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Direct oligonucleotide synthesis onto super-paramagnetic beads

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Abstract

Super-paramagnetic beads (SPMB)s used for a variety of molecular diagnostic assays are prepared by attaching pre-synthesized oligonucleotides to the surface via a cumbersome and low efficient method of carbodiimide-mediated amide bond formation. To mainstream the process, we describe a novel procedure of direct oligonucleotide synthesis onto the surface of SPMBs (e.g. MyOne Dynabeads). With the many challenges surrounding containment of paramagnetic beads (≤ 1 μm) during automated oligonucleotide synthesis, we show that by applying a magnetic force directly to the SPMBs we prevent their loss caused by high-pressure drain steps during synthesis. To date we have synthesized 40mers using a Spacer 9 phosphoramidite (triethylene glycol) coupled to the surface of hydroxylated SPMBs. HPLC analysis shows successful product generation with an average yield of 200 pmoles per sample. Furthermore, because of the versatility of this powerful research tool, we envision its use in any laboratory working with conventional synthesis automation, as employed for single columns and for multi-well titer plates. In addition to direct synthesis of oligodeoxynucleotides (DNA) onto SPMBs, this platform also has the potential for RNA and peptide nucleic acid synthesis.

INTRODUCTION

Micro- and nanometer-sized beads with surface-modifications to bind particular ligands (chemical group or compound), have been used extensively for a wide variety of molecular diagnostic assays (1–4). The beads can either be applied to non-target-specific (e.g. carboxyl groups – nucleic acids and streptavidin-biotin) or target-specific purposes via surface coupling of biomarker molecules (e.g. nucleic acids and antibody/antigens) (5–8). For nucleic acid ligands, the oligonucleotide is pre-synthesized with end modifications to fit the bead-attachment chemistry used; the biomarker ligand will then bind a corresponding anti-target site to be selected for in downstream processing (9, 10).

Super-paramagnetic beads (SPMB)s compared to other bead-types (e.g. acrylamide, sepharose, silica and polystyrene) have a major advantage in the sample preparation methodology due to the inherent ease of separating particles in solution with an external

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SUPPLEMENTARY DATA

Supplementary Data are available at Journal of Biotechnology Online: Supplementary Figures S1–S4.

magnet (2). Examples where SPMBs have been used for various types of sample preparation and detection platforms include but are not limited to a) Pyrosequencing (2, 11), b) Luminex (10, 12), c) Applied Biocode, d) SOLID (13, 14), and e) Mag Array (15–17).

An alternative to full strand attachment to the SPMB is direct oligonucleotide chemical synthesis onto the bead surface. The advantage of this method lies in streamlining the process, where the middle-step of bead-surface modification is rendered unnecessary. Other benefits include a) the possibility to scale-down the size of the actual SPMBs allowing for a higher surface display of the synthesized oligonucleotides, b) higher DNA copy number per bead, c) lower production costs, d) consistent quality assessments, e) increased production speed, and f) massive parallelization.

Applications using SPMB nucleic acid capture and isolation are becoming more sophisticated often requiring nano-sized particles (15–17). Since traditional solid-phase chemical synthesis is a filter-based platform (18), retaining micro- and nanometer-sized beads is not possible. Commercial solid supports for oligonucleotide synthesis (e.g. controlled-pore glass (CPG) particles and polystyrene (PS) beads) have a median diameter of 100 μm, and where filters used to retain these supports have a median porosity of 40 μm allow only passage of reagents during wash and drain steps of the synthesis cycle; however, this is too porous for MyOne beads, for example, which are only 1 μm in diameter. As such, filters with < 1 μm porosity greatly restrict reagent flow-through, and therefore cannot be used. Due to these reasons past investigations of chemical synthesis on SPMBs have had only limited, small-scale success proving a major challenge in adapting to higher throughput synthesis automation (19).

Instead, by applying a magnetic force directly to the SPMBs, we prevent bead loss caused by high-pressure drainage during reagent purge-to-waste steps in the synthesis cycle. This is accomplished by using a permanent magnet (e.g. sphere or disc) to which the beads are attracted and remain bound (Figure 1).

With readily available on-the-market supplies (e.g. MyOne SPMBs and magnetic spheres/discs) we envision this method for use in any laboratory working with conventional synthesis automation such as those for single columns (Applied Biosystems AB394 and AB3900 instruments) and those for multi-well titer plates (96, 384, and 1,536). The option to synthesize directly onto SPMBs will vastly improve workflow in the lab from synthesizer to sequencer; therefore, time-consuming and expensive oligonucleotide pre-functionalization, bead attachment and laborious washing procedures are avoided.

MATERIALS AND METHODS

Direct oligonucleotide synthesis onto super-paramagnetic beads (SPMB)s

Hydroxyl coated MyOne Dynabeads were kindly provided by Life Technologies. The beads were washed three times with 1X wash/storage buffer (PBS, supplemented with 0.2% Tween-20 (pH 7.7)) using a standard DYNAL Invitrogen bead separator magnetic rack (Life Technologies, Carlsbad, CA) to remove any excess bovine serum albumin (BSA); BSA is an additive to prevent clumping during storage. Single magnetic spheres (~3 mm diameter, Neodymium Iron Boron (NdFeB), K&J Magnetics) were then placed inside blank synthesis columns (Biosearch Technologies, Novato, CA); a bottom filter is used only to retain the sphere. Thirty μl of the original MyOne stock solution (~10^7 beads/μl) were used to coat the surface of the magnetic sphere. Prior to synthesis, magnetic sphere-bound MyOne beads were then washed repeatedly with acetonitrile to remove excess water and residual BSA. Using the manufacturer's recommended synthesis protocol (for Applied Biosystems AB3900DNA synthesizer) all samples were synthesized onto the hydroxylated surface of the

MyOne beads with a non-cleavable spacer (9-O-Dimethoxytrityl (DMT)-triethylene glycol, 1-[(2-cyanoethyl)-(N, N-diisopropyl)]-Spacer 9 Phosphoramidite (S9)) (10-1909-xx, Glen Research, Sterling, VA) (Supplementary Figure S1).

For control samples, 5′-DMT thymidine succinyl hexamide phosphoramidite (CLP-2244, Chem Genes, Wilmington, MA) was added before the first base of each sequence (Supplementary Figure S2); the succinyl linkage allows for cleavage of the target strand during ammonolysis. This was done for strand characterization using reverse-phase high performance liquid chromatography (HPLC), sequencing analysis (Pyro- and Sanger), and for measuring optical density (OD) to determine final product yield. All sequences were synthesized at the Stanford Genome Technology Center (SGTC) using a default 50 nmole-scale protocol (for Applied Biosystems AB3900 DNA synthesizer). Synthesis reagents included: Trichloroacetic acid (TCA) (3% TCA/dichloromethane) (American International Chemicals (AiC), Framingham, MA), acetonitrile (AiC, Framingham, MA), 0.02 M oxidizing solution (Sigma Aldrich, St. Louis, MO), cap A/B (Glen Research, Sterling, VA), 0.1 M solutions of phosphoramidites (3′ → 5′) dA, dC, dG and dT (Thermo Fisher Scientific, Waltham, MA), 0.1 M solution of phosphoramidites (5′ → 3′) dA, dC, dG and dT (Glen Research, Sterling, VA), and 0.25 M 5-benzoylthio-1H-tetrazole (AiC, Framingham, MA). An inert layer of nitrogen gas was maintained over the reaction wells during synthesis and for high pressure drains steps.

For post-synthesis processing (base deprotection and strand cleavage where applicable), oligonucleotide-bound SPMBs + spheres were placed in 1.5 ml eppendorf tubes where they were immersed in 80 μl ammonium hydroxide (NH_4OH, 28–30% ammonia in water) for incubation (15 hr) at 55°C. Samples were then separated from the spheres into solution by manually swiping the eppendorf tube (10 times) over an external permanent magnet; this caused the spheres to flip poles rapidly, displacing the SPMBs long enough to aspirate by pipette.

Those samples which remained attached to the beads (for fluorescent verification of oligonucleotide capture on SPMBs) were then added to a 1.5 mL eppendorf tube containing 160 μl of 1X wash/storage buffer (PBS, supplemented with 0.2% Tween-20 (pH 7.7)). Three rounds of washing were performed with 200 μl 1X wash/storage buffer (PBS, supplemented with 0.2% Tween-20 (pH 7.7)) using a standard DYNAL Invitrogen bead separator magnetic rack. The final bead pellet was then resolved in 20 μl 1X wash/storage buffer (PBS, supplemented with 0.2% Tween-20 (pH 7.7)).

For samples where oligonucleotides were released from the SPMBs during ammonolysis (for further sequencing verification, HPLC analysis and yield determination), eppendorf tubes containing only SPMB-bound oligonucleotides in NH_4OH were placed inside the bead separator for five minutes; the supernatant was then collected by pipette, added to a clean eppendorf tube, lyophilized and stored at 4°C until later use.

To determine approximate final yield (total pmoles of synthesized DNA on SPMBs), five magnetic spheres coated with SPMBs were weighed before and after synthesis (40 cycles) using an analytical balance (Sartorius CPA623S, Mark Court, California). The average values we obtained for oligonucleotides released from the SPMBs (via succinyl linkage post-synthesis) were as follows: Starting material was 3.0×10^8 beads/sphere (30 μl * 10^7 beads/μl stock solution), while final yield was 2.6×10^8 beads/sphere following synthesis ($(1 – 0.143) * 3.0 \times 10^8$); this translates into an average bead loss of 14.3%. OD values were measured with the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) at 260 nm, which were used to calculate an average total yield of 200 pmoles or 7.7×10^{-7} pmoles/SPMB ($200/2.6 \times 10^8$).

Fluorescent verification of oligonucleotide capture on SPMBs

Primer A′ (SPMB—S9—TTTTTTTTTTTTTTGTCGGAGACACGCAGGGATGAGATGG-3′) was synthesized (5′ → 3′) directly onto the hydroxylated MyOne SPMBs then base-deprotected as described above (S9 (Spacer 9 phosphoramidite) was first coupled to surface followed by Primer A′ synthesis). Oligonucleotide-bound SPMBs were then washed three times with 1X wash/storage buffer (PBS, supplemented with 0.2% Tween-20 (pH 7.7)) using a standard DYNAL Invitrogen bead separator magnetic rack. Remaining beads were resuspended in a 3 μl aliquot of 1X wash/storage buffer (PBS, supplemented with 0.2% Tween-20 (pH 7.7)). As a reference, Primer A′ was also synthesized with a 5′ amino modifier C6 (Integrated DNA Technologies, Coralville, IA) and covalently attached to MyOne carboxylic acid beads (Life Technologies, Carlsbad, CA) via a traditional 2-step carbodiimide chemical protocol (5–8) using EDC (Sigma-Aldrich, St. Louis, MO), 25 mM MES, pH 6 (Sigma-Aldrich, St. Louis, MO), and washed with 50 mM Tris pH 7.5 (Sigma-Aldrich, St. Louis, MO). SPMBs containing captured fluorinated oligonucleotides were then measured in-house (SGTC) with the Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA) using the Qubit ssDNA Assay kit (Life Technologies, Carlsbad, CA) with OliGreen fluorescent stain. For control samples, 3 μl of non-synthesized hydroxylated MyOne beads (~10^7 beads/μl) and 3 μl non-attached carboxylated MyOne bead (~10^7 beads/μl) were used as negative controls and also measured in the Qubit 2.0 Fluorometer with the Qubit ssDNA Assay kit. Each measured component (including Qubit 2.0 measurement) was repeated in triplicate.

Primer A (5′-CCATCTCATCCCTGCGTGTCTCCGAC-3′) (Integrated DNA Technologies, Coralville, IA) was synthesized as a template positive control with a complementary sequence to Primer A′. Annealing of Primer A to A′ was performed in the GeneAmp PCR system 9700 (Life Technologies, Carlsbad, CA) at 95°C for 2 min followed by 37°C for 2 minutes and allowed to cool to room temperature. The beads were subsequently washed three times with 1X wash/storage buffer (PBS, supplemented with 0.2% Tween-20 (pH 7.7)) using a standard DYNAL Invitrogen bead separator magnetic rack, before verification with the Qubit dsDNA HS assay kit (Life Technologies, Carlsbad, CA) with PicoGreen fluorescent stain on the Qubit 2.0 Fluorometer. As a negative control sample, 3 μl of non-synthesized hydroxylated MyOne beads (~10^7 beads/μl) were measured in the Qubit 2.0 Fluorometer. Each measured component (including Qubit 2.0 measurement) was repeated in triplicate.

Strand verification of oligonucleotides synthesized onto SPMBs

To verify our target product was generated on SPMBs, Primer A′ (SPMB—S9—X—TTTTTTTTTTTTTGTCGGAGACACGCAGGGATGAGATGG-5′, where X is thymidine succinyl hexamide phosphoramidite, and S9 is Spacer 9 phosphoramidite), was synthesized (3′ → 5′), cleaved from the SPMBs and base deprotected (as described above) then analyzed on a Transgenomic Wave HPLC System (Transgenomic, Omaha, NE) (Figure 2a). A control sample of identical sequence (Figure 2b) was synthesized in parallel using standard CPG support (Biosearch Technologies, Novato, CA) to match with the target chromatogram peak. HPLC setup consisted of a DNASep C-18 column at 80°C with UV detection at 260 nm; running buffers were a mixture of water, acetonitrile, triethyl ammonium acetate and EDTA.

For additional sequence verification using Pyro- and Sanger sequencing, the Template strand (SPMB—S9—X—GGTAGAGTAGGGACGCACAGAGGCTG-[AGTC-TGCTC]-TAGTGGCTGACGGGTATCTCTCCGACTCTGACGGTTCCGTGTGTCCCCTATCC-5′) was synthesized (3′ → 5′) directly onto SPMBs bound to a permanent magnet (3 mm NdFeB sphere); Primer A′ and B sequences are underlined (3′ → 5′) with target sequence in brackets [key-insert]) (Supplementary Figures S3a and b). Samples were cleaved and

deprotected as described above. The Template strand was then PCR amplified using Primer A and 5′ biotinylated Primer B (5′-CCTATCCCCTGTGTGCCTTGGCAGTCTCAG) to a final volume of 50 μl. Reactions contained 1 μl Template strand, 1X PCR Buffer II (Life Technologies, Carlsbad, CA), 2.5 mM $MgCl_2$ (Life Technologies, Carlsbad, CA), 0.12 mM dNTPs (Fermentas, Hanover, MD), 2.5 units AmpliTaq Gold DNA polymerase (Life Technologies, Carlsbad, CA), and 0.2 mM of each primer A and B. Cycling conditions using a GeneAmp PCR system 9700 (Life Technologies, Carlsbad, CA) were as follows: 95°C/10 min | 30 * [95°C/45 sec | 58°C/45 sec | 72°C/30 sec]. Single-strand template preparation of the PCR products was carried out as previously described (20). Pyrosequencing (with Primer A) was then performed with a cyclic *de novo* sequencing dispensation (ACGT) using a PSQTM HS96A DNA sequencing system (Biotage, Uppsala, Sweden) (Supplementary Figure S3c).

The Template strand was also used for the Sanger sequencing method (Supplementary Figure S3d). Samples were first PCR amplified with Primers A and B to generate double-stranded DNA, then 5′ adenylated (PCR extension 72°C for 10 min with regular Amplitaq polymerase), ligated and cloned with a TOPO TA Cloning kit (Life Technologies, Carlsbad, CA), and then plasmid purified (Qiagen R.E.A.L. Prep 96 plasmid kit (Qiagen Sciences Inc. USA, Germantown, MD). Samples were sequenced (GenScript USA Inc., Piscataway, NJ) then trace analyzed in-house (SGTC).

SPMB dispersion post-synthesis

In order to investigate the SPMBs dispersion effects (e.g. possible bead clumping in solution), MyOne Dynabeads Streptavidin C1, Carboxylic Acid, and hydroxylated (Life Technologies, Carlsbad, CA) were visually inspected with an Axiovert 40 CFL microscope (Zeiss, Oberkochen Germany). The stock solutions (~10^7 beads/μl) were diluted 1:10,000; 5 μl (~100 beads/μl) droplets of which were then placed on a microscope slide (Thermo Fisher Scientific, Waltham, MA). Each image was taken with a 10X lens with an AxioCam MRN CCD camera (Zeiss, Oberkochen Germany). The pixel-to-micron conversion rate was 0.645, which provided the reference for the scale bar.

RESULTS AND DISCUSSION

Direct oligonucleotide synthesis onto super-paramagnetic beads (SPMB)s

We developed the magnetic approach to oligonucleotide synthesis because containment of micro- and nanometer-sized beads is not possible using a filtered well or column (as in traditional DNA synthesis (18)), due to flow restrictions and excess sample loss. It would require a filter porosity of < 1 um to retain the beads; however, oligonucleotide synthesis reagents (e.g. acetonitrile, TCA and pyridine) will not pass through the filter even under elevated pressure. Furthermore, super-paramagnetic beads (SPMB)s have the potential to clog the filter pores increasing resistance to reagent flow. Therefore, using an internal/external magnetic source (e.g. NdFeB sphere) showed to be the preferred alternative (Figure 1).

For our research, we used SPMBs (MyOne Dynabeads), which are coated with hydroxylated polystyrene (PS). Because it is chemically inert, PS is an ideal polymer for use in oligonucleotide synthesis. To generate DNA strands directly onto the surface, a spacer is required to avoid steric hindrance associated with neighbouring base-base interaction; phosphoramidites are bulky, and coupling in close proximity results in extremely poor yields. From the commercially available spacers (e.g. C3, S9 and S18), we chose to work with S9 based on previous literature showing this to have ideal spacer properties (21). Moreover, the S9 spacer is non-cleavable during ammonolysis allowing oligonucleotides to remain attached to the surface of SPMBs post-synthesis.

While there is flexibility to synthesize in several different column and plate configurations, we focused on the column-based AB3900 used in routine synthesis (50 nmole-scale) as our target instrument for generating oligonucleotides on SPMBs. To test the magnetic stability through repeated wash and drain steps, we subjected five test samples to a 40-cycle round of synthesis (Figure 1). Here we added hydroxylated beads to the spheres by pipette, and with an initial (average) loading of 3.0×10^8 beads we were left with 2.6×10^8 beads at the end of the run (14.3% loss); an average final yield of 200 pmoles (7.8×10^{-7} pmoles/bead) was calculated. The reason for product loss is most likely explained by a decreased magnetism of the outer SPMB layers. Because the surface of the sphere is smooth, beads stack on top of one another during the loading process; as such, some beads are stripped from the surface during high-pressure drain steps. To circumvent this, we are investigating means of micro patterning on NdFeB spheres (22). Here grooves or pockets (2–4 μm) are etched into the surface of the magnet; this allows MyOne SPMBs (~1 μm), for example, to fill the pockets with a more uniform monolayer dispersion. And since they are imbedded, SPMBs are much less susceptible to dislodging from high-pressure reagent drainage. Furthermore, sonication (SPMBs + spheres in solution) may facilitate bead settling over the etched surface.

From visual observation of the spheres (under 40X magnification), we found surface appearance before and after 40 synthesis cycles identical; while this may suggest NdFeB is chemically resistant, we have not tested the spheres beyond minimal synthesis exposure. And because it may be desired that the spheres are recycled for repeat use in mainstream production, we are exploring avenues of further protecting the spheres from potential corrosion. The primary candidate is plasma-enhanced chemical vapor deposition, which is used to treat material with an inert hydrophobic polymer such as Teflon, perfluoroalkoxy, fluorinated ethylene propylene or PS.

The S9, when coupled to the hydroxylated surface of SPMBs, has the advantage of being a small non-charged molecule; after S9 addition, individual nucleoside phosphoramidites are coupled to the growing chain one at a time. In this case, the overall charge of the oligonucleotide is neutral because of the a) cyanoethyl protecting groups of the sugar-phosophate backbone, b) nucleoside cyclic amine protection (dA, dG and dC) and c) DMT groups (5′ or 3′ hydroxyl protection). Conversely, in the traditional carbodiimide approach a synthetic amino-modified oligonucleotide is made separately and then attached. This fully processed (deprotected) molecule is much larger than S9 and carries a highly negative charge due to the phosphate backbone (charge is proportional to length). Because the surface charges of the carboxylated MyOne beads are also negative, oligonucleotides in close proximity are naturally repelled. This may explain the lower overall yields of the carbodiimide variants compared with direct synthesis onto the SPMBs (Table 1). However, the carbodiimide approach has certain large-scale advantages, when the main interest is to indiscriminately attach one oligonucleotide sequence to the SPMBs.

Because the S9 linkage of the bead is universal (no pre-attached base), any nucleoside phosphoramidite or modifier can be coupled upstream of the target strand. This provides for the option to synthesize in either $3' \rightarrow 5'$ or $5' \rightarrow 3'$ directions. In the latter case, for example, a primer annealing to the target strand will create a 3′ hydroxyl recess, which is necessary for polymerase extension. And while the S9 linkage is non-cleavable, there may be instances where it is required that the strand be released from the SPMB. This can be accomplished either by enzymatic or chemical means, and may include any one of the following designs: a) succinyl linker (described above) where target strands are released into solution during routine ammonolysis, b) single/double-strand restriction site (cut with sequence-specific endonuclease), and c) introduction of uracil (strands are cleaved at this position with uracil DNA glycosylase (23)).

Fluorescent verification of oligonucleotide capture on SPMBs

To verify the presence of synthesized Primer A′ on the hydroxylated MyOne beads, the OliGreen fluorescent stain was used to bind to single-stranded DNA. Post-synthesis SPMBs were extensively washed to remove any non-attached nucleic acid molecules; native non-synthesized SPMBS were used as a comparator. When matching the native hydroxylated MyOne SPMBs to those from post-synthesis, there is a clear difference in DNA concentration, 9.71 ng/ml vs 29.8 ng/ml, respectively (Table 1a). As a reference, carboxylated MyOne beads were coupled to pre-synthesized amino-modified Primer A′ oligonucleotide molecules. When compared with non-coupled carboxylated MyOne beads, there is also a clear difference in DNA concentration, 20.3 ng/ml vs 6.99 ng/ml (Table 1b). When subtracting the background noise effects, the data indicates a higher DNA concentration for samples using direct oligonucleotide synthesis 20.9 ng/ml vs 13.31 ng/ml (Table 1a and b), which might account for an improved efficiency for the described production method. In a second experiment the same SPMBs with synthesized Primer A′ oligonucleotides were subjected to annealing of the complementary primer A. The PicoGreen fluorescent stain was used to bind to double-stranded DNA in order to verify that primer hybridization event had occurred. When compared with native non-synthesized beads (with Primer A annealing) the post-synthesis SPMBs showed a higher DNA concentration with 4.07 ng/ml vs 0.82 ng/ml (native beads with Primer A annealing) (Table 1c).

Strand verification of oligonucleotides synthesized onto SPMBs

To demonstrate full-length product generation onto SPMBs, we synthesized and released Primer A′ from the surface for analysis using HPLC (Figure 2a). As a control, we synthesized the same strand on standard support (CPG) (Figure 2b). Peak retention times are 4.817 and 4.819 minutes, respectively. By this we conclude that product synthesized onto SPMBs is comparable in size and purity to product synthesized on standard support.

For additional strand verification, the Template strand was synthesized ($3' \rightarrow 5'$) then released from the SPMBs for sequencing using both Pyro- and Sanger methods. Here we show a pyrogram (Supplementary Figure S3c) of the target insert-key (TCAGACGAG) as well as indirect sequence validation using the Sanger method (Supplementary Figure S3d); the target insert-key is highlighted in the chromatogram.

SPMB dispersion post-synthesis

We were initially concerned about the potential of the SPMBs to clump during and following synthesis, which may affect read resolution on downstream instrumentation. BSA was used in the buffer solution of the hydroxylated MyOne beads to prevent clumping; however, based upon visual inspection using a 10X magnification, we found no observable traces of bead clumping prior to or following synthesis (Supplementary Figure S4).

CONCLUSION

The use of super-paramagnetic beads (SPMB)s in sample preparation and detection (1–4) has many advantages. However conventional methods of bead attachment require that the oligonucleotide be pre-synthesized with specific end modifications; consequently, this is laborious and expensive, demanding many hours of preparation. Therefore to mainstream the process, we have introduced a method of direct oligonucleotide synthesis onto SPMBs (e.g. MyOne). But since bead diameters are ≤ 1 μm, traditional filter-based solid-phase DNA synthesis is not possible. Instead, by applying a magnetic sphere to which the beads are attracted and bound, synthesis can occur with minimal loss of product during wash and drain steps of each cycle. To date we have demonstrated proof-of-concept that 40 bp oligonucleotides can be generated on SPMBs using single columns, which was validated by

fluorescence detection and HPLC analysis. And because Spacer 9 creates a universal linkage between the SPMB support and target oligonucleotide strand, any number of phosphoramidite modifiers can be introduced, which may allow for either permanent attachment or strand release by chemical or enzymatic means. Furthermore, we anticipate higher demand for future applications where small volume, multi-well titer plates (96, 384 and 1536 platforms) will greatly increase throughput for large-scale projects. In addition to use with oligodeoxynucleotides (DNA), we envision a broader application with RNA as well as peptide nucleic acid direct synthesis onto SPMBs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

1. Oligonucleotide generation successful on paramagnetic beads during automated synthesis
2. Fluorescent verification of oligonucleotide capture on paramagnetic beads
3. HPLC verification of 40 bp strands on paramagnetic beads
4. Secondary verification of 89mer generated on paramagnetic beads (Pyro and Sanger sequencing)
5. Product generation on average of 200 pmoles/$2.7(10)^8$ beads
6. Method can be used on 96, 384 and 1536-well synthesis platforms as well as for RNA and PNA

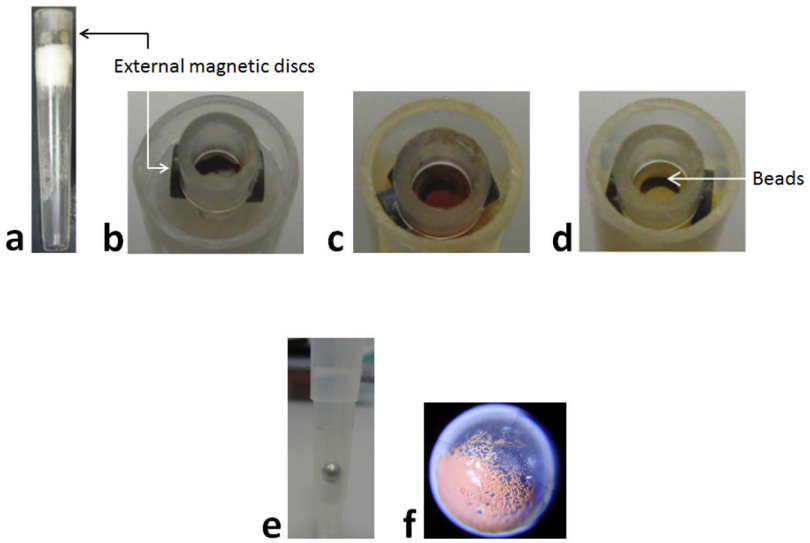

Figure 1.

shows options for magnetic binding of super-paramagnetic beads (SPMB)s during automated oligonucleotide synthesis. (**a**) Side view of a modified synthesis column (AB3900-instrument style) with external magnets around an inner column where SPMBs are bound to the walls. (**b**) Top view of column showing SPMBs bound to the wall before oligonucleotide synthesis. (**c**) Oligonucleotide synthesis (oxidation step); SPMBs are immersed in oxidizing reagent. (**d**) Post-synthesis showing SPMBs are still bound to column wall. (**e**) AB3900-style column with magnetic sphere inside. (**f**) Sphere coated with SPMBs before synthesis.

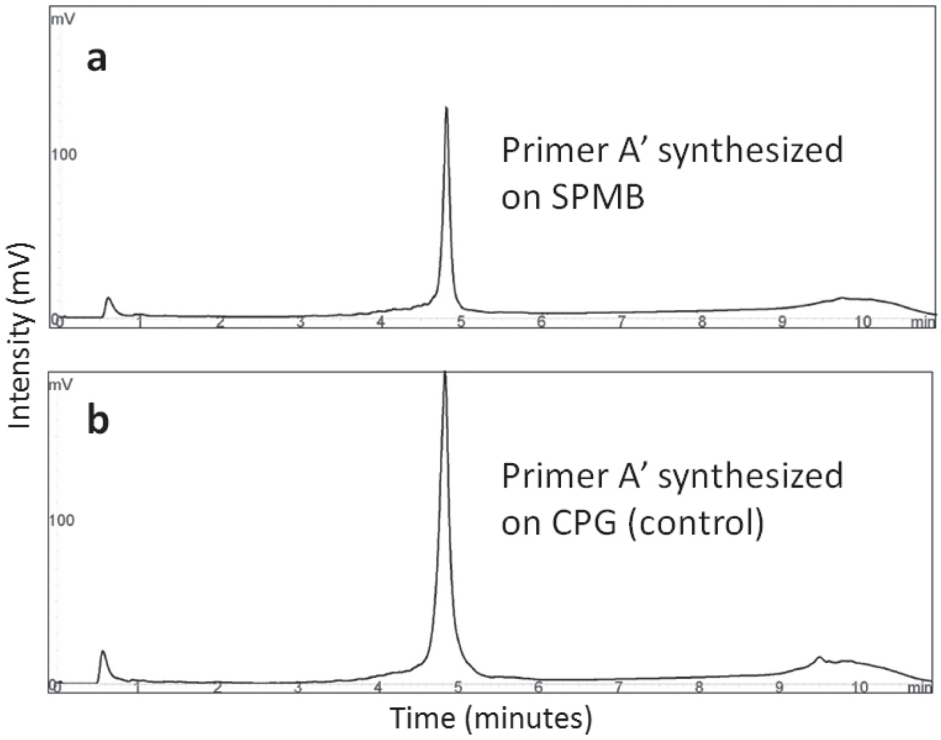

Figure 2.

shows HPLC chromatograms of (**a**) Primer A′ (40 bp) synthesized on super-paramagnetic beads (SPMB)s, and (**b**) Primer A′ synthesized on CPG support as a control. Peak retention times are 4.817 and 4.819 minutes, respectively.

Table 1

Fluorescent verification of carboxylated and post-synthesis hydroxylated super-paramagnetic beads (SPMBs) with and without oligonucleotide capture

	BEAD TYPE	MODIFICATION	STAIN	RESULT	NOISE	DNA CONC.
a.	Hydroxylated	Post-synthesis	OliGreen (ssDNA)	29.8 ng/ml	9.71 ng/ml	20.9 ng/ml
b.	Carboxylated	Coupled ligand	OliGreen (ssDNA)	20.3 ng/ml	6.99 ng/ml	13.31 ng/ml
c.	Hydroxylated	Post-synthesis and hybridization	PicoGreen (dsDNA)	4.07 ng/ml	0.82 ng/ml	3.25 ng/ml

[a]) Post-synthesis hydroxylated MyOne SPMBs (with the synthesized Primer A′ oligonucleotide) were washed after oligonucleotide synthesis. The oligonucleotide was not released from the SPMBs. As a negative control native hydroxylated MyOne SPMBs (washed only) were used to check for background noise. OliGreen fluorescent stain was used to verify presence of single-strand DNA (ssDNA).

[b]) Carboxylated MyOne SPMBs to which the amino modified synthetic oligonucleotide Primer A′ was chemically coupled to. After coupling, the beads were washed. As a negative control native carboxylated MyOne SPMBs (washed only) were used to check for background noise. The OliGreen fluorescent stain was used to verify presence of ssDNA.

[c]) The same post-synthesis hydroxylated MyOne SPMBs as in (**a**), but with annealing of the complimentary Primer A oligonucleotide. As a negative control native hydroxylated MyOne SPMBs (with Primer A annealing) were used to check for background noise. Verification of Primer A hybridization was based on the fluorescent PicoGreen stain to verify the presence of double-strand DNA (dsDNA).