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pH-Responsive Nanometric Polydiacetylenic Micelles Allow for Efficient Intracellular siRNA Delivery

Manon Ripoll,^a Patrick Neuberg,^{a} Antoine Kichler,^a Nassera Tounsi,^c Alain Wagner,^b and Jean-Serge Remy^{a*}*

^a Laboratory Vectors: Synthesis and Therapeutic Applications, and Labex Medalis, CAMB UMR7199 CNRS-Université de Strasbourg, Faculté de Pharmacie, Illkirch, France.

^b Laboratory of Functional Chemo Systems, and Labex Medalis, CAMB UMR7199 CNRS-Université de Strasbourg, Faculté de Pharmacie, Illkirch, France.

^c Laboratory of Therapeutic Innovation, UMR7200 CNRS-Université de Strasbourg, Faculté de Pharmacie, Illkirch, France.

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3 KEYWORDS
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6 Micelles, photo-polymerization, siRNA, imidazole, proton sponge effect
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10 ABSTRACT
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14 A novel generation of pH-responsive Photopolymerized Diacetylenic Amphiphile (PDA)
15 micelles with a diameter of 10 nm was designed and optimized for the intracellular delivery of
16 siRNAs. Dialysis and photo-polymerization of the micelles allowed a strong reduction of the
17 cytotoxicity of the nanovector, while the hydrophilic histidine headgroup permitted enhancing
18 the siRNA delivery potential by improving the endosomal escape via imidazole protonation.
19 These PDA-micellar systems were fully characterized by DLS, TEM, and DOSY-NMR
20 experiments. The resulting bioactive complexes of PDA-micelles with siRNA were shown to
21 have an optimal size below 100 nm.
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3 Over the past few years different types of nanoparticles like liposomes, stable nucleic acid
4 lipid particles (SNALPs) micelles, dendrimers, and polymers such as polyethylenimine have
5 been explored for the delivery of genes and/or siRNAs into cells.¹⁻³ In particular, the delivery of
6 siRNAs has revealed highly interesting results because of its great therapeutic potential in all
7 pathologies in which down-regulation of a specific mRNA leads to a beneficial effect, paving the
8 way for various clinical trials for the treatment of diseases such as cancers, viral infections and
9 genetic disorders.⁴⁻⁸

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11 Among novel nanostructured materials, our group reported recently that UV-irradiated
12 cationic polydiacetylenic micelles are efficient gene delivery vehicles.⁹ The small, 10-nm
13 diameter PDA-micelles were obtained by self-assembly of surfactants composed of a photo-
14 polymerizable diacetylenic motif within a C₂₅-hydrophobic chain and a cationic polar headgroup.
15 While efficient for DNA delivery, this first-generation vector however did not permit siRNA
16 delivery. In the meantime, Doris' group has shown the strong potential of neutral
17 polydiacetylene micelles as cargo for targeted drug delivery.¹⁰⁻¹³

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19 Due to their interesting properties and significant potential, we decided to push forward our
20 development of PDA-micelles for siRNA delivery, which has to date remained uninvestigated.
21 Our guideline to overcome the limitation of this first generation was to take advantage of the
22 polymerized core of the PDA-micelles and design the hydrophilic headgroup of the diacetylenic
23 surfactant in such a manner that it can improve the delivery and intracellular release of the
24 siRNA.

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26 When mixed with siRNA at the optimal ratio, the positively charged nanoparticles are taken
27 up by the cells through an endocytosis-mediated process. However, efficient cell uptake is not
28 sufficient since endosomal escape is also a crucial step for achieving gene silencing. Indeed, the

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siRNA has to be delivered intact into the cell cytoplasm in order to be able to incorporate into the RNA-Induced Silencing Complexes (RISCs). To achieve the escape from the endosomes and avoid degradation, various strategies have been developed, among which is the use of chemical functions with favorable pKa values such as imidazole, which induce the so-called ‘proton sponge’ effect^{14–16}. Indeed, the membrane-bound ATPase proton pumps actively translocate protons into the endosomes. Buffering of the endosomes through compounds like PEI¹⁷ (polyethylenimine) or imidazole, leads to a massive proton accumulation concomitant with passive chloride influx. These events eventually cause osmotic swelling and subsequent endosome disruption (a phenomenon called the ‘proton sponge’ effect by J-P. Behr).^{18,19} Based on this knowledge, we wanted to translate this concept to the PDA-micellar delivery systems for siRNA delivery. Here we synthesized a cationic monomer molecule with a histidine linked to the trioxatridecane linker (Figure 1A). To demonstrate the role played by the histidine group for siRNA delivery, we decided to also evaluate surfactant **2** (Figure 1B), an analogue of **1** bearing only a primary amine instead of a histidine group (see SI for surfactant synthesis).

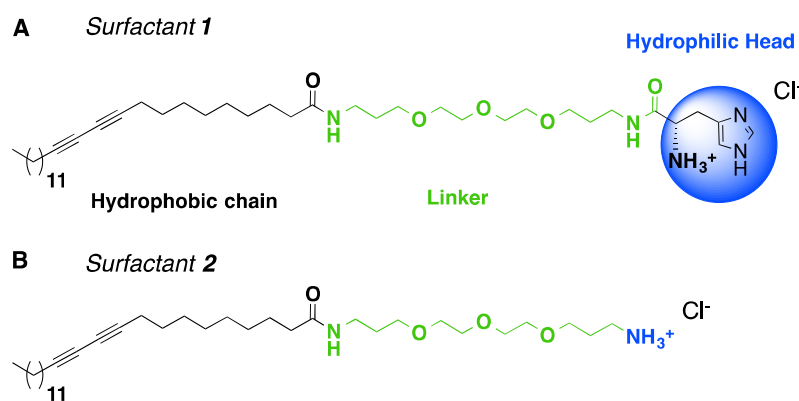


Figure 1. (A) Structure of surfactant **1** at neutral pH. (B) Structure of surfactant **2** at neutral pH.

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3 The non-polymerized micelles (NPM) abbreviated Hist-NPMs and NH₂-NPMs for surfactant **1**
4 and **2** respectively were formulated under their chlorhydrate form. Then, they were polymerized
5 under UV-irradiation at 254 nm in order to obtain the polymerized micelles (PM), abbreviated
6 Hist-PMs and NH₂-PMs, which permits reducing the cytotoxicity.²⁰ The polymerized Hist-PMs
7 were characterized by Dynamic Light Scattering (DLS), Transmission Electron Microscopy
8 (TEM) and UV-spectroscopy (Figure 2A and 2B; S3 and S4). DLS and TEM showed that both
9 Hist-NPMs and Hist-PMs are spherical objects of ~10 nm diameters, whatever the
10 polymerization time (Table S1). We then evaluated the capacity of the micelles to complex
11 siRNAs. To favor the formation of small particles, the complexes between the cationic histidine
12 photopolymerized micelles and the siRNA were generated in low salt conditions (HBG: Heps
13 Buffered Glucose). The complexation of siRNA was followed by gel retardation assay with a
14 Tris-Acetate (TA) buffer (pH=7.5) as a running buffer (Figure S14). Complete complexation of
15 the nucleic acids took place at low N/P ratio (=2) as expected, since at physiological pH the
16 primary amines of the histidine group are protonated (N/P: primary amine versus phosphate
17 groups of siRNA). The size of the particles was measured by DLS and TEM, and the results
18 showed that the siRNA-micelles complexes remained homogeneous and smaller than 100 nm for
19 N/P ratios above 10 (Figures 2C, 2D and S6).

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22 The intracellular uptake of the complexes was then investigated using Cy5-labeled siRNA.
23 Fluorescence microscopy showed that the Cy5-siRNA was able to penetrate into the cells when
24 complexed to Hist-PMs (Figure S20). We next evaluated how the histidine residue impacts the
25 biological activity. Surfactants **1** and **2** were compared in the same experiment using the same
26 formulation. Transfection assays were performed on cells which stably expressed the luciferase
27 gene (A549-luc cells) using low concentrations of siRNA (10 nM) targeting the luciferase
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3 mRNA (Luc-siRNA) (see SI). After the first series of biological assays, only the micelles
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5 polymerized for 4 hours were further studied, because this polymerization time resulted in the
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7 best balance between cytotoxicity and efficiency (Figure S17). From luciferase expression,
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9 specific inhibitions were calculated by measuring the silencing obtained with the Luc-siRNA
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11 compared to the control siRNA. The results obtained with the micelles polymerized for 4 hours
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13 show that the nature of the headgroup plays a crucial role in siRNA delivery. The results indicate
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15 that almost 80 % of specific inhibition was reached with 25 μg micelles/mL (N/P = 83.3) of
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17 compound **1**, whereas the NH_2 -system **2** only poorly performed in these experiments with a
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19 maximum specific inhibition of 25 % (Figure S18, bars).
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25 However, despite extended UV irradiation times of the photopolymerized micelles, a moderate
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27 toxicity of Hist-PMs on cells still remained (Figure S18, triangles) – it is assumed this is due to
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29 the remaining monomers.²⁰ This issue has been solved by an optimized procedure consisting of
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31 dialysis steps as described below.
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35 In order to gain further insights into the molecular form of the photopolymerized micelles, we
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37 chose to analyze the surfactant assembly by DOSY-NMR experiments (Figure 2E). The Hist-
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39 NPMs were evaporated to dryness then dissociated into molecular form in deuterated methanol.
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41 A control monomer solution was dissolved in deuterated methanol and showed discrete diffusion
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43 constants in coherence with dissociated molecules (Figure S12). The DOSY-NMR experiments
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45 done on the Hist-NPMs also showed that all the micelles were able to dissociate into the
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47 monomers reflecting an absence of covalent linkage formation in this non-polymerized form
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49 between the dyine motifs. The same protocol and analysis applied to the photopolymerized Hist-
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51 PMs revealed two separate populations, one corresponding to the monomer with diffusion
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53 constant equal to $D=760 \cdot 10^{-12} \text{ m}^2/\text{s}$ and the other with a lower diffusion constant ($D=213 \cdot 10^{-12}$
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m^2/s) corresponding to oligomer structures. Thus, the DOSY-NMR experiments clearly showed that even after 4 hours of polymerization, a small population of monomers - estimated to be around 25 % - still remained.

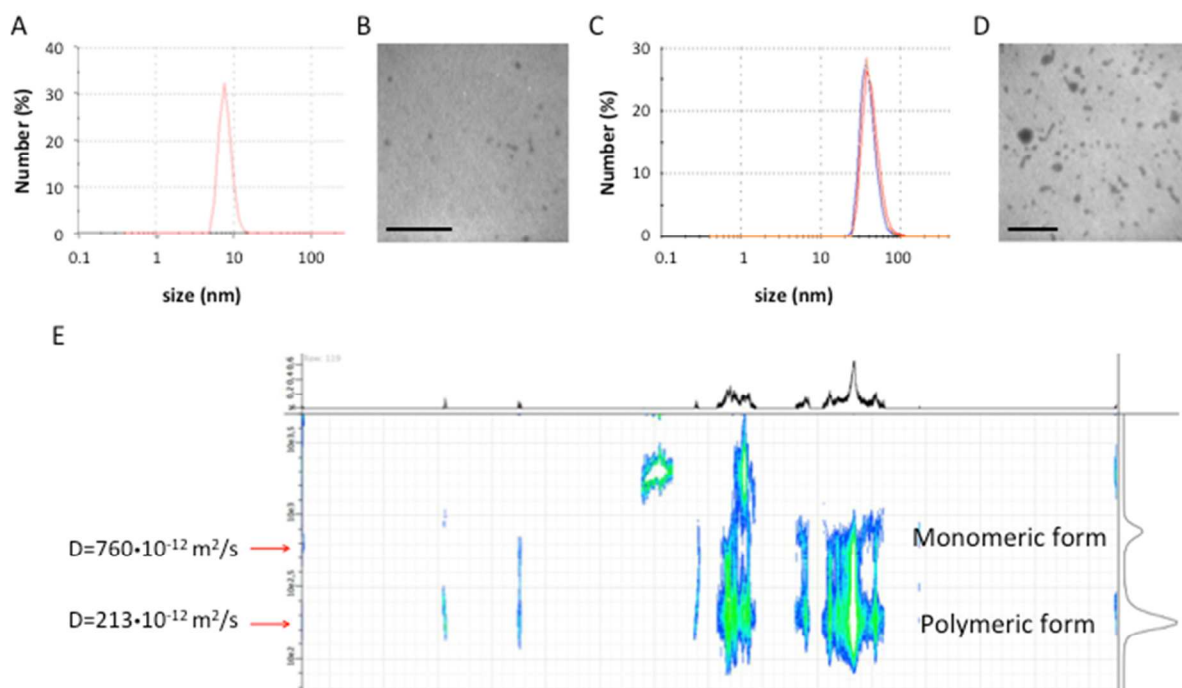
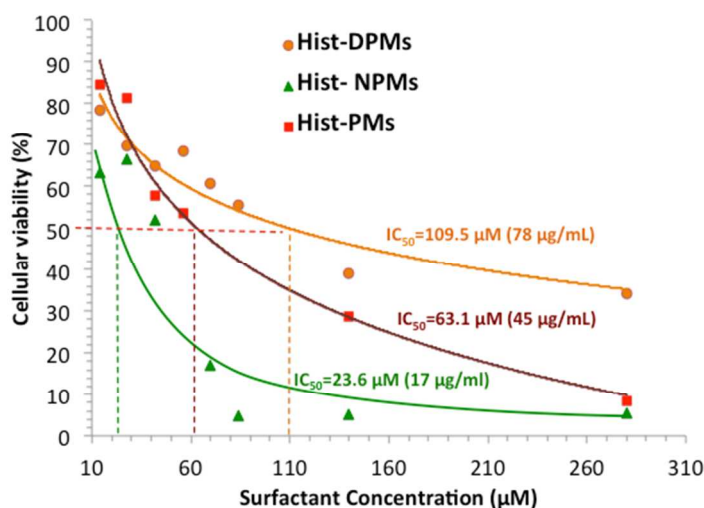


Figure 2: (A) Size distribution by number of the 4h-Hist-PMs (PDI = 0.287). (B) TEM image of 4h-Hist-PMs in Hepes buffer (10 nM, pH = 7.5). Scale bar: 500 nm. (C) Size distribution by number of the complexes formed in low salt condition (HBG) between siRNA and Hist-PMs (70 ng/ μL siRNA, 20 $\mu\text{g}/\text{mL}$ Hist-DPMs) (PDI = 0.259). (D) TEM image of the siRNA-4h-Hist-DPMs in Hepes buffer (10 nM, pH = 7.5). Scale bar: 500 nm. (E) DOSY-NMR analysis of the 4h-Hist-PMs after evaporation under vacuum and suspension in deuterated methanol.

This result concerning the remaining monomer was also confirmed by the critical micelle concentrations (CMC) of non-polymerized Hist-NPMs and polymerized Hist-PMs measured by the Pyrene inclusion method. Hist-NPMs showed CMC of 0.1 mM, while Hist-PMs showed higher CMC values (0.7 mM) (Figures S7 and S8). The higher CMC of the Hist-PMs can be explained by the fact that the polymerized lipid does not contribute to efficient pyrene inclusion. Thus, the measured CMC is the minimum concentration of total lipid where the remaining

monomer starts interacting with the hydrophobic pyrene probe. As the concentration of monomer in the polymerized systems is lower as compared to Hist-NPMs, the calculated CMC appears higher, proving indirectly a partial conversion of this monomer surfactant.¹⁶

In order to further reduce toxicity, we adopted a complementary strategy; the micelles were extensively dialyzed in order to get rid of the remaining monomer (see SI). The real concentration of the Dialyzed-Polymerized Micelles abbreviated Hist-DPMs measured by primary amine quantification using the TNBS method was estimated to be 5 mM by taking the 7 mM non-dialyzed Hist-PMs as a reference (Figure S9). To validate the hypothesis that the remaining monomers accounted for the toxicity, cytotoxicity assays were performed on A549-luc cells. Cell viability was quantified using an MTT assay (see SI). The results on the A549-Luc showed that non-polymerized micelles ($IC_{50}=24 \mu\text{M}$) were almost three times more toxic than the polymerized ones ($IC_{50}=63 \mu\text{M}$) (Figure 3), which confirmed the interest of the polymerization. A similar reduced toxicity of the polymerized micelles was also observed on human cervical cancer cells (HeLa; Figure S19).



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3 **Figure. 3** MTT cytotoxicity assay performed 48h after surfactant addition on A549-Luc cells. Cellular viability is
4 given for non-polymerized micelles (hist-NPMs), 4h-polymerized micelles (hist- PMs) and dialyzed and 4h-
5 polymerized micelles (Hist-DPMs).
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10 Then, the dialyzed samples were compared in toxicity tests as well as in siRNA transfections
11 assays. The dialyzed 4h-polymerized micelles (Hist-DPMs) presented the lowest toxicity ($IC_{50}=$
12 $110\ \mu\text{M}$), as compared to the non-dialyzed one ($IC_{50}=63\ \mu\text{M}$). In order to gain physical evidence
13 of the loss of monomer, we again performed DOSY-NMR experiments on the dialyzed sample
14 (Figure S13). Once again, two separated populations were observed. One was characteristic of
15 the monomer with $724\cdot 10^{-12}\ \text{m}^2/\text{s}$ as a diffusion constant, and the other one corresponded to the
16 polymeric form with $185\cdot 10^{-12}\ \text{m}^2/\text{s}$ as a diffusion constant. Areas under the curves showed that
17 only 10 % of monomers remained, which is in accordance with the reduced toxicity of the PDA-
18 micelles.
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31 The siRNA delivery potential of the Hist-DPMs was then evaluated on A549-luc cells to study
32 whether the reduced amount of remaining monomer impacts the biological activity. The
33 inhibition efficiency was calculated relative to the non-transfected A549-luc cells and compared
34 to a control siRNA (siCTL). The efficiency of the optimized nanovector obtained by dialysis is
35 shown in Figure 4A. Up to 80 % of inhibition of the luciferase gene expression was obtained
36 with $12\ \mu\text{g}/\text{mL}$ (N/P = 39) of Hist-DPMs. Interestingly, in comparison to the non-dialyzed
37 system, the dialyzed sample allowed reaching the same maximal specific inhibition while using
38 less surfactant and while lowering cytotoxicity. It is worth noting that, in good agreement with
39 the results obtained with peptidic¹⁶ and polymeric¹⁷ transfection agents, higher N/P ratios are
40 needed for efficient PDA-mediated siRNA delivery as compared to the N/P ratio which allows
41 total complexation of siRNA in gel mobility shift assays. One explanation of the need for a
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3 higher N/P for transfection is that the size of the siRNA-Hist-PMs complexes varies with the N/P
4 values (Figure S6). In fact, higher N/P values ensure small sizes of the complexes and allow for
5 efficient stabilization of the complexes in the presence of competing anionic molecules, such as
6 serum proteins and sulfated polysaccharides (Figure S15 and S16).
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11 To evidence that these compounds presented a favorable pKa, the titration profiles of the
12 monomer and the Hist-DPMs were determined (Figures 4B, S10 and S11). The results show the
13 monomers to have important buffering abilities in the pH range that occur in the endosomal
14 cellular compartments (from 4.0 to 6.0), which is essential to efficiently protect siRNA during
15 delivery. Half the equivalent of NaOH per amine is needed to raise the pH of the solution from
16 4.0 to 6.0. By comparison, the potentiometric curve of the Hist-DPMs indicates that the
17 polymerization permitted increasing the buffering abilities of the system two-fold in the pH
18 range of interest. This increased capacity is due to the fact that when micelles are polymerized, a
19 fully condensed conformation is adopted. Hence, primary amines are more closely packed
20 resulting in an increase in the mutual electrostatic repulsion leading to a decrease of their pKa
21 values.
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38 This experiment permits concluding that surfactant **1** has a favorable pKa value that can even
39 be improved by polymerization.
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43 Finally, to gain insight into the biological mechanism of siRNA release in the cytoplasm, we
44 evaluated the effect of bafilomycin A1 on the transfection activity of the Hist-DPMs.
45 Bafilomycin is an antibiotic that selectively inhibits vacuolar type H⁺ ATPase (V-ATPase).
46 These V-ATPases cause acidification of the internal space of several organelles of the vacuolar
47 system including endosomal compartments.^{21,22} The assays were realized on A549-luc cells by
48 using 175 nM of bafilomycin A1 and 10 nM siRNA. As shown on Figure 4C, we observed a
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3 significantly lower specific inhibition when the transfection was realized in the presence of
4 bafilomycin A1. These experiments evidence that acidification of the endosomes plays an
5 important role in the mechanism of action of histidine-containing PDAs. Protonation of the
6 imidazole group probably allows a ‘proton sponge’-like effect that ultimately results in an
7 enhanced cytosolic delivery of siRNA^{14,16,23}. It is worth noting that, in the case of polymers with
8 high buffering capacities such as fractured dendrimers and polyethylenimines (two compounds
9 for which a proton sponge mechanism is widely accepted¹⁸), an increase of the polymer volume
10 has been observed with decreasing pH due to electrostatic repulsion between protonated
11 amines²⁴. Here we asked whether endosomal acidification could also lead to a swelling of the
12 PDA/siRNA complexes, entailing endosomal membrane destabilization. The results show that, at
13 neutral pH (7.4), complexes have a diameter of 60 nm. When decreasing the pH up to one unit
14 by acid addition, the size of the siRNA-Hist-PM increases two-fold (from 60 nm to 110 nm),
15 which can be explained by increased protonation of the histidine groups leading to charge
16 repulsion (Figure S21).
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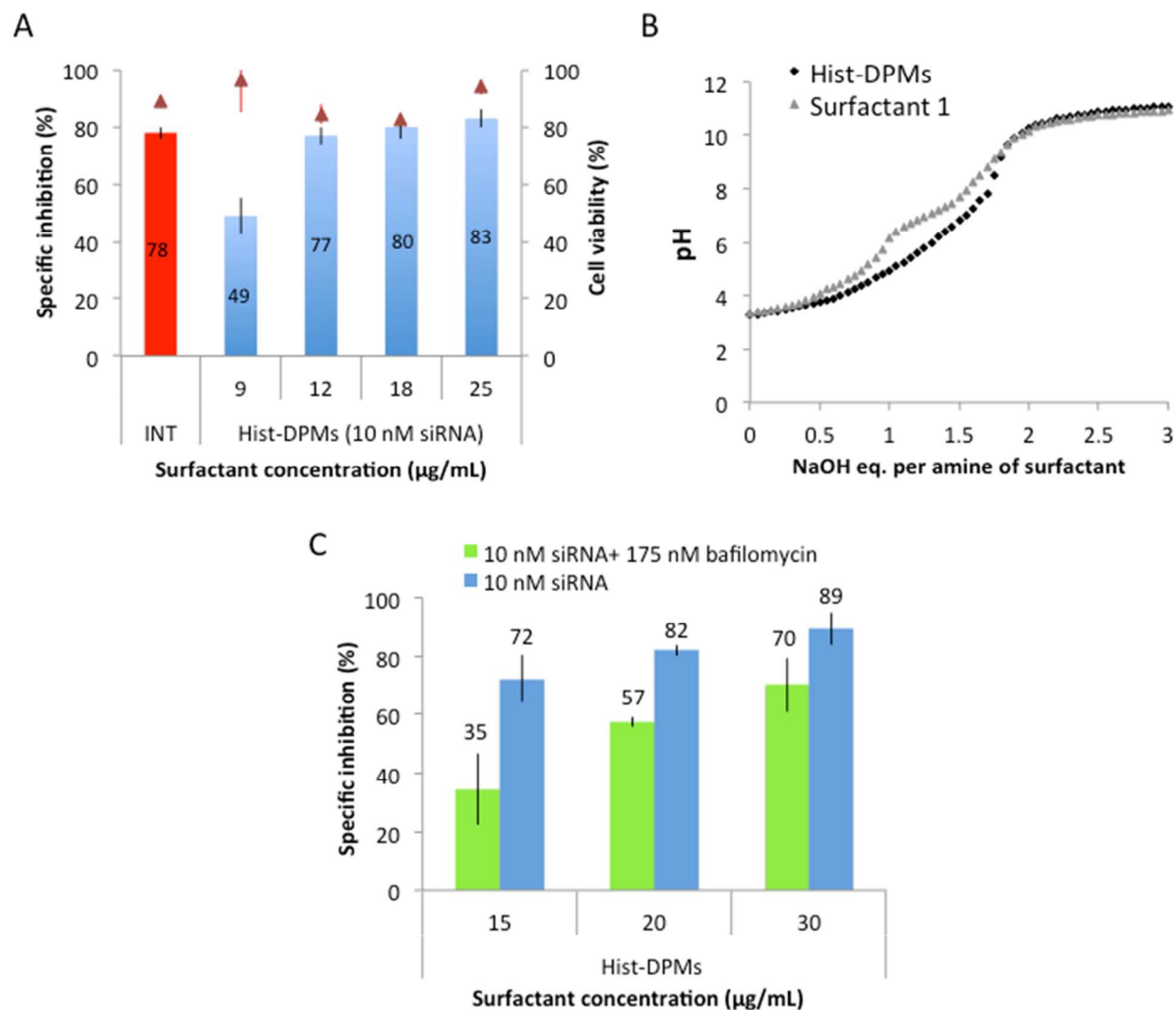


Figure 4. (A) siRNA delivery experiments performed on A549-luc cells using various concentrations of surfactant 1 with 10 nM siRNA. Specific inhibition compared to A549-luc cells transfected with negative control siRNA after 48h of transfection are given for commercial positive control (INTERFERin™) and Hist-DPMs in 70% ethanol resuspended in water (bars). Cell viability was measured by total protein assay and represented here compared to non transfected cells (triangles). (B) Potentiometric titration profiles of surfactant 1 (grey triangles) and of the Hist-DPMs (dark diamonds) showing extended buffering from pH=6 to pH=4 (C) Effect of bafilomycin A1 on Hist-DPMs transfection activity on A549-luc cells after 48h of transfection. Specific inhibition of luciferase expression as compared to siRNA control is given for Hist-DPMs in the presence of 10 nM siRNA +/- 175 nM bafilomycin A1.

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3 In summary, a new generation of photopolymerized micelles based on a diacetylene surfactant
4 was synthesized and optimized for intracellular siRNA delivery. PDA-micelles have previously
5 been used for drug or gene transfection, but not for siRNA delivery. Here, we show that by fine-
6 tuning the headgroup properties, the PDA-micelles system becomes a promising nanovector for
7 siRNA delivery with more than 80 % of specific inhibition in reporter gene assays. In particular,
8 the use of the histidine group as a hydrophilic polar head permits drastically improving the
9 specificity it is assumed by improving the siRNA release into the cytoplasm, through the ‘proton
10 sponge’ effect and a swelling of the complexes in the endosomal compartment following cellular
11 uptake. Whereas polymerization already permitted reducing the toxicity and stabilizing the
12 system by cross-linking, dialysis of the micelles allowed for a further reduction the cytotoxicity
13 while preserving excellent siRNA delivery potential. Finally, the nanometric size range of these
14 siRNA-nanoobjects opens up interesting prospects for *in vivo* studies in the field of cancer
15 research, since small particles (<100 nm) are reported to better diffuse through blood vessels and
16 reach deeper into the tumor tissues.²⁵
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3 ASSOCIATED CONTENT
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7 **Supporting Information**
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9 Experimental section, surfactant characterizations, UV spectra, DLS figures, CMC curves,
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11 DOSY-NMR spectra, gel retardation assay, siRNA transfection results and fluorescence
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13 microscopy images.
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16 This material is available free of charge. (PDF)
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20 AUTHOR INFORMATION
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23 **Corresponding Author**
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25 * E-mail: remy@unistra.fr and neuberg@unistra.fr
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29 **Notes**
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31 The authors declare no competing financial interest.
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ABBREVIATIONS

PDA, Photopolymerized Diacetylenic Amphiphile; HBG, Hepes buffered glucose; DLS, dynamic light scattering; TEM, transmission electron microscopy; DOSY, Diffusion-Ordered Spectroscopy; siRNA, small interfering RNA; CMC, critical micelle concentration.

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