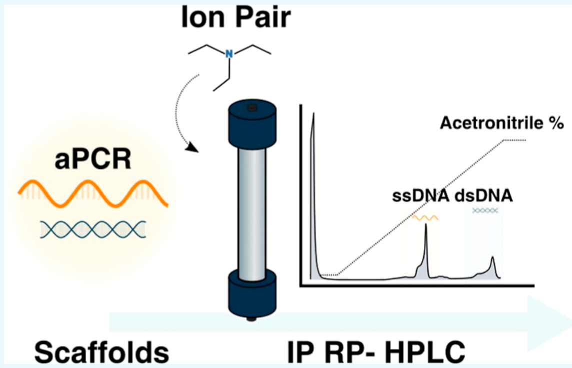
ACS OMEGA
http://pubs.acs.org/journal/acsodf
Article

Quantification of ssDNA Scaffold Production by Ion-Pair Reverse Phase Chromatography

A. Rita Silva-Santos, Sara Sousa Rosa, Marco P. C. Marques, Ana M. Azevedo, and Duarte Miguel F. Prazeres*

Cite This: *ACS Omega* 2024, 9, 22619−22624

ABSTRACT: DNA origami is an emerging technology that can be used as a nanoscale platform in numerous applications ranging from drug delivery systems to biosensors. The DNA nanostructures are assembled from large single-stranded DNA (ssDNA) scaffolds, ranging from hundreds to thousands of nucleotides and from short staple strands. Scaffolds are usually obtained by asymmetric PCR (aPCR) or *Escherichia coli* infection/transformation with phages or phagemids. Scaffold quantification is typically based on agarose gel electrophoresis densitometry for molecules obtained by aPCR, or by UV absorbance, in the case of scaffolds obtained by infection or transformation. Although these methods are well-established and easy-to-apply, the results obtained are often inaccurate due to the lack of selectivity and sensitivity in the presence of impurities. Herein, we present an HPLC method based on ion-pair reversed-phase (IP-RP) chromatography to quantify DNA scaffolds. Using IP-RP chromatography, ssDNA products (449 and 1000 nt) prepared by aPCR were separated from impurities and from the double stranded (ds) DNA byproduct. Additionally, both ss and dsDNA were quantified with high accuracy. The method was used to guide the optimization of the production of ssDNA by aPCR, which targeted the maximization of the ratio of ssDNA to dsDNA obtained. Moreover, ssDNA produced from phage infection of *E. coli* cells was also quantified by IP-RP using commercial ssDNA from the M13mp18 phage as a standard.



■ INTRODUCTION

The DNA nanotechnology explores Watson−Crick base pairing to self-assemble nucleic acids into complex nanostructures.[1−3] In particular, a strategy denominated "scaffolded DNA origami" can be used where a long single-stranded DNA (ssDNA) molecule (the scaffold) is folded into a target shape with the help of short oligonucleotides (the staples).[4−6] Due to its programmable design, high loading capacity, multifunctionality, biocompatibility, biodegradability, stability, protection of encapsulated drugs against enzymatic degradation and harsh environmental conditions, controlled drug release, and scalability in the manufacturing process, the DNA origami technique has been increasingly studied in a wide range of fields of study, like drug delivery systems[7,8] and biosensors,[9] surpassing conventional systems.

The production of scaffold DNA for DNA origami manufacturing is usually performed by the purification of phage-derived single-stranded genomic DNA[10,11] or with asymmetric PCR (aPCR) from DNA templates.[5,12] Scaffold quantification is typically based on absorbance at 260 nm or on agarose gel electrophoresis densitometry. Although both methods are well-established and are easy-to-apply, the results obtained are often inaccurate due to a lack of selectivity due to the presence of impurities. When *Escherichia coli*/phage systems are used to produce ssDNA, phage-derived impurities like proteins, and cell-derived impurities like genomic DNA and cell debris, will increase absorbance at 260 nm, thus contributing to overestimate the scaffold concentration.[13,14] On the other hand, the use of densitometry analysis of DNA bands in agarose gels will typically underestimate the ssDNA concentration since the commonly used dye molecules for gel staining intercalate less on ssDNA compared to double-stranded DNA (dsDNA).[15] While different fluorescent dyes can be used to quantify ssDNA with high sensitivity (1 ng/mL), they lack sensibility and present fluorescence enhancement when bound to dsDNA and RNA. To overcome these limitations, we present a novel method based on ion-pair reversed-phase (IP-RP) chromatography to quantify DNA scaffolds throughout the biomanufacturing process.

HPLC is extensively used as an analytical tool due to its high selectivity, sensitivity, and reproducibility. In particular, IP-RP

Received: December 31, 2023
Revised: March 6, 2024
Accepted: March 8, 2024
Published: May 15, 2024

https://doi.org/10.1021/acsomega.3c10533
ACS Omega 2024, 9, 22619−22624

is widely described for the separation of nucleic acids, namely, DNA fragments[16,17] and ssRNA.[18] For example, IP-RP is used to analyze RNA quality in terms of size,[19] presence of dsRNA,[20] or poly-A size.[21] To achieve separation, an ion-pair must form between the quaternary ammonium compounds present in the mobile phase (eluent) and the negatively charged sugar−phosphate backbone of the DNA. This ion-pair will then interact with the stationary phase of the chromatography column. Elution of bound molecules is performed using an adequate solvent, such as acetonitrile. When applied to nucleic acids, IP-RP separation is dependent on the number of sugar−phosphate groups in each molecule.

In this work, IP-RP is first explored as a methodology to separate and quantify ssDNA scaffolds (449 and 1000 nt long) and the corresponding dsDNA impurities obtained by aPCR. To characterize the method, several attributes are determined, namely, the limit of detection (LOD), limit of quantification (LOQ), decision limit (CCα), and detection capability (CCβ). The method is further employed to optimize the primer ratios that maximize the aPCR production of ssDNA relative to dsDNA. Furthermore, the established method was successfully used to quantify the ssDNA genome of the M13mp18 phage, a circular ssDNA molecule widely used as a scaffold for the DNA-origami production of large objects.[22,23] Overall, an analytical method that is selective and sensitive was implemented for the separation and quantification of ssDNA molecules, which can be used in multiple steps of the scaffold biomanufacturing process.

EXPERIMENTAL SECTION

Materials. All chemicals and reagents were of analytical grade. Capto Q resin was obtained from GE Healthcare (Uppsala, Sweden). The producer *E. coli* strain K12 ER2738, M13mp18 RF I DNA, and M13mp18 ssDNA were obtained from New England Biolabs (Massachusetts, USA). AccustartTM Taq DNA Polymerase Hifi was obtained from Quantabio (Massachusetts, USA). Primers and oligonucleotides were obtained from StabVida (Caparica, Portugal). DNAPac RP column and X2 DNAPacRP guard column were obtained from ThermoFisher (Massachusetts, USA).

Scaffold Production. *M13mp18 ssDNA.* M13mp18 ssDNA was produced according to Silva-Santos et al.[24] Briefly, after *E. coli* K12 ER2738 transformation with M13mp18 RFI DNA (GenBank: X02513.1), a blue plaque was selected and used for infection of an *E. coli* preculture at an optical density of 0.5 in 5 mL of 2× YT medium supplemented with 10 μg/mL tetracycline (Sigma-Aldrich, Missouri, USA). After 2 h at 37 °C and 250 rpm, the cells were transferred to flasks containing 750 mL 2× YT supplemented with 10 μg/mL tetracycline. The phage expansion occurred overnight under the same conditions. Phage purification and genomic ssDNA extraction were performed according to Kick et al.[11]

aPCR Mixtures. The target 449 and 1000 nt ssDNA scaffolds were synthesized by aPCR with different ratios of forward to reverse primers. The reaction conditions used were in accordance with Veneziano et al.[12,24] Briefly, a forward primer with a concentration ranging between 4 μM and 500 nM (GTCGTCGTCCCCTCAAACT for 449 nt and GTCTCGCTGGTGAAAAGAAA for 1000 nt), a reverse primer with a concentration ranging between 20 and 500 nM (ATTAATGCCGGAGAGGGTAG), 30 ng of purified M13 ssDNA template, 200 nM deoxynucleotide triphosphates (dNTPs), and 1 U of Accustart Taq DNA polymerase were mixed in a final volume of 50 μL. The aPCR program consisted of an initial denaturation step of 1 min at 94 °C and 35 thermal cycles of (i) 94 °C for 20 s, (ii) 55 °C for 30 s, and (iii) 68 °C for 60 s per kilobase amplification.

HPLC. DNA was quantified using a 2.1 nm × 100 nm DNAPac RP column (3 nm × 10 nm) and a guard column (ThermoFisher, USA). 15 μL of the sample was diluted with 1 M triethylammonium acetate (TEAA) to a final concentration of 0.1 M and injected into the column pre-equilibrated with 0.1 M triethylamine acetate (TEAA) (ThermoFisher, USA). Gradient elution was performed using 0.1 M TEAA (ThermoFisher, USA) with 25% (v/v) acetonitrile (Fisher, USA). Runs were performed at 60 °C at a flow rate of 0.4 mL/min, and the absorbance was monitored at 260 nm. The run conditions are presented in the Supporting Information Table S1. M13mp18 ssDNA standards (1 to 4 μg/mL) were prepared with commercially available ssDNA from New England Biolabs (USA) and used to obtain a calibration curve. Products of 20 pooled aPCR reactions purified with a Capto Q ImpRes anion exchanger according to the method described in Silva-Santos et al.[25] were used to obtain calibration curves for ssDNA (0.2 and 20 μg/mL) and dsDNA (0.2 and 25 μg/mL). Calibration curves and standard chromatographic profiles for each of the pure samples are shown in the Supporting Information. Replicates were performed independently.

Statistical Analysis. Validation of the method was performed by calculating the LOD, LOQ, CCα, and the CCβ, according to the European Union regulation 2002/657/EC guidelines.[26] The limits were calculated based on the standard error of the intercept (σ) and the slope (S) of the calibration curves at a signal-to-noise ratio of 3.3 (LOD) and 10 (LOQ) according to

$$\mathrm{LOD} = \frac{3.3\sigma}{S} \tag{1}$$

$$\mathrm{LOQ} = \frac{10\sigma}{S} \tag{2}$$

Calibration curves were performed twice with independent replicates. CCα (eq 3) and CCβ (eq 4) were calculated considering a 2.33 factor, which corresponds to 1% of false positive risk, and a 1.64 factor, which corresponds to a 5% false negative risk with regard to CCα,[26,27] respectively.

$$\mathrm{CC}\alpha = \frac{2.33\sigma}{S} \tag{3}$$

$$\mathrm{CC}\beta = \mathrm{CC}\alpha + \frac{1.64\sigma}{S} \tag{4}$$

RESULTS AND DISCUSSION

An ion-pair reverse phase chromatography analytical method was developed for the quantification of the DNA scaffolds. Using this method, ssDNA scaffolds (449 and 1000 nt) were quantified directly from samples collected from aPCR reaction mixtures. Typical chromatograms (Figure 1a) display a flowthrough peak that contains process-related impurities, as demonstrated by injecting control samples with polymerase and dNTPs (Figure S3). Upon elution with the increased acetonitrile concentration, two peaks with ill-defined shape emerge at 12−15 min and at 18−22 min (Figure 1a). Chromatograms were similar whether aPCR mixtures contained ssDNA scaffolds with 449 or 1000 nt (Figure 1a), albeit

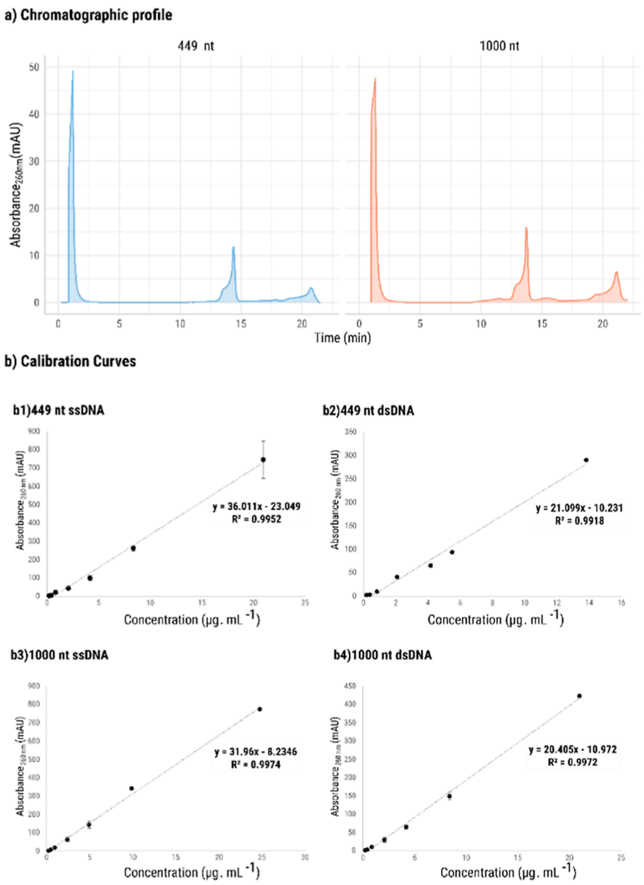


Figure 1. (a) Representative IP-RP chromatograms of samples collected from aPCR reaction mixtures used to produce 449 and 1000 nt ssDNA scaffolds. (b) Calibration curves and trendlines obtained for: [b(1)] 449 nt ssDNA; [b(2)] 449 bp dsDNA; [b(3)] 1000 nt ssDNA; and [b(4)] 1000 bp dsDNA (n = 2).

residence times varied slightly. These results shows that the proposed method is more sensitive to DNA conformation (single or double) than to differences in size. To determine the identity of the molecules in the elution peaks, ssDNA scaffolds and the corresponding dsDNA impurities were first isolated from aPCR mixtures by preparative anion exchange chromatography,[28] analyzed by agarose gel electrophoresis (Figure S1b), and then injected in the IP-RP column. Results show that samples containing ssDNA scaffolds eluted with retention times corresponding to the first peak [Figure S1a(1), (3)], whereas samples containing dsDNA eluted with retention times corresponding to the second peak [see Figure S1a(2), (4)].

IP-RP chromatography is a well-described technique that explores differences in hydrophobicity and is widely used in the separation of nucleic acids.[29] Optimization of the eluent gradient and column temperature are considered the key factors for the separation of dsDNA and ssDNA. Temperature is also an important factor on the separation as it can affect the physical properties of the DNA.[30] For DNA molecules to form an ion-pair with a cationic ion-pair reagent (IPR) such as TEAA, phosphate groups in the backbone must be exposed. Then, the carbon atoms of the alkyl chains of the IP will form a hydrophobic bond with the stationary phase.[31] Exposure of the phosphate groups is facilitated if one interferes with the secondary structure of the DNA, for example, by performing the separation at a temperature of 60 °C. Since dsDNA contains twice the number of phosphate groups when compared with the corresponding ssDNA, the binding with the solid phase will be stronger. Concomitantly, a higher concentration of acetonitrile is required for elution, as observed in Figure 1a.

To validate the analytical chromatography method proposed, several attributes were calculated, namely, the LOD, LOQ, CCα, and CCβ, using the calibration curves represented in Figure 1b. The LOD corresponds to the smallest concentration of the analyte that can be confidently quantified by the method, while the LOQ is the smallest concentration that can be quantified with a given level of confidence.[32] The results for both the 449 and 1000 bp scaffolds are shown in Table 1. Although the TEAA buffer used is known to suppress the absorbance signal,[33] the method presents LODs of 0.89 μg/mL (ssDNA) and 0.79 μg/mL (dsDNA) for the 449 nt scaffold, and 0.78 μg/mL (ssDNA) and 0.68 μg/mL (dsDNA) for the 1000 nt scaffold. The difference in the LOD observed between the different length scaffolds and between ss and ds DNA can be correlated to the molar mass of each analyte. Analytes with a higher molar mass will be detected at lower concentrations.

Table 1. Quantification Attributes of the IP-RP Chromatography for the 449 and 1000 bp Scaffolds, Single- (ssDNA) and Double-Stranded (dsDNA), in Terms of Limit of Detection (LOD), Limit of Quantification (LOQ), the Decision Limit (CCα), and the Detection Capability (CCβ)

	449 nt/bp		1000 nt/bp	
	ss	ds	ss	ds
LOD (μg/mL)	0.89	0.79	0.78	0.68
LOQ (μg/mL)	2.70	2.40	2.36	2.07
CCα (μg/mL)	0.63	0.56	0.55	0.48
CCβ (μg/mL)	1.07	0.95	0.94	0.82

The CCα and CCβ are described by the European Commission Decision 2002/657/EC as the limit at and above which a sample is considered to be noncompliant, with an error probability of α, and the smallest analyte quantity that may be detected, identified, or quantified with an error probability of β, respectively.[26] The CCβ and CCα values for both ss and dsDNA can be found in Table 1. As previously observed, molecules with higher mass (449 and 1000 nt dsDNA) presented lower values for CCα and CCβ.

The IP-RP HPLC method was tested next to optimize the production of ssDNA scaffolds by aPCR. In this amplification method, a molar excess of the forward primer, which will originate the ssDNA scaffold, is used over the reverse primer, so as to maximize scaffold production.[12,34] The optimization was performed by varying the molar ratio between the forward and reverse primers from 1 to 200 (w/w) and then evaluating the ratio of the mass of ssDNA to mass of dsDNA produced (Figure 2). The highest amount of ssDNA relatively to dsDNA is obtained with a 50-molar excess of forward primer for both DNA sizes −2.84 ± 0.08 ratio for 449 bp and 3.81 ± 0.18 for 1000 bp. Increasing the primers ratio above 50 negatively impacted the aPCR yield. These results are in line with the aPCR results obtained by Veneziano et al.,[12] which were determined on the basis of gel electrophoresis analysis.

The adequacy of the IP-RP method to quantify ssDNA from M13mp18 phage was also evaluated. A typical chromatogram of a standard used displays a peak with ill-defined shape eluting

https://doi.org/10.1021/acsomega.3c10533
ACS Omega 2024, 9, 22619−22624

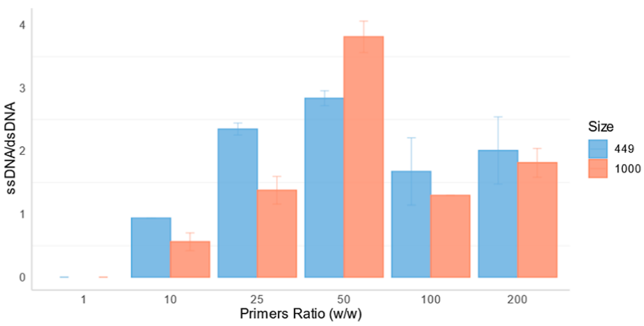
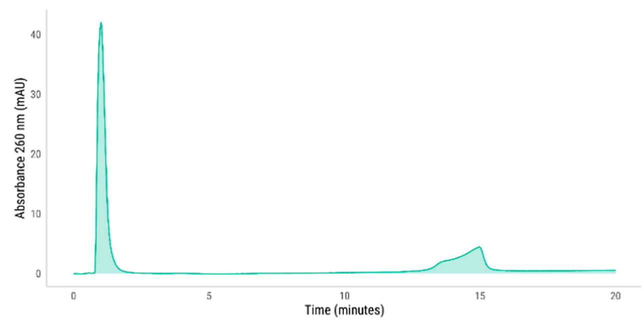


Figure 2. Optimization of ssDNA scaffold production by aPCR. Optimization was performed so that the highest yield possible of ssDNA is obtained relative to the dsDNA production. The ssDNA/dsDNA ratio was determined using the IP-RP chromatography method described (n = 3).

at 13−15 min (Figure S2). This reinforces the hypothesis that the method is neither sensitive to size differences nor to the linear or cyclic nature of the ssDNA. The method was validated by calculating LOD, LOQ, CCα, and CCβ. As observed in Figure 3b,c, LOD and LOQ of 1.45 and 4.4 μg/mL were obtained, respectively. In the case of CCα and CCβ, the values obtained were 1.03 and 1.75 μg/mL.

The method was then used to quantify ssDNA extracted from the M13mp18 phage. A concentration of 126.25 ± 8.60 μg/mL was obtained (Figure 3a). This value is lower than the one obtained when quantitation was performed by UV absorbance at 260 nm (198.15 ± 2.95 μg/mL). Nevertheless, this difference is expected due to the lack of specificity presented by spectrophotometric techniques, which will not have the ability to separate signals originating from impurities from those ascribed to the analyte.

a) Chromatographic profile



b) Attributes

LOD (μg/mL)	LOQ (μg/mL)	CCα (μg/mL)	CCß (μg/mL)
1.45	4.40	1.03	1.75

c) Calibration curve

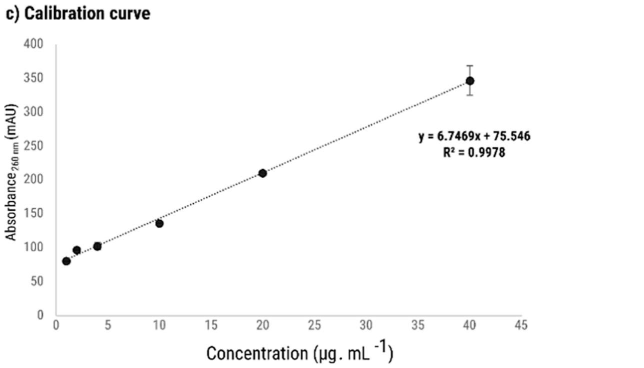


Figure 3. (a) Representative IP-RP chromatogram of ssDNA extracted from the M13mp18 phage. (b) Attributes of the ssDNA extracted from the M13mp18 phage, in terms of LOD, LOQ, CCα, and CCβ. (c) Calibration curve for the M13mp18 ssDNA (n = 2).

CONCLUSIONS

DNA scaffolds is a crucial step for the DNA-origami technology. A sensitive quantification of DNA scaffolds is critical for accurate determination of DNA-origami production yields. Additionally, optimization of scaffold production at a large scale must rely on sensitive analytical methods that can accurately quantify ssDNA and that can be applied at-line. However, current quantification methods rely on the use of techniques that are neither specific nor sensitive enough, leading to erroneous estimations of the actual scaffold quantities and therefore of the amount of DNA-origami nanostructures that can be produced. In this work, a HPLC method based on IP-RP was used to quantify different size scaffolds produced either by aPCR techniques or by infecting *E. coli* cells with the M13 phage. IP-RP is advantageous when compared with traditional quantification methods, such as absorbance at 260 nm and agarose gel densitometry. First, it presents a higher selectivity as complete separation between dsDNA and ssDNA is achieved. Additionally, the quantification is neither impacted by impurities nor user dependent. The method is sensitive, accurate, and specific and delivers reliable titer measurements, with LOD below 1.5 μg/mL for all the DNA species evaluated, making it a powerful tool for quantification of ssDNA scaffolds regardless of the production system used.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c10533.

IP-RP HPLC gradient method and IP-RP HPLC chromatograms of control samples (PDF)

AUTHOR INFORMATION

Corresponding Author

Duarte Miguel F. Prazeres − *iBB—Institute for Bioengineering and Biosciences, Department of Bioengineering, Instituto Superior Técnico, Universidade de Lisboa, 1049-001 Lisboa, Portugal; Associate Laboratory i4HB−Institute for Health and Bioeconomy at Instituto Superior Técnico, Universidade de Lisboa, 1049-001 Lisboa, Portugal;* orcid.org/0000-0002-6928-692X; Email: miguelprazeres@tecnico.ulisboa.pt

Authors

A. Rita Silva-Santos − *iBB—Institute for Bioengineering and Biosciences, Department of Bioengineering, Instituto Superior Técnico, Universidade de Lisboa, 1049-001 Lisboa, Portugal; Associate Laboratory i4HB−Institute for Health and Bioeconomy at Instituto Superior Técnico, Universidade de Lisboa, 1049-001 Lisboa, Portugal;* orcid.org/0000-0001-6025-0114

Sara Sousa Rosa − *iBB—Institute for Bioengineering and Biosciences, Department of Bioengineering, Instituto Superior*

Técnico, Universidade de Lisboa, 1049-001 Lisboa, Portugal; Associate Laboratory i4HB—Institute for Health and Bioeconomy at Instituto Superior Técnico, Universidade de Lisboa, 1049-001 Lisboa, Portugal; orcid.org/0000-0003-4415-1364

Marco P. C. Marques − *Department of Biochemical Engineering, University College London, London WC1H 0AH, U.K.*

Ana M. Azevedo − *iBB—Institute for Bioengineering and Biosciences, Department of Bioengineering, Instituto Superior Técnico, Universidade de Lisboa, 1049-001 Lisboa, Portugal; Associate Laboratory i4HB—Institute for Health and Bioeconomy at Instituto Superior Técnico, Universidade de Lisboa, 1049-001 Lisboa, Portugal*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.3c10533

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. A.R.S.-S. and S.S.R. contributed equally.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was funded by national funds from FCT—Fundação para a Ciência e a Tecnologia, I.P., in the scope of the project UIDB/04565/2020 and UIDP/04565/2020 of the Research Unit Institute for Bioengineering and Biosciences−iBB, the project LA/P/0140/2020 of the Associate Laboratory Institute for Health and Bioeconomy—i4HB and project PTDC/NAN-MAT/29460/2017, cofinanced by FEDER under the PT2020 Partnership Agreement, and supported by the Future Biomanufacturing Research Hub (grant EP/S01778*X*/1), funded by the Engineering and Physical Sciences Research Council (EPSRC) and Biotechnology and Biological Sciences Research Council (BBSRC) as part of UK Research and Innovation. A. Rita Silva-Santos acknowledges FCT for PhD grants PD/BD/135138/2017 (BIOTECnico program) and COVID/BD/151841/2021. Sara Sousa Rosa acknowledges FCT for the PhD grant SFRH/BD/158437/2019.

■ REFERENCES

(1) Seeman, N. C.; Sleiman, H. F. DNA nanotechnology,. *Nat. Rev. Mater.* **2017**, *3*, 17068.
(2) Rothemund, P. W. K. Folding DNA to create nanoscale shapes and patterns,. *Nature* **2006**, *440* (7082), 297−302.
(3) Zhang, F.; Nangreave, J.; Liu, Y.; Yan, H. Structural DNA nanotechnology: State of the art and future perspective,. *J. Am. Chem. Soc.* **2014**, *136* (32), 11198−11211.
(4) Shih, W. M.; Quispe, J. D.; Joyce, G. F. A 1.7-kilobase single-stranded DNA that folds into a nanoscale octahedron,. *Nature* **2004**, *427* (6975), 618−621.
(5) Veneziano, R.; Ratanalert, S.; Zhang, K.; Zhang, F.; Yan, H.; Chiu, W.; Bathe, M. Designer nanoscale DNA assemblies programmed from the top down,. *Science* **2016**, *352* (6293), 1534.
(6) Castro, C. E.; Kilchherr, F.; Kim, D. N.; Shiao, E. L.; Wauer, T.; Wortmann, P.; Bathe, M.; Dietz, H. A primer to scaffolded DNA origami,. *Nat. Methods* **2011**, *8* (3), 221−229.
(7) Douglas, S. M.; Bachelet, I.; Church, G. M. A Logic-Gated Nanorobot for Targeted Transport of Molecular Payloads,. *Science* **2012**, *335* (6070), 831−834.
(8) Yan, J.; Hu, C.; Liu, X.; Zhong, J.; Sun, G.; He, D. Recent Developments of New DNA Origami Nanostructures for Drug Delivery,. *Curr. Pharm. Des.* **2015**, *21* (22), 3181−3190.
(9) Pei, H.; Lu, N.; Wen, Y.; Song, S.; Liu, Y.; Yan, H.; Fan, C. A DNA Nanostructure-based Biomolecular Probe Carrier Platform for Electrochemical Biosensing,. *Adv. Mater.* **2010**, *22* (42), 4754−4758.
(10) Praetorius, F.; Kick, B.; Behler, K. L.; Honemann, M. N.; Weuster-Botz, D.; Dietz, H. Biotechnological mass production of DNA origami,. *Nature* **2017**, *552* (7683), 84−87.
(11) Kick, B.; Behler, K. L.; Severin, T. S.; Weuster-Botz, D. Chemostat studies of bacteriophage M13 infected Escherichia coli JM109 for continuous ssDNA production,. *J. Biotechnol.* **2017**, *258* (January), 92−100.
(12) Veneziano, R.; Shepherd, T. R.; Ratanalert, S.; Bellou, L.; Tao, C.; Bathe, M. In vitro synthesis of gene-length single-stranded DNA,. *Sci. Rep.* **2018**, *8* (1), 6548.
(13) Shen, C.-H. Detection and Analysis of Nucleic Acids. *Diagnostic Molecular Biology*; Academic Press, 2019; pp 167−185.
(14) Behler, K. L.; Honemann, M. N.; Silva-Santos, A. R.; Dietz, H.; Weuster-Botz, D. Phage-free production of artificial ssDNA with Escherichia coli,. *Biotechnol. Bioeng.* **2022**, *119* (10), 2878−2889.
(15) Green, M. R.; Sambrook, J. Analysis of DNA by agarose gel electrophoresis,. *Cold Spring Harbor Protocols* **2019**, *2019* (1), pdb.top100388.
(16) Eriksson, S.; Glad, G.; Pernemalm, P. Å.; Westman, E. Separation of DNA restriction fragments by ion-pair chromatography,. *J. Chromatogr. A* **1986**, *359* (C), 265−274.
(17) Huber, C. G.; Oefner, P. J.; Bonn, G. K. Rapid and Accurate Sizing of DNA Fragments by Ion-Pair Chromatography on Alkylated Nonporous Poly(Styrene-Divinylbenzene) Particles,. *Anal. Chem.* **1995**, *67* (3), 578−585.
(18) Karikó, K.; Muramatsu, H.; Ludwig, J.; Weissman, D. Generating the optimal mRNA for therapy: HPLC purification eliminates immune activation and improves translation of nucleoside-modified, protein-encoding mRNA,. *Nucleic Acids Res.* **2011**, *39* (21), No. e142.
(19) Kuwayama, T.; Ozaki, M.; Shimotsuma, M.; Hirose, T. Separation of long-stranded RNAs by RP-HPLC using an octadecyl-based column with super-wide pores,. *Anal. Sci.* **2023**, *39* (3), 417−425.
(20) Currie, J.; et al. Stability Indicating Ion-Pair Reversed-Phase Liquid Chromatography Method for Modified mRNA. *ChemRxiv*..
(21) Gilar, M.; Doneanu, C.; Gaye, M. M. Liquid Chromatography Methods for Analysis of mRNA Poly(A) Tail Length and Heterogeneity,. *Anal. Chem.* **2023**, *95* (38), 14308−14316.
(22) Kick, B.; Praetorius, F.; Dietz, H.; Weuster-Botz, D. Efficient Production of Single-Stranded Phage DNA as Scaffolds for DNA Origami,. *Nano Lett.* **2015**, *15* (7), 4672−4676.
(23) Douglas, S. M.; Dietz, H.; Liedl, T.; Högberg, B.; Graf, F.; Shih, W. M. Self-assembly of DNA into nanoscale three-dimensional shapes,. *Nature* **2009**, *459* (7245), 414−418.
(24) Silva-Santos, A. R.; Oliveira-Silva, R.; Sousa Rosa, S.; Paulo, P. M. R.; Prazeres, D. M. F. Affinity-Based Magnetic Particles for the Purification of Single-Stranded DNA Scaffolds for Biomanufacturing DNA-Origami Nanostructures,. *ACS Appl. Nano Mater.* **2021**, *4* (12), 14169−14177.
(25) Silva-Santos, A. R.; Paulo, P. M. R.; F Prazeres, D. M. Scalable purification of single stranded DNA scaffolds for biomanufacturing DNA-origami nanostructures: Exploring anion-exchange and multi-modal chromatography,. *Sep. Purif. Technol.* **2022**, *298* (June), 121623.
(26) Commission, E. Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results,. *Off. J. Eur. Communitiee* **2002**, *50*, 8−36.
(27) Van Loco, J.; Jànosi, A.; Impens, S.; Fraselle, S.; Cornet, V.; Degroodt, J. M. Calculation of the decision limit (CCα) and the detection capability (CCβ) for banned substances: The imperfect

marriage between the quantitative and the qualitative criteria. *Anal. Chim. Acta* **2007**, *586* (1−2), 8−12.

(28) Silva-santos, A. R.; Paulo, P. M. R.; F Prazeres, D. M. Scalable purification of single stranded DNA scaffolds for biomanufacturing DNA-origami nanostructures: exploring anion-exchange and multimodal chromatography,. *Sep. Purif. Technol.* **2022**, *298*, 121623.

(29) Premstaller, A.; Oberacher, H.; Huber, C. G. High-performance liquid chromatography-electrospray ionization mass spectrometry of single- and double-stranded nucleic acids using monolithic capillary columns,. *Anal. Chem.* **2000**, *72* (18), 4386−4393.

(30) Devaney, J. M.; Girard, J. E.; Marino, M. A. DNA microsatellite analysis using ion-pair reversed-phase high- performance liquid chromatography,. *Anal. Chem.* **2000**, *72* (4), 858−864.

(31) Kaczmarkiewicz, A.; Nuckowski, Ł.; Studzińska, S.; Buszewski, B. Analysis of Antisense Oligonucleotides and Their Metabolites with the Use of Ion Pair Reversed-Phase Liquid Chromatography Coupled with Mass Spectrometry,. *Crit. Rev. Anal. Chem.* **2019**, *49* (3), 256−270.

(32) Vial, J.; Jardy, A. Experimental comparison of the different approaches to estimate LOD and LOQ of an HPLC method,. *Anal. Chem.* **1999**, *71* (14), 2672−2677.

(33) Gilar, M.; Fountain, K. J.; Budman, Y.; Holyoke, J. L.; Davoudi, H.; Gebler, J. C. Characterization of Therapeutic Oligonucleotides Using Liquid Chromatography with On-line Mass Spectrometry Detection,. *Oligonucleotides* **2003**, *13* (4), 229−243.

(34) Wooddell, C. I.; Burgess, R. R. Use of asymmetric PCR to generate long primers and single-stranded DNA for incorporating cross-linking analogs into specific sites in a DNA probe,. *Genome Res.* **1996**, *6* (9), 886−892.

https://doi.org/10.1021/acsomega.3c10533
ACS Omega 2024, 9, 22619−22624