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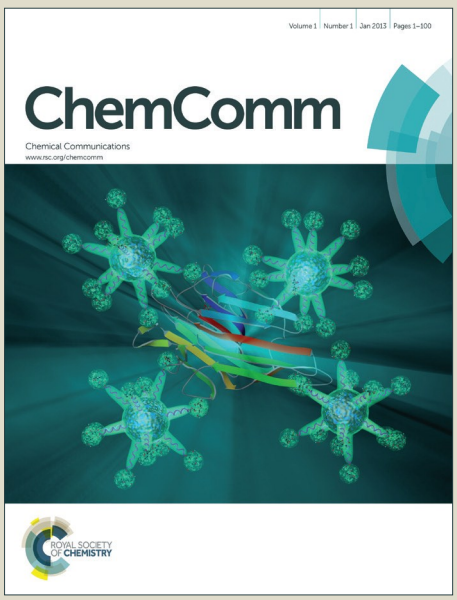

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Dr Alan Holder
Publishing Editor ChemComm
Royal Society of Chemistry

Dear Dr Holder
On behalf of the authors of the manuscript entitled “Tools of gene transfer applied for the intracellular delivery of non-nucleic acid polyanionic drugs” (Manuscript ID CC-COM-09-2015-008011)

I would like to thank you for considering our work for publication.

We are very pleased to see that the reviewers found our revision efforts satisfactory and the new data convincing.

The only remaining point we need to address pertains to a microscopy image in the Supporting Information.

According to the accepted policy, the data in the Main text have to be convincing and solid on their own and publishable without the Supporting Information. Our data in the main text are fully convincing – in the words of the Reviewer.

As we have written in the Response to reviewers, we were simply un-able to obtain a better image for this experiment in the Supporting Information – and I apologize for this. We kindly ask that this image is published as is – or, alternatively, we are prepared to remove the image and the mention of this data from the manuscript.

We sincerely hope that with otherwise fully positive comments from the Reviewer(s), our manuscript is publishable in Chemical Communications.

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Tools of gene transfer applied for the intracellular delivery of non-nucleic acid polyanionic drugs

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We report the first successful implementation of transfection agents to facilitate the delivery of non-nucleic acid based anti-inflammatory and anti-viral drugs. In doing so, we illustrate a new paradigm in the intracellular delivery of polyanionic drugs and also extend the scope and utility of successful tools of gene transfer into a new area of biomedical research.

We report the first successful implementation of transfection agents to facilitate the delivery of non-nucleic acid based anti-inflammatory and anti-viral drugs. The tools of gene transfer are among the most well established tools in biomedicine and additional tremendous efforts are being invested into their progression from lab to clinic.[1,2] Over the past years, we developed polyanionic antiviral macromolecular (pro)drugs (PAMP)[3-6] which exhibited high efficacy but sub-optimal potency in their therapeutic effect, the latter being readily explained by the restricted cell entry of these agents. We hypothesized that delivery of PAMP can be optimized using the existing tools so well developed for delivery of highly anionic polymeric drugs, namely nucleic acids. Below we demonstrate that commercial polymer and lipid based transfection agents facilitate the cell entry of PAMP resulting in a near 100-fold increase in their potency. In doing so, we illustrate a new paradigm in the delivery of macromolecular polyanionic drugs and also extend the scope and utility of successful tools of gene transfer into a whole new area of biomedical research.

The association of transfection agents with nucleic acids is typically electrostatic by nature whereby the negatively charged DNA or RNA form an electrostatic complex with a positively charged polymer, lipid, nanoparticle, etc.[1,2] The thermodynamic gain of this association is due to increased entropy, owing to a high number of counterions liberated from the polymers into solution. For this reason, the same polycation can interact with nucleic acids and other polyanions alike.[7,8] While polyanionic drug are many – hyaluronic acid,[9] heparin,[10] chondroitin sulfate[11], etc – and have diverse therapeutic targets and effects, surprisingly, virtually solitary examples exist of use of tools of gene transfer for the delivery of polyanionic drugs. Multi-layered polyelectrolyte thin films initially established for gene delivery[12,13] were successfully optimized at a later point to deliver anionic polysaccharides to the surrounding tissue.[14] In another example, cationic liposomes were used as carriers for inhalable formulations of heparin.[15] To our knowledge, there are no examples of intracellular delivery of polyanionic drugs using tools of gene transfer.

In our recent work, we used PAMP based poly(acrylic acid), PAA, and ribavirin (RBV), the latter being a broad spectrum antiviral agent[16] with a pronounced anti-inflammatory effect in cultured macrophages.[17] We used these PAMP as efficacious anti-inflammatory (anti-hepatitis) and anti-viral (anti-HIV and HCV) drugs that required intracellular delivery but were plagued with poor rates of cell entry.[3-6] To overcome this, herein we attempted to use the existing, commercially available polymer and lipid based tools of gene transfer, poly(ethylene imine),[18] PEI, and lipofectamine, LF[19,20]. When mixed with PEI at a 2:1 weight ratio (PEI to polyanion), PAA and PAMP formed particles with an average size of 900-1100 nm regardless of the presence of RBV, as established via the dynamic light scattering measurements (typical polydispersity < 0.25). These data are supported by the transmission electron microscopy images (Figure 1,A) which illustrates spherical polymer aggregates sizes marginally smaller than reported by the DLS readings – reflecting shrinking of particles upon drying for TEM visualization. Zeta-potential measurements confirmed an expected overall positive surface potential of the particles (+18±2 mV). Lowering the polymer ratio to 1:1 (PEI to polyanion by weight) rendered surface potential negative (-22±5 mV) but did not result in drastic changes in the particle size (880±50 nm). DLS measurements for LF showed that lipid-polyanion complexes were larger (~1.4±0.2 μm) and had near neutral surface potential and (-7±4 mV). We note that micrometer size of aggregates with PEI and LF is beneficial and likely to facilitate uptake by macrophages.

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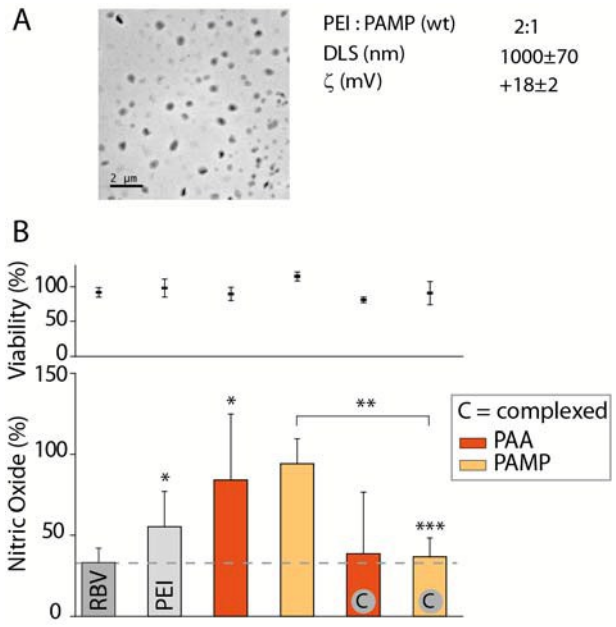

Figure 1. Inhibition of NO production and associated cytotoxic effect in lipopolysaccharide stimulated macrophages after 24 h incubation with PEI, pristine PAA and PAA-RBV, and complexes of PEI and pristine PAA or PAA-RBV respectively (final polymer conc. 1 mg/L). RBV control (10 μM). Results shown are average of triplicate experiments, reported as mean ± SD (n=3). Statistical significance: is given if *P<0.05, **P<0.01, ***P<0.001.

PAA has an inherent, structure-related activity in the anti-inflammatory assays in macrophages[4] providing a platform to quantify delivery of this polyanion. In contrast to efficacy, potency of PAA and PAMP derived thereof was quite poor and for effective treatment, high doses of the polymer were required.4 In agreement with this, when PAA and RBV containing PAMP were administered at a concentration of 1 mg/L (thus being 100-fold lower than used in our previously reported experiments),[4] the polymers revealed minor if any anti-inflammatory activity, Figure 1,B. When pre-mixed with PEI, administration of PAMP resulted in an anti-inflammatory effect matching that of 10 μM RBV (therapeutically relevant drug concentration).[16] A similar effect was observed with the use of PAA and surprisingly, the data in Figure 1,B also reveal that cationic PEI suppresses production of NO by stimulated macrophages as well. In this system, each of the three components – PEI, PAA, and RBV – appears to contribute to the overall anti-inflammatory effect. This observation may prove beneficial for the design of potent anti-inflammatory formulations based on synthetic polymers. However, it also hinders the desired visualization of the use of transfection agents for the delivery of PAMP.

To overcome this, we used the tools of direct quantification of the interaction of drug carriers with mammalian cells, namely fluorescently labelled polymers and flow cytometry as the analysis technique. Supporting the data presented in Figure 1, flow cytometry revealed that at low concentration of PAA or PAMP,

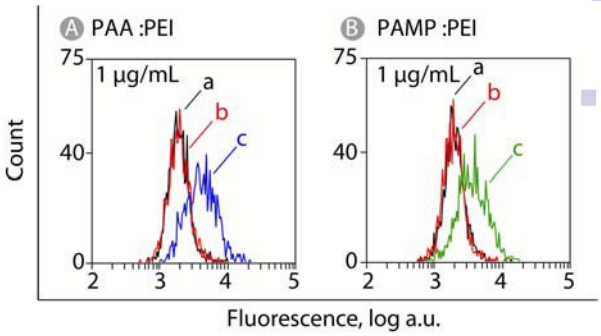

Figure 2. Flow cytometry data on the fluorescence of macrophages upon their incubation with A) PAA, B) PAMP, or complexes thereof with PEI (2:1 PEI to PAA/PAMP charge ratio) over 24 h at polyanion concentration 1 mg/L: a) untreated cells, b) cells incubated with PAA (left) or PAMP (right); c) cells incubated with PEI complexes of PAA (left) or PAMP (right).

cultured macrophages hardly increased in associated level of fluorescence upon incubation with the polymer (Figure 2). In stark contrast, when formulated with PEI and despite such a low concentration, for both PAA and PAMP there was a full population shift and a significant increase in the cells fluorescence thus revealing pronounced level of interaction of the complexes with mammalian cells. These data provide direct evidence for the PEI-assisted enhancement of interaction of non-nucleic acid based polyanionic drugs with cells.

The above data were obtained at a low polymer concentration and to observe pronounced increase in cells fluorescence, extended incubation of the cells with the administered polyelectrolyte complexes was required (24 h). At a higher PAMP content, 10 mg/L, pronounced increase in cells fluorescence was observed already upon 1 h of incubation, Figure 3,A. Furthermore, qualitatively, the data were similar when PAMP was formulated with LF in which case too, macrophages revealed pronounced increase in fluorescence, Figure 3,B. For both transfection agents, increase in the cells fluorescence was significant whereas administration of PAMP alone led to a minor increase in fluorescence. Interestingly, formulations with PEI and LF had a similar effect despite a different sign of the surface potential (positive for PEI and negative for LF). This demonstrates that particle size in the micrometer range is an important factor to facilitate interaction with macrophages whereas surface potential is of secondary importance. Quantitatively, the levels of mean cells fluorescence were dependent on the ratio between the cationic carrier and PAMP and were also different for PEI and LF revealing that optimization is required to achieve optimal translocation results (Figure 3,C), as is the case for gene transfer and is recommended by the vendors of commercial transfection reagents. Interestingly, for PEI, 1:1 complexes with PAMP (characterized with a negative surface potential) demonstrate a greater degree of cell association during 1 h incubation than the 2:1 complexes (exhibiting a positive surface charge). Confocal laser scanning microscopy imaging of the polyplexes or lipoplexes of PAMP upon incubation with macrophages confirmed localization of fluorescence inside the cells, that is, successful internalization of PAMP when facilitated through the use of commercial transfection

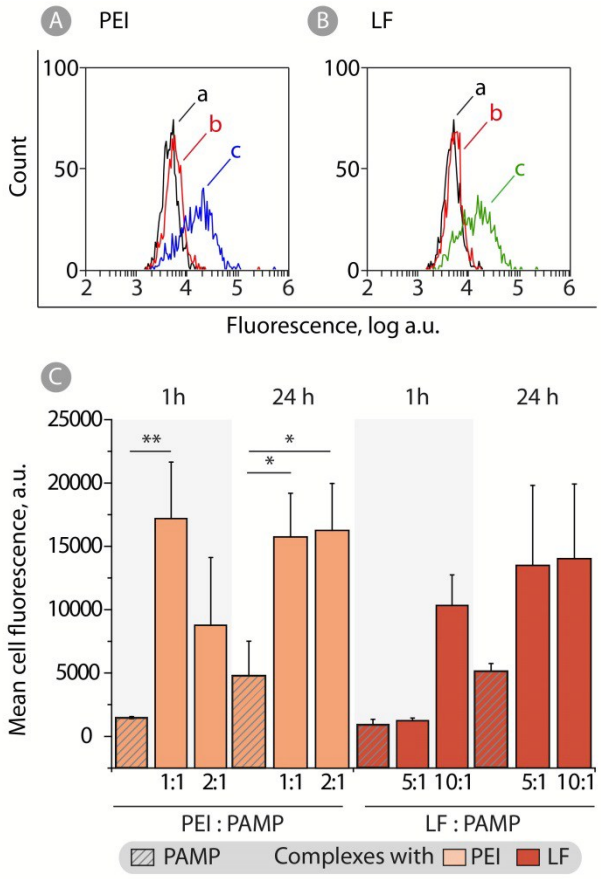

Figure 3. A,B): Experimental flow cytometry data on the interaction of macrophages with PAA, PAMP, or complexes thereof with PEI (A) or lipofectamine (B): a) untreated cells; b) PAMP; c) PAMP polyplex with PEI (2:1 PEI to PAMP charge ratio) or lipoplex with LF. PAMP concentration 10 mg/L; incubation time 1 h. C): Mean cells fluorescence of macraphages incubated with PAMP or its complexes with PEI (polyplex) or lipofectamine (lipoplex) after 1 h and 24 h incubation at PAMP concentration of 10 mg/L. Results shown are the average of triplicate experiments, reported as mean ±SD (n=3).

agents (Figure S4). Initial rationale of formulation of RBV into PAMP aimed to overcome the origin of the main side effect of this drug, namely hemolytic anemia, through suppressed internalization into red blood cells.[3] This effect was preserved and no hemolysis was observed when red blood cells were incubated with PAMP formulated with the tools of gene transfer, Figure S5. Together, results in Figure 2 and 3 illustrate a successful use of tools of gene transfer to facilitate the delivery of non-nucleic acid based polyanionic (pro)drugs to mammalian cells. Further optimization may be performed through the use of polycations with lysosomal escape capacity and/or biodegradable polymers, which is the subject of ongoing research.

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