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Permeabilization of Lipid Membranes and Cells by a Light-Responsive Copolymer

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Membrane permeabilization is achieved via numerous techniques involving the use of molecular agents such as peptides used in antimicrobial therapy. Although high efficiency is reached, the permeabilization mechanism remains global with a noticeable lack of control. To achieve localized control and more gradual increase in membrane perturbation, we have developed hydrophobically modified poly(acrylic acid) amphiphilic copolymers with light-responsive azobenzene hydrophobic moieties. We present evidence for light triggered membrane permeabilization in the presence azobenzene-modified polymers (AMPs). Exposure to UV or blue light reversibly switches the polarity of the azobenzene (cis−trans isomerization) in AMPs, hence controlling AMP-loaded lipid vesicles permeabilization via *in situ* activation. Release of encapsulated probes was studied by microscopy on isolated AMP-loaded giant unilamellar vesicles (pol-GUVs). We show that in pH and ionic strength conditions that are biologically relevant pol-GUVs are kept impermeable when they contain predominantly cis-AMPs but become leaky with no membrane breakage upon exposure to blue light due to AMPs switch to a trans-apolar state. In addition, we show that AMPs induce destabilization of plasma membranes when added to mammal cells in their trans-apolar state, with no loss of cell viability. These features make AMPs promising tools for remote control of cell membrane permeabilization in mild conditions.

Introduction

Lipid bilayers provide the basic structure of cell membranes and act as an impermeable barrier to the passage of most molecules, though internal specific cellular transport processes exist to allow them to cross the cell membrane.[1] Membranes perform key cellular functions including regulation of transmembrane exchanges, transport of nutrients, and information that are determinant for cell survival.[2] In turn, this regulation of bilayer properties makes controlled infusion of external agents, such as drug molecules, bioactive peptides, or polynucleotides a very difficult practice.[3,4] Designing novel synthetic agents that are able to affect membrane properties and induce a controlled permeabilization while keeping the integrity of membrane is hence a major challenge for academic chemistry and biophysics, applications in pharmaceutical research, and biotechnologies. This goal can be reached by a remote control of a chemical gate in the membrane, as triggered by an external stimulus. For instance, light-addressable nanoscale devices based on the chemical modification of protein channels by photochromic ligands trigger permeability to specific ions upon light irradiation.[5−7] Early photochromic ligands could bind only to ion channels that had been genetically modified. To date, Trauner and colleagues have tailored azobenzene molecule that can photosensitize native voltage-gated potassium channels in neurons.[8] All these devices stands out as an exquisite achievement in the field but address the question of permeabilization to specific molecules in cells that express the corresponding protein channel.

In contrast, nonspecific permeabilization (i.e., inducing lipid bilayer permeabilization to any large or highly hydrophilic compounds) is not trivial, in particular when cell survival is required. So far, nonspecific permeabilization is achieved by supplementation with strong membrane permeabilizers such as detergents and amphiphilic peptides.[9−11] Surfactants (dubbed detergents in biology) are widely used in membrane biology, and it is well-known that they solubilize membrane proteins and often induce membranes disruption.[10] Their interactions with cells is difficult to control as they ultimately induce cell membrane disruption and hence lead to cell death.[11] On the other hand, amphiphilic peptides are mostly used as antimicrobial peptides which can specifically destabilize and disrupt bacteria and fungus outer membranes.[12] They typically induce the formation of pores that cause the cellular content to leak out leading to high cytotoxicity, including on mammalian cells.[11,13] The use of light-responsive amphiphiles improves control on the membrane response via the use of external triggers and hence is a method of

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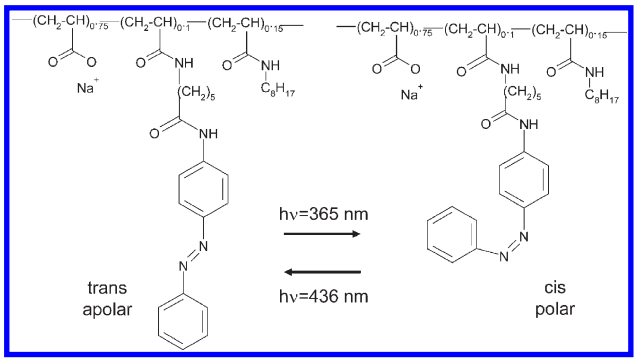

Figure 1. Structures of hydrophobically modified poly(acrylic acid) amphiphilic copolymers modified octyl side chains (C8) and azobenzene which can undergo cis−trans photoconversion. Here the polymer is a terpolymer, with integration levels of 15% C8 and 10% azobenzene side group. A change in the polarity of the azobenzene moiety is induced from a cis/polar to a trans/apolar conformation by UV (λ = 365 nm) and blue light irradiations (λ = 436 nm), respectively.

choice for transient membrane permeabilization.[14] To this aim, surfactants have been imparted with light-responsiveness. Photoisomerizable cholesterol derivatives or lipid-like molecules containing azobenzene in their apolar tail bring photomodulation of the (hours-long) leakage of small liposomes, though their efficiency depends markedly on their amount in the membrane.[15,16] The concentration of surfactant in membranes is however difficult to control in applications, especially *in cellulo*. Overloads make the membranes permanently leaky and finally induce instability and breakage of the bilayers.

Here we propose the use of milder permeabilizers: azobenzene-modified polymers (AMPs) whose hydrophobicity is specifically modulated by light irradiation. The azobenzene moiety in AMPs responds to both near-UV and visible light irradiations (λ = 365 nm and λ = 436 nm, respectively)[17] by reversible cis (polar)−trans (apolar) rearrangements, which can be translated in terms of AMPs polarity (Figure 1). AMPs belong to the family of hydrophobically modified poly(acrylic acid)(HMPA) polymers which were recently reported as inducing agents for controlled membrane permeabilization.[18] One of the main advantages to use HMPAs is their ability to permeabilize membranes with no disruption. HMPAs avidly bind to lipid bilayers via noncovalent hydrophobic interactions and form transient nanochannels of well-defined diameters with no disruption of the membrane structure.[19] Their efficiency to induce membrane permeabilization relies on their degree of ionization and hydrophobicity, which can be modulated by a change in both pH and external ionic strength. Accordingly, no permeabilization was observed at pH 7.5 where the polymer is in an ionized state but permeabilization was triggered at pH 6.8 where the polymer is more hydrophobic.[20] On the mechanistic insight, HMPAs binding to lipid bilayers affects lipid segregation,[21] and because they form micelle-like assemblies[22,23] in water, they presumably favor high local curvatures, which might influence pore formation and stabilization. An appropriate hydrophobic balance is thus critical for membrane permeabilization[14] and for the control of the association/dissociation features of AMPs on amphiphilic assemblies upon light irradiation.[17,24]

In this report, we show specific and controlled AMPs induced membrane permeabilization. We examined their potential to induce giant unilamellar vesicles (GUVs) local destabilization and permeabilization at different pH and ionic strength conditions upon UV and visible light irradiation. On the basis of leakage experiments monitored by fluorescence microscopy on isolated AMP-enriched vesicles (pol-GUVs), we present the first report of controlled membrane permeabilization with no membrane breakage achieved via photostimulation in biologically relevant conditions. We then investigated the effects of irradiated AMPs interaction with cell membranes of mammalian origin by monitoring both plasma membrane integrity and cell viability. Our results indicate that AMP trans apolar state induced membrane destabilization but maintained cell viability. AMPs are therefore promising tools for effective cell membrane permeabilization through rapid, remote, noninvasive, and spatially accurate control.

Materials and Methods

Materials. 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) was purchased from Avanti Polar Lipids Inc. in lyophilized powder form and used with no further purification. Fluorescein isothiocyanate (FITC), dextran FD4 (M_w = 4000 g/mol, R_H = 14 Å), and dipalmitoylphosphatidylethanolamine sulforhodamine B (DPPE rhodamine) were purchased from Sigma-Aldrich, France.

Polymer Synthesis. Polymers were obtained by coupling a short poly(acrylic acid) (M_w 7500 g/mol, Aldrich) in *N*-methylpyrolidone to first octylamine and next the azobenzene chromophore, 6-amino-*N*-(4-phenylazophenyl)hexanamide, as previously described.[22] One equivalent aliquot of octylamine was added to attach 15 mol % of octyl side groups (relative to the molar amount of carboxylic groups). Two equivalents were required to attach 10 mol % of the azobenzene moieties, linked by an amide bond to acrylate units. At variance with procedure found in ref 22, purification of the polymer was not achieved by precipitation in alcohol because the hydrophobically modified chains were soluble in most organic solvents, including chloroform. We submitted instead the polymers to three cycles of solublization in NaOH−water solution (pH > 13), filtration (Millex seringe filters), precipitation by dropwise addition of 10 M HCl (down to pH < 3), and washing with water. In the final solution, pH was adjusted to 8.0 prior to lyophilization. The degrees of integration of octyl and azobenzene were obtained by ^{1}H NMR and by absorbance measurements using extinction coefficient of the chromophore in water of $\varepsilon_{347\,nm}$ = 2.32 L mol^{-1} cm^{-1}.

pol-GUVs and DOPC GUVs Preparation. Giant unilamellar vesicles were formed using the Nanion Prep Pro stand alone station. Typically, DOPC, DPPE rhodamine (0.5 mol %), and AMPs at a 0.1 g/g lipid ratio were dissolved in chloroform:methanol (3:1). The mixture was then deposited onto ITO slides. The solvent was removed by nitrogen stream and dried under vacuum for 1 h. The lipid−polymer mixture was hydrated using a buffer solution made of 0.3 M sucrose and 0.02 M Tris-HCl (pH 8.5) containing 150 μM FITC dextran at 15 °C. An ac field was applied and linearly increased over 20 min to 3 V at 5 Hz. The vesicles were allowed to grow for 3 h before the field was linearly decreased over 20 min. Vesicles were then collected by gentle pipet

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aspiration and used for fluorescence microscopy experiments. Control DOPC GUVs were similarly prepared but without addition of polymer. Vesicles were kept at 4 °C and used within 1 week after their preparation.

Vesicle Preparation for Fluorescence and Phase Contrast Microscopy. Stock solutions of giant vesicles encapsulating FITC dextran and having a diameter in the range 5−50 μm were prepared by electroformation in sucrose buffer as described above. To enable vesicle observation, the vesicles were diluted 50 times either in buffer containing 0.3 M glucose, 0.02 M Tris-HCl at pH7 or 8.5 or in buffer containing glucose 0.05 mM, 0.02 M Tris-HCl, 150 mM NaCl at pH 7 or 8.5 on a pretreated bovine serum albumin (BSA) glass plate. The observation condition was slightly hyperosmotic outside the vesicle, but this did not affect vesicle integrity. The encapsulated sucrose made the vesicle denser than the outside phase which allowed them to settle down on the bottom of the observation plate for an easier observation. While diluted in glucose buffer, the vesicles (pol-GUVs and DOPC GUVs) were irradiated under UV light (370 ± 10 nm) at a power of 1 mW/cm^2 for at least 3 min. This step allowed the AMPs to predominantly switch to a cis state in the bilayer. Then the vesicles were homogeneously irradiated at 436 nm to induce the polymer to switch to a trans-apolar state. Vesicles were observed by fluorescence and phase contrast microscopy using a LEICA DM-IRE2 inverted microscope. The fluorescence of GUVs membrane was excited at 515−560 nm, while the fluorescence of the internal compartments was excited at 460−500 nm. The signals were recorded on a CoolSnap monochrome camera (Roper Scientific, Germany) under fixed exposure conditions (typical exposure time 500 ms for fluorescence and 250 ms for phase contrast, binning 2 × 2, which corresponded to a resolution of ~0.5 μm). Vesicle permeabilization was followed by FITC dextran and sucrose leakage kinetics where one image was taken every 5 min for 1 h. For experiments in the dark, vesicles were irradiated under UV light for 3 min and then kept in the dark for 1 h. Then the vesicles were briefly irradiated under white light (< 1 min). (N.B.: We checked that about 90% of AMPs switch to a trans conformation at these irradiation conditions.) Kinetics were recorded as described above. Permeabilization percentage was assessed for both conditions by counting manually 50 unilamellar vesicles.

Cell Culture. COS cells were grown in Dulbecco's Modified Eagle cell culture medium (DMEM) supplemented with 10% fetal calf serum and 2 mM glutamine at 37 °C and 5% CO_2. Cells were routinely passed when they were 90−95% confluent.

AMPs-Induced Cell Membrane Destabilization. AMPs (1 μg/mL) were UV irradiated for 15 min in serum-free DMEM and added to preseeded (24 h) COS cells (40 000 cells) on glass coverslips in a 24-well plate. UV-irradiated AMPs were left to incubate with the cells in the dark for 5 min at 37 °C in a 5% CO_2 atmosphere incubator. The preparation was then either kept in the dark for 1 h or irradiated at 436 nm to induce the polymer's trans configuration (quoted t0). Prior to cell observation, the polymer was withdrawn at time (20, 40, or 60 min), and membrane destabilization was assessed immediately as follows: cells were washed three times with phosphate buffer, and membrane destabilization was monitored by using the Annexin-V-Cy3 apoptosis detection kit (Sigma) according to the fabricant's protocol. Externalization of the inner phosphatidylserine (PS) was detected via red fluorescently labeled annexinV-Cy3 observed as red fluorescence. On the other hand, cell viability was assessed by adding the nonfluorescent compound 6-carboxyfluorescein diacetate (6-CFDA) to the cells. When 6-CFDA enters the cells, esterases present in the cytoplasm hydrolyze it into the fluorescent carboxyfluorescein (CF) observed as green fluorescence. Membrane destabilization and cell viability were also determined after AMP removal and incubation in serum supplemented culture medium for 24 h at 37 °C and 5% CO_2. Briefly cells were washed in binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.5), after which AnnexinV-Cy3 and 6CFDA solutions were added to the cells for 10 min in the dark. To remove excess label, cells were washed in binding buffer three times. Cells were then fixed in 4% paraformaldehyde pH 7.4 for 20 min at 4 °C, and nuclei were DAPI stained. Coverslips were mounted in mowiol on glass slides for fluorescence microscopy.

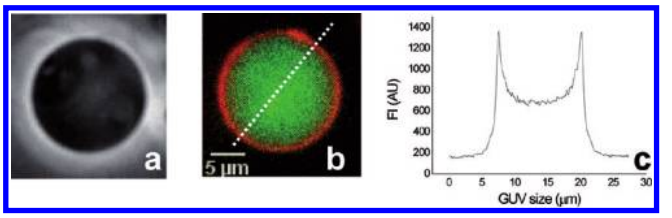

Figure 2. AMP-enriched impermeable giant unilamellar vesicles (pol-GUVs) detected by phase contrast and epifluorescence microscopy (×63): (a) phase contrast signals come from the encapsulated sucrose observed in glucose buffer, whereas fluorescence signals (b) come from the membrane labeled with PE-Rho (λ_{exc} = 557 nm, λ_{em} = 571 nm) and the encapsulated FITC dextran (λ_{exc} = 490 nm, λ_{em} = 519 nm). The solid line corresponds to the fluorescence profile of PE-Rho across the diameter shown in (c).

Image Analysis. Images were analyzed with Image J software to measure fluorescence and phase contrast intensities. For both techniques, the background intensity was subtracted to the intensity measured inside the vesicle for every single frame. Leakage curves were plotted accordingly.

Results

Polymer-Enriched Impermeable Giant Unilamellar Vesicles (pol-GUVs). AMP-enriched GUVs (pol-GUVs) were formed by the electroformation technique which is routinely used to yield vesicles with a diameter greater than one micrometer. Most of the vesicles formed were found as unilamellar vesicles as it is expected for electroformation grown vesicles.[25] The pol-GUVs were selected according to their unilamellarity from fluorescence profiles across the diameter.[20] The vesicles were formed in a buffer at pH 8.5, in which the polymer is in an ionized nonpermeabilizing state. Figure 2 shows a typical vesicle observed by phase contrast and fluorescence microscopy obtained by this method. Phase contrast and fluorescence signals coming from the encapsulated sucrose and fluorescent dextran respectively (λ_{exc} 460−500 nm) were both detected and found homogeneously distributed inside the vesicle. Vesicles are symmetrical and spherically shaped, with the membrane fluorescently labeled with PE rhodamine (λ_{exc} 515−560 nm). This was unexpected since adsorption of polyelectrolyte and other surfactants onto membrane vesicles usually leads to local membrane disorders, defects, and protrusion.[21,26] Here no defects were detected at 0.1 g/g polymer to lipid ratio whereas vesicles grown at a ratio of 0.2 g/g led to the formation of distorted vesicles with detectable membrane defects, and no vesicles were formed at higher ratio of 1 g/g at pH 8.5. Vesicles formed at pH 8.5 and 0.1 g/g AMP/lipid ratio remained stable for several hours, but no vesicles were formed at 0.1 g/g at pH 7 conditions in which the polymer is partially neutralized and thus more hydrophobic. This indicates that the polymer is likely to affect bilayers stability at low pH and above a critical AMP:lipid ratio of ca. 0.2 g/g. The AMP:lipid ratio of 0.1 g/g corresponds to a total amount of 1 alkyl side groups of the polymer per 10 lipids in the bilayer.

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Delayed (Light-Triggered) Permeabilization. We used fluorescence intensity measurements inside the vesicles to follow the leakage of dextran molecules as a function of time after light irradiation. The pol-GUVs preparation was first irradiated under UV light for 3 min. This step allowed the polymer to organize in a predominant cis polar state. Then, cis pol-GUVs were diluted in a glucose buffer containing 20 mM Tris-HCl, 50 mM glucose, and 150 mM NaCl at pH 8.5 or 7. The composition of the diluting buffer was adapted so the osmolarity of the preparation would remain close to the one before dilution. In a first set of experiments, the preparations were kept in the dark for 1 h prior to a brief exposure to blue light at time quoted "60 minutes" (light exposure <1 min), at a wavelength (436 nm) at which the azobenzene in the polymer switches to a trans-apolar state. Fluorescence signals coming from the encapsulated FITC dextran in an isolated pol-GUV were recorded over 1 h (with samples maintained at 15 °C in the dark between successive observations). Fluorescence labeling of the membrane indicated that pol-GUVs remained intact; this enabled us to count only unilamellar vesicles.[20] Studies on a group of 50 vesicles revealed that about 80% of cis pol-GUVs retained their impermeability in the dark, until the time 60 min (Figure 3a), where about 20% dextran-loaded cis pol-GUVs emptied is similar to that of DOPC vesicles devoid of AMPs (Figure 3a, $t = 60$ min). Therefore, this should be considered as indicative of the absence of leakage. Rapidly after the brief (1 min) irradiation at 436 nm, dextran leakage is observed: at time 90 min (30 min after exposure to blue light), about 70% in a population of 50 pol-GUVs have leaked. This percentage of 70% emptied vesicles remains stable up to 1 h after irradiation. 100% of empty pol-GUVs was not achieved up to time 3 h (data not shown). The pol-GUVs that were studied at pH 8.5 did not present any leakage with their signals being similar to that of controls with no polymer at the same pH, and no leakage was observed either in DOPC GUVs controls (no polymer in the membrane) at pH 8.5 and pH 7 after 2 h incubation in similar conditions (Figure 3b).

As for the lack of 100% leakage in pol-GUVs at pH 7, we considered as a hypothesis that some pol-GUVs may contain more than the average amount of inserted polymer, leaving the rest of the vesicles devoid from permeabilizer. In order to test this hypothesis, we carried out similar experiments with lower average polymer/lipid ratio (0.05 and 0.01 g/g were tested). A lower amount of AMPs led however to the same observations and results comparable with the higher ratio of 0.1 g/g: the pol-GUVs were not leaky during the initial incubation in the dark, with AMPs in their cis form, but encapsulated probe release by permeabilized GUVs occurred in about 70% of pol-GUVs at time 90 min (30 min after exposure to blue light, in fixed conditions of pH 7.0 and 150 mM ionic strength) (Figure 3c). Irrespective of the composition of pol-GUVs, the final population of empty vesicles happen to coexist with loaded vesicles and the time to empty the population of leaky GUVs being shorter than 30−40 min. Altogether, the data shown above indicate that pol-GUVs are essentially maintained impermeable for 1 h with AMPs in their cis form. Their permeability is then triggered by exposure to light at 436 nm in less than 30−40 min.

To bring additional proof of photocontrol, pol-GUVs were exposed to blue light with no delay after exposure to UV and dilution. Leakage then started immediately, with no delay (Figure 3d). Here, studies were conducted on three independent samples and groups of 50 pol-GUVs (respectively on controls with no polymer) that were counted to estimate experimental errors as shown in Figure 3d. There is a noticeable permeabilization of pol-GUVs after irradiation of the sample at 436 nm where about 30% of the vesicles are permeabilized at pH 7 at $t = 20$ min. The percentage of permeabilization increased up to 50% at $t = 40$ min.

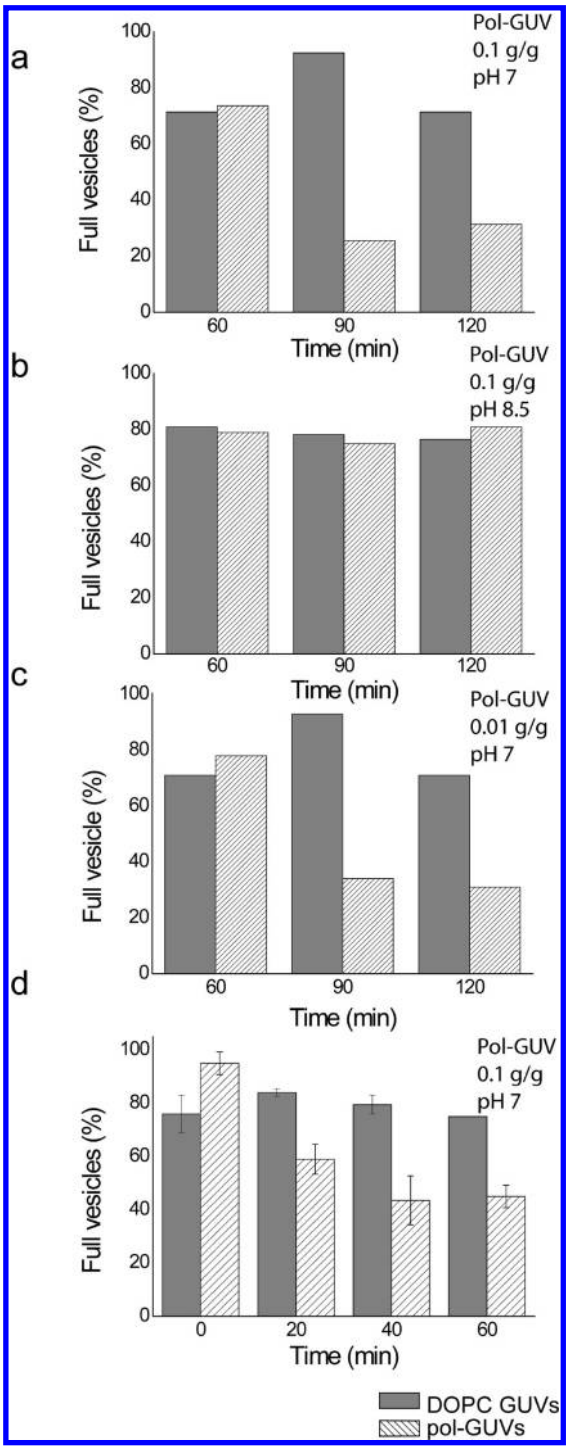

Figure 3. Histograms representing the percentage of full pol-GUVs (dashed columns) and DOPC control GUVs (gray column) with varying AMP:DOPC ratio, pH, and irradiation conditions as a function of time. (a) 0.1 g/g pol-GUVs and DOPC GUVs were initially irradiated at 365 nm for 3 min at pH 7. The vesicles were maintained in the dark for 60 min. At $t = 60$ min cis pol-GUVs were briefly (1 min) irradiated at 436 nm. The percentage of empty vesicles was followed over 1 h at pH 7. (b) Same conditions as (a) at pH 8.5. (c) Same conditions as (a) at a ratio of AMP:DOPC of 0.01 g/g. (d) Same condition as (a) with 0.1 g/g pol-GUVs and DOPC GUVs irradiated at 365 nm for 3 min at pH 7 and immediately irradiated at 436 nm. The error bars represent higher and lower values from three sets of experiments.

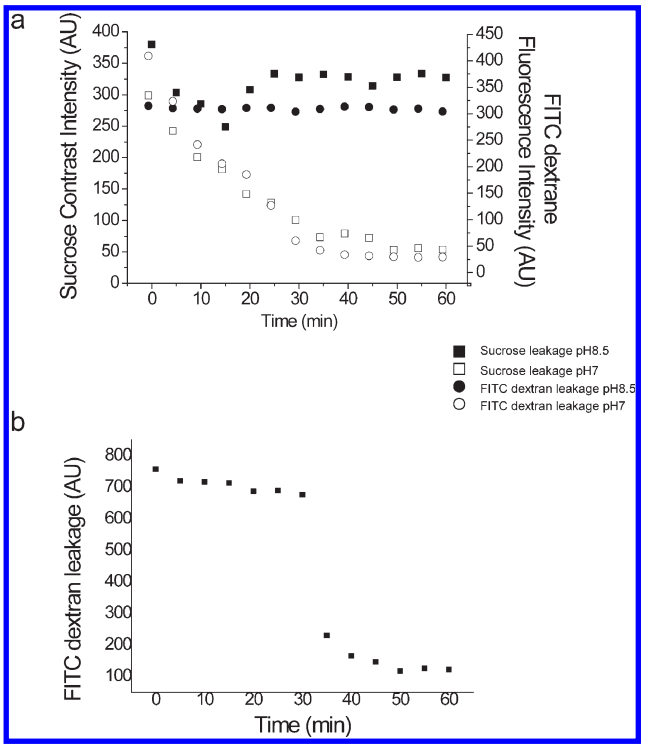

Figure 4. Leakage profiles of three isolated pol-GUVs. (a) Sucrose leakage kinetics at pH 8.5 and 7 (full and empty squares, respectively) and FITC dextran leakage kinetics at pH 8.5 and 7 (full and empty circles, respectively). (b) FITC-dextran leakage profile of another isolated pol-GUV at pH 7 as a function of time.

Beyond 40 min, the amount of empty GUVs remained stable up to 180 min (data not shown). In comparison with the lack of leakage found in the kinetics performed over 1 h incubation with AMP under its cis form (Figure 3a, time 60 min), leakage in Figure 3d points out the effect of cis to trans isomerization of AMPs. Also controls with no AMPs in GUVs or with pol-GUVs at higher pHs showed no leakage (populations of pol-GUVs diluted in pH 8.5 and DOPC GUVs both at pH 8.5 and pH 7 shown in Figure 3b,d). Kinetics were also performed in systems where pol-GUVs were diluted in glucose buffer at pH 8.5 or pH 7 in the absence of salts. No leakage was observed for pol-GUVs diluted in pH 8.5 and pH 7 buffer over an hour. This is in accordance with previous results obtained with HMPAs containing conventional (nonresponsive) hydrophobic modifications.[18,20] As for ionic strength, it is expected that high NaCl concentrations decrease charge repulsions in polyelectrolytes and tighten the binding of AMP polymers onto lipid membranes.[14] These observations point out polymer efficiency at pH and ionic strength conditions that are biologically relevant. Altogether, our data indicate also that vesicle permeability is minimal with cis-AMPs configuration and that leakage takes place upon exposure to visible light (436 nm) upon cis to trans conversion of the azobenzene moiety.

Leakage of Isolated pol-GUVs. From micrographs of pol-GUVs diluted in pH 7 buffer after irradiation at 436 nm, we recorded the decrease in internal fluorescence (FITC-dextran) and contrast intensities (sucrose) of isolated GUVs over 1 h (Figure 4a). In the example shown, the fluorescence intensity linearly decreases from time ~0 up to 35 min, time at which the intensity goes back to baseline. The same profile was also observed for sucrose leakage which indicates that both encapsulated molecules have leaked out with similar rates. On the other hand, the fluorescence signal coming from the PE rhodamine that labels the membrane remained constant during the whole experiment, which suggests that the integrity of the bilayer remains after the vesicle has leaked. The time needed to empty one isolated vesicle is however highly fluctuating. In another example shown in Figure 4b, the fluorescence intensity of an isolated GUV remained stable for several tens of minutes and then went rapidly back to baseline (< 5 min). This in accordance with previous observations of GUV supplemented with alkyl-modified polymers, which pointed out the existence of long lag times before the opening of transient pores of nanometric diameter, consistent with rapid individual leakage but a marked variability of the time of "opening" of the first pore.[19] Finally, kinetics performed on several isolated pol-GUVs, even in the same sample, provided different times for the onset of leakage and different half-time of leakage. Important variabilities in half-leakage times reveal a possible heterogeneity, in either pore diameters or the number of pores formed in a GUVs. It was however not our purpose to investigate in detail the origin of these variabilities.

Control experiments on isolated pol-GUVs were conducted at pH 8.5 (in 20 mM Tris-HCl, 150 mM NaCl) in order to fully ionize AMPs and accordingly make the polymer chain markedly more hydrophilic than at pH 7. As expected, no leakage of the fluorescent dye or the sucrose was observed at pH 8.5 over an hour (Figure 4a), since AMPs are not hydrophobic enough. The same results were observed for control GUVs made of DOPC with no polymer both at pH 7 and 8.5 with 150 mM NaCl (data not shown) in the dilution buffer. The fluorescence signals remained constant over the duration of the experiment; this rules out that bleaching contributes to our experimental leakage signals. Therefore, we assess here that AMP hydrophobicity controls pol-GUV leakage.

AMPs-Induced Cell Membrane Destabilization. We investigated by fluorescence microscopy the effects of AMPs under their cis and trans configuration on preseeded COS cells (Figure 5). Phosphatidylserine (PS) translocation in the cell plasma membrane is associated with cellular stress and can be detected via fluorescently (Cy3) labeled annexin V binding.[27] In our conditions, we could establish that PS externalization occurred in cells that were in contact with AMPs stabilized in their trans configuration immediately after irradiation at 436 nm at t0 (Figure 5b). A gradual increase in the fluorescence intensity associated with the PS externalization is visible over 60 min in these samples (Figure 5d). On the other hand, cells that interacted with AMPs stabilized in their cis state and kept in the dark for 1 h showed only minor PS externalization similar to that of the control with no AMP (Figure 5c,e,f). This minor PS externalization could also be associated with the stress coming from the lack of serum in the medium used for the 1 h long incubation. Cell viability was also monitored in parallel and extended to over 24 h (see Materials and Methods section). Basically, the green fluorescence associated with the cytoplasm (Figure 5) coming from the hydrolysis of the 6-CFDA by cytosolic esterases indicated that cells remained viable upon addition and incubation with AMPs (Figure 5d). After AMPs removal and 24 h incubation in serum supplemented culture medium, cells remained viable, with only minor PS externalization detected and persistence of cell division. We can conclude that AMPs polarity as switched by light irradiation affects the organizational state of the cell bilayer and induces a more obvious destabilization under its trans form compared to its cis form. Trans-AMPs induce PS translocation from the inner to

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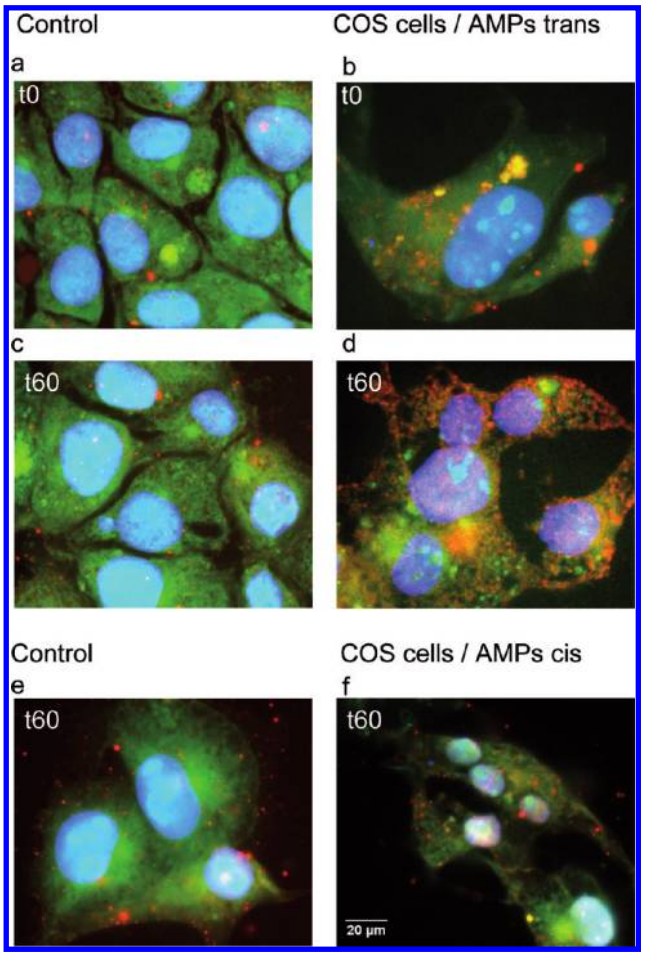

Figure 5. Effects of trans-irradiated AMPs on COS cell membrane organization and viability just after (a, b) and 60 min after polymer addition (c, d). Effects of cis-irradiated AMPs on COS cell membrane and viability after interaction in the dark for 60 min (e, f).

the outer leaflet of the bilayer without membrane breakage, and in the same time cell esterase activity in the cytosol is preserved. Although externalization of PS in the outer leaflet is a signal for apoptosis, COS cells remain viable after interaction with AMPs; this indicates that PS translocation might be a transient phenomenon that could correlate membrane destabilization and/or transient pore formation in the bilayer with weak or no leakage of the cytosolic content.

Discussion

The results presented in this report show for the first time that azobenzene-modified polymers (AMPs) are capable of triggering controlled membrane permeabilization upon visible light irradiation in pH and ionic strength conditions that are biologically relevant. AMPs belong to the class of weak polyacids whose permeabilizing features are modulated by their apparent hydrophobicity, i.e., by external conditions. Here, AMPs hydrophobicity is specifically increased by irradiation under visible light by undergoing cis (polar)−trans (apolar) isomerization. A cis-polar configuration of AMPs in the membrane preserves vesicle impermeability. Irradiation at 436 nm however induces AMPs to switch to a trans-apolar conformation; this leads to an increase in AMPs hydrophobicity which causes membrane destabilization and permeabilization, while membrane integrity is preserved as detected at the micrometer scale by optical microscopy. A tentative representation of light-induced permeabilization is

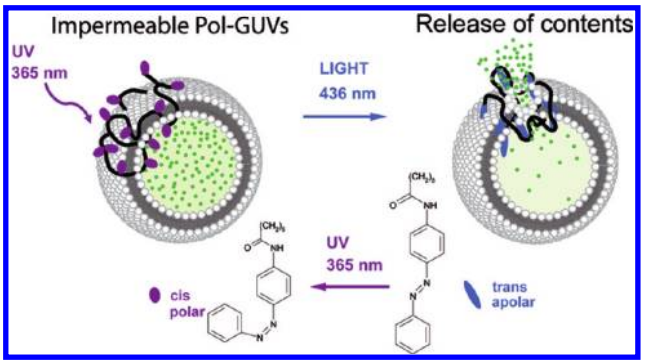

Figure 6. Illustration of light-induced membrane permeabilization. The pol-GUVs maintained impermeable when irradiated under UV light become permeable and release their internal content when irradiated at 436 nm.

schematically drawn in Figure 6. This effect is comparable to that of HMPAs with increasing hydrophobic modifications. For example, at fixed pH and ionic strength, the rate of leakage increases with increased integration level of alkyl side groups: a polymer modified with 5% octyl (C8) is less permeabilizing than a polymer with 25% C8 or a 25% C8 40% isopropyl (C3).[14] Here, AMPs have a total hydrophobic modification of 25%, which consists of 15% C8 and 10% of azobenzene graft, and their permeabilizing efficiency is in addition modulated by a change of azobenzene isomerization state. Using light irradiation allows a fine-tune of the polarity of AMP below or above a critical hydrophobicity, which depends on pH and ionic strength conditions. In our system at pH 8.5 (i.e., full ionization of the carboxylic groups in the polymer) no leakage was observed, irrespective of cis/trans isomerization. AMPs used in this study are modified with a density of hydrophobes with critical hydrophobic balance that is effective at physiological conditions.

AMPs appear remarkably efficient tools at low density of inserted alkyl groups into lipid, and this may be a reason why they only induce mild effects on membranes. Photoresponsive pol-GUVs (i.e., containing 0.1−0.01 g/g polymer/lipid ratio) are spherically shaped with no membrane defects detected. Their composition corresponds to a molar ratio of 1 to 10 octyl chains for every 100 lipids. The polymer:lipid ratio used here is thus much lower than ratios needed with surfactants to permeabilize lipid membranes (1:1 or 1:2).[20,28] A low fraction of hydrophobic anchor adsorbed in the lipid bilayers is an advantage in practice to preserve membrane stability. In addition, long sequences of hydrophilic nature hamper polymers to cross the bilayer, though they do bind tightly to the membrane interface by hydrophobic and/or amphiphilic segments. A first consequence of controlled penetration of polymers in membranes is that optimized polymers show low toxicity to cells, though they can bind tightly to them. A group of short amphiphilic copolymers called amphipols was successfully used in membrane biology for solubilizing and handling integral membrane proteins. In particular, amphipols induced only mild toxicity when added to fibroblasts.[29] Several amphiphilic copolymers have already been successfully used as delivery systems in mixed lipid/drug cargoes,[23,30] with the

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polymer corona increasing the encapsulated drug circulation lifetime *in vivo*.[31]

In practice, all this makes polymers quantity in membranes easier to handle than other permeabilizers such as surfactants that can rapidly desorb and equilibrate with solution. In particular, when it comes to cell-based assays, using AMPs as permeabilizing agents opens promising outcomes for chemical design and *in vivo* applications. When added to cells, AMPs induce membrane destabilization detected by externalization of phosphatdylserines (PS) from the inner layer to the outer layer but preserve cell viability with no toxic effects in particular after AMPs removal and cells immersion in serum supplemented medium. By doing so, AMPs could facilitate cellular uptake of molecules controlled by light irradiation, i.e., with a highly localized effect, while minimal cell toxicity is achieved. We may also reasonably expect AMPs to contribute to the design and formulation of photoresponsive objects such as stealth liposomes, which would allow local release of active compounds upon light irradiation. Because the light is easily focused, individual cells may be targeted in a population or a tissue culture.

On the mechanistic insight, it is known that polymer association induces local effects on the bilayer such as lateral lipid segregation, membrane thinning, and changes in the membrane's spontaneous curvature.[20] Here, AMPs hydrophobic side groups are attached to the polymer coil whose penetration in the hydrophobic core of the bilayer would depend on the isomerization state of the azobenzene grafts. The azobenzene cis−trans conformational change might destabilize the membrane by dragging hydrophobic groups of a surface-bound AMP into the membrane, up to a local amount of alkyl/lipid that locally disrupt the lipid self-assembly. It was recently reported that azobenzene derived lipids induce reversible vesicular budding and membrane deformation upon cis−trans irradiation.[32] Also, as described for HMPAs, it is likely that AMPs undergo pore formation.[19,33] Studies involving HMPA interactions with egg-PC lipids by current measurements through black lipid membranes pointed out a typical pore size of about 10 nm.[19] In pol-GUVs systems, the encapsulated sucrose and FITC dextran leak out with similar rates, which indicates that both of their molecular sizes are smaller than the size of the pore.

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Conclusion

The work presented here shows for the first time that membrane permeability can be induced by external light irradiation, with no membrane breakage. We have shown that AMP-loaded giant vesicles irradiated upon visible light (436 nm) are specifically turned from impermeable into permeable state. In cell cultures supplemented with AMPs under their cis form, PS externalization on cell membrane is induced via the trans-apolar isomerization of the azobenzene upon exposure to blue light. In these systems, permeabilization is dictated by the phototriggered increase of AMPs hydrophobicity. On the other hand, membrane impermeability is preserved while AMPs are in a cis-polar state. The cis form can be prepared by pre-exposure of AMPs solutions to UV light and is maintained for hours in the dark, until samples are exposed to blue light, which enables them to be used for biological applications. These features make the AMPs versatile tools for photopermeabilization based on polymer hydrophobicity balance and polymers integral to lipid membranes, including for light-controlled permeabilization of cellular membranes.

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