Electronic Supporting Information

Measuring Viscosity inside Mesoporous Silica Using Protein-Bound Molecular Rotor Probe

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S.1. Synthesis of MC2 and MCF12

Pluronic™ P123 was dissolved in 1.6 M HCl at room temperature. After that TMB was added and the temperature was raised to 40 ºC under vigorous stirring for 2 hours, followed by the addition of TEOS. After 20 hours, 0.11g of NH_4F was added and the suspension was transferred to a Teflon-lined stainless-steel autoclave and aged during 24 hours at 100 ºC. The samples were filtered, washed with distilled water and dried in air at room temperature. The products were calcined in air at 550 ºC for 8 hours (heating rate of 1 ºC·min^{-1}).[1]

S.2. Circular dichroism experiments on free enzyme, labelled enzyme in phosphate buffer and 70% glycerol

Circular dichroism spectra were recorded on a Chirascan, Applied photophysics spectrophotometer in the wavelength region 198-260 nm, which is sensitive to protein secondary structure composition, in order to detect any conformational changes of the MML enzyme at the highest concentration of glycerol used in this study (70% mass fraction in buffer). Figure S.1 shows the average spectra between five independent measurements of each sample.

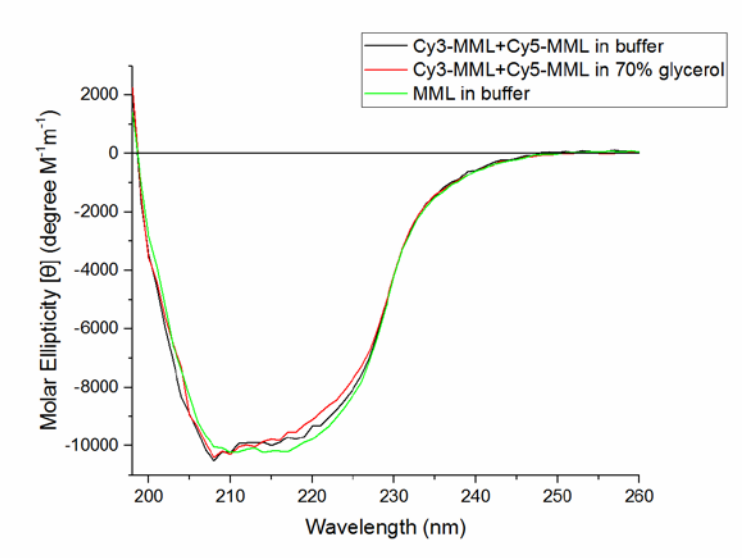


Figure.S.1. CD spectra of lipase and the mixture of single-labelled lipase (Cy3-MML+Cy5-MML) in buffer and 70% glycerol.

S.3. Calculation of pore filling

In addition to protein loading P_{LD} (mass of protein per mass of particles) we use two measures of the amount of immobilized protein which provide a more direct, molecular picture. The average number of proteins per gram of particle $N_{prot/g\ of\ MPS}$ was calculated from the P_{LD} value as[2]

$$N_{prot/g\ of\ MPS} = N_A \cdot P_{LD} / M_W \qquad \text{Eq.S1}$$

Where N_A is Avogadro's number and M_w the molecular weight of the protein (Table 1 of main text).

The actual protein concentration in the pores was expressed in terms of the pore filling P_f defined as the fraction of the available pore volume which is occupied by proteins, and calculated as[2]

$$P_f = (N_{prot/g\ of\ MPS}.\ V_{prot})/V_{pore} \qquad \text{Eq.S2}$$

where $N_{prot/g\ of\ MPS}$ is the number of proteins per g of MPS, V_{pore} is the total pore volume per gram of the particles (see Table 3 of main text), and V_{prot} the volume of protein per gram of particles is calculated assuming each protein is a sphere with a radius equal to the hydrodynamic radius R_H (Table 1 of main text).

S.4. Absorption spectra of labeled enzyme

Figure S.2. Shows the absorption spectra of single-labeled lipase individually (with either Cy3 or Cy5) and comparing with the mixture of single-labelled lipase in the same concentration of Cy3, Cy5 and lipase. It shows that the absorption spectra do not change after mixing, and also that at 483 nm Cy5 hardly absorbs any light and so excitation at this wavelength will only excite Cy3. Therefore, for FRET experiments, we used an excitation wavelength of 483nm.

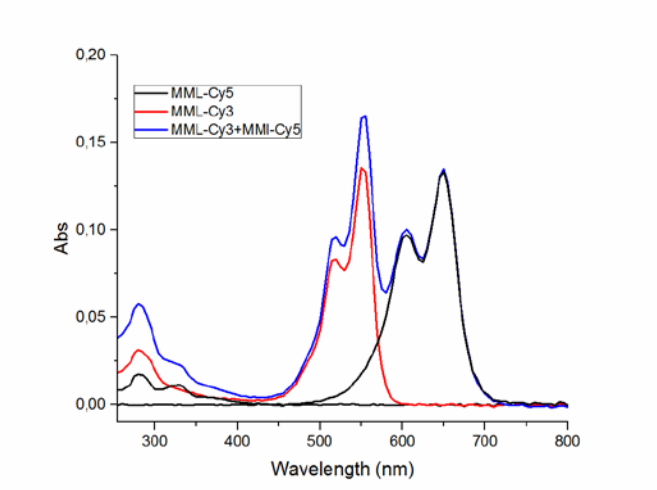


Figure.S.2. absorption spectra of single-labeled lipase individually and the mixture of single-labelled lipase in the same concentration.

S.5. Time-resolved (TC-SPC) measurements: checking for FRET in the immobilized samples

FRET is a process in which energy is transferred non-radiatively via long-range dipole-dipole coupling from an excited donor fluorophore to another dye molecule, the acceptor. Cy3 and Cy5 constitute a good donor-acceptor pair because the fluorescence emission spectrum of the donor Cy3 has a large spectral overlap with the absorption spectrum of the Cy5 acceptor chromophore. To observe FRET the orientation of the transition dipole moments of the two chromophores must be approximately parallel to each other. [3] Secondly, the donor and acceptor dye must be in close proximity of each other, as characterized by the Förster distance R_0 which is given by [4]

$$R_0 = 0.211(\kappa^2 n^{-4} \Phi_D J(\lambda))^{1/6} \qquad \text{(Eq. S3)}$$

Experimentally the strength of the FRET is presented as transfer efficiency (E) which is a direct measure of the fraction of photons absorbed by the donor that is transferred to an acceptor. It is commonly measured as the relative lifetime of the donor in presence (τ_{DA}) and absence (τ_D) of the acceptor, [5]

$$E = 1 - (\tau_{DA} / \tau_D) \qquad \text{(Eq. S4)}$$

which has the advantage in turbid samples such as our particle-based system that it does not involve measurements of absolute intensity. Equation (Eq. S5) shows that E depends on the inverse of the sixth power of the distance (r) between the two fluorophores

$$E = R_0^6 / (R_0^6 + r^6) \qquad \text{(Eq. S5)}$$

which has made FRET a useful and sensitive method to measure distances between fluorophores, for example in protein-protein interaction studies.[6, 7] It is seen that the Förster distance R_0 represents the distance between the two chromophores where the transfer efficiency is 50% for a given fluorophore pair. For the Cy3/Cy5-pair, R_0 is approximately 6 nm.[8]

a) Unmodified silica particles (SBA-15, MCF2, MCF12)

To make sure that there is no energy transfer between dyes after immobilization in the MPS particles, (see Methods) TC-SPC measurements were performed on each protein-particle sample after immobilization, and in free solution. Figure S.3a (blue and red) shows the time-resolved intensity profiles in the phosphate buffer. The curve with only Cy3-MML (blue) has the same decay-profile as when Cy5-MML is added (red). This observation shows there is no energy transfer between Cy3-MML and Cy5-MML in free confirming the steady-state intensity observation for the single-labelled case in main text Figure 3b. (as expected from Eq. S5 because the average distance between the Cy3 and Cy5 labeled proteins are typically μm in the dilute free solutions and much larger than R_0),

The same observation is made after immobilization in SBA-15 (green and black in Figure S.3a), the intensity decay when only Cy3-MML is immobilized (green) is the same as when Cy5-MML is co-immobilized with Cy3-MML (black). (The immobilized concentration of Cy3-MML were approximately the same in both samples). The conclusion is that there is no FRET also after the Cy3-MML+Cy5-MML sample immobilized in SBA-15. Secondly the decays are seen to be faster in free solution than in the presence of particles, which is consistent with a higher steady state Cy3-emission intensity in the presence of the particles. This observation supports that the effective viscosity is higher in the pores than in free solution,[9] although a decay curve at a single wavelength cannot distinguish between viscosity and polarity effects as the steady state intensity ratio-method is capable of.

Similar results were obtained with MCF2 and MCF12 (See Figure S.3b and S.3c). It can be seen that there is no detectable FRET for any of the non-modified particle types at the highest protein concentrations (60 μg/mg) studied here. It is noteworthy that the TC-SPC measurements were performed for all three protein concentrations, but only the decays related to highest

amount of protein concentration are shown here. The main reason is to show that even for the highest amount of immobilized labeled MML, FRET cannot be detected and also lifetime decay measurements are independent to protein concentration.

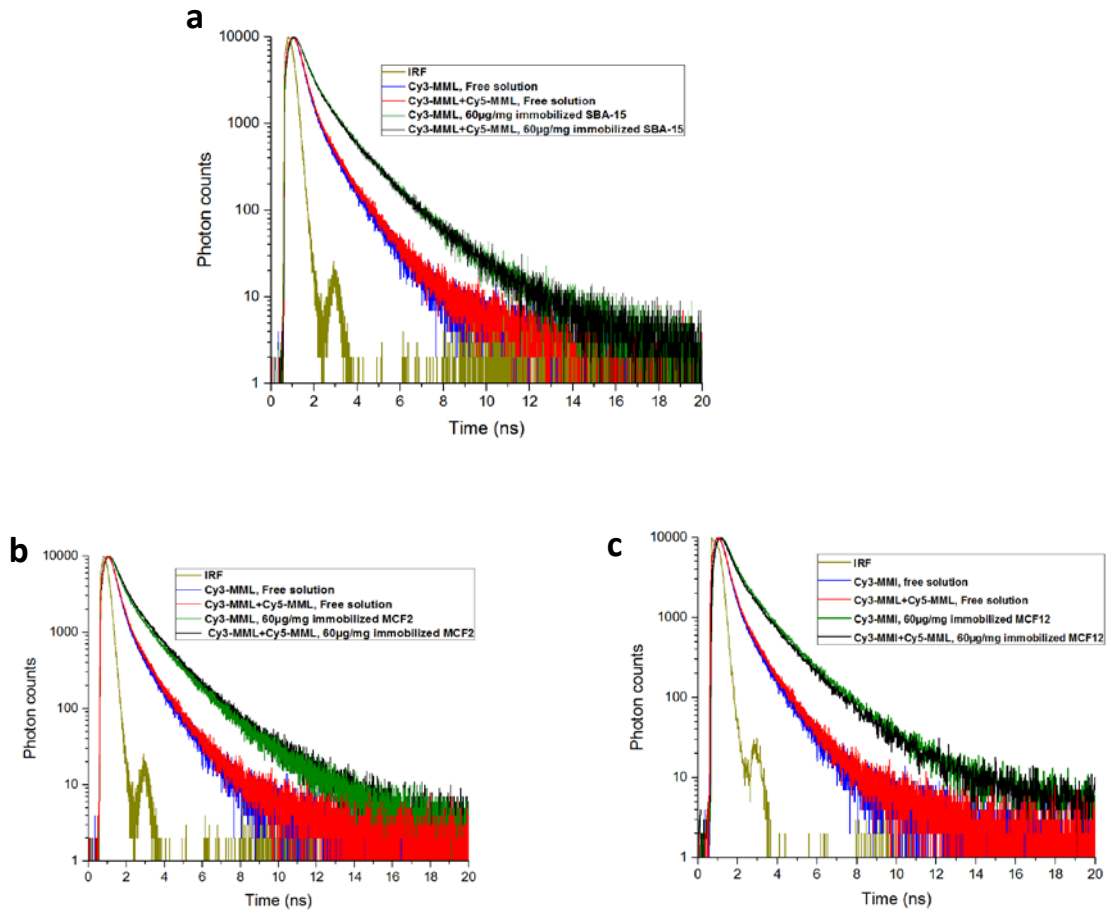


Figure S.3. Time-resolved intensity measurements of protein attached dyes in free solution (no particle) and immobilized in SBA-15 (a), MCF2 (b) and MF12 (c). Each pair of curves shows the fluorescence intensity decay of Cy3-MML in the absence and presence of Cy5-MML. The blue and red curves are in free solution (blue is without Cy5-MML), and green and black curves after immobilization in the specific particle (green is without Cy5-MML). Excitation at 483 nm and emission at 565 nm. The golden curve is the instrument response function (IRF), i.e. the decay of the excitation lamp pulse intensity. The decays were reproducible for three independent experiments.

b) OC-modified MCF particles

Figure S.4 shows the time-resolved intensity profiles after immobilization in the MCF2-OC (Figure S.4a) and MCF12-OC (Figure S.4b) particles. The faster intensity decay for Cy3-MML

when Cy5-MML is co-immobilized (black) compared to when only C3-MML is immobilized by itself (red) shows that the addition of Cy5 introduces another quenching mechanism which is attributed to FRET between Cy3 and Cy5. It is noteworthy that the TC-SPC measurements were performed for all three protein concentration, but only the decays related to lowest amount of protein concentration (20µg/mg) are shown here, the main reason is to show that FRET can be seen even for the lowest amount of labeled proteins.

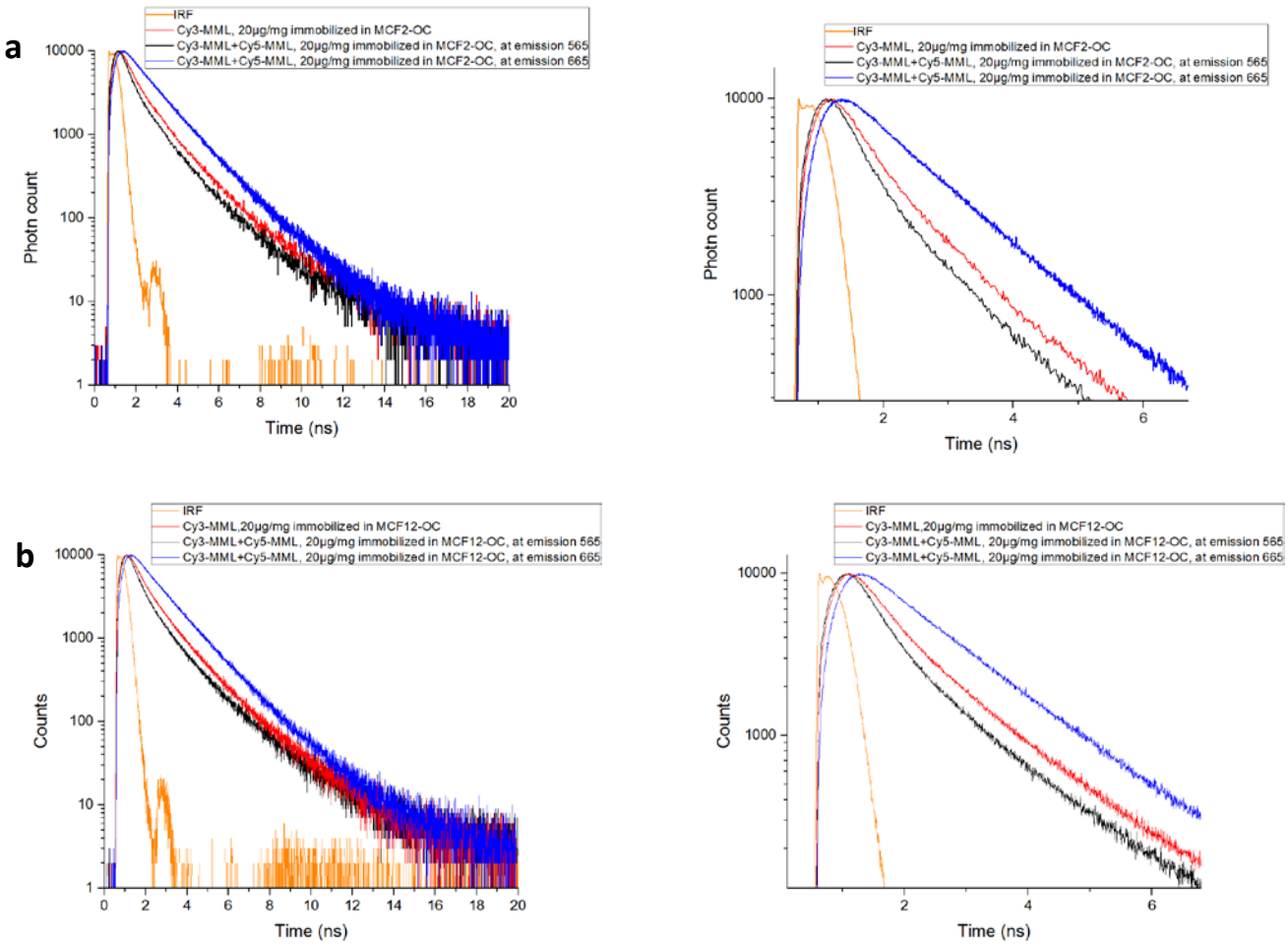


Figure S.4. Time-resolved fluorescence intensity measurements with MCF2-OC (a) and MCF12-OC (b). Time resolved fluorescence intensity decay of Cy3-MML in the absence and presence of Cy5-MML. The red curve shows the particles with only Cy3-MML immobilized in silica particles, black curves when Cy3-MML and Cy5-MML has been co-immobilized in the same particles; excitation at 483 nm and emission at 565 nm. The blue curves when Cy3-MML and Cy5-MML has been co-immobilized in the same particles, excitation at 483 nm and emission at 665 nm. The orange curve is the instrument response function (IRF), i.e. the decay of the excitation lamp pulse intensity. The decays were reproducible for three independent experiments. The right figures are the zoom-in view of the left figures in order to show the presence of FRET.

S.6. Time-resolved fluorescence anisotropy for free and immobilized labelled MML

Figure S.5 shows time-resolved fluorescence anisotropy curves *r*(t) for Cy3-MML, either in free solution (black curves) or immobilized in one of the particle types studied here (see figure legend). There is a minimum in *r*(t), and this kind of "dip-and-rise" behavior has been observed previously for dyes in the presence of dendrimers or silica nanoparticles.[10]

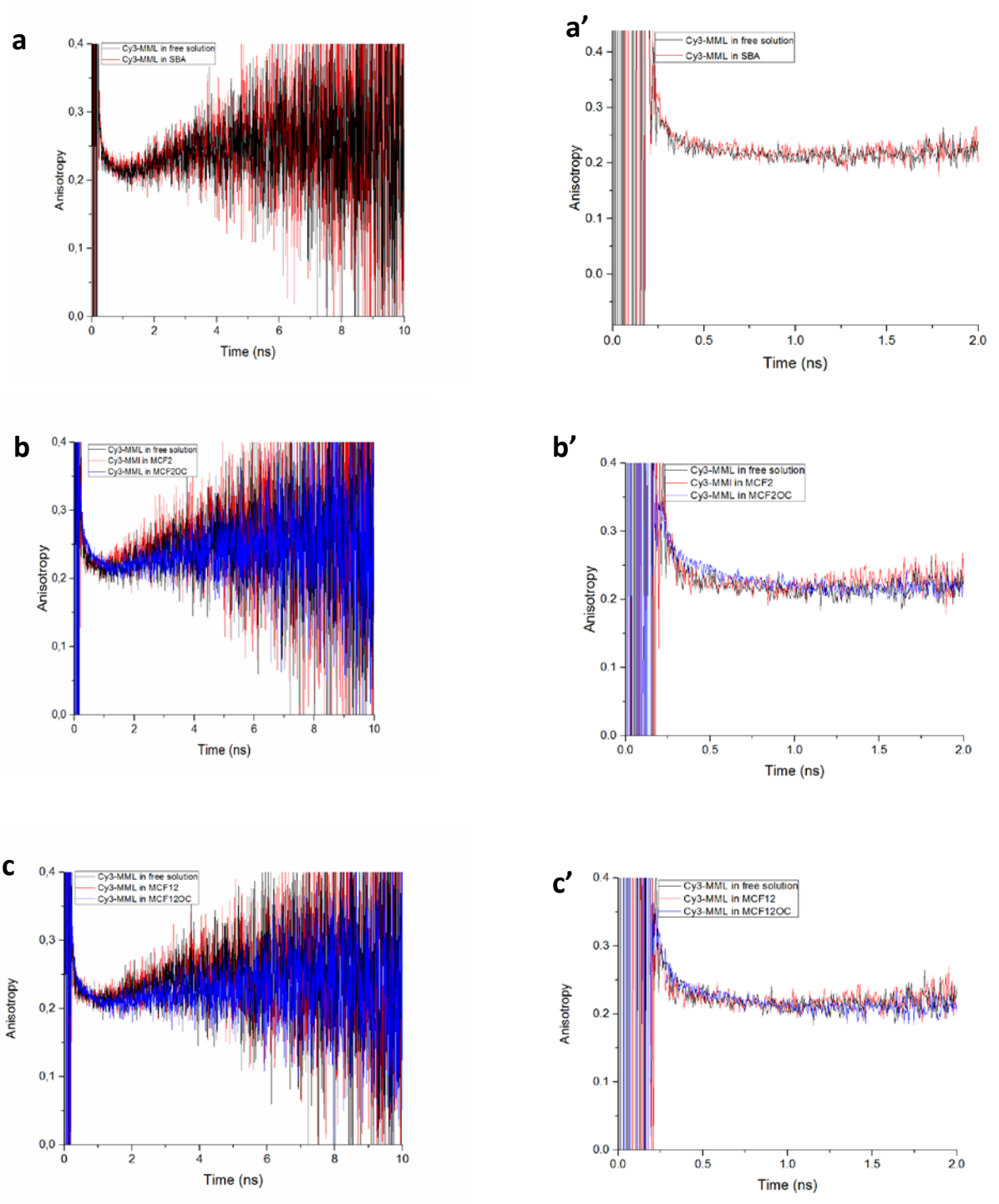


Figure S.5. Comparison of the fluorescence anisotropy of Cy3-MML in free soution (black), and in the pores in the five particle types used in this study. a) SBA-15 (red), a') zoom-in view of figure (a); b) MCF2 (red) and MCF2 OC (blue), b') zoom-in view of Figure b; c) MCF12 (red) and MCF12 OC (blue), c') zoom-in view of Figure c. The decays were reproducible for five independent measurements.

The most likely explanation is that the dye exists in two states, one free to rotate around the covalent linker (low anisotropy) and one state where the dye is associated with the protein also by intermolecular interactions yielding a higher anisotropy due to the slow rotation of the protein on the time scale of the excited state life time. If the associated dye state has longer life time than the non-associated state, the low-anisotropy contribution of the latter to $r(t)$ will diminish with time, and the r-value averaged over the two states will increase.

For the present study the important observation in Figure S.5 is that for all of the five particle types the anisotropy curve is indistinguishable from the case when the protein-dye complex is free outside the particle. The high similarity in the complex anisotropy response means that the rotational behavior of the MML-attached Cy3 is not affected by the immobilization, and that the calibration curve in free solution (Fig.5 in main text) also can be used for immobilized MML. Secondly, it can be concluded that the dyes do not bind to the silica pore wall. The anisotropy value of Cy3-MML is approximately 0.21±0.05 in free solution and different types of particles and compared to free Cy3 in free solution (approximately 0.0±0.05)[11], it can be concluded that the rotational behavior of Cy3 is changed after attaching to enzyme but not after immobilization.

S7. Emission spectra of Cy3-MML + Cy5-MML sample in MCF-particles

Figure S.6 shows representative steady state emission spectra of Cy3-MML+Cy5-MML immobilized in MCF2 (a), MCF12 (b), MCF2-OC (c) and MCF12-OC (d) particles (see Figure 6 in the main text for corresponding spectra with SBA-15 particles). It is seen that the emission peaks are somewhat red-shifted in the OC-modified particles compared to the same unmodified particles, and more so for Cy5 than Cy3. This observation shows that proteins labelled with

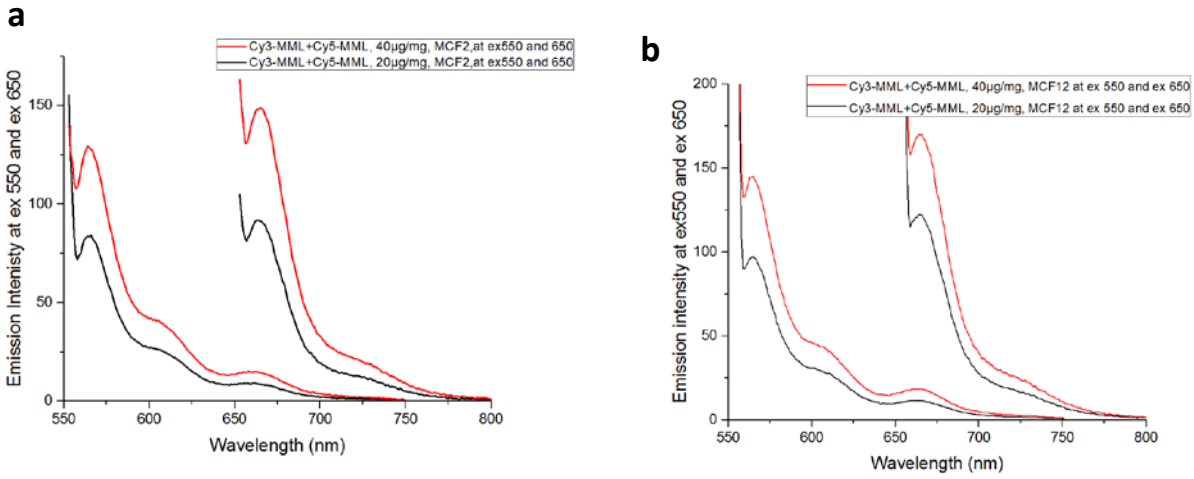
Cy3 and Cy5 can be used to monitor the polarity of the pore environment, even though at the moment there is no ratio method to evaluate the dielectric constant independently of viscosity changes.

The calculated intensity ratios (R) are given in Table 7 of main text for unmodified particles and in Table S.1 for the OC-modified particles but no effective viscosities are reported because of the presence of FRET.

Table S.1. Intensity ratio R and effective viscosity inside OC-modified mesoporous silica particles[a]

	MCF2-OC (24.8nm)[b]		MCF12-OC (32.7nm)[b]	
Added protein-conc.	R	Viscosity[c] (cP)	R	Viscosity[c] (cP)
20μg/mg	1.12	N.A.	1.05	N.A.
40μg/mg	1.30	N.A.	1.22	N.A.
60μg/mg	1.43	N.A.	1.35	N.A.

a. Intensity ratio and effective viscosity using the 1:1 Cy3-MML+Cy5-MML sample, uncertainty ±0.2 for three independent experiments
b. Pore diameter of OC-modified MCF particles
c. NA: Not applicable due to presence of FRET.



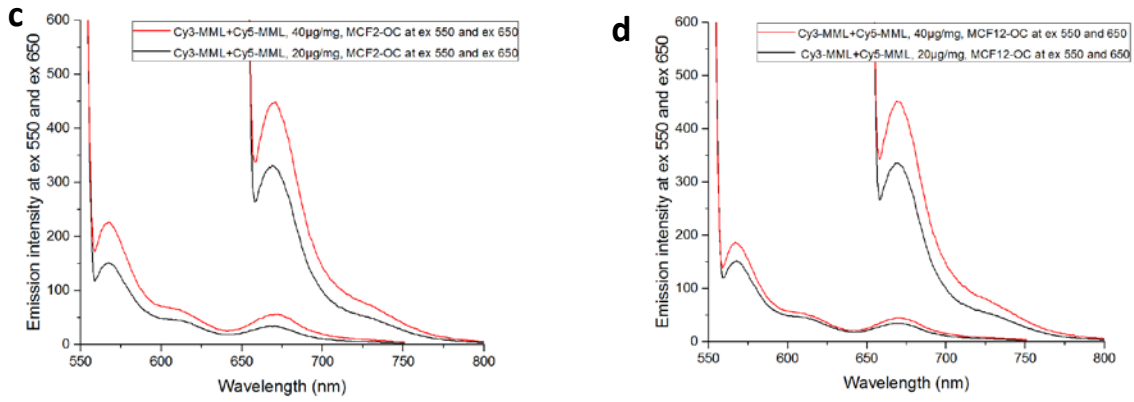


Figure S.6. Steady state emission spectra of the 1:1 mixture of Cy3-MML and Cy5-MML immobilized in MCF2 (a), MCF12 (b), MCF2-OC (c), MCF12-OC (d) at two different amounts of added protein (20µg (black) and 40 µg (red) total protein per mg of particles). Excitation wavelengths were 550 nm for Cy3 and at 650 nm for Cy5.

S.8. Lifetime of Cy3-MML in unmodified and OC-modified MCF

Table S.2 shows the fluorescence lifetimes of Cy3-MML immobilized in the different MCF particles of this study and fitting the intensity decays to mono-, bi- or tri-exponential functions was performed using Fluofit Pro v.4 software (PicoQuant GmbH). It is seen that the Cy3 lifetime values in the modified particles (MCF2-OC and MCF12-OC) are somewhat higher compared to the corresponding unmodified particles (MCF2 and MCF12, respectively). The effect is somewhat stronger in the MCF2-particles, but there is no clear trend with the amount of added protein. This observation suggests that the OC-layer increases the effective viscosity sensed by the Cy3-labeled MML, although not quantification is possible in the absence of a calibration curve.

Table S.2. Average of lifetime (ns) for donor samples (Cy3-MML) in different particles.[a]

Added protein	MCF2	MCF2-OC	r(MFC2)[b]	MCF12	MCF12-OC	r(MFC12)[b]
20µg/mg	0.71±0.2	0.87±0.2	1.23	0.76±0.2	0.81±0.2	1.07
40µg/mg	0.69±0.2	0.88±0.2	1.28	0.78±0.2	0.83±0.2	1.06
60µg/mg	0.71±0.2	0.90±0.2	1.27	0.78±0.2	0.83±0.2	1.06

a. Uncertainties correspond to 5 independent experiments
b. Ratio between OC-modified and non-modified particles

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