In Vitro Investigations of Smart Drug Delivery Systems Based on Redox-Sensitive Cross-Linked Micelles

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Redox-sensitive micelles are designed by using block copolymers of different architectures composed of a hydrophilic block of poly(ethylene oxide), and hydrophobic blocks of poly(e-caprolactone) and poly(α-azide-ε-caprolactone). Stability of these micelles is insured in diluted media by cross-linking their core via the addition of a bifunctional cross-linker, while redox sensitivity is provided to these micelles by inserting a disulfide bridge in the cross-linker. The potential of these responsive micelles to be used as nanocarriers is studied in terms of cytotoxicity and cellular internalization. The release profiles are also investigated by varying the environment reductive strength.

1. Introduction

These last years, nanoparticles, liposomes, and polymer micelles have been largely investigated as drug nanocarriers.[1–4] The main common way of administration of these drug delivery systems is the direct systemic injection, which already bypasses most of the natural barriers. Nevertheless, prolonged circulation time of nanocarriers is required to reach passive targeting of tumor tissues, that is, the spontaneous accumulation of the nanoparticles in tumors by the enhanced permeability and retention (EPR) effect.[5,6] Indeed, nanocarriers are generally rapidly recognized by the mononuclear phagocytic system (MPS) which removes foreign bodies of the bloodstream to direct them to the liver or the spleen.[7,8] The coating of the nanocarriers by poly(ethylene oxide) (PEO) prevents their too fast recognition by the macrophages and insures the stealthiness of the nanocarriers, due to its hydrophilic nature but also by its rapid conformational changes.[9,10] Moreover, the control of the nanocarrier size is essential to reach long circulating particles. Too small nanoparticles are rapidly removed from the body via the renal system while nanoparticles with larger radius than 200 nm are less stealthy and are rapidly directed to the liver.

Besides passive targeting, a more active targeting of the nanocarriers can be achieved by grafting targeting moieties exposed at the nanocarrier surface and able to recognize specific receptors over-expressed by cancer cells. This would fasten the internalization which is of prime importance since most of the drugs used to treat cancers act intracellularly.[11] However, the presence of a ligand at the surface of the nanocarriers is detrimental to the
The development of more complex and adaptive systems is thus today investigated, for example, systems with a controlled exposition of the targeting units only in the surrounding of tumor cells. This allows for stealthiness.

The stability of the drug loaded nanocarriers is also a key point to avoid prematured release of the drug or Burst effect upon injection. For example, in case of micelles, when they are submitted to high dilutions, their destabilization might occur by crossing the critical micellar concentration (CMC) of the copolymers which causes the drug release before reaching the tumor site. In order to insure the nanocarrier stability against dilution and strongly entrapment of the drug inside, polymer micelles systems are usually cross-linked. Nevertheless, after internalization by tumor cells, the drug needs to be released to perform its intracellular action. Thus, the development of reversibly cross-linked micelles was investigated over the recent years. In this context, it was already shown that by using redox sensitive disulfide bridges to cross-link micelles, the stability of such nanocarrier is assured under a weakly reductive environment such as the bloodstream. In the cytoplasm, the higher level of glutathione (1000-fold more concentrated than in the bloodstream), a tripeptide containing glutamic acid, cysteine, and glycine, the reduction of the disulfide bridges occurs hence the encapsulated drug is intracellularly delivered.

In this framework, some of us have already investigated redox-sensitive core cross-linked micelles built from the three block copolymers, that is, PEO-b-poly(CL-co-αN3CL) (1), PEO-b-PCL-b-poly(αN3CL) (2), and PEO-b-poly(αN3CL)-b-PCL (3) (Scheme 1) that were cross-linked by a bis-alkyne containing a disulfide bridge as described in the Scheme 2. While the stealthiness of all these nanocarriers thanks to the hydrophilic PEO shell was previously demonstrated by CH50 test, their in vitro cytotoxicity and internalization properties are the purpose of the present paper. The interest of using three amphiphilic copolymers of the same composition but with different architectures (Scheme 1) lies in the possible difference in cross-links distribution within the micellar core, with potentially an effect on the drug release profiles. These release profiles are thus also investigated here by means of a hydrophobic dye, chosen as a model for a drug, in relation of the environment reductive strength and micelles structure.

2. Experimental Section

2.1. Materials

5-(and 6)-carboxyFluorescein succinimidyl ester (NHS-Fluorescein) was purchased from Thermo Scientific. 1,4-Dithiothreitol (DTT), Nile Red and a solution of 0.5 M ethylenediaminetetraacetic (EDTA) (pH 7) were purchased from Sigma–Aldrich. Dimethylformamide (DMF) was dried on molecular sieves. Deionized water was obtained from a Milli-Q plus system (Millipore). All other chemicals were used as received.

2.2. Copolymer Synthesis

The block copolymers of PEO-b-poly(CL-co-αN3CL), PEO-b-PCL-b-poly(αN3CL), and PEO-b-poly(αN3CL)-b-PCL were synthesized by ring-opening polymerization (ROP). Details of the copolymer synthesis were already reported elsewhere. Briefly, ROP of ε-caprolactone (εCL) and α-chloro-ε-caprolactone (αCL) was...
initiated from monomethoxy PEO macroinitiator in refluxing toluene for 48 h in presence of a tin-based catalyst. For the random block copolymer, a mixture of the two lactones was copolymerized while a sequential monomer addition was followed for the synthesis of the two triblock architectures. The substitution of the chloride atoms by azide functions was carried out in presence of sodium azide in DMF for one night.

The molecular weight of the copolymers was calculated by $^1$H NMR spectroscopy while polydispersity index ($\overline{M}_w$/\overline{M}_n) was determined by size exclusion chromatography (Table 1).

### 2.3. Copolymer Labeling

0.3 g of PEO-b-poly(CL-co-αN3CL)-OH ($\overline{M}_n$ = 7100 g mol$^{-1}$, 4.2 $\times$ 10$^{-5}$ mol) (or PEO-b-poly(αN3CL)-OH or PEO-b-poly(αN3CL)-b-PCL-OH) and 0.1 g NHS-Fluorescein (2.1 $\times$ 10$^{-4}$ mol) were dissolved in 4 ml of anhydrous DMF and stirred for 48 h at room temperature. The final product, PEO-b-poly(CL-co-αN3CL)-Fluorescein were dialyzed against 50/50 water/DMF until a colorless dialysis medium was obtained and finally against water before recovery by lyophilization. After this process, the yield of labeling was estimated to be between 5 and 10 mol-% of the chains.

### 2.4. Micelle Preparation and Cross-linking

Aqueous dispersions of micelles were prepared by addition of water to the copolymer solution in an organic solvent (DMF) chosen as a good solvent for all the hydrophilic and hydrophobic blocks. Their cross-linking was simultaneously achieved by copper alkyne-azide cycloaddition (CuAAC) of the pendant azide functions of the hydrophobic micelle core with 2-(2-pent-4-ynoyloxyethyl)dithiobenzyl(ethyl pent4-ynoate, i.e., a bis-alkyne cross-linker containing a disulfide bridge, following an already described process.[23]

Briefly, a 1% stock solution of the copolymer was prepared in DMF in presence of the cross-linker (0.6 mol equiv. vs. azide groups). 10 mL of Milli-Q water were added to 2.5 mL of this organic solution under vigorous stirring for 1 d. CuSO$_4$ (0.25 mol equiv. vs. azide groups) and ascorbic acid sodium salt (0.25 mol equiv. vs. azide groups) were added to the mixture. The cross-linking reaction was allowed to proceed for 1 d at room temperature. 2 mol equiv. versus Cu of solution of EDTA pH 7 was added to the solution to complex the copper. Then, the core cross-linked copolymer micelles were purified by dialysis overnight against 1 L of water using cellulose dialysis membrane (Spectrapor, cut-off 3500).

In order to prepare fluorescent micelles, a mixed solution of Fluorescein-labeled and unlabeled copolymers (50/50) were used in the procedure described above.

In order to get dye loaded micelles, 2.5 mg of Nile Red were also added to the initial copolymer solution in DMF before following the same procedure of micellization and cross-linking.

The size distribution of the micelles was estimated based on the CONTIN method.

### Table 1. Main characteristics of the copolymers used in this study and of the corresponding cross-linked micelles.

<table>
<thead>
<tr>
<th>Copolymer architecture</th>
<th>$\overline{M}_n$, total [g mol$^{-1}$]$^a$</th>
<th>$\overline{M}_w$/\overline{M}_n$^b$</th>
<th>$D_h$ [nm]$^c$</th>
<th>PDI$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEO$<em>{114}$-b-poly(CL$</em>{10}$-co-αN$_3$CL)$_7$ (1)</td>
<td>7 100</td>
<td>1.08</td>
<td>37 (32)$^e$</td>
<td>0.07</td>
</tr>
<tr>
<td>PEO$<em>{114}$-b-PCL$</em>{11}$-b-poly(αN$_3$CL)$_6$ (2)</td>
<td>7 200</td>
<td>1.08</td>
<td>43 (29)$^e$</td>
<td>0.16</td>
</tr>
<tr>
<td>PEO$_{114}$-b-poly(αN$_3$CL)$<em>6$-b-PCL$</em>{10}$ (3)</td>
<td>6 900</td>
<td>1.08</td>
<td>36 (33)$^e$</td>
<td>0.11</td>
</tr>
</tbody>
</table>

$^a$Experimental molar mass determined by $^1$H NMR; $^b$Polydispersity index measured in THF by size exclusion chromatography (polystyrene calibration); $^c$Apparent hydrodynamic mean diameter of the micelles determined by DLS in water; $^d$Polydispersity index measured by DLS; $^e$In bracket is given the $D_h$ value for the micelles before cross-linking (bearing azide).
2.5. Cell Culture

The human melanoma line MEL-5 was obtained from De Giovani (University of Liege, Belgium). MEL-5 cells were grown at 37 °C under humidified air containing 5% CO₂ in Dulbecco modified Eagle medium (DMEM) with glucose containing 5% vol of foetal bovine serum (FBS), 1% vol GlutaMax, 1% vol HEPES, 1% vol of penicillin/streptomycin [10 000 units of penicillin (base) and 10 000 units of streptomycin (base)/mL using penicillin G (sodium salt) and streptomycin sulfate in 0.85% saline].

The murine melanoma line B16 was obtained from ATCC. B16 cells were grown at 37 °C under humidified air containing 5% of CO₂ in minimum essential medium (MEM) Alpha medium with 10% vol FBS, 1% vol GlutaMax, 1% vol penicillin/streptomycin.

2.6. Micelle Cytotoxicity

The cytotoxicity of the micelles was evaluated by determining the viability of B16 and MEL-5 cells after incubation with different concentrations of micelles (from 0.5 to 2 mg mL⁻¹) for 24 and 48 h. The number of viable cells was determined by estimation of their dehydrogenase activity using the tetrazolium-based colorimetric method (MTT conversion test). B16 and MEL-5 cells were seeded in 96-well plates at the density of 6 000 viable cells per well and incubated 24 h to allow cell attachment. At the end of incubation period with micelles, cells were incubated with 10 μL of an MTT solution (5 mg mL⁻¹) for at least 1 h at 37 °C until crystal formation. After rinsing, 200 μL of DMSO were then added in order to dissolve the formazan crystals. The absorbance of the solubilized formazan crystals was measured spectrophotometrically at 580 nm. Cell viability was expressed as the ratio between the amount of formazan determined for cells treated with the micelles and for control non-treated cells. The optical density values were measured using Powerwave X multiwell-scanning spectrophotometer.

2.7. Internalization Studies by Flow Cytometry

Nile Red loaded cross-linked micelles were diluted in culture media (final concentrations: from 1 to 0.25 mg mL⁻¹) and were incubated with MEL-5 cells for 1 and 4 h. After incubation, removal of attached micelles was accomplished by washing cells two times with culture medium and two times with phosphate buffered saline solution (PBS without Ca⁺⁺ and Mg⁺⁺). Cells were then detached by trypsinization (trypsin in PBS). After centrifugation the pellet was suspended in PBS. Analyses of the internalized micelles were then performed using a fluorescence spectrometer Perkin Elmer VICTOR3 1420.

2.8. Internalization Studies by Fluorescence Microscopy

MEL-5 and B16 cells were seeded in a 12-well plate with 2 mL of culture medium. After 48 h, medium was replaced by Fluorescein-labeled micelles loaded with Nile Red at a concentration of 0.5 mg mL⁻¹. After 0.5 h of incubation, removal of attached micelles was accomplished by washing the cells with culture medium and twice with PBS. Cells were then fixed and nuclei stained with 4% formaldehyde/Hoechst (10 μg) in PBS solution for 15 min at 4 °C in the dark. Finally, after aspiration, 0.5 mL of formaldehyde solution in PBS were added. Analyses of nuclei and internalized micelles were performed using fluorescent microscope Olympus IX81.

2.9. Internalization Studies by Fluorescence Spectrometry

Fluorescein-labeled cross-linked micelles loaded with Nile Red were diluted in culture media (final concentration: 0.5 mg mL⁻¹) and were incubated with MEL-5 cells for 0.5 h. After incubation, removal of attached micelles was accomplished by washing cells two times with culture medium and two times with PBS (without Ca⁺⁺ and Mg⁺⁺). Cells were then detached by trypsinization (trypsin in PBS). After centrifugation the pellet was suspended in PBS. Analyses of the internalized micelles were then performed using a fluorescence spectrometer Perkin Elmer VICTOR3 1420.

2.10. Statistical Analyses

Cell culture experiments were performed in triplicates. Results are presented as mean ± standard deviation. Statistical analyses of the data were performed using the unpaired, two-tailed, Student’s t-test. Statistical significance was determined at a p < 0.01.

3. Results and Discussion

3.1. Copolymer Synthesis and Core Cross-linked Micelle Formation

In order to insure the stability of the PEO_{14-b-PCL_{17}} spherical micelles deeply studied and well-suited for drug delivery systems, the cross-linking of the PCL core was targeted by introducing some reactive azide groups along the hydrophobic segment, while keeping unchanged the
hydrophilic/lipophilic balance. By the living ring-opening copolymerization of \(\varepsilon\)CL and \(\alpha\)ClCL from a PEO macro-initiator, followed by the substitution of chloro atoms by azide functions during the micellization process. Depending on the location of azide functions in the hydrophobic block, the cross-linking distribution within the hydrophobic core might be different having may be an impact on the drug release profile. The copolymer synthesis and cross-linking were previously reported in details.\[20,21\] The Table 1 summarizes the main characteristics of the copolymers used in the present study together with the size and size distribution of their corresponding cross-linked micelles that includes a disulfide bridge in order to provide redox sensitive cross-linking. A quantitative cross-linking was obtained for the three different copolymers. As already mentioned in a previous paper,\[21\] the cross-linking of the micelles slightly increases the micelles size, leading to cross-linked micelles of similar sizes for copolymers 1 and 3 with a diameter of about 37 nm while cross-linked micelles of copolymer 2 are slightly bigger with a size of 43 nm. Moreover, these last micelles were not as well defined as micelles of copolymers 1 and 3. The location of the azide group as an end-block in copolymer 2 rather than more central or distributed along the hydrophobic block might thus affect more the initial micellar structure than with the two other copolymers. After purification by dialysis, these cross-linked spherical micelles as was evidenced by transmission electron microscopy images (as already shown elsewhere)\[23\] were then used for the following in vitro studies.

### 3.2. Cytotoxicity

To evaluate the in vitro cytotoxicity of the cross-linked micelles, MTT test was performed using B16 and MEL-5 melanoma cells. Figure 1A and B show the cell viability of B16 cells treated with 0.5–2 mg mL\(^{-1}\) of the three kinds of micelles after 24 and 48 h, and the same analyses on Figure 2A and B for MEL-5 cells. All these micelles showed similar in vitro cytotoxicity. A concentration for the micelles of 1 mg mL\(^{-1}\) leads to a percentage of survival of 40–60% after 24 h while the ideal concentration of 0.5 mg mL\(^{-1}\) leads to very high level of survival of 80–100%. After 48 h, the percentage of survival falls to about 30% for the concentration of 1 mg mL\(^{-1}\) while a diminution of cell viability (60–80%) was recorded for the concentration of 0.5 mg mL\(^{-1}\). These cytotoxicity results are comparable to other polymer micelles of similar size benefiting of a PEO corona.\[25\] In accordance to literature data,\[26\] the core cross-linked micelles benefit from a still flexible corona of PEO which keeps low their cytotoxicity.\[27\] From these first data, no cytotoxicity really results from the core cross-linking process whatever the considered micellar system and for both kinds of studied cells lines.

### 3.3. Micelle Uptake

The in vitro micelle uptake was evaluated on B16 and MEL-5 cells after different concentrations and incubation times. Firstly, in order to quantify by flow cytometry the cell uptake, the cross-linked micelles have been loaded before cross-linking with Nile Red, a fluorescent dye mimicking a poorly water-soluble drug, easily detected by the FACS. Flow cytometry study was performed after 1 and 4 h of incubation of Nile Red loaded micelles at concentration of...
1 and 0.5 mg mL\(^{-1}\) on MEL-5 cells in order to quantify the uptake. Data obtained after 1 h of incubation for the lower concentration of 0.5 mg mL\(^{-1}\) already confirmed the rapid internalization of the dye loaded micelles despite the absence of targeting moieties at the surface of the micelles. Indeed, 100% of cells were positive to the Nile Red according to flow cytometry data whatever the type of cross-linked micelles used. The same results were obtained for a lower concentration of 0.25 mg mL\(^{-1}\) and for a reduced incubation time of 0.5 h (Figure 3).

Nevertheless, if the flow cytometry allows a quantitative determination of the fluorescent cells, it does not clearly discern whether the dye is still in the micelles or not and if it is externally adsorbed at the cell surface or actually internalized. In order to clarify the situation, some Fluorescein-labeled copolymers were used to form the micelles loaded with Nile Red and cross-linked. Thus, having one encapsulated red dye and one green dye covalently attached to the copolymer micelles would afford information of the location of both the micelles and the encapsulated dye, independently. Fluorescein-labeled cross-linked micelles loaded with Nile Red were thus incubated with B16 and MEL-5 cells at a concentration of 0.5 mg mL\(^{-1}\) for 0.5 h. Then, the cell nuclei were stained with Hoechst dye after fixation. Fluorescence microscopy observations of the fixed cells clearly show the successful internalization of the cross-linked micelles after short incubation times (Figures 4, 5). The red image is the signature of Nile Red, while the green image is the one of the micelles labeled by the Fluorescein. The blue image shows the cells nuclei. Figure 4 compares the internalization of the three different kinds of copolymer micelles into B16 cells. Uniform distribution of the Nile Red was observed into the cytoplasm of the cells but not inside the nuclei. This was also observed for the green micelles except that the nuclei of the cells were also marked by the Fluorescein especially with the copolymer 3 where dense structures (nucleoli) in the nuclei are visible (Figure 4, 3-C). Micelles of copolymer 2 lead to a high green and red fluorescence homogeneous in the cytoplasm and strong ring around nuclei. The presence of the Fluorescein in the cells allows confirming the internalization of the Nile Red loaded micelles and not only of free Nile Red previously released from the micelles. The fluorescence
The intensity of the Fluorescein needed to be increased comparing to the Hoechst and Nile Red contributions in Figures 4, 5, due to the low content of Fluorescein on the polymer chains and the low concentration in the micelles.

The same experiments performed on MEL-5 cells with the three kinds of core cross-linked micelles have led to similar conclusions. Results obtained with the cross-linked micelles of copolymer 3 on MEL-5 cell are illustrated in Figure 5 and allowed to highlight the difference of location of the Nile Red dye and the Fluorescein-labeled copolymer. Indeed, the Nile Red is only localized in the cytoplasm of the cell (Figure 5, 3-A) while Fluorescein is also localized in the nuclei (Figure 5, 3-B). That difference is mainly underlined on Figure 5, 3-D, where the green and red filters are combined and compared to Figure 5, 3-C. The substitution of the blue coloration by the green one confirms the presence of the copolymer in the cell nucleus. One possible explanation for this observation is that after the uptake of the loaded micelles by the cells, the reductive environment of the cytoplasm allows the reduction of the disulfide bridges which cross-linked the micelle core. As a consequence, the encapsulated Nile Red dye is released by destabilization of the micelles and freely diffuses in the cytoplasm. This hypothesis is supported by the uniform distribution of the Nile Red in the cytoplasm and the location of the green copolymer in the nucleus independently of the Nile Red, which tend to confirm the micelles dissociation. The phenomenon appears mainly with micelles of copolymer 3, that is, with the cross-linking closest to the hydrophilic block and thus probably the most sensitive to the reductive environment needed for the rupture of the disulfide cross-links.

Finally, the micelles uptake by MEL-5 cells was quantified by fluorescence spectrometry. The percentage of internalization was calculated from the fluorescence intensity of the cells after incubation in presence of Fluorescein-labeled cross-linked micelles loaded with Nile Red. Micelle uptake typical for micelles exhibiting a PEO shell at their external periphery has been measured for the three systems, as reported in Figure 6 that shows the percentage of internalization after 0.5 h of incubation at a concentration of 0.5 mg mL$^{-1}$. Similar values were obtained for the three kinds of copolymer micelles. This shows that they have all similar propensities to enter the cells, which is not surprising since they are all exhibiting the same PEO shell at their external periphery. Interestingly enough, different behaviors have been observed when the same experiment is performed on the Nile Red quantification. In that case, a significantly higher Nile Red internalization is measured on the micelles of copolymer 2. That observation could be explained by a less efficient encapsulation of Nile Red in the cross-linked micelles of copolymer 2 that are less efficiently preventing the red dye to diffuse out of the cell. 

In Vitro Investigations of Smart Drug Delivery Systems Based...
micelle core and hence leading to some release of the Nile Red before the micelle internalization. This prematurely released Nile Red would thus be internalized independently of the micelles which would explain the higher Nile Red dye content as compared to the fluorescein-labeled micelles (Figure 6). Micelle internalization was also investigated after 1 h of incubation and with a higher micelle concentration of 1 mg mL\(^{-1}\). Increasing the incubation time keeps constant the percentage of internalization while increasing of the micellar concentration even decreases the percentage of internalization because similar amount of micelles are internalized for a higher concentration at the same incubation time. Internalization appears thus kinetically limited by the PEO external shell which would be improved by decorating with a specific ligand\(^{[29]}\) or with positive charges.\(^{[30]}\)

### 3.4. Redox-Dependent Release of Nile Red from Cross-Linked Micelles

Redox-dependent release profile of the core cross-linked micelles was investigated using Nile Red as fluorescent probe, mimicking a hydrophobic drug. The Nile Red was encapsulated in the micelles by incubation with the micelles in DMF before the cross-linking step. The Nile Red loaded and cross-linked micelles were incubated in phosphate buffer at two different concentrations and in presence or not of dithiothreitol (DTT), a reducing agent for the disulfide bridges. The release of the dye was quantified versus time by fluorescence spectrometry. Results are shown in Figures 7, 8.

The Figure 7A compares the Nile Red release profiles of the three cross-linked micellar systems at high concentration (1 mg mL\(^{-1}\)) against PBS buffer. At first look, the release profiles were quite similar for the three kinds of cross-linked micelle systems. A faster release of the Nile Red is however observed for the micelles of cross-linked copolymer 2 as compared to the two other systems. In line with the above explanation given for the Nile Red uptake, we can again conclude that for this cross-linked micellar system, the network does not efficiently entrap the dye which might diffuse out of the hydrophobic core, leading to faster release. Similar profiles with limited release of the dye is observed for the two other cross-linked micelle systems showing that the cross-linking appears in these cases as an efficient barrier preventing diffusion and release of more than 75% of the dye. Figure 7B shows the same experiment performed at lower concentration (0.35 mg mL\(^{-1}\)), that is, a concentration that would lead to micelles destabilization if they would not be cross-linked by the disulfide bridges (the CMC of noncross-linked micelles is \(\approx 0.35\) mg mL\(^{-1}\)). In these conditions, even if the release appears slightly faster than at higher concentration, it remains diffusion limited and below 50% after 1000 min. This clearly evidences the strong effect of the cross-linking on the release profile whatever the micellar systems. At very low concentration, cross-linking keeps the compartmentalization of the systems which
allows the capture of the dye by hydrophobic interactions with the micelle core avoiding Burst effect.

Finally, the dye release profile studied against PBS was compared to the same experiment performed against PBS containing DTT as reducing agent (Figure 8). For all the three studied systems, the presence of DTT clearly triggers the Nile Red release in case of diluted solutions (0.35 mg mL\(^{-1}\)). A very fast release was observed that reached about 80% after 19 h. In the reductive DTT environment, the reduction of the disulfide bridges is breaking the cross-links allowing the destabilization the micelles below the CMC and causing a rapid delivery of the Nile Red. It is noteworthy that the release profiles in reductive environment but at a concentration above the CMC (1 mg mL\(^{-1}\)) are similar for all the three systems and typical for a diffusion limited release from noncross-linked micelles.

Figure 9 shows an overlay of the release profile in reduced environment of diluted micelles evidencing a slightly quicker release of the dye from micelles of copolymer 3 as compared to copolymer 1. This is in line with the conclusion of a faster micelles destabilization observed for micelles of copolymer 3 after 30 min. of incubation in internalization experiments. The cross-links being close to the hydrophilic part of the micelle due to the structure of copolymer 3 might be the reason why cleavage of the disulfide bridges is faster for these micelles made of copolymer 3.

In summary, these experiments evidenced that a fast drug release could be expected in the cell cytoplasm which is a more reductive environment than extracellular media. Moreover, it was demonstrated that micelle cross-linking not only insures high micelles stability in diluted environment but also offers an efficient barrier towards
dye diffusion particularly in case of cross-linked micelles of copolymers 1 and 3. These two systems appear thus quite relevant for the development of adaptive drug delivery systems. Particularly the system of copolymer 3 allows the rapid trigger of the drug release by fast disulfide cleavage in reductive environment thanks to the most external location of the cross-links. Nevertheless, we observed for all these core-crosslinked micelles that the cross-linking leads to low loading efficiencies for both the model drug, and paclitaxel, an antimitotic drug.

4. Conclusion

In this study, the potential of novel redox-sensitive core cross-linked micelles to act as smart drug delivery systems has been demonstrated. Three kinds of cross-linked micelles differing by the reactive-groups distribution along the hydrophobic block of the involved amphiphilic copolymer have been developed and compared. All the three systems exhibit in vitro low cytotoxicity and are able to be internalized without the presence of a targeting moiety. Thus, after their intravenous administration, these new nanocarriers would be able to be internalized in solid tumors by EPR effect. Moreover, the presence of the disulfide bridges insures the micelle stabilization in the bloodstream and limits the release of the encapsulated dye even at very low concentration, particularly in case of copolymers 1 and 3. After their internalization, the reduction of these bridges triggers the release of the dye most rapidly in case of copolymer 3 which exhibits the reactive groups for cross-linking in the closest position to the hydrophilic block. Based on these data, cross-linked micelles of the PEO114-b-poly-(aN20CL)6-b-PCL10 appear to us as the most promising candidate for drug delivery applications. The length of the copolymer 3 so as its composition is currently under optimization in order to improve the drug loading efficiency and study the effect of such nanomedicine on tumor growth.

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