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Polyplex-embedding in polyelectrolyte multilayers for gene delivery

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Abstract

In this work, incorporation of plasmid DNA, pre-complexed with PEI, into polyelectrolyte multilayers has been studied to further develop platforms for local gene delivery. Polyplex embedding in synthetic and naturally degradable architectures was efficient for transfection of human hepato-cellular carcinoma cells.

Keywords: Polyelectrolyte multilayer; Gene delivery; Polyplex

Polyelectrolyte multilayers represent a new type of interface to functionalize biomaterials. Generated by alternate adsorption of polyanions and polycations, they have provided the opportunity to develop platforms for delivery of bioactive molecules such as signal molecules and drugs [1–9], the mode of incorporation of which (simple adsorption, chemical grafting, complexation with nanoparticles) depends on the chemical characteristics of the molecule (charge, size, solubility). Recently, multilayered assemblies made of naked plasmid DNA and a degradable polyamine were shown to be efficient in the release of plasmid DNA and subsequent in vitro transfection of adherent COS-7 cells [10,11]. The sequential assembly of plasmid DNA and poly(ethyleneimine) (PEI) has also been described, applied to the fabrication of transfected cell microarrays [12]. Such a loading of DNA onto the surface of an electrode was stable under physiological conditions and efficient transfection of DNA was achieved by applying a short electric pulse to the cell–electrode system. As an alternative to these systems, we have evaluated the incorporation of plasmid DNA, pre-complexed with PEI to form polyplexes, into multilayered architectures made of natural degradable and synthetic polelectrolytes. Our hypothesis was that embedding of polyplexes would further be exploited to generate assemblies able to sustain DNA availability thanks to the sequential incorporation of complexes at different levels of the architecture. The further possibility to tailor such polyelectrolyte assemblies with additional functionalities would also constitute a major advantage of the system.

Before incorporation in the multilayer, plasmid DNA (pEGFP-C1 expressing a stable variant of GFP) (Clontech BD Science) was complexed with 22 kDa linear polyethylenimine (PEI) (Polyplus Transfection) to form positively charged polyplexes. pEGFP-C1 was amplified in *Escherichia coli* DH5α strain, and purified using the "Endofree plasmid Maxi kit" from Qiagen. Polyplexes were formed by diluting plasmid and polycation separately in HEPES buffer (20 mM HEPES, NaCl 0.15 M). An appropriate volume of PEI diluted to a concentration of 10 mM of nitrogen atom was added to the plasmid solution (1 μg in 100 μL). The solution was vortexed and incubated for 15 min before using for build-up of functionalized multilayers. Over the N/P ratio of 5, polyplexes were positively charged (data not shown) and were expected to adsorb on polyanions. Polyelectrolyte multilayers were prepared on glass coverslips (CML, France) pretreated with 10^{-2} M SDS and 0.12 N HCl for 15 min at 100 °C, and then extensively rinsed with deionised water. Glass coverslips (2 cm^2 in diameter) were deposited in 24 well plates (Nunc, Danemark). Poly-(L-lysine) hydrobromide (PLL, MW = 30.3 kDa), poly-(L-glutamic acid) (PGA, MW = 54 kDa), chitosan (CHI, MW = 100 kDa), hyaluronan (HA, MW = 400 kDa), poly-(allylamine hydrochloride) (PAH, MW = 50 kDa) and poly-(sodium-4-styrene-sulfonate) (PSS, MW = 70 kDa), purchased from Sigma, were used without any further purification. Architectures made of *n*(PAH-

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PSS), *n*(PLL/PGA) and *n*(CHI-HA) bilayers were built by alternated immersion of the pre-treated coverslips during 10 min in polyelectrolyte solutions (300 μL) at the concentration of 1 mg/mL for PAH, PSS, PLL, PGA and HA and 0.5 mg/mL for CHI, in HEPES buffer, at pH = 7.4 for PAH/PSS and PLL/PGA, and pH = 5 for CHI/HA architectures, respectively. 300 μL of a solution of PEI/pEGFP-C1 complexes prepared at the N/P of 12, containing 1 μg of pDNA, was added on polyanion-ending architectures for 1 h. The amount of pDNA effectively deposited on PLL/PGA- and CHI/HA- based architectures during this step was 50 and 65 ng (25 and 30 ng/cm^2), respectively. After polyplex deposition, alternate polyelectrolyte adsorption was pursued for polyplex embedding. After each deposition, the coverslips were rinsed three times during 10 min with HEPES buffer.

In order to take into account possible rearrangements of the complexes with polyanions [13] during the build-up process, we preliminary analyzed the interaction of polyanions and polyplexes by gel electromobility shift assays. An equal volume of PEI/pEGFP-C1 polyplex solution (N/P = 12) and increasing concentrations of HA, PSS, or PGA in HEPES buffer were incubated together during ten min before being submitted to electrophoresis in 1% agarose gels for 1 h at 77 V. PSS and PGA shifted the complexes even at low concentration, the threshold being between 20 μg/mL and 50 μg/mL, but HA did not (Fig. 1). Considering that 100 μg/mL was necessary for the homogenous adsorption of polyelectrolyte at each adsorption step, one could assume that PSS or PGA could lead to destabilisation of polyplexes during the build-up process. In opposition, incorporation of HA would not induce DNA shift from PEI/DNA complexes but would ensure polyplexes a conserved structure. Consistently, further build-up of architectures made of PAH/PSS and PLL/PGA bilayers should include the deposition of HA after complexes deposition to preserve the pre-complexation of DNA with PEI. To check the feasibility of this approach, charge surface measurements were done on polyplexes (N/P = 12) after adsorption of HA at different concentrations. Surface charge switched from +(16.6 ± 1.3) mV to −(32.6 ± 0.7) mV after deposition of HA at the concentration of 1 mg/mL, signing the coating of polyplexes by HA.

The build-up of polyplex-functionalized multilayers was analyzed by Fourier transform infrared spectroscopy (FTIR) in the attenuated reflexion mode using an Equinox 55 FTIR spectrometer (Brucker, Germany) with a liquid-nitrogen-cooled MCT detector in buffers made from D_2O as usually done in the conformational study of polypeptides [14]. Reference and sample spectra were taken by collecting 512 interferograms at 2 cm^{-1} resolution in the totally attenuated reflexion mode. The multilayers were built up layer by layer on ZnSe crystal as in previous studies [15]. Fig. 2 shows spectra obtained during the build-up of a $(PAH/PSS)_6$((PEI/pEGFP-C1)/HA)$(PLL/PGA)_3$ architecture. Generally, the infrared spectra of DNA exhibit characteristic bands of base pairs at 1602 cm^{-1} (adenine), 1650 cm^{-1} (thymine), 1684 cm^{-1} (guanine) and 1481 cm^{-1} (cytosine) [16] in addition to bands attributed to the phosphate groups. In this study, it was

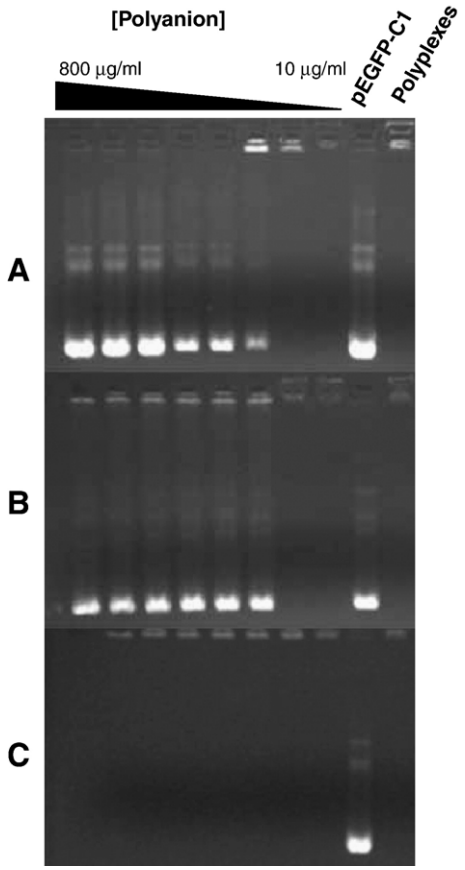

Fig. 1. Polyanions exchange with polyplexes. PEI/pEGFP-C1 polypexes were pre-incubated with PGA (A), PSS (B), HA (C) in a concentration range of 10 to 800 μg/mL indicated by the arrow and submitted to electrophoresis. Control patterns on the right resulted from the electrophoretic mobility of pEGFP-C1 and PEI/pEGFP-C1 polyplexes, respectively, in the absence of pre-incubation with polyanion.

only possible to use the bands attributed to the phosphate groups of DNA close to 1250 cm^{-1} to ascertain the presence of PEI/pEGFP-C1 complexes when they were embedded under a HA-$(PLL\text{-}PGA)_3$ architecture since PLL and PGA contribute significantly to the infrared spectrum in the spectral region between 1400 and 1700 cm^{-1} (amide II and amide I regions). The (PEI/pEGFP-C1) complexes on $(PAH/PSS)_6$ showed the characteristic band at 1223 cm^{-1} of DNA which is assigned to the anti-symmetric stretching of phosphate groups (ν_{as}). This band is characteristically found at 1235 cm^{-1} for DNA in the B conformation but a frequency shift to smaller wave numbers, as it is seen here, is frequent when DNA is complexed to cationic polyelectrolytes [17–20]. Build-up of $HA(PLL/PGA)_3$ over (PEI/pEGFP-C1) complexes was effective as demonstrated by the adsorption increase both in the amide II region between 1400 and 1500 cm^{-1} and in the amide I region between 1600 and 1700 cm^{-1}. After $HA(PLL/PGA)_3$ deposition, the band centered at 1223 cm^{-1} is practically unchanged, with the exception of the occurrence of a shoulder at 1235 cm^{-1}. This modification of the spectrum could sign the displacement of a part of PEI from the PEI/pEGFP-C1 complexes initially adsorbed on top of the $(PAH/PSS)_6$ film, according to the shift of the complexes observed in gel retardation assays. A part

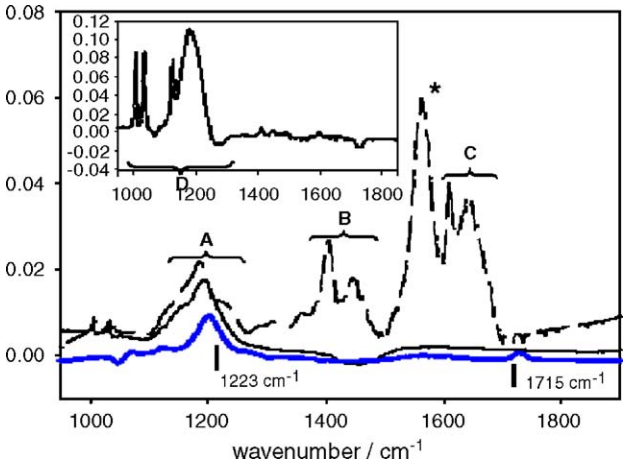

Fig. 2. Infrared analysis of the build-up of $(PAH/PSS)_6((PEI/pEGFP-C1)/HA)(PLL/PGA)_3$. Infrared spectra in the totally attenuated reflexion mode of PEI/pEGFP-C1 complexes directly adsorbed on the bare ZnSe crystal (—), of the same sample deposited on a $(PAH/PSS)_6$ multilayer (−), and after embedding of the complexes under a $HA/(PLL/PGA)_3$ multilayer (- - -) were collected. The latter two spectra are displayed as difference spectra between the respective spectra of $(PAH/PSS)_6$ (PEI/pEGFP-C1), $(PAH/PSS)_6$ (PEI/pEGFP-C1) $HA/(PLL/PGA)_3$, and $(PAH/PSS)_6$. The insert displays the spectrum of $(PAH/PSS)_6$. The regions denoted A–D correspond respectively to that of DNA (the vertical line at 1223 cm^{-1} shows the peak maximum position attributed to the phosphate backbone of DNA, of the amide II′ region (between 1400 and 1500 cm^{-1}), the amide I region of $HA/(PLL/PGA)_3$ (between 1600 and 1700 cm^{-1}) and the characteristic peaks of a PAH/PSS multilayer. The vertical line at 1715 cm^{-1} is the maximum peak position related to the base carbonyl of DNA. The asterisk in the (PEI/pEGFP-C1) $HA/(PLL/PGA)_3$ spectrum indicates the peak attributed to the deprotonated carboxylic groups of PGA.

of PEI would still be displaced upon the deposition of PGA even with HA deposited on top of the complexes before $(PLL/PGA)_3$. However, as the absorbance under the band centered at 1223 cm^{-1} can be related to the amount of DNA actually adsorbed, it proves that DNA has not been significantly desorbed upon embedding under the additional $HA(PLL/PGA)_3$ film. Moreover, the phosphate band maximum still remains at 1223 cm^{-1} after embedding of the complexes. This means that a big part, but not all, of DNA was still complexed with a cationic polyelectrolyte. AFM measurements confirmed for the most part the integrity of complexes incorporated in $(PLL/PGA)_6$ (PEI/pEGFP-C1)/HA $(PLL/PGA)_6$ architectures (not shown). Complexes appeared 500 nm wide which is coherent with the value preliminary determined by Dynamical Light Scattering for the N/P ratio of 12. The lateral distribution of the complexes was non homogenous. The mean values of roughness of PLL/PGA-based architectures measured by AFM were respectively of 2.3 nm, 2.38 nm and 2.6 nm for $(PLL/PGA)_{10}$, $(PLL/PGA)_6$ (PEI/pEGFP-C1) and $(PLL/PGA)_6$ (PEI/pEGFP-C1/HA) $(PLL/PGA)_6$ architectures. Similar values were measured for PSS/PAH-based architectures.

Transfection ability of functionalized architectures was tested with the human hepato-cellular carcinoma cell line Huh-7 [21]. Huh-7 cells were cultivated on functionalized multilayers, in DMEM (4.5 g/L glucose, L-pyruvate, L-glutamine) supplemented with 10% fetal bovine serum and 1% MEM vitamins during 2 days. The day of transfection, cells were washed with PBS and

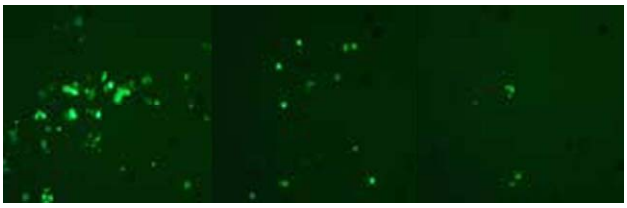

Fig. 3. Cell transfection from CHI/HA architectures. Transfection of Huh-7 cells cultivated during 2 days on functionalized architectures, from the left to the right, $(CHI/HA)_{10}$(PEI/pEGFP-C1), $(CHI/HA)_{10}$(PEI/pEGFP-C1)$(HA/CHI)_5$, and $(CHI/HA)_{10}$(PEI/pEGFP-C1)$(HA/CHI)_{10}$, was observed by fluorescence microscopy (magnification ×10).

seeded on architectures at a concentration of 3×10^5 in 500 μL of complete medium. After incubation at 37 °C in 5% CO2 for 48 h, transfected cells were observed in an inverted light microscope (Zeiss Axiovert 10, Germany) equipped with a 10× dry objective connected to a video camera (SSC6M3, Sony, Japan). As shown in Fig. 3, polyplexes embedded in CHI/HA architectures were able to transfect Huh-7 cells even under 10 (HA/CHI) bilayers. Although this was to a lesser extent than with polyplexes embedded under 5 bilayers and, a fortiori, adsorbed at the surface, this result demonstrated the feasibility of DNA delivery with polyplexes embedded in degradable architectures.

The cell transfection ability of polyplexes embedded in architectures in which, a priori, polyplexes could be displaced was also checked. With PLL/PGA and PAH/PSS architectures, no transfection event was observed even if polyplexes were just covered by one layer of PGA or PSS respectively (not shown). The effect of the addition of HA on the top of polyplexes embedded in these architectures was evaluated. When the unmodified architectures did not allow cell transfection, HA deposit on the top of polyplexes restored transfection ability (Fig. 4), demonstrating that HA could preserve embedded DNA in a functional form. After 4 days of culture, transfection rates, quantified by using the plasmid pIV1066 expressing luciferase activity, were 2.5×10^8, 1.8×10^8 and 2.37×10^5 RLU/mg protein with $(PAH/PSS)_6$PEI/pIV1066, $(PAH/PSS)_6$PEI/pIV1066/HA and $(PAH/PSS)_6$ (PEI/pIV1066/HA)$(PLL/PGA)_3$ respectively, which indicates decreasing availability of the complexes with the embedding depth.

In summary, we have shown that polyplexes embedded in polyelectrolyte multilayers could be functional for cell transfection. Depending on the nature of constituent polyelectrolytes,

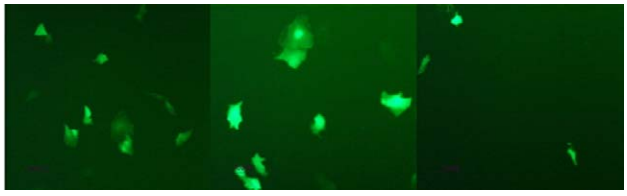

Fig. 4. Cell transfection from PAH/PSS- based architectures. Transfection of Huh-7 cells cultivated during 2 days on functionalized architectures, from the left to the right, $(PAH/PSS)_6$ (PEI/pEGFP-C1)/HA, $(PAH/PSS)_6$ (PEI/pEGFP-C1)(HA/PLL), $(PAH/PSS)_6$ (PEI/pEGFP-C1)/HA$(PLL/PGA)_3$, was observed by fluorescence microscopy (magnification ×40).

polyplexes might be shifted, indicating that the choice of the architectures should take into account possible exchanges and rearrangements between polyelectrolytes and polyplexes. The incorporation of polyplexes between $(PAH/PSS)_6$ and $HA(PLL/PGA)_3$ was possible without a significant change of DNA concentration neither significant change in DNA conformation, demonstrating that the deposition of a HA monolayer protects significantly PEI/pEGFP-C1 complexes from PGA-triggered displacement.

In conclusion, the feasibility of embedding of polyplexes functional for cell transfection has been demonstrated. The approach could be optimized in terms of transfection ability by embedding polyplexes at different levels during the multilayer build-up in order to ensure transfection efficiency over long periods, and by adding other functionalities such as sequences that would provide vectorial intracellular trafficking to the nucleus for instance. Additionally, the possibility to graft cell targeting ligand-sequences on the ending polyelectrolyte layer to enhance gene transfer via receptor-mediated uptake of the complexes could be also beneficial to the transfection efficiency of such systems which could be advantageously applied to gene delivery from implanted materials and medical devices and also in the development of DNA vaccines using patches for instance.

Acknowledgments

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