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Article

Reversible and Fully Controllable Generation of Organo-Soluble DNA (osDNA)

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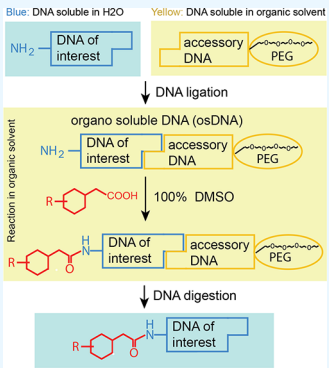
ABSTRACT: The present work describes a complete and reversible transformation of DNA's properties allowing solubilization in organic solvents and subsequent chemical modifications that are otherwise not possible in an aqueous medium. Organo-soluble DNA (osDNA) moieties are generated by covalently linking a dsDNA fragment to a polyether moiety with a built-in mechanism, rendering the process perfectly reversible and fully controllable. The precise removal of the polyether moiety frees up the initial DNA fragment, unaltered, both in sequence and nature. The solubility of osDNA was confirmed in six organic solvents of decreasing polarity and six types of osDNAs. As a proof of concept, in the context of DNA-encoded library (DEL) technology, an amidation reaction was successfully performed on osDNA in 100% DMSO. The development of osDNA opens up entirely new avenues for any DNA applications that could benefit from working in nonaqueous solutions, including chemical transformations.



INTRODUCTION

The world of nanoscience is quickly evolving.[1−4] As DNA-related costs (synthesis and sequencing) are increasingly accessible, nanotechnological methods using DNA, and their applications, are burgeoning and far-reaching,[5] ranging from well-known rather simple applications (e.g., barcoding and data storage)[6,7] to highly complex ones (e.g., random number generation, logic gates and circuits, and cryptography).[8] One deleterious limiting factor is the intrinsic nature of DNA that requires aqueous working environments. To solve that problem, we introduce the concept of organo-soluble DNA (osDNA) and propose a method describing a reversible DNA transphilicity switch that induces DNA solubility in organic solvents. The extension of this approach will allow any nucleic acid or nucleic acid-like containing moiety to be modified independently of the experiments, needs, and chemical conditions required.

Nucleic acids and dsDNA in particular are highly polar due to the presence of negative charges associated with each phosphate group decorating the DNA strands. These nucleic acid phosphate groups create an overall polar backbone that has a pK_a value near zero. They are fully ionized and negatively charged at pH 7.0, which qualifies them as acid molecules. Altogether this means that DNA is hydrophilic, soluble only in aqueous solutions, and insoluble in anhydrous organic solvents. In the field of organic chemistry, a large number of chemical reactions require anhydrous organic conditions as chemical building blocks (BBs) are mostly insoluble in aqueous medium[9−18] and cannot be carried out in an aqueous solution. For these reasons and because nucleic acids are a central part of nanotechnologies, there is a great need, and an astonishing potential benefit, for breaking the strict relationship that exists between DNA and H_2O.

The DNA-encoded library (DEL) technology, developed for large-scale drug discovery programs, belongs to the high-power screening (HPS) technologies.[19,20]

A DEL is a mixture of large numbers (millions to billions) of drug-like molecules of small molecular weight, where each molecule is conjugated to a specific and unique DNA-barcode that encodes its chemical structure.[21−23] A typical DEL molecule is a hybrid drug-like molecule harboring a DNA label attached with a chemical linker and that was generated by the serial addition of BBs onto a scaffold, using a combinatorial approach (e.g., split-and-pool protocol). The combinatorial approach gives rise to libraries containing millions to billions of compounds,[24−29] and importantly, it requires that the chemical reactions happen in the presence of DNA, and therefore in aqueous solution. Due to this important limitation, the DNA-compatible chemical space is fairly limited.

The equation to solve this problem became counteracting the overall negative charge of the DNA, without denaturing the DNA of interest (any kind of DNA and especially double-strand DNA [dsDNA], of any length) and in a reversible and controllable way. Methods were proposed to dissolve very short DNA fragments, mostly single strands, in organic

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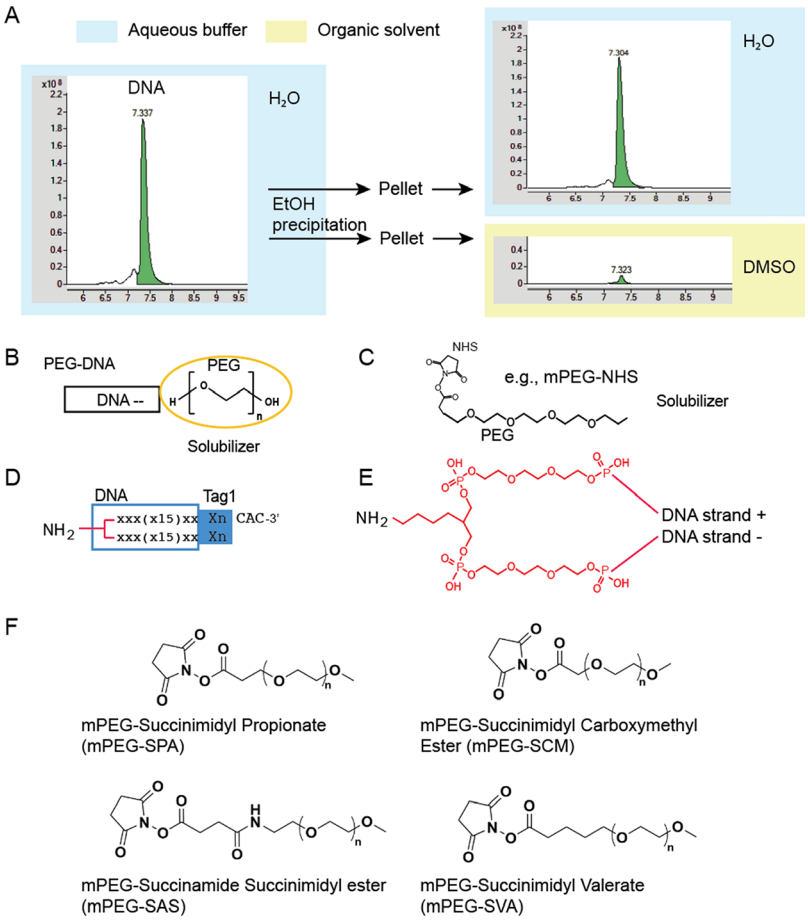

Figure 1. DNA solubility in water. (A) Pellets of DNA can be resuspended back in H_2O (light blue background) but not in organic solvent (e.g., DMSO) (light yellow background). (B) Binary entity that is a hybrid between a DNA fragment (rectangle) and a chemical compound (orange oval). An example is shown where the chemical compound is polyethylene glycol (PEG). (C) Example of modified PEG that can directly be added to DNA. (D) Example of DNAoi that can be reversibly altered harboring a dsDNA sequence shown as Xs (blue open rectangle) and a tag1 barcoding for example for a chemical block (dark blue rectangle). (E) Detailed structure of the chemical modification linking the two DNA strands covalently and providing a free reactive NH2 group. (F) Four examples of mPEG-NHS are presented: PEG-SCM: PEG-succinimidyl carboxymethyl ester; PEG-SPA: PEG-succinimidyl propionate; PEG-SAS: PEG-succinamide succinimidyl ester; PEG-SVA: PEG-succinimidyl valerate. Color background: Light blue background indicates solubility in H_2O. A light yellow background indicates solubility in organic solvents. Red line: covalent linkage of DNA strands.

solvents.[10,12−14,16,18,30−34] These include strategies involving complexation of DNA with cationic surfactants like cetyltrimethylammonium bromide (CTAB) and didodecyldimethylammonium bromide (DDAB),[10,18,33] nanoparticles formed by DNA and random cationic copolymer,[13] addition of a single polymer to DNA to increase the solubility in organic solvents,[31,34] and reactions on solid supports.[35−38] These strategies have only been validated for short oligonucleotides, mostly for single-strand DNA (ssDNA), and present severe shortcomings such as the use of strong acidic treatment, limited nucleotide compatibility, solubility, and separation issues. This would not be suitable for most technologies involving DNA including DEL for example. Furthermore, there is no report of these strategies being used for constructing a DEL or any other type of DNA library.

Having explored various options, a hybrid molecule made of a removable dsDNA fragment (engineered to be ligated to the dsDNA of interest) and a long polyether (polyether glycol or polyol) presented all of the necessary characteristics as a possible solution to our solubility problem. Indeed, we have now discovered a novel reversible strategy, where a hybrid DNA-PEG (polyethylene glycol) molecule can be fused reversibly to a DNA of interest (DNAoi) to form an osDNA, altering DNA's intrinsic properties and allowing its solubilization in organic solvents.

In summary, the present work describes a successful strategy to alter reversibly the intrinsic properties of DNA by reducing its hydrophilicity and creating an osDNA. The validated steps necessary to add a PEG moiety of six different sizes to a given DNA fragment are presented. Ethanol was used first with a short dsDNA fragment to validate the concept. After the validation, a dsDNA fragment corresponding to a DEL partial DNA label was used. In these conditions, two different PEG lengths were used, and 6 different organic solvents of different polarity were successfully tested. Amidation,[39−42] a chemical reaction well-known in the DEL field and that cannot happen in 100% organic solvent in the presence of DNA, was successfully performed in 100% DMSO. Indeed, a chemical block was added onto an osDNA scaffold, as it happens when constructing a DEL. Finally, the DNAoi was fully released from its modification and a built-in mechanism allowed selection, for future steps, of only the DNA that was modified. For practical reasons, this study was limited to validating our proof-of-concept using the amidation reaction. In conclusion, chemical

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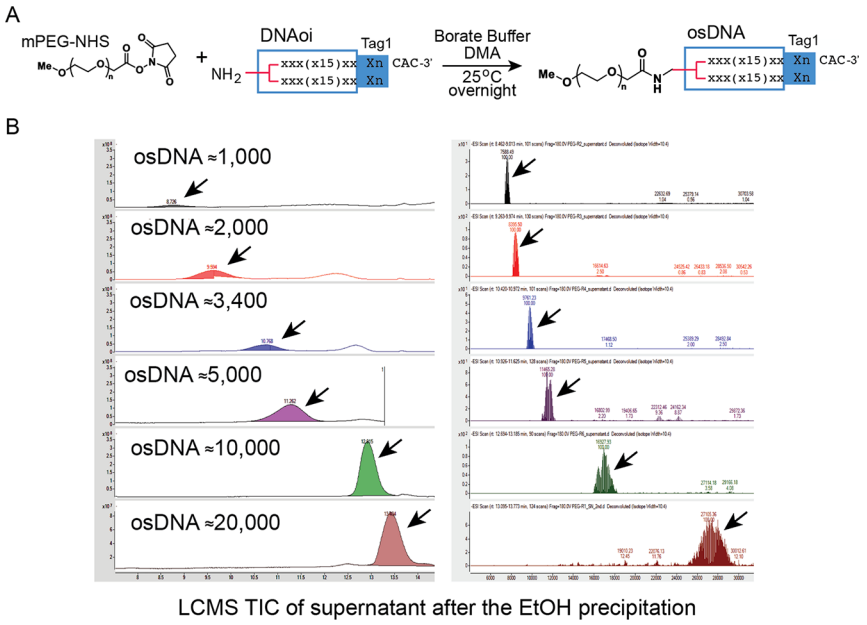

Figure 2. Test of osDNA solubility with PEG molecules of increasing size.(A) Chemical reaction used to add a PEG molecule onto a DNAoi (blue rectangle). (B) Six different PEG molecules (MW ∼1,000; ∼2,000; ∼3,400; ∼5,000; ∼10,000 and ∼20,000) were added onto a dsDNA fragment, and the amount of osDNA present in the ethanol (100%) supernatant following ethanol precipitation was analyzed by LCMS. The spectra on the left correspond to UV spectra and those on the right to deconvoluted total ion chromatograms. The osDNA molecules were detected (see arrows) in all 6 cases with a gradual increase in parallel to the PEG size. PEG ∼5,000 (purple peaks), PEG ∼10,000 (green peaks), and PEG ∼20,000 (burgundy peaks) were particularly efficient. The largest PEG molecule (MW ∼20,000) led to the highest quantity of DNA solubilized in pure ethanol.

reactions that were not previously possible in the presence of DNA, and/or using chemical blocks and systems that are not water-soluble are now achievable. This strategy offers great potential to significantly increase the chemical space accessible to DNA-related endeavors.

RESULTS AND DISCUSSION

Lyophilized DNA can be fully resuspended in water but not in organic solvents (e.g., DMSO) due to the phosphate groups that decorate nucleic acids and create an overall polar backbone that is strongly hydrophilic. The goal was to engineer a strategy to permit DNA solubilization in an organic milieu and ideally in a reversible and controllable way. The successful strategy is presented below.

To measure the impact of DNA modifications on solubility, a short dsDNA fragment (the dsDNA of interest) was evaluated under various conditions and systematically compared to an identical DNA sample (origin, quality, and quantity) by aliquoting DNA and ethanol precipitating it. Each tube containing a lyophilized DNA pellet was then treated. A schematic representation is presented, and an example of LC−MS results is shown (Figure 1A). The starting material solubilized in H_2O was systematically analyzed by LC-MS to ensure initial quality (left spectrum, green peak). Resuspension of a DNA pellet in H_2O postprecipitation, as observed following bioanalyzer quantification and confirmed by LC−MS analysis (top right spectrum), led to identical results as with initial DNA (left spectrum), while resuspension in DMSO (low right spectrum) confirmed the absence of resuspension. For simplicity, aqueous conditions are highlighted in light blue and organic conditions are in yellow.

Schematic representations of essential components are presented in Figure 1B−E. The component inducing organic solubility, to be covalently linked to a dsDNA of interest, will be called a "solubilizer" (Figure 1B). A solubilizer is a hybrid DNA (black rectangle) designed to be partially complementary to the DNA of interest and to harbor the polyether (yellow oval) to be tested for its organic solubilizing capacity. The main polyether compounds used in this study were polyethylene glycol-based (e.g., mPEG-NHS) (Figure 1C). An example of a DNA fragment of interest (called DNAoi) used in this study is shown in Figure 1D. The chemical bridge covalently linking two DNA strands and presenting a free-NH_2 group will be indicated in red, as shown in Figure 1D, both for the DNAoi and the solubilizer. The DNAoi can be designed with a 3-nucleotide overhang sequence (e.g., CAC) for DNA ligation purposes. An example of the chemical linker used is presented with molecular resolution (Figure 1E) as well as four different PEG molecules (Figure 1F).

The short DNA fragment presented above (Figure 1D) resuspended in borate buffer was linked covalently, via the free amine (NH_2) group, to six different mPEG-SCM molecules (MW: ∼1,000; ∼2,000; ∼3,400; ∼5,000; ∼10,000; ∼20,000) harboring approximately 20−450 PEG units (Figure 2A). Of note, due to the enzymatic nature of PEG synthesis, the MW of PEG molecules is approximative and indicative of the average size; as a consequence of being a mixture of molecules, the LC-MS spectra appear as diffuse peaks. A representative example is presented in Figure S1. After the addition of methoxy-PEG-*N*-hydroxysuccinimide (mPEG-NHS) ester to the DNA, the samples were ethanol-precipitated, the supernatant separated, the pellets dried, and a solubility test was carried out by measuring the pellet content and retention in the supernatant, where no DNA is expected to be found. LC−MS analysis of the supernatant revealed that DNA is actually found in the ethanol phase (Figure 2B, arrows), including for the smallest PEG molecule added (top spectra), indicating that

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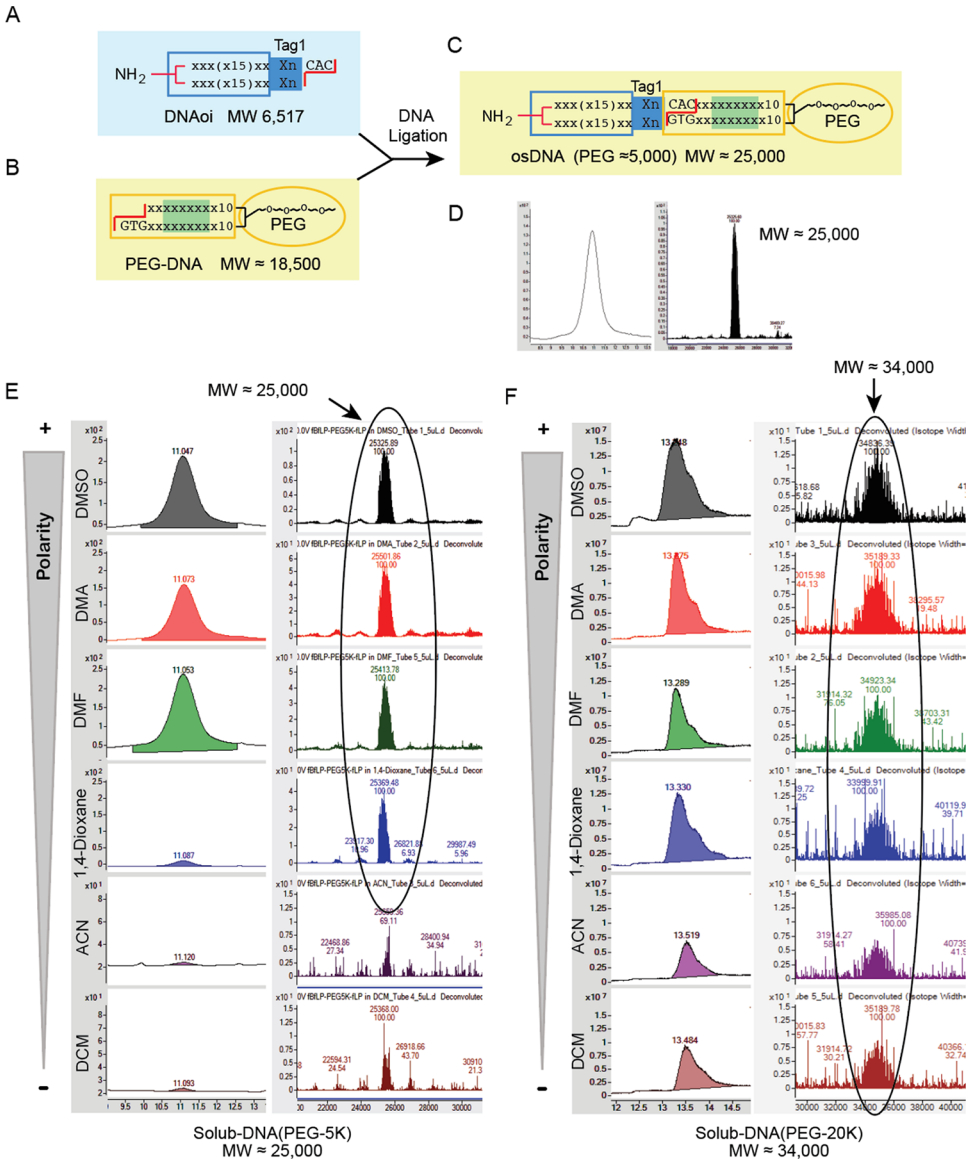

Figure 3. Test of DNA solubility using a larger dsDNA fragment and compatible with DEL construction. The DNAoi (blue open rectangle and dark blue rectangle) (A) is covalently ligated with T4 DNA ligase to a PEG-DNA (MW ∼5,000) (B) and the overall MW of the resulting osDNA (MW ∼25,000) (C) was confirmed by LCMS (D). (E) osDNA was dissolved following ethanol precipitation in 6 different organic solvents of decreasing polarity (DMSO (black), DMA (red), DMF (green), 1,4-dioxane (blue), ACN (purple), and DCM (burgundy)). The expected molecular weight is approximately 25,000. The open black oval indicates a positive detection. The spectra on the left correspond to UV spectra and on the right to deconvoluted total ion chromatograms. (F) osDNA construct containing a PEG moiety of MW ∼20,000 was dissolved in 6 different organic solvents of decreasing polarity (DMSO (black), DMA (red), DMF (green), 1,4-dioxane (blue), ACN (purple), and DCM (brown)). The expected molecular weight is approximately 34,000. The open black oval indicates positive detection. (E, F) The left spectra of each panel correspond to UV spectra and the right spectra to deconvoluted total ion chromatograms. Abbreviations: DMSO: dimethyl sulfoxide; DMF: dimethylformamide; DMA: dimethylacetamide; ACN: acetonitrile; DCM: dichloromethane.

20 PEG units (MW ∼1,000) are sufficient to solubilize DNA partially. More importantly, the solubility of DNA in ethanol increased proportionally to the size of the PEG molecule fused with the DNA fragment (Figure 2B, from the top to bottom spectra). These results demonstrate that a PEG moiety linked to DNA significantly alters its nature to generate a functional osDNA. Additional peaks observed at retention times between 12 and 13 min correspond to unreacted mPEG-NHS compounds.

After the validation of the concept of osDNA, a more elaborated system was designed using a longer dsDNA fragment generated by ligating two dsDNA fragments, with reversibility in mind. The DNAoi can be similar to the one shown in Figure 1D (see also Figure 3A), and the other DNA fragment linked to the solubilizer (PEG) will be called PEG-DNA (Figure 3B). As expected, the DNAoi is soluble in water and not in organic solvents, including ethanol (100%). The two fragments have compatible ends for DNA ligation (e.g., CAC/GTG) and to generate the osDNA following a T4 DNA ligase step (Figure 3C). The LC−MS analysis indicates that the expected MW of 25,000 is obtained and clearly different from each of the individual fragments. This design is compatible with the construction of a DEL, where Tag1 could represent the first tagging step of a split-and-pool

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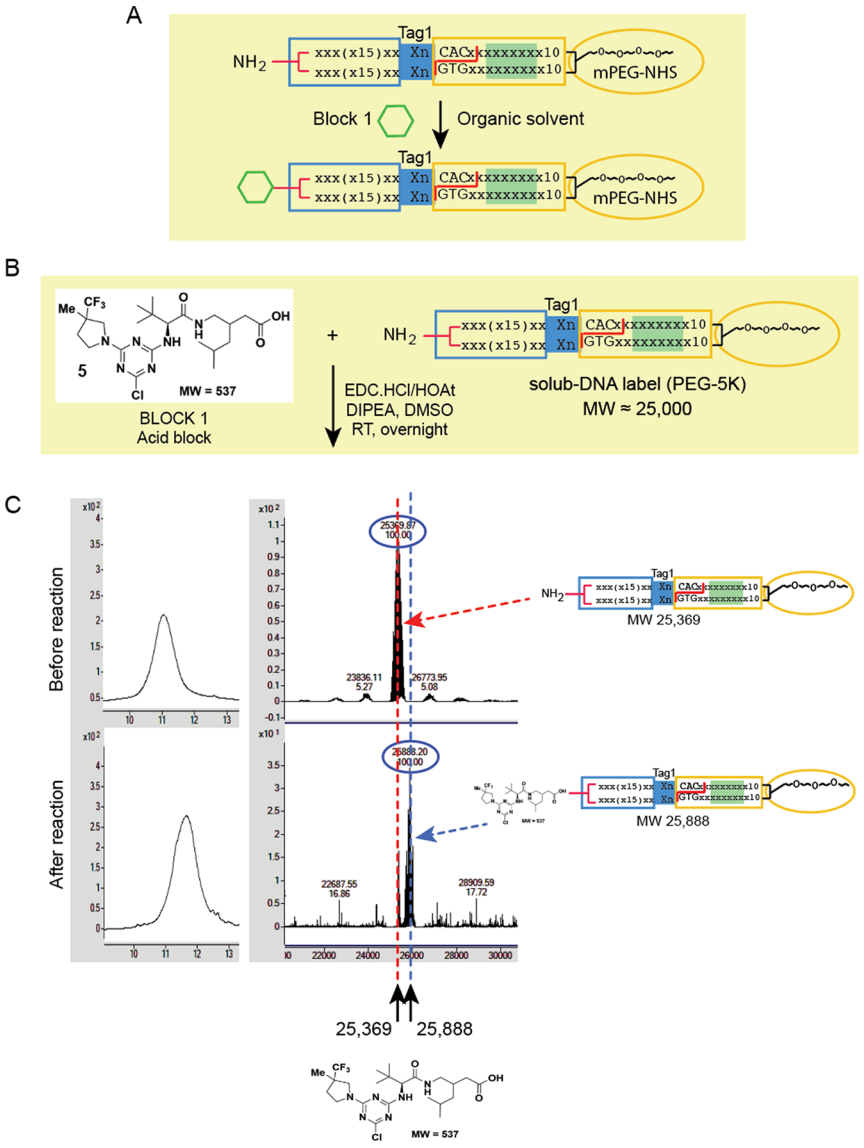

Figure 4. Amidation reaction in organic solvent of an osDNA fragment. (A) Schematic of a chemical block (green hexagon) being added onto osDNA. (B) Compound presented (MW 537) is added onto osDNA (PEG MW ~5,000) using an amidation reaction in 100% DMSO. This reaction would not be possible in aqueous solution. (C) LCMS spectra analysis is presented before and after the reaction. The shift in molecular weight due to the addition of the chemical block is highlighted with dotted lines (red before the reaction and blue after the reaction).

strategy; of note, any sequence and tag numbers could replace the Tag1 sequence.

The solubility of osDNA was evaluated first in ethanol. After ethanol precipitation, analysis of the ethanol phase by LC-MS confirmed that osDNA was well soluble in ethanol (Figure 3D). The osDNA was then purified by HPLC, aliquoted, and lyophilized fully. The solubilized DNA ("osDNA") can be simply separated from the nonligated material (unreacted starting material) and excess DNA tags via dissolution in an organic solvent and filtration. These osDNA pellets were then treated individually with 6 different solvents of decreasing polarity (DMSO, DMA, DMF, 1,4-dioxane, ACN, and DCM). After overnight resuspension, the solubilities of these samples were analyzed using LC-MS-UV spectra. This osDNA is totally soluble in DMSO, DMA, and DMF, partially soluble in 1,4-dioxane but not soluble in ACN and DCM (Figure 3E). Positive results are highlighted (open oval shape) in the deconvoluted spectra. More importantly, similar experiments were repeated using the largest PEG moiety previously tested (MW ~20,000, for a total MW of ~34,000) as described above (Figure 3A–D and in these conditions, osDNA was fully soluble in all 6 solvents tested as highlighted in Figure 3F (oval shape).

Based on these results, having validated the solubility of osDNA in organic solvents, the next step was to apply this new technology to a tangible question such as performing a chemical reaction on DNA that cannot otherwise be performed in aqueous solution. The free NH_2 group of osDNA was used as a substrate to covalently link a chemical block (e.g., Acid 1, green hexagon, Figure 4A) using organic conditions. As a proof of concept, an amidation reaction was carried out between osDNA and a chemical acid block (**A1**) in 100% DMSO (for details, see also Supporting Information, Figures S2–S5). The osDNA in DMSO was mixed with 100 equiv of the acid block (MW 537) also in DMSO followed by the addition of 100 equiv of EDC.HCl/HOAt and 300 equiv of DIPEA in DMSO (RT, overnight, with agitation at 900 rpm) (Figure 4B). LC-MS analysis of the crude mixtures (Figure 4C), before (top)

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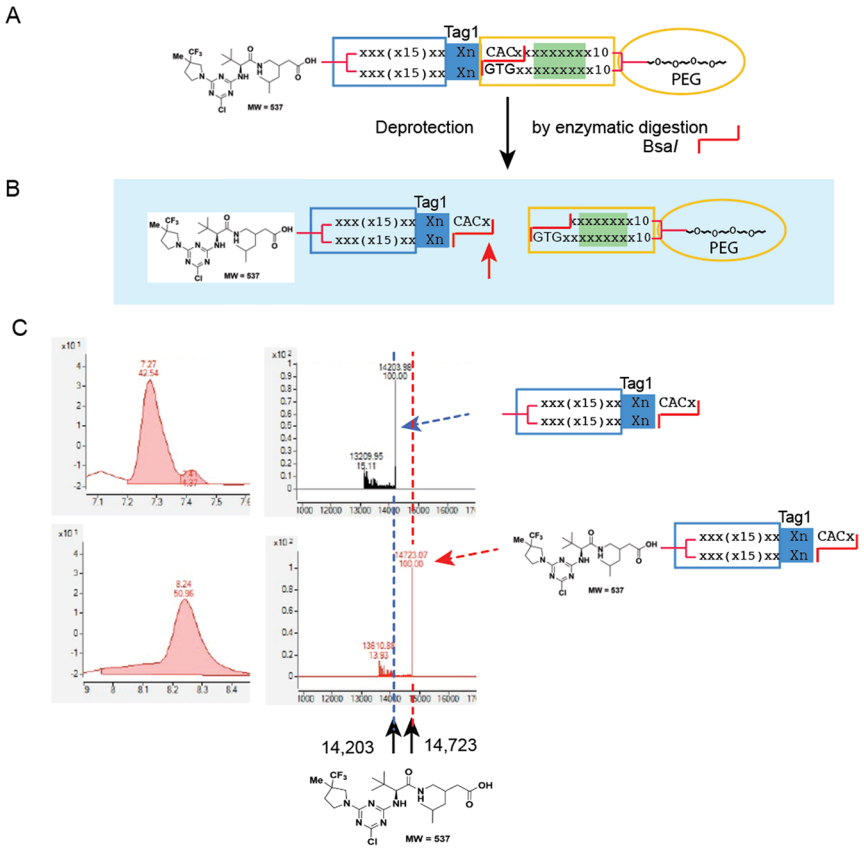

Figure 5. Reversibility of osDNA and confirmation of added compound molecular weight. (A) Following the addition of the desired chemical block, the PEG-DNA can be removed by enzymatic digestion represented by the red line. (A, B) Less frequently used enzyme (type 2) will cut the DNA outside the recognition sequence (red line outside the green box) and will lead to only 1 extra nucleotide being added to the DEL label (red arrow). (C) Results of the reaction and LCMS spectra analysis are presented after digestion. The expected shift of 537 is highlighted by the two dotted lines (blue: starting material; red: product).

and after the reaction (bottom), indicated that the amidation reaction happened successfully in 100% DMSO as demonstrated by the presence of a complete shift of the main peak from MW 25,369 to MW 25,888. The shift is highlighted in red- and blue-dotted lines. This was successfully performed and confirmed using PEG MW ~20,000. It is important to note that this work is limited to a single chemical modification; we anticipate that it would work equally well for other types, but this could not be tested at this time.

The next steps, after the chemical block was added, were to reverse the process, deprotect, and evaluate the DNAoi quality. The dual solubility of PEG in organic solvents and aqueous solutions was another important parameter integrated into the design of this approach. Following the chemical reaction in DMSO, the samples were first diluted 90% in water ($DMSO:H_2O$ = 1:9) and then processed for enzymatic digestion using standard conditions.

DNA digestion with the enzyme Bsa*I* allowed for accurate DNA cleavage and removal of PEG-DNA, with minimal DNA scaring. Bsa*I* was chosen in the initial design because it is a type IIS enzyme that cleaves DNA outside of their DNA recognition site sequence 1 and 5 nucleotides away from the recognition site (Figure 5A; red line; CACx/GTGx), guaranteeing minimal DNAoi alteration, as only 1 extra nucleotide is added to the overhang sequence (see red arrow, Figure 5B). It is important to note that the Bsa*I* site is located on the PEG-DNA side and is fully removed after digestion. Therefore, the method can be applied more than once. Indeed, by using Bsa*I* only and 1 PEG-DNA specific for each tag step, the process can easily be repeated for each step. LC-MS and gel analysis of the crude mixtures, obtained with and without reaction, indicated that the enzymatic cleavage proceeded perfectly as demonstrated by the presence of a shift from MW 14,203 to MW 14,723 (Figure 5C), also further confirming the block addition. The shift is highlighted with red and blue dotted lines to emphasize the difference in size.

To evaluate the scope and generality of this coupling reaction, 11 different acids were selected, and similar experiments were performed (Table 1). The reaction was facile with aliphatic acids **A2** and **A3**, resulting in yields of 60 and 90%, respectively. The coupling reaction of aromatic acids **A4**−**A8** with PEGylated DNA proceeded well with very good yields. Finally, heteroaromatic carboxylic acids **A9** (Indole-5-carboxylic acid) and **A10** (pyrazole carboxylic acid) also reacted and produced very good yields. The benzothiophene carboxylic acid **A11** gave a 45% yield. In conclusion, it is possible to use 100% organic solvent (e.g., DMSO) as the reaction solvent for PEGylated DNA. This has important applications for water-sensitive organic reactions on DNA.

Following the cleavage, the DNAoi has been reversed with the exception of a 1 nucleotide scar. Alternatively, it is ready for another DNA ligation, for example, with Tag2 (Figure 6A, red rectangle) for the next modification step in the context of DEL. The existence of 4 (3 + 1 new one) overhang nucleotides after cleavage (Figure 6A) has an added advantage as it differentiates DNAoi that has not been processed (has only 3

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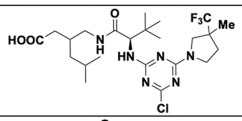
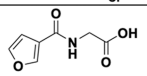
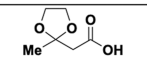
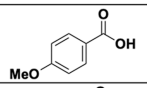
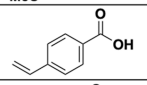
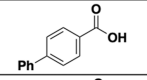
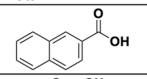
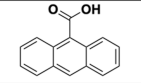
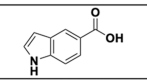
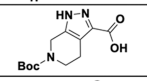
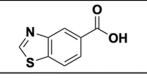
Table 1. Scope of the Amidation Reaction of PEGylated DNA with Various Acids[a]

Entry	Acid structure	% Yield[b]
A1		48%
A2		60%
A3		90%
A4		95%
A5		91%
A6		69%
A7		86%
A8		80%
A9		>95%
A10		79%
A11		45%

[a]Reaction condition: PEG-DNA (10 μL, 1 mM in 100% DMSO), acid block (10 μL, 100 mM in DMSO), EDC.HCl (10 μL, 100 mM in DMSO), HOAt (10 μL, 100 mM in DMSO), and DIPEA (10 μL, 300 mM in DMSO), RT, overnight. [b]Yields calculated from LCMS.

nucleotides overhang) (Figures 1Dand 6C) and cannot ligate to Tag2 (Figure 6D). After DNA ligation of the modified-DNAoi with Tag2 (Figure 6B), the new DNAoi can be ligated again to a PEG-DNA with compatible cohesive ends leading to a new osDNA (Figure 6E) and the same steps can happen again to add for example a block 2, in organic conditions, onto the block 1 (Figure 6B, green hexagon, red arrow). Of note, the PEG-DNA used was longer than the length obtained if tags 1, 2, and 3 were incorporated with a shorter PEG-DNA. In other words, the PEG-DNA length could be adjusted as needed based on the step; alternatively, a shorter version could be used for all three steps.

CONCLUSIONS

In summary, we have created a novel type of DNA that we call osDNA for organo-soluble DNA. This approach allows DNA solubilization in organic solvents ranging from ethanol to DCM. It represents an entirely new avenue to chemically modify DNA, and DNA-containing moieties, as we demonstrated with an amidation reaction in the context of building a DEL. This work demonstrates the considerable chemistry potential that can now be performed in the presence of nucleic acids, dramatically opening up the chemical space DNA-compatible, both in terms of solvent compatibility and chemical block solubility. Furthermore, this versatile approach applicable to any DNA is reversible, fully controllable, and does not alter the initial DNA in any way. This contribution will foster the development of novel methods that are important for nanoscience and might have a broad impact. Further studies on expanding the scope and application of osDNA are now underway in our laboratory.

MATERIALS AND METHODS

Reagents. The following commercially available reagents were used: acetonitrile (HPLC grade, cat# 34851, Sigma-Aldrich, USA), methanol (HPLC grade, cat# A452SK-4, Fisher Scientific, USA), *N*,*N*-dimethyl formamide (DMF) (HPLC grade, cat# 588725, Sigma-Aldrich, USA), *N*,*N*-dimethyl acetamide (DMA) (HPLC grade, 99.5%, cat# 22916, Alfa Aesar, USA), 1,4-dioxane (HPLC grade, cat# 296309, Sigma-Aldrich, USA), dimethyl sulfoxide (DMSO) (≥99.5%, cat# D5879, Sigma-Aldrich, USA), 1,1,1,3,3,3-Hexafluoroisopropyl alcohol (99.9%, cat# 00080, Chem-Impex International, Inc., USA), Triethylamine (≥99%, cat# T0886, Sigma-Aldrich,

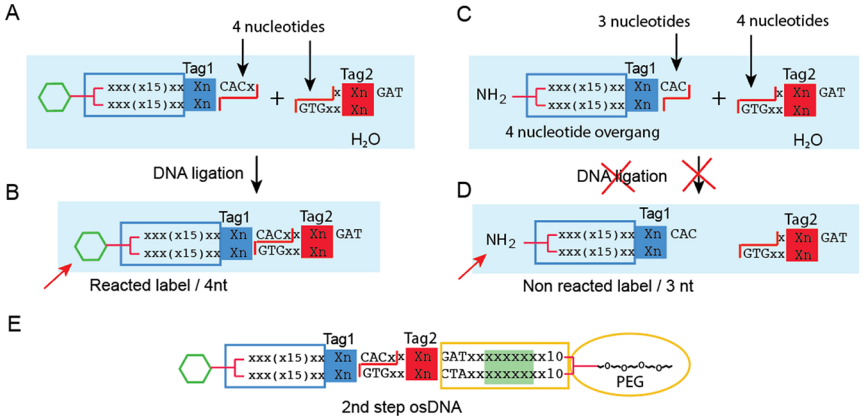


Figure 6. Use of modified DNAoi for the next preparative step.(A) Following the removal of PEG-DNA by enzymatic digestion (red line), the modified DNAoi is ready for future applications. (B) For example, in the case of DEL, the next ligation step which consists in adding another DEL tag (e.g., tag2; red box) can be performed. (C, D) Due to the nature of the overhang DNA sequences, any residual nonmodified DNAoi will not interfere with the next step as the 3 versus 4 nucleotide overhang ends will not ligate. (E) Final product from B can then be used as a new DNAoi and be modified by ligation to a novel PEG-DNA and so forth.

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USA), and *N,N*-diisopropylethyl amine (≥99%, cat# T0886, Sigma-Aldrich, USA). UltraPure distilled water (DNase, RNase free, cat# 10977-015, Invitrogen, USA) and sodium hydroxide solution (BioUltra, 10 M in H_2O, cat# 72068, Sigma-Aldrich, USA) were used for buffer preparation. Deionized water was used for the LC-MS mobile phase preparation. Activated polyethylene glycol mPEG-SCM (PEG-NHS ester, molecular weight of PEG ∼5,000) was purchased from Biopharma PEG Scientific Inc., USA. The other PEG-NHS esters of different molecular weights (∼1,000; ∼2,000; ∼3,400; ∼10,000; ∼20,000) were also purchased from Biopharma PEG Scientific Inc., USA.

DNA Design and Preparation. All DNA fragments used in the present work were designed in-house, custom-made, and synthesized by Integrated DNA Technologies, Inc. (IDT, Coralville, Iowa, USA). Lyophilized DNA samples were resuspended in Tris-EDTA (TE) buffer pH 8.0 at 1 mM (or unless specified at lower concentration and/or in H_2O), tested for quality purposes by mass spectrometry (LC−MS), quantified, and stored at −20 °C. The sequence and modifications of the DNA presented and used in Figure 1E are as follows: 5′-/5Phos/GTCAGACT/iSp9//iUniAmM//iSp9/AGTCTGACGCT-3′. iSp9 is an internal triethylene glycol Spacer 9. The internal Uni-Link (iUniAmM) is an amino-modifier phosphoramidite harboring a free primary amine attached via an aliphatic spacer arm.

LC−MS Instrumentation. LC-MS analyses were performed using an Agilent LC-MS system (LC-MS-TOF 6230B) (Agilent, Santa Clara, CA, USA) according to the manufacturer instructions consisting of LC parts, a multisampler (model number G7167A), binary pump (model number - G7112B), column compartment (model number - G7116A), UV/MWD detector (model number G7165A), and MS TOF (model number - G6230B).

LC−MS Analysis Conditions. The mobile phase consisted of 100 mM HFIP and 8.9 mM TEA in deionized water (A) and MeOH (B). The samples were injected onto a reverse phase chromatography column (Targa C18, 5 μm, 50 × 2.1 mm, 120 A°), and gradient elution was as follows: 1% B hold for 1 min; 1−95% B for 15 min and set the post time for 3 min to equilibrate; at a flow rate of 0.4 mL/min and the column temperature at 40 °C. The dual ESI negative mode polarity was used with a scan range of 500−3200 Da. The source conditions were as follows: Drying gas flow of 12 L/min at 325 °C and a nebulizer pressure of 30 psi. The capillary voltage was set to 4000 V.

LC−MS Data Acquisition and Analysis. The data for each DNA sample were acquired using Agilent mass hunter workstation data acquisition software, and the data were analyzed using Agilent mass hunter qualitative analysis B.07.00. The quality and estimated yield of DNA samples were determined by examination of the UV absorbance traces at 260 nm and total ion chromatogram (TIC) traces corresponding to the peaks after deconvolution.

PEG-DNA Synthesis. DNA oligonucleotides (250 nmol or 250 μL of 1 mM) resuspended in 150 mM borate buffer (pH 9.5) were added to mPEG-NHS ester (20 mM) in DMA at room temperature, and the reaction mixture was stirred (ThermoMixer C) at 900 rpm at 25 °C overnight. The resulting product was purified by reversed-phase high-performance liquid chromatography (Gemini C18 column, 100 × 10 mm inner diameter, 5-μm particle size, Phenomenex, USA). The acetonitrile/MeOH concentration was increased from 1 to 95% over 15 min and from 95 to 100% over 20 min. Unreacted DNA was eluted at around 4−5 min, and PEGylated DNA fragments were eluted at around 8−10 min for (PEG MW∼5,000) and 10−12 min for (PEG MW∼20,000). The solution containing PEG-DNA was collected selectively and lyophilized overnight.

DNA Ligation. Standard molecular techniques were used for DNA ligation. Commercially available T4 DNA ligase (NEB, Biolabs) was purchased and tested using an in-house linear plasmid. DNA ligations were performed for 1 h at room temperature using the ligase buffer provided with the enzyme. The ligation was monitored by agarose gel electrophoresis and also by LC−MS when a high sensibility was needed.

Solubility of osDNA in Organic Solvents. The various osDNAs (10 nmol or 10 μL of 1 mM) were lyophilized in 1.5 mL Eppendorf tubes and dissolved in 40 μL of solvent [dimethylsulfoxide, dimethylformamide, dimethylacetamide, 1,4-dioxane, acetonitrile, and dichloromethane]. Five microliters of this stock solution were directly injected and analyzed by LC-MS. All organic solvents (HPLC grade) were purchased from Sigma-Aldrich (USA). LC−MS analyses were performed using an Agilent LC−MS system (LC-MS-TOF 6230B) (Agilent, Santa Clara, CA, USA)

Reaction Protocol for Coupling a Chemical Block with osDNA. The PEG-DNAs (10 μL, 1 mM in DMSO) in a 1.5 mL Eppendorf tube were mixed with 100 equiv of a chemical block (acid block; 10 μL, 100 mM in DMSO), EDC.HCl (10 μL, 100 mM in DMSO), and HOAt (10 μL, 100 mM in DMSO). Finally, 300 equiv of DIPEA (10 μL, 300 mM in DMSO) were added. The reaction mixture was stirred (ThermoMixer C) at 900 rpm at room temperature overnight, and the crude reaction mixture was checked by LC−MS.

DNA Digestion of osDNA for Deprotection. Standard molecular techniques were used for reversing the osDNA to DNAoi by enzymatic cleavage. Commercially available enzymes (NEB, Biolabs) were purchased and tested by using an in-house plasmid known to contain target restriction sites. Enzymatic digestion was performed for 1 h at room temperature in the appropriate buffer. Deprotection following digestion was monitored by agarose gel electrophoresis and confirmed by LC−MS when a high sensitivity was needed.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c06755.

NMR spectral data; reaction procedure for the synthesis of the chemical block (**A1**); and LC−MS spectra of the reaction products (PDF)

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Notes

The authors declare the following competing financial interest(s): M.F. is a founder/board member, and equity holder of Jillion Therapeutics. A pending patent application related to this published study has been submitted to the US Patent and Trademark Office by The Rockefeller University.

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