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Derivatization of a Protein with Fluorescamine Utilizing the Tube Radial Distribution Phenomenon of Ternary Mixed Carrier Solvents in a Capillary Tube

Yuji Masuhara, Naoya Jinno, Masahiko Hashimoto, and Kazuhiko Tsukagoshi*
Department of Chemical Engineering and Materials Science, Doshisha University, Kyotanabe, Kyoto 610-0321

(Received April 21, 2011; CL-110338; E-mail: ktsukago@mail.doshisha.ac.jp)

The derivatization of bovine serum albumin with fluorescamine was examined utilizing the tube radial distribution phenomenon of the ternary mixed solution of water–acetonitrile–ethyl acetate (3:15:8 volume ratio) in a fused-silica capillary tube. Double capillary tubes having different inner diameters (100 and 250 μm i.d.) were designed to promote the tube radial distribution phenomenon. The smaller tube was inserted into the larger one through a T-type joint. A water–acetonitrile mixture (3:1 volume ratio) including bovine serum albumin was delivered into the large tube from the inside through the small tube, and an acetonitrile–ethyl acetate mixture (7:4 volume ratio) including fluorescamine was delivered from the outside through the joint. The solutions were mixed through the large tube to perform the derivatization reaction. The fluorescence intensity of the fluorescamine-derivatized bovine serum albumin obtained under the tube radial distribution phenomenon was greater than that obtained through a batch-reaction using a homogeneous solution of water–acetonitrile (1:5 volume ratio).

Microflow systems are an active area of research in chemistry and biochemistry. Novel components of microflow systems are frequently sought after to improve performance for different types of uses and users. Capillary tubes with inner diameters less than several hundred micrometers that provide a microflow are also known to exhibit interesting and useful physical or hydrodynamic phenomena, such as electroosmotic flow and laminar flow. The electroosmotic flow in a capillary tube promotes capillary electrophoresis[1,2] and capillary electrochromatography,[3] while laminar flow conditions enable hydrodynamic chromatography.[4,5]

Recently, our group reported the tube radial distribution phenomenon of carrier solvents,[6–10] which we call the "tube radial distribution phenomenon (TRDP)." When the ternary mixed carrier solvents of water–hydrophilic/hydrophobic organic solvent mixtures are delivered into a microspace, such as a microchannel or a capillary tube under laminar flow conditions, the carrier solvent molecules are radially distributed in the microspace, generating inner and outer phases. For example, when a carrier solution of water–acetonitrile–ethyl acetate (3:8:4 volume ratio) was fed into a fused-silica capillary tube (75 μm i.d.), an organic solvent-rich major phase was generated around the middle of the tube as an inner phase far from the inner wall while a water-rich minor phase formed near the inner wall as an outer phase or capillary wall phase. A novel capillary chromatography system where the outer phase functions as a pseudo-stationary phase under laminar flow conditions has been developed based on the TRDP. We call it "tube radial distribution chromatography (TRDC)".[6–10]

The TRDP creates a phase interface or kinetic aqueous–organic interface in a microspace under laminar flow conditions. In this study we applied the specific phase interface created through the TRDP to a chemical reaction space in a microflow system. A chemical reaction that takes place at the kinetic aqueous–organic interface created under the TRDP will be called a "tube radial distribution reaction (TRDR)" for convenience. The derivatization reaction of bovine serum albumin (BSA) with fluorescamine (FR) was carried out in the present TRDR system as a model.

Water was purified with an Elix UV 3 system (Millipore Co., Billerica, MA). All reagents used were obtained commercially and were of analytical grade. BSA was purchased from Sigma-Aldrich, Japan (Tokyo). FR, perylene, Eosin Y, acetonitrile, and ethyl acetate were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Fused-silica capillary tubes were purchased from GL Science (Tokyo, Japan).

The schematic diagram of the present TRDR system is shown in Figure 1. The smaller tube (100 μm i.d.) was inserted into the larger one (250 μm i.d., 150 cm length) through a T-type joint. A water (10 mM borate buffer, pH 9.0)–acetonitrile mixture (3:1 volume ratio) including 25 μM BSA was delivered into the large tube from the inside through the small tube, and an acetonitrile–ethyl acetate mixture (7:4 volume ratio) including 1 mM FR was delivered from the outside through the joint. They were then mixed through the large tube to perform the derivatization reaction in the water–acetonitrile–ethyl acetate (3:15:8 volume ratio) solution. The BSA and FR solutions were fed into the tubes with microsyringe pumps at flow rates of 5 and 10 μL min^{-1}, respectively. The solution including FR-derivatized BSA was collected at the outlet of the large tube until 1 mL was obtained for fluorescence spectroscopy (FP-6500, JASCO Corporation, Tokyo, Japan).

The large capillary tube (250 μm i.d.) was set up for the fluorescence microscope-CCD camera system (Figure 1) in order to confirm the tube radial distribution of the carrier solvents. The

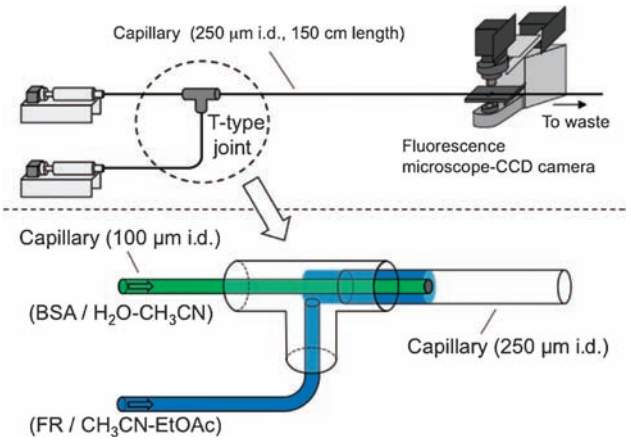

Figure 1. Schematic diagram of the present TRDR system with fluorescence microscope-CCD camera.

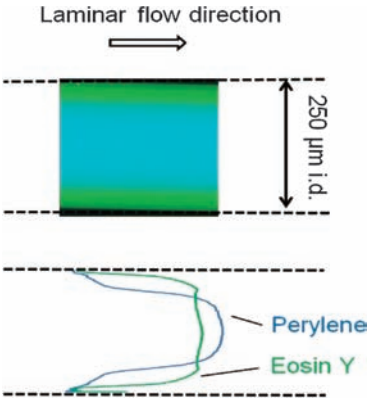

Figure 2. Fluorescence photograph and profiles of the fluorescent dyes dissolved in the ternary mixed carrier solvents at 30 cm from the capillary outlet. Conditions: carrier, water–acetonitrile–ethyl acetate mixture (3:15:8, v/v/v), including 67 μM perylene and 0.33 mM Eosin Y; flow rate, 15 μL min^{-1}.

water–acetonitrile mixture (3:1 volume ratio) including 1 mM Eosin Y and acetonitrile–ethyl acetate mixture (7:4 volume ratio) including 0.1 mM perylene solutions were delivered into the large capillary tube at the flow rates of 5 and 10 μL min^{-1}, respectively. The fluorescence in the capillary tube was monitored at a position 30 cm from the outlet using a fluorescence microscope (BX51; Olympus, Tokyo, Japan) equipped with a Hg lamp, a filter (U-MWU2, ex 330–385 nm, em >420 nm), and a CCD camera (JK-TU53H).

Figure 2 shows the fluorescence photograph and profiles observed for the capillary tube in which the fluorescent dye-containing aqueous–organic solvent carrier solution was delivered. The photograph and profiles showed that the hydrophobic perylene molecule (blue) was distributed around the middle of the tube and away from the tube inner wall, whereas the hydrophilic Eosin Y molecule (green) was distributed near the tube inner wall. Thus, the kinetic liquid–liquid interface created through the TRDP was clearly confirmed in the present TRDR system using double capillary tubes.

FR, which has no fluorescence itself before derivatization, is commonly used as a fluorogenic reagent. The reagent reacts readily under alkaline conditions with primary amines to form fluorescent substances, providing the basis for a rapid and sensitive assay of amino acids, peptides, proteins, and other primary amines.[11] FR is also quickly hydrolyzed in aqueous solution to become fluorescently inactive. An aqueous solution including a biomolecule such as a protein and an organic solution including FR are mixed and stirred for the derivatization reaction. FR competitively reacts with BSA and water (hydrolysis) in an aqueous–organic solvent mixture solution.

First, the derivatization reaction of BSA with FR was confirmed in a batch vessel using an aqueous–organic solvent solution in the usual manner, as a reference. The 10 mM borate buffer solution (1.0 mL, pH 9.0) of 50 μM BSA was mixed with the acetonitrile solution (5.0 mL) of 0.8 mM FR and stirred in the batch vessel (water–acetonitrile; 1:5 volume ratio). The fluorescence due to the derivative in the solution was measured by fluorescence spectroscopy ($\lambda_{ex} = 390$ nm). The fluorescence spectrum exhibited a maximum around 30 min after mixing, which then gradually decreased. The obtained spectrum at 30 min is shown in Figure 3. The maximum intensity was ca. 200

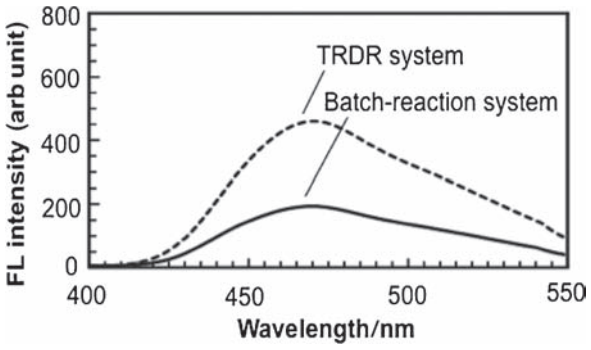

Figure 3. Fluorescence spectra of the FR-labeled BSA. The solutions included 8.3 μM BSA and 0.67 mM FR for the derivatization in the systems. Dotted line, TRDR system (water–acetonitrile–ethyl acetate; 3:15:8 v/v/v) and solid line; batch reactor (water–acetonitrile; 1:5 v/v).

(arbitrary unit) ($\lambda_{ex} = 390$ nm, $\lambda_{em} = 470$ nm). The derivatization reaction of BSA with FR was not performed in a batch vessel using aqueous–organic solvent solution (water–acetonitrile–ethyl acetate; 3:15:8 volume ratio) due to protein deposition.

The fluorescence spectrum for the solution collected through the capillary tube in the TRDR system (water–acetonitrile–ethyl acetate; 3:15:8 volume ratio) is also shown in Figure 3. The maximum intensity was ca. 490 (arbitrary unit) ($\lambda_{ex} = 390$ nm, $\lambda_{em} = 470$ nm). The migration time in the capillary tube of 150 cm length was 4.6 min, and the collecting time at the outlet of the tube was 67 min. The fluorescence intensity of the FR-derivatized BSA obtained under the TRDP in the capillary tube was about two times as great as that obtained in the batch-vessel using a water–acetonitrile homogeneous solution. The greater fluorescence intensity in the TRDR system must be attributed to the specific reaction area, i.e., the kinetic aqueous–organic phase interface created through the TRDP in the capillary tube.

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