Protein corona formation around biocatalytic nanomotors unveiled by STORM

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ABSTRACT: The interaction of nanoparticles with biological media is a topic of general interest for drug delivery systems and among those for active nanoparticles, also called nanomotors. Herein, we report the use of super resolution microscopy, in particular stochastic optical reconstruction microscopy (STORM), to characterize the formation of protein corona around active enzyme-powered nanomotors. First, we characterize the distribution and number of enzymes on nano-sized particles and characterized their motion capabilities. Then, we incubated the nanomotors with fluorescently labelled serum proteins. Interestingly, we observed a significant decrease of protein corona formation (20 %) and different composition, which was studied by a proteomic analysis. Moreover, motion was not hindered, as nanomotors displayed an enhanced diffusion regardless of protein corona. Elucidating how active particles interact with biological media and maintain their self-propulsion after protein corona formation will pave the way of the use these systems in complex biological fluids in biomedicine.

Inspired by nature, catalytic nanomotors that convert chemical energy into motion have been developed in the last decade.¹ Particularly, enzymes are highly efficient natural catalysts that constitute a very promising strategy to build biocompatible micro- and nanoswimmers that self-propel using bioavailable fuels such as glucose,² H₂O₂³ or urea^{4,5} without the need from external power sources. This has opened new possibilities in the use of nanomotors for biomedical applications, and several milestones have been already reached, including enhanced anti-cancer drug delivery,⁶ improved cell uptake,⁷ sensing⁸ and medical imaging. Despite these exciting outcomes, not all enzymes can generate active motion in a highly efficient manner, being urease one of the most powerful enzymes to promote self-propulsion for micro- and nanoparticles.⁹ Urease-powered nanomotors have already been used to enhance anti-cancer drug delivery,⁶ and promote a more efficient cell uptake¹⁰ targeting and penetration of 3D spheroids⁷. Additionally, urease-nanomotors are capable of self-propel within biological fluids such as urine⁷ and blood.¹⁰

Despite these exciting outcomes, some concerns have been raised for their use in biological media and potential biomedical application.¹¹ To tackle this issue, the challenges that all nanoparticle delivery systems face when they enter the organism should be considered. Upon nanoparticle administration, proteins, lipids and small metabolites present within the biological fluids instantly adsorb to the nanoparticle surface, forming a protein corona that confers a new biological identity to the nanoparticle,^{12,13} altering their targeting capabilities,^{14,15} cellular uptake,^{16–19} circulating time and biodistribution.²⁰ The protein corona composition and assembly strongly depends on the intrinsic properties of nanoparticles, including the size, shape and surface charges.^{21–23} Additionally, the characteristics of the surrounding environment can also modulate the protein corona formation, such as the

pH²⁰ or shear stress and flow.^{24,25} Several techniques have been used so far to analyze protein corona,^{26,27} being mass spectrometry-based proteomics^{28,29} and gel electrophoresis³⁰ the most extended approaches. Using these techniques, the protein corona analysis is performed in bulk. Recently, super resolution microscopy and, in particular, STORM, is emerging as a powerful tool to characterize the properties of nanomaterials,³¹ including the dynamics of protein corona formation around nanoparticles at the single particle level.^{32,33} Furthermore, STORM is the only current approach that allows visualizing the number and position of individual proteins allowing for a precise spatial mapping. This is particularly relevant in the case of enzyme powered micro- and nanomotors, since their enzymatic distribution is intrinsically heterogeneous, and a certain degree of heterogeneity in the formation of protein corona around them may also be expected.³⁴

Herein, we aim at studying the formation of a protein corona around active urease-powered nanomotors using STORM with two main objectives: i) to study how nanomotor activity affects the formation of protein corona and ii) to study how protein corona formation affects the self-propulsion of nanomotors.

To this end, we synthesized mesoporous silica nanoparticles by using a modified Stöber method, as previously reported,⁷ where cetyltrimethylammonium bromide (CTAB) was employed to generate the mesopores, and triethanolamine (TEOA) and fluorescein isothiocyanate conjugated to (3-aminopropyl)triethoxysilane (FITC-APTES) were used as precursors (6:1 volume). The resulting particles were characterized using Scanning Electron Microscopy (Figure 1A) which showed monodisperse particles with a mean diameter of 434 ± 2 nm (mean $\pm$ s.e.m, N= 200). The inner cylindrical porous structure was verified with transmission electron microscopy (TEM, Figure 1B) where an average pore size of 3 nm was determined. To functionalize the particles with urease, their surface was first modified with aminopropyltriethoxysilane (APTES) to provide them with available -NH₂ functional groups. Urease was subsequently conjugated using glutaraldehyde (GA) as a linker (Figure 1C). Nanomotors self-propel thanks to the conversion of urea into ammonia and carbon dioxide ($(\text{NH}_2)_2\text{CO} + \text{H}_2\text{O} \rightarrow \text{CO}_2 + 2\text{NH}_3$) by urease (Figure 1D).^{34,35} To characterize the different functionalization steps, the electrophoretic mobility of the particles was monitored. Dynamic light scattering (DLS) showed the initial unmodified particles to have a surface ζ -potential of -29.5 ± 0.34 mV (Smoluchowski), as expected from the presence of silanol groups (Figure 1E). After their modification with GA and urease, the ζ -potential of the particles was modified. As expected from the isoelectric points of the surface groups^{36,37}, amine modified silica yielded positive surface ζ -potentials and enzyme conjugation recovered the negative surface ζ -potential, confirming the presence of urease on the surface. Urease activity was determined by using a commercial enzyme activity assay kit (Figure 1F).

Figure 1. Fabrication and characterization of urease-powered nanomotors. A) SEM micrograph of mesoporous silica nanoparticles. Scale bar = 500 nm. B) Representative TEM micrograph of a mesoporous silica nanoparticle. Scale bar= 100 nm. C) Schematic representation of the functionalization approach. D) Schematic representation of the self-propulsion upon enzyme catalysis. E) ζ -

potential characterization of the particles along the functionalization process. F) Enzyme activity of the particles before and after their functionalization with urease.

To generate self-propulsion, an asymmetric distribution of the catalyst is always necessary in order to avoid a net compensation of forces. Recent works have reported that stochastic binding of enzymes onto micron-sized particles results in a non-homogeneous, patchy-like distribution leads to the self-propulsion of biocatalytic micromotors without the need for physical asymmetries like the case of Janus particles.^{34,38} While this parameter has been thoroughly studied for micron-sized particles, which display a ballistic type of motion, little is known about nano-sized motors, which display a different type of motion known as enhanced diffusion.⁵ For this type of motion dynamics, several asymmetric^{2,39,40} and non-asymmetric structures have been reported.^{6,41,42}

Figure 2. STORM characterization of urease molecules around the nanomotors. A) STORM recorded localisations of Cy5-labelled enzymes are superimposed to the FITC fluorescence image for better visualization. The colour of a localisations represents the frame in which it was recorded. Scale bars are 300 nm. B) Representative STORM images showing urease localizations in a nanomotor. C) Corresponding images showing the localization density, where the distribution of urease can be visualized. D) Histogram of the number of urease localisations per analysed nanomotor. Mean $\pm$ SD ($n = 41$).

However, a robust and systematic analysis of the enzyme distribution onto nano-sized motors is missing. This is particularly relevant since two factors have already been demonstrated to be crucial for self-propulsion of urease micromotors: an asymmetric distribution and the number of enzymes on the motor surface.³⁴ Here, we used STORM to detect urease molecules bound to nanomotors surface. For this, urease was previously labelled with Cy5 fluorophore and a calibration to estimate the number of localisations recorded per single labelled urease was performed (Figure S1).³⁴ Nanomotors functionalized with 25% labelled urease were placed onto a glass slide and allowed to precipitate until immobilized. Unbound nanomotors were washed out by replacing the remaining dispersion in PBS solution by the STORM buffer. Figure 2A (right) shows the result of merging both conventional fluorescence and STORM images. Figure 2B, 2C and S2, show representative examples of urease localizations and enzyme density, respectively. Generally, a non-uniform urease coating of nanomotors was observed (Figures 2B and 2C), similarly to what has been previously observed for micron-sized urease motors³⁴. Using STORM, we were able to determine the amount of urease molecules bound to the nanomotor surface by using a custom-made python-based code, analyzing a minimum amount of 40 nanoparticles per case. First, particle size was determined by the information provided by fluorescence images, being the mean nanoparticle radii 254 ± 10 nm (mean $\pm$ s.e.m, $N = 50$) being these results consistent with both TEM/SEM analysis. STORM images enabled the quantification of urease molecules bound to the nanoparticle surface, with a mean of 584 ± 91 (mean $\pm$ s.e.m, $N = 40$).

Motion dynamics was studied by recording the nanomotors either with or without urea, at 100 mM in phosphate buffered saline solution (PBS 1x) which is the optimal concentration of urea at which nanomotors move.^{6,9,41} Bright field videos were recorded for 30 s at a 25 FPS rate. Nanomotors trajectories were tracked using a custom-made python-based code. Figure 3A and 3B show the trajectories of the nanomotors with and without urea, respectively. From the trajectories, we extracted the Mean Squared Displacement (MSD), represented in Figure 3C. Both with and without fuel, a linear MSD was observed, which denotes a diffusive type of motion dynamics.

However, in the case of nanomotors in 100 mM urea, the slope of the MSD was significantly higher, indicating enhanced diffusion (Figure 3C).^{5,43,44}

Figure 3D shows the distribution of obtained diffusion coefficients, being them significantly higher ($P=0.0035$) in the case of 100 mM urea.

Figure 3. Motion dynamics of urease nanomotors. A) Trajectories of nanomotors in the absence of fuel. B) Trajectories of nanomotors in the presence of 100 mM urea. C) Comparison between the MSD in the presence or absence of fuel. Results are shown as the mean $\pm$ s.e.m (n=20). D) Diffusion coefficient of the nanomotors calculated from the MSD. Results are shown as the mean $\pm$ s.e.m (n=20).

Addressing the performance of biomedical micro- and nanomotors in complex biological fluids is a critical aspect, since certain physiological conditions may hamper their motility. In this study, we monitored the motility of particles using PBS, which contains different salts and ionic species at a physiologically relevant concentration. The presence of ionic species has been recently demonstrated to impede the motion of micron-sized motors powered by urease.⁴⁵ However, in the case of nano-sized motors, their motion persists in different types of media, including PBS,^{6,46} urine⁴¹ and blood¹⁰, probably due to their different type of motion mechanism. Nonetheless, biological fluids not only contain salts and ionic species and other components such as proteins might interact with the nanomotor surface. Here, to investigate the effect of the activity of nanomotors on the formation of protein corona in a quantitative manner, we incubated urease-nanomotors with Cy5-labelled serum proteins, either in the presence or absence of 100 mM urea (Figure 4A). For this, urease nanomotors were incubated for 30 min in a solution containing non-labelled Fetal Bovine Serum (FBS), 5% Cy5 labelled FBS and 0 or 100 mM urea for the control and active samples, respectively (Figure A). As in the previous experiments, green fluorescence was used to localize the nanomotor boundary and the images were superimposed with the STORM images (Figure 4B and 4C), where labelled serum proteins were quantified. Figure 4D shows a histogram depicting the distribution of FBS detections per nanomotor. In the presence of 100 mM urea, the peak of the histogram is significantly shifted to the left, indicating a lower average FBS detection per nanomotors, compared to the nanomotors without fuel. Figure 4E shows a comparison of the average detected FBS points per nanomotor. Surprisingly, around 20% reduction on the FBS localizations was found between control (615 ± 24 localizations, mean $\pm$ s.e.m.) and active nanomotors (486 ± 21 localizations, mean $\pm$ s.e.m.). These results indicate a significant reduction ($P=0.002$) of protein corona formation around nanomotors when they are active. This effect could be explained by two factors. First, urease activity of micromotors has been already reported to modify the local surrounding environment, leading to a pH increase.^{8,47} The formation and stability of protein corona has shown sensitivity to different pH⁴⁸⁻⁵⁰ and particle surface charges.⁵¹ The changes in pH induced by urease activity could thus result in an alteration of the physicochemical properties of the particle surface and the serum proteins, ultimately affecting the interactions between both. Second, nanomotors chemical activity often leads to the creation of a shear flow around them.⁴⁴ This is also a very interesting feature, since protein corona has shown to be dependent not

only on the physicochemical properties of the particles but also on the presence of shear stress and shear flows.^{24,25,52}

Figure 4. Protein corona formation around active nanomotors. A) Schematic representation of the experimental approach, where fluorescent nanoparticles (FITC-MSNP) were functionalized with non-labelled urease and incubated with Cy5-labelled serum proteins either in the presence or absence of fuel. B) Representative STORM images of nanomotors after their incubation with labelled FBS in the absence of fuel, showing FBS localizations (top) and FBS density (bottom). C) Representative STORM images of nanomotors after their incubation with labelled FBS in the presence of fuel, showing FBS localizations (top) and FBS density (bottom). D) Histogram of the FBS detections per nanomotor distribution. E) Comparison of the average detected FBS proteins in nanomotors with and without urea. F) Comparison of the diffusion coefficients of the nanomotors in different conditions. Results are shown as the mean $\pm$ s.e.m.

Since the self-propulsion of nanomotors is generated by the decomposition of urea catalyzed by urease, the presence of a protein corona on the particle surface could hamper the substrate and product exchanges, limiting the motion performance. For this reason, we analyzed the motion of nanomotors after the formation of protein corona. For this, we incubated the nanomotors with FBS as described previously, following the same procedure as for the STORM imaging experiments. Then, the motors were resuspended in PBS 1x and their motion was analysed as described above. Results showed a significant increase ($P=0.047$) in the diffusion coefficient of the nanomotors in 100 mM urea, constituting an increase of 18 % respect to the control (0 mM urea). This increase was slightly lower than the one observed for the nanomotors swimming in PBS, without being previously incubated with FBS, where a 44 % increase in the diffusion coefficient was found. These results indicate that urease powered nanomotors are able to swim regardless of the diminished protein corona formation around their surface. However, their performance is slightly limited compared to the bare nanomotors, indicating that future strategies to further reduce protein corona might be desired.

Figure 5. Proteomic analysis of protein corona composition around nanomotors when incubated 30 min with FBS. A) Venn diagram representing the number of proteins only present in the nanomotors exposed to urea, no urea and the number of proteins present in both conditions. B) Dotplot displaying the most abundant proteins, according to their intensity. In each case, results are shown as the mean $\log_2(\text{Intensity})$ of three independent biological replicates. nU: no urea; U: 100 mM urea. C) Fold-change of the most significant proteins for the 100 mM urea conditions (light orange) with respect to no urea (light blue) condition. Results are shown as the mean $\pm$ SD.

Next, we investigated whether the activity of the nanomotors resulted in a change in protein corona composition. For this, after incubating nanomotors with FBS for 30 min with or without fuel, we performed a proteomic analysis by digesting adsorbed proteins using a trypsin treatment, followed by protein identification using HPLC-Mass Spectrometry analysis. We identified a total amount of 321 proteins, from which 236 protein groups were quantified. A differential expression analysis between 100 mM urea and no urea conditions was performed. Figure 5A shows a venn diagram of the proteins found for each condition and in common for both conditions. When we selected the 20 most abundant proteins for each condition (Figure 5B), the changes in abundance were non-significant, although the tendency was to find a lower abundance for most proteins in the case of urea condition. Interestingly, for the non-urea condition, the heat shock cognate 71 kDa protein and Heat shock 70 kDa protein 1-like were among the most abundant, which was not the case for urea condition. Other examples are Apolipoprotein C-II and ALB protein, which were among the 20 most abundant proteins in urea conditions but not in non-urea conditions.

Moreover, several significant proteins were identified using standard cutoffs for fold change ($|FC| > 1.5$) and adjusted p-value ($\text{padj} < 0.05$) when comparing urea and non-urea conditions (Figure S3). In this regard, 13 proteins were shown to be up-regulated and 21 down-regulated (Figure 5C).

This study reports for the first time the relationship behind enzyme-driven motion and protein corona, highlighting the relevance of extending these studies to the micro- and nanomotors community, since the effects on their propulsion capabilities might depend on the particle properties and their interaction with biological media. We made use of superresolution microscopy to unveil the protein corona formation on nanomotors in the presence and absence of chemical fuel, and we identified the type and quantified its variation by proteomics analysis. Moreover, our observations show that, active nanomotors can reduce the protein corona formation, which might be desirable for certain biomedical applications. Noteworthy, the changes in protein corona are not only qualitative but also quantitative, where the impact in different biological interactions such as cell-nanoparticle interactions will need to be considered in the near future.

ASSOCIATED CONTENT

Supporting Information.

Supporting Information: Materials and methods, characterization of nanomotors enzymatic activity, characterization of Cy5-urease labelling by STORM. (PDF) (file type, i.e., PDF)

brief description (file type, i.e., PDF)

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