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Shear Dependent LC Purification of an Engineered DNA Nanoswitch and Implications for DNA Origami

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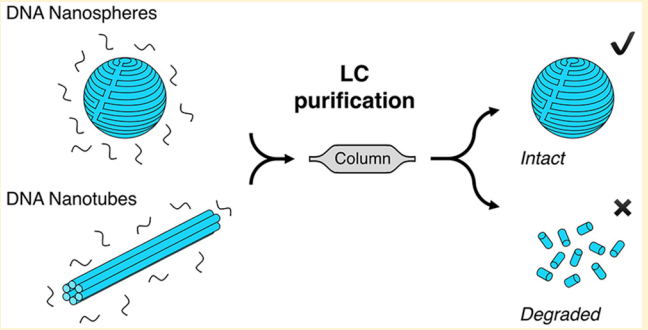
Supporting Information

ABSTRACT: As DNA nanotechnology matures, there is increasing need for fast, reliable, and automated purification methods. Here, we develop UHPLC methods to purify self-assembled DNA nanoswitches, which are formed using DNA origami approaches and are designed to change conformations in response to a binding partner. We found that shear degradation hindered LC purification of the DNA nanoswitches, removing oligonucleotides from the scaffold strand and causing loss of function. However, proper choice of column, flow rate, and buffers enabled robust and automated purification of DNA nanoswitches without loss of function in under a half hour. Applying our approach to DNA origami structures, we found that ∼400 nm long nanotubes degraded under the gentlest flow conditions while ∼40 nm diameter nanospheres remained intact even under aggressive conditions. These examples show how fluid stresses can affect different DNA nanostructures during LC purification and suggest that shear forces may be relevant for some applications of DNA nanotechnology. Further development of this approach could lead to fast and automated purification of DNA nanostructures of various shapes and sizes, which would be an important advance for the field.



Bottom up construction using DNA has enabled the fabrication of complex nanoscale structures,[1] with emerging applications such as biosensing[2] and drug delivery.[3] As the field moves toward applications, fast and robust purification of nanostructures becomes an increasingly important challenge.[4] DNA-based construction methods sometimes result in low yields[5] of the desired structures and may contain various byproducts including excess staple strands, proteins, and misfolded or incomplete structures. Removal of unreacted strands can be vital for hierarchical assembly and is often desirable in DNA origami structures as well. For functional DNA structures such as the DNA nanoswitches used in our group,[6−8] removing these byproducts is important to achieving the desired biosensing function.

There are two significant challenges in the purification of DNA nanostructures. First, DNA nanostructures are relatively large on the scale of molecular purifications, often with MegaDalton (MDa) molecular weights and linear dimensions of ∼10 nm to a few micrometers. Second, the structures are held together by relatively weak noncovalent interactions. While the total number of paired bases can range from hundreds (e.g., DNA tetrahedron[9]) to many thousands (e.g., DNA origami objects[10,11]), individual connections can contain as little as a few base pairs.[10] Several methods have been proposed for purification of such structures, including rate-zonal centrifugation,[12,13] PEG-based separation,[13−16] size exclusion columns,[13,17] spin filters,[18] magnetic bead capture,[13] and the routinely used method based on agarose gel electrophoresis extraction.[13,19] Many of these methods suffer from drawbacks including long processing times, low throughput, low repeatability, low recovery, and large sample dilutions.

One attractive method that overcomes some of these purification challenges is liquid chromatography, which can be high-throughput, selective, fast, automated, and highly reproducible. Chromatographic methods are already well established for the isolation and purification of nucleic acids,[20−22] but retaining structural features is not often a

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major consideration. Some recent studies have shown successful retention of structured nucleic acids in LC-based purifications under nondenaturing conditions,[23−25] and a recent FPLC method successfully purified functionalized DNA origami structures.[13]

Here, we aim to develop UHPLC-based methodology for purification of DNA nanoswitches (Figure 1) that can

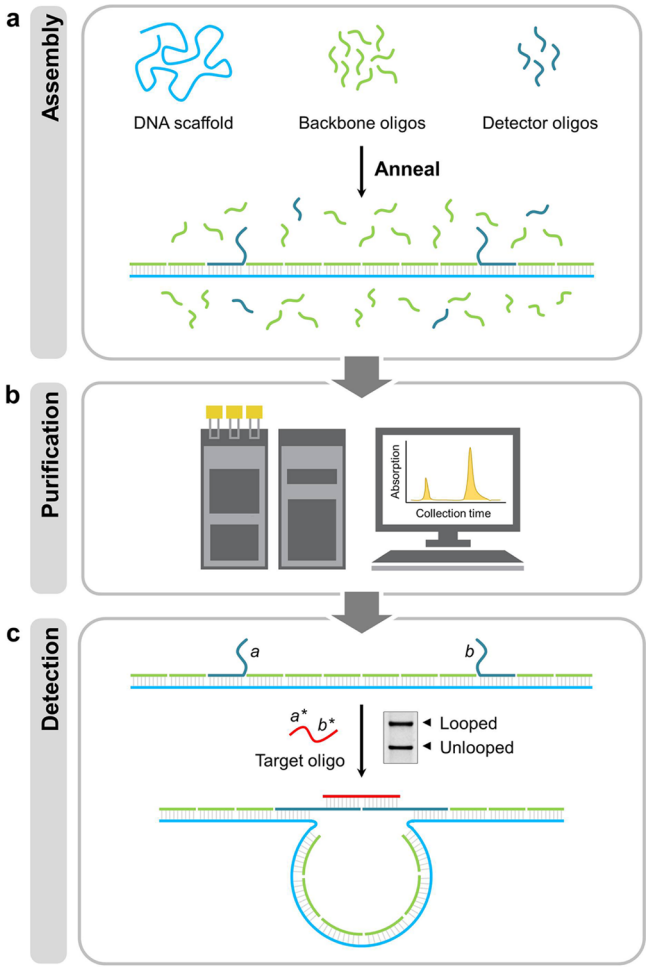

Figure 1. Cartoon of DNA nanoswitch assembly, purification, and target sequence detection. (a) Nanoswitches self-assemble under thermal annealing conditions, (b) LC-UV purification is intended to remove unreacted components, (c) a purified nanoswitch can be used for downstream biosensing applications.

potentially be applied to other DNA nanostructures. Constructed using DNA origami techniques,[10] the nanoswitches are comprised of a linear duplex with an inducible loop that is triggered by molecular detection.[6,7] We used DNA nanoswitches designed to form a single loop upon interaction with a target DNA oligonucleotide (Figure S1 and Tables S1 and S2). In this arrangement, interaction of the target DNA oligo with excess strands competes with loop formation and reduces our detection signal. Our current purification method to deplete excess oligos is based on PEG purification,[16] which we find to be less than ideal due to manual processing, significant material loss, and poor repeatability.

To develop our purification method, we used a Waters Acquity H-class Bio UPLC equipped with a quaternary pump, an autosampler, and an analytical, microliter scale fraction collection manager. We chose a macroporous polystyrene-divinylbenzene (PS-DVB) column (Agilent PLRP 150 × 2.1 mm), with 8 μm particles and a relatively large pore size of 4000 Å. Previous studies have shown an increase in resolution by increasing the size of the pore from 300 to 4000 Å for large rRNAs, ∼5000 nt.[26] The autosampler and fraction collector were maintained at 4 °C and the column at 25 °C with monitoring at 260 and 280 nm.

We first evaluated ion-pair reverse phase chromatography using 10 mM hexylammonium acetate (HAA) and 10 μM ammonium phosphate as the aqueous mobile phase and acetonitrile (ACN) as the organic mobile phase. Using a linear gradient (Supplemental Methods), we achieved separation of the DNA nanoswitches from the excess oligos in ∼10 min (Figure 2a). The oligos had an elution peak at ∼46% ACN

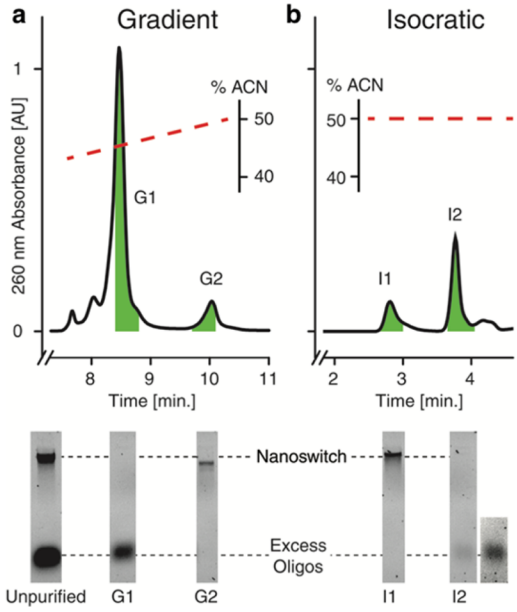

Figure 2. LC method development. (a) Under a linear gradient of increasing acetonitrile from 35% to 50%, the oligos are released from the column first, followed by the nanoswitch. (b) At a constant 50% acetonitrile, the order is reversed with the nanoswitch eluting first and the oligos second. The additional gel panel has enhanced contrast to show oligos.

while the nanoswitches had an elution peak at ∼50% ACN. When tested for activity, however, we found that the purified nanoswitches were unable to form loops in the presence of the target DNA oligo. Noting a shift in the gel migration and decreased brightness of the purified nanoswitches, we hypothesized that oligos were being removed from the linear scaffold as a result of shear stresses due to flow. To minimize shear, we used an isocratic method with 50% ACN to enable fast elution of the nanoswitch with minimal interactions with the column particles. Under these conditions, reverse order separation was achieved with the nanoswitches eluting first with the flow-through followed by the excess oligos (Figure 2b). This chromatographic behavior resembles that of size exclusion, with large nanoswitches passing through with minimal to no interaction with the column. Most importantly, the nanoswitches in the isocratic mode retained some of their functionality.

To confirm that the loss in activity was due to shear degradation, we performed additional experiments (Figure S2).

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First, we tested the nanoswitches for degradation by organic solvent alone and found that incubation of the nanoswitches in LC elution buffer containing acetonitrile did not significantly affect nanoswitch performance. Next, we experimentally verified that oligos were being removed from the nanoswitches by mixing them with an oligo that is complementary to two regions in the M13 scaffold where the "detector" strands normally bind (see Figure 1A). In this case, loops can only form if both of the detector strands are removed. The "purified" nanoswitches formed loops under this condition, but the unpurified ones did not, confirming that strands are removed as a result of the flow during the LC process.

On the basis of promising initial results from the isocratic mode, we further optimized the method to minimize the activity loss of the nanoswitch. We first sought to reduce acetonitrile in the elution, since acetonitrile is known to weaken base pairing,[27] which could exacerbate shear degradation. To accomplish this, we switched the aqueous phase from the ion-pairing HAA to a 10 mM ammonium acetate buffer. This change reduced the interaction between the DNA and the column due to the decrease of hydrophobic interactions. Using ammonium acetate, we achieved fully functional nanoswitch elution with as little as 10% acetonitrile. Under these conditions, we directly investigated the effect of shear stress by varying the flow rate between 0.2 and 0.01 mL/min (Figure 3a). We found that decreasing the flow rate increased the

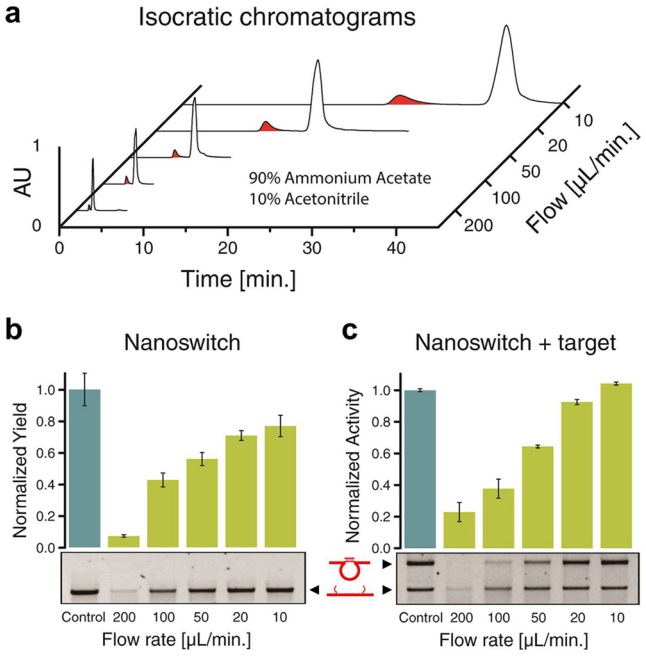

Figure 3. Shear effects. (a) Using 90%, 10 mM ammonium acetate and 10% acetonitrile, chromatograms are shown for 5 flow rates with nanoswitch collection areas shaded in red. (b) The apparent yield of the nanoswitches normalized to an unpurified control. (c) The activity of the nanoswitches when reacted with the target oligo at high concentration (25 nM), normalized to an unpurified control.

apparent yield of the nanoswitch from a few percent to ~80% (Figure 3b) and increased the nanoswitch activity from ~20% to ~100% (Figure 3c). Both of these results are consistent with oligos being removed due to shear stresses imparted from the flow in the column. The low apparent yield under high flow conditions is likely to be mostly due to the reduced efficiency of the intercalating dye for single-stranded DNA compared to double-stranded DNA.[28] Nanoswitches purified at the lowest flow rate outperformed PEG-purified nanoswitches in a low concentration detection test (Figure S3).

Following successful nanoswitch purification, we sought to apply our approach to other DNA nanostructures (Figure 4).

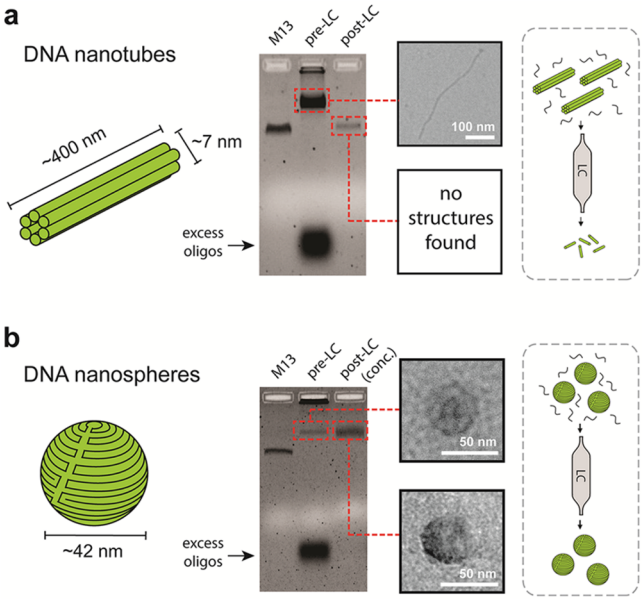

Figure 4. Application to other DNA nanostructures. (a) An ~400 nm long nanotube is significantly degraded from LC purification at a 0.01 mL/min flow rate. (b) An ~40 nm diameter sphere remains intact following purification under the same conditions.

To best understand the effects of fluid stresses, we chose two nanostructures that represent somewhat opposite extremes of shape and size: a compact ~40 nm diameter DNA sphere[29] and a long (~400 nm) 6-helix nanotube (Supplemental Methods; Tables S3 and S4). We tested each under the gentlest conditions that we applied to the DNA nanoswitches (isocratic mode, 0.01 mL/min) and collected samples from the peaks (full chromatograms and gels in Figure S3). Gel electrophoresis indicated that the nanotubes were degraded, migrating alongside the naked M13 scaffold (Figure 4a). The nanospheres, however, appeared unchanged and intact (Figure 4b). These results were confirmed with TEM imaging, where nanotube structures were only found in the unpurified sample, while intact nanospheres were found in both purified and unpurified samples. Noting that some nanospheres eluted with the oligo fraction in isocratic mode (Figure S4), we additionally tried separating them with a gradient method and retrieved intact nanospheres at a 10× higher flow rate of 0.1 mL/min (Figure S5).

As demonstrated here, purification of DNA nanostructures requires overcoming the sometimes significant challenge of shear degradation. While shear degradation in LC purification has not been a major concern in the literature,[30] shear effects on macromolecules including DNA are well documented.[31−33] As LC technology has progressed to smaller particles to increase the resolution, shear rates have become potentially damaging as we have shown. DNA nanostructures are especially vulnerable due to the relatively weak noncovalent base pairing that holds the structures together. Furthermore, this weak

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structural "glue" is particularly strained due to the large sizes of some of the structures.

For the DNA nanoswitch (~2.5 μm long), we can draw comparisons to von Willebrand Factor (vWF), a large blood protein. In previous work, it was estimated that an ~10 μm vWF molecule could experience forces exceeding 10 pN from a shear rate of 5000/s.[34] This level of shear overlaps with our estimates in the column (see Supplemental Note 1), and such a force level has been shown to be sufficient for removing a strand of DNA from its complement.[35] For the DNA nanotubes (~400 nm long), tensile forces are expected to be lower due to their shorter length, but their rigidity may cause them to suffer from additional bending forces or to become partially stuck between the beads or within the pores. For the DNA nanospheres (~40 nm diameter), their compact shape and symmetry will cause them to experience dramatically lower stresses that are likely to be well distributed over the structure.

Forces imparted from the fluid on DNA nanostructures can act to accelerate unbinding of DNA and also to prevent the rebinding of DNA. Considering that the force scale for unbinding is on the order of several picoNewtons,[36,37] we speculate that the prevention of rebinding plays a larger role. In solution, if a single staple strand in a DNA origami partially unbinds, it will be likely to rebind as the rest of the structure will remain largely intact. Under stress, however, strand detachment could result in a deformation that biases against rebinding. This effect could be something of a downward spiral as stresses could increase due to new exposed surface area, and the stresses would be redistributed among fewer staples, increasing the force acting on each DNA connection. In addition to physical forces, the LC also introduces physical separation of oligos from scaffold, buffers that may weaken base pairing interactions, and complex interfaces between column particles and nanostructures. It is likely that all of these factors play a role in the degradation.

When shear degradation is overcome, LC-based purification of DNA nanostructures offers many attractive benefits such as speed, automation, repeatability, and minimal dilution. Over our range of flow rates, purification can be completed in 10−30 min compared to over 1 h using PEG-purification. Relatedly, the automated LC process improves throughput by enabling processing of multiple samples (up to 96) without user intervention. The automation also increases reliability with sample-to-sample deviation of <1% in retention time and <5% in peak area (Figure S6). Other methods such as precipitation, magnetic beads, gel electrophoresis, and centrifugation-based techniques can require a high level of user expertise and practice. Furthermore, a notable advantage is high sample recovery with minimal dilution due to relatively slow flows but still high peak resolution. The method can be optimized to achieve near perfect recovery or to achieve minimal dilution by injecting and collecting the same volume.

Our results mark advances for chromatographic separation of large DNAs and RNAs that are often complicated by the high charge present. Increased temperature, often used to improve chromatographic resolution,[20,26] is likely to denature large nucleic acids. The use of alternative chromatographic modes such as size exclusion or ion-exchange run in series or in mix-mode conditions has shown to help in the purification of nucleic acids.[22] However, these methods increase cost, require user expertise, and use high concentration salts and other agents that require further sample processing for downstream applications such as mass spectrometry.[38] We have overcome some of these challenges, achieving selective chromatographic separations while maintaining low column temperature (25 °C) to help maintain native structures. Furthermore, we introduce an isocratic method on a common reverse phase column that may act as an alternative to size exclusion columns. These separations can be achieved using only low organic content and low volatility weak pairing agent that make it especially attractive for immediate downstream use without further processing.

Despite its advantages, our approach has some drawbacks and room for improvement. The LC method introduces organic solvent (in this case 10% acetonitrile), which in certain applications may need to be removed (by ethanol precipitation, drying, or dialysis, for example). We found this unnecessary since we had only ~1% acetonitrile after diluting to our working concentrations. While our purification is relatively fast, speeds may be further improved with shorter or thinner columns. Furthermore, larger bead sizes would increase the interstitial space, allowing a faster flow rate for a given shear rate. With further optimization, it is likely that an order of magnitude increase in speed could be realized. This could reduce our nanoswitch purification to a few minutes and potentially enable purification of difficult structures such as the nanotubes.

Robust, rapid, and high-throughput purification techniques are needed for DNA nanotechnology as the field matures from building static objects to constructing more complex active devices. The method we present is an important step in that direction, showing successful DNA nanostructure purification and offering important lessons for LC purification of various DNA structures and large macromolecules, such as vWF, and shear sensitive proteins and enzymes.[30] Success with the DNA nanosphere suggests that purification of a wide range of DNA nanostructures may be possible with further development, but conditions may need to be tuned according to size and shape of the object. Interestingly, our method may provide comparative assessment of the mechanical stability of DNA nanostructures, which will become important for biomedical applications that may subject structures to significant stresses. Such assessments performed in LC buffers may not be directly comparable to physiological counterparts but still offer insight into relative stability of different structures and potential for degradation in other contexts. Our results already suggest that some DNA origami constructs could potentially disintegrate when exposed to physiological fluid flow in the human bloodstream, which may complicate proposed efforts in drug delivery.[39] This could also prove important for microinjection of DNA nanostructures into cells,[40] use of DNA nanostructures in microfluidic channels,[41] or actuation,[42] where structural support will be required to perform mechanical work.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.7b00791.

Detailed experimental methods, supplemental note on estimation of shear rate, illustration of nanoswitch construction, control experiments demonstrating shear degradation, comparison of PEG and LC purification, chromatograms and gels from LC purification of

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nanostructures, LC repeatability test, and list of all sequences used (PDF)

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Notes

The authors declare the following competing financial interest(s): A.R.C. and K.H. have pending patent applications on aspects of DNA nanoswitch technology.

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