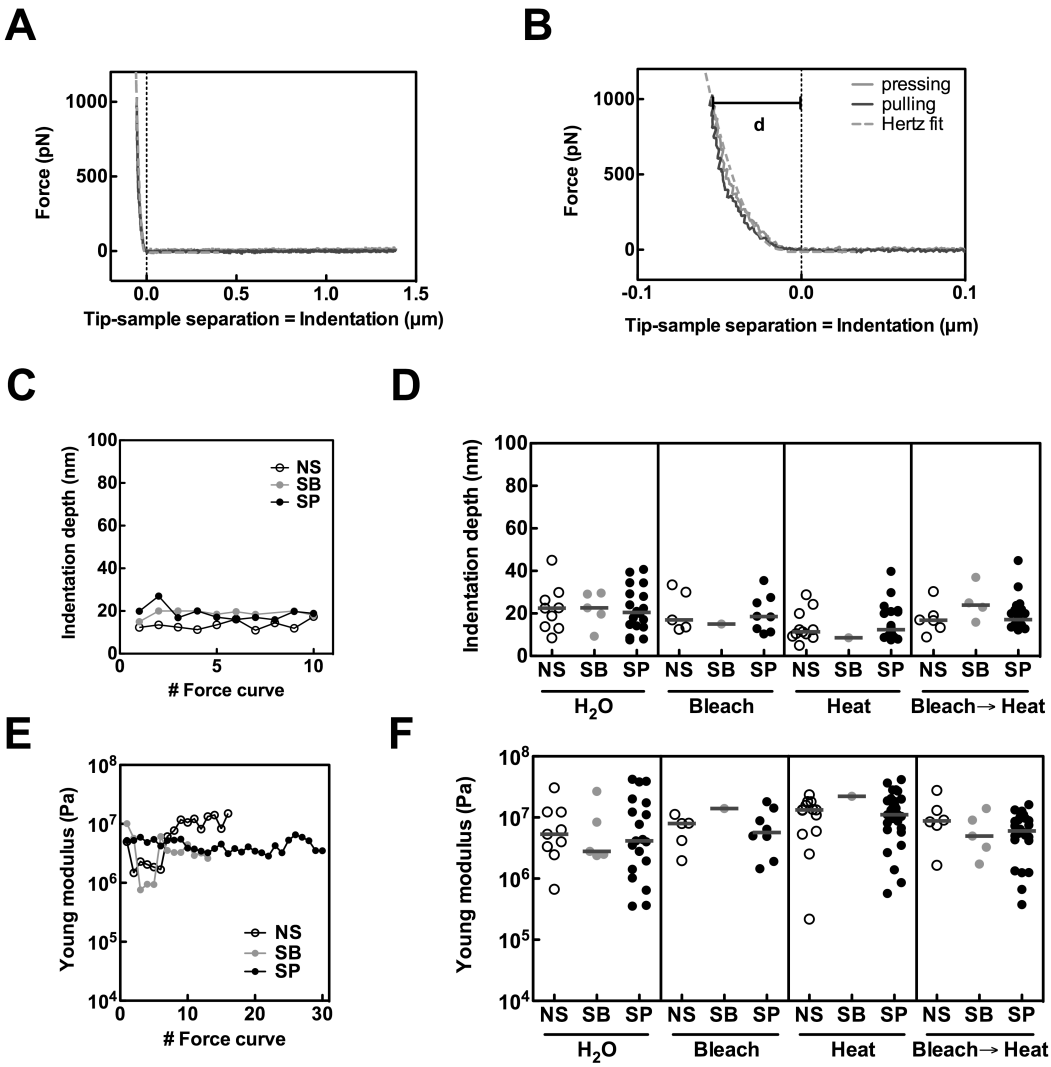
A
Force (pN)
1000
500
0
0.0
0.5
1.0
1.5
Tip-sample separation = Indentation (µm)
B
pressing
pulling
Hertz fit
d
Force (pN)
1000
500
0
-0.1
0.0
0.1
Tip-sample separation = Indentation (µm)
C
Indentation depth (nm)
100
80
60
40
20
0
NS
SB
SP
0
5
10
Force curve
D
Indentation depth (nm)
100
80
60
40
20
0
NS SB SP NS SB SP NS SB SP NS SB SP
H2O
Bleach
Heat
Bleach→ Heat
E
Young modulus (Pa)
10^8
10^7
10^6
10^5
10^4
NS
SB
SP
0
10
20
30
Force curve
F
Young modulus (Pa)
10^8
10^7
10^6
10^5
10^4
NS SB SP NS SB SP NS SB SP NS SB SP
H2O
Bleach
Heat
Bleach→ Heat

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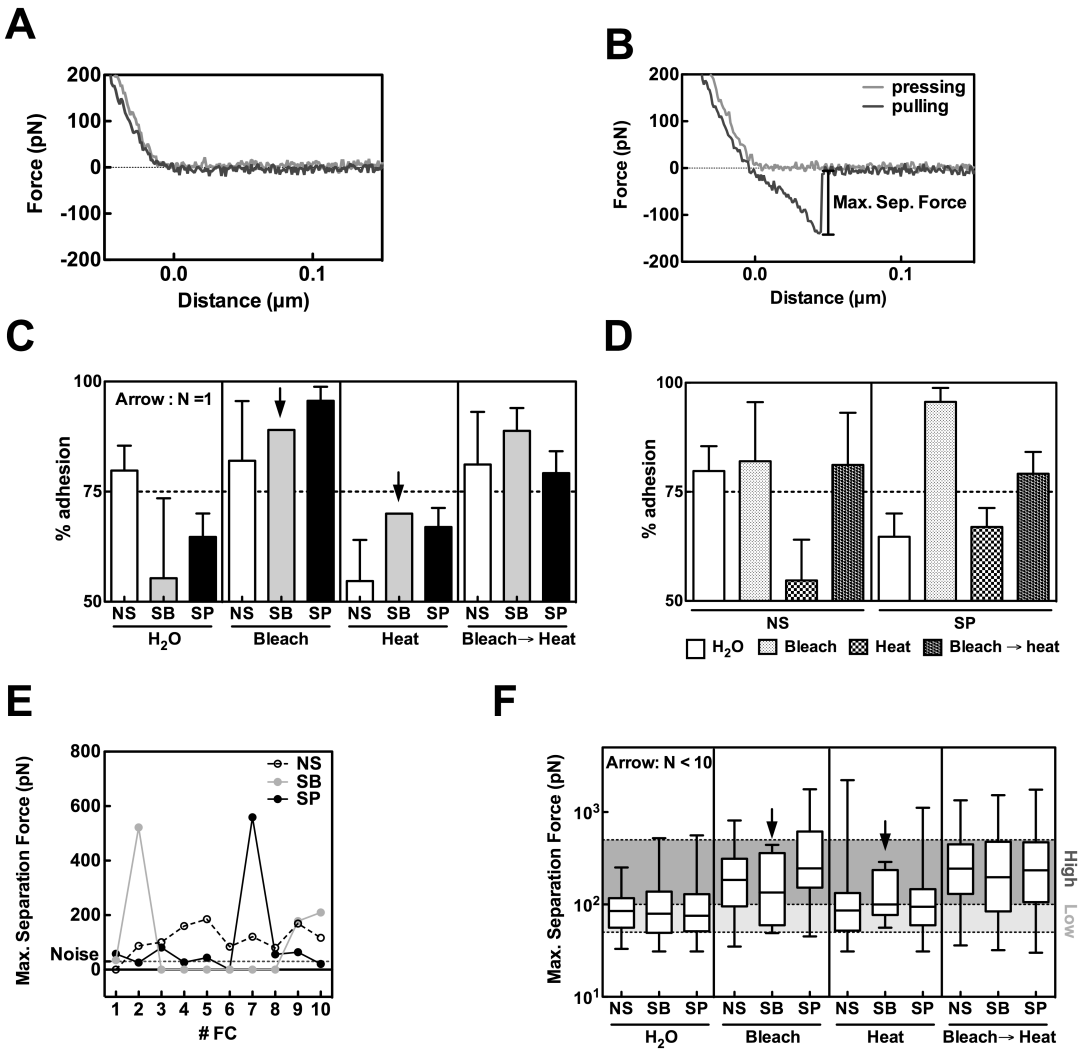
Figure 4: Measuring non-specific adhesive properties of *T. gondii* oocysts. (A) Typical force curve showing no adhesion event. (B) Typical force curve showing adhesion event. We present here the zoom on the force curve near the contact / adhesion zone, with the recorded maximal adhesion force. Note that the noise is far below the forces measured (typically 15 to 30 pN). (C) The fraction of adhesion as a function of oocyst subtypes and treatments (mean +/- SD over the different days of experiments). Arrows show cases where only one object for a given condition was observed over different repetitions. Dotted line has been placed at 75 % adhesion as a guide for the eye. (D) Same data as in C, showing the comparison of NS and SP oocysts as a function of treatment. (E) Example of maximal separation force for untreated oocysts of different subtypes, showing no tendency upon repeated indentation / pulling, indicating that no strong pollution of, or transfer of material, to the lever tip was observed. Note that the dotted line corresponds to the average observed noise on the baseline of the force curves. (F) Distribution of adhesion forces as whisker plots for oocysts of different subtypes and treatments. The significant differences correspond to the high / low adhesion separation on the graph (*SI Appendix*, Tables S3 and S4).


A
Force (pN)
Distance (μm)
B
pressing
pulling
Max. Sep. Force
C
Arrow : N =1
% adhesion
NS SB SP
H2O
Bleach
Heat
Bleach→ Heat
D
NS
SP
H2O Bleach Heat Bleach → heat
E
Max. Separation Force (pN)
Noise
FC
F
Arrow: N < 10
High
Low

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Supplement Information (SI) Appendix

Materials and Methods

Oocyst Purification and Sporulation. Oocysts of the genotype II TgNmBr1strain of *T. gondii* (1) were harvested from feces of cat 6-8 days after feeding infected mouse tissues to a *T. gondii* free cat. Oocysts were collected by flotation at 4°C from cat feces on a 1.15 specific gravity sucrose solution without phenol. Concentrated oocyst pellets were then resuspended in 5 mL of distilled water and sent with cold packs within 48 hours by FedEx from Beltsville, Maryland, USA to Marseille, France, for sporulation and further experiments. Upon arrival, oocysts were washed three times in distilled water, pelleted by centrifugation at 5000 g for 5 min at room temperature (RT, 20-22°C), then resuspended in 7 mL of distilled water, and transferred in a 100 mL small plastic container. Oocysts were allowed to sporulate at RT for 5 days under adequate aeration and gentle continuous shaking. Sporulation progress was monitored daily by examining a fraction of the suspension by using bright field and UV microscopy as described elsewhere (2, 3). Oocyst suspension was stored in distilled water at 4°C until used within 3 months.

Immunofluorescence Assay (IFA). The effects of bleach and heat treatments on the integrity of the oocyst wall were evaluated by IFA combined with the autofluorescent signal (AF). We used a monoclonal antibody (IgM mAb 4B6), which was previously shown to react mainly with the inner layer of the oocyst wall (4, 5). This labeling was performed on oocysts in suspension rather than on air-dried parasites because drying on slides frequently induces the opening of the oocyst wall, which invariably gives to the mAb 4B6 an access to the inner layer (4, 5).

Briefly, untreated (H_2O-stored), heat-inactivated and bleach-treated oocysts were allowed to react with mAb 4B6 diluted at 1:100 in PBS for 30 min at 37°C. Oocysts were subsequently washed three times in PBS by gentle centrifugations at 5000 g for 5 min prior to incubation with a goat fluorescein-isothiocyanate (FITC) conjugate anti-mouse IgM+IgG (50-011, Argene, France) diluted to 1:100 in PBS. After that, parasites were washed again three times in PBS using the same protocol. Samples are then mounted and

examined on a BX51 microscope (Olympus, France) equipped with suitable epifluorescence filters for FITC and UV autofluorescence (AF) and 40x lens. Bright field, FITC and AF images were acquired using the fluorescence imaging system Cell^{A} (Olympus, France) and quantified using ImageJ 1.46m. The normally blue AF signal (Fig. S1B) was placed in the red channel and the FITC in the green channel for more convenient merging when performing fluorescence colocalization (Fig. S2).

Oocyst Immobilization on Glass Coverslides. Coverslides were cleaned using a 10% v/v Helmanex (Helma) solution in water in an ultrasonic bath for 30 min at 60°C, subsequently separately rinsed using alternating baths of ethanol and MQ water (three of each). Then, a supplementary cleaning in MQ water in an ultrasonic bath for 30 min at 60°C was performed before a last rinsing step with MQ water prior to drying under an air flow. Coverslides were stored away from humidity and dust for up to two weeks before use. For coating with poly-L-lysine (PLL), clean coverslides were activated for 1 min using a residual air-based plasma cleaner and a PDMS stamp with a circular 8 mm in diameter hole was stuck on them. 100 µL of 0.01% PLL in water was added and incubated for 45 min to 1 hr at RT. Three rinsing with PBS were performed before gluing a plastic ring with vacuum grease after removal of the PDMS stamp. The resulting chamber was then filled with 1 mL PBS and 100 µL of untreated, heat-inactivated, or bleach-treated oocyst suspension were seeded onto the PLL treated zone and let to settle for 45min to 1 hr at RT. Three gentle rinsing steps, with 1mL PBS, were performed before mounting on the AFM. We observed that this procedure did not grossly affect the different sub-populations ratios as compared to the original suspension (Fig. S5B). Observation lasted for 2 hr maximum at RT with occasional supplementations of water to counteract the evaporation.

SI Appendix Figures

Figure S1: *Toxoplasma gondii* oocyst subpopulations obtained after 5 days of sporulation in water. The suspension contained a mixture of different maturing stages of oocysts. Observed unsporulated oocysts (NS, small arrowhead) were spherical and contained a single granular mass (which corresponds to the zygote (6)), almost filling the oocyst. Maturing oocysts containing two sporoblasts (SB, large arrowhead) were ovoid and harbored two spherical sporoblasts, each filled with granular material. Fully sporulated (SP) oocysts containing sporocysts (large arrow, and C) were ovoid in shape and had two fully developed sporocysts containing four sporozoites each. Additionally, oocysts at the SB-SP transition (small arrow), i.e. containing one sporoblast and one sporocyst, were sometimes observed and were further recorded as SB oocysts (e.g. in AFM experiments). Oocysts were observed under bright field (A) or UV excitation for recording their autofluorescence pattern (B). Note the presence of fecal contaminants on the bright field image. Scale bars = 10 µm. (C) Fully sporulated *Toxoplasma gondii* oocysts, under bright field, harboring two sporocysts containing four banana-shaped sporozoite forms of the parasite. At least one sporozoite is very distinct in the picture (arrow). Scale bar = 5 µm.

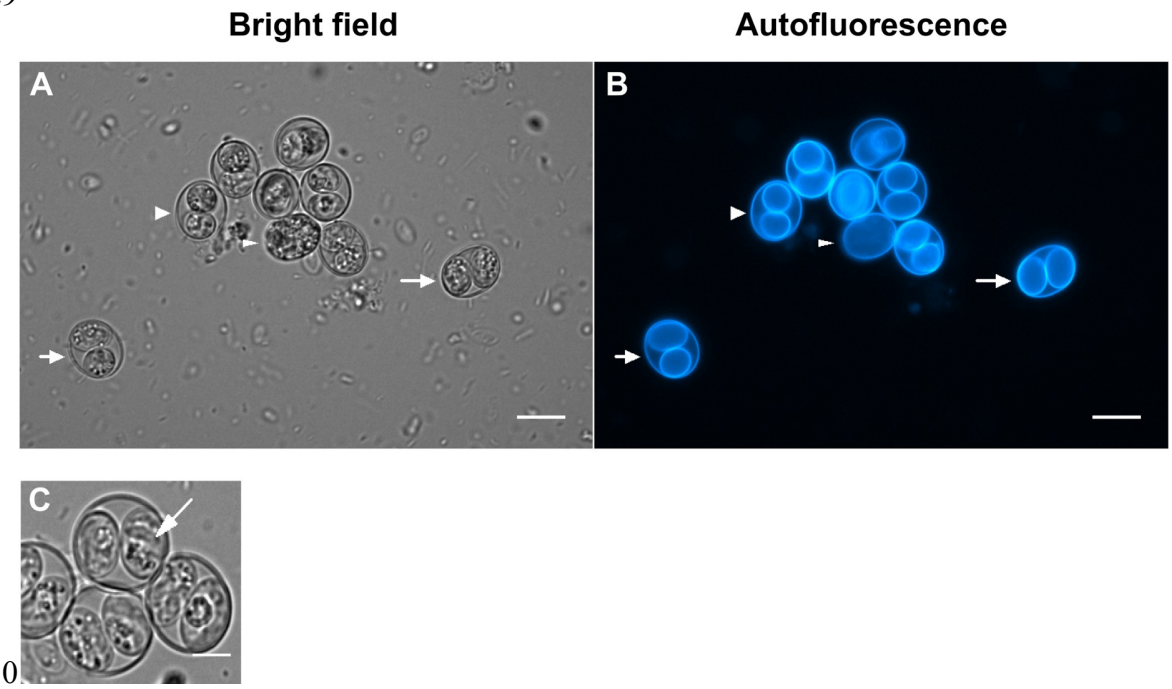

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Figure S2: Fluorescence patterns of the wall of *T. gondii* oocysts exposed solely to H_2O (A), heated at 80°C (B), treated by bleach solution (C), or bleach- and then heat-treated (D) as described in the materiel and methods section. Oocysts were then allowed to react with 4B6 antibody specific to their inner wall (4, 5). BF, bright field; AF, autofluorescence under UV excitation (red channel); FITC corresponds to 4B6 fluorescence (green channel). Merge presents overlay between AF and FITC channel. Scale bars = 5 μm.

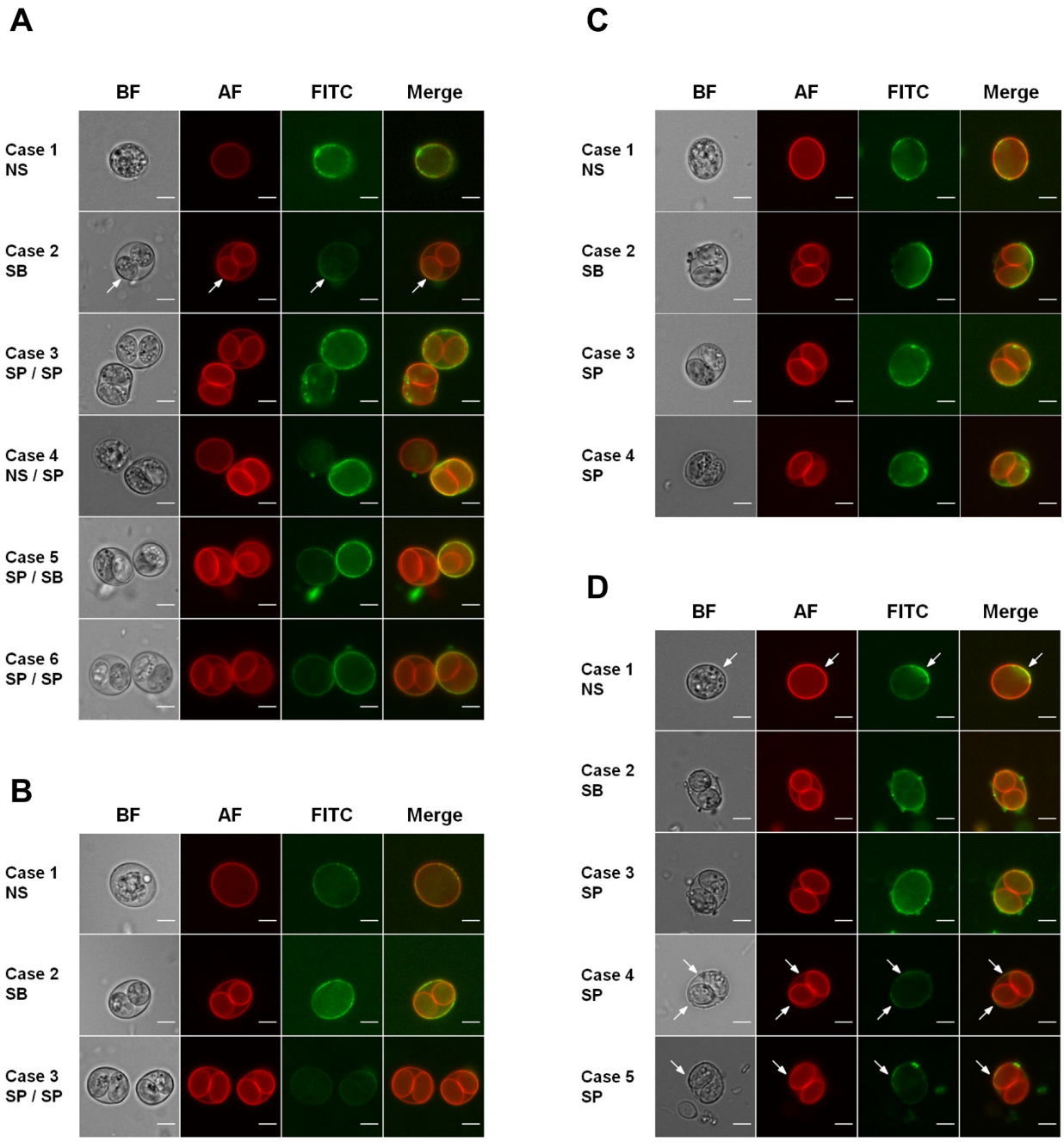

A
BF
AF
FITC
Merge
Case 1
NS
Case 2
SB
Case 3
SP / SP
Case 4
NS / SP
Case 5
SP / SB
Case 6
SP / SP
B
BF
AF
FITC
Merge
Case 1
NS
Case 2
SB
Case 3
SP / SP
C
BF
AF
FITC
Merge
Case 1
NS
Case 2
SB
Case 3
SP
Case 4
SP
D
BF
AF
FITC
Merge
Case 1
NS
Case 2
SB
Case 3
SP
Case 4
SP
Case 5
SP

Figure S3: Fluorescein Infiltration Assay. (A) The permeability of the wall of *Toxoplasma gondii* oocysts exposed or not to bleach treatment was assessed by incubating oocysts with fluorescein isothiocyanate. BF, bright field; AF, autofluorescence under UV excitation (red); FITC, fluorescein isothiocyanate fluorescence (green). Scale bars = 5 µm. (B) Percents of FITC permeable oocysts. No statistical difference was noted between control and bleach-treated oocysts at any maturing stage.

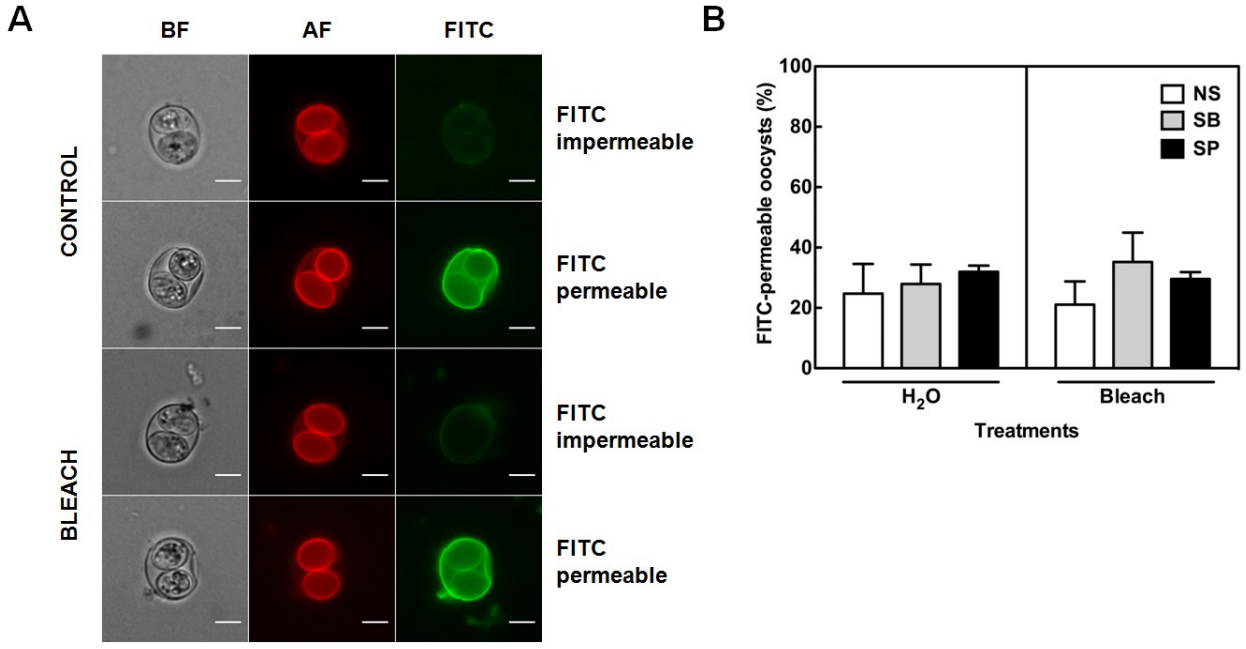


Figure S4: Quantification of the oocyst wall autofluorescence. (A) Control, heat- or bleach-treated oocysts were randomly examined microscopically under bright field (BF) and UV excitation (AF) (typically 10-35 oocysts per maturing stage and treatment condition). Their respective AF pattern was recorded as gray scale images. The relative oocyst wall AF intensity values were obtained by recording pixel gray values along a straight line (in yellow) arbitrarily set up across the width of each oocyst type. Values were plotted as a function of the pixel position along the selection line, and then normalized with regard to background gray value of each image (yellow square). Red circles indicate gray values of the oocyst wall that then were plotted in graph B. Scale bars= 5 µm. (B) Distribution of the relative autofluorescence intensity of the oocyst wall. The line is the median of the distribution. Significant differences were observed when comparing NS vs. NS/bleach ($p<0.001$), SB vs. SB/bleach ($p<0.001$), SP vs. SP/bleach ($p<0.001$), SB/heat vs. SB/bleach ($p<0.001$), and SP/heat vs. SP/bleach ($p<0.001$). No statistical difference was noted between control and heated oocysts at any maturing stage.

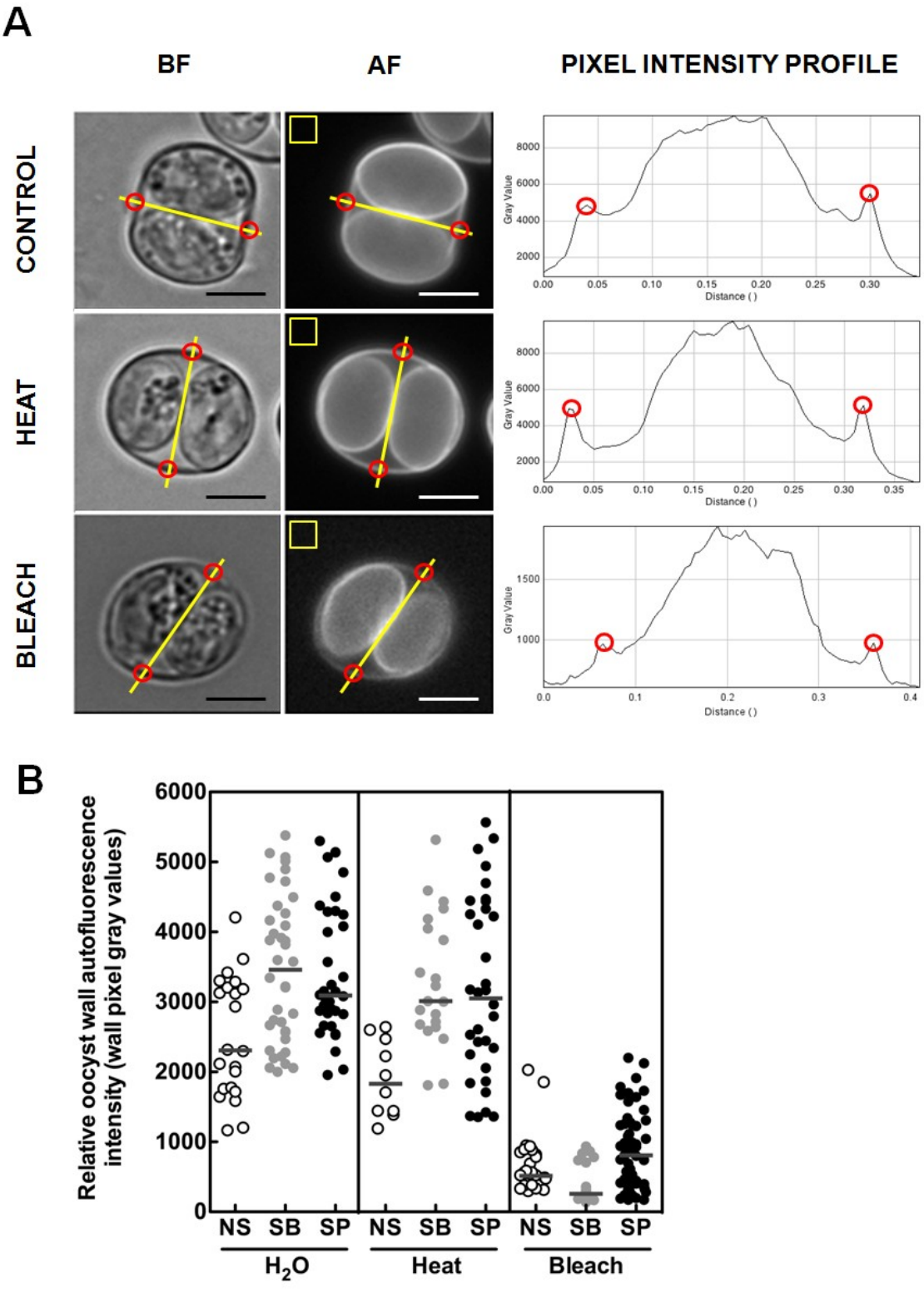

A
BF
AF
PIXEL INTENSITY PROFILE
CONTROL
HEAT
BLEACH
Gray Value
Distance ( )
B
Relative oocyst wall autofluorescence intensity (wall pixel gray values)
NS
SB
SP
H2O
Heat
Bleach

Figure S5: Attachment of the *Toxoplasma gondii* oocysts onto Poly-L-Lysine (PLL)-coated glass slides and positioning of the AFM tip on top of the adhered oocysts. (A) Schematics of the procedure. (B) Conservation of subpopulations of oocysts after transfer to PLL-coated surface compared to the subpopulations of the parasites from the original suspension, as a fraction of total observed objects. (C) NS (left), SB (middle) and SP (right) oocysts were imaged together with the AFM cantilever (i.e. the dark triangular-shape object on the pictures), showing that one can distinguish them easily while doing AFM. The presence of other fecal objects such as yeasts or larger objects (mainly fiber-like debris, arrow) invariably occurred because the oocysts we used for AFM experiments were extracted and stored in water with no additional chemicals to limit bacterial proliferation. Such non-target objects could be located near the oocysts, at the same focal plane, however they did not affect the overall AFM cantilever motions, except on rare occasions. In these latter cases, the corresponding force curves were not processed for further analyses. Scale bars = 10 µm. (D) Zoom on the same SP oocyst as in panel C. The calibrated AFM cantilever was positioned using micrometer screws on top of it (e.g. over the two sporocysts of SP oocysts and between them (mid position)). (E) Distribution of the Young modulus at each of the three positions of the AFM tip. The red line is the median of the distribution. No significant difference was observed (Kruskall-Wallis test, $p>0.05$). No trace of the indentation can be seen in our optical magnification on the oocyst surface following indentation repetition. (F, G) Oocyst mechanics explored at higher contact forces. (F) Repeated measured values of Young modulus of 3 heated oocysts of two subtypes for heated oocyst samples, showing that for a given object no tendency can be observed when indenting with a maximal force 30 to 120 times the one used in our study. Note that the value of 10^4 Pa used as the lower limit of the scale corresponds to the values recorded for hard eukaryotic cells as found in literature. (G) Young modulus as a function of oocyst subtype. Each point is the value obtained for a single force curve. The red line is the median of the distribution. No significant difference is observed between the two cases and the measured medians are similar to the ones measured at 1 nN.

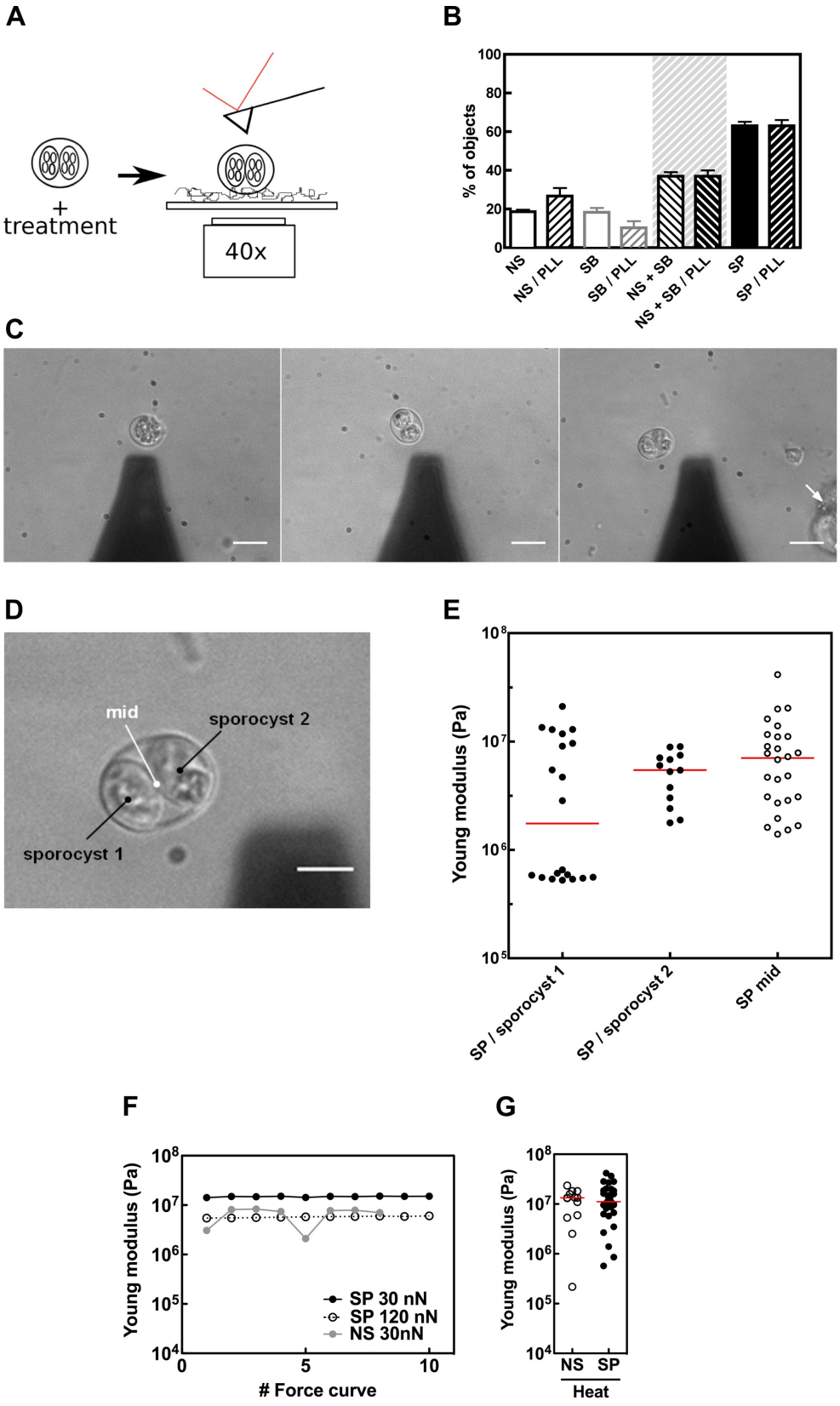
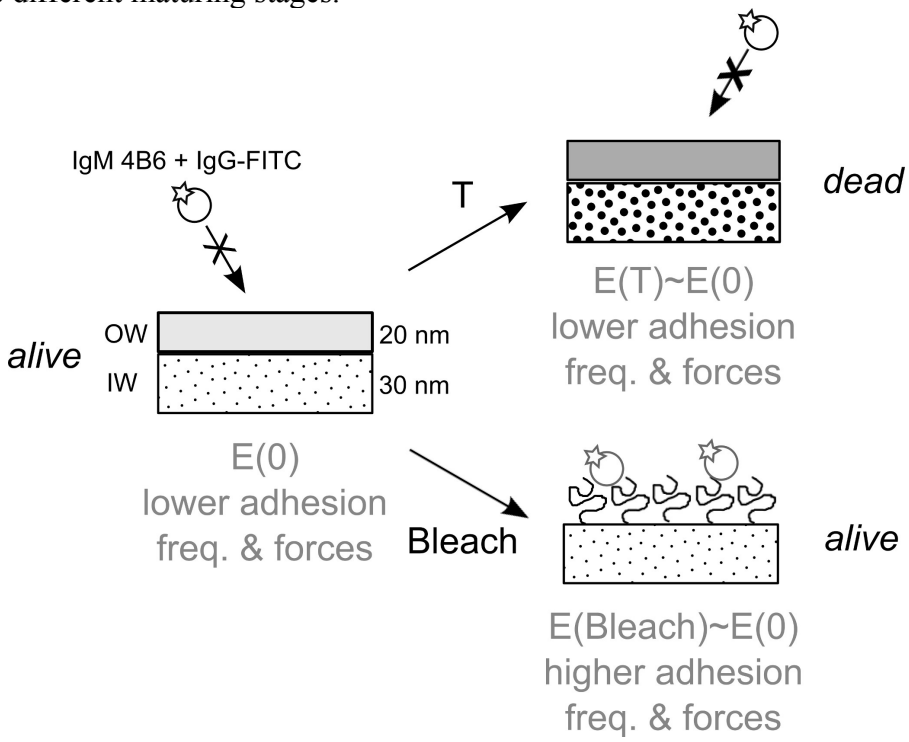

A
+
treatment
40x
B
% of objects
100
80
60
40
20
0
NS
NS / PLL
SB
SB / PLL
NS + SB
NS + SB / PLL
SP
SP / PLL
C
D
mid
sporocyst 2
sporocyst 1
E
Young modulus (Pa)
10^8
10^7
10^6
10^5
SP / sporocyst 1
SP / sporocyst 2
SP mid
F
Young modulus (Pa)
10^8
10^7
10^6
10^5
10^4
SP 30 nN
SP 120 nN
NS 30nN
0
5
10
Force curve
G
Young modulus (Pa)
10^8
10^7
10^6
10^5
10^4
NS
SP
Heat

Figure S6: Proposed structure of the bi-layered wall of the *Toxoplasma* oocyst in terms of mechanics and adhesive properties. Temperature and bleach treatments have no effect on the oocyst wall mechanics but have opposite effect on wall adhesion and accessibility to inner wall by a specific antibody: bleached oocysts exhibit higher adhesion frequency and forces than heated or control oocysts. Little differences are observed among the different maturing stages.



SI Appendix Tables

Table S1: Statistical analyses of the maximal indentation under a force of 1 nN as a function of oocyst subtype and treatment (see Fig. 3D). Data obtained with Prism 6 (GraphPad).

Table Analyzed	Indentation @ 1nN			
Kruskal-Wallis test				
P value	0,0145			
Exact or approximate P value?	Approximate			
P value summary	*			
Do the medians vary signif. (P < 0.05)	Yes			
Number of groups	10			
Kruskal-Wallis statistic	20,60			
Data summary				
Number of treatments (columns)	10			
Number of values (total)	115			
Number of families	1			
Number of comparisons per family	45			
Alpha	0,05			
Dunn's multiple comparisons test	Mean rank diff,	Significant?		Adjusted P Value
NS vs. SB	-2,622	No	ns	> 0,9999
NS vs. SP	1,861	No	ns	> 0,9999
NS vs. NS / T	33,46	No	ns	> 0,9999
NS vs. SP / T	30,20	No	ns	0,8379
NS vs. NS / bleach	1,778	No	ns	> 0,9999
NS vs. SP / bleach	7,340	No	ns	> 0,9999

NS vs. NS / bleach / T	13,94	No	ns	> 0,9999	
NS vs. SB / bleach / T	-15,35	No	ns	> 0,9999	
NS vs. SP / bleach / T	5,369	No	ns	> 0,9999	
SB vs. SP	4,483	No	ns	> 0,9999	
SB vs. NS / T	36,08	No	ns	> 0,9999	
SB vs. SP / T	32,83	No	ns	> 0,9999	
SB vs. NS / bleach	4,400	No	ns	> 0,9999	
SB vs. SP / bleach	9,963	No	ns	> 0,9999	
SB vs. NS / bleach / T	16,57	No	ns	> 0,9999	
SB vs. SB / bleach / T	-12,73	No	ns	> 0,9999	
SB vs. SP / bleach / T	7,991	No	ns	> 0,9999	
SP vs. NS / T	31,60	No	ns	0,5983	
SP vs. SP / T	28,34	No	ns	0,2352	
SP vs. NS / bleach	-0,08333No	ns	> 0,9999		
SP vs. SP / bleach	5,479	No	ns	> 0,9999	
SP vs. NS / bleach / T	12,08	No	ns	> 0,9999	
SP vs. SB / bleach / T	-17,21	No	ns	> 0,9999	
SP vs. SP / bleach / T	3,508	No	ns	> 0,9999	
NS / T vs. SP / T	-3,256	No	ns	> 0,9999	
NS / T vs. NS / bleach	-31,68	No	ns	> 0,9999	
NS / T vs. SP / bleach	-26,12	No	ns	> 0,9999	
NS / T vs. NS / bleach / T	-19,52	No	ns	> 0,9999	
NS / T vs. SB / bleach / T	-48,81	No	ns	0,5487	
NS / T vs. SP / bleach / T	-28,09	No	ns	> 0,9999	
SP / T vs. NS / bleach	-28,43	No	ns	> 0,9999	
SP / T vs. SP / bleach	-22,86	No	ns	> 0,9999	
SP / T vs. NS / bleach / T	-16,26	No	ns	> 0,9999	
SP / T vs. SB / bleach / T	-45,55	No	ns	0,4857	

SP / T vs. SP / bleach / T	-24,84	No	ns	0,4285
NS / bleach vs. SP / bleach	5,563	No	ns	> 0,9999
NS / bleach vs. NS / bleach / T	12,17	No	ns	> 0,9999
NS / bleach vs. SB / bleach / T	-17,13	No	ns	> 0,9999
NS / bleach vs. SP / bleach / T	3,591	No	ns	> 0,9999
SP / bleach vs. NS / bleach / T	6,604	No	ns	> 0,9999
SP / bleach vs. SB / bleach / T	-22,69	No	ns	> 0,9999
SP / bleach vs. SP / bleach / T	-1,972	No	ns	> 0,9999
NS / bleach / T vs. SB / bleach / T	-29,29	No	ns	> 0,9999
NS / bleach / T vs. SP / bleach / T	-8,576	No	ns	> 0,9999
SB / bleach / T vs. SP / bleach / T	20,72	No	ns	> 0,9999

Table S2: Statistical analyses of the Young modulus as a function of oocyst subtype and treatment (see Fig. 3F). Data obtained with Prism 6 (GraphPad).

Table Analyzed	Young Modulus			
Kruskal-Wallis test				
P value	0,0790			
Exact or approximate P value?	Approximate			
P value summary	ns			
Do the medians vary signif. (P < 0.05)	No			
Number of groups	10			
Kruskal-Wallis statistic	15,46			
Data summary				
Number of treatments (columns)	10			
Number of values (total)	127			
Number of families	1			
Number of comparisons per family	45			
Alpha	0,05			
Dunn's multiple comparisons test	Mean rank diff,	Significant?		Adjusted P Value
SP / T vs. NS / T	2,998	No	ns	> 0,9999
SP / T vs. SP / bleach	25,15	No	ns	> 0,9999
SP / T vs. NS / bleach	28,15	No	ns	> 0,9999
SP / T vs. SP	25,97	No	ns	0,6446
SP / T vs. SB	30,15	No	ns	> 0,9999
SP / T vs. NS	26,65	No	ns	> 0,9999
SP / T vs. SP / bleach / T	29,03	No	ns	0,1485

SP / T vs. SB / bleach / T	29,75	No	ns	> 0,9999
SP / T vs. NS / bleach / T	11,32	No	ns	> 0,9999
NS / T vs. SP / bleach	22,15	No	ns	> 0,9999
NS / T vs. NS / bleach	25,15	No	ns	> 0,9999
NS / T vs. SP	22,97	No	ns	> 0,9999
NS / T vs. SB	27,15	No	ns	> 0,9999
NS / T vs. NS	23,65	No	ns	> 0,9999
NS / T vs. SP / bleach / T	26,03	No	ns	> 0,9999
NS / T vs. SB / bleach / T	26,75	No	ns	> 0,9999
NS / T vs. NS / bleach / T	8,321	No	ns	> 0,9999
SP / bleach vs. NS / bleach	3,000	No	ns	> 0,9999
SP / bleach vs. SP	0,8158	No	ns	> 0,9999
SP / bleach vs. SB	5,000	No	ns	> 0,9999
SP / bleach vs. NS	1,500	No	ns	> 0,9999
SP / bleach vs. SP / bleach / T	3,875	No	ns	> 0,9999
SP / bleach vs. SB / bleach / T	4,600	No	ns	> 0,9999
SP / bleach vs. NS / bleach / T	-13,83	No	ns	> 0,9999
NS / bleach vs. SP	-2,184	No	ns	> 0,9999
NS / bleach vs. SB	2,000	No	ns	> 0,9999
NS / bleach vs. NS	-1,500	No	ns	> 0,9999
NS / bleach vs. SP / bleach / T	0,8750	No	ns	> 0,9999
NS / bleach vs. SB / bleach / T	1,600	No	ns	> 0,9999
NS / bleach vs. NS / bleach / T	-16,83	No	ns	> 0,9999
SP vs. SB	4,184	No	ns	> 0,9999
SP vs. NS	0,6842	No	ns	> 0,9999
SP vs. SP / bleach / T	3,059	No	ns	> 0,9999
SP vs. SB / bleach / T	3,784	No	ns	> 0,9999
SP vs. NS / bleach / T	-14,65	No	ns	> 0,9999

SB vs. NS	-3,500	No	ns	> 0,9999
SB vs. SP / bleach / T	-1,125	No	ns	> 0,9999
SB vs. SB / bleach / T	-0,4000	No	ns	> 0,9999
SB vs. NS / bleach / T	-18,83	No	ns	> 0,9999
NS vs. SP / bleach / T	2,375	No	ns	> 0,9999
NS vs. SB / bleach / T	3,100	No	ns	> 0,9999
NS vs. NS / bleach / T	-15,33	No	ns	> 0,9999
SP / bleach / T vs. SB / bleach / T	0,7250	No	ns	> 0,9999
SP / bleach / T vs. NS / bleach / T	-17,71	No	ns	> 0,9999
SB / bleach / T vs. NS / bleach / T	-18,43	No	ns	> 0,9999

Table S3: Statistical analyses of the distribution of adhesion forces as whisker plots for oocysts of different subtypes and treatments (see Fig. 4F). Data obtained with Prism 6 (GraphPad).

Table Analyzed	Adhesion Force			
Kruskal-Wallis test				
P value	< 0,0001			
Exact or approximate P value?	Approximate			
P value summary	****			
Do the medians vary signif. (P < 0.05)	Yes			
Number of groups	12			
Kruskal-Wallis statistic	216,2			
Data summary				
Number of treatments (columns)	12			
Number of values (total)	913			
Number of families	1			
Number of comparisons per family	66			
Alpha	0,05			
Dunn's multiple comparisons test	Mean rank diff,	Significant?		Adjusted P Value
NS vs. SB	-15,39	No	ns	> 0,9999
NS vs. SP	-4,319	No	ns	> 0,9999
NS vs. NS / T	-42,15	No	ns	> 0,9999
NS vs. SB / T	-142,8	No	ns	> 0,9999
NS vs. SP / T	-60,13	No	ns	> 0,9999
NS vs. NS / bleach	-212,6	Yes	**	0,0017
NS vs. SB / bleach	-138,8	No	ns	> 0,9999
NS vs. SP / bleach	-332,5	Yes	****	< 0,0001

NS vs. NS / bleach / T	-308,4	Yes	****	< 0,0001
NS vs. SB / bleach / T	-237,1	Yes	****	< 0,0001
NS vs. SP / bleach / T	-278,8	Yes	****	< 0,0001
SB vs. SP	11,07	No	ns	> 0,9999
SB vs. NS / T	-26,76	No	ns	> 0,9999
SB vs. SB / T	-127,4	No	ns	> 0,9999
SB vs. SP / T	-44,74	No	ns	> 0,9999
SB vs. NS / bleach	-197,2	No	ns	0,0629
SB vs. SB / bleach	-123,4	No	ns	> 0,9999
SB vs. SP / bleach	-317,1	Yes	****	< 0,0001
SB vs. NS / bleach / T	-293,1	Yes	****	< 0,0001
SB vs. SB / bleach / T	-221,8	Yes	**	0,0087
SB vs. SP / bleach / T	-263,4	Yes	****	< 0,0001
SP vs. NS / T	-37,83	No	ns	> 0,9999
SP vs. SB / T	-138,4	No	ns	> 0,9999
SP vs. SP / T	-55,81	No	ns	> 0,9999
SP vs. NS / bleach	-208,2	Yes	***	0,0003
SP vs. SB / bleach	-134,5	No	ns	> 0,9999
SP vs. SP / bleach	-328,2	Yes	****	< 0,0001
SP vs. NS / bleach / T	-304,1	Yes	****	< 0,0001
SP vs. SB / bleach / T	-232,8	Yes	****	< 0,0001
SP vs. SP / bleach / T	-274,4	Yes	****	< 0,0001
NS / T vs. SB / T	-100,6	No	ns	> 0,9999
NS / T vs. SP / T	-17,98	No	ns	> 0,9999
NS / T vs. NS / bleach	-170,4	No	ns	0,0985
NS / T vs. SB / bleach	-96,67	No	ns	> 0,9999
NS / T vs. SP / bleach	-290,4	Yes	****	< 0,0001
NS / T vs. NS / bleach / T	-266,3	Yes	****	< 0,0001

NS / T vs. SB / bleach / T	-195,0	Yes	*	0,0109
NS / T vs. SP / bleach / T	-236,6	Yes	****	< 0,0001
SB / T vs. SP / T	82,64	No	ns	> 0,9999
SB / T vs. NS / bleach	-69,79	No	ns	> 0,9999
SB / T vs. SB / bleach	3,946	No	ns	> 0,9999
SB / T vs. SP / bleach	-189,7	No	ns	> 0,9999
SB / T vs. NS / bleach / T	-165,7	No	ns	> 0,9999
SB / T vs. SB / bleach / T	-94,38	No	ns	> 0,9999
SB / T vs. SP / bleach / T	-136,0	No	ns	> 0,9999
SP / T vs. NS / bleach	-152,4	Yes	*	0,0298
SP / T vs. SB / bleach	-78,69	No	ns	> 0,9999
SP / T vs. SP / bleach	-272,4	Yes	****	< 0,0001
SP / T vs. NS / bleach / T	-248,3	Yes	****	< 0,0001
SP / T vs. SB / bleach / T	-177,0	Yes	**	0,0011
SP / T vs. SP / bleach / T	-218,6	Yes	****	< 0,0001
NS / bleach vs. SB / bleach	73,73	No	ns	> 0,9999
NS / bleach vs. SP / bleach	-120,0	No	ns	0,7992
NS / bleach vs. NS / bleach / T	-95,88	No	ns	> 0,9999
NS / bleach vs. SB / bleach / T	-24,59	No	ns	> 0,9999
NS / bleach vs. SP / bleach / T	-66,22	No	ns	> 0,9999
SB / bleach vs. SP / bleach	-193,7	No	ns	> 0,9999
SB / bleach vs. NS / bleach / T	-169,6	No	ns	> 0,9999
SB / bleach vs. SB / bleach / T	-98,33	No	ns	> 0,9999
SB / bleach vs. SP / bleach / T	-139,9	No	ns	> 0,9999
SP / bleach vs. NS / bleach / T	24,08	No	ns	> 0,9999
SP / bleach vs. SB / bleach / T	95,36	No	ns	> 0,9999
SP / bleach vs. SP / bleach / T	53,74	No	ns	> 0,9999
NS / bleach / T vs. SB / bleach / T	71,29	No	ns	> 0,9999

NS / bleach / T vs. SP / bleach / T	29,67	No	ns	> 0,9999
SB / bleach / T vs. SP / bleach / T	-41,62	No	ns	> 0,9999

Table S4: Summary of median values of indentation, *E* moduli, and adhesion force of *Toxoplasma* oocysts at different maturing stages following different surface treatments. n = conserved data points.

	H_2O			Heat			Bleach			Bleach→heat		
	NS	**SB**	**SP**	**NS**	**SB**	**SP**	**NS**	**SB**	**SP**	**NS**	**SB**	**SP**
Indentation (nm)	22,5	22,6	20,5	11,4	8,60	12,4	17,0	15,0	18,5	16,8	23,9	17,1
n (oocysts)	9	5	18	11	1	27	5	1	8	6	4	22
***E* modulus (MPa)**	5.3	2.8	4.2	11.1	22.2	11.1	7.9	14.0	5.7	8.8	5.0	6.0
n (oocysts)	9	5	19	13	1	33	5	1	8	6	5	24
Adhesion force (pN)	85,0	79,5	75,5	86,0	100,0	94,5	184,5	134,5	246,0	244,5	197,0	234,0
n (force curves)	67	34	124	51	7	186	46	8	90	60	53	187

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