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Development of a capillary electrophoretic separation of an *N*-(substituted)-glycine-peptoid combinatorial mixture

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Abstract

Capillary electrophoresis was used for the separation of a combinatorially synthesized *N*-(substituted)-glycine (NSG) peptoid mixture. This mixture consisted of 24 trimeric compounds sharing a common backbone structure but differing in the side chain attached at the *N*-terminal residue. Standards of the individual components were unavailable so that development of the separation was based on the mixture. A variety of buffer additives were investigated to enhance the CE resolution of this diverse mixture. Ion-pairing agents, cyclodextrins and organic modifiers were all evaluated as buffer additives. The best separations were achieved using a combination of buffer additives, each serving a different purpose in the separation. Heptane sulphonic acid (HSA) was used to reduce hydrophobic intramolecular interactions. Methyl-β-cyclodextrin was used to provide host–guest interactions in order to resolve the very hydrophobic components of the NSG-peptoid mixture. The optimized run buffer consisted of 250 m*M* sodium phosphate buffer, pH 2.0, with 25 m*M* HSA and 40 mg/ml BCD and resulted in the resolution of 21 peaks for the 24 peptoids in the combinatorial mixture.

Keywords: Peptoid combinatorial mixture; Glycine

1. Introduction

The use of combinatorial chemistry methodologies is a rapidly emerging area in the biopharmaceutical industry. This novel approach is capable of synthesizing vast numbers of very diverse compounds for evaluation as drug entities [1]. Compounds are synthesized by robotic devices capable of generating thousands of compounds daily [2]. Due to this massive increase in the rate of compounds synthesized, there is a resulting increase in the demand to rapidly analyze and characterize these libraries.

One example of combinatorial chemistry are the oligo *N*-(substituted)-glycine (NSG) peptoids. NSG-peptoids are synthetic oligomers with the peptide backbone structure. They differ from peptides in that functional groups are attached at the amide nitrogen instead of the α-carbon. An important feature of these peptoids is their resistance to cleavage by proteases which is a major advantage for their potential bioavailability [3,4]. Several

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potent ligands to therapeutic targets have been discovered from a library containing approximately 5000 synthetic NSG-peptoids [5].

The utility of capillary electrophoresis (CE) for the analysis of combinatorially synthesized mixtures was evaluated by developing a CE separation of a representative mixture of NSG-peptoids prepared by combinatorial synthesis. The mixture investigated was CHIR 4580; a mixture containing 24 physicochemically diverse compounds that range in molecular mass from 392–699 with calculated pK_a values ranging from 3.4 to 10.11, calculated pK_b values ranging from 7.6 to 9.8 and log *D* ranging from −2.5 to 4.5. The components of this mixture differ by the side chain at the *N*-terminal position with this side chain imparting diverse physicochemical properties to the peptoid defined by functional group, topology indices, receptor recognition groups and lipophilicity [6]. The general structure of the NSG-peptoids and the range of side chain groups is shown in Fig. 1. With reversed-phase high-performance liquid chromatography (HPLC), separation of physicochemically diverse mixtures presents a significant challenge. The separation in CE is based on differences in charge and size and provides significantly higher efficiencies than typical with HPLC. In addition, the sample requirement is considerably smaller for CE relative to HPLC. CE was therefore thought to provide advantages for the analysis of peptoid based combinatorial mixtures.

The diversity of the peptoid mixture necessitated the use of background electrolyte (BGE) additives to enhance the range of compounds separated by CE. Terabe and coworkers [7,8] first introduced the idea of using BGE additives to enhance CE separations of hydrophobic and neutral molecules. Organic modifiers and ion-pairing agents have been used to enhance the separation of hydrophobic molecules including peptides [9–11]. Cyclodextrins (CDs) have become extremely popular for the separation of chiral molecules [12–16], but have also been used to enhance the separation of achiral compounds such as basic drugs [17] and polyaromatic hydrocarbons in soil samples [18]. In this report, a separation of the peptoid mixture was developed using multiple BGE additives to enhance resolution.

2. Experimental

2.1. Chemicals

CHIR 4580 was synthesized at Chiron (Emeryville, CA, USA) [5] as previously described and supplied as a solution of 10 mg/ml in ethanol. This stock was further diluted ten-fold with NANOpure water. Methyl-β-CD (average degree of substitution=1.8) was obtained from Aldrich (Milwaukee, WI, USA) and 1-heptane sulphonic acid (HSA) was obtained from Sigma (St. Louis, MO, USA). Orthophosphoric acid (85%), ammonium acetate, sodium borate, sodium phosphate (monobasic), sodium phosphate (dibasic) and all organic solvents were obtained from Fisher (Fair Lawn, NJ, USA). All chemicals were reagent grade or better and used as received.

2.2. Capillary electrophoresis system

The CE apparatus was laboratory-made using a Spellman high-voltage d.c. (0–30 kV) CE 1000R power supply (Spellman High Voltage Electronics, Plainview, NJ, USA). The anodic (high voltage) end of the capillary was isolated in a plexiglass box fitted with a safety interlock device. A 50-μm fused-silica capillary from Polymicro Technologies (Tuscon, AZ, USA) with a total length of 60 cm and a 40 cm length to the detection window was used for separation of the mixture. A voltage of 15 kV was used for all experiments. The capillary was first flushed with 0.1 *M* sodium hydroxide for 20 min followed by conditioning of the capillary with the BGE for a further 30–45 min prior to use. The peptoid mixture was hydrodynamically injected into the capillary by applying a pressure of 17 kPa for 5–8 s which injects a volume of 2 to 5 nl. The injection volume was determined by determining the time to fill the separation capillary to the detector. Detection was performed at 210 nm using an ISCO CV^4 CE UV absorbance detector connected to a Hewlett-Packard 3396 Series II Integrator for data acquisition.

2.3. Preparation of buffer solutions

Sodium phosphate buffer, pH 2.0, was prepared by diluting the appropriate volume of concentrated

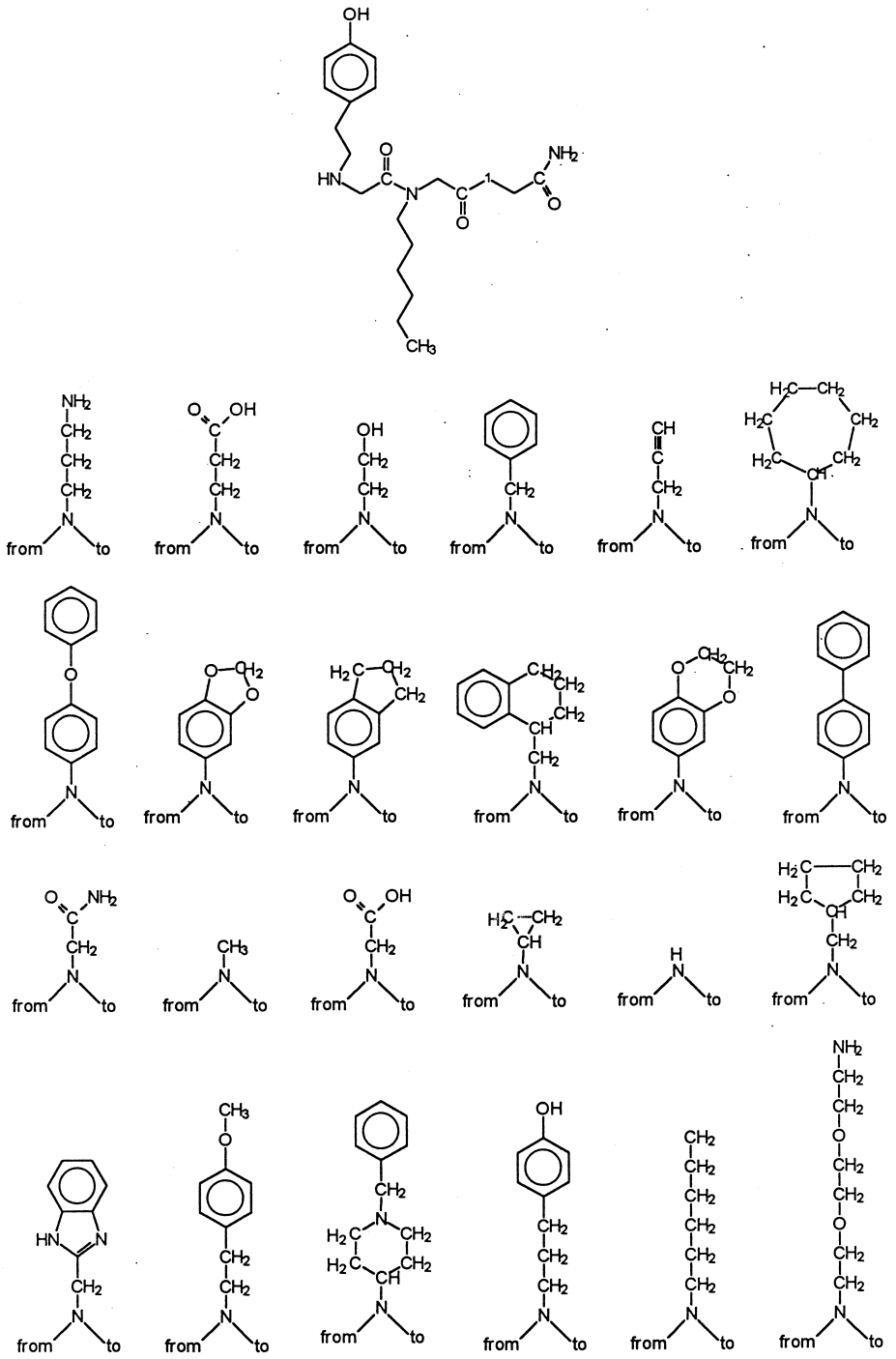

Fig. 1. Chemical structure of the NSG-peptoid mixture CHIR 4580.1 and the 24 side chains which are attached at the position denoted by the number 1.

phosphoric acid to 250 m*M* and adjusting the pH with 0.1 *M* sodium hydroxide. The pH was monitored daily and adjusted as needed with 0.1 *M* sodium hydroxide. Phosphate buffer with ion-pair reagent was prepared by dissolving the appropriate amount of HSA in 250 m*M* sodium phosphate buffer. The solution was sonicated for 30 min to insure complete dissolution of the HSA and the pH then adjusted to 2.0 with 0.1 *M* sodium hydroxide. Sodium phosphate buffer with CD was prepared by dissolving the appropriate amount of methyl-β-CD in 250 m*M* sodium phosphate buffer and the pH adjusted to 2.0 with 0.1 *M* sodium hydroxide. All solutions were filtered through a 0.2-μm nylon Acrodisc 13-mm syringe filter (Fisher) prior to use.

2.4. Physicochemical characterization

Predictions of apparent 1-octanol–buffer distribution coefficient (log $D_{o/b}$) and pK_a were performed using PROLOGD v2.0 and PKALC v3.1 software, respectively from CompuDrug (Budapest, Hungary).

3. Results and discussion

3.1. Effect of pH

The net charge on a peptoid molecule can be manipulated by modification of the BGE pH, particularly near the pK_a values of the amino terminus or side chain. The test peptoid mixture investigated, CHIR 4580, had a predicted pK_a range from 3.4 to 10.1 and a predicted pK_b range from 7.6 to 9.8 [6]. The BGE pH also effects the electroosmotic flow (EOF) with larger flow at higher pH. BGE pH over the range of 2 to 10 was investigated (Fig. 2). At pH greater than 11 the peptoid mixture has an overall negative charge and electrophoretic migration towards the anode. However, the very fast EOF at high pH overwhelms the electrophoretic migration of the peptoids resulting in short elution times and poor separation. Using a borate buffer at pH 10, most of the peptoids eluted as a single broad peak at 10 min. Over the pH range of 4 to 8 the NSG-peptoids are zwitterions with minimal net charge. Electrophoresis

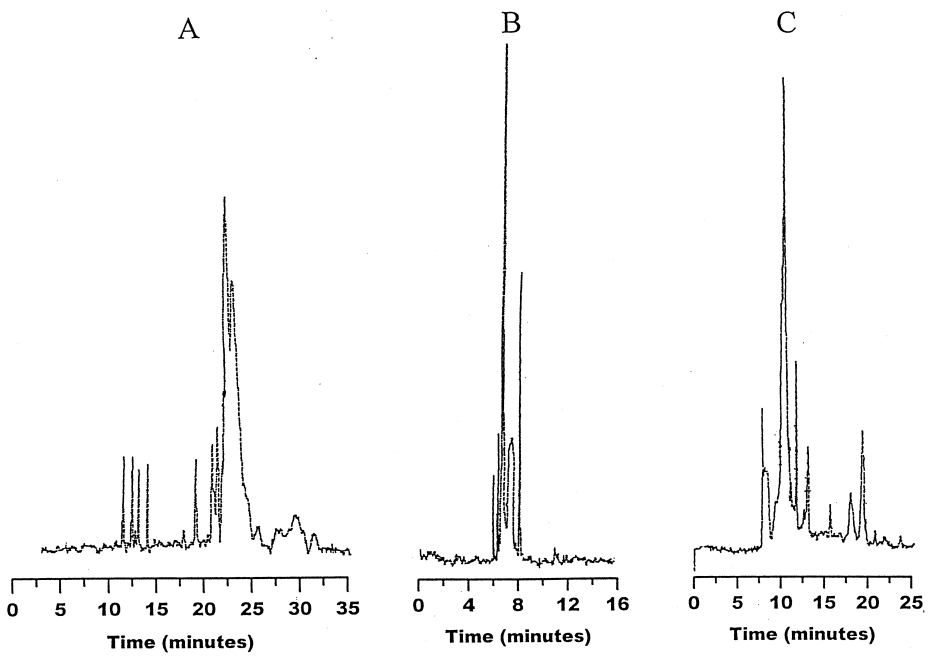

Fig. 2. Effect of pH on the separation of NSG-peptoid mixture CHIR 4580.1. The BGEs were: (A) 100 m*M* sodium phosphate buffer, pH 2.0; (B) 100 m*M* sodium phosphate buffer, pH 7.0; (C) 100 m*M* sodium borate buffer, pH 10.

is not suitable for separation of the peptoids in this pH range.

At pH less than 3 the peptoids have an overall positive charge and electrophoretic migration toward the cathode. While the EOF is also toward the cathode, at this pH the EOF is very slow resulting in relatively long times to achieve separation. Better resolution is achieved when the main transport process is electrophoresis rather than EOF. Sodium phosphate buffer at both pH 2 and 3 resulted in good separation of the test mixture. A pH of 2.0 was selected for this method to insure protonation for a range of peptoid libraries.

3.2. Effect of ionic strength

Increasing the ionic strength of the BGE improved resolution by decreasing the EOF. This effect can be seen in Fig. 3 where various concentrations of sodium phosphate buffer, all at pH 2.0 were used. The upper limit in usable ionic strength was determined by Joule heating. Higher ionic strength BGEs have higher conductivities and therefore higher electrophoretic currents. With this system, increasing the BGE concentration from 150 m*M* to 250 m*M* sodium phosphate buffer, pH 2.0, doubled the electrophoretic current. The optimum ionic strength was 250 m*M* for maximum efficiency while not inducing significant Joule heating problems. Using a BGE of 250 m*M* sodium phosphate buffer, pH 2.0, 14 individual peaks were identified from the test mixture containing 24 compounds (Fig. 3). Because of insufficient resolution of this complex combinatorial mixture, BGE additives were investigated to increase the resolving power of CE. Organic solvents, ion-pairing agents and CDs were investigated as suitable BGE additives for the resolution of this peptoid mixture.

3.3. Effect of ion-pair agents

Recently, ion-pairing agents, such as HSA, have been used in CE analysis for the separation of hydrophobic molecules such as proteins and peptides [9,10]. McLaughlin et al. [10] have reported that in CE the ion-pair agent can form a hydrophobic

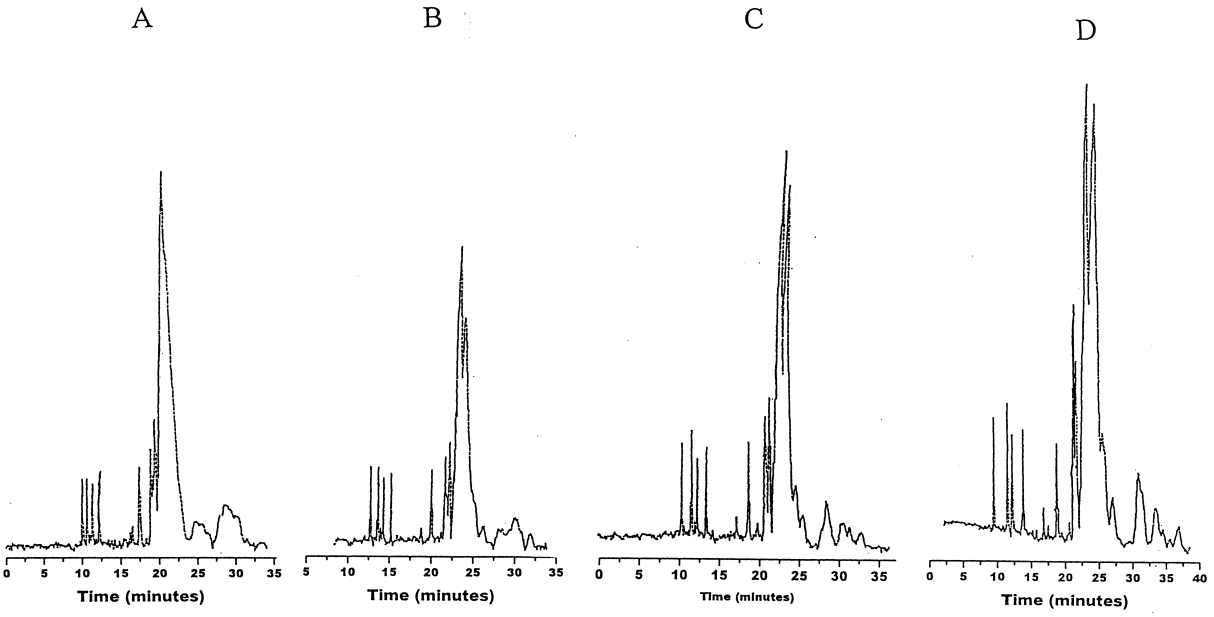

Fig. 3. Effect of ionic strength on the separation of NSG-peptoid mixture CHIR 4580.1. (A) 50 m*M* sodium phosphate buffer, pH 2.0; (B) 100 m*M* sodium phosphate buffer, pH 2; (C) 150 m*M* sodium phosphate buffer, pH 2.0; (D) 250 m*M* sodium phosphate buffer, pH 2.0.

interaction between its alkyl chain and hydrophobic regions of the analyte molecules. This interaction is believed to decrease self-aggregation and eliminate interactions of the analytes with the silica wall. In addition, due to the sulphonic acid groups of the HSA, the HSA increases the electronegativity of the adduct which results in increased migration times. HSA can also form classic ion-pairs with positively charged peptoids, thus decreasing the positive charge of the adduct and decreasing any interactions with the negatively charged capillary wall. Which mechanism is more important is likely a function of the properties of the specific peptoid. The net result is a significant improvement in the electrophoretic separation.

Upon addition of 25 m*M* HSA, several more components of the combinatorial mixture were resolved when compared with the separation obtained with phosphate buffer alone (Figs. 3 and 4). The resolution of the large band of late eluting molecules was improved quite substantially. Increasing the concentration above 25 m*M* provided only a slight improvement in separation. Even at 75 m*M*, HSA alone was not sufficient to completely resolve the band of late eluting compounds. A consequence of increasing the HSA concentration was an increase in the electrophoretic current and, hence, the Joule heating. Increasing the concentration from 25 m*M* to 75 m*M*, increased the current from 30 μA to 78 μA. A 25 m*M* HSA concentration was chosen for further experiments to minimize Joule heating without significantly affecting resolution. This concentration of HSA resulted in 15 peaks for the 24 NSG-peptoids (Fig. 4).

3.4. Effect of cyclodextrin

CDs have been used extensively as CE BGE additives to add a partitioning component to the separation [12–18]. Because the poorly resolved peptoids were thought to be hydrophobic, the addition of methyl-β-CD was investigated to determine the effect on the separation of the peptoid mixture at acidic pH. Because it is neutral, methyl-β-CD migrates with the EOF while the protonated peptoids migrate electrophoretically towards the detector (cathodic end). Interaction of a peptoid with the methyl-β-CD will cause the peptoid to have a net slower migration rate. The greater the concentration of methyl-β-CD the slower the overall elution time of the peptoid mixture and the greater the resolution of the later eluting peaks. However, above 60 mg/ml no further increase in resolution was observed. Nishi et al. [13] have previously reported that resolution

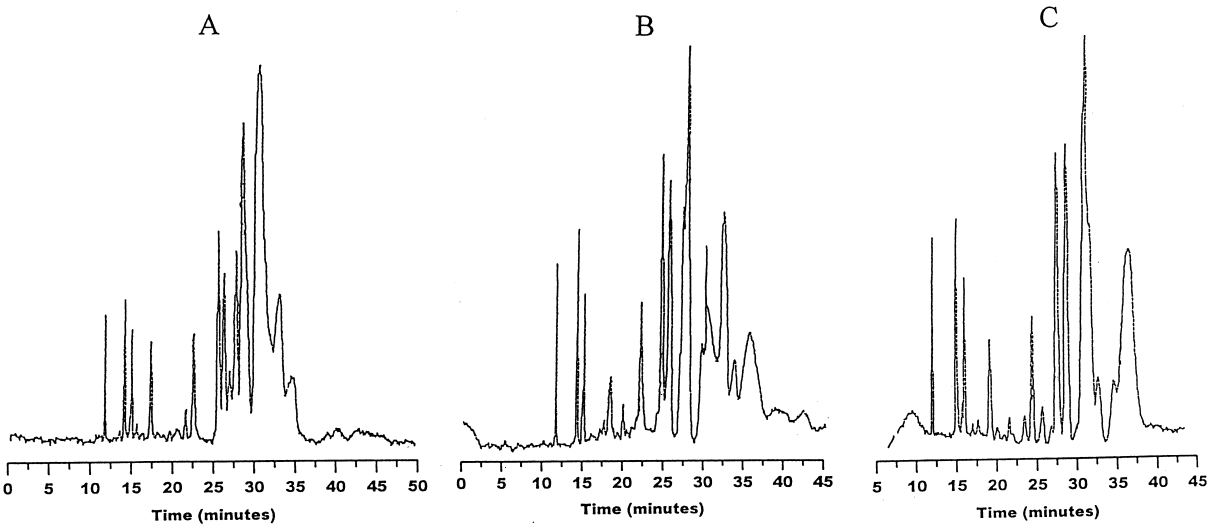

Fig. 4. Effect of the ion-pairing agent, 1-heptane sulphonic acid, on the separation of NSG-peptoid mixture CHIR 4580.1. All run buffers were 250 m*M* sodium phosphate buffer, pH 2.0. (A) 25 m*M* HSA; (B) 50 m*M* HSA; (C) 75 m*M* HSA.

deteriorates at high CD concentration because of decreased efficiency as a result of increased elution time. Using a BGE of 250 m*M* sodium phosphate buffer, pH 2.0, with 60 mg/ml methyl-β-CD the separation of the test peptoid mixture resulted in approximately 17 peaks for the 24 NSG-peptoids (Fig. 5).

3.5. Optimized separation

The greatest separation efficiency of the peptoid mixture was obtained when methyl-β-CD was employed in conjunction with HSA (Fig. 6). It is presumed that the role of the HSA was to solubilize the large hydrophobic molecules and reduce the self-aggregation of these peptoids, while the role of the methyl-β-CD was to add a partitioning mechanism to the separation. Hydrophilic peptoids remain separated by an electrophoretic mechanism as the early part of the electropherogram is similar under all separation conditions. The conditions which gave optimum resolution of this mixture were 250 m*M* sodium phosphate buffer, pH 2.0, with 25 m*M* HSA and 40 mg/ml methyl-β-CD. Using these optimized conditions 21 peaks were detected for a mixture of 24 peptoids (Fig. 6).

4. Conclusions

This report demonstrates the applicability of CE as a separation tool for NSG-peptoid combinatorial mixtures. A multiple BGE additive strategy provided enhanced selectivity over the additives used individually [9]. An ion-pairing agent, HSA, presumably reduced the propensity to form strong intramolecular interactions between the peptoids and disrupted electrostatic interactions with the capillary wall. Methyl-β-CD imparted a partitioning component to the separation to aid in the resolution of the more hydrophobic peptoids. The addition of organic solvents to the BGE electrolyte, alone and in conjunction with HSA, has been shown to enhance the separation of peptides [9–11]. In this study the use of both acetonitrile and methanol were investigated. Acetonitrile had no effect on the separation of the peptoid mixture. Addition of methanol altered the viscosity of the BGE resulting in slower EOF and longer migration times for all components of the

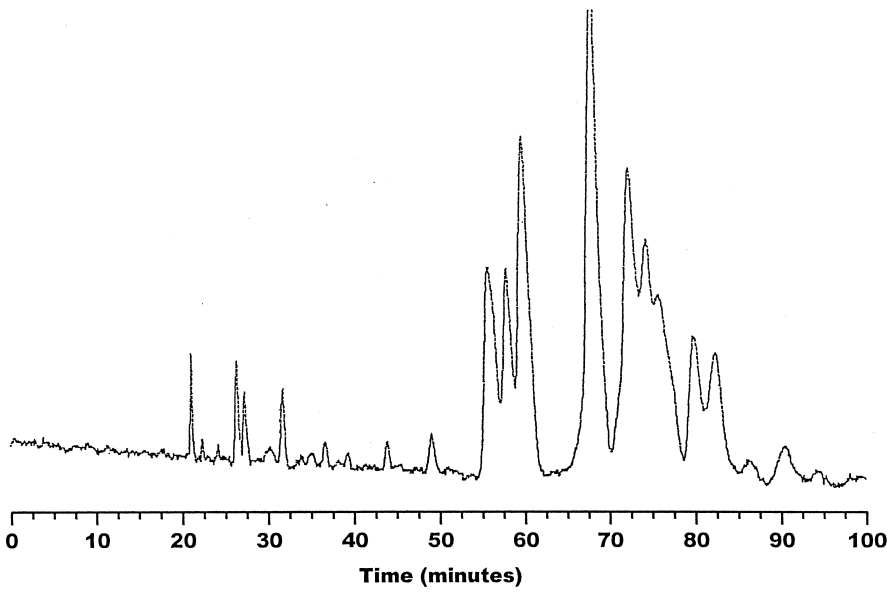

Fig. 5. Effect of CD host–guest interactions on the separation NSG-peptoid mixture CHIR 4580.1. Conditions: 250 m*M* sodium phosphate buffer, pH 2.0, with 60 mg/ml methyl-β-CD.

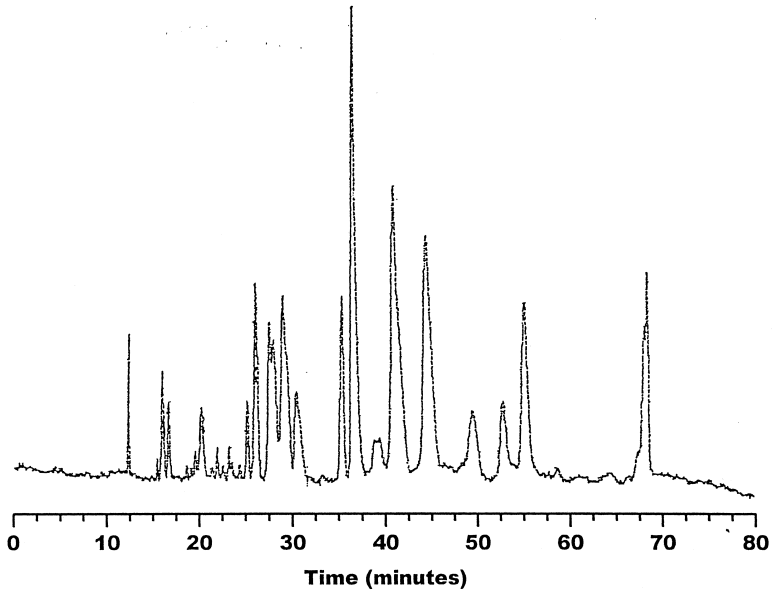

Fig. 6. Optimized separation of NSG-peptoid mixture CHIR 4580.1. Conditions: 250 m*M* sodium phosphate buffer, pH 2.0, with 25 m*M* HSA and 40 mg/ml methyl-β-CD; current=72 μA.

mixture. However, no significant change in resolution was observed using acetonitrile alone or in conjunction with HSA.

CE offers potential advantages over traditional HPLC for the analysis of complex peptoid mixtures. Only nanoliters of sample are injected for each analysis and as little as 5 μl of sample is needed for reproducible injection. This can be a tremendous advantage when extremely small amounts of sample are available as is typical with combinatorial chemistry. Separations are inherently based on charge-to-size ratios and partitioning can be incorporated providing greater flexibility in developing a separation. Methods, such as this, which provide rapid separation and analysis could be of significant utility in the growing field of combinatorial chemistry.

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