Titania Nanoparticles Regulate Innate Immunity

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Abstract

Titanium dioxide (TiO_2) nanoparticles, widely incorporated in everyday products such as food additives (E171), cosmetics, confectionary and surgical implants, represent one of the most prevalent forms of engineered metal-based nanoparticles (MNPs) in modern environments. Despite their ubiquity, safety data on their immunological impact remains limited. Of particular concern are sub-5 nm TiO_2 nanoparticles, which possess the capacity to cross cellular barriers and disrupt protein function. This study investigates the interaction between 3.5 nm triethanolamine-terminated TiO_2 nanoparticles and complement C3, a central protein in the human innate immune system. Using cryo-electron microscopy, we reveal direct binding of TiO_2 nanoparticles to the surface of C3, leading to structural restrictions that hinder its activation and subsequent complement cascade progression. Biochemical and haemolytic assays confirm that these nanoparticles act as alternative pathway-specific inhibitors, impairing normal immune function. These findings underscore the potential immunomodulatory effects of TiO_2 nanoparticles and highlight the need for thorough evaluation of their biological impact, particularly given their widespread human exposure.

Introduction

Throughout evolution, humans have been exposed to natural metal and metalloid nanoparticles formed during extreme environmental events such as volcanic eruptions.[1] However, engineered nanoparticles, such as silver, gold, zinc and titanium oxides, are products of modern science and have only been introduced into the environment within the last few decades. In particular, titanium dioxide (TiO_2) nanoparticles have usage in an immense range of everyday products such as a whitening food additive (E171), confectionary, paint, hygiene and personal care products.[2-5] Despite the recent increase in anthropogenic metal-based nanoparticles (MNPs) and increased daily exposure, very

limited safety information is available concerning their biological effect.[6] Synthesised TiO_2 nanoparticles can range from a couple of nanometres in diameter through to several hundred nanometres, with each size class capable of eliciting distinct biological responses.[7] Toxicity considerations are particularly relevant for TiO_2 nanoparticles <5 nm in diameter due to their ability to cross the blood-brain barrier and penetrate cellular walls.[8, 9] A non-exhaustive list of MNP effects on proteins ranges from misfolding, enzymatic inhibition, dysregulation, aggregation and structural alteration.[10-13] Regardless of their biological influence, the ultimate fate of MNPs is bio-clearance and destruction by the host immune system. Yet, some metal nanoparticles have shown to compromise important proteins in the immune system, specifically, within the complement system.[14-16]

Over the course of 500 million years, the complement system in vertebrates has evolved into the highly advanced and intricate defence mechanism found in humans today. This system involves the coordination of ~50 different immune regulation factors and proteins.[17-19] The complement system functions in tissue, plasma, intracellularly and demonstrates exceptional sensitivity to antibodies, foreign microbial sugars and pathogens.[20] The C3 zymogen, an inactive enzyme precursor that requires activation to function, is perhaps the most significant protein in the entire complement cascade. It exists as the most abundant complement protein found in the blood of healthy humans at concentrations above 1.0 mg/mL.[21] For activation of C3 to occur, a small 9 kDa domain (C3a) must be cleaved from the main 176 kDa chain (C3b) by a C3 convertase or serine proteases.[22-24] This promotes a significant conformational shift of C3b and the exposure of a highly reactive thioester which is responsible for the opsonisation (tagging) of foreign particles for destruction by downstream immune system machinery.[25]

Our groups have previously shown that triethanolamine terminated titania (TATT), consisting of size-uniform 3.5 nm TiO_2 nanoparticles, upregulate the coagulant system, a system intricately linked with the complement pathways.[26-28] Consequently, these nanoparticles demonstrated excellent wound healing properties resulting from their impact on blood platelet aggregation.[28] Other research indicates that TATT can either trigger or inhibit blood platelet aggregation in vitro and in vivo.[29, 30] Notably, macro-scale titania from implanted surfaces or environmental exposure can be gradually etched away by bodily fluids into smaller nanoparticle oxides, potentially contributing to systemic nanoparticle exposure.[31-33] Given the increased human exposure to TiO_2, and the close crosstalk between the complement and coagulant systems, [27, 34] we investigated whether the titania nanoparticles imparted any effect on the structure and function of C3.

Here we present the seminal cryo-EM structure of a TiO_2 nanoparticle bound to wild-type human complement C3. Biochemical and haemolytic assays show that these nanoparticles are responsible for C3 immune activity in blood serum and function as an alternative pathway-specific inhibitor. These findings are further supported by structural investigations which reveal an outer-shell interaction between the nanoparticles and C3, restricting protein motion and impeding the formation of complement complexes necessary for continued immune function.

Results

Biophysical analyses of complement C3

Prior to structural analyses, baseline characterisations of native C3 were undertaken, including melting temperature (T_m) and hydrodynamic radius (R_h) at physiological and extreme conditions. DLS experiments showed that within the range of 25 – 55 °C, the protein maintained a constant diameter of ~12 nm (120 Å) which, surprisingly, is 40 Å smaller than reported inactive C3 structures (Figure 1a).[35, 36] Polydispersity values were consistently low and the hydrodynamic size remained monodispersed and unchanged after 90 minutes. Thermal stability tests demonstrated that heating the sample incrementally produced notable and rapid C3 aggregation at around 58 °C, with 6 nm cumulative radii protein reaching 100 nm at 62 °C. This result was supported by nanoDSF measurements which showed fast protein denaturation and a resulting T_m of 60 °C (Figure 1b).

SDS-PAGE and size-exclusion chromatography were used to confirm whether the smaller-than-expected protein diameter was due to missing or cleaved domains besides the 9 kDa anaphylatoxin domain of C3a. Despite possessing the same number of domains, the inactive and hydrolysed states of complement C3 have been shown to elute at significantly different times simply owing to considerable inter-domain reorientation.[37-39] Following protein digestion under reducing conditions, the gel showed all bands and their corresponding domains were accounted for. The size-exclusion chromatogram further confirmed that all domains were present with the 2 separate elution peaks being attributed to both inactive and hydrolysed C3. Importantly, no smaller peaks or contaminants were detectable after the final C3-associated elution (ESI Figure S1).

Although all biophysical and purity analyses showed the C3 sample as homogenous, conflicting evidence suggested the protein was significantly smaller than the crystallographic and cryo-EM structures already reported. To explain this discrepancy, we turned to single-particle cryo-EM and determined the structure of native complement C3 under the same physiological conditions as all

100 previous analyses. To our surprise, we observed several states of the complement protein achieving
101 an extreme range of inter-domain motion.
102

103 Full-range of C3 flexibility revealed

104 The most compact state, shown in Figure 2a, appears as the dominant species in solution and
105 represents the inactive state conformation. This arrangement shows the thioester domain (TED)
106 compressed and adjacent to the macroglobulin ring (MG) domains of MG2 and MG6. The interactions
107 between the TED and MG domains are facilitated mainly through dipole contacts rather than strong
108 ionic interactions. This feature allows for the movement experienced by the MG domains to proceed
109 freely. In fact, of the three interacting domains in this region, there are almost no electrostatic
110 interactions or hydrogen bonds formed. Instead, a select few complementary hydrophobic and
111 hydrophilic pockets of amino acids are enough to stabilise this conformation (Figure 3).
112
113 3D Flexibility analysis shows two distinct states attributed to MG ring domains 1 - 6 which possess the
114 ability to locally swivel and pivot in-place (ESI Figure S2). This was an unexpected finding, as the
115 structurally well-characterised macroglobulin ring domain is reported as rigid.[40, 41] Combining both 3D
116 variability and 3D flexibility analyses, the global hinge-like motion between the contracted and
117 expanded states could be resolved in addition to the flexible local motions experienced by each
118 domain throughout the full movement. The MG domain movement between each conformational
119 endpoint was found to reach up to 28 Å, with the MG2 and MG6 connecting arm region shifting by 12
120 Å. Minor classes found in 2D classification suggest that the extension may actually be greater than
121 this, however these views were too rare to be reliably incorporated into the 3D variability constructions.
122 In biological systems, the thioester domain acts as a "warhead" to connect to the detected foreign
123 particle. The current structural series shows the MG2 and MG6 domains assist with this tagging
124 process by uncovering the thioester region and exposing it for binding. These domains act as an
125 extending arm which moves the bulky MG regions away from the TED by roughly 30°. Furthermore,
126 the MG7 domain exhibits a slight bending motion which affords more solvent-accessible space for the
127 linker domain to extend into.
128
129 Each "compact" and "stretched" conformation could be reconstructed to a $FSC_{0.143}$ of 3.0 Å and 4.0 Å,
130 respectively (ESI Supplementary Table 1). The local resolution range varied more depending on the
131 degree of flexibility demonstrated in each conformational state. The alpha chain of C3 remained rigid
132 with local resolution reconstructions of 2.5 Å possible. The flexible MG ring domains could be
133 reconstructed to approximately 5 Å in the extended pose while a higher resolution of 3.2 Å was
134 achievable in the contracted, more rigid conformation.

Biophysical assays suggest TiO_2 nanoparticles interact with human Complement C3

The alternative pathway of complement consists of continuously activated C3b and inactive C3 at low concentrations. Therefore, it presented as an attractive testing ground for assessing the effect of TATT nanoparticles on the immune system. Repeating the DLS and thermal unfolding experiments on TATT in the same buffered system as C3 proved unsuccessful as the presence of salt promoted the near-immediate aggregation and gelation of the nanoparticle colloid throughout a range of concentrations (ESI Figure S3a). Instead using only filtered water, the nanoparticles showed no signs of aggregation which was confirmed by DLS as the TiO_2 particles were found to be of a homogenous diameter and stable at room temperature for 2 hours (ESI Figure S3b).

After confirming that C3 was also amenable in only filtered water, several concentrations of TiO_2 nanoparticles were added to differing aliquots of 0.4 mg/mL C3 for DLS and nanoDSF analysis (ESI Figure S3c). DLS results showed no appreciable indications of protein or nanoparticle aggregation. NanoDSF showed near identical T_m inflection points for C3 in both water and buffered solution over a range of temperatures. Importantly, the addition of TiO_2 resulted in an increase of T_m by ~7 ℃ for several separate concentrations of nanoparticle, demonstrating a meaningful interaction and significant thermostabilising effect of the TiO_2 species on C3.

Biochemical assays show TiO_2 nanoparticles downregulate C3 activity

To test the effect TATT may have on C3 function, the formation of the alternative pathway (AP) convertase, C3bBb was considered. In the presence of Mg^{2+} ions, factor B (FB) binds to C3b forming the foundation of an AP convertase, allowing for the action of factor D (FD) to cleave FB into fragments Ba and Bb. In turn, C3bBb is formed with a substrate specificity for native C3, the convertase cleaves off C3a generating C3b. Using C3b as a positive control, native C3 (1 μM) was incubated with and without TATT (1 μM) prior to the addition of FB and FD, relying on C3(H_2O) contamination or spontaneous hydrolysis occurrence to enable convertase formation and Bb generation. To analyse the effect of TATT, the ability for FB binding to native C3 was assessed by comparing peak area values of FB and fragment Bb generation detected by a monoclonal antibody against Factor Bb neoepitopes. The capillary electrophoresis data generated combined area for Factor B and Factor Bb that were converted to percentage area in the Compass for Simple Western (v 6.1.0) programme after peak naming. Figure 4A conveys the percent of Factor B remaining, indicating a reduction in the AP convertase formation and activity, reducing Factor Bb generation. In the presence of TiO_2, after 5 minutes, 95.1% FB remains (Native C3; 71.8%, C3b; 64.3%), 30 minutes 69.6% remains (Native C3; 13.9%, C3b; 15.6%) and 60 minutes 18.5% remains (Native C3; 11.9%, C3b; 8.9%).

Haemolysis of red blood cells by C3 is inhibited with TiO_2

Building on the AP inhibition, the capacity for native C3 to maintain its haemolytic properties was tested. Native C3 was incubated with incrementally increasing concentrations of TATT (0.5, 1, 2, 4, 8 and 16 μM). Rabbit red blood cells (RBCs) were used in this experiment as they fail to inhibit human complement and therefore become rapidly opsonised with C3b, enabling AP convertase and subsequent membrane attack complex formation (MAC) assembly, leading to haemolysis. In this serum dependant assay, an absorbance at 540 nm was recorded from native serum (total lysis), C3 depleted serum (blank) and the test samples of C3 with or without nanoparticles. The test samples are reported as a percentage of total haemolysis and show a significant reduction in haemolysis as nanoparticle concentration increases compared to neat native C3 (4 μM TATT p= 0.0187; 8 μM TATT p= 0.0268; 16μM TATT p= 0.003) as shown in Figure 4B.

Direct observation of TiO_2 nanoparticles interacting with human C3

Using these optimal conditions, C3 and TiO_2 at differing ratios were applied to Quantifoil EM grids and plunge frozen in liquid ethane in preparation for cryo-EM. Despite their modest size of 3.5 nm, the nanoparticles were readily visible as intense white spots on the micrograph with the ~185 kDa protein in close proximity, barely visible as a blurred grey signal (ESI Figure S4a). Additional sample processing in RELION 5.0 provided more discernible particles of both C3 protein, TiO_2 nanoparticles as well as classes which showed both in close proximity (ESI Figure S4b, S4d). Several ATLAS and eucentric height scans on different grids suggested that both particle distribution and contrast were favourable in thicker ice. It was immediately obvious from the micrographs that the nanoparticles further contributed to sample heterogeneity as they did not appear to interact with the protein in a uniform manner. This became evident from particle picking and 2D classification as several potential classes showed up to 3 nanoparticles may be interacting with a single protein, although at low alignment resolutions, this may be attributed to increased particle densities in the thick ice regions. Using a non-standard processing strategy in RELION 5.0 (Materials and Methods), we obtained a low-resolution electron density map suitable for protein and nanoparticle fitting.

Despite a loss of resolution, the C3 core particle density, comprising of the thioester and surrounding domains, matched very well with the previously-collected apo-C3 (amino acids ~550-1641). Similarly to apo-C3, the macroglobulin ring density remained challenging to capture, however the location of the missing domains could be approximated as the MG2 and MG6 domain densities connecting the TED and MG ring, could be accounted for. The predominant pose of the C3 protein appears to be in the most compressed state, where the MG2 and MG6 domains rest directly parallel to the thioester

203 domain. Several charged residues along the linker region form complementary interactions between
204 oppositely charged residues on the external face of the thioester domain, further stabilising this
205 conformation (Figure 5A, B). The vast majority of 2D classes also support this as we were unable to
206 locate any rare views of extended protein in close proximity to nanoparticle density. In fact, it seems
207 as though these extended forms of C3 are only observable in the absence of TiO_2.
208
209 Despite the lack of a defined binding site, protein flexibility and alignment challenges from spurious
210 classes, a clear density corresponding to the diameter of TiO_2 was located adjacent to the C3 electron
211 density. Owing to the signal thresholding during data processing, the most intense signal from the
212 interior of the nanoparticle has been eliminated, leaving just the periphery which resulted in a density
213 with a hollow interior. The pseudo-spherical density can be confidently attributed to the nanoparticle
214 rather than the missing macroglobulin ring domains as the TiO_2 density not only appears as the most
215 intense after map contouring compared to protein signal, but is approximately 40 Å from the expected
216 location of the macroglobulin ring. Furthermore, this region is situated well outside the latent axis of
217 motion displayed by the apo-C3 structure.
218
219 Overlaying the reported inactive structure with the nanoparticle-bound C3 shows that the majority of
220 the structure is unchanged, however producing a calculated RMSD of 4.27 Å relative to the MG7
221 domains of the native and TiO_2 structures (Figure 6A, B). This variability is largely due to the apparent
222 outward shift of the MG7 domain (residues 807-911), as the RMSD of the remaining protein is 0.912
223 Å. This macroglobulin domain is connected by two linker regions and is reported to move during
224 activation of C3, demonstrating a rotation of approximately 35°. This movement is facilitated by the
225 excision of the polypeptide chain between MG7 and MG8 by factor I, thus allowing the two MG
226 domains to nearly exchange positions.[36] Within an inactive C3, this domain is situated between the
227 linker and thioester domains, and has already been shown to move within the inactive state in the apo-
228 C3 structure. The location of MG7 in the current structure shows an outward shift of approximately 9
229 Å in the opposing direction to MG8. As the linkers are still intact, a slight rotation of this domain is
230 afforded to form an interaction with the nanoparticle which is positioned 6 Å adjacent to this domain.
231 Given the significant electronegative nanoparticle, it was anticipated that any interaction formed
232 between the species would occur at an acidic or phosphorylated region of the protein. A close
233 inspection of the solvent-accessible residues on MG7 indeed confirm that the nanoparticle is forming
234 an outer-sphere complexation with several electropositive residues (His846, Lys857, Arg858, Arg859,
235 His860) in addition to hydrogen bonding interactions with several more residues (Cys851, Thr855,
236 Thr856, Gln862).
237

Discussion/Conclusion

At present, this work represents the first example of a protein-nanoparticle interaction using a protein which lacks an engineered binding site specificity for the nanoparticle substrate. As such, this had evident repercussions on particle alignment and 2D classification challenges for non-specific interactions between the protein and nanoparticles.[42-44] Following a non-standard processing strategy for the C3-TiO_2 particles, a low-resolution reconstruction shows the interaction between C3 and a single TiO_2 nanoparticle. Despite the lack of a dedicated binding site for the nanoparticle, the location of TiO_2 does not appear to be random as it is situated adjacent to an electropositive, solvent-accessible region of the MG7 domain. This location demonstrates a charge complementarity between the highly electronegative, oxygen-coated, surface of the titania nanoparticle and the solvent-accessible arginine and lysine residues on MG7. Although the resolution is not sufficient to unambiguously determine the conformation of the amino acids participating in the interaction, the location of the nanoparticle throughout all classification steps remained consistent, indicating this interaction as non-transient and stable. Notably, the C3 has remained in an inactive conformation as the thioester domain, during activation, would produce a C3 diameter of ~160 Å. In the current structure, the compact density produced a diameter of 100 Å, much more similar to the size of reported inactive C3.[36] These structural findings were supported through the combination of Wes and haemolysis results: a significant functional deactivation of the C3 is imparted by the nanoparticle. In the context of the alternative pathway, this can be explained by the binding site obstruction for the C3 convertase, C3bBb. In the reported C3 convertase structure (2WIN),[45] the MG7 domain and C-terminal domain of C345C form a junction where Factor B is able to bind. Analysis of these inter-domain interfaces shows that they are stabilised mainly through hydrophilic interactions, particularly hydrogen bonds and polar side chain contacts, rather than by hydrophobic or charged interactions. Following this, Factor D cleaves bound into Bb and Ba, with the immediate expulsion of Ba. This leaves Bb attached to C3 to form the full C3 convertase of C3bBb, prepared to activate neighbouring C3 zymogens. In the current structure, the titania nanoparticle is situated almost exactly where Factor B would normally interact with C3 to activate the zymogen and form the convertase. Furthermore, the metal oxide nanoparticle, albeit not much larger than a Bb domain, is an extremely electronegative species as the titanium core is coated by oxygen. This results in a steric and electrostatic repulsive effect on the binding of Factor B to C3, producing no interaction between the two species. This was supported with a consistent melting temperature from nanoDSF of Factor B in water, buffer and in the presence and absence of TATT nanoparticles (ESI Figure S5). Additionally, the close proximity of the titania nanoparticles to MG7, a central domain involved in protein flexibility, has implications for the native motion of C3. The presence of the nanoparticles may sterically hinder conformational shifts in MG7 or disrupt intramolecular

interactions necessary for the propagation of structural changes, potentially altering the activation or regulation of the complement cascade. Relatedly, a recent report managed to deny the translocation of C3a and rearrangement of C3 by binding a specific nanobody to native C3.[46] To achieve this, the nanobody was found to bind at the MG7 domain in the same position as the TiO_2 nanoparticles. These interferences with the dynamic regions upon the protein could impair the ability of C3 to undergo the structural rearrangements required for its conversion to C3b or subsequent binding events. Although the C3-TiO_2 data are low resolution, it is clear by the predominant protein conformation that C3 indeed remains in a native state. Recent evidence from our lab suggests that C3 can undergo conformational shifts and adopt a C3b-like state even in the absence of Factors B and D, which promote cleavage. Evidence of this can be extrapolated from both DLS and cryo-EM results. Particle radii of C3 from DLS measurements remained reliably compact in the presence of nanoparticles when compared to apo C3. This was further supported by cryo-EM electron densities, as all obtained classifications, both 2D and 3D, showed the inactive (compact) form of C3. No evidence from the microscopy or biophysical data suggests titania nanoparticles are associated with C3 in the extended conformation or that they promote a major conformational shift to an active C3b-like state. Accordingly, given the inhibitory biochemical and structural results, it is likely that these small, TATT nanoparticles are recognised as a ligand inhibitor by C3, rather than as a foreign surface.[47, 48] Further experiments are necessary to confirm whether this perception is due to the size, composition or charge of the MNPs. Interestingly, the Compstatin family of cyclic peptide inhibitors, specific for C3, are shown to bind in a completely separate area of the protein. These organic inhibitors are all documented to bind between the MG4 and MG5 domains, approximately 60 Å away from the observed TiO_2 binding site at the MG7 domain. Yet, Compstatin binding does not arrest the conformation of C3, but instead prevents cleavage by convertases (C4b2a in the classical/lectin pathway and C3bBb in the alternative pathway). Accordingly, the titania nanoparticles may act to stabilise C3 in a compact, inactive form by preferentially binding to flexible domains like MG7, thereby biasing the conformational landscape toward a closed state. Such stabilisation could have functional implications, potentially dampening C3 activation by limiting the structural transitions required for convertase formation or cleavage.

The complement and coagulation systems are deeply intertwined, functioning not as isolated cascades but as a coordinated network that responds dynamically to tissue injury. Crosstalk between these systems ensures rapid haemostasis, immune cell recruitment, and tissue repair - functions that are tightly regulated to prevent chronic inflammation or thrombosis. Our findings of TATT nanoparticles inhibiting C3 activity introduce a novel mechanism by which these particles may influence the balance between inflammation, coagulation, and regeneration, for example at wound sites. Our data suggest that TiO_2 exerts active immunomodulatory effects by suppressing complement activation at the level

of C3. Furthermore, these results raise pertinent questions about the immunological effects these nanoparticles may have on human immune systems. The inhibition of C3 by TATT nanoparticles implies a downstream dampening of both the classical and alternative complement pathways, which could translate into decreased anaphylatoxin generation (C3a, C5a), reduced opsonisation, and eventual diminished membrane attack complex (MAC) formation. This modulation could directly alter immune cell recruitment and activation at injury sites, potentially shifting the local environment from a pro-inflammatory to a more regenerative state.

These findings reframe TiO_2 not just as a scaffold or surface-modifying agent, but as a bioactive nanomaterial capable of modulating complex immune-coagulation networks. Our findings align with emerging evidence that other metal or metal oxide-based nanoparticles, such as silver, zinc oxide, cerium oxide, and gold, also exhibit immunomodulatory properties through diverse mechanisms.[49-51] These insights support a paradigm shift in the use of nanomaterials in regenerative medicine, positioning them not merely as passive scaffolds or antimicrobial agents, but as tuneable modulators of host immune and haemostatic responses.

Materials and Methods

C3 isolation, purification and storage

Native C3 was isolated and purified in-house from human plasma according to previous reports.[52] The final step of purification involved cation exchange chromatography which was performed using a Mono S 5/50 GL column (GE Healthcare, Bio-Sciences AB, Uppsala, Sweden). The column was equilibrated with 5 column volumes (CV) buffer A (20 mM phosphate buffer pH 6.8), followed by sample application (0.25 CV). The column was washed with 3 CV of buffer A and then the elution was started using a linear gradient of buffer B (20 mM phosphate buffer pH 6.8, ranging from 0 to 0.85M NaCl). The protein elution was monitored with an UV detector at 280 nm and fractions of 0.5 mL were collected during the elution procedure. C3 was precipitated in a low salt and pH buffer for storage by dialysis against a 40 mM phosphate buffer with ionic strength 0.05 (mS) and pH 6.0 at +4°C with a buffer volume at least 10-fold higher than the volume of C3. After 24 h the buffer was replaced with fresh buffer and dialysis continued for another 24 h until a visible precipitate was formed. The stock solution of precipitated native C3 was then suspended (without centrifugation) and aliquoted before storage at -80°C. In order to dissolve the precipitated C3, 1-part VB^{++} (5x stock solution) was added to 4-parts of precipitated C3 immediately before use.[37]

341 ## Preparation of sol-gel titania

342 The synthesis of the uniform titania colloids used in this work was performed by following a previously
343 reported preparation.[53] The precursor of titanium ethoxide (5 mL) was dissolved in anhydrous ethanol
344 (5 mL) followed by the addition of 1.5 mL of triethanolamine during continuous stirring. A hydrolysing
345 solution (1 mL) was produced by mixing nitric acid (0.5 M) with ethanol (2 mL). The resulting lightly-
346 yellow, transparent solution contained 120 mg/mL TiO_2 according to TGA measurements.[28] The
347 obtained product was of high-enough purity to not require additional purification.
348

349 ## Biophysical measurements

350 DLS, thermal unfolding and nanoDSF measurements were performed on Prometheus Panta
351 (NanoTemper, Munich, Germany) in technical triplicate. For apo-protein experiments, 0.5 mg/mL of
352 C3 in filtered MQ water or buffered solution was added to separate capillaries at room temperature.
353 For TiO_2 experiments, nanoparticles in a stock solution of 1 mM were suspended in filtered MQ water,
354 briefly incubated with C3 and added to capillaries. All raw data were exported for analysis and graph
355 generation in GraphPad Prism version 10.0.
356

357 ## Biochemical assays

358 For capillary electrophoresis analysis by Wes immunoassay and alternative pathway haemolysis,
359 native C3 (1 μM) was preincubated with varying micromolar concentrations of TiO_2 nanoparticles in
360 VBS^{++} for 30 minutes at 37°C.
361

362 For capillary electrophoresis, 70 μg/mL of C3, C3b and C3 incubated with nanoparticles were added
363 to a preparation of Factor B (100 μg/mL) (Complement Technology Inc) and Factor D (0.5 μg/mL)
364 (Complement Technology Inc) in VBS^{++} and incubated over a time course of 5, 30 and 60 mins.
365 Individual time points were stopped with the addition of EDTA to a final concentration of 10 mM. The
366 Wes immunoassay was performed under reducing conditions with a primary mouse monoclonal anti-
367 human complement Factor Bb antibody (2 μg/mL) (Bio-Rad Laboratories).
368

369 The alternative pathway haemolysis assay was set up and adapted according to previous methods,[54,
370 55] where 25 μL of C3 (200 μg/mL) preincubated with 0.5, 1, 2, 4, 8 and 16 μM of TiO_2 nanoparticles
371 were added to 50 μL C3 depleted serum (Complement Technology Inc) followed by the addition of
372 100 μL 50% rabbit erythrocytes (v/v) in GVB Mg EGTA. These samples were then incubated in a
373 shaking water bath for 20 mins at 37°C at 200 rpm before being stopped with the addition of cold VB-

374 EDTA. The samples were then centrifuged at 860 g for 10 mins at 4°C and absorbance measured at
375 540 nm on a CLARIOstar Plus (BMG Labtech).
376

377 Cryo-EM structure determination of native C3

378 C3 protein buffer was exchanged from VB^{++} to filtered MQ water and concentrated to 0.4 mg/mL before
379 grid freezing. Cryo-EM samples were prepared on UltrAuFoil R1.2/1.3 300 mesh holey gold grids
380 (Quantifoil Microtools). Once the grids were glow discharged with a PELCO easiGlow instrument (20
381 mA, 0.4 mBar, 1.5 min), 3 µL of C3 was applied to the glow-discharged grids. Vitrification was
382 performed with a Vitrobot Mark IV (Thermo Fisher Scientific) at 4 °C and 95 % humidity. Grids were
383 blotted in duplicate for 3 seconds and plunged into liquid ethane. Following vitrification, the grids were
384 clipped into autogrid cartridges (Thermo Fisher Scientific) for use with autoloader systems.
385
386 All grids were ATLAS screened on a Glacios 200 kV cryo-electron microscope mounted with a Falcon
387 III direct electron detector. Data were acquired in EER format at a pixel size of 0.7463 Å/pixel with a
388 dose rate of 0.91 electrons $pixel^{-1}$ sec^{-1}. Approximately 1200 micrographs had 2x binning applied and
389 were processed using exposures fractionated into 50 frames for motion correction. This enabled for a
390 rapid assessment of the sample quality, particle density and distribution before high-resolution data
391 were collected. For full data collections, a 300 kV Titan Krios G2 (Thermo Scientific) transmission
392 electron microscope equipped with a K3 detector (Gatan) and a 20 eV BioQuantum energy filter
393 (Gatan), was used. Data were collected with the following settings: 0.648 Å/pixel (190 kx
394 magnification), 51 $e^{-}/Å^{2}$ total electron dose, −0.5 to −2.5 µm defocus range. Automated collection was
395 performed using the EPU software (Thermo Scientific) with 30° stage tilting. A total of 17,420 movies
396 were collected.
397
398 During data collection, cryoSPARC live (cryoSPARC v4.1.1) was employed for on-the-fly data
399 processing for the first few hundred micrographs using the same settings as for the initial screening
400 dataset. Processing was performed in cryoSPARC v4.6.0[56] and included initial Patch Motion Correction
401 and Patch CTF being performed before micrograph curation. Micrographs were manually curated
402 based on CFT estimations, total frame motion, defocus, ice thickness and contamination. Using the
403 obtained template from the screening dataset, all particle picking was performed with template picker
404 and the Topaz[57] wrapper template picker in cryoSPARC. Once data collection was completed, the
405 template-based picks totalled ~5 million and were 2x downsampled and extracted with a 400 x 400
406 box size before being Fourier-cropped to 250 x 250 pixels. Particles were classified into 150 2D
407 classes (10 Å minimum separation distance, 170 - 200 Å circular mask inner-diameter, 2 final full

408 iterations, 40 online-EM iterations, 400 batch size per class). Following 2D class selection, this
409 classification/selection procedure was repeated twice more which resulted in ~1.9 million particles
410 suitable for *Ab-initio* reconstruction. *Ab-initio* reconstruction was followed by heterogenous refinement
411 using a few poor density "dummy" classes as seeds for junk particles. Two further rounds of
412 heterogenous refinement seeding resulted in 818,343 particles which displayed discrete
413 heterogeneity, which was elucidated using 3D variability analysis.[58] Masked refinement and
414 reconstruction jobs reliably produced densities at resolutions of ~2.5 Å. 3D variability analysis
415 demonstrated the inherent continuous disorder of the particles and was used to separate individual
416 latent dimensions of motion attributed to approximately 3 states experienced by the particles. Particles
417 responsible for each state were isolated and reconstructed independently to produce each individual
418 C3 pose.
419
420 A model was constructed by using the reported inactive C3 structure (PDB 2A73)[36] as a starting model.
421 Owing to the large conformational differences between the current particles and the reported structure,
422 the domains were individually docked and fitted into the electron density with UCSF ChimeraX version
423 1.9.[59] These hand-fitted domains were then quantitatively fitted into the density and refined using the
424 DockEM[60] and Refmac Servalcat[61] packages within CCP-EM 1.6.0.[62] The Coot[63] program was used for
425 manual rebuilding and inspection of the model in the electron density before a final real-space
426 refinement run in PHENIX.[64] The relevant structural validation metrics are shown in supplementary
427 Table 1.
428

429 ## Cryo-EM structure determination of C3-TiO_2

430 As previous, C3 protein buffer was exchanged from VB^{++} to filtered MQ water and concentrated to 0.4
431 mg/mL before grid freezing. Cryo-EM samples were prepared on UltrAuFoil R1.2/1.3 300 mesh holey
432 gold grids (Quantifoil Microtools). While the grids were glow discharged with a PELCO easiGlow
433 instrument (20 mA, 0.4 mBar, 1.5 min), C3 was incubated with nanoparticles at an equimolar
434 concentration for 5 minutes on ice before 3 μL of the mixture was applied to the glow-discharged grids.
435 Vitrification was performed with a Vitrobot Mark IV (Thermo Fisher Scientific) at 4 °C and 95 % humidity.
436 Grids were blotted in duplicate for 3 seconds and plunged into liquid ethane. Following vitrification,
437 the grids were clipped into autogrid cartridges (Thermo Fisher Scientific) for use with autoloader
438 systems.
439
440 A 300 kV Titan Krios G2 (Thermo Scientific) transmission electron microscope equipped with a K3
441 detector (Gatan) and a 20 eV BioQuantum energy filter (Gatan), was used for the full data collections.
442 Data were collected with the following settings: 0.648 Å/pixel (190 kx magnification), 51 $e^-/Å^2$ total

electron dose, −0.5 to −2.5 μm defocus range. Automated collection was performed using the EPU software (Thermo Scientific) and a total of 17,706 movies were collected. Processing was performed in cryoSPARC v4.6.0 and included initial Patch Motion Correction and Patch CTF being performed before micrograph curation. Micrographs were manually curated based on CFT estimations, total frame motion, defocus, ice thickness and contamination.

A visual inspection of the micrographs and blob-picked classes in cryoSPARC suggested there was a clear picking bias which made further classification and alignment impossible (ESI Figure S4a). As the electron dosage charged the TiO_2, the nanoparticles appeared as bright hotspots on the micrographs which almost completely overpowered signals attributed to protein. Attempts to use the previous C3 reconstruction as a picking template were unsuccessful due to the picking algorithm being unable to locate any signal related to protein. Analysis of the protein and TiO_2 relative intensities in EMAN2[65] showed that there was approximately a 1:10 intensity disparity between the protein and nanoparticles, respectively (ESI Figure S4c). Attempts to enforce non-negativity, solvent-clamping and template picking using native C3 prior to 2D classification proved unsuccessful as the picking was strongly biased toward the most intense signals of the nanoparticles. To the best of our knowledge, cryoSPARC does not have the ability for the user to manually threshold and normalise intensities, and for this reason we attempted to complete the data processing using RELION 5.0.[66] Following a standard pre-processing strategy up to CTF estimation, Topaz was trained on a small subset of particles and once training converged, particles were extracted from all micrographs. The extraction job settings were not default: particle box size 384 pixels, diameter background circle 40 pixels, stddev white dust removal -1.0, stddev black dust removal 3.0, with normalisation on and a minimum FOM of 1.0. The relion image handler tool was used to threshold a series of incremental values from 1.0 – 3.0 in order to find which cutoff yielded the best SNR of both protein and nanoparticle. After 2D classification was run on each thresholded particle stack, normalisation was run using the relion stack create and relion preprocess tools. Performing this step was found to improve particle alignment as well as provide an intensity distribution favoured by RELION. A low-resolution map was obtained from the thresholded and normalised particle stack after running through 2D classification, 3D reconstruction and masked refinement.

The obtained density showed most domains present excluding those in the macroglobulin head region (MG1-4). Excluding this missing area, the domains were individually docked and fitted into the electron density with UCSF ChimeraX version 1.9, which conformed to the inactive state C3 conformation.

Structural and statistical image preparation

All cryo-EM images were prepared using ChimeraX. All biophysical and biochemical graphs and analyses have been prepared, fitted and displayed using the program GraphPad Prism version 10.0.

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Author contributions

M.S, K.N.E, B.N and V.G.K conceptualised the research. G.A.S synthesised the pure titania colloidal nanoparticles. D.E and K.F purified the C3 protein and performed all biochemical and haemolysis assays. J.J.W performed all biophysical characterisations with C3 and TiO_2. J.J.W prepared all TEM grids, performed data collection, processing, model refinement and validation of all cryo-EM structures. J.J.W and D.E compiled and analysed the data, wrote the original manuscript and prepared all figures and tables. Editorial assistance was provided by V.G.K, G.A.S, M.S, B.N and K.N.E. All authors have reviewed, edited and approved the final version of the manuscript.

Competing interests

The authors declare no conflicts of interest.

Data availability

Original data points are shown in the manuscript. Additional information can be obtained from the corresponding author upon request.

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