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Selective Nascent Polymer Catch-and-Release Enables Scalable Isolation of Multi-Kilobase Single-Stranded DNA

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Abstract: *Scalable methods currently are lacking for isolation of long ssDNA, an important material for numerous biotechnological applications. Conventional biomolecule purification strategies achieve target capture using solid supports, which are limited in scale and susceptible to contamination owing to nonspecific adsorption and desorption on the substrate surface. We herein disclose selective nascent polymer catch and release (SNAPCAR), a method that utilizes the reactivity of growing poly(acrylamide-co-acrylate) chains to capture acrylamide-labeled molecules in free solution. The copolymer acts as a stimuli-responsive anchor that can be precipitated on demand to pull down the target from solution. SNAPCAR enabled scalable isolation of multi-kilobase ssDNA with high purity and 50–70 % yield. The ssDNA products were used to fold various DNA origami. SNAPCAR-produced ssDNA will expand the scope of applications in nanotechnology, gene editing, and DNA library construction.*

DNA plays a pivotal role in nature, as it encodes the genetic information in all living organisms. Besides its biological importance, DNA has intriguing properties for materials science and nanotechnology.[1] In particular, the DNA-origami[2] method uses multiple-kilobase-long single-stranded (ss) DNA "scaffolds" that fold into custom nanostructures.[2,3] DNA origami have promising applications as drug delivery vehicles,[4] plasmonic systems,[5] devices for detection,[6] computing,[7] and diagnostics.[8]

Even though there are scalable methods for production of double-stranded DNA (dsDNA), almost all applications in DNA nanotechnology require precursors in single-stranded form. ssDNA is also an advantageous template for CRISPR/Cas9 gene editing via homology directed repair, as its increased knock-in efficiency in comparison to dsDNA may be crucial for overcoming current gene-editing challenges.[9–11] Moreover, formulation as ssDNA is required for production of functional DNAzyme and aptamer libraries, DNA-based therapeutics, for sequencing, and efficient in vitro transcription.

Despite the high demand, a general method for isolation of long ssDNA with arbitrary sequence at low cost, high purity, and large quantity remains elusive. Phosphoramidite chemistry is limited to synthesis of strands up to 200 nt.[12] Longer strands can be derived from M13 phage or phagemids, which is the method of choice for production of DNA origami scaffolds.[13] However, phage-derived ssDNA restricts the choice of sequences to those that can be stably propagated in bacteria, and the product strand typically contains a large domain of vector DNA. Alternative methods are asymmetric PCR,[14,15] rolling circle[16] and strand displacement[17] amplifications, as well as enzymatic digestion approaches.[18,19] These methods are well suitable for ssDNA isolation on small scales, but modest yields, laborious purification steps, or requirement of expensive enzymes hamper their application for routine ssDNA preparations on large scales.

One important class of isolation approaches employs solid substrates, such as microparticles, to bind, purify, and release target molecules (Figure 1 a).[20–22] This approach is widely used in biomolecular pull-down assays. For ssDNA isolation, a dsDNA precursor is first bound to the substrate and subsequently denatured to selectively release one ssDNA strand into solution.[20,22] Classical heterogeneous-phase pull-down-assays have several limitations for large-scale preparations: most of the mass of the particles is not exposed to its surface, and therefore cannot participate in target binding. The small fraction of reactive material sets limitations to its binding capacity. Moreover, large macromolecules tend to adsorb to solid surfaces, thus reducing specificity of binding, as well as the ability of the substrate to efficiently release the product back into solution.[23,24]

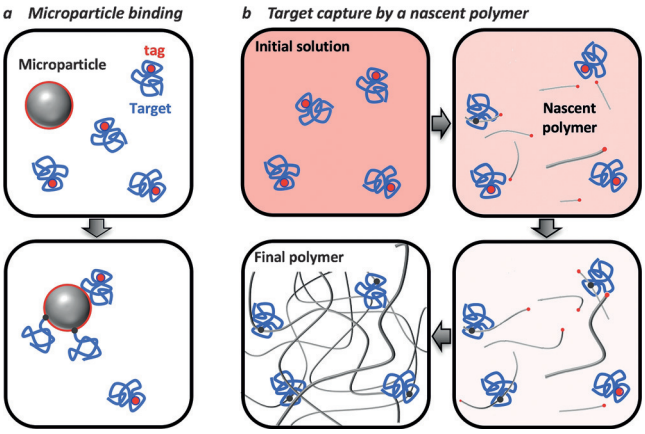

Figure 1. Target binding by microparticles versus SNAPCAR. a) Microparticles: Target molecules (blue) are captured at the solid–liquid interface. Reactive groups: red; inactive material: gray. b) SNAPCAR: Target molecules are captured by the reactive ends of nascent polymer chains. Free monomers in solution (red background) fuel the growth of the polymer chains.

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Herein we report a new approach, called selective nascent polymer catch and release (SNAPCAR), which enables selective binding and controlled release of nucleic acid targets. In short, the method relies on covalent capture (anchoring) of an acrylamide (AA)-labeled DNA duplex by copolymerization[25–27] with acrylic monomers. The polymer anchor is then used to pull down the target from solution. After separation from contaminants, the polymer-anchored duplex is denatured, which releases one of its two single strands into solution. The complementary strand remains covalently linked to the copolymer anchor and can be pulled down again to isolate the free ssDNA complement.

We show that SNAPCAR enables inexpensive and scalable isolation of ssDNA with strand lengths of up to several thousand nucleotides. The template with the sequence information for the dsDNA precursor is generated either by PCR, Gibson assembly, direct ligation, or it can be commercially obtained in the form of plasmids or gene fragments. AA-tagged dsDNA was obtained by PCR amplification of the template, yielding between 5 μg (in a PCR tube) to 1.5 mg (in 96-well plates; see Supporting Information).

SNAPCAR is conceptually related to conventional solid-substrate-based strategies, but has fundamental advantages owing to its homogeneous-phase nature (Figure 1):

1. The target can be efficiently captured at a large range of concentrations, as the polymer binding capacity is not limited by a finite solid–liquid interface;
2. The absence of solid surfaces also circumvents the pervading challenge of nonspecific adsorption;
3. Flexible nascent polymer chains, as opposed to solid surfaces, may have better access to partially obscured reactive tags on the target.

We tested this concept with short oligonucleotides as well as multi-kilobase PCR amplicons. In agreement with our premise, target binding was selective (Figure 3a; Supporting Information, Figure S3) and efficient for long and short ssDNA and dsDNA alike (90–97%; Supporting Information, Figures S3–S5). The anchoring yield remained over 90% even for DNA concentrations of 1 mg mL^{-1}, confirming that SNAPCAR provides high binding capacity (Supporting Information, Figure S6).

In initial experiments, we employed a mixture of AA and bis(acrylamide) (BAA) monomers, which yielded a cross-linked copolymer (X; Supporting Information, Figure S1). After denaturation of the DNA, X could be removed from the solution by filtration. This strategy was successfully used to retrieve a 1650 bp ssDNA product from an AA-labeled dsDNA precursor with 72% yield, and without traces of dsDNA or primer contaminations (Supporting Information, Figure S11a).

Despite the encouraging result, polymer X revealed several shortcomings: 1) long polymerization time ($\geq$16 h), very low initiator concentration, and meticulous exclusion of oxygen were needed to achieve massive molecular weight, which was required for size-selective removal of X; 2) scalability was limited due to rapid clogging of filtration membranes by X; 3) UV/Vis-detectable amounts of residual polyacrylamide contaminated the product (Supporting Information, Table S1).

To overcome these limitations, we developed an improved route (Figure 2). A linear copolymer (L) was formed by copolymerization of AA-labeled DNA in the presence of 5% w/v AA and 0.05% w/v acrylate (A). After brief nitrogen-bubbling and subsequent incubation for 3 h, the generated poly(AA-*co*-A) chains had an average molecular weight of about 1.2 MDa with a polydispersity of 1.27 (Figure 3b; Supporting Information, Figure S1).

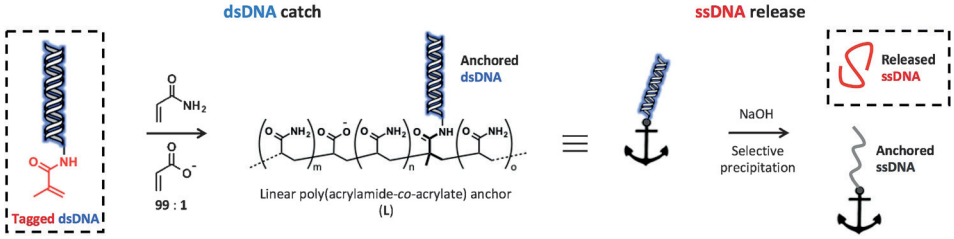

Figure 2. ssDNA isolation by SNAPCAR. Copolymerization of target dsDNA in presence of 99:1 (wt/wt) AA/A produces a linear copolymer (L). ssDNA is released from the copolymer by alkaline denaturation, and then isolated by selective precipitation of its anchored complement.

We found that addition of one volume of methanol (MeOH) to a solution of L selectively precipitated poly(AA-*co*-A) while leaving free DNA (250–10,000 bp) in solution (Supporting Information, Figures S8, S9). We note that, in contrast, a linear polymer of 100% AA (that is, poly(AA)) nonspecifically co-precipitates DNA. Including 1% of negatively charged A in the polymer recipe was crucial to suppress undesired DNA co-precipitation (Supporting Information, Figure S9). Precipitation was efficient and selective under native and denaturing conditions alike, but was sensitive to ionic strength: precipitation at low ionic strength ($<$20 mM) is incomplete, whereas high ionic strength ($>$60 mM) causes co-precipitation of free DNA. Therefore, precipitation steps were carried out within the ionic strength window of 30–50 mM (see the Supporting Information).

For isolation of ssDNA, L was first precipitated with MeOH and briefly centrifuged (step I, Figure 3c,d). Anchored dsDNA was dragged into the pellet, whilst unreacted dsDNA, short copolymer chains, and other contaminants remained in the supernatant. In a second step, the polymer pellet was re-dispersed under alkaline denaturing conditions to release ssDNA. L was then precipitated with MeOH and removed from the solution by centrifugation, leaving free ssDNA in the supernatant (step II, Figure 3c,d).

Agarose gel electrophoresis (AGE) of the supernatants from steps I and II showed that both solutions contain together $>$95% of DNA, demonstrating that target loss due to co-precipitation or nonspecific binding is low (Figure 3d;

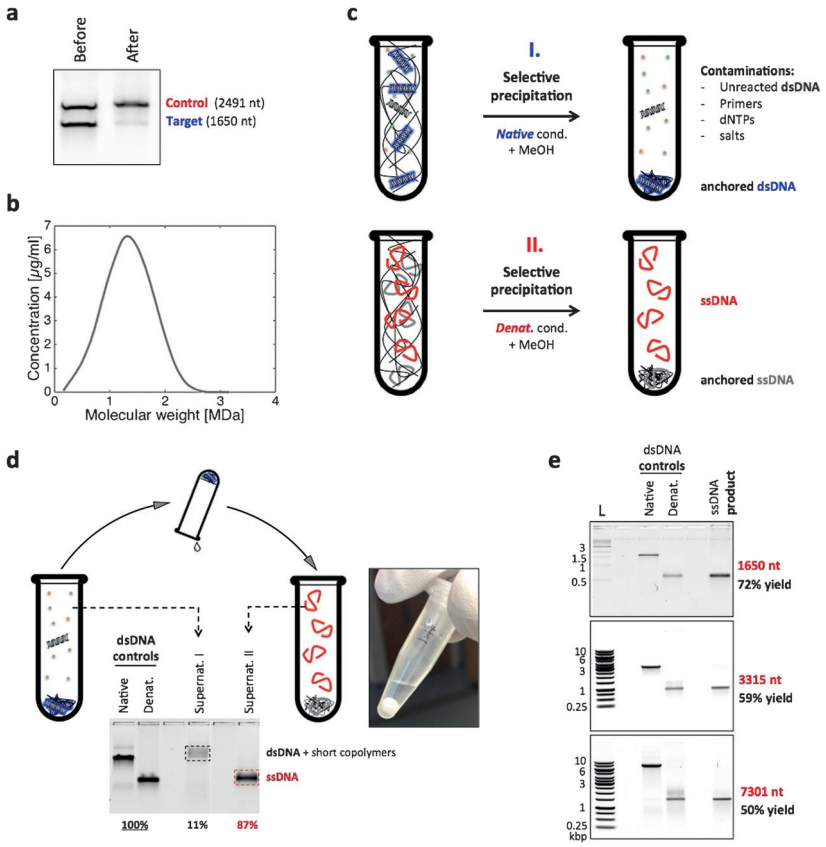

Figure 3. Selective binding of AA-tagged dsDNA and isolation of ssDNA. a) Agarose gel electrophoresis (AGE) of filtrates of an AA-labeled 1650-nt target dsDNA in the presence of an unlabeled 2491-nt dsDNA control strand before and after capture. b) Molecular weight distribution of linear polymer L. c) Isolation of ssDNA by two-step precipitation: Precipitation of L under native conditions removes unreacted dsDNA and other contaminations (step I). A second precipitation of re-dispersed L under denaturing conditions separates released ssDNA from the polymer (step II). d) AGE of supernatants obtained in steps I and II. Right side: photograph of the polymer pellet. e) AGE of various ssDNA products in TE buffer.

Supporting Information, Figure S5). A small fraction of L is too short to precipitate in step I. Since this fraction is discarded with the first supernatant, it does not contaminate the ssDNA product in step II.

Figure 3e shows AGE analysis of three SNAPCAR-produced strands with 1650 nt (ss1650), 3315 nt (ss3315), and 7301 nt (ss7301) length, in TE buffer. The dsDNA precursor in TBE or formamide buffer served as native and denatured control, respectively. The ssDNA products were obtained with 50–70% yield and high purity. UV absorbance (OD) measurements showed ideal OD_{260}/OD_{280} and OD_{260}/OD_{230} ratios and no spectral signs of contamination (Supporting Information, Figure S13, Table S1).

For comparison, we also tested DNA binding and release by magnetic microparticles.[22] These showed significant nonspecific binding of unlabeled multi-kilobase DNA and lower ssDNA product yield and purity (Supporting Information, Figure S12). However, it should be noted that the use of magnetic particles is faster on small scales, and can be automated for high-throughput assays.

An initial concern of the SNAPCAR approach was that the free radical polymerization could cause DNA backbone breakage. To assess potential radical damage, we exposed supercoiled pBR322 plasmid (4361 bp) to SNAPCAR conditions. No significant backbone breakage was detected, even in the presence of 10-fold increased concentration of the radical initiator. When nitrogen bubbling was omitted under the same conditions, 6% of plasmids had a backbone lesion (Supporting Information, Figures S14, S15). This finding indicates that under standard SNAPCAR conditions, DNA damage is negligible because 1) the concentration of radicals is low, and 2) exclusion of oxygen from the reaction prevents formation of reactive oxygen species, which could otherwise damage the target.[28]

To demonstrate application of the produced ssDNA, we folded several DNA origami (Figure 4): a barrel[29] was folded from ss3315; a rectangle[2] and a 6-helix bundle[8,30] were folded from ss7301. All reactions proceeded with quantitative conversion of the ssDNA scaffold, as revealed by a band shift in AGE. DNA origami structures were observed by transmission electron microscopy (TEM), both in the crude folding mixture and when extracted from the major product band, thus confirming efficient folding (Figure 4; Supporting Information, Figure S16).

The diameter of the folded barrels was 30.7 ± 1.0 nm, in good agreement with the expected value of about 31 nm. The measured lengths of rectangle and 6-helix bundle were 90.1 ± 3.5 nm and 405 ± 11 nm, respectively. These values matched the anticipated values of about 90 nm and about 400 nm, respectively. Overall, our experiments generated the designed structures with high fidelity and yield, comparable to M13-derived scaffolds.

In conclusion, SNAPCAR is a new method for selective capture, isolation, and controlled release of DNA-based targets. SNAPCAR utilizes the reactivity of growing poly(AA-*co*-A) chains to bind AA-tagged targets within a homogeneous phase. The absence of solid surfaces and the tunability of the polymer composition allows efficient and selective binding with high capacity and negligible product loss by nonspecific adsorption. SNAPCAR is greatly scalable (Supporting Information, Figure S17), since polymerization and precipitation are applicable to large sample volumes and high target concentrations. SNAPCAR enabled preparative-scale isolation of multi-kilobase ssDNA directly from crude PCR amplicons, achieving 50–70% product yield, without the need for chromatographic or electrophoretic purification. The estimated cost of SNAPCAR reagents is $0.35 per 1 nmol ssDNA product, three orders of magnitudes lower

than widely used streptavidin-coated microparticles, and even less expensive than the dsDNA precursor itself. In this study, we used single PCR-amplified precursors, but SNAPCAR is likewise applicable to libraries derived from multiplex-PCR or isothermal amplification products. The ssDNA products were suitable for folding of two- and three-dimensional DNA origami. This method will find immediate application for design of new types of DNA origami with fully programmable scaffold sequences. It will also enable large-scale production of custom nucleic acid therapeutics, generation of repair templates for CRISPR/Cas9 gene editing, and ssDNA libraries via systematic evolution of ligands by exponential enrichment (SELEX).[19] While we have applied SNAPCAR for ssDNA isolation, its principle may be adapted for selective capture of other macromolecular targets.

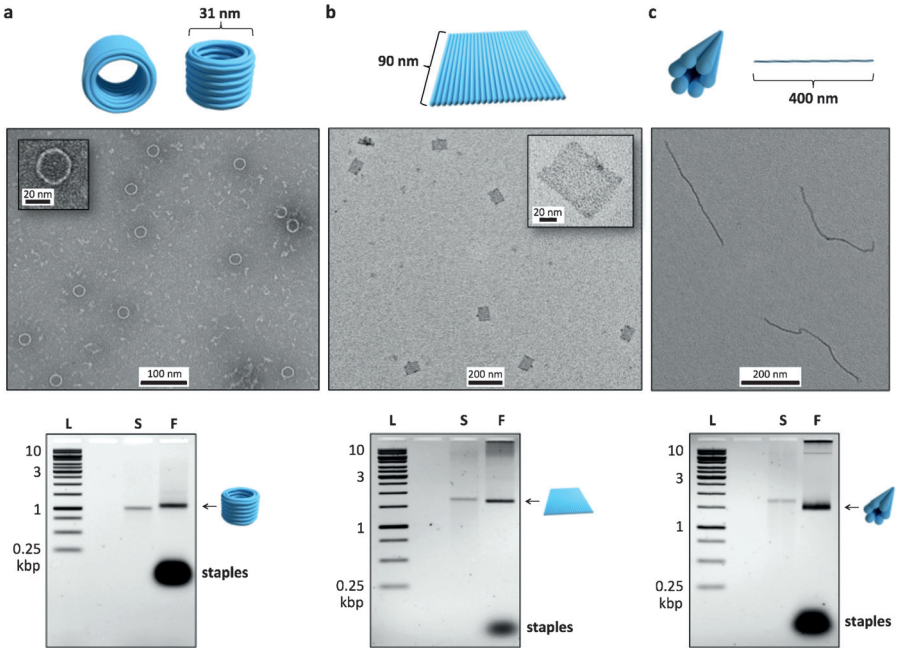

Figure 4. DNA origami folded from SNAPCAR-produced scaffold. Design (top), TEM images (center), and corresponding agarose gels (bottom) of folded barrels (crude product mixture; a), rectangles (excised from major gel band; b), and 6-helix bundles (excised from major gel band; c). L = dsDNA ladder, S = scaffold, F = folded structure.

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Conflict of interest

The authors have filed a provisional patent on this technology.

Keywords: DNA · molecular biology · nanotechnology · polymerization · synthetic methods

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