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A Simple Thermoplastic Substrate Containing Hierarchical Silica Lamellae for High Molecular Weight DNA Extraction

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Graphical Abstract

Inexpensive, magnetic thermoplastic nanomaterial is developed utilizing a hierarchical layering of micro- and nanoscale silica lamella to create a high surface area and low shear substrate capable of capturing vast amounts of ultra high molecular weight DNA. Extraction is performed via a simple 45 minute process and is capable of achieving binding capacities up to 1,000,000-times greater than silica microparticles.

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Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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The ability to obtain large quantities of high molecular weight (HMW) and high quality DNA is of rising importance as genomic analysis tools become increasingly sophisticated and the implications of long-range genomic information are realized. In DNA sequencing, 3rd generation technologies, such as Pacific Biosciences' SMRT technology and Oxford Nanopore's MinION technology, offer long read lengths greatly surpassing Sanger sequencing that facilitate de novo sequence assembly and the deciphering of complex repeat sequences.^[1] Barcoding technologies such as 10X Genomics' GemCode offer the potential to link myriad short reads in Mb-sized long-reads^[2]. Recent genome mapping methods such as BioNano Genomics' Irys technology and OpGen's Argus technology require DNA that is hundreds of kb to Mb in length in order to characterize chromosomal rearrangements and long-range genetic information such as copy number variation and inversions.^[3]

Despite rapid advances in such genomic tools, few advances have been made in DNA extraction since silica extraction technology was first introduced.^[4] To achieve high DNA binding capacities (i.e. extracted DNA per unit area) and extraction yields (i.e. total amount of extracted DNA), these methods rely on the adsorption of DNA to micro- and nanostructured materials with high surface area to volume ratios, such as magnetic microparticles^[5–9] or spin columns containing porous materials.^[8, 10] These methods have enjoyed widespread adoption due to their speed, ease-of-use, and generally high performance (i.e. high purity and efficiency). However, the shear forces imposed by the existing silica extraction methods tend to fragment DNA such that tedious precipitation methods (e.g. phenol-chloroform or salting out) and multi-day gel plug extractions^[11] remain the only option when the highest DNA quality is required.

We have taken a conceptually different approach to silica DNA extraction by developing a novel thermoplastic substrate containing a hierarchical layering of microscale folds and nanoscale silica lamella called Nanobind. In contrast to microparticles and spin columns where nucleic acids can be sheared by particle mixing or by flow through porous matrices, Nanobind high surface area, nonporous structure protects DNA from shear force-induced fragmentation by harboring it in a condensed, tentacle conformation that enables the extraction of vast amounts of ultra high molecular weight (UHMW) DNA. This gentle process ensures rapid isolation (<45 minutes) of high quality, megabase (Mb) DNA, containing minimal damage such as nicks and abasic sites, which can be used to generate high quality, single molecule sequencing libraries capable of superior read lengths. Furthermore, Nanobind is easily magnetized to facilitate sample processing.

Herein, we probe Nanobind's unique binding mechanism, the fabrication parameters affecting silica lamella surface topography, and characterize the DNA extraction performance. We then perform a thorough comparison against the three most commonly used DNA extraction methods and conclude by using Nanobind extracted DNA for long-read sequencing using Pacific Biosciences single molecule, real-time (SMRT) sequencing technology.

Nanobind's high surface area lamella structures are fabricated via a simple and inexpensive 2-step process comprising evaporation of silicon dioxide onto both sides of polyolefin (PO) film and heat-shrinking in an oven (Figure 1a). Film stresses generated by the heat shrinking process lead to a hierarchical layering of microscale polyolefin folds and nanoscale silica lamella that can act as a high surface area, low shear substrate for DNA extraction. Optionally, a magnetic sandwich layer, which imparts a dark gray hue to the white substrate, may be deposited inline to facilitate magnetic manipulation. In this case, silicon dioxide is used to sandwich an iron layer such that DNA adsorption can continue to occur on both sides of the Nanobind substrate while also passivating the metallic iron layer from unwanted buffer interactions. DNA isolation is performed using a standard bind, wash, and elute protocol that parallels spin column and magnetic microparticle extraction (Figure 1b) and can be completed in less than 45 minutes using either non-magnetic or magnetic processing. Magnetic processing can further be performed in either manual or automated format using standard robotic instruments (Figure S1, Supporting Information).

Nanobind DNA is captured via a novel tentacle binding mechanism in which a macromolecular entanglement of DNA is tethered to the silica substrate (i.e. the white disk) at a single end and extends deep into solution in a manner akin to jellyfish tentacles (Figure 1c). The low shear format prevents fragmentation and allows Nanobind to act as a seed upon which high molecular weight DNA tentacles grow. As cellular input increases, the DNA assembles into sheet- and sack-like structures, enabling a small area of substrate to capture vast amounts of DNA. SEM images (Figure S2, Supporting Information) show that HMW genomic DNA assembles into sheet-like structures and that tether across silica lamellae surface.

High surface area is created by the hierarchical layering of nanoscale silica lamella and microscale polyolefin folds. As seen in Figure 2, the surface topography can be fine-tuned

by varying the amount of silicon dioxide that is deposited prior to heat shrinking. With 2 nm of silicon dioxide, the oxide layer largely conforms to the microfolds of the underlying polyolefin film. With 20 nm and 50 nm of silicon dioxide, nanoscale silica wrinkles and lamella begin to develop on top of the microfolds as the film stresses begin to overwhelm the adhesion between the oxide and underlying polyolefin. With 100 nm, 150 nm, and 200 nm of oxide, a high density of silica lamella begin to form and progressively increase in size and number as film stresses cause the silicon dioxide to delaminate and fracture. In addition, rather than fully detach and release, the silica lamella remain stably anchored in place by a combination of mechanical interlocking, residual adhesive portions, and lamina-lamina adhesion. Proper design of the layer structures further ensures that addition of the optional magnetic sandwich does not affect silica lamella surface topography (Figure 2g). SEM analysis also shows that the lamella remain stably anchored before and after DNA extraction with no observable degradation (Figure S3, Supporting Information).

This hierarchical layering of features provides a substantial increase in surface area over a planar substrate. Using Brunauer-Emmett-Teller (BET) gas adsorption, we measured the surface area of the 100 nm and 200 nm oxide Nanobind substrates. Each 6 mm diameter disk resulted in BET surface areas of 9.33 cm² and 9.74 cm², respectively, a 35-fold increase over the planar substrate (Figure S4, Supporting Information).

To investigate the effects of silica lamella topography on DNA binding, we performed extractions using the 0 nm oxide (i.e. no oxide control), 20 nm oxide (i.e. low density lamella), 100 nm oxide (i.e. high density lamella), 200 nm oxide (i.e. high density lamella), and unshrunk 100 nm oxide (i.e. flat oxide control) Nanobind substrates. As expected based on SEM and BET analysis, DNA extraction efficiency progressively grew with oxide thickness and the expected surface area (Figure 3a). Both the 100 nm and 200 nm oxide Nanobind substrates had high efficiency and recovered nearly all of the input DNA. In contrast, the unshrunk 100 nm oxide Nanobind substrate recovered only moderate amounts of DNA while the 0 nm oxide Nanobind substrate recovered very little DNA, demonstrating that a high density of silica lamella is critical for high extraction yields. The non-magnetic and magnetic Nanobind substrates can be used interchangeably with no noticeable difference in performance (Figure S5, Supporting Information).

Next, we attempted to measure Nanobind binding capacity by performing extractions on cultured cells. As seen in Figure 3b, extraction yield increased linearly across the entire range of cell inputs. With 25×10⁶ MCF-7 cells, the 6 mm Nanobind extracted over 175 µg of DNA, representing 100% efficiency and a binding capacity of 188,000 µg/m². This binding capacity is 100–1,000,000-times greater than previously reported values for silica microparticles (<0.03 µg/m² to 2,100 µg/m²).^[5–10] The true Nanobind binding capacity likely exceeds this as binding saturation had not yet been reached. It is noteworthy that with 25×10⁶ cells input, over 600 µg of RNA was also co-purified, further illustrating the high binding capacity.

A direct comparison against commercial spin columns and magnetic microparticles showed that with moderate cell input (Figure S6a, Supporting Information), Nanobind obtained twice the amount of DNA, but with a high cell input (Figure S6b, Supporting Information),

Nanobind yielded 10 to 30-times the amount of DNA. We predict this difference would continue to grow with further increases in cell input. The 1.5 mL format spin column and microparticle processes used herein are capable of high efficiency when extracting 10 to 20 μg of DNA but decrease in efficiency at higher inputs as the binding surfaces become saturated. Using Nanobind, over 200 μg of DNA could be extracted and eluted into a volume as small as 50 μL , resulting in DNA concentrations as high as 4 $\mu\text{g}/\mu\text{L}$. Nanobind also demonstrated high repeatability with extraction-to-extraction CV of only 12%.

Despite the increased extraction yield, the Nanobind surface area (9.33 cm^2) was actually less than that typically used in microparticle extractions (45–275 cm^2).^[5] Thus, we postulated that the extreme binding capacity was actually a result of the unique tentacle binding mechanism. Thus, large genomic DNA fragments, which more easily form macromolecular tentacles than short DNA fragments, should result in higher extraction yields. To test this, we measured Nanobind binding capacity using λ DNA that had been heat fragmented from 48 kb progressively down to 400 bp (Figure S7, Supporting Information). As seen in Figure 3c and S7, Nanobind is exceptionally efficient at capturing large DNA. Full length λ DNA exhibited a binding capacity 80-times greater than 400 bp DNA. The true difference is potentially even greater as the full length λ DNA had not reached binding saturation at 100 μg of input. This dramatic increase in large DNA binding capacity can be attributed to the decrease in surface area necessary to tether a few pieces of large DNA as opposed to many pieces of small DNA.

As evident in Figure 1c and 2g, the bound genomic DNA does not lie in a strictly prone conformation. Previous studies have modeled the physics of DNA adsorption^[5, 6, 12] while attempting to untangle complex electrostatic, salt-bridging, and entropic binding effects.^[5, 6, 8] The simplest models assume that DNA lies prone against the silica substrate, bound by multiple contact points along the length of the molecule.^[5] This mechanism implies that binding capacity is limited predominantly by substrate surface area and DNA packing density, with a maximum surface coverage of 20–30%.^[6, 9] Studies have further proposed that binding conformation can be affected by parameters such as buffer conditions, surface properties, packing density, and, potentially, DNA length.^[5] Through the use of the low shear, planar, non-porous Nanobind, we are able to bias the DNA binding away from the typical prone conformation towards a tethered conformation that is particularly efficient for high molecular weight DNA extraction. It is possible that due to this unique mechanism binding saturation may never reasonably occur.

We then compared Nanobind (NB) against phenol-chloroform (PC), spin column, and magnetic microparticles by extracting genomic DNA from 3×10^6 cultured MCF-7 cells using all four methods. First, we compared the length of the extracted DNA using single molecule free solution hydrodynamic separation (SML-FSHS)^[13]. SML-FSHS uses single molecule sensitivity to enable accurate determination of large DNA size and concurrent detection of minute amounts of DNA fragmentation. As shown in Figure 3d, Nanobind and phenol-chloroform DNA had high molecular weights with length centered around 100 kb. Nearly all of the DNA was greater than 50 kb with substantial portions greater than 100 kb. Magnetic particle DNA showed increased fragmentation with very little DNA >90 kb while spin column DNA displayed obvious shearing and appeared as a broad smear. Agarose gel

electrophoresis confirmed these trends (Figure S8, Supporting Information) while pulsed field gel electrophoresis further showed that Nanobind extracted DNA up to 300 kb with an average length around 150 kb (Figure 3e). Under UV absorbance analysis, all four systems showed high purity with 260/280 ratios between 1.99 to 2.04 and 260/230 ratios between 2.10 and 2.25 (Figure 3f and Figure S9 in Supporting Information). When qPCR was used to analyze 10-fold serial dilutions of the previously extracted DNA, all 4 samples exhibited the expected log-linear relation between DNA input and threshold cycle C_t (Figure 3g and Figure S10 in Supporting Information). At all dilutions, Nanobind and phenol-chloroform extracted DNA resulted in lower C_t values than the microparticle or column extracted DNA. As the same number of cells was input into each extraction, the lower C_t value is likely a combination of factors such as higher extraction yield, lower amounts of carryover PCR inhibitors (e.g. residual organic solvents, salts, etc), and lower DNA damage.

We selected Nanobind and phenol-chloroform extracted DNA to create long-insert SMRTbell libraries for single molecule sequencing as these samples exhibited the highest molecular weights. For Nanobind DNA, an on-substrate RNase digestion and wash were added to remove RNA contamination and obtain pure genomic DNA. Both samples were capable of supporting libraries with a 30 kb lower cutoff, the maximum cutoff possible using the current Blue Pippin size-selection method (Figure S11, Supporting Information). The mean maximum subread lengths per zero-mode waveguide (ZMW) produced by the phenol-chloroform and Nanobind libraries were 20.6 kb and 20.1 kb, respectively. The subread N50 values, based on the longest reads obtained per ZMW, were 29.4 kb for phenol-chloroform (Figure 4a) and 28.0 kb for Nanobind (Figure 4b), indicating that 50% of the sequenced bases are in subreads greater than 29.4 and 28.0 kb, respectively. For both samples, approximately 10% of subreads were in excess of 40 kb, and the longest reads were near 70 kb. Consistent with these outstanding subread length distributions, the frequency of damage-related sequencing terminations in these libraries is estimated to be less than one in 500 kb, indicating five- to ten-fold less damage than that typically observed in libraries of this size.

To further test the capabilities of Nanobind, we developed an UHMW process using a custom buffer set and protocol to further reduce shear. Using this process, extracted DNA size rose from 300 kb up to greater than 5.7 Mb (Figure 4c and Figure S12 in Supporting Information). To our knowledge, this is the largest DNA ever extracted using a standard, silica-based bind, wash, and elute protocol. The UHMW DNA was then used to generate a SMRTbell library with 30 kb cutoff as before and subjected to single molecule sequencing. The resultant library produced a mean maximum subread length of 16.7 kb, a subread N50 value of 24.6 kb, and maximum subread length of 73.7 kb (Figure 4d, e). While these sequencing metrics are not as outstanding as the first set of sequencing data, they are consistent with libraries of the highest quality, and the differences likely reflect variability in template preparation and chip loading.

Nanobind extracted DNA has ultra high MW exceeding Mb in length, has low damage, and high purity. This is evidenced by both its high performance in qPCR and in long-read single molecule sequencing. Using Nanobind DNA, we prepared the highest quality long-insert libraries and generated extremely long single molecule reads, with 50% of the sequenced bases contained in subreads greater than 28 kb, and maximum reads in excess of 70 kb.

These read length gains have important implications for genome assembly, as there is a tradeoff between read length, coverage, and expected contig length in a genome assembly. These gains can be roughly predicted by the Lander-Waterman model^[14], a widely applied model from the Sanger sequencing era. This model predicts that, under ideal conditions in an assembly in which all overlaps have been detected perfectly, expected contig length for a given coverage level should scale linearly with increasing read lengths (Figure S13a, Supporting Information). Thus, increasing read length from 15 to 30 kb is predicted to increase the average contig length by two-fold. Looking at it another way, the amount of coverage needed to achieve a comparable expected contig length decreases with increasing read length (Figure S13b, Supporting Information). In practice, the simplifying assumptions made by this model, particularly the assumption that the genome is free of repeats, mean that this model effectively gives an upper bound on what is achievable. In reality, the relationship between read length, coverage, and contig size is dependent on the number and length of repeats in a given genome. Longer reads additionally allow for sequencing through longer repeat regions, potentially allowing access to previously inaccessible regions of a genome and improving genome assembly.

These data suggest that Nanobind is an excellent alternative to phenol-chloroform and gel plug extraction when the highest quality genomic DNA is required. Whereas these methods can take 1–3 days and require substantial manual skill, the equivalent Nanobind extraction can be performed in less than 45 minutes. Nanobind offers a unique combination of high binding capacity and high molecular weight DNA that cannot be achieved with any existing solid phase technology. The high binding capacity enables high extraction yield while the short processing time and non-porous format render it easily incorporated into small volume, microfluidic, and automated extraction systems at μL to mL scales. Additionally, the ability to re-suspend into small volumes is useful in obtaining high concentration DNA. This suggests that Nanobind can also be used as a plug-and-play substitute for spin columns and microparticles in diverse research, clinical, and production applications beyond high molecular weight. Furthermore, by engineering Nanobind to contain a magnetic sandwich on one side, we are able to process samples manually using a magnetic rack or automated using a robotic instrument.

Film stresses in polymer substrates have been previously used to create surfaces with controlled wrinkle structures^[15] while heat shrinking of thermoplastic films has been used to create precision microfluidic channels^[16] and micropatterned surfaces.^[17] Alternatively, stresses within layer-by-layer deposition processes have been used to create superhydrophobic, hierarchical metal nanoflake surfaces.^[18] In this work, we integrate these principles to create a novel hierarchical silica lamella surface. The underlying polyolefin film costs pennies per square foot, and the fabrication can be easily scaled using industrial roll-to-roll processes to create a nanostructured DNA extraction substrate that is exceptionally inexpensive to produce. The extraction chemistry incorporated herein used off-the-shelf buffers and obtained a mixture of both DNA and RNA but high efficiency extractions have also been performed using our own custom buffers. For example, in the UHMW process, custom buffers and protocols were able to push extracted DNA length north of 5.7 Mb, while retaining a short 45 minute process time. Future work will integrate Nanobind into other molecular analysis workflows including genome mapping, methylation

analysis, and microRNA profiling from a broad array of samples including bacterial and plant cells, tissues, body fluids, and formalin-fixed paraffin embedded (FFPE) samples.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

High molecular weight DNA extraction using the magnetic thermoplastic Nanobind. a) The hierarchical silica lamella are fabricated by depositing a thin layers of silica onto a thermoplastic polyolefin (PO) film, heat shrinking in an oven, and punching out a 6 mm disk of material. Alternatively, an silica-iron-silica sandwich may be deposited on the backside to facilitate magnetic processing. The white disk is a non-magnetic Nanobind substrate while the dark gray disk is a magnetic Nanobind substrate. b) DNA extraction is performed using a standard bind, wash, and elute protocol that can be performed with or without magnetic handling. c) Images of genomic DNA captured by non-magnetic Nanobind and tethered onto silica surface after cell lysis and mixing with IPA. The high surface area and low shear forces enable large amounts of high molecular weight DNA to be captured via a tethered binding mode where macromolecular tentacles of DNA extend from the Nanobind substrate deep into solution. From left to right are images of Nanobind disks bound with genomic DNA from low (2×10^6), moderate (7×10^6), and high (25×10^6) cell inputs.

Figure 2.

SEM images of Nanobind surface topography. The heat-shrinking process leads to a hierarchical structure of microscale polyolefin folds layered with nanoscale silica lamella that can be fine-tuned via oxide deposition thickness. SEM analysis was performed on non-magnetic Nanobind with a) 2 nm, b) 20 nm, c) 50 nm, d) 100 nm, e) 150 nm and f) 200 nm of deposited silicon dioxide. g) SEM images of magnetic Nanobind show a similar structure of microscale folds and nanoscale lamella. Each pair of images shows the same material under low (**left**) and high magnification (**right**).

Figure 3.

Nanobind DNA extraction characterization and comparison. a) Extraction efficiency was measured as a function of Nanobind silicon dioxide thickness. The 200 nm and 100 nm silica Nanobind substrates contain a high density of silica lamella and achieve high extraction efficiencies when compared to the 0 nm oxide and planar oxide controls. (n=4) b) DNA extraction yield was measured as a function of cell input. No saturation was seen even at 25×10^6 cells input, demonstrating that the binding capacity exceeds $188,000 \mu\text{g}/\text{m}^2$. c) Nanobind binding capacity was measured as a function of DNA length. Binding capacity was significantly enhanced for full length λ DNA versus highly fragmented λ DNA. (n=3) d) Single molecule free solution hydrodynamic separation and e) pulsed field gel electrophoresis were performed on genomic DNA extracted using phenol-chloroform (PC), Nanobind (NB), magnetic microparticles and spin column. Red dotted lines represent the median length of DNA extracted with each method. Phenol-chloroform and Nanobind DNA have the highest molecular weight with average length around 150 – 200 kb. Spin column DNA is highly fragmented (<50 kb) while magnetic particles show moderate shearing with DNA with average length around 75 kb. f) UV absorbance shows that all 4 methods obtain high purity nucleic acids. g) qPCR was performed on serial dilutions of the extracted DNA. Nanobind and phenol-chloroform DNA consistently resulted in the lowest C_t values. (n=3)

Figure 4.

SMRT sequencing data for phenol-chloroform (PC), Nanobind (NB) and ultra-high molecular weight Nanobind (UHMW) extracted DNA. >30 kb insert SMRTbell libraries prepared using a) PC and b) NB extracted DNA resulted in exceptional read lengths with N50 values of 29.2 kb and 27.7 kb, respectively. c) An UHMW extraction process was developed that increased extracted DNA size from 300 kb up to Mb range. d) The UHMW DNA was then used to generate >30 kb insert SMRTbell libraries that resulted in robust long reads with N50 values of 24 kb and longest reads of 70 kb. e) SMRT sequencing read metrics for all libraries. All data is based on the max subread length obtained per zero-mode waveguide.