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Article

Encapsidation of RNA–Polyelectrolyte Complexes with Amphiphilic Block Copolymers: Toward a New Self-Assembly Route

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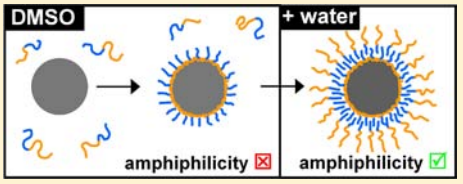
Supporting Information

ABSTRACT: Amphiphilic block copolymers are molecules composed of hydrophilic and hydrophobic segments having the capacity to spontaneously self-assemble into a variety of supramolecular structures like micelles and vesicles. Here, we propose an original way to self-assemble amphiphilic block copolymers into a supported bilayer membrane for defined coating of nanoparticles. The heart of the method rests on a change of the amphiphilicity of the copolymer that can be turned off and on by varying the polarity of the solvent. In this condition, the assembly process can take advantage of specific molecular interactions in both organic solvent and water. While the concept potentially could be applied to any type of charged substrates, we focus our interest on the design of a new type of polymer assembly mimicking the virus morphology. A capsid-like shell of glycoprotein-mimic amphiphilic block copolymer was self-assembled around a positively charged complex of siRNA and polyethyleneimine. The process requires two steps. Block copolymers first interact with the complexes dispersed in DMSO through electrostatic interactions. Next, the increase of the water content in the medium triggers the hydrophobic effect and the concomitant self-assembly of free block copolymer molecules into a bilayer membrane at the complex surface. The higher gene silencing activity of the copolymer-modified complexes over the complexes alone shows the potential of this new type of nanoconstructs for biological applications, especially for the delivery of therapeutic biomolecules.



1. INTRODUCTION

The simplest form of a virus is a genomic nucleic acid (DNA or RNA) enclosed in a capsid shell made of multiple copies of similar or identical protein subunits. In general, the encapsidation of genetic material follows a self-assembly process governed by free-energy minimization that can be reproduced to some extent in the laboratory if proper components and physicochemical conditions are provided.[1] From this perspective, there has been a continuous interest in using tools of supramolecular chemistry to design virus-like assemblies from natural or synthetic molecules.[2] The most straightforward way toward virus mimicking is obtained with viral coat protein subunits that can spontaneously self-assemble into nanocages around a genetic material. In this context, the expression "virus-like particles" (VLPs) is used to describe types of nanoparticles made of viral proteins. Nowadays, VLPs represent a platform for many applications in medicine and materials development.[3] Besides, among the different methods used to pack DNA into nanostructures without using virus subunits, the formation of polyelectrolyte complexes of DNA with cationic polymers or lipids is by far the most common approach, giving rise to so-called polyplexes and lipoplexes, respectively.[4a] This method, which relies on DNA condensation through electrostatic interactions, has been the central point of an extensive research in the framework of gene therapy.[4b−g]

A further step toward the mimicking of the virus structure is the formation of a capsid-like shell around the condensed DNA core. Beyond the biomimetic aspect, such a coating may have a beneficial impact on the stability of DNA complexes in physiological conditions preventing them from aggregating or breaking apart. In this respect, a first approach consists of forming core−shell polyplex micelles from diblock copolymers comprising a positively charged block that can electrostatically associate DNA or RNA and an uncharged hydrophilic block with stabilizing properties such as polyethylene glycol (PEG).[5] Alternately, the surface of polyelectrolyte complexes of DNA can also be covalently modified with semitelechelic or multivalent reactive polymers to create a hydrophilic shell around complex particles.[6] A second approach that mimics more closely the virus structure is the encapsulation of precondensed DNA particles within liposomes. This can be performed by complexing DNA molecules with a cationic agent and subsequently mixing them with a liposomal suspension, the

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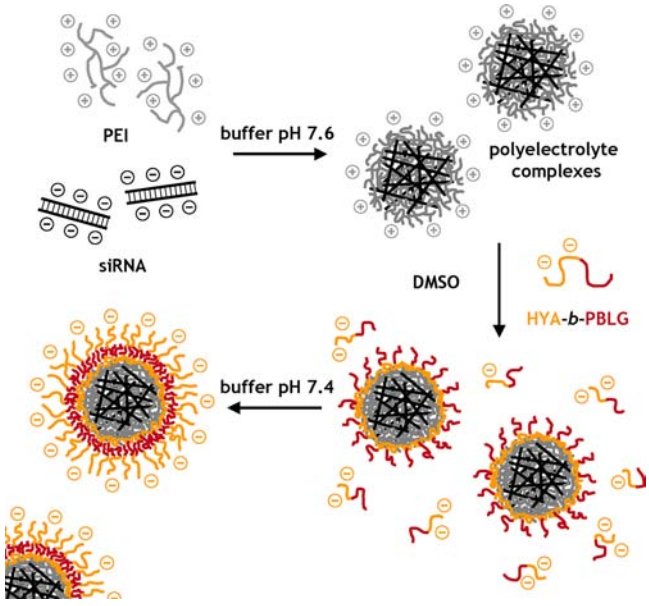
lipid membrane being sufficiently fluid to accommodate the surface of the complexes.[7]

Considering the fact that capsid proteins have a molecular weight in the range of 10−100 kDa, an even closer mimic of the virus structure would be the encapsulation of the charged DNA core within a shell made of polymers having properties similar to their protein counterparts. From that point of view, amphiphilic block copolymers are probably the best suited candidates regarding their capacity to self-assemble into vesicles (polymersomes) with membrane properties close to those of viral capsids in terms of elasticity and permeability.[8] Specifically, we showed in a previous work that polysaccharide-*block*-polypeptide copolymers, which may be considered as basic analogues of glycoproteins self-assemble into robust vesicles and as such can be used to build a synthetic capsid.[9] However, and contrary to liposomes, the direct encapsulation of DNA or RNA polyplexes within polymersomes is not possible by a simple mixing of the two species due to the high stability of the polymer vesicle membrane. Other, more sophisticated encapsulation techniques could be used, but we shall consider primarily that the genomic core in viral assemblies is in close interaction with the protein capsid. On the basis of this consideration, we studied for the first time the possibility of combining RNA−polyelectrolyte complexation and block copolymer self-assembly in a single process to build a (bi)layer of amphiphilic copolymer around a charged RNA core. The complexation of polyelectrolytes being achieved in aqueous solutions, the main difficulty is to control the amphiphilicity of the copolymer, here the hyaluronan-*block*-poly(γ-benzyl L-glutamate) (HYA-*b*-PBLG), to avoid its premature self-assembly in vesicles. Therefore, we set up a new approach (Scheme 1), which consists of (i) preparing positively charged RNA−polycation complexes of ∼150 nm in diameter in buffer solution, (ii) neutralizing the excess surface charges by complexing them with negatively charged hyaluronan blocks in DMSO, which is a good solvent for both hyaluronan and PBLG blocks, and (iii) switching to aqueous buffer to self-assemble a membrane of coat copolymer molecules surrounding the RNA core. The goal of the present work is to investigate this original self-assembly strategy by carefully analyzing the morphology and the stability of the polymer assemblies at each step of the process.

Scheme 1. Design of Virus-like Polymer Particles by Self-Assembling Amphiphilic Block Copolymer Molecules around RNA-Based Polyelectrolyte Complexes[a]



[a]See text for abbreviations of molecules.

2. MATERIALS AND METHODS

2.1. Synthesis and Self-Assembly of Hyaluronan-*block*-PBLG Copolymer. The details of the synthesis and characterization of hyaluronan-*block*-poly(γ-benzyl L-glutamate) were reported elsewhere.[10] In the present study, the number-average molecular weight (M_n) of each block was 5000 ± 300 g/mol. For fluorescence experiments with the copolymer, the amine end-group of the PBLG block was labeled with 5(6)-carboxy-fluorescein-*N*-hydroxysuccinimide ester (Thermo Scientific) following standard conjugation procedures.

The block copolymer alone was self-assembled into polymersomes through a solvent-displacement method. The block copolymer was first dissolved in DMSO, and water (or 10 mM Tris buffer at pH 7.4) was added at 0.15 mL/min to induce the formation of polymer vesicles. In a typical experiment, an initial copolymer concentration of 1 mg/mL was used, and the final water/DMSO content was set to 9/1 (v/v). In a separate experiment, the water content was varied from 0 to 90% to determine the onset and end of the self-assembly process by light scattering analysis. The dispersions were dialyzed against 10 mM Tris buffer pH 7.4 before multiangle light scattering measurements or against water before transmission electron microscopy (TEM) analysis using a Spectra/Por Float-A-Lyzer (MWCO = 20 kDa, Spectrum Laboratories) device. Experimental details about light scattering and TEM measurements are provided in the Supporting Information.

2.2. siRNA−PEI Polyelectrolyte Complexes. *Complex Formation.* PEI (branched, weight-average molecular weight (M_w) = 25 000 g/mol, M_w/M_n = 2.5) and siRNA (23 base pairs, M = 14 600 g/mol) were purchased from Aldrich. Hepes buffer (10 mM, pH 7.6) filtered onto 0.1 μm membrane was used to prepare stock solutions of PEI and siRNA at 2.98 and 2.25 g/L, respectively. The pH of the PEI solution was adjusted to 7.6 by addition of 1.0 M HCl, and the polymer concentration was corrected by the dilution factor. The concentration of siRNA in the stock solution was accurately determined with a Nanodrop 1000 (Thermo Scientific). The complexes were prepared at various N/P ratios by mixing two equal volumes of PEI and siRNA solution in a 0.6 mL microtube. The concentration of the siRNA solution was set to 0.875 g/L (=60 μM), and the required concentration of the PEI solution was calculated as follows:

$$C_{PEI} = \frac{(N/P)m_{0,PEI}V_{siRNA}C_{siRNA}2n_{bp}}{V_{PEI}M_{siRNA}}$$

with M_{siRNA} = 14 600 g/mol, $m_{0,PEI}$ = 43 g/mol (repetitive unit), n_{bp} = 23 (number of base pairs), V_{PEI}, V_{siRNA} are the volumes of PEI and siRNA solution (V = 25 μL in most experiments, but larger volumes were also used for zeta potential measurements), and C_{PEI}, C_{siRNA} are the mass concentrations of PEI and siRNA solution. Both siRNA and PEI solutions were prepared by dilution of the stock solution with Hepes buffer (10 mM, pH 7.6). The solutions were then rapidly mixed by repeated pipet aspiration and ejection during 30 s. Importantly, the addition of siRNA to PEI was preferred to keep an excess of positive charges in the medium (the reverse order of addition implies the crossing of the isoelectric point of the system located at N/P ≈ 2).

siRNA−PEI complexes prepared at various N/P ratios were analyzed with a Malvern Zetasizer (Nano ZS90). Mean hydrodynamic sizes and polydispersity indexes were derived through a cumulant analysis of the scattering autocorrelation function. The zeta potential of complexes was measured with the same instrument using the phase analysis light scattering (PALS) technology. All of the measurements were the average of at least five runs performed at 25 °C. Complexes

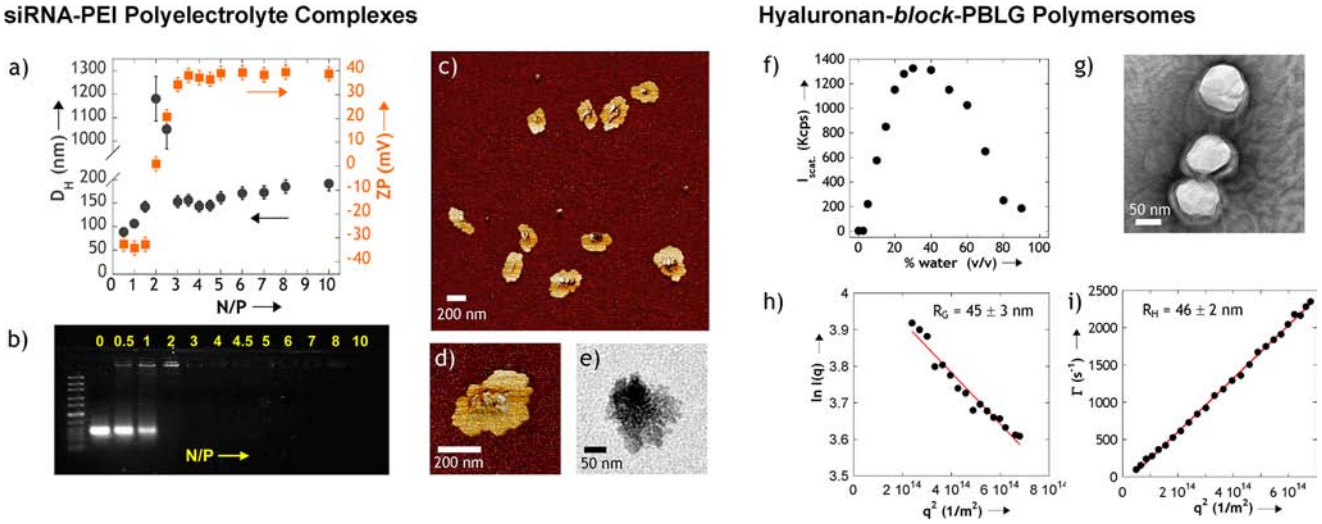


Figure 1. Physico-chemical characterization of siRNA–PEI polyelectrolyte complexes and hyaluronan-*b*-PBLG vesicles in aqueous solutions. (a) Hydrodynamic diameters (D_H) and zeta potential (ZP) values of siRNA–PEI complexes prepared at various nitrogen to phosphate (N/P) ratios in 10 mM Hepes buffer at pH 7.6 and with a final concentration of 30 μM siRNA. (b) Agarose gel electrophoresis of siRNA–PEI complexes prepared under the same conditions as in (a). The N/P ratio used to form each complex is given above the corresponding lane. The first lane corresponds to 10–300 bp DNA ladder. (c,d) 3D AFM imaging of siRNA–PEI complexes prepared at N/P = 4. (e) TEM picture of complexes prepared in same conditions. (f) Light scattering intensity (I_{scat}) of a 0.1% (w/v) hyaluronan-*b*-PBLG solution in DMSO at various water contents. (g) TEM picture of block copolymer vesicles. (h,i) Light scattering analysis of block copolymer vesicles in 10 mM Hepes buffer pH 7.4. (h) Static mode: guinier representation giving the radius of gyration (R_G). (i) Dynamic mode: dependence of the decay rate (Γ) on the square of the scattering vector (q^2) from which the value of the hydrodynamic radius (R_H) is derived. The R_G/R_H ratio is close to the unity, which is in agreement with a vesicular morphology.

were imaged by TEM after 1:200 dilution with water and without staining agent. The complexation of PEI and siRNA was also visualized by gel retardation assay. The complexes prepared at various N/P ratios were submitted to electrophoresis in TBE 1X agarose (4%, Fermentas) gel with SYBR Green (Invitrogen) at 100 V for 30 min and visualized under UV light. An ultra low range DNA ladder (10–300 bp, O'gene ruler, Fermentas) was used as reference.

Atomic Force Microscopy (AFM) Analysis of Polyelectrolyte Complexes. AFM measurements were performed at room temperature using a Veeco Dimension Icon AFM system equipped with a Nanoscope V controller. Both topographic and phase images of individual complexes were obtained in tapping mode using a rectangular silicon cantilever (AC 160-TS, Atomic Force, Germany) with a spring constant of 42 N m^{-1}, a resonance frequency lying in the 290–320 kHz range, and a radius of curvature of less than 10 nm. The scan rates were in a range of 0.6–0.8 Hz. Measurements of height, width, and spacing were taken using the section analysis tool provided with the AFM software (Nanoscope Analysis V1.20 from Bruker).

The aqueous dispersions of siRNA complexes were diluted 10^5 times with water before AFM analysis. To reduce the strong interaction with negative charges of mica substrate, freshly mica sheets were dipped into an aqueous solution of polyethylenimine at 3 g/L adjusted at pH 7.4 for 1 h, rinsed two times with deionized water, and dried under a nitrogen stream. One droplet of 10 μL of solution of siRNA complexes was then deposited on positively charged mica surface and allowed to dry overnight at room temperature.

2.3. Hyaluronan- and Copolymer-Modified siRNA–PEI Complexes. The typical protocol used to modify the surface of siRNA–PEI complexes is given in Figure 2. A fresh preparation of complexes (N/P = 4) in 10 mM Hepes buffer at pH 7.6 was used for each experiment. After each mixing step, the dispersions were homogenized by repeated pipet aspiration and ejection during 30 s. When DMSO solutions of hyaluronan or copolymer were mixed with the aqueous dispersions of complexes, the resulting solution was left to stand at room temperature for 1 h because of the temperature increase related to the exothermic behavior of DMSO/water mixtures. The same protocol of preparation of copolymer-modified complexes was used with fluorescein-labeled copolymer and cyanine 5-modified siRNA (Thermoscientific). The whole process was followed by means of dynamic light scattering and zeta potential measurements (see Figure 2). The values of viscosity, refractive index, and dielectric constant of DMSO/water mixtures were found in the literature.[11]

For AFM analysis and biological experiments, the dispersions were dialyzed against water to remove the salts and the DMSO. One droplet of 10 μL of dispersion diluted ten times with water was then deposited onto freshly cleaved mica and dried overnight at room temperature. The same conditions were used with naked glycopolypeptide vesicles. Fluorescence microscopy experiments were performed with a Leica DMR (Leica Microsystems, Wetzlar, Germany) epi-fluorescence microscope using a HCX PL APO 63× oil NA 1.32 objective. This microscope is equipped with an excitation and emission filter wheel. The camera used on this system is a CoolSnap HQ (Photometrics, Tucson, AZ).

2.4. Biological Experiments. *Cell Culture.* The human HeLa cell line was grown as monolayer in DMEM (Dulbecco's modified Eagle medium) supplemented with 10% fetal calf serum and 2 mM L-glutamine at 37 °C in a 5% CO_2 atmosphere. The cell line was routinely subcultured at a 1/10 split ratio after dissociation of the cell layer by a trypsine/EDTA solution. All tissue culture reagents were purchased from Invitrogen.

Fluorescence Microscopy. Eight × 10^4 cells per well were cultured in a 24-well plate containing a glass coverslip per well. The following day, they were incubated with 50 nM of siRNA–PEI complexes modified with different concentrations of copolymer in the complete growth medium. After 24 h, the cell layers were rinsed twice with phosphate-buffered saline (PBS) and fixed in 3% formaldehyde in PBS for 15 min at room temperature. The nuclei were stained with 1 μg/mL 4′,6′-diamidino-2-phenylindol (DAPI) in PBS for 5 min at room temperature. The glass coverslips were then mounted on glass slides using Vectashield (Vector Laboratories) mounting solution. The slides were observed on a Leica confocal microscope.

Cell Transfection and siRNA-Mediated Gene Silencing. Eight × 10^4 cells per well were cultured in a 24-well plate. The following day, cells were cotransfected with 1 μg/well GL4.15-SV40 firefly luciferase (Fluc) reporter harboring the firefly *luc2p* gene under the control of the SV40 promoter (Promega) and 100 ng/well pRLSV40 *Renilla*

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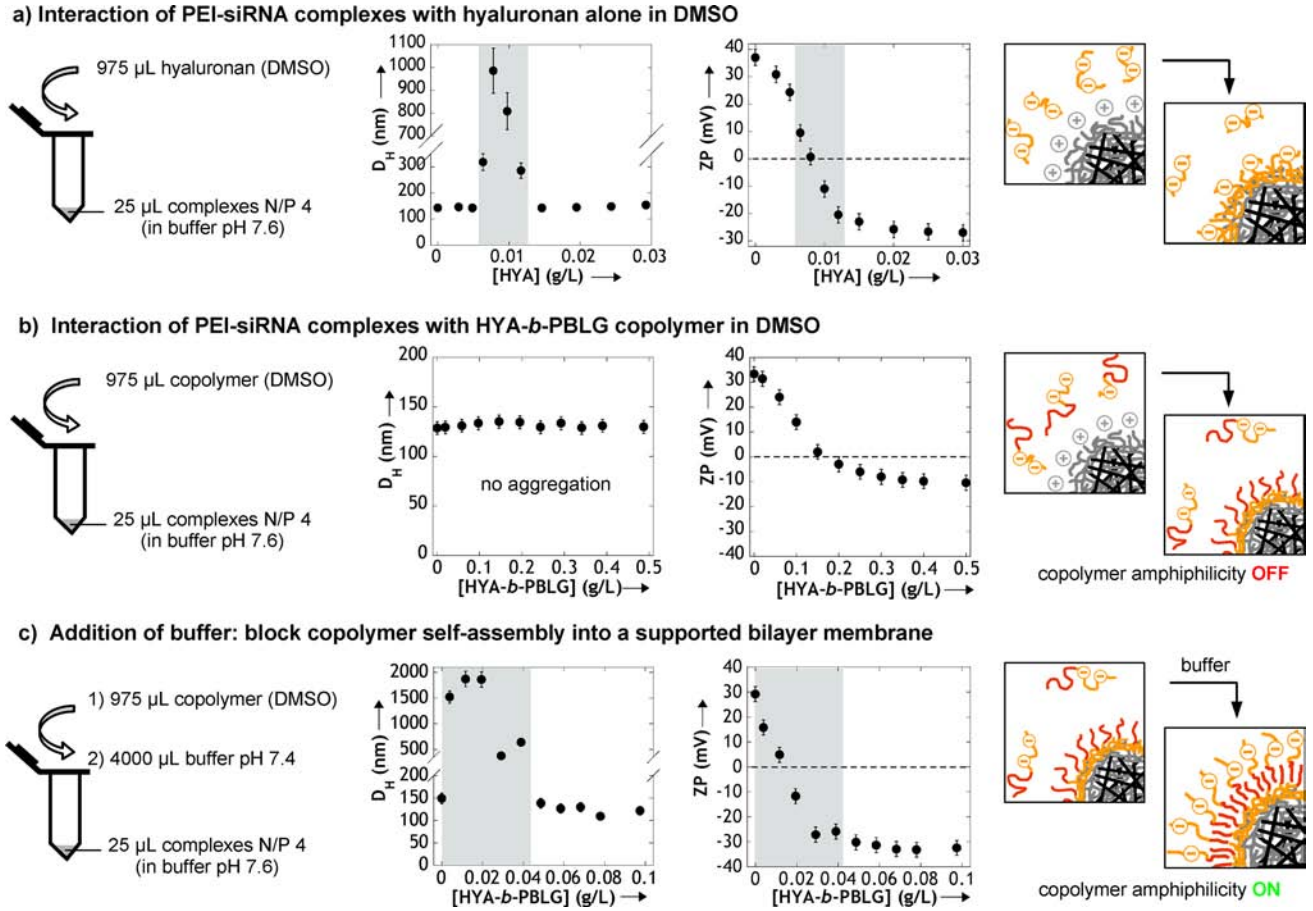

Figure 2. (a) Interaction of hyaluronan (HYA) with siRNA–PEI complexes (N/P = 4, C_{siRNA} = 30 μM) in DMSO/Hepes buffer (97.5/2.5) (v/v). (b and c) Two-step assembly of HYA-*b*-PBLG copolymer around siRNA–PEI polyelectrolyte complexes. First step (b): Interaction of the copolymer with complexes in DMSO/Hepes buffer (97.5/2.5) (v/v). Second step (c): Addition of Hepes buffer in the medium up to a final DMSO/buffer composition of 20/80 (v/v). The mean hydrodynamic diameter (D_H) and zeta potential (ZP) of resulting particles are plotted as a function of the concentration of hyaluronan (a) or copolymer (b and c). Similar plots of the scattering intensity (I_{scat}) and the polydispersity index (PDI) can be found in the Supporting Information. The gray area represents the domain of colloidal instability where particles sediment. Importantly, the amphiphilicity of the copolymer is turned OFF and ON when changing from DMSO to buffer.

luciferase (Rluc) reporter vector (internal control; Promega) using Lipofectamine 2000 (Invitrogen) as recommended. The transfected cells were incubated for 18 h at 37 °C, washed with culture medium, and incubated at 37 °C with siRNA–PEI complexes at different concentrations of specific anti-*luc2p* siRNAs (5′-GCAGCUUGCAA-GACUAUAATT-3′; 5′-GCCCUGAUCAUGAACAGUATT-3′; 5′-CUGGUGCCCACACUAUUUATT-3′) or control siRNA containing four mismatches (5′-UCCAUGAGCAUGACCAGUATT-3′), with or without different copolymer formulations, in the complete growth medium. After a 4 h incubation, the cell layers were washed again and further incubated at 37 °C for 48 or 72 h. The cells were then rinsed twice with ice-cold PBS and lyzed in 100 μL of lysis buffer (Promega). Firefly and *Renilla* luciferase activities were measured on 10 μL of cell lysate using the Dual-Luciferase system (Promega) on a BERTHOLD luminometer. The specific firefly luciferase silencing was determined by the ratio $(Fluc/Rluc)_{anti\text{-}luc2p\ siRNA}/(Fluc/Rluc)_{control\ siRNA}$.

3. RESULTS AND DISCUSSION

Branched polyethyleneimine (PEI, M_w = 25 000 g/mol) and small-interfering RNA (siRNA, 23 base pairs) were selected as model polyelectrolytes to build the positively charged electrostatic core in aqueous solvent (10 mM Hepes buffer pH 7.6).[12] A combination of dynamic light scattering, zeta potential, and gel electrophoresis measurements was used to follow the complexation at various PEI nitrogen to RNA phosphate (N/P) ratios. Stable and positive colloidal complexes are obtained above the isoelectric point located at N/P = 2 (Figure 1a). The absence of siRNA release in this range of N/P indicates strong electrostatic interactions between siRNA and PEI (Figure 1b). The hydrodynamic diameter of the complexes slightly increases from 150 nm (N/P = 3) to 185 nm (N/P = 10), supporting the fact that the complexation stoichiometry is probably not constant when the N/P ratio increases. The structure of siRNA–PEI complexes at N/P = 4 was observed by TEM and AFM (Figure 1c–e). Both imaging techniques show a core–shell structure resulting from the segregation of complexed segments into particles stabilized by free unpaired segments of excess PEI. This type of structure is probably also obtained for polyelectrolyte complexes formed at higher N/P ratios. Importantly, neither DLS nor AFM analysis put in evidence the presence of free PEI in the medium at N/P = 4, which tends to demonstrate that the amount of uncomplexed PEI must be relatively low. Another possibility is that free PEI chains are physisorbed on complex particles through non-electrostatic interactions. Colloidal particles of polyelectrolyte complexes are usually characterized by a rather high size-dispersity in relation with the formation mechanism where kinetically frozen aggregates are favored over thermodynamically equilibrated ones.[13] Here, however, we found that a small

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pH change of the complexation medium significantly influences the size distribution of complexes; particles are better defined at pH 7.6 than pH 7.2 (see the Supporting Information). The lower charge density of PEI at pH 7.6[14] probably favors molecular rearrangements toward a better ion-pairing within complexes resulting in a closer-to-equilibrium system. The hyaluronan-*block*-PBLG copolymer ($M_{n,HYA}$ = $M_{n,PBLG}$ = 5000 g/mol) was chosen as biomimetic component to build the synthetic capsid around siRNA−PEI complexes. This amphiphilic glycopolypeptide copolymer cannot be self-assembled by direct dissolution in water because of the high cohesive energy between PBLG blocks.[10] Therefore, the copolymer must be first dissolved in DMSO, which is a good solvent for both blocks, and then aqueous buffer (10 mM Hepes at pH 7.4) is added dropwise to the solution, resulting in a poorer solvent for the PBLG blocks. The phase separation in the copolymer solution starts from a critical water content of 5% (v/v) as determined by light scattering (Figure 1f) measurements at increasing concentrations of water. Such a low tolerance of the copolymer to water arises from the highly hydrophobic character of the PBLG blocks, the hyaluronan segments being fully soluble in buffer. TEM and light scattering analyses evidence the block copolymer self-assembly into vesicles of 90 nm in diameter (Figure 1g−i).

Following the strategy toward virus-like assemblies devised in Scheme 1, anionic hyaluronan blocks of copolymer have to interact electrostatically in DMSO with free positive charges of PEI present at the surface of polyelectrolyte complexes. Importantly, this step must be achieved without self-assembling the copolymer; that is, the water content in the medium must be lower than 5%. In an organic solvent such as DMSO, we expect a moderate strengthening of Coulomb forces with decreasing dielectric constant of the solvent (ε_{DMSO} = 47, ε_{water} = 81) and a weakening of hydrophobic forces.[15] Both effects may affect the stability of polyelectrolyte complexes and their interaction with charged hyaluronan blocks. Therefore, we first investigated the behavior of complexes in DMSO in the presence of hyaluronan homopolymer. In a typical experiment, 975 μL of a DMSO solution of hyaluronan, varying in concentration, was added to 25 μL of siRNA−PEI complexes prepared in Hepes buffer (pH 7.6) at N/P = 4, so that the final water content was 2.5% (v/v). Figure 2a shows that the structure of complexes is likely to be preserved in DMSO-rich solution as the values of hydrodynamic size and zeta potential are similar to the ones previously determined in Hepes buffer (see Figure 2a at [HYA] = 0 g/L).[16] It is worth noting that the complexes remain positively charged in DMSO despite the relatively low protonation degree of PEI and the basic character of DMSO molecules. Because the structure of the complexes seems not to be affected by the DMSO, it is reasonable to assume that siRNA molecules keep their bioactivity. When the concentration of hyaluronan increases an aggregation/dispersion phenomenon concomitant to the charge inversion of the system is observed (Figure 2a). This suggests a predominantly electrostatic interaction between the polysaccharide in solution and the positive amino groups at the surface of the complexes. Such interaction requires deprotonation of the hyaluronan, which was acidified prior to solubilization in DMSO. Therefore, the proton transfer from the hyaluronan carboxylic acids to the uncharged amino groups of PEI through acid−base reaction is likely to occur in DMSO solution. A similar mechanism of interaction was proposed by Cui et al. with poly(acrylic acid) and diamine in THF/water mixtures.[17] However, the interaction process is certainly more complex, and specificities related to secondary interaction like hydrogen bonding, peculiar solvency properties of DMSO,[18] and charge regulation effects between oppositely charged groups should be considered as well to get a better description of the system. The colloidal stability of modified particles of complexes observed on both sides of the isoelectric point is in line with a predominantly electrostatic stabilization. Eventually, the strong negative value of zeta potential obtained with excess hyaluronan (ZP = −27 mV, Figure 2a) indicates an overcharging of the particles of complexes, which is a typical effect obtained with oppositely charged macroions.[19]

The interaction of hyaluronan with siRNA−PEI complexes in DMSO being established, the next step consists of studying the interaction of HYA-*b*-PBLG glycopolypeptide in same conditions. Figure 2b shows a similar charge reversal of the particles of complexes when the concentration of copolymer increases, the remarkable difference from previous experiments with hyaluronan being that absolutely no particle aggregation could be detected at the isoelectric point by dynamic light scattering. Hence, this supports a mechanism of copolymer adsorption onto complexes where the driving force is the electrostatic interaction of hyaluronan blocks with the positive surface charge of the complexes, while the PBLG blocks that have no affinity for PEI extend into the DMSO-rich solution and form a steric layer, thus imparting stability. In this respect, the moderate surface charge of the complexes in the presence of excess copolymer (ZP = −10 mV, Figure 2b) confirms the predominance of a steric stabilization of copolymer-modified complexes over an electrostatic one.

In the last step, the water content is increased from 2.5% to 80% (v/v) by dropwise addition of Hepes buffer at pH 7.4 in the above dispersion of complexes. Appearances notwithstanding, this operation is not trivial due to heat released by the mixing of water with DMSO, which may affect the stability of complexes in solution.[20] However, we verified the integrity of native siRNA/PEI complexes in terms of hydrodynamic size, polydispersity, and charge surface after addition of the buffer in the DMSO solution (see Figure 2c at [HYA-*b*-PBLG] = 0 g/L). In the presence of copolymer, the dispersion of complexes exhibits a very different behavior from the one observed in DMSO. Here, the surface charge inversion of complexes at increasing concentration of copolymer is accompanied by a marked phenomenon of aggregation/dispersion as seen by the variation of the hydrodynamic size (Figure 2c). Also, the zeta potential reaches a much lower value (ZP = −33 mV) than previously in DMSO before addition of buffer. It is believed that both results traduce the formation of a bilayer of amphiphilic copolymer with stabilizing properties around the core of siRNA−PEI complexes. The proposed mechanism is the following: the first layer of adsorbed copolymer at the surface of complexes is preserved when the system is switched from DMSO to buffer. In this condition, the complexes are not stabilized because PBLG blocks are highly hydrophobic. However, from a certain concentration of HYA-*b*-PBLG, the slow addition of buffer into DMSO solution can initiate the self-assembly of excess free copolymer chains that coexist with adsorbed ones. As for any surfactant, the amphiphilic molecules of glycopolypeptide start to adsorb at interfaces, here, at the surface of complexes that displays PBLG blocks. Consequently, the formation of a copolymer bilayer through mutual hydrophobic interactions between PBLG blocks is the most likely process to occur at the particle surface. In this view, the

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highly negative zeta potential of modified complexes confirms the buildup of an outer layer of copolymer featuring hyaluronan blocks that are fully deprotonated at pH 7.4. The dissociation of aggregates into small and well-stabilized particles at $C_{copolymer}$ = 0.05 g/L points out the completion of the bilayer self-assembly and its positive impact on the particle stabilization (Figure 2c). The strong increase of the scattering intensity observed at the same copolymer concentration also supports a profound modification of the optical properties of the complex particles, which is in line with the formation of a copolymer membrane (see the Supporting Information). Above $C_{copolymer}$ = 0.05 g/L, glycopolypeptide-modified complexes must coexist with naked polymer vesicles. However, the size of the two types of particles is too close to be discriminated by dynamic light scattering,[21] and consequently no significant increase of the size-polydispersity was determined. Importantly, we verified by gel electrophoresis experiments that no siRNA was released during the assembly process, which evidences the stability of the polyelectrolyte core in such condition (see the Supporting Information).

AFM imaging shows that vesicles of HYA-*b*-PBLG have a collapsed structure in agreement with previous studies on PBLG-based copolymers (Figure 3a).[10a,22] Even if PBLG is usually considered as a rigid block, the capillary forces between the copolymer vesicles and the mica surface account for the vesicle flattening. Also, it is likely that the residual water inside the vesicles plasticizes to some extent the chains of PBLG. The fact that glycopolypeptide complexes assume the same morphology as the naked vesicles strongly supports the formation of a copolymer membrane around the polyelectrolyte complexes (Figures 3b). The similar height profile obtained for copolymer vesicles and copolymer-modified complex particles suggests that siRNA–PEI complexes behave as relatively soft structures embedded in the copolymer shell (see the height profile of the complexes alone in the Supporting Information). Besides, the larger dimensions of the copolymer-modified complexes can be attributed to a templating effect of the siRNA–PEI complexes whose initial size is in the 150 nm range while the vesicle dimension is only 90 nm as seen previously. The PBLG thickness in the membrane is estimated to be 3.5 nm if one assumes, in a first approximation, that PBLG blocks adopt an α-helical conformation and are stacked in a strictly antiparallel orientation.[9] By using fluorescent-tagged molecules of copolymer and siRNA, fluorescence microscopy also confirms the colocalization of both components within same aggregates at $C_{copolymer}$ = 0.05 g/L (Figure 3c), which is the concentration required to self-assemble the copolymer membrane around the complexes (see Figure 2c).

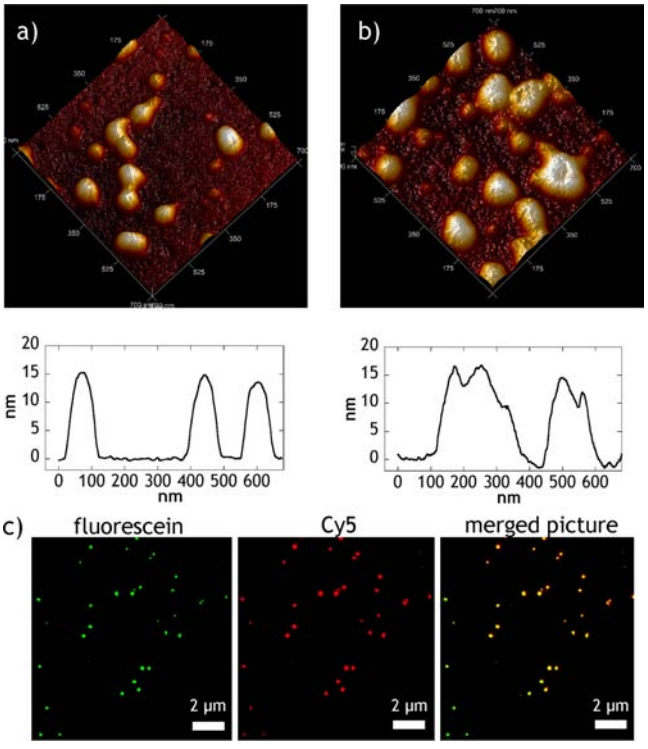

Figure 3. 3D AFM pictures (700 nm × 700 nm) and height profiles of naked glycopolypeptide vesicles (a) and glycopolypeptide-modified siRNA–PEI complexes (b).The high length to height ratio of the particles is in agreement with a soft vesicle-like morphology that has collapsed during the drying step. (c) Fluorescence microscopy analysis of glycopolypeptide complexes with fluorescein-labeled copolymer (λ_{ex} = 490 nm) and cyanine 5-modified siRNA (λ_{ex} = 649 nm). The concentration of copolymer is set to 0.05 g/L in both experiments, that is, the concentration required to self-assemble a copolymer bilayer at the complex surface (see Figure 2c).

The dual structure of the copolymer-coated complexes of PEI–siRNA was also highlighted through simple biological experiments. There is a clear correlation between the concentration of the copolymer used to modify the surface of complexes and the amount of siRNA taken up by HeLa cells. Above $C_{copolymer}$ = 0.05 g/L where empty copolymer vesicles start to coexist with copolymer-modified complexes, the fluorescence intensity of the Cy5-labeled siRNA decreases in the cytoplasm, while the level of fluorescence of fluorescein-labeled HYA-*b*-PBLG remains high (Figure 4). This observation supports a dilution effect of the particles of complexes by an excess of free copolymer vesicles. It also confirms the existence of a threshold copolymer concentration needed for the membrane formation around complexes. The same behavior applies when considering the gene silencing activity of siRNA. Indeed, Figure 4b shows a typical siRNA concentration-dependent knockdown of the firefly luciferase activity with siRNA–PEI complexes modified with copolymer at C = 0.05 g/L, whereas no specific activity of siRNA is detected at higher concentration of copolymer. Importantly, higher levels of inhibition of firefly luciferase expression are obtained with siRNA–PEI complexes embedded in a membrane of glycopolypeptide than naked complexes (Figure 4a). It is hypothesized that the co-complexation of PEI with hyaluronan blocks in the inner layer of the membrane facilitates the dissociation of the polyelectrolyte core and the concomitant release of siRNA molecules in the cytoplasm.

4. CONCLUSION

In summary, we report both a new method to self-assemble amphiphilic block copolymers in solution and a new type of colloidal polymer assemblies mimicking roughly the virus structure. While amphiphilic block copolymers are typically self-assembled in water into micelles or vesicles that may possibly be adsorbed onto solid surfaces, we propose here a way to self-assemble them into a bilayer membrane at the surface of a charged substrate. The method that consists of two steps exploits the fact that the amphiphilicity of the copolymer can be turned off and on by varying the polarity of the solvent. In a first step, the copolymer is solubilized in DMSO where it cannot self-assemble but still has the possibility to interact

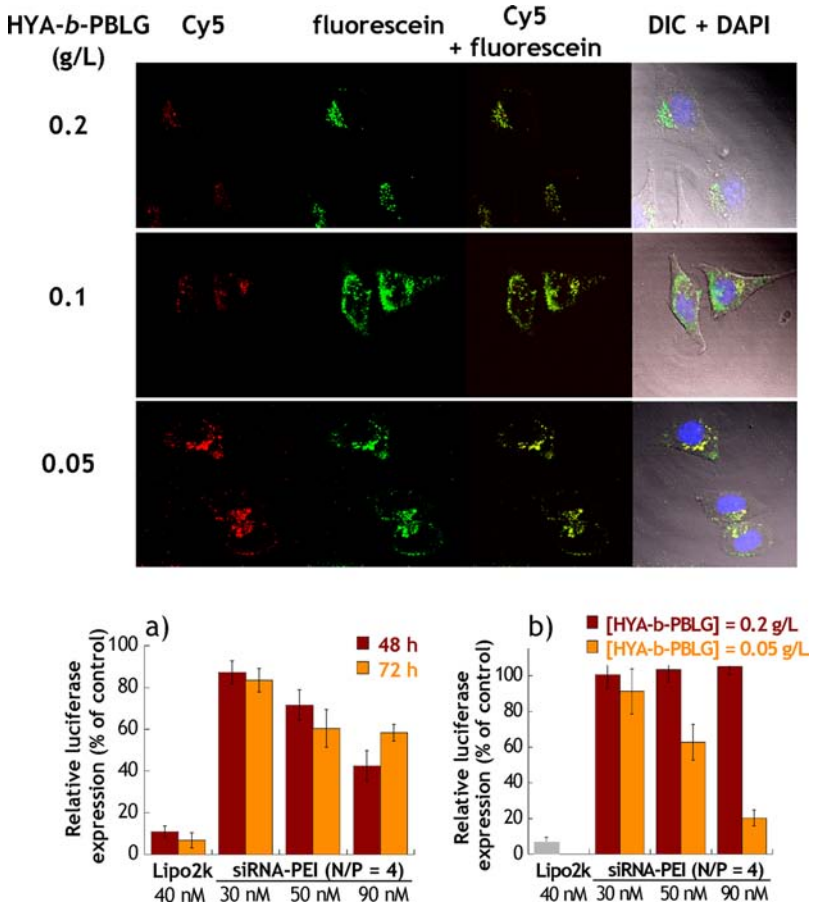

Figure 4. (Top) Uptake and intracellular distribution of HYA-*b*-PBLG-modified siRNA–PEI complexes prepared at different concentrations of copolymer into HeLa cells after 24 h of incubation ([siRNA] = 50 nM). Copolymer and siRNA molecules are labeled with fluorescein and Cy5, respectively. Last column: Overlay of DIC (differential interference contrast) and fluorescence images (blue: nuclei stained with DAPI). (Bottom) Relative gene expression of firefly luciferase with Renilla luciferase as internal control after incubation of HeLa cells with siRNA complexes prepared in different conditions. (a) siRNA–PEI complexes alone (N/P = 4) at varying concentrations of siRNA and comparison with lipofectamine 2000 (Lipo 2k). (b) siRNA–PEI complexes modified with HYA-*b*-PBLG copolymer (results at 72 h). The data represent means ± SD and are representative of three independent experiments.

electrostatically with an oppositely charged substrate. This leads to the formation of a first layer of adsorbed copolymer. Next, in a second step, the amphiphilicity of the copolymer is turned on by adding water in the medium. This triggers the copolymer self-assembly; that is, polymer chains interact with the first adsorbed layer to form a supported bilayer membrane. From a conceptual point of view, we believe that this finding is an important advance in block copolymer self-assembly. The process that has some analogy to the surface layer-by-layer approach is easy to perform and could be applied to a large range of amphiphilic copolymers and charged substrates including micro-, nanoparticles and flat surfaces. Here, the method was applied to build up a membrane of amphiphilic glycopolypeptide molecules around an electrostatic complex of RNA and polycation. While the vesicles of amphiphilic block copolymers (polymersomes) have been often compared to viral capsids, this is the first evidence of the successful encapsidation of a charged particle of complexed nucleic acid within such structures. Interestingly, siRNA–PEI complexes coated with the copolymer have a higher gene silencing activity than naked complexes. This atypical behavior probably results from the formation of a ternary complex between siRNA, hyaluronan, and PEI, which in turn favors the release of siRNA molecules in the cell cytoplasm.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Characterization of HYA-*b*-PBLG vesicles, siRNA-PEI polyelectrolyte complexation at various pH's, AFM analysis of complexes, scattering intensity and polydispersity index of hyaluronan- and copolymer-modified complexes, and stability of copolymer-coated complexes. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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