Multistep Synthesis on SU-8: Combining Microfabrication and Solid-Phase Chemistry on a Single Material.

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ABSTRACT: SU-8 is an epoxy-novolac resin and a well established negative photoresist for microfabrication and microengineering. The photopolymerized resist is an extremely highly crosslinked polymer showing outstanding chemical and physical robustness with residual surface epoxy groups

amenable for chemical functionalization. In this paper we describe, for the first time, the preparation and surface modification of SU-8 particles shaped as microbars, the attachment of appropriate linkers and the successful application of these particles to multistep solid-phase synthesis leading to oligonucleotides and peptides attached in an unambiguous manner to the support surface.

KEYWORDS: SU-8, epoxy resin, photolithography, microfabrication, solid-phase synthesis, peptides, DNA, oligonucleotides, multistep synthesis, functionalization.

MANUSCRIPT TEXT

Introduction

SU-8 is a Novolac-Epoxy resin well established in the field of microfabrication and micropatterning as a negative photoresist.¹⁻³ Commercially available formulations (Shell Chemical, MicroChem) provide a macromonomer **1** (Scheme 1) dissolved in γ -butyrolactone at different concentrations.¹ These different formulations are spin-coated onto a wide range of substrates giving rise to a range of film thicknesses in the range 1 to 100 μ m, depending on the viscosity of the original solution and the spin speed.¹⁻³ They also contain a photoacid to initiate polymerization of the epoxide groups upon UV exposure.¹⁻³ The resulting polymerized resin is a highly crosslinked polymer, the hardness of which depends on the time and energy of the UV exposure and post-exposure baking times and temperatures.¹⁻³ Photolithographic masks are used to pattern the SU8, producing well defined structures with high aspect ratio (ratio height/width) and excellent mechanical properties.^{2,3} Thus, SU-8 has been successfully fabricated into a range of microstructures including microfluidic structures,^{4,5} moulds and masters for microembossing,^{6,7} probes for microscopy,^{8,9} and biosensors.¹⁰ Because of its outstanding performance in microfabrication, optical transparency in the visible range, and excellent physical and chemical stability, SU-8 has recently attracted attention for use in bioanalytical applications as a support for the direct attachment of biomolecules.¹¹⁻¹⁶ After microfabrication, residual surface epoxy groups are suitable to act as reactive sites for surface functionalization.^{11,12}

Unpolymerized SU-8 has been used in chip technology for DNA hybridization assays coated on glass.¹³⁻
¹⁵ More recently, photopolymerized and structured SU-8 has been used for DNA hybridization.¹⁶
Immobilization of DNA probes was carried out by spotting either amino-modified or unmodified oligonucleotides on the surface of SU-8 films and structures microfabricated through photolithography. This resulted in an uncertain mode of chemical attachment and indeterminate orientation of oligonucleotide probes on the SU-8 surface.¹⁶ In this paper we describe, for the first time, the preparation and surface modification of SU-8 particles shaped as microbars, the attachment of appropriate spacers in combination with linkers and the successful application of these particles to multistep synthesis leading to oligonucleotides and peptides attached in an unambiguous manner to the support surface. While SU-8 will probably not become a successful support for synthesis on its own right competing with optimized supports for synthesis, its establishment in microfabrication coupled with the feasibility of carrying out multistep synthesis enables access to interesting applications in bioanalytical sciences (e.g. microfabricated encoded carriers, microfluidic devices with in built molecular probes, etc).

Results and discussion

Fabrication of SU-8 microparticles

The fabrication of SU8 microparticles suitable for multistep synthesis required significant optimization. The mask for the photolithographic process was designed to produce microparticles (cross section: 20 μm x 10 μm) comparable in size to the beaded polymer supports used in conventional multistep solid phase synthesis. For a typical film height (3-4 μm) this results in 10 mg of particles per wafer. The microfabrication process exposes the particles to a wide range of physical and chemical conditions, some of which could potentially modify the surface properties of the particles and render them unsuitable for further chemistry.¹⁻³ Therefore an important feature of this work was to determine whether the fabricated microparticles remained suitable for synthetic chemistry.

SU-8 microparticles were prepared by conventional photolithography (Figures 1 and 2a). In order to allow the release of microparticles from the substrate after microfabrication, a sacrificial layer was incorporated in the process, as shown in Figure 1. The selection of the sacrificial layer was based on good adhesion properties towards SU-8, ease of handling and simplicity of etching process.¹⁷⁻²⁰ The last variable is critical and requires that the sacrificial layer is efficiently and rapidly removed, releasing the SU-8 particles into suspension without compromising the presence of residual surface epoxy groups to be used for functionalization.

Initially, hard baked positive photoresist, S1813 was selected as a sacrificial layer.¹⁷ However, during the SU8 processing, some of the microparticles were released suggesting that the S1813 layer was insufficiently stable. Hard baking at an elevated temperature of 175°C for 1 h resulted in a significant improvement in the stability of the S1813 layer, but increased the difficulty of releasing the particles in the final lift-off phase.¹⁷

As an alternative, the use of aluminum was investigated.¹⁸ A thin layer was evaporated onto a substrate, followed by spin coating with Ti primer prior to SU-8 processing, which resulted in excellent adhesion of the SU-8. After photopolymerization and a post-exposure bake, the non exposed areas were developed and the sacrificial layer etched. Clean etching of the aluminum layer was successful using an ultrasonic bath with commercial developer MF 319 (tetramethylammoniumhydroxide solution, TMAH). In all cases, the released SU-8 microparticles were collected by centrifugation, washed with methanol and then dried under vacuum to produce dry SU-8 support (Figure 2b). The yield of particles was 8 mg per wafer (80 %).

Two batches of SU-8 microparticles were prepared by using two different sacrificial layers; one batch obtained using aluminum-coated wafers **2a** and another obtained using S1813 as the sacrificial layer **2b** (Scheme 2). The suitability of these two batches for peptide and oligonucleotide synthesis was then investigated.

Chemistry on SU-8

Although optimization of the microfabrication was essential for optimal synthesis on SU8, chemical functionalization was first studied using large pieces of SU-8 prepared on S1813-coated glass. Films of SU-8 were produced this way by overexposure under UV and grinding the final film resin (100 μm thick) to small pieces (visually not more than 1 mm). This simpler process rapidly provided large quantities of material (SU-8, **3**) for preliminary studies on the functionalization chemistry as shown in Scheme 2. It was anticipated that the straightforward chemistry of functionalization could subsequently be applied to well-defined and carefully microfabricated particles. The residual epoxy groups present on the surface of SU-8 were reacted with bisamines to introduce free amino groups selected (Scheme 2) using a similar procedure to that applied to other epoxide-containing polymer supports such as GMA (glycidyl methacrylate supports).^{21,22} 1,3-diaminopropane (**4a**) and Jeffamine₈₀₀ (**4b**) were selected as suitable amines and provided spacers of different lengths between the SU8 surface and the primary amine. The conditions resulting in the highest loading levels were found to be overnight stirring in acetonitrile at 65 °C. Test washes with hot and cold acetonitrile and analysis of the washings suggested that the excess bisamine was thoroughly removed by series of simple washing/centrifugation cycles with acetonitrile at room temperature, although the possibility of permanent physical entrapment of the bisamines in the polymer network cannot be excluded. The loading levels of the amino supports (**5**) were approximately quantified using the ninhydrin test (Table 1). The use of 1,3-diaminopropane (**4a**) resulted in a loading level of 20 $\mu\text{mol/g}$, whilst Jeffamine₈₀₀ (**4b**) gave a loading level of 9 $\mu\text{mol/g}$. The lower loading levels observed with Jeffamine₈₀₀ (**4b**) may be rationalized in terms of the reduced accessibility of epoxide groups on the polymer to this bulkier nucleophile.

The resultant amino groups were then coupled to *N*-Fmoc-6-aminohexanoic acid using standard carbodiimide/*N*-hydroxybenzotriazole (HOBt) chemistry.²³ Quantitation of this reaction by release of the Fmoc group with piperidine in *N,N*-dimethylformamide (DMF) showed that while Jeffamine-derived aminosupport **5b** led to quantitative coupling (relative to the initial loading levels), 1,3-diaminopropane-

derived support **5a** showed much lower coupling efficiency (Table 1).²³ It may be that the smaller bisamine, whilst derivatizing the less accessible epoxy sites on the support, leads to amino groups that are not homogeneously reactive (e.g. amide formation). For this reason the use of 1,3-diaminopropane was abandoned and all further synthetic studies made use of Jeffamine for the functionalization of the SU-8 resin.

Having demonstrated the feasibility of functionalizing SU-8 on this model material, further studies were carried out with microfabricated SU-8 particles. Microparticles prepared using aluminum (**2a**) and S1813 (**2b**) as sacrificial layers were treated with Jeffamine (**4b**) at 65 °C in acetonitrile overnight. Ninhydrin assay of the amino particles showed that **2b** gave rise to higher loading amino-SU-8 (**8b**, 66 µmol/g) compared to the particles developed on aluminum (**8a**, 22 µmol/g) (Table 1). This difference was consistent from batch to batch of SU-8 with **8b** in the range of 40-70 µmol/g and **8a** in the range of 15-20 µmol/g amino groups. These low loading levels were expected as a consequence of surface-only functional sites. This is caused by the high levels of crosslinking and the fact that polymerization occurs in a thin film on the solid state with the absence of any porogen leaving accessible epoxy groups on the surface of the monolithic particles (See Figure 2).

The initial functionalization was extended by attachment of a second spacer, *N*-Fmoc-6-aminohexanoic acid followed by capping the residual nucleophiles on the surface of the particles (Scheme 2). By cleaving the Fmoc group from a small aliquot of particles, the efficiency of this coupling reaction to form **9a** and **9b** (Scheme 2) could be determined. This experiment demonstrated that the microfabrication method had a profound impact on the reproducibility of subsequent chemistry: loading levels for **8a** prepared with aluminum as sacrificial layer proceeds quantitatively (within experimental error). On the other hand, the use of S1813 as sacrificial layer (**2b**, **8b**, **9b**) led to poorly reproducible results, although sometimes the reactions were relatively high yielding. It was also observed that the SU8 prepared with an S1813 sacrificial layer appeared to have a higher loading level after the coupling of *N*-Fmoc-6-aminohexanoic acid (as quantified by the Fmoc test of **9b**) than had been observed by the

ninhydrin test of **8b** (Table 1). This can be most simply explained by the presence of additional non-amine functionalized groups that undergo acylation by *N*-Fmoc-6-aminohexanoic acid. These results indicate that SU-8 particles prepared on Al as a sacrificial layer are more suitable for the development of robust and reliable solid-phase chemistry.

Peptide synthesis

To test the performance of SU-8 in multistep synthesis, solid-phase peptide synthesis was chosen as a well-established methodology,²⁴ and the resultant peptides are attractive targets in a myriad of biotechnological assays and applications.²⁵ Leucine-Enkephalin was selected as a simple test sequence. It is a pentapeptide (Tyr-Gly-Gly-Phe-Leu) that is routinely used as a model for testing materials as supports for synthesis.²⁶⁻²⁸ To permit Fmoc-chemistry, 4-hydroxymethylphenoxyacetic acid (HMPA) was attached as a linker using carbodiimide/HOBt chemistry at room temperature.²³ (Scheme 3). The first amino acid (*N*-Fmoc-Leu) was coupled onto the resin using carbodiimide/*N,N*-dimethyl-4-aminopyridine (DMAP) at room temperature.²³ A quantitative Fmoc test showed efficient loading of the first amino acid (Table 1). Subsequent couplings were carried out using (*N,N,N',N'*-tetramethyl-*O*-benzotriazol-1-yl)uronium tetrafluoroborate (TBTU) as coupling reagent.²³ Throughout the peptide synthesis, SU-8 prepared on aluminum (**11a**) showed a marked superiority over SU-8 microfabricated on S1813 (**11b**). The latter (SU-8/S1813, **11b**) repeatedly gave inconclusive ninhydrin tests and couplings had to be repeated several times (Table 1). After the synthesis, the peptides were cleaved from the support using TFA/phenol (98:2). The available quantities of microparticles limited the synthesis to a very small scale (50 mg of SU-8 particles, 850 nmol scale synthesis) and this scale restricted the range of techniques available for product characterization. However, HPLC-MS analysis required very small quantities of material and allowed comparison of the synthetic peptide samples with a commercial sample of Leu-Enkephalin (Figure 3a,b). SU-8 microfabricated on aluminum (**11a**) produced Leucine-Enkephalin (**12a**) essentially as a single peptide product (Figure 3c,d). The purity of **12a** was low as can be seen from HPLC analysis but no other peptide impurities were detected (Figure 3a,b). Further

optimization will be required to minimise the level of impurities present, which we associate with leach of the support during TFA cleavage. Although necessary at this stage of evaluation, this step will not be carried out in analytical applications of microfabricated supports. The yield of product (**12a**) with respect to the loading of the first aminoacid was estimated to be 5 % by chromatographic comparison with a commercial standard (Figure 3a, c). Although somewhat disappointing, this yield highlights the practical difficulties in isolating polar compounds such as peptides from manual nanomolar scale syntheses. A commercial sample of Leu-Enkephalin showed identical mass spectrometric and chromatographic properties to those found for **12a**. SU-8 **11b**, prepared on S1813, yielded a mixture of peptides (**12b**) including some with a higher than expected molecular weight, with mass differences from Leu-Enkephalin consistent with the addition of extra Tyr and Gly residues (Table 1, Figure 3e,f). These LCMS results, combined with the observed inconclusive results from ninhydrin tests during the coupling reactions may indicate partial cleavage of Fmoc groups during couplings, leading to oligomerization of the same aminoacid onto the growing peptide chain, especially when couplings were repeated. With this result, it was concluded that the SU-8 microfabricated on S1813 (**2b**) was not suitable for use in multistep synthesis. In contrast, SU-8 patterned on aluminum (**2a**) showed excellent properties as a support for multistep synthesis throughout the chemical transformations studied. Synthesis of a larger peptide was then explored to confirm the suitability of SU-8 prepared on Al for multistep synthesis. A nonapeptide (HIV-Protease 1 substrate), was chosen as the target (**13**, Val-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln).²⁹⁻³¹ Nonapeptide **13** was synthesized on SU-8 prepared on Al (**11a**) since this had given the best results for the synthesis of Leu-Enkephalin (**12**) (Scheme 3). As peptide **13** was not commercially available, a synthetic standard of **14** was prepared by standard SPPS using standard PS resin with a Wang linker, **15**. The synthesis of **13** proceeded smoothly requiring only single couplings.²³ The product peptides were analyzed by HPLC-MS after cleavage and the results are shown in Figure 4 and Table 2. The yield of crude peptide **13** was higher than in the previous case (40 %) although equally impure. However, the major impurities are not peptide side products but support leach as stated before. As expected the crude product from conventional SPPS, **14**, was relatively pure with a single minor

impurity. MS of the impurity was consistent with a dehydration product (possibly dehydration of Asn or Gln) (Figure 4c,d, Table 2). The nonapeptide synthesized on SU-8 (**13**) contained the same minor impurity. Identity of the sample prepared on SU-8 was further confirmed by co-elution of the crude **13** with HPLC purified **14**.

Although the final yields from the nanoscale synthesis may not be comparable to well-established supports for solid-phase chemistry, the most important parameter for this study was the purity of target peptides in comparison to other peptide products and this was certainly satisfactory in the case of SU-8 microfabricated on aluminum (**11a**). This proves the feasibility of using microfabricated SU-8 for the direct synthesis of analytical probes. The general purity of the products, though, was not satisfactory. However, as explained before, we believe that major impurities are based on support leach during cleavage conditions required at this stage for product analysis but avoided in analytical applications of the microfabricated surfaces. Further optimization to improve this situation will also be carried out.

Oligonucleotide synthesis

To demonstrate the flexibility of SU-8 as a support for solid-phase synthesis, oligonucleotides were targeted through the phosphoramidite approach.^{32,33} Amino-SU-8 (**10a** and **10b**) was functionalized with succinimidyl nucleoside **16** to produce amide **17** (Scheme 4). The loading level was measured by release of dimethoxytrityl cation under acidic conditions and found to be 25 $\mu\text{mol/g}$.³² SU-8 **17a** and **17b** were used in the synthesis of the following oligonucleotides: T₁₅C **19a,b** and GCTTATGCTTCTTC **20a,b**. These structures were not based on natural sequences. In parallel to this synthesis the same oligonucleotide sequences were also prepared on pre-packed CPG columns (**18**, 1000 Å pore size, 50 nmol scale from Bioautomation), producing oligonucleotides **19c** and **20c**). For the synthesis an automatic MerMade192 synthesizer (Bioautomation) was used following manufacturer protocols optimized for the synthesis on column CPG (**18**) and performing double phosphoramidite couplings on the SU-8 supports. After conventional synthesis (DMT off) and cleavage of the oligonucleotides from the support, the oligonucleotides were characterized by capillary electrophoresis (shown in Figure 5) and

MALDI-TOF MS.³⁴⁻³⁶ In agreement with earlier observations, SU-8 microfabricated on a S1813 layer **17b** was not suitable for synthesis, giving extremely low yields and poor purity of oligonucleotides **19-20b** (less than 3 % or not detected, data not shown). On the other hand, SU-8 prepared on an aluminum sacrificial layer **17a** produced oligonucleotides **19a** and **20a** with results comparable to those obtained from CPG as shown in Figure 5 and Table 3. This indicates that SU-8 is suitable for oligonucleotide synthesis, although further improvements in the purity and yield can be anticipated once the oligonucleotide synthesis protocols are optimized for this substrate.

Given the purity of products, though, it is important to note that SU-8 seems, at this stage of development, to be appropriate for the synthesis of short oligonucleotides. Larger oligonucleotides will result in an increased number of deletion sequences and even lower purity levels.

Conclusions

Fabrication of releasable SU-8 microparticles on silicon wafers was achieved using standard photolithography techniques. In this process, aluminum proved to be a suitable sacrificial layer for the release of bars from the silicon substrate using basic conditions leading to microparticles with good yielding performance in subsequent chemistry.

The use of SU-8, a well-established material for microfabrication, has been demonstrated as a feasible support for peptide and oligonucleotide synthesis with good purity compared to possible side products, but low purity overall due to leach during cleavage of products. While at this stage we do not envisage SU-8 as a possible competitor for established supports, these important results demonstrate the combination of microfabrication and multistep solid-phase synthesis. This useful combination of properties is very suitable for application in a wide range of bioanalytical systems and processes.

Experimental

Microfabrication of SU-8 particles: The photoresist SU-8 (SU-8-2, SU-8-25 and SU-8-50) and developers (Microposit EC and propyleneglycol methyl ether acetate, PGMEA) were supplied by

Chestech Ltd, UK. Rohm and Haas S1813 and Microposit MF-319 (tetramethylammoniumhydroxide, TMAH) were obtained from Shipley Europe Ltd. The universal Ti primer and single sided polished 4 inch (100 mm) silicon wafers (thickness: $525 \pm 25\mu\text{m}$) were purchased from Microchem Corp. and Si-Mat Silicon Materials, Germany respectively. A Headway Research spinner and a SUSS Microtech Mask Aligner MA6 (lamp HG 1000 DC) were used for spinning and exposing the SU-8. The photo-mask (dark field) was designed to produce rectangular bars ($20 \times 10 \mu\text{m}$) separated by a $10 \mu\text{m}$ spacing. It was generated using a CAD package (L-Edit 11.0) and printed on a glass-chromium photomask by Compugraphics International.

Crosslinked SU-8 for functionalization studies: SU-8-50 (1 ml) was spun on thin objective microscope cover glass slides at 2000 rpm for 2 min. The slides were soft-baked at 95°C for 30 min and exposed to UV light (100 W UV-vis bulb, cut-off filter 365 nm) for 5 min. The exposed slides were soft-baked at 95°C for 30 min and the SU-8 was easily lifted off the glass slides by gently bending the slides. The crosslinked SU-8 films were ground to particle sizes not bigger than several hundreds μm (visually less than 1 mm).

SU-8 microfabrication by photolithography: Standard photolithographic methods were used to fabricate the SU-8 micro-particles onto silicon wafers (4 inch) (Figure 1). A six step process was optimized as follows: (1) selection and coating of sacrificial layer, (2) optimization of adhesion of SU-8, (3) spin coating of SU-8, (4) photolithography, (5) development, and finally (6) the lift off process. Silicon wafers were cleaned by immersion in fuming nitric acid for 20 minutes and then rinsed twice in water. The acid cleaned wafers were then spun dry and baked at 200°C in a convection oven overnight. The wafers were then coated with sacrificial layers, either Al (50 nm) or S1813 resist. The S1813 (5-6 ml) was spin coated onto pre-cleaned silicon wafers at 2000 rpm for 30 seconds. The wafers were then baked for 30 minutes at one of three temperatures: 95°C , 115°C or 175°C . A 50 nm thick layer of Al was coated on to pre-cleaned silicon wafers using an E-Gun evaporator; the coated wafers were washed with acetone and isopropanol and blow dried. Then the wafers were then baked at 200°C for 1 hour to

ensure dryness and a universal Ti primer (sufficient to coat the entire wafer, ~3 ml) was then spin coated onto the Al-coated wafers at 2500 rpm for 30 seconds. The resultant wafers were then baked at 120° C for 10 minutes in an oven.

SU-8-2 was spin coated onto pre-cleaned and primed wafers using the spin cycle started from a spread cycle at 500 rpm for 5 seconds at an acceleration of 100 rpm per second followed by a final cycle at 1500 rpm for 30 seconds at an acceleration of 300 rpm per second. A similar process was applied for spin coating SU-8-5 except that the final cycle was at 2500 rpm. After applying the SU-8 layer, the wafers were soft baked at 65° C for 3 minutes, and then the temperature was increased at 4 °Cmin⁻¹ to 95° C, then the temperature maintained for 5 minutes for SU-8-2 and 10 minutes for SU-8-5.

The SU-8 coated wafers were exposed to 365nm light, optimized by varying the exposure time from 3-10 seconds with increments of 1 second (at the rate of 20.1 mW cm⁻²) with exposure doses between 90-110 mJ cm⁻². After exposure, the wafers were baked at 65° C for 1 minute, and then the temperature was increased at 4° Cmin⁻¹ to 95° C for 1 minute for SU-8-2 and 3 minutes for SU-8-5 respectively. The wafers were then left to cool to room temperature. The wafers were developed in PGMEA for 2 min with agitation, then thoroughly rinsed with isopropyl alcohol and blow dried. The sacrificial layer was removed by sonicating the wafers in TMAH (Microposit MF-319) at room temperature for 10 mins. The released microparticles were collected by centrifugation (13000 rpm for 1 min) and then washed in methanol (1 ml x 8 times) and dried under vacuum at room temperature for 4 h. The yield of isolated particles was typically 8 mg per wafer (80 %).

Chemistry on SU-8: All reactions were carried out in microcentrifuge tubes (1.5 ml) and separation of the support from solutions carried out by centrifugation at 13200 rpm for 1-3 minutes in an accuSpin™ Micro microcentrifuge (Fisher Scientific). Ninhydrin, Fmoc and trityl tests were carried out by UV spectrophotometry as reported in the literature.^{33,37,38}

Functionalization of fragmented SU-8: SU-8 (100 mg) was treated with Jeffamine₈₀₀ (500 mg, neat) and acetonitrile (500 µl) and heated to 65 °C in an oven overnight. The support was washed with acetonitrile (7 x 800 µl) followed by methanol (7 x 800 µl) and dried under vacuum at room temperature for 4 h to yield **8a** and **8b**. N-Fmoc-6-aminohexanoic acid (5.0 mg, 14 µmol) was dissolved in DMF (100 µl) and *N,N'*-diisopropylcarbodiimide (DIC) (2 µl, 13 µmol) was added. The mixture was shaken for 8 min at room temperature, HOBt (2 mg, 15 µmol) was added and the mixture was shaken for 5 min at room temperature. The mixture was added to amino SU-8 (1.50 µmol based on free –NH₂ groups) suspended in DMF (300 µl) and the mixture was heated to 60 °C for 1h. The support was washed with DMF (7 x 800 µl) followed by THF (7 x 800 µl). The support was then suspended in a freshly prepared solution of THF (1 ml) containing acetic anhydride (10 % v/v), 2,6-lutidine (11 % v/v) and *N*-methylimidazole (16 % v/v) and shaken for 15 min at room temperature. The support was then washed with THF (2 x 800 µl) and the treatment with acetic anhydride, 2,6-lutidine and *N*-methylimidazole was repeated. The support was washed with THF (7 x 800 µl) followed by methanol (7 x 800 µl) and then dried under vacuum at room temperature for 4 h to yield **6a**, **6b**, **9a** and **9b**. Fmoc-SU-8 (less than 200 mg) was suspended in piperidine (20 % in DMF, 1 ml) and shaken at room temperature for 20 min. The treatment with piperidine/DMF was repeated. The support was washed with DMF (10 x 800 µl) followed by tetrahydrofuran (THF) (8 x 800 µl) and diethyl ether (3 x 800 µl) and dried under vacuum at room temperature for 4 h to yield **7a**, **7b**, **10a** and **10b**.

Attachment of HMPA linker (Synthesis of 11a and 11b): HMPA (8 mg, 45 µmol) was dissolved in DMF (100 µl) and DIC (7 µl, 45 µmol) was added. The mixture was shaken for 8 min at room

temperature, HOBt (6 mg, 45 μmol) was added and the mixture was shaken for 5 min at room temperature. The mixture was added to amino SU-8 (50 mg, 850 nmol based on free $-\text{NH}_2$ groups) suspended in DMF (100 μl) and the mixture was shaken for 1 h at room temperature. The support was washed with DMF (7 x 800 μl) and the procedure was repeated. The support was washed with DMF (7 x 800 μl) followed by methanol (7 x 800 μl) and dried under vacuum at room temperature for 4 h.

Attachment of first aminoacid: N-Fmoc-Leucine (12 mg, 33 μmol) was dissolved in DMF (50 μl) and DIC (5 μl , 33 μmol) was added. The mixture was shaken for 8 min at r.t. DMAP (0.5 mg, 3 μmol) was added and the mixture was added to HMPA SU-8 (850 nmol based on loss of amino groups after the attachment of the linker) suspended in DMF (100 μl) and the mixture was shaken for 1h at room temperature. The support was washed with DMF (7 x 800 μl) and the procedure was repeated twice. The support was washed with DMF (7 x 800 μl) followed by methanol (7 x 800 μl) and dried under vacuum at room temperature for 4 h.

Peptide synthesis: The following aminoacids were required: *N*- α -Fmoc-Leu, *N*- α -Fmoc-Gly, *N*- α -Fmoc-Phe, *N*- α -Fmoc-Val, *N*- α -Fmoc-Gln, *N*- α -Fmoc-Asn, *N*- α -Fmoc-Pro, *N*- α -Fmoc-Ile, *N*- α -Fmoc-(*O*-Trt)-Ser and *N*- α -Fmoc-(*O*-2-Cl-Trt)-Tyr. *N*- α -Fmoc-aminoacid (8 μmol) was dissolved in DMF (50 μl) and TBTU (3 mg, 9 μmol), HOBt (0.3 mg) and *N,N*-diisopropyl-*N*-ethylamine (DIPEA) (1.5 μl , 9 μmol) were added. The mixture was shaken for 2 min, then the mixture was added to the deprotected SU-8 (850 nmol based on $-\text{NH}_2$ groups) suspended in DMF (100 μl) and the mixture was shaken for 1h at r.t. The support was washed with DMF (3 x 800 μl) followed by methanol (2 x 800 μl) and diethyl ether (3 x 800 μl). The completeness of the reaction was monitored by ninhydrin test. After a negative ninhydrin test the N-terminus Fmoc group was removed. The peptide was cleaved from the support by treatment with TFA/phenol (98/2 % v/w, 25 ml/g resin) for 90 min at room temperature. The support was filtered and washed with TFA (3 x 1 ml). The combined filtrates were evaporated under vacuum

and the residual oil was triturated with diethyl ether. The solid that precipitated was washed with diethyl ether and dried under vacuum at room temperature for 4 h.

LC-MS analysis of peptides: dry samples were dissolved in acetonitrile/H₂O (50/50 % v/v) (0.5 mg/ml). Analytical HPLC (Gilson) was monitored at 215 and 280 nm using a Phenomenex C18 column (150 x 4.5 mm, 5 µm, 300 Å pore size). Gradient: 10-100 % B in A gradient over 32 minutes at 1 ml/min, where A: 0.1 % TFA /10 % acetonitrile/water and B: 0.1 % TFA/acetonitrile. The injection volume was 200 µl. ESI-MS (Surveyor MSQ) was coupled online with the HPLC separation by a splitter (1/4 split) and mixing the chromatographic outlet with 0.3 % formic acid / 50 % acetonitrile / 50 % H₂O in a third pump (1 ml/min). See main text for results.

HIV Protease I substrate, **13**, was further characterized by NMR spectroscopy using a Bruker spectrometer operating at either 400 MHz (¹H) or 100 MHz (¹³C). ¹H NMR (D₂O) δ 7.06 (m, 2H, *J* = 8.7 Hz), 6.78 (d, 1H, *J* = 8.6 Hz), 6.75 (d, 1H, *J* = 8.2 Hz), 4.74 (dd, 1H, *J* = 8.6, 5.6 Hz), 4.58 (dd, 1H, *J* = 7.9, 6.0 Hz), 4.45 (t, 1H, *J* = 6 Hz), 4.36 (dd, 1H, *J* = 7.0, 6.4 Hz), 4.31 (dd, 1H, *J* = 9.0, 5.0 Hz), 4.24 (dd, 1H, *J* = 8.4, 5.6 Hz), 4.11-4.03 (m, 2H), 3.79 (m, 3H), 3.67 (dd, 1H, *J* = 7.1, 16.5 Hz), 3.48 (dd, 1H, *J* = 6.4, 16.0 Hz), 3.01 (dd, 1H, *J* = 5.0, 14.6 Hz), 2.79 (m, 1H), 2.68 (m, 1H), 2.56 (m, 2H), 2.29 (m, 3H), 2.17 (m, 4H), 2.00-1.76 (m, 7H), 1.44 (m, 1H), 1.13 (m, 1H), 0.95 (m, 6H), 0.89-0.78 (m, 12 H). ¹³C NMR (D₂O) δ 178.0, 174.9, 174.6, 174.1, 173.9, 173.5, 172.1, 171.8, 171.5, 171.4, 169.8, 154.8, 131.1, 130.9, 128.4, 61.4, 60.7, 59.7, 58.8, 58.7, 55.8, 53.4, 53.3, 52.4, 50.6, 48.3, 36.6, 36.4, 35.9, 31.5, 31.3, 30.6, 30.3, 29.6, 27.2, 26.8, 25.1, 25.0, 18.7, 18.3, 18.2, 18.0, 17.1, 15.2, 10.5

Synthesis of 17a and 17b: 3'-*O*-Succinimydyl-5'-*O*-dimethoxytrityl-*N*-benzoyl-deoxycytidine (8.0 mg, 11 µmol) was dissolved in DMF (500 µl). HOBt (0.3 mg, 2 µmol), TBTU (3.5 mg, 11 µmol) and DIPEA (1.8 µl, 11 µmol) were added and the mixture stirred at room temperature for 5 min. The mixture was added to amino SU-8 **10a** (200 mg, 3.5 µmol based on -NH₂ groups) and stirred vigorously at room temperature for 30 min. The support was washed with DMF (3 x 800 µl) and the procedure was

repeated. The support was washed with DMF (7 x 800 μ l) followed by THF (7 x 800 μ l) and dried under vacuum at room temperature for 4 h.

Oligonucleotide synthesis, purification and analysis:^{36,39} A MerMade 192 automatic synthesizer (Bioautomation Inc.) was used for oligonucleotide synthesis according to the manufacturer protocol for a 50 nmol scale synthesis optimized for MerMade CPG columns (1000 Å pore size, 50 nmol, standard CPG loaded with 3'-C). For SU-8 particles a modified protocol was used involving phosphoramidite double couplings. After the synthesis, the oligonucleotides were cleaved from the support using ammonia solution (35 %, 0.88 g/ml, 1 x 150 μ l for 15 min, followed by 3 x 100 μ l for 15 min, filtering and collecting the filtrate each time). The combined filtrates were heated in a sealed plate at 65 °C for 6 hours. The solutions were freeze dried overnight and the residue dissolved in H₂O (150 μ l). The oligonucleotide solutions were purified by HPLC. Preparative HPLC (Gilson) was monitored at 254 and 280 nm. Separation was carried out using a Phenomenex Jupiter C18 column (50 x 4.60 mm, 5 μ m, 300Å pore size). Gradient: 0-40 % B in A gradient over 8 minutes, isocratic at 40 % B in A for 30 sec, then 40-0 % B in A in 30 sec and isocratic at 100 % A for a minute, at 1 ml/min, where A: 0.1 M ammonium acetate pH 7 in water and B: 0.1 M ammonium acetate pH 7 in 50 % acetonitrile in water. The injection volume was 120 μ l. Isolated peak detection allowed for collection of single peak fractions between 2-7 min. After freeze drying of the fractions they were analyzed by Capillary Electrophoresis using a Beckman-Coulter P/ACE MDQ Capillary Electrophoresis System following the manufacturers protocol using a Beckman eCAP ssDNA 100-R kit. Separation carried out in Tris-Borate urea (7M) buffer using an eCAP DNA 100 μ m ID capillary (20 cm). Fresh eCAP ssDNA gel was loaded into the capillary under pressure (60 psi x 15 min) followed by equilibration of the capillary immersed in Tris-Borate urea (7M) buffer at constant voltage (3kV, 0.17 min ramp, normal polarity x 5 min, followed by 9kV, 0.17 min ramp, normal polarity x 10 min). Capillary temperature was set at a constant 30 °C. The sample (diluted to 0.5 OD at 254 nm) was loaded at constant voltage (10 kV x 2 sec, reverse polarity) and then separated at constant voltage (9 kV x 60 min, 0.17 min ramp, reverse polarity). UV monitoring

was carried out at 254 nm. Migration times were compared to a standard oligonucleotide ladder (Beckman-Coulter) and confirmed by co-migration of samples. Oligonucleotides were further characterized using a DYNAMO MS MALDI-TOF spectrometer as described elsewhere.³⁶ Samples of T₁₀, T₁₅ and T₂₀ were used as internal standards.

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Supporting Information Available: MS and NMR of peptides.

FIGURES

Figure 1

Figure 2

a)

b)

c)

Figure 3

Figure 4

Figure 5

a)

b)

c)

d)

SCHEMES:

Scheme 1

Scheme 2

Scheme 3

Scheme 4

FIGURE CAPTIONS

Figure 1. Microfabrication of SU-8 particles by photolithography: (1) Add sacrificial layer (either Al or S1813); (2) Add SU-8 and bake; (3) Expose to UV and bake; (4) Develop non crosslinked SU-8; (5) Etch sacrificial layer.

Figure 2. Scanning Electron Microscopy Analysis of SU-8 microparticles prepared on an Al sacrificial layer: (a) SU-8 particles on Si-wafer after microfabrication (size bar: 30 μm); (b) SU-8 particles after functionalization with Jeffamine (size bar: 20 μm). (c) Close section of SU-8 particle showing monolithic structure with lack of porosity.

Figure 3. HPLC-MS Analysis of Leu-Enkephalin: (a) HPLC standard Leu-Enkephalin; (b) total ion current (TIC) standard Leu-Enkephalin; (c) HPLC **12a** (synthesis on SU-8 (Al)); (d) TIC **12a** (synthesis on SU-8 (Al)); (e) HPLC **12b** (synthesis on SU-8 (S1813)); (f) TIC **12b** (synthesis on SU-8 (S1813)). Note: HPLC trace corresponds to UV detection at 215 nm. The arrow indicates the target material.

Figure 4. HPLC-MS Analysis of HIV-Protease I substrate: (a) HPLC **14** (Wang resin product); (b) total ion current (TIC) **14** (Wang resin product); (c) HPLC **13** (SU8 product); (d) TIC **13** (SU8 product). Note: HPLC trace corresponds to UV detection at 215 nm. The arrow indicates the target material.

Figure 5. Capillary Electrophoresis of oligonucleotides. (a) Sequence **19** synthesized on **18** (column CPG); (b) sequence **20** synthesized on **18** (column CPG); (c) sequence **19** synthesized on **17a** (SU-8); (d) sequence **20** synthesized on **17a** (SU-8).

SCHEME TITLES:

Scheme 1. Polymerization of SU-8

Scheme 2. Functionalization of SU-8

Scheme 3. Peptide synthesis on SU-8

Scheme 4. Oligonucleotide synthesis on SU-8

TABLES:

Table 1

Reaction type/Process stage	Loading levels of Supports (method of measurement, observations)			
Starting material	3	3	2a	2b
	5a,	5b,	8a,	8b,
Functionalization with bisamine	20 ± 3 μmol/g (Ninhydrin)	9 ± 3 μmol/g (Ninhydrin)	15-25 μmol/g (Ninhydrin)	40-70 μmol/g (Ninhydrin)
	6a,	6b,	9a,	9b,
Attachment of Fmoc-Aminohexanoic acid	4 ± 2 μmol/g (Fmoc)	9 ± 2 μmol/g (Fmoc)	15-19 μmol/g (Fmoc)	50-100 μmol/g (Fmoc)
			10a,	10b,
Deprotection of amino groups	7a	7b	16-18 μmol/g (Ninhydrin)	50-60 μmol/g (Ninhydrin)
Attachment of HMPA linker			11a	11b
Attachment of first aminoacid (Leu)			15 μmol/g (Fmoc)	60 μmol/g (Fmoc)
Attachment of Phe			Single coupling	Coupling repeated
Attachment of Gly			Single coupling	Single coupling
Attachment of Gly			Single coupling	Coupling repeated, intensity of ninhydrin test is faint after deprotection of Fmoc group
Attachment of Tyr			Single coupling	Coupling repeated 3 times
Peptide cleavage			12a , 0.5 mg crude isolated (expected 0.5 mg, but only 5 % calculated from HPLC trace). ESI-MS: Found 556.5 (MH ⁺) (Expected 556.63)	12b , 1.0 mg crude peptide isolated (30 % calculated from HPLC trace). ESI-MS: Found 613.57 (MH ⁺ +Gly), 719.68 (MH ⁺ +Tyr), 776.68 (MH ⁺ +Tyr+Gly), 833.73 ((MH ⁺ +Tyr+2Gly),

Table 1. Functionalization of SU-8. Solid-phase synthesis of Leu-Enkephalin

Table 2

HIV Protease I substrate (support)	Yield	% Overall Purity of nonapeptide	Overall % of Elimination product	% Nonapeptide with respect to other peptides (ESI-MS)	% Elimination product with respect to other peptides (ESI-MS)	% Main peptide impurity ^c (ESI-MS)
13 (SU-8 11a)	40 % ^a	29	11	58 (ESI-MS: Found 1046.9(M ⁺) (Expected 1047.19)	15 (ESI-MS: Found 1028.6(M ⁺) (Expected 1029.17)	15 (ESI-MS: Found 610.0 possible Val-Ser-Gln- Asn-Tyr (M ⁺) (Expected 609.64)
14 (Wang 15)	87 % ^b	67	23	88 (ESI-MS: Found 1046.7(M ⁺) (Expected 1047.19)	12 (ESI-MS: Found 1028.9(M ⁺) (Expected 1029.17)	0

Table 2. Solid-phase synthesis of HIV-Protease I substrate. Notes: ^a Estimated from integration of HPLC trace compared to calibrated data with pure **14**. ^b Estimated from weight of crude. ^c Excluding elimination product.

Table 3

Results				
	Oligonucleotide sequence (support)	Purity of target oligonucleotide (%) ^a	Mass by MALDI-TOF (Expected) ^b	% of principal impurity (length in bases) ^a
1	19a (17a)	47	4791.5 (4791.2)	15 (13)
2	19c (18)	77	4791.1 (4791.2)	6 (15)
3	20a (17a)	56	4196.6 (4196.8)	20 (5) ^c
4	20c (18)	91	4196.6 (4196.8)	3 (11)

Table 3. Solid-Phase Synthesis of oligonucleotides on SU-8. Comparison with CPG. Notes: ^a Estimated from CE data ^b Corresponds to (MH)⁺ ^c Estimated from HPLC data (not shown).

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SYNOPSIS TOC

SU-8 particles
microfabricated by
photolithography

Peptide
synthesis

VSQNYPIVQ

DNA
synthesis

GCTTATGCTTCTTC