Convenient and Efficient Synthesis of a Lanthanide^{3+}-Coordinated, Diethylene Triamine Pentaacetic Acid Labeled Biopolymer as an Assay for the Cholecystokinin B Receptor

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ABSTRACT: To develop an assay for the cholecystokinin B receptor with an Eu^{3+}-labeled cholecystokinin peptide via a diethylene triamine pentaacetic acid chelating linker, a commercial dianhydride diethylene triamine pentaacetic acid precursor was directly attached to the N-terminus of cholecystokinin peptides by a solid-phase synthesis method with a satisfactory yield and purity after reverse-phase high-performance liquid chromatography separation. Lanthanide was then coordinated to the peptide via a diethylene triamine pentaacetic acid bifunctional agent. This method is a useful approach to the large-scale synthesis of lanthanide^{3+}-coordinated, diethylene triamine pentaacetic acid labeled biopolymers. This research provides not only a simple and convenient method for the preparation of lanthanide-based peptide ligand libraries but also possible lanthanide-based high-throughput screening of peptide receptors with a time-resolved fluorescence assay system. Five biopolymers were synthesized and characterized with high-resolution electrospray ionization in this study. © 2007 Wiley Periodicals, Inc. J Appl Polym Sci 106: 2683–2688, 2007

Key words: biopolymers; fluorescence; high performance liquid chromatography (HPLC); peptides; synthesis

INTRODUCTION

Two decades of peptide research have yielded a lot of information on the design, synthesis, and characterization of the structures of peptides.[1–4] Various polymer supports for the solid-phase synthesis of peptides have been studied.[5,6] The recent development of the incorporation of biopolymers with time-resolved fluorescence (TRF) for the imaging and therapy of tumors has become a stimulus for the design and synthesis of novel peptide polymers.[7–9] TRF assay systems are widely employed in high-throughput applications.[10–13] The quality of high-throughput screening is fairly related to the photophysical nature of the resolved compounds, that is, the contrast compounds, which play the key role of discriminating between normal and pathological tissues. Regular organic fluorescence dyes, such as fluorescein, rhodamine, and cyanine dyes, are chosen as contrast agents for high-throughput applications. However, their photophysical properties, short fluorescence lifetimes (1–20 ns), and lack of obvious Stokes redshifts limit their applications for TRF. Moreover, the contrast agents may show some autoluminescence and thereby falsify the results of biological measurements. Because of the short fluorescence lifetime of the dyes, the background (i.e., noisy) fluorescence of the assay solution itself may significantly hurt the sensitivity and veracity of an assay format. In contrast, lanthanides, such as europium, terbium, samarium, and dysprosium, offer long fluorescence lifetimes (ms) and a large Stokes redshift; therefore, their fluorescence can be measured by a microsecond fluorescence lifetime technique.

Cholecystokinin (CCK) peptide acts as a neurotransmitter and neuromodulator in the central nervous system. The CCK peptide exists in various isoforms, including cholecystokinin 5 (CCK5) and cholecystokinin 8 (CCK8), which play important roles in the periphery and display a high affinity for CCK receptors cholecystokinin A-receptor (CCKA-R) and cholecystokinin B-receptor (CCKB-R), which belong to the G-protein coupled receptor superfamily and are localized in the cell membrane.[14–16] Both receptors are very promising targets for specific contrast agents because of their overexpression in many

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tumors. The ligand–receptor specific interactions hold significant promise for molecule-based drug discovery and development and have stimulated research for the imaging and therapy of tumors in the past few years.[17] The research of ligand–receptor interactions has been focused on the radiolabeled assay approach;[18–21] however, this has produced many problems concerning protection against and disposal of radio biohazard materials. In recent years, a high-throughput assay system has been established through a combination of dissociation enhanced lanthanide fluoroimmunoassay technology and TRF.[9–12]

A multistep synthesis of precursors to link diethylene triamine pentaacetic acid (DTPA) with a biopolymer has been achieved in a liquid phase,[22–26] but this limits the large-scale production of the target ligand. One of the objectives of this study was to establish a convenient and efficient way of adding DTPA to a CCK biopolymer with a commercial DTPA dianhydride through solid-phase synthesis to bind a europium atom as a substitute assay for the cholecystokinin B (CCKB) receptor by TRF.

EXPERIMENTAL

Materials

All organic solvents, europium(III) chloride, 1-hydroxybenzotriazole (HOBt), *O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, and dianhydride DTPA were purchased from Aldrich (Milwaukee, WI) and used as received without further purification. The rink resin and *N*-α-9-fluorenylmethyloxycarbonyl (N^{α}-Fmoc)/*tert*-butyl protecting amino acids were obtained from Novatis (East Hanovers, NJ).

Analysis of the biopolymer

The protecting groups of the rink resin were removed with piperidine in dimethylformamide. The N^{α}-Fmoc amino acid was coupled with preactivated HOBt ester. When the reaction was finished, the same procedure was repeated for the next amino acid until all the amino acids in the sequence were attached. After the final amino acid was incorporated, the Fmoc or allyloxycarbonyl group was deprotected, and the free amine group was acetylated with preformed HOBt ester of DTPA. The resin was washed with dimethylformamide, tetrahydrofuran, and CH_2Cl_2. A cleavage mixture consisting of trifluoroacetic acid (TFA), water, 1,2-ethandithiol, and thioanisole was injected into the resin and stirred for 3 h at room temperature. The product was precipitated with cold ether. The peptide pellets were washed three times with cold ether and were then lyophilized. The lyophilizate was dissolved in 0.5*M* ammonium carbonate (adjusted to pH 8.0) and was treated with 10 equiv of europium(III) chloride. The purification of the compounds was achieved with a Hewlett–Packard 1100 series high-performance liquid chromatography (HPLC, Wilmington, DE) instrument with a reverse-phase column (Vydac, Columbia, MD; 10 × 220 mm, 10 μm, 300 Å). Europium-labeled peptides were eluted with a linear acetonitrile/0.1*M* ammonium acetate gradient at a flow rate of 3.0 mL/min. Separations were monitored at 230 and 280 nm with a Hewlett–Packard 110 series UV detector and were integrated with a Hewlett–Packard 3396 series III integrator. The structures were characterized with electrospray ionization (ESI; Thermoquest LCQ ion-trap instrument, Finnigan, San Jose, CA) or matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF). The purity was detected with analytical HPLC (a Hewlett–Packard 1100 series HPLC instrument or a Waters Alliance instrument, Milford, MA) with a reverse-phase column (YMC, Yamamura Chemical Corp., Kyoto, Japan; 4.6 × 150 mm, 5 μm, and 120 Å). Condition A was a linear gradient from 10 to 40% B over 30 min (where A was 0.1% TFA and B was acetonitrile); condition B was a linear gradient from 17 to 67% B over 30 min (where A was 0.1*M* ammonium acetate in water and B was 0.1*M* ammonium acetate in 60% aqueous acetonitrile).

RESULTS AND DISCUSSION

All the biopolymers were synthesized by standard solid-phase technology with a manual synthesizer with N^{α}-Fmoc/*tert*-butyl chemistry. The DTPA

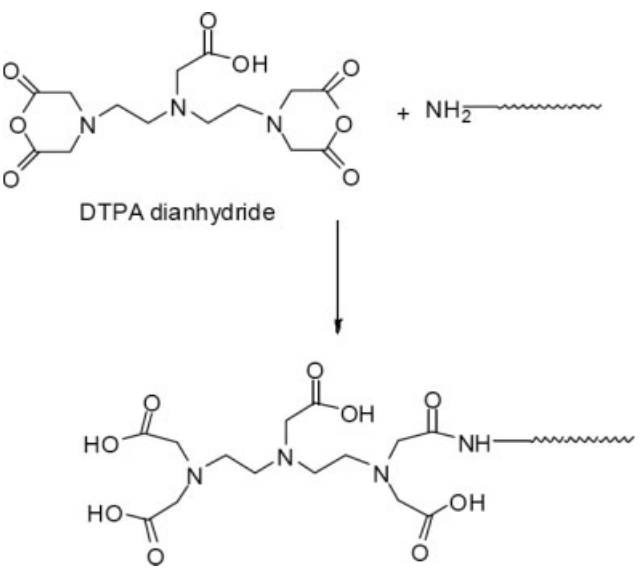

Figure 1 Ring-opening reaction of the DTPA dianhydride and free amine group.

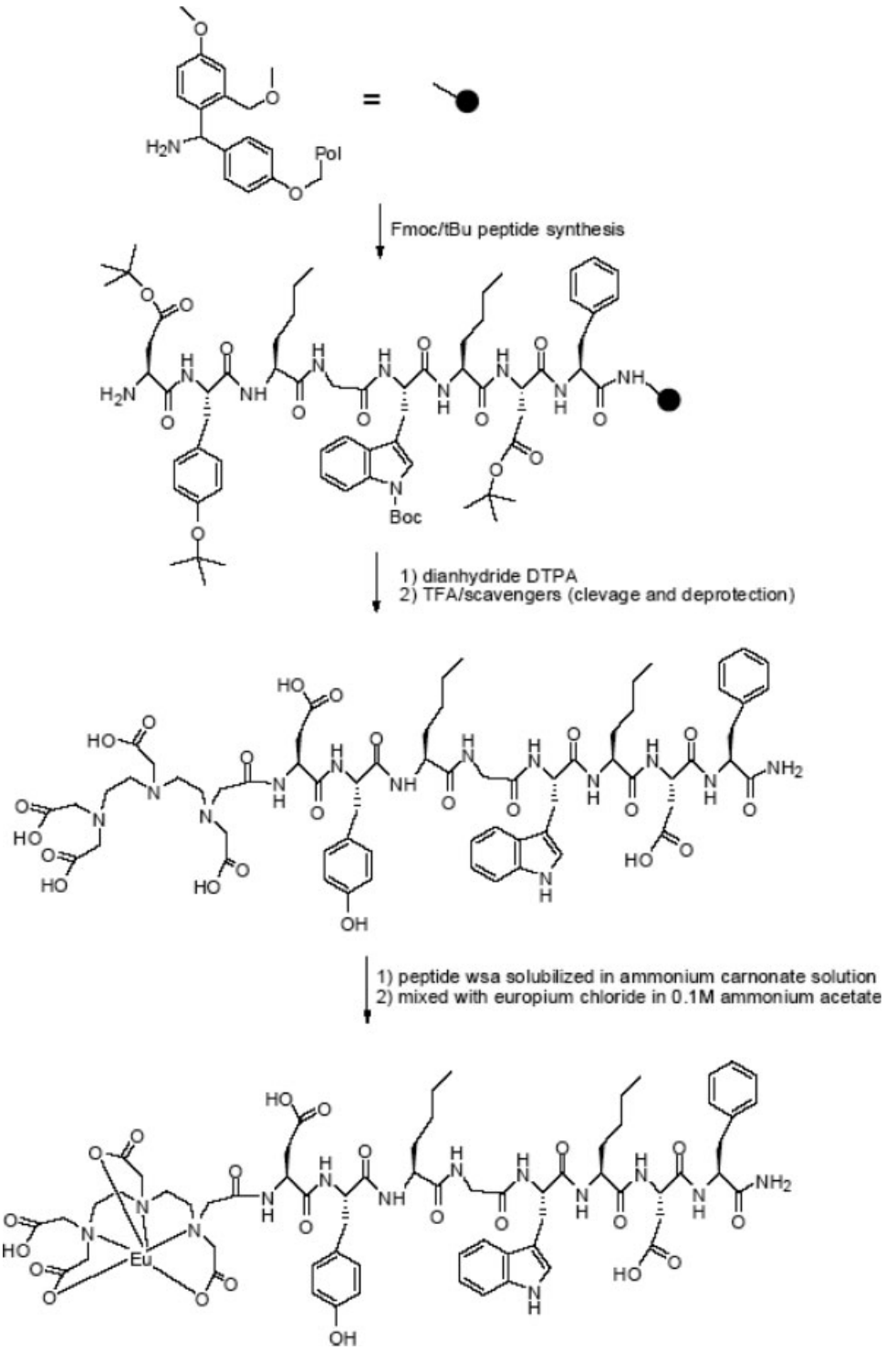

Figure 2 Solid-phase synthesis of the Eu–DTPA–CCK8 biopolymer.

chelator was attached to the N-terminus of the peptide resin through the opening of the ring of the DTPA dianhydride (see Fig. 1).

As shown in Figures 1 and 2, as the last amino acid sequence was coupled to the CCK biopolymers, the protecting group was removed with piperidine, and the free amine group then easily reacted with the DTPA anhydride via an open ring; DTPA–CCK biopolymers were then formed. Normally, a small yield of a di-DTPA–CCK byproduct was produced (the ratio of the byproduct to the main product was 5 : 95). The biopolymers were finally cleaved from the resin together with the protecting groups by the TFA mixture, as shown in Figure 2. The compounds

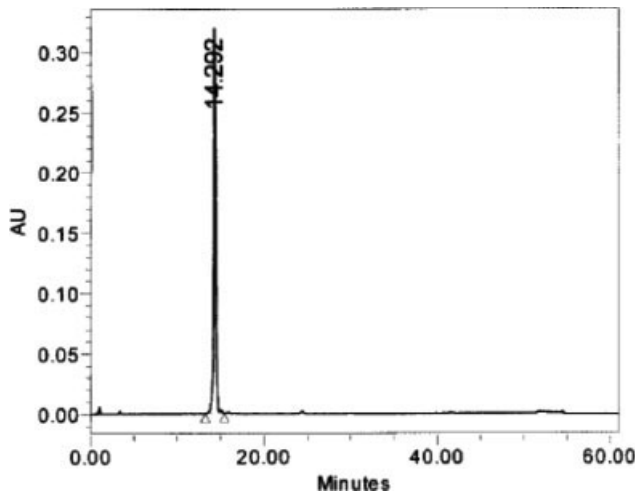

Figure 3 Typical HPLC spectrum of the biopolymer (DTPA–CCK8) after purification.

were purified by reverse-phase HPLC, and the purity was determined by thin-layer chromatography and analytical HPLC with a reverse-phase column. The structures were characterized with high-resolution ESI or MALDI-TOF. A typical HPLC spectrum of a DTPA–CCK biopolymer is shown in Figure 3.

The synthetic DTPA–CCK biopolymer could be solubilized only in a weakly basic (pH 8.0) ammonium carbonate buffer solution because of the free carboxylic groups of DTPA. When the solution was mixed with a europium chloride solution (in 0.1*M* ammonium acetate), a large precipitate was produced. The precipitate could be solubilized in a weakly basic (pH 8.0) ammonium carbonate solution as well and was further analyzed and purified by reverse-phase HPLC (Fig. 4). HPLC and mass spectrometry analysis showed that the Eu-labeled peptide was mainly contained in the precipitate. As shown in Figure 2, the Eu–DTPA complex was formed through the loss of hydrogen atoms of carboxylic groups of DTPA, and Eu was coordinated with O and N atoms of DTPA. Consequently, the stability of the complex highly depended on the pH value of the medium. Therefore, the pH value of the solution had to be kept around neutral conditions (pH = 6–8) during the preparation and purification of the metal-labeled biopolymers because the complex decomposed in a strongly basic solution (pH = 10–12), and this induced the formation of $Eu(OH)_3$ easily. It is readily understood that an Eu–DTPA–CCK complex is hard to form in a strongly acidic (pH = 1–3) solution.

HPLC spectra of the DTPA–CCK biopolymers always showed highly overlapping double peaks, which were characterized by the same mass spectrum; this indicated that the double peaks stood for fairly similar chemical structures with only different configurations. A very reasonable explanation is shown as Figure 5. The Eu(III)-coordinated DTPA biopolymers should exist as two isomers: A and B. Consequently, the HPLC spectra of the Eu(III)-coordinated DTPA biopolymers always displayed highly overlapping double peaks. A similar phenomenon was observed by Selvin et al.[27] for a small luminescent molecule linked with DTPA.

All the compounds were purified by reverse-phase HPLC with 0.1*M* ammonium acetate for elution, and the solid products were obtained after lyophilization. As shown in Table I, the structures were characterized with high-resolution ESI, and the results of the overall yield after HPLC purification are listed there. The results suggest that the yields were similar to

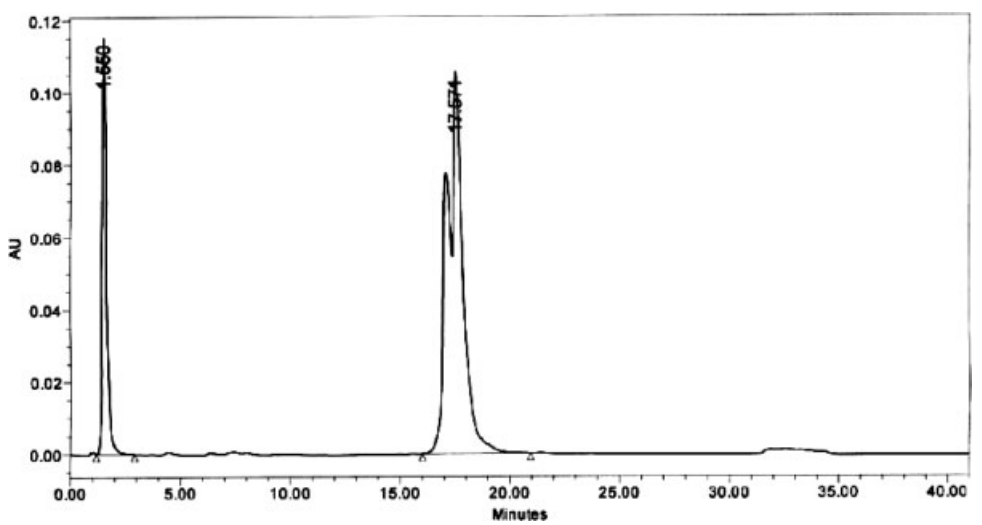

Figure 4 HPLC spectrum of the Eu–DTPA–CCK biopolymer after reverse-phase HPLC purification.

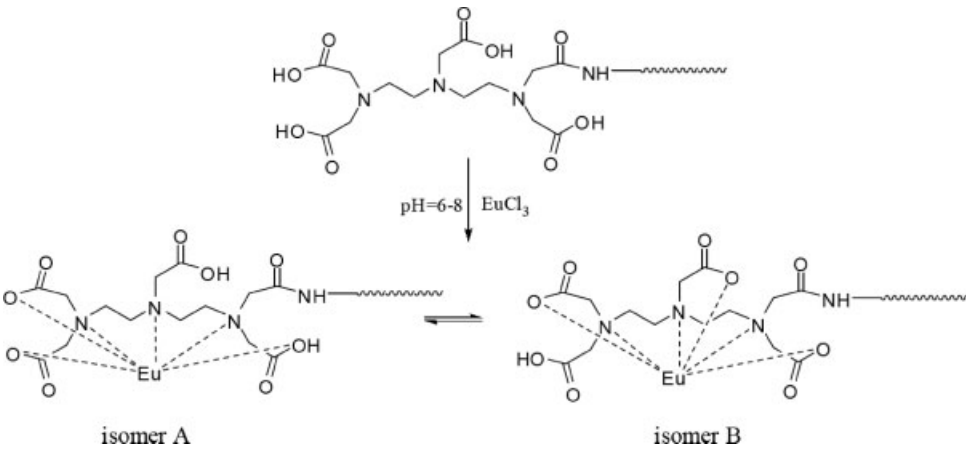

Figure 5 Structural balance of the Eu(III)-coordinated DTPA biopolymers.

TABLE I
Analytical Data of the Synthetic Biopolymers

Ligand[a]	HPLC purity (%)[b]	tR (min)	ESI Calculated	ESI Found	Overall yield after HPLC purification (%)[e]
Ac–CCK5	100	23.6[c]	692.1, 692.9	692.1, 692.9 [(M + 1)$^+$]	35.8
DTPA–CCK5	100	20.3[d]	1025.3, 1026.2	1025.3, 1026.2 [(M + 1)$^+$]	30.1
CCK8	100	22.7[c]	1027.3, 1028.2	1027.3, 1028.2 [(M + 1)$^+$]	20.6
DTPA–CCK8	100	14.3[d]	1402.4, 1403.3	1402.4, 1403.3 [(M + 1)$^+$]	16.8
Eu–DTPA–CCK8	100	26.2[d]	1552.2, 1553.2	1552.2, 1553.2 [(M + 1)$^+$]	15.6

tR, retention time.
[a] For CCK5, H–Gly–Trp–NMeNle–Asp–Phe–NH_2; for CCK8, H–Asp–Tyr–Nle–Gly–Trp–Nle–Asp–Phe–NH_2.
[b] After size exclusion chromatography.
[c] HPLC conditions: A was 0.1% TFA, and B was acetonitrile.
[d] HPLC condition: an ammonium acetate solution.
[e] Based on the amount of the rink resin used.

those of other biopolymers developed by various laboratories via solid-phase synthesis,[28] and this demonstrates that this is an efficient approach to the preparation of Eu(III)-coordinated DTPA biopolymers. Affinity effects with CCKB receptors for these biopolymers suggest that lanthanide-labeled DTPA–CCK biopolymers are good assay candidates for CCKB receptors.

CONCLUSIONS

To develop an assay for CCKB receptors with a lanthanide-coordinating, DTPA-labeled CCK biopolymer, a simple and efficient synthesis using commercial DTPA has been developed. A satisfactory yield and good purity for DTPA-conjugated biopolymers via a ring-opening reaction of the dianhydride DTPA and the free amine groups of the biopolymers have been obtained. This provides the possibility of the large-scale production of Eu(III)-coordinated biopolymers. All the synthetic biopolymers have been characterized with HPLC and high-resolution ESI.

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