Tuning preparation conditions towards optimized separation performance of thermally polymerized organo-silica monolithic columns in capillary liquid chromatography

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Tuning of preparation conditions, such as variations in the amount of a porogen, concentration of an aqueous acid catalyst, and adjustment in polymerization temperature and time, towards optimized chromatographic performance of thermally polymerized monolithic capillaries prepared from 3-(methacryloyloxy)propyltrimethoxysilane has been carried out. Performance of capillary columns in reversed-phase liquid chromatography was assessed utilizing various sets of solutes. Results describing hydrophobicity, steric selectivity, and extent of hydrogen bonding enabled comparison of performance of hybrid monolithic columns prepared under thermal (TSG) and photopolymerized (PSG) conditions. Reduced amounts of porogen in the polymerization mixture, and prolonged reaction times were necessary for the preparation of monolithic columns with enhanced retention and column efficiency that reached to 111 000 plates/m for alkylbenzenes with shorter alkyl chains. Both increased concentration of catalyst and higher temperature resulted in faster polymerization but inevitably in insufficient time for pore formation. Thermally polymerized monoliths produced surfaces, which were slightly more hydrophobic (a methylene selectivity of 1.28 ± 0.002 TSG vs 1.20 ± 0.002 PSG), with reduced number of residual silanols (a caffeine/phenol selectivity of 0.13 ± 0.001 TSG vs 0.17 ± 0.003 PSG). However, steric selectivity of 1.70 ± 0.01 was the same for both types of columns. The batch-to-batch repeatability was better using thermal initiation compared to monolithic columns prepared under photopolymerized conditions. RSD for retention factor of benzene was 3.7% for TSG capillaries (n=42) vs. 6.6% for PSG capillaries (n=18). A similar trend was observed for columns prepared within the same batch.

1. Introduction

High performance liquid chromatography (HPLC) is the most widely utilized analytical separation technique due to its universal applicability to quantitative and qualitative analysis of a wide range of compounds. This technique can be used for separation of analytes varying greatly in polarity and charge. In contrast to gas chromatography, limited thermal stability and volatility of analytes is of less concern in HPLC. Large numbers of columns, commercial and custom-made, are available enabling these separations, even though combination of columns with diverse chemistry might be required for complex mixtures.

In recent years the quest for acceleration of analyses, while preventing the loss in column efficiency, led to development of highly permeable monolithic stationary phases. This concept was first envisioned by Mould and Synge in 1952 [1]. As with traditional particle-packed columns, developing formulas for preparation of monolithic columns based on organic polymers [2-5] and silica [6-10] attracted the most attention. Silica-based monolithic columns are prepared using sol-gel technology whereas free radical polymerization of suitable organic monomer is most often used for formation of organic polymer monoliths. Structure porosity and surface chemistry have been shown to control chromatographic performance of both types of monolithic columns. The size of through-pores and silica skeletons is controlled in silica-based monoliths independently by controlling composition of reaction mixture, and the concentration of silane and polyethylene glycol. Furthermore, thermal post-gelation treatment in basic conditions leads to formation of mesopores within the silica skeletons [11, 12]. In the case of polymer monoliths, porosity is optimized by adjusting the percentage of the porogen and cross-linking monomer in

the polymerization mixture, as well as temperature and duration of the polymerization reaction [13, 14].

A fair number of manuscripts were devoted to the preparation of hybrid organo-silica monoliths [15-20]. These monoliths have also been subject of our attention, specifically those prepared using 3-(methacryloyloxy)propyltrimethoxysilane. In our early work inspired by research of Dulay *et al.* [15-17], we have optimized conditions for the preparation of photopolymerized sol-gel (PSG) monoliths, and evaluated their chromatographic properties utilizing capillary liquid chromatography [18]. We have reported that these stationary phases have free silanols as well as organic moieties available on the pore surface, and therefore could be further modified with various organic functionalities via silanization or graft polymerization. In addition, these materials exhibited an interesting potential for the separation of large molecules such as proteins. Hence, these separation media were promising in proteomics applications.

In this manuscript we extended our early findings mainly by switching from photoinitiation to thermally initiated polymerization. We assumed that temperature will simultaneously increase the rate of hydrolysis and polycondensation of methoxysilanes as well as the rate of initiation of free radical polymerization of methacrylate groups of the starting monomer. This should ultimately result into formation of monoliths with different porosity and surface chemistry compared to photopolymerized monoliths. Further, we have evaluated the effect of variations in the amount of the porogen, the concentration of the aqueous acid catalyst, polymerization temperature, and time on the column permeability, retention, and efficiency. Subsequently, several sets of solutes were separated to assess chromatographic performance of thermally (TSG) and photopolymerized (PSG) sol-gel monoliths.

2. Material and methods

2.1 Chemicals and Materials

3-(Methacryloyloxy)propyltrimethoxysilane (MPTMS, 98%), 2,2'-azobisisobutyronitrile (AIBN, 98%), anhydrous toluene, together with high purity HPLC solvents, methanol, ethanol, tetrahydrofuran, acetonitrile, and probe compounds of at least 98.5% purity were purchased from Sigma Aldrich (St. Louis, MO).

2.2 Preparation of sol-gel monoliths

Monolithic capillaries were prepared by mixing 575 μL of 3-(methacryloyloxy)propyltrimethoxysilane and 100 μL of 0.15 mol/L HCl for 30 min. Simultaneously, in a separate dark brown vial, 30 mg AIBN (5% w/v) was stirred with 420 μL toluene (70% v/v). Next, 180 μL MPTMS/HCl solution was added to the solution of initiator in toluene. The solution was vortexed for 3 min, and purged with nitrogen for 30 s. UV-transparent fused silica capillaries (25 cm) were filled with the final mixture using compressed nitrogen, were plugged with a rubber septa and were either heated (TSG, 80 $^{\circ}\text{C}$) or irradiated (PSG, 365 nm, 900 mJ/cm^2). The temperature in the instrument used for irradiation was not controlled but did not exceed 29 $^{\circ}\text{C}$, i.e. a temperature that does not start thermally initiated polymerization. After the polymerization was completed, the capillaries were rinsed with toluene for removal of unpolymerized components, and their length reduced to 18 cm.

2.3 Instrumentation

Thermal polymerizations were carried out in an Isotemp oven (Fisher Scientific, Pittsburgh, PA). Irradiation of the capillaries was performed using the XL-1500A UV crosslinker with six 365 nm tubes (both Spectroline, Westbury, NY). Chromatographic performance was evaluated using a 1200 Series capillary pump (Agilent Technologies, Wilmington, DE), connected to an Agilent 35900E A/D interface, a Rheodyne MXP7980-000 injector (Chrom Tech Inc., Apple Valley, MN) equipped with 8 nL injection loop (injection time 0.1 sec), and a Spectra System UV 2000 detector (Thermo Separation Products) equipped with a 50 μ m I.D. UV transparent capillary as the detection cell. Fourier transform infrared spectroscopy (FT-IR) was carried out using a 100 Series FT-IR spectrometer (Perkin Elmer) equipped with attenuated total reflectance (ATR). For this purpose monoliths were prepared in 4 mL vials at 80 °C, washed several times with ethanol to remove unreacted components, and dried in the vacuum oven to eliminate residual solvent and moisture. Liquid samples were analyzed without any processing.

2.4 Chromatographic conditions

For separation of alkylbenzenes under isocratic conditions an aqueous acetonitrile solution ranging from 35 to 60% was employed as a mobile phase. The solution of a mixture of alkylbenzenes consisted of uracil (0.7 mg/mL), benzene, toluene, ethylbenzene, propylbenzene, butylbenzene, pentylbenzene, and hexylbenzene (each 20 μ L/mL in 80% aqueous acetonitrile). Analytes were eluted at a flow rate of 1 μ L/min and were detected at a wavelength of 214 nm. Each injection was repeated 5 times. For evaluation of the presence of free silanols on the pore surface in the monoliths, separation of a mixture of uracil (0.5 mg/mL), caffeine (3.3 mg/mL), phloroglucinol (3.2 mg/mL), resorcinol (2.9 mg/mL), and phenol (3.0 mg/mL) in 20% aqueous acetonitrile was carried out at a flow rate of 1 μ L/min, and detection wavelength of 214 nm. For evaluation of the steric selectivity, a mixture of thiourea (1.2 mg/mL), *o*-terphenyl (3.6 mg/mL), and triphenylene (0.4 mg/mL) in a mobile phase consisting of 70% aqueous acetonitrile was separated at a flow rate of 1.5 μ L/min and detected at a wavelength of 254 nm.

3. Results and Discussion

3.1 Acid catalyzed hydrolysis/polycondensation and free radical polymerization of MPTMS

Our initial work aimed at rapid preparation of photopolymerized sol-gel hybrid organo-silica monoliths to obtain separation media for applications in reversed-phase capillary liquid chromatography [18]. This study expands the early effort aiming at tuning preparation conditions towards optimized separation performance of thermally polymerized sol-gel monoliths, and further comparison of chromatographic properties of both types of columns.

Organo-silica hybrid monoliths were prepared using 3-(methacryloyloxy)propyltrimethoxysilane as the single monomer, hydrochloric acid as an aqueous catalyst, toluene as a porogen, and 2,2'-azobisisobutyronitrile as commonly available initiator. The MPTMS monomer used as the starting material contains both inorganic and organic functionality, thus enabling, under specific reaction conditions, preparation of hybrid monoliths containing siloxane and carbon-carbon chains. It is known that in the presence of an aqueous acid the methoxy groups of the methoxysilane (Si-OCH₃) undergo simultaneous hydrolysis and polycondensation leading to a

formation of silanols (Si-OH) and siloxane chains (Si-O-Si). Simultaneously, methacrylate functionalities undergo free radical polymerization reaction initiated by decomposition of AIBN using heating or exposure to UV light.

Our preliminary experiments included analysis of monoliths prepared under various conditions using Fourier transform infrared spectroscopy (FT-IR). Acquired spectra aided in structure characterization (Fig. 1) by providing information about functional groups present in the monoliths.

The IR spectrum of the starting material MPTMS (Fig. 2A) confirms the presence of methacrylate functionality (C=C stretch and =CH₂ twisting at 1638 and 1296 cm⁻¹, C=O stretch at 1717 cm⁻¹) and the presence of methoxysilane (asymmetric and symmetric C-H stretching in -OCH₃ at 2944, 2841 cm⁻¹ and Si-O stretch at 1078 cm⁻¹).

Using aqueous hydrochloric acid catalyst alone in the absence of an AIBN initiator, a transparent liquid became highly viscous within 4 h of heating as a result of siloxane polymerization. However, formation of monolith was not observed even after 96 h. Similar results were observed by Bersani *et al.* [21]. The IR spectrum in Fig. 2B confirmed that hydrolysis and polycondensation of methoxysilane functionalities took place resulting in formation of silanols (-OH at 3400 cm⁻¹) and siloxane chains (Si-O-Si, 1000-1100 cm⁻¹). Evidently, polymerization of methacrylate functionalities did not occur as vinylidene signals originating from the MPTMS monomer were still present.

A similar trend was observed while using an AIBN initiator in the absence of the acid catalyst. The IR spectrum of the resulting transparent liquid (Fig. 2C) confirmed the presence of methoxysilane originating from the MPTMS and the absence of C=C signals, confirming that the free radical polymerization reaction of methacrylate double bonds occurred without hydrolysis of methoxy groups.

In the presence of both acid catalyst and initiator, solid white opaque monolith formed within 30 min of heating at 80 °C. Signals originating from free silanols, siloxane linkages, and methylene functionalities can be observed in the IR spectrum (Fig. 2D). This result demonstrates that the simultaneous presence of aqueous hydrochloric acid and 2,2'-azobisisobutyronitrile is needed for formation of porous monoliths. The aqueous HCl catalyzes inorganic sol-gel transition while the azo initiator is instrumental for free radical polymerization of double bonds.

Photoinitiated polymerization resulted in a transparent monolith that was produced in 10 min of irradiation at 365 nm. This monolith turned white after 2 h. This confirms that photoinitiation is much faster compared to thermally initiated polymerization of methacrylate functionalities, and that hydrolysis and condensation of alkoxysilane is slower than the polymerization reaction. In contrast, polycondensation of methoxysilanes is much faster than thermally initiated polymerization of methacrylates as we conclude from evaluation of chromatographic performance (*vide infra*).

3.2 Effect of preparation conditions on column performance

Retention factor and column efficiency were used to evaluate the effect of reaction variables on the chromatographic performance of the monolithic capillary columns. Elution times and peak

widths at half height for unretained thiourea and retained benzene were measured at a flow rate of 0.5 $\mu\text{L}/\text{min}$ using 50% aqueous acetonitrile as the mobile phase. The first variable whose effect we investigated was the amount of porogen toluene in the reaction mixture ranging from 80 to 60% v/v. As expected, a decrease in the percentage of toluene and therefore an increase in the percentage of MPTMS monomer in the reaction mixture resulted in higher column efficiency for benzene and its longer retention time since more polymer was available in the column for the interaction with this analyte. TSG monolithic capillaries prepared with 60% v/v of toluene were impermeable. Those made in the presence of 65% v/v toluene were permeable but required high pressure to achieve flow through. The best result was obtained with a mixture containing 70% toluene. A similar trend was observed for PSG monoliths [16, 18]. However, a higher percentage of MPTMS (35% v/v) in the reaction mixture was needed to achieve retention factor for benzene similar to that observed for a TSG column.

The concentration of hydrochloric acid was varied in a range of 0.12-1.00 mol/L. Reaction mixtures containing 0.12, 0.15 and 0.20 mol/L HCl were transparent. In contrast, formation of cloudy solutions, which separated in two layers upon standing due to immiscibility of toluene and silanol rich aqueous layer, was observed at higher concentrations (0.40, 0.60 and 1.00 mol/L). White opaque monoliths were produced upon heating to 80 $^{\circ}\text{C}$ that exhibited improved mechanical strength with increasing HCl concentration. An increase in HCl concentration from 0.12 to 0.20 mol/L did not result in a significant effect on performance defined in terms of the retention factor of benzene. However, higher back pressure was observed in columns prepared with 0.20 mol/L HCl, and capillary columns prepared using a HCl concentration of 0.40 mol/L or more were impermeable since the faster rate of sol-gel transition compared to phase separation did not allow pore formation.

Temperature is known to accelerate both sol-gel transition and decomposition of AIBN. The latter leads to formation of a higher number of free radicals that increase the rate of initiation of the free radical polymerization. The fast polymerization at 100 $^{\circ}\text{C}$ was completed in 10 min but resulted in the formation of impermeable monoliths. Slightly higher retention of benzene was observed after 3 h reaction at 80 $^{\circ}\text{C}$ compared to that carried out at 65 $^{\circ}\text{C}$. Consequently, 80 $^{\circ}\text{C}$ was selected as optimal temperature.

We also varied polymerization time. The progression of conversion of MPTMS to the monolith formation was carried out in vials. Monoliths formed at different times were washed with toluene, and dried in oven. The conversion percent was calculated using the mass of monolith to mass of the MPTMS in monomer solution. Near 100% conversion was achieved in about 1 h. This does mean that at least one of the polymerizable functionalities, i.e. methoxysilane or methacrylate, reacted. Thus, the complete conversion only confirms that all MPTMS monomer units were included in the polymer. However, this inclusion can be achieved via only one type of functionality. This monolith then contains a multiplicity of functionalities that can continue reacting without observing any increase in the weight of the monolith. However, this process changes porous properties and polarity of the pore surface.

Continuation of the reactions is confirmed by an increase in retention factor of benzene observed for columns using significantly longer polymerization times as shown in Fig. 3. Clearly, reaction time must be extended considerably beyond one hour to produce monolithic columns with the desired separation performance.

3.3 Chromatographic performance: comparison of TSG and PSG monoliths

3.3.1 Effect of mobile phase composition, methylene selectivity, repeatability

The evaluation of chromatographic performance of monolithic columns prepared under optimized polymerization conditions was carried out using the standard mixture of a homologous series of n-alkylbenzenes. Solvent-strength selectivity is a useful tool for determining adequate retention and resolution. Plotting the logarithm of retention factor for each analyte versus volume percent of acetonitrile ranging from 35 to 60% revealed that 35-40% and 45-50% are the most suitable mobile phases (determined as $\log k$ in the range 0-1) for separation of mixture of alkylbenzenes for PSG and TSG column, respectively (plots not shown). Chromatograms in Fig. 4 confirm the separation follows the order of increasing hydrophobicity with hexylbenzene retained most. As previously mentioned, the retention factor of benzene calculated for PSG column was smaller than that for TSG when the capillary columns were prepared from mixtures of similar composition. Consequently, faster elution, considering the same mobile phase composition, flow rate, and capillary length, was achieved using PSG column. This can be attributed to the presence of fewer hydrocarbon functionalities at the pore surface due to faster free radical polymerization reaction of methacrylate groups under photopolymerization conditions in comparison to hydrolysis and polycondensation of methoxysilanes that forms a layer on the top of the polymerized chains.

The plots of $\log k$ versus the number of methylene groups in the alkyl chain shown in Fig. 5 were linear with the average correlation factors (R^2) for each mobile phase of 0.9996 ± 0.0003 . The nominal value of methylene selectivity (α_{CH_2}), calculated from the slope at varying compositions of mobile phase, decreased as the strength of the mobile phase increased confirming the reverse phase separation mechanism. In comparison to other monolithic columns, such as typical C18 modified silica (1.39 ± 0.01 in 80% ACN) [22], our hybrid columns exhibited smaller methylene selectivity (1.20 ± 0.002 for PSG and 1.28 ± 0.002 for TSG in 50% ACN). This suggests that the surface of the hybrid monolith is decorated with hydrophobic functionalities but it is not as hydrophobic as the C18 modified silica monolith.

The relative standard deviation of the retention factor for all alkylbenzenes using 50% aqueous acetonitrile was in the range of 0.8 – 3.2% for five consecutive runs. The relative standard deviation for the retention factor of benzene was 4.4% when calculated for 266 consecutive runs. This data confirms good run-to-run repeatability. An improvement in batch-to-batch repeatability was also observed for columns prepared using thermal initiation compared to photoinitiation with relative standard deviation for retention factor of benzene at 3.7% for TSG capillaries (n=42) and 6.6% for PSG capillaries (n=18). A similar trend was observed for columns prepared within the same batch.

Van Deemter plots presented for PSG and TSG columns in Fig. 6 are almost identical and similar minimum plate height was observed at the optimal mobile phase velocity confirming their similar column efficiencies. The TSG capillary columns (plots not shown) exhibited the minimum plate height of 9.0 μm representing a quite good efficiency of 111 000 plates/m for alkylbenzenes with shorter alkyls at a flow velocity of 0.27 mm/s. Greater dispersion of curves observed at increasing flow velocity can be attributed to the larger effect of mass transfer for larger molecules (C-term).

3.3.2 Silanol effect and steric selectivity

The starting material 3-(methacryloyloxy)propyltrimethoxysilane contains inorganic as well as organic groups. Accordingly, the IR spectrum of the hybrid monolith confirms the simultaneous presence of signals originating from inorganic (silanols) and organic (hydrocarbon) functionalities. The ability to retain and separate alkylbenzenes attested to the hydrophobic nature of the surface. The existence of polar silanols on the pore surface is demonstrated by the separation of a mixture of polar analytes with varying partition coefficient (P). Fig. 7 shows that the compounds are eluting according to their $\log P$ values with the least polar phenol (the highest $\log P$) retained most. In the case of a thermally-polymerized monolith, baseline separation of caffeine and phloroglucinol was not achieved until the amount of acetonitrile in the mobile phase was only 5%. The caffeine/phenol selectivity ($\alpha_{C/P}$) which is commonly utilized [23] to assess the extent of the hydrogen bonding interaction of analytes with the stationary phase was measured in 20% aqueous acetonitrile. A slightly higher value of 0.17 ± 0.003 was observed in case of photopolymerized hybrid monolith compared to 0.13 ± 0.001 for its thermally polymerized counterpart. This trend confirms that hydrolysis and condensation of methoxysilane units is slower under photoinitiated conditions, resulting in the larger number of free silanols located on the pore surface of the PSG monolith.

Triphenylene and *o*-terphenyl, i.e. compounds of similar hydrophobicity and size but differing in planarity, were used for evaluation of steric selectivity of the stationary phase. Separation of both probes using 70% aqueous acetonitrile shown in Fig. 8 demonstrates that the planar triphenylene is more retained compared to the nonplanar *o*-terphenyl. Significantly better column efficiency and smaller retention factors are observed for both compounds using PSG column. The extent of steric selectivity was expressed as the ratio of separation factors of triphenylene and *o*-terphenyl ($\alpha_{T/O}$) [23]. For both, TSG and PSG monolith prepared under optimized conditions $\alpha_{T/O}$ was similar (1.70 ± 0.01 , $n=10$, TSG and 1.72 ± 0.03 , $n=5$, PSG). This indicates that both columns discriminate planar and nonplanar analytes to the same extent.

4. Conclusions

This study demonstrates that highly efficient hybrid monolithic columns can be prepared in a single step via simultaneous sol-gel transition and free radical polymerization of 3-(methacryloyloxy)propyltrimethoxysilane. We optimized conditions including the amount of porogen, polymerization time, temperature, and concentration of aqueous catalyst for the preparation of thermally polymerized organo-silica hybrid monolithic columns. Excellent column performance characterized by efficiencies exceeding 110,000 plates/m was achieved using prolonged polymerization time, and lower amount of porogen in the reaction mixture. Promising results were achieved thanks to the shift from originally developed photopolymerization to thermally initiated polymerization. Thermally initiated monolithic sol-gel columns exhibited better batch-to-batch repeatability and their pore-surface was decorated with additional hydrophobic hydrocarbon chains while the number of hydrophilic silanols was reduced. This information is useful when considering future approaches to surface modifications using bonding with organosilane reagents or grafting with organic monomers.

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Figure 1. Structures of 3-(methacryloyloxy)propyltrimethoxysilane (A) and polymer formed at 80 °C in the absence of 2,2'-azobisisobutyronitrile initiator (B), absence of aqueous HCl catalyst (C), simultaneous presence of initiator and catalyst (D).

Figure 2. Infrared spectrum of 3-(methacryloyloxy)propyltrimethoxysilane (A), polymer formed at 80 °C in the absence of 2,2'-azobisisobutyronitrile initiator (B), absence of aqueous HCl catalyst (C), simultaneous presence of initiator and catalyst (D).

Figure 3. Effect of polymerization time on retention factor of benzene. Numerical values displayed correspond to average values and numbers in parentheses represent number of monolithic columns tested. Chromatographic conditions: mobile phase 50% aqueous acetonitrile, flow rate 0.5 µL/min, detection wavelength 214 nm; Preparation conditions: 28% v/v 33-(methacryloyloxy)propyltrimethoxysilane, 0.15 mol/L HCl, 5% w/v 2,2'-azobisisobutyronitrile in toluene/monomer solution, 80 °C.

Figure 4. Effect of percentage of acetonitrile in the mobile phase on separation of alkylbenzenes using PSG (A data from ref. [18]) and TSG (B) monolithic columns. Chromatographic conditions: mobile phase 50% aqueous acetonitrile, flow rate 1 µL/min, detection wavelength 214 nm, analytes in order of elution: uracil, benzene, toluene, ethylbenzene, propylbenzene, butylbenzene, amylbenzene, hexylbenzene.

Figure 5. Effect of the number of methylene groups in the alkyl chain of alkylbenzenes on their retention expressed as logarithm of retention factor ($\log k$) at different percentages of acetonitrile in the mobile phase. (A) PSG (taken from ref. [18]) (B) TSG column. For conditions see Fig. 4.

Figure 6. Van Deemter plots demonstrating effect of linear mobile phase flow velocity on column efficiency expressed as height of theoretical plates (HETP) of benzene using PSG and TSG columns. Conditions: mobile phase 50% aqueous acetonitrile, detection wavelength 214 nm.

Figure 7. Effect of silanol functionalities. (A) PSG (taken from ref. [18]) (B) TSG column. Conditions: mobile phase 20% aqueous acetonitrile, flow rate 1.0 µL/min, detection wavelength 214 nm, analytes: uracil (1), caffeine (2), phloroglucinol (3), resorcinol (4), phenol (5).

Figure 8. Demonstration of steric selectivity of the monolithic stationary phase. (A) PSG (taken from ref. [18]) (B) TSG column. Conditions: mobile phase 70% aqueous acetonitrile, flow rate 1.5 µL/min, detection wavelength 254 nm, analytes: thiourea (1), *o*-terphenyl (2), triphenylene (3).

FIGURE 1

FIGURE 2

FIGURE 3

FIGURE 4A

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FIGURE 4B

FIGURE 5A

FIGURE 5B

FIGURE 6

FIGURE 7A

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FIGURE 7B

FIGURE 8A

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FIGURE 8B