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[1] *A new delivery date was approved by the Project Officer due to the delay caused by COVID-19 outbreak.*

Overview/Abstract

A comparison of the interactions of polymeric micelles with biological media and cells. Using enzymatically cleavable micelles with three widely used hydrophilic shells (polyethylene glycol, poly(2-ethyl oxazoline) and polyacrylic acid) a spectral self-reporting mechanism allowed us to study how hydrophilic shells greatly affect the efficacy of micelles as nanocarriers.

Explanation for large delay in submitting deliverable

N/A

Led by

Name	Lorenzo Albertazzi	**Partner**	IBEC	**Date**	22/12/20
Name	Alis Olea (ESR7)	**Partner**	IBEC	**Date**	22/12/20

Reviewed by

Name	Roey Amir	**Partner**	TAU	**Date**	25/12/20
Name	Rosa Miralles	**Partner**	IBEC	**Date**	29/12/20

Document Control

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Description of the structure-activity relations of the material-cell interactions

1. Introduction

Enzymatically responsive polymeric micelles have great potential as drug delivery systems, allowing the encapsulation of hydrophobic drugs and their selective release at the tumor site. A great variety of hydrophobic cores and enzyme responsive moieties has been studied in recent years. However, few studies address the effect of the outer hydrophilic shell on the micelle properties and their interaction with cells or biological media. We compared the effects of three different hydrophilic shells, namely polyethylene glycol (PEG), poly(2-ethyl oxazoline) (PEtOx) and polyacrylic acid (PAA), on the properties of the micelle and their cellular interactions. For a comprehensive comparison, we used dendrons as hydrophobic blocks, with either 6 carbons ("Hex") or 9 carbons chains ("Non"), which formed the core of the micelles and have the advantage of high molecular precision. Furthermore, the amphiphiles contained a 7-(Diethylamino)coumarin-3-carboxylic acid (7-DEAC) dye, which shows an emission spectral shift between assembled (micelle) and disassembled state (unimer)[1].

2. Objectives

The aim of this deliverable was to observe the effect of specific nanomaterial characteristics onto their interactions with cells and stability in biological environment, with the purpose of modulating the delivery of nanocatalysts or prodrugs to the tumor site.

3. Results and discussion

3.1. Micelles stability in biological media

In order to assess the stability of different micelle formulations in a biologically relevant environment and their interactions with serum proteins, we incubated micelles with bovine serum albumin (BSA), fluorescently labeled with Cyanine 5 (Cy5). The fluorescence spectra under 405nm excitation revealed the assembly state of the amphiphiles as well as close interactions with BSA-Cy5, which serves as FRET receptor for micelle fluorescence (and much less for unimer's fluorescence) (Figure 1A, B).

The results show that increasing the hydrophobicity decreases interactions between unimers and BSA, indicating an increase in micellar stability (Figure 1C). PEtOx and PEG micelles showed overall a similar behavior, with slightly weaker interactions of PEtOx micelles with BSA indicated by a smaller peak of FRET signal. For both PEtOx and PEG hexyl micelles, there was moderate increase in the emission of unimers, suggesting a moderate destabilization from the BSA interaction (Figure 1C). Almost no changes were observed for PEtOx and PEG nonyl micelles, as there was no increase in the unimer peak. On the other hand, PAA-Hex showed complete disassembly due to interaction with BSA as indicated by the total disappearance of micelle fluorescence, as well as a substantial increase in unimer signal intensity (Figure 1C, D). This

increase might also be caused by a change in 7-DEAC microenvironment that can influence its quantum yield dramatically. PAA-Non also showed an increase in the unimer signal intensity, but micelle signal is still observed, and Cy5 FRET signal is the most intense of all the micelles, suggesting the strongest micelle-BSA interaction among the different polymers. To monitor the destabilization of the micelles over time, we calculated the unimer to micelle fluorescence ratio (data not shown). All the ratios were almost constant during 5 hours, except for PAA-Non, which showed an increase in the unimer/micelle ratio. Overall, this suggests that for all the micelles the interaction with BSA happens almost immediately after addition, leading to various types and degrees of interactions. While the more hydrophobic nonyl micelles generally interact less with BSA, the PAA-Non showed significantly stronger interaction of both micelles and unimers with the BSA.

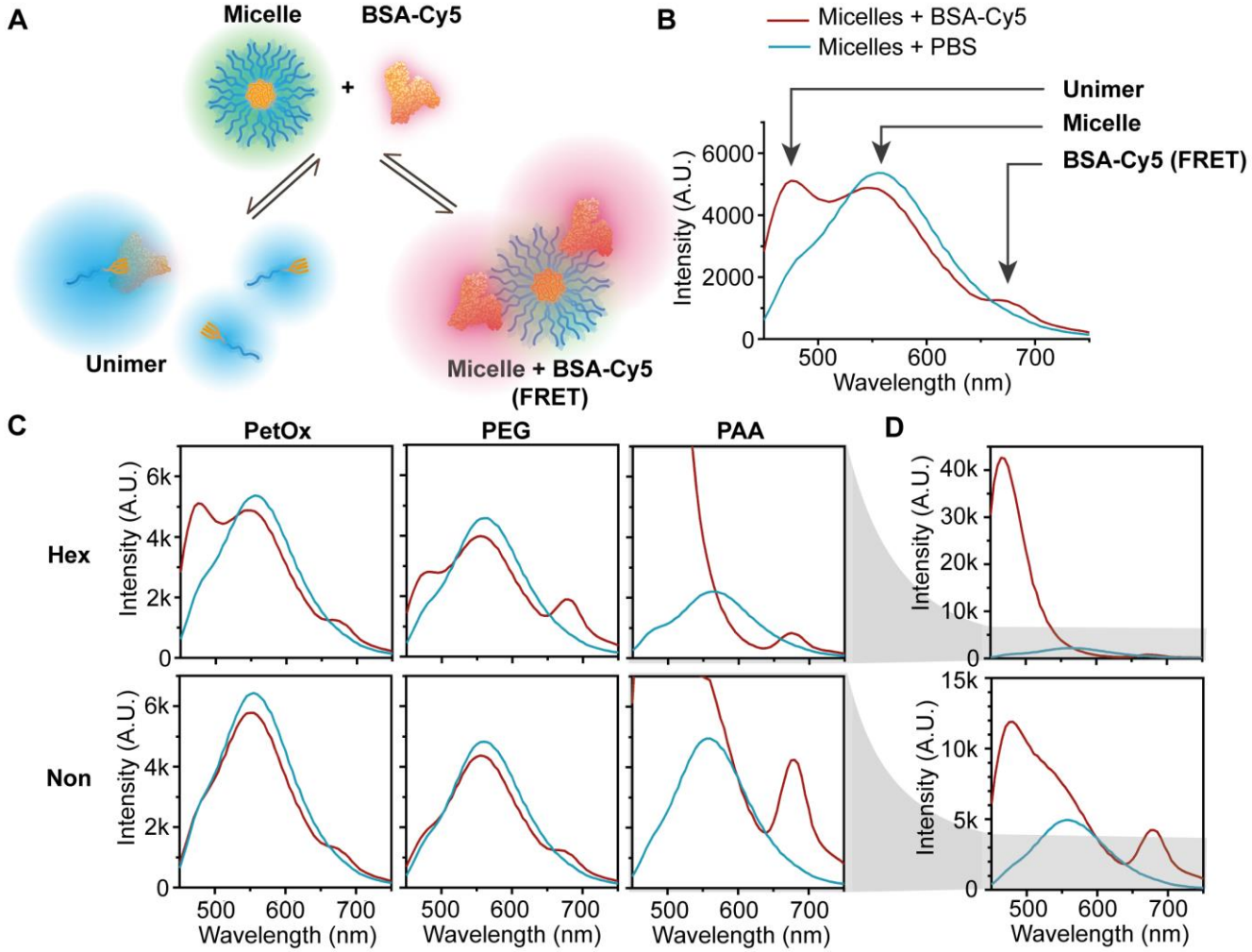

Figure 1 – (A) Illustration of the two main possible interaction pathways with Cy5 labeled BSA: interaction of BSA either with unimer (left) or micelle (right). (B) Selected fluorescence emission spectrum with arrows highlighting the contribution of the different species to the spectrum. (C) Fluorescence spectra of the micelles with (red lines) and without (blue lines) BSA-Cy5 and (D) Zoom out into PAA amphiphiles emission spectra. [amphiphile] = 160 µM, [BSA-Cy5] = 5.5 mg/ml, λ_{Ex} = 420 nm.[2]

3.2. Cell internalization

After assessing micellar stability in the presence of BSA, the next step was to follow micelles internalization into HeLa cells. Micelles were incubated with cells in full DMEM medium (with

10% fetal bovine serum) at a final micelle concentration of 160 μM and imaged with confocal microscopy.

Using 405 nm excitation, we were able to differentiate between the assembly states, by separating the signal into two distinct channels: one for unimer signal (400-500 nm) and one for the micelles signal (500-700 nm). We obtained total fluorescence and ratiometric images, by dividing unimer to micelle signal after background removal. Images with total fluorescence allowed a direct comparison of internalization efficiency of different amphiphiles as unimers and/or micelles, also showing the distribution inside different cellular compartments. Ratiometric images allowed the visualization of the assembly state of the amphiphiles in any given pixel, enabling a deep understanding of the behavior of the different micelles.

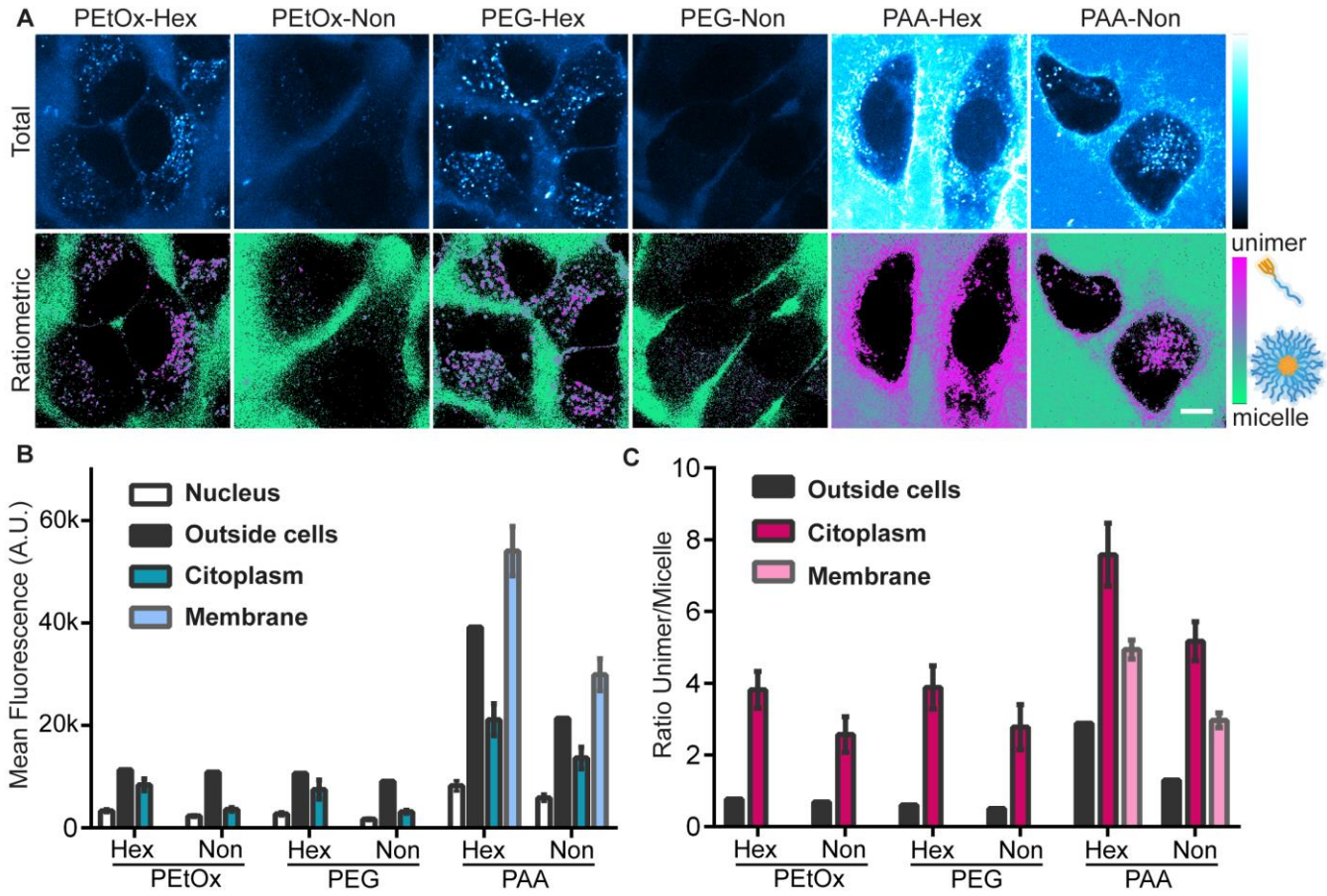

Figure 2 – Internalization of micelles into HeLa cells after 1 hour incubation in DMEM with 10% FBS. Images show total fluorescence signal with 405nm excitation (A, top row) or ratiometric images of unimer/ micelle pixel ratio after background removal (A, bottom row). Green color indicates micellar form and magenta the unimer form. Scale bar is 10 μm. The median fluorescence for control areas (inside nucleus as negative control and in solution outside cells as positive control) were plotted along with the mean fluorescence inside the cytoplasm or in the membrane area (n=8-10 cells) for either total fluorescence (B) or unimer/micelle ratio (C).[2]

Looking at the total intensity inside cells, we observed a remarkable difference in the degree of internalization (Figures 2A top row and 2B). The micelles of PEtOx-Non and PEG-Non, that were shown to be more stable in BSA, showed a very weak signal inside cells. PEtOx-Hex and PEG-Hex had similar distributions in intracellular vesicles, while PAA-Hex and PAA-Non bound mostly to

the cell membrane and had the most intense fluorescence emissions, similar to the trend observed for the incubation with BSA. Thus, we can assume that the hydrophilic block directs the cellular fate of micelles towards the endolysosomal compartment for PEtOx and PEG, and membrane-bound for PAA. Interestingly, the relatively small change in hydrophobicity causes a notable decrease in internalization efficiency for the nonyl micelles. To assess if this difference is due to disassembly of the micelles outside or inside the cells, we analyzed the ratiometric images (Figure 2A bottom row). For all amphiphiles except PAA-Hex, the ratiometric analysis indicated the presence of micelles outside the cells, while inside cells or on the cell membrane, all amphiphiles were mostly in their unimer form (Figure 2C). However, slightly more micelles were observed inside the cells for the more stable PEtOx-Non and PEG-Non (Figures 5B and 5C). We can assume that the less stable PAA micelles disassembled in the solution more readily into unimers that could intercalate into the plasma membrane. For the polymers that were localized in endosomal vesicles, it may be that they internalized as micelles and very rapidly disassembled inside the vesicles into unimers.

3.3. Cargo release mechanism

Perhaps even more important than micelle internalization inside cells is their capacity to release a molecular cargo in the presence of cells. Two possible mechanisms for the release of physically encapsulated cargo from polymeric micelles can be envisioned: (i) spontaneous leakage or (ii) disassembly and release. Spontaneous leakage would leave the micelles intact, while the cargo would exit and accumulate inside cells. On the other hand, in disassembly-based release the micelles would break outside the cells and polymers would get internalized together with the released cargo. Based on the inferior stability noted in all previous experiments for the PAA micelles, we expected that the release of encapsulated cargo will be significantly faster as great percentage of PAA micelles disassembled in the presence of HeLa cell cultures.

In order to observe how their hydrophilic shells affect their cargo release kinetics, we chose the more stable nonyl amphiphiles. A hydrophobic Cy5 derivative served as a model for a physically encapsulated lipophilic drug, which would accumulate spontaneously inside cells upon release from the micelles. The three types of micelles were loaded with Cy5 and incubated with HeLa cells. In a confocal microscope, a group a cells was imaged during 1 hour of micelle incubation, measuring the fluorescence of the unimer and micelle (excitation at 405 nm) as well as directly following the Cy5 (excited at 640 nm, Figure 3A). For every time point, the fluorescence was quantified inside the cytoplasm of 10 cells and the mean values were compared to the signal outside cells (Figure 4B, C). Regarding the assembly state of the micelles, as shown in the unimer/micelle ratio (Figure 3C), PAA-Non micelles showed significant disassembly over time, both outside and inside cells, which was in line with previous experiments. As expected, PEtOx-Non and PEG-Non were much more stable and almost no disassembly was noted, indicated by almost constant unimer/micelle fluorescence ratio. Therefore, looking at the accumulation of Cy5 into cells, it was surprising to observe that the release and internalization of Cy5 was slower for PEtOx-Non and PEG-Non micelles than for PAA-Non (Figure 3A, B). While both PEtOx-Non and PEG-Non samples showed steep increase in Cy5 signal inside the cells, PAA-Non showed delayed

and more gradual increase, even though its micelles tendency to disassemble is much higher. These results demonstrate that lower micellar stability does not always correlate with faster release kinetics, and that disassembly of the carrier is not essential for cargo release.

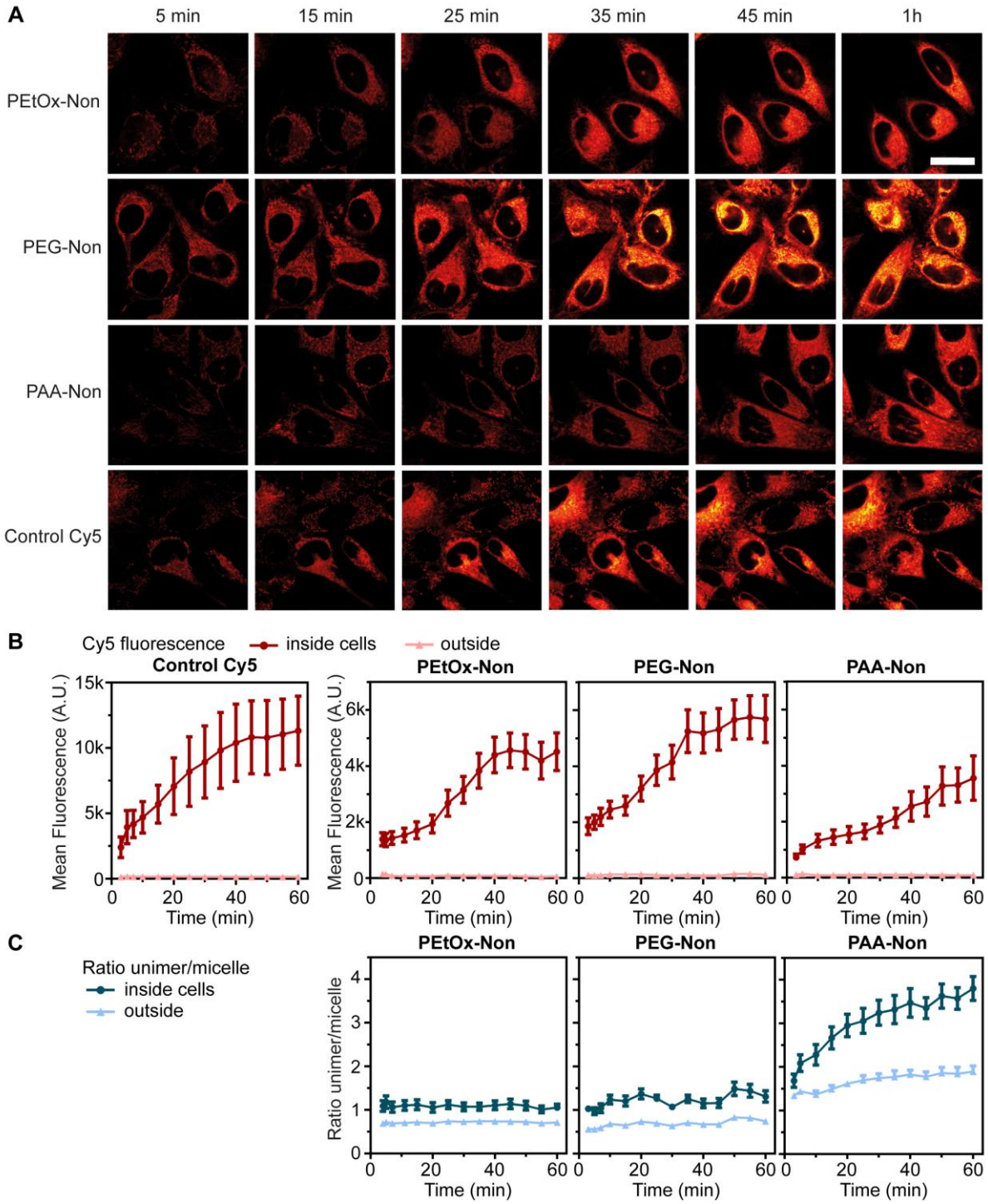

Figure 3 – Release of encapsulated Cy5 onto HeLa cells over time. (A) Fluorescence with 640 nm excitation is shown inside HeLa cells at different time points for hydrophobic Cy5 encapsulated in micelles or free, in full DMEM (10% FBS), scale bar is 20 μm. The quantification of fluorescence from confocal images (C) as mean fluorescence intensity in the Cy5 channel, with 640 nm excitation, or (B) as ratio of unimer/micelle signal with 405 nm excitation. Regions of interest were manually drawn around cell cytoplasm, including the cell membrane and excluding the nucleus; n=10 cells. [amphiphile] = 160 μM, [Cy5] = 4 μM.[2]

4. Conclusions

In summary, we compared the effects of three different hydrophilic shells (PEtOx, PEG and PAA) on the cellular interactions of enzymatically cleavable polymeric micelles, taking into account also the hydrophobicity of the micellar core. We observed that micelles with PEtOx or PEG shells were more stable in the presence of BSA than PAA micelles. Furthermore, the hydrophilic shell was indicative of the cellular fate of the micelles in HeLa cells experiments, in which PEtOx had a similar distribution with PEG micelles inside endocytic vesicles, while PAA amphiphiles were mostly membrane-bound. Then, we used a physically encapsulated hydrophobic Cy5 derivative for testing the cargo release mechanism. In this case, PEtOx and PEG micelles showed a faster cargo release than PAA, suggesting that leakage of cargo from intact micelles can be a faster cargo-release mechanism (for PEtOx and PEG) than micelle disassembly (for PAA). Overall, these experiments helped us understand better the complexity behind the choice of hydrophilic shell for micelle-based nanoassemblies and can serve as a starting point for more advanced nanocatalyst delivery studies.

5. References

(1) Feiner-Gracia, N.; Buzhor, M.; Fuentes, E.; Pujals, S.; Amir, R. J.; Albertazzi, L. Micellar Stability in Biological Media Dictates Internalization in Living Cells. *J. Am. Chem. Soc.* **2017**, *139* (46), 16677–16687. https://doi.org/10.1021/jacs.7b08351.

(2) Slor, G.; Olea, A.; Pujals, S.; Tigrine, A.; De la Rosa, V.; Hoogenboom, R.; Albertazzi, L.; Amir, R. Judging enzyme responsive micelles by their covers: direct comparison of dendritic amphiphiles with different hydrophilic blocks. *Biomacromolecules.* Manuscript under revision.