In Situ Functionalized Polymers for siRNA Delivery

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Post-polymerization for siRNA delivery

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"*In situ*" Functionalized Polymers for siRNA Delivery **

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Abstract: *Here we report a new methodology to screen for the biological activity of functional polymers across a consistent degree of polymerization and in situ, i.e. in aqueous conditions and without purification/isolation of candidate polymers. In brief, the chemical functionality of a poly(acryloyl hydrazide) scaffold was activated under aqueous conditions using readily available aldehydes to obtain amphiphilic polymers. The transport activity of the resulting polymers can be in situ evaluated using model membranes and living cells without the need of tedious isolation and purification. This technology allowed the rapid identification of a supramolecular polymeric vector with excellent efficiency and reproducibility for the delivery of siRNA into human cells (HeLa-EGFP). The reported methodology constitutes a blueprint for the high-throughput screening and future discovery of new polymeric functional materials with relevant biological applications.*

Polymers are emerging as one of the most promising scaffolds for the multivalent presentation of relevant biological information.[1,2] The polymeric display of chemical motifs triggers new opportunities for cargo conjugation and delivery of the resulting covalent and/or supramolecular nanocomposites.[3] Polymers have been suggested as one of the best nanomaterials for drug delivery.[3,4] Along these lines, the delivery of exogenous small interfering RNA (siRNA),[5,6] is one of the potential therapies where polymers have attracted great attention.[7] RNAi can regulate gene expression in a catalytic manner, and as such, offers several advantages over other gene therapies.[8] However, the potential biosafety problems associated with viral vectors and the intrinsic limitations of siRNA (e.g. nuclease digestion) strongly hinder the development of suitable therapies.[6] It is therefore crucial to innovate and to identify new synthetic vectors for the delivery of functional polynucleotides.

Current progress in polymer synthesis allows the preparation of materials with multiple functionalities capable of mimicking some of the desired characteristics of viral vectors for gene delivery.[7,9] To speed up this discovery process, screening strategies have been developed.[10-14] However, in most of these cases the monomer composition strongly affects the outcome of the polymerization results.[15] Furthermore, these platforms are often compromised by the lack of strategies that allow evaluation of a range of chemical compositions across a consistent molecular weight and/or polymer length. Moreover, there are even less protocols that allow the *in situ* evaluation of the generated polynucleotide vectors.[12] Therefore, purification/isolation steps have to be implemented, even for inactive candidates, increasing this way the time and cost of the discovery process. Unfortunately, as the sophistication in polymer design increases, so does the synthetic effort required to prepare polymer vectors.

In this paper we report the synthesis of poly(acryloyl hydrazide)s for their straightforward functionalization with aldehydes to afford amphiphilic polymers that can be *in situ* (i.e. in aqueous conditions and without further purification) screened for the activated transport of nucleotides across lipid membranes (Scheme I). Optimization of these hydrazone-activated polymers can be performed in aqueous conditions and without purification, minimizing the synthetic effort and the time required to identify novel candidates for polynucleotide delivery. This versatile technology allowed the rapid identification of a single component formulation for the delivery of siRNA with better performance than one of the best commercial reagents (lipofectamine RNAiMAX®).

Post-polymerization functionalization is an ideal strategy to develop and to evaluate polymer compositions across a consistent degree of polymerization.[16] Post-polymerization often relies on highly efficient reactions (e.g. cycloadditions, reversible carbonyl chemistry, thiol-ene) to modify polymer properties.[16] Of these, we anticipated that using a poly(acryloyl hydrazide) scaffold (**P1**, Scheme 1) would give the required solubility in water to be able to screen for siRNA delivery without the need to purify the candidate amphiphiles. Poly(hydrazides) are weakly protonated at neutral pH[10] and can readily react with aldehydes to form acyl hydrazones that are sufficiently stable under physiological conditions.[17] Accordingly, hydrazone formation has been widely used in biological settings including drug delivery,[18] sensing,[19,20] or even in the synthesis of polynucleotides delivery vectors.[21,22] However, the use of poly(hydrazide) as a "clickable" and versatile scaffold has been limited and only a set of examples report its use to synthesize glycopolymers or for pH responsive drug delivery.[16,23,24] Alternative elegant strategies for multi-hydrazone formation have also been developed in the context of DNA or protein templated dynamic combinatorial libraries. [25-28]

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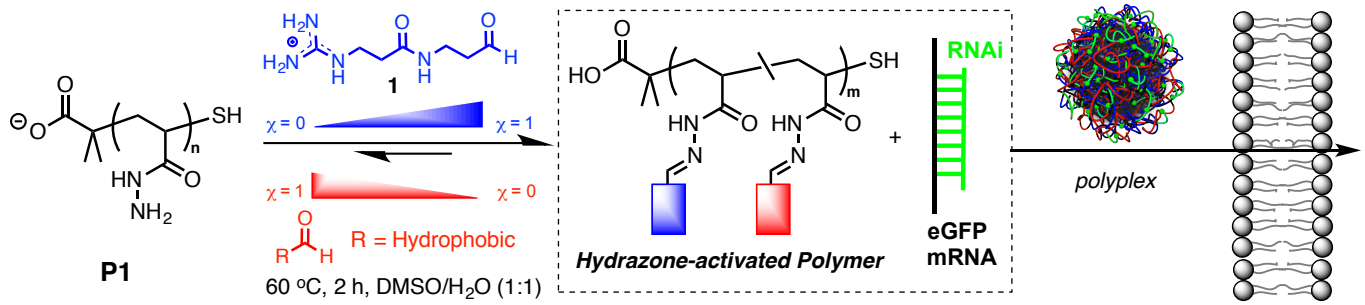

Scheme 1. Post-polymerization functionalization with cationic and hydrophobic aldehydes is followed by supramolecular conjugation of activated polymers with cargo (siRNA) and polyplex delivery.

The proposed poly(acryloyl hydrazide) scaffold (**P1**) was prepared using controlled free radical polymerization (Supporting Information, SI). **P1** was highly water-soluble and *aqueous* hydrazone formation was readily confirmed by employing the UV-active 4-imidazolecarboxaldehyde. ^{1}H NMR analysis of **P1** incubated (< 1 h) with 4-imidazolecarboxaldehyde (0.3-0.9 eq in 100 mM AcOH in D_2O) showed a broadening of the aromatic peaks of the aldehyde (Figure S3). Comparison of the residual aldehyde peak against the overall amount of protons revealed that the loading of 4-imidazolecarboxaldehyde on **P1** was ~70% (Figure S3).[23] No increase in loading was observed with higher equivalents of aldehyde (**Im**), longer times (up to 4h), different solvents or even heating. Further characterization was obtained from GPC (Figure 1). A clear polymer signal [**P1**(**Imidazol**), R_t 18 min] was already detected with only 0.25 eq 4-imidazolecarboxaldehyde added to **P1**, confirming chromophor attachment to **P1** (Figure 1). Increasing amounts of 4-imidazolecarboxaldehyde resulted in an increase in the intensity of the peak at 18 min, with that signal reaching a maximum at around 0.75 eq. Further equivalents simply increased the amount of free 4-imidazolecarboxaldehyde (R_t 31 min), validating the NMR estimation of a maximum polymer loading of ~70% (Figure S3).

Having established the feasibility of modifying **P1** functionality with a model aldehyde, we explored the use of **P1** for the *in situ* screening of membrane active polymers. A close inspection of some of the structural motifs commonly found in membrane active polymers (e.g. antimicrobial or cell-penetrating polymers) highlights a high prevalence of amphiphilic structures with the presence of both cationic (e.g. guanidinium) and hydrophobic moieties (e.g. isopropyl, benzyl).[19,29,30] The potential of establishing bidentate hydrogen bonding and the stable protonation under physiological conditions, makes the guanidium group (p*K*a ~ 12.5) the optimal cationic moiety for membrane penetration. Thus, we decided to investigate a small library of aldehydes where the cationic aldehyde (**1,** Scheme 1) was maintained constant and independently combined with different hydrophobic aldehydes (Figure 2 and SI).

Activated polymers for membrane transport were thus prepared by combining an aqueous stock solution of **P1** with a DMSO solution containing the cationic and the corresponding hydrophobic aldehydes in different molar ratios (SI for details). Hydrazone formation of **P1** with long hydrophobic aldehydes (≥ 8 carbon atoms) lead to the rapid precipitation of the resulting polymers, making these combinations inappropriate for further evaluation. However, combination of a series of short hydrophobic aldehydes (e.g. isovaleraldehyde (**2**)) with **1** afforded water soluble and stable hydrazone polyhydrazones nanoparticles (Figure S4). DLS analysis revealed suitable sizes for membrane transport (~ 200 nm) at a molar ratio of cationic/hydrophobic aldehydes = 0.85:0.15 (Figures S4 and S17).[21] Neighbouring effects (i.e. cation repulsion) could impact the final composition of the final hydrazone-modified polymer. Therefore, to further characterize the post-polymerization reaction, we measured DLS and zeta potential of **P1** combined with different ratios of guanidinium aldehyde (**1**) and isovaleraldehyde (**2**). These measurements showed that increasing the molar fraction of hydrophobic aldehyde increased the size and thus the cationic character of the resulting polymeric nanoparticles (Figure S4b). The maximum increase was observed around at molar fraction of χ**2** = 0.3-0.4 and further increase caused an important decrease in both the size and the zeta potential (Figure S4B). These results confirmed that changes in the aldehyde molar ratios are directly translated into the composition of the hydrazone-modified polymer.

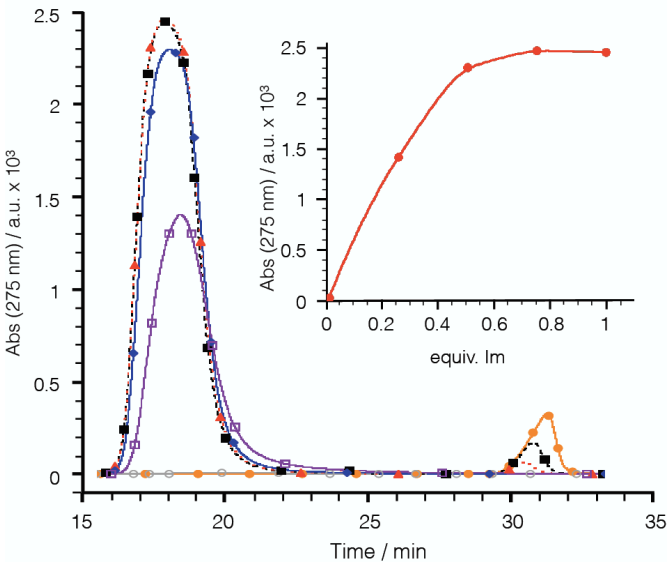

Figure 1. GPC (λ_{Abs} = 275 nm) analysis of **P1** incubated with (1 (■), 0.75 (▲), 0.5 (◆), 0.25 (□), 0 (○) equiv and pure (●) 4-imidazolecarboxaldehyde; Conditions: (100 mM acetic acid, pH 2.9, 2 h). *Inset*: Increase in absorbance of the high molecular weight peak (R_t 18 min) with increasing amounts of 4-imidazolecarboxaldehyde.

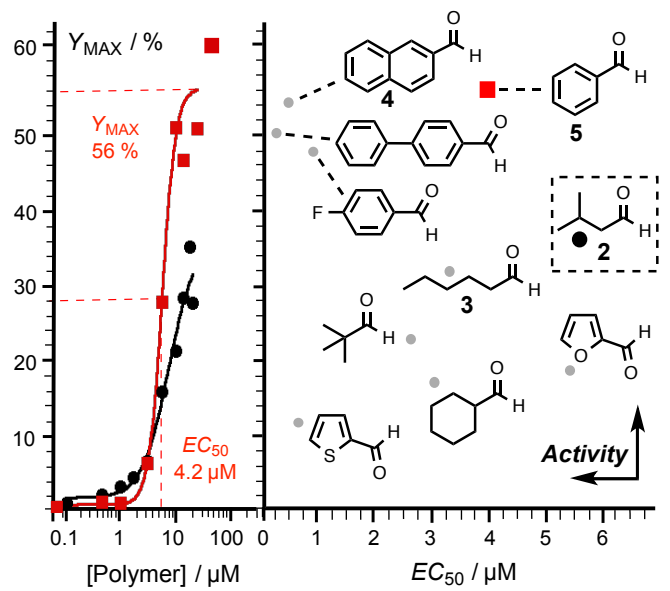

Figure 2. Representation of Y_{Max} *vs* EC_{50} for the DNA transport experiments in EYPC-LUVs⊃HPTS/DPX for hydrazone-activated polymers at χ**Hydrophobic**: 0.15 and χ**1**: 0.85.

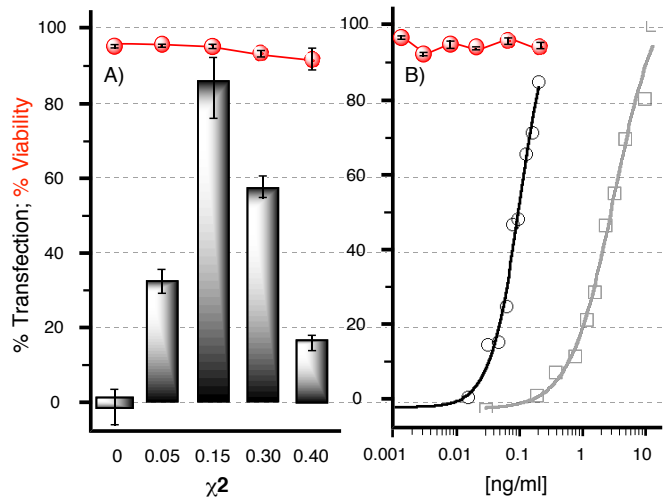

Figure 3. A) Transfection efficiency (grey bars) and cell viability (red circles) in HeLa-EGFP, at a constant siRNA (14 nM) and **P1(1)$_{85}$(2)$_{15}$** (4 µM) concentrations and prepared with different molar fractions of **2**. B) Dose-response curves of lead candidate **P1(1)$_{85}$(2)$_{15}$** (black circles, EC_{50} = 0.09 ng/mL) and Lipofectamine RNAiMAX® (grey squares, EC_{50} = 2.5 ng/mL) and cell viability for **P1(1)$_{85}$(2)$_{15}$** (red circles).

Lead hydrazone-activated polymers from DLS analysis were then evaluated in supramolecular DNA transport experiments using large unilamellar vesicles (Egg yolk L-α-phosphatidylcholine) loaded with 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt and *p*-xylene-bis-pyridinium bromide (EYPC LUVs⊃HPTS/DPX).[19,20] This routine assay reports on internal dye release as an increase in the HPTS fluorescence (Figure S5). This model allows the quick identification of inactive formulations as well as compositions that lead to significant membrane damage. In these experiments, a heterogeneous mixture of short dsDNAs (Herring DNA) was selected to model siRNA. Transport experiments in these fluorogenic vesicles revealed isovaleraldehyde (**2**), hexanal (**3**) and 2-naphthaldehyde (**4**), and benzaldehyde (**5**) as the leading hits for the aliphatic and the aromatic series respectively (Figures 2 and S8, Table S2). Increasing the molar fraction of hydrophobic aldehydes over (χ**Hydrophobic** > 0.15) afforded amphiphilic polymers with membrane disrupting behaviors (Figure S6). Control experiments confirmed lack of activity for the parent hydrazide **P1** either pure or independently combined with hydrophilic (**1**) or hydrophobic aldehydes (Figure S7).

Following the synthesis and *in situ* screening of polyhydrazones for membrane activity and DNA transport, the aldehyde lead candidates from fluorogenic assays, **2**, **3**, **4** and **5**, were taken forward for their evaluation in siRNA delivery (GFP knockdown) in HeLa-EGFP cells (Figure 3, see SI for details).[31] Again, isolation and purification of the synthesized activated polymers was not required and transfection experiments were performed by simply diluting freshly prepared polyhydrazones (buffer/DMSO, see SI for details) in culture media. In these experiments, activated polymers with aldehydes **3**, **4** and **5** showed no activity for siRNA delivery in cells (Figure S11). Aliphatic isovaleraldehyde (**2**) however showed efficient EGFP knockdown with an accurate reproducibility in all the transfection replicates measured (Figures S9-S14).

The biocompatible experimental conditions for hydrazone-activated polymers preparation allowed the straightforward optimization of this polymeric vector. We evaluated **P1** with different molar ratios of isovaleraldehyde (χ**2** = 0-0.4) and different polymer concentrations to maximise transfection efficiency and cell viability (Figures 3 and S15). Interestingly, the optimum molar ratio of the hydrophobic aldehyde (χ**2** = 0.15, Figure 3A) correlated well with the molar ratio identified in vesicle transport experiments (Figures 2, S6 and S8). Maximum transfection efficiency for **P1(1)$_{85}$(2)$_{15}$** (where the subindex indicate the percentage of the corresponding aldehyde) could be achieved at a concentration of 4 µM (Figure S12). Under these conditions, we could confirm the formation of supramolecular polyplexes (150 nm) with a positive ζ-potential of + 7 mV (Figure S17). Additionally, gel electrophoresis showed complete complexation of RNA into the polymeric polyplexes with as little as 0.12 µM of **P1(1)$_{85}$(2)$_{15}$** (Figure S18).

Cell viability was optimal for the parent polymer **P1** and for all the range of molar fractions and concentrations of the active **P1(1)$_{85}$(2)$_{15}$** (Figure 3A, Figure S15 and S16). However, increasing the molar ratio of the hydrophobic aldehyde (χ**2** = 0.4) caused a slight decrease in cell viability together with an increase in the standard deviation of the assay (Figure 3A, red circles). This observation could be related with the membrane detergent behavior detected for highly hydrophobic polyhydrazones in vesicles experiments (Figure 3A and Figure S15, Figure S6). Remarkably, the comparison of the dose response curves of transfection efficiency revealed that the polymeric vector performed with almost equal efficiency using more than ten times less concentration than the commercial reagent lipofectime RNAiMAX® (Figure 3B). Furthermore, the stability of all the components involved in the preparation of this polymeric cytofectin, allowed the storage of the stock solutions for months while keeping intact their transfection efficiency.

In conclusion, we have developed a novel strategy for the *in situ* (i.e. in aqueous conditions and without purification) evaluation of polymers with biological activity. Poly(hydrazide) functionality has been *"activated"*, under aqueous conditions, to yield amphiphilic functional polymers that did not require any further purification for their evaluation in relevant biological assays. This

protocol allowed the rapid identification of a single component supramolecular polymeric formulation for siRNA transfection with better performance than the current gold standard for siRNA delivery. We believe that the reported methodology is not limited to the screening of polynucleotide delivery and that it can be easily adapted (through an informed choice of aldehydes) for the high-throughput screening of polymers with complex chemical functionalities and different biological relevance. The control over the distribution and sequential arrangement of the aldehyde groups onto the polymer scaffold will be a great future challenge that will allow the extraction of intriguingly topology/activity relationships. Our efforts in these directions will be reported in due course.

Acknowledgements

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Keywords: Supramolecular Chemistry, Polymer Chemistry, Vesicle Transport Experiments, siRNA delivery, Lipid Bilayer Membrane.

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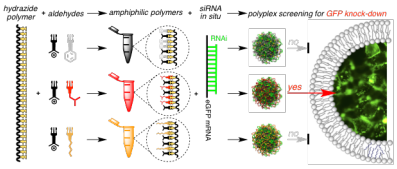
Entry for the Table of Contents (Please choose one layout)

Layout 1:

COMMUNICATION

The chemical functionality of a polyhydrazide scaffold is modified by an appropriate choice of aldehydes to produce amphiphilic vectors for supramolecular polynucleotide delivery. This methodology allowed the "*in situ*" and quick identification of a single component formulation with better efficiency than the current gold standard for siRNA delivery.



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