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Oligocarbonate Molecular Transporters: Oligomerization-Based Syntheses and Cell-Penetrating Studies

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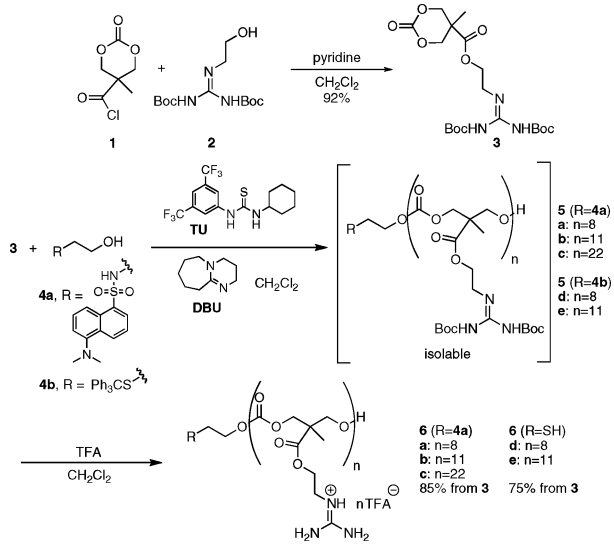
New strategies, devices, and agents that enable or enhance the passage of drugs or probes across biological barriers are required to address a range of major challenges in chemotherapy, imaging, diagnostics, and mechanistic chemical biology.[1] In 2000, we reported that the cellular uptake of the Tat_{49-57} peptide could be mimicked by homooligomers of arginine.[2] Uptake was shown to be a function of the number and array of guanidinium groups, observations that led to the design and synthesis of the first guanidinium-rich (GR) peptoids,[2] GR-spaced peptides,[3] GR-oligocarbamates[4] and GR-dendrimeric molecular transporters (MoTrs).[5] Noteworthy subsequent studies from several groups showed that a variety of other scaffolds, including beta-peptides, carbohydrates, heterocycles, and peptide nucleic acids, upon perguanidinylation, exhibit cell-penetrating activity.[6] GR MoTrs have been shown to carry a variety of cargos into cells, including small molecules, probes, metals, peptides, proteins, siRNA, morpholino-RNAs, and DNA plasmids.[7] Activatable MoTrs have been reported for targeted therapy and imaging,[8] a releasable octaarginine-drug conjugate has been shown to overcome Pgp-mediated resistance in animal models of cancer,[9] and a drug-heptaarginine conjugate has been advanced to phase II human clinical trials.[10]

While octaarginine MoTrs have been made on scale under GMP conditions and a step-saving segment doubling approach has been introduced,[11] the length and associated costs of these syntheses preclude some anticipated applications. A solid phase synthesis of octaarginine requires ≥16 steps, while the segment doubling approach involves 9 steps.[11] We report herein a new family of oligocarbonate GR MoTrs that can be flexibly and efficiently assembled in a one-step organocatalytic ring-opening oligomerization process that also allows for concomitant probe (or drug) attachment and control over transporter length.

We have previously shown that a metal-free, organocatalytic ring-opening polymerization (ROP)[12] of cyclic carbonates[13] initiated by a variety of nucleophiles, including alcohols, amines and thiols, provides narrowly dispersed polymers of predictable molecular weights and end-group fidelity.[14] We reasoned that if cyclic carbonates incorporating a guanidinium side chain could be used in this process, and if the initiator could be a drug or probe, then one-step assembly of oligocarbonate MoTr-drug or -probe conjugates could be realized. Significantly, unlike solid or solution phase syntheses of oligomeric MoTrs in which step count increases with transporter length, this controlled catalytic oligomerization strategy would provide access to various lengths in one step simply through adjustment of the initiator-monomer ratio.[15] Moreover, the metal-free nature of the catalysts and low catalyst loadings (typically 5%) are anticipated to avoid the cytotoxicity associated with catalyst residues.

The new guanidine-protected monomer **3** was prepared by coupling the cyclic carbonate **1** and 1,3-di-Boc-2-(2-hydroxyethyl) guanidine **2**. It is noteworthy that alcohol **2** does not initiate oligomerization of monomer **3** in the absence of catalyst. However, when the alcohol-tagged dansyl fluorophore initiator **4a** or protected sulfur alcohol **4b** (Scheme 1) is mixed with monomer **3** in the presence of the bifunctional thiourea/amine catalyst TU/DBU,[16] ring-opening oligomerization readily occurs. This catalyst exhibits exquisite selectivity for ring-opening oligomerization; no transesterification is observed. This exquisite control stems from the high selectivity of this catalyst combination toward the strained cyclic carbonate of the monomer relative to the acyclic carbonate and ester moieties of the oligomers.[14,16] Moreover, oligomers of various lengths are generated by simply controlling the monomer-to-initiator ratio ($[M]_o/[I]_o$). Oligomers **5a**−**e** exhibit well-defined molecular weights and narrow polydispersities (M_n = 3800, 5200, 10 000, 3900, 5100; M_w/M_n = 1.16, 1.11, 1.15, 1.16, 1.16, respectively). With a 5 mol % catalyst loading ($[M]_0$ = 1M), full conversion is reached in 1.25 h at room temperature. The process is highly reproducible over the range of scales studied (50 mg to 2.5 g). ^{1}H NMR spectroscopy showed that each oligomer was end-labeled with the initiator, and the overlay of the GPC traces from the RI and UV detectors confirms quantitative initiation and predictable molecular weights (see SI). Removal of the Boc groups by simple

Scheme 1. Oligomerization Strategy



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exposure of **5a**−**e** to TFA gave oligocarbonate MoTr conjugates **6a**−**e** in high yields from **3**.

The new MoTr conjugates **6a**-**e** incorporate a backbone scaffold (carbonate) and side chain spacing (1,7) previously unexplored in cell uptake studies.[3] A distinguishing feature of these molecular transporters is their stability profile; while they are stable for months as solids at room temperature or in buffer (PBS) at ≤4 °C, they degrade under physiologically relevant conditions (Hepes buffered saline, pH 7.4) with a half-life of ∼8 h at 37 °C. This affords excellent shelf stability, but also the novel ability to degrade after cellular uptake. Additionally, the MoTrs are nontoxic at concentrations required for uptake analysis (5 min incubation, EC_{50} **6a** = 160 μM; **6b** = 48 μM, for additional toxicity and stability information see SI). Like analogous oligoarginines, these transporters are highly water-soluble, but as shown for **6a** and **6b**, they readily partition into octanol when treated with sodium laurate (1.2 equiv per charge) (Supporting Information).[17]

The ability of GR oligocarbonate MoTrs **6a**-**c** to enter cells was initially determined by flow cytometry with Jurkat cells that had been incubated for 5 min at 23 °C with the dansylated oligomers, washed with PBS to remove the remaining oligomers, and resuspended in PBS for analysis (Figure 1). The uptake of **6a**-**c** was compared to that of a dansylated octaarginine derivative (r8, see SI for synthesis) as a positive control and the dansyl initiator **4a** as a negative control using the same 5 min pulse strategy.

The dansyl probe initiator **4a** alone does not enter Jurkat cells. In striking contrast, dansyl-oligocarbonate conjugates **6a** and **6b** exhibited rapid and concentration-dependent uptake similar to that of the dansylated r8 positive control. The extended oligomer **6c** showed uptake but also cell−cell adhesion behavior and was excluded from further analysis (Supporting Information). The significant increase in uptake observed for **6b** relative to **6a** at higher concentrations is consistent with the increase in uptake observed for MoTrs with increasing guanidinium content (up to $n = 15$)[18] and provides further evidence that the GR-oligocarbonates are functionally analogous to oligoarginines.

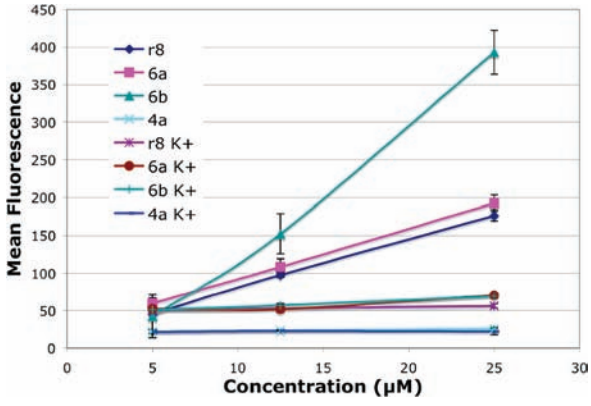

Figure 1. Flow cytometry determined cellular uptake of oligocarbonates **6a** and **6b**, dansylated-r8, and dansyl initiator **4** in either PBS or high [K+] PBS. Jurkat cells were incubated with the various transporters or positive and negative controls for 5 min at 23 °C. Cell viability was >80% as determined by propidium iodide analysis.

Not unlike the behavior of other GR MoTrs, the uptake of **6a** and **6b** was drastically decreased when cells were incubated with modified PBS in which all sodium ions were replaced with potassium ions (Figure 1), a protocol used to decrease the voltage potential across the cell membrane.[17] Additionally, incubating cells with NaN_3, conditions known to interfere with ATP dependent processes,[19] led to a decrease in uptake (Supporting Information). Finally, decreased uptake (18−37%) was observed with cells incubated at 4 °C, suggesting a mixed mechanistic pathway in which endocytosis could play a role (Supporting Information).[20]

In addition to flow cytometry studies, fluorescence microscopy using a two-photon excitation method established that both **6a** and **6b** were internalized into Jurkat cells upon incubation for 5 min at 23 °C (Figure 2).

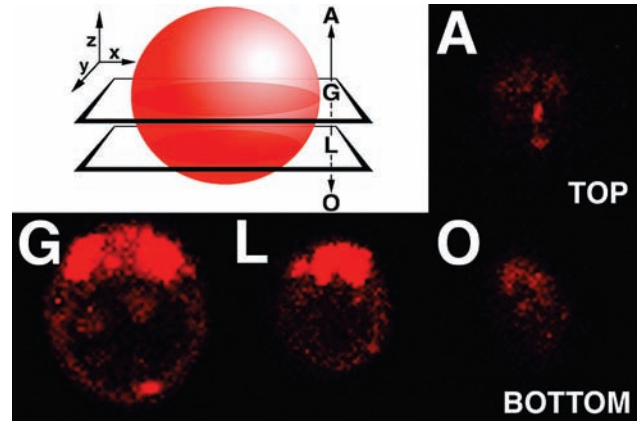

Figure 2. Fluorescence microscopy images showing internalization of **6b** throughout various layers (0.9 μm wide) of a Jurkat cell (5 min incubation, 25 μM at 23 °C). Panels A, G, L, and O show a series of z-cuts through the cell as illustrated in the diagram at top left (see Supporting Information for full z-cut series).

To further probe the ability of the oligocarbonate MoTrs to function as delivery vectors, experiments examining the delivery of the bioluminescent small molecule luciferin were conducted. In this recently introduced assay,[21] the ability of a conjugate to enter cells and release its luciferin cargo is measured by the light emitted when luciferin is converted by luciferase to oxyluciferin and a photon of detectable light. Only free luciferin is measured and the analysis is independent of the mechanism(s) of entry, providing a real-time measure of drug/probe availability. A new strategy to access thiol-terminated oligomers **6d** and **6e** (Scheme 1, Supporting Information) enabled the facile synthesis of disulfide-releasable luciferin conjugates **7a** and **7b** (Figure 3). The ability of **7a** and **7b** to deliver luciferin into HepG2 cells expressing click beetle luciferase was analyzed with a cooled charge-coupled device camera (photon count). Importantly, alkylated luciferin is not a substrate for the luciferase enzyme,[22] and all light observed is therefore derived from the intracellular release and turnover of free luciferin.

Figure 4 shows the uptake and delivery of luciferin for **7a**, **7b**, an analogous D-cysteine-r8 conjugate,[21] and luciferin alone in

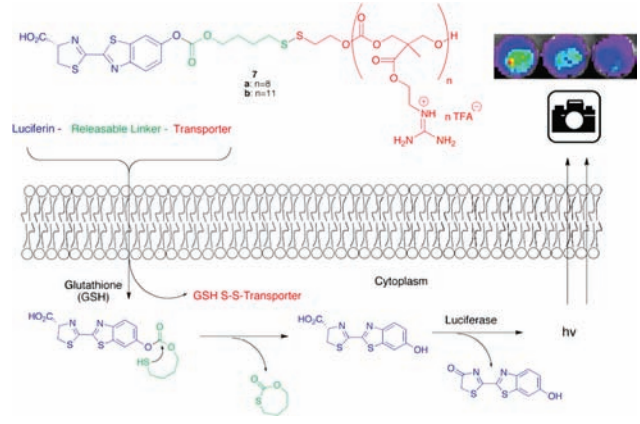

Figure 3. Assay for measurement of intracellular luciferin delivery.

Ringers (140 mM NaCl, 5 mM KCl, 10 mM HEPES, 10 mM D-glucose, 2 mM $MgCl_2$, and 2 mM $CaCl_2$) and high [K+] Ringers (70 mM NaCl, 75 mM KCl, 10 mM HEPES, 10 mM D-glucose, 2 mM $MgCl_2$, and 2 mM $CaCl_2$) solutions, imaging buffers which contain a variety of ions and glucose to maintain healthy cells during longer imaging times. Following a 5 min incubation of the luciferase-expressing cells with transporter, both oligocarbonate MoTr conjugates **7a** and **7b** continuously release free luciferin over a period of about one hour. This behavior is in contrast to the r8 control, which exhibits much faster release kinetics both in cells (over about 20 min, Figure 4), and when treated with DTT in buffer, as observed by analytical HPLC analysis (see Supporting Information). The ability of these MoTrs to release cargo over time offers several advantages, including the potential to avoid bolus effects associated with administration of a free drug alone. The oligocarbonate MoTrs are able to deliver free luciferin in a concentration-dependent manner (Supporting Information) that is inhibited by high [K^+] conditions associated with a diminished membrane potential. Free luciferin alone, while marginally cell permeable, exhibits negligible light output after a 5 min incubation. Taken together, these data demonstrate that the novel oligocarbonate MoTrs are able to not only penetrate the cell membranes of multiple cell types, but also efficiently deliver and release small molecule cargos where they are available for turnover by intracellular targets.

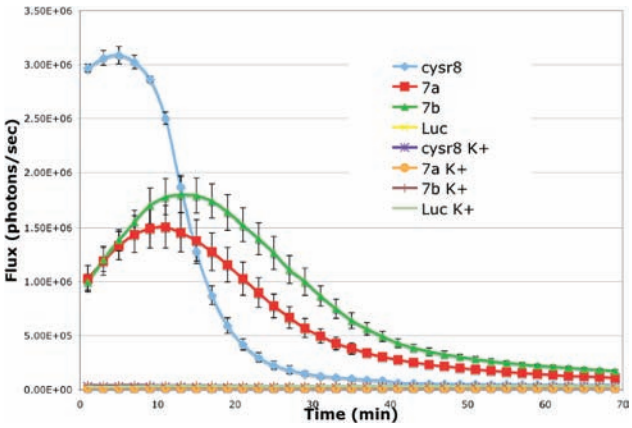

Figure 4. Observed bioluminescence from HepG2 cells expressing click beetle luciferase following a 5 min incubation with 25 μM **7a**, **7b**, cysr8 luciferin derivative, or luciferin alone in either Ringers or [K^+] Ringers solutions.

In conclusion, an expedient one-step, metal-free oligomerization route to a new family of MoTrs is described. This strategy enables the direct conjugation of probes and, by analogy, drug moieties as part of the oligomerization process. The monomers could thus be used as "kit" reagents for transporter-conjugate synthesis. Importantly, these oligocarbonate MoTrs show low cytotoxicity and exhibit uptake comparable to or better than that of the parent oligoarginines as determined by flow cytometry and fluorescence microscopy. In addition, their ability to intracellularly deliver and release the bioluminescent small molecule probe luciferin was demonstrated, confirming the intracellular availability of the free cargo to interact with its target enzyme. The facile cellular uptake exhibited by these new MoTrs, the ease with which short to long oligomers (and presumably mixed oligomers) can be prepared, and their ability to degrade after uptake offer many advantages for drug/probe delivery, particularly for biological and macromolecular cargos.

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Supporting Information Available: Experimental procedures, flow cytometry and concentration dependent uptake data, NMR data and fluorescence microscopy images. This material is available free of charge via the Internet at http://pubs.acs.org.

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