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Monitoring of the conjugation reaction between human serum transferrin and fluorescein isothiocyanate by capillary electrophoresis

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Abstract

Labeling of iron-free human serum transferrin by an amine-reactive probe, fluorescein isothiocyanate (FITC) was monitored with different dye to protein ratios. The degree of labeling was followed and determined by capillary electrophoresis. Depending on the number of the bound FITC, a shift in the electropherogram was observed, but the conjugated-transferrin forms were resolved from the unbound FITC in zone electrophoresis. The lowest protein concentration that resulted in detectable transferrin conjugate was 0.13 μM, and the limit of detection of the conjugated protein was 0.13 nM (10 ng/ml) with a signal to noise ratio of one to three.

Keywords: Transferrin; Fluorescein isothiocyanate; Proteins; Glycoproteins; Derivatization, electrophoresis

1. Introduction

Capillary electrophoresis is a powerful tool for monitoring reactions in which the mobility of an analyte changes upon the interaction [1]. One of the drawbacks of this technique, is the limited sensitivity when using UV absorbance detection due to the short optical path length within the capillary. Fluorescence derivatization of compounds (e.g., proteins) provides high sensitivity, and especially laser-induced fluorescence (LIF) detection produces outstanding results [2,3]. However, loss of efficiency and resolution can be observed upon multiple labeling of the protein molecules [4]. The electrophoretic mobility of the labeled molecules might change, causing broad, overlapping peaks.

A lot of fluorescent probes are in the market which can be attached through covalent or non-covalent interactions [5] to produce fluorescent proteins. Perhaps, the most widely used fluorophore in protein conjugation is the amine-reactive probe, fluorescein isothiocyanate (FITC). Using this probe, picomolar concentrations of stained amino acids have been detected [6]. However, the labeling has to be done at high concentration, because the chemistry of FITC derivatization of primary amines is slow and strongly concentration dependent. This limitation is also apparent for protein derivatization. Thus, the limit of detection (LOD) of real samples can be given in two ways, i.e., the lowest protein concentration with satisfactory derivatization, or the lowest concentration of the conjugated substance detected by a fluorescent detector [7]. For amine-reactive probe, fluorescein, conjugation to proteins occurs almost exclusively through nucleophilic attack by the ε-amino group of lysine residues and the N-terminal amino group forming fluorescein thiocarbamyl derivatives [8]. The reaction depends on several parameters, e.g., pH of the buffer, concentration of protein, and dye, the molar excess of dye to protein, etc. Although, lysine is a hydrophilic amino acid and usually takes exposed positions at the surface of proteins, the distribution and accessibility of the ε-amino groups differ from protein to protein. The general protocols available for conjugation should be set to keep the biological activity of the protein and to provide stable conjugates.

Transferrin is a monomeric serum glycoprotein (M_r 76,000 D) that binds two ferric ions for delivery to vertebrate cells through receptor-mediated endocytosis. Once, iron-carrying transferrins are inside endosomes, the acidic environment results in dissociation of iron from the transferrin–receptor complex. Following the release of iron, the apotransferrin is released from its receptor to bind iron

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again [9]. Transferrin is able to bind other irons, such as aluminium(III), gallium(III), indium(III), thallium(III) [10], vanadium(V) [11], and zirconium(IV) [12]. It is also applied for delivering different drugs into cells (drug targeting), since transferrin uptake of cancer cells is increased [13,14]. Transferrin deficiencies are characteristic for alcoholism [15,16]. It was also found to be as a chiral selector suitable in capillary electrophoresis for enantiomer separations [17,18]. Our aim was to monitor the labeling procedure of transferrin with FITC, since the labeled protein can be used as a receptor-mediated endocytosis marker [19,20].

2. Experimental

2.1. Chemicals

All reagents used in the study, were of analytical grade. $NaHCO_3$, NaOH and H_3BO_3 were purchased from Reanal (Budapest, Hungary). FITC isomer I was obtained from Sigma-Aldrich (Steinheim, Germany). Iron-free transferrin was accessed from Behring Werke AG (Marburg, Germany). All solutions were prepared in double-distilled water. Microspin G-25 columns for sample purification were obtained from Amersham Biosciences (USA).

2.2. Conjugation of FITC to transferrin

A 2 mM stock solution of FITC isomer I was prepared in 100 mM $NaHCO_3$ buffer pH 9 and was immediately used for derivatization. A dilution series was made from 2.6×10^{-4} M to 2.6×10^{-7} M of transferrin (20–0.02 mg/ml). Reaction mixtures were prepared to obtain 10, 5, 1, 0.5, 0.1, 0.05, 0.01 mg/ml (0.13 mM to 0.13 nM) protein, and 1, 0.1, 0.01 mM FITC final concentrations and were allowed to react for 24-h long at room temperature in darkness. For controls 100 mM $NaHCO_3$ buffer pH 9 was added instead of the protein samples resulting in the same FITC concentrations as the reaction mixtures had.

2.3. Molar ratios of conjugated FITC to transferrin

The degree of labeling was checked by measuring the UV absorbance recommended in the product manual of FITC [5]. The samples were purified by spinning over a Microspin G-25 column in a tabletop centrifuge (Eppendorf Centrifuge 5415 D) with 3000 rpm for 2 min to remove the unreacted dye. This way it was not needed to dilute the samples before this purification. The removal of the dye was checked by capillary electrophoresis. The UV absorbance of the purified sample was determined at 280 and 494 nm (Specord 50, Analytik Jena, Jena, Germany). The FITC to protein ratios were calculated according to the following equation: ratio = $A_{494} \times \varepsilon_{\mathrm{Tf,280\,nm}} / \{\varepsilon_{\mathrm{FITC,494\,nm}} [A_{280} - (A_{494} \times 0.30)]\}$, where 0.30 is a correction factor which is included to compensate for absorption of the fluorophore at 280 nm [5], and $\varepsilon_{\mathrm{Tf,280\,nm}}$ is the molar extinction coefficient of the iron-free transferrin (72,500 M^{-1} cm^{-1}) at 280 nm (pH 8), $\varepsilon_{\mathrm{FITC,494\,nm}}$ is the molar extinction coefficient of the dye (68,000 M^{-1} cm^{-1}) at 494 nm (pH 8).

2.4. Instrumentation and separation conditions

An automated $HP^{3D}CE$ system (Hewlett-Packard, Waldbronn, Germany) equipped with a diode array detector coupled with a broadband fluorescence detector (Argos 250B, 240–400 nm, broadband excitation filter and a 495 nm cut off emission filter, Flux Instruments, Basle, Switzerland) was applied in CZE experiments. Uncoated 75 μm i.d. fused-silica capillary with 59 cm total length (effective length 41 cm for fluorescence detection and 50.5 cm for UV detection) was used in a standard HP cassette at 20 °C. Samples were injected with 100 mbar.s, runs were conducted with 20 kV. Data acquisition, storage and analysis were performed, using Agilent ChemStation Plus software. LIF detection of capillary electrophoresis runs of conjugated samples were performed with Crystal model 310 (Unicam, The Netherlands) equipment equipped with a Zetalif detector (Picometrics, Ramonville, France), and a 488 nm wavelength argon-ion laser. Data collection, processing and analysis of these results were performed, using the Axxiom Chromatography Model 717 software (Axxiom Chromatography, Moorpark, USA). Uncoated fused-silica 65 cm (effective length 47 cm) × 50 μm i.d. capillary was used. Samples were injected hydrodinamically, with 20 mbar for 15 s. Separation voltage was 25 kV. Boric acid (100 mM) was used as background electrolyte adjusted to pH 8.3 with sodium hydroxide.

3. Results

Iron-free transferrin samples in the range 0.13–0.13 nM were labeled with FITC in the presence of 1, 0.1 and 0.01 mM (final concentration) dye as it is described in the Section 2. Broadband fluorescent detection and laser-induced fluorescent detection were used to monitor the course of labeling. The electrophoretic patterns of labeled samples without purification (i.e., the unbound dye was not removed) are shown in Fig. 1. The dye itself provides a main peak and several minor ones which are also seen in the electropherogram of FITC alone with broadband fluorescence or LIF detection (not shown), although in a diluted sample only the main component appears that would suggest only a single peak. Fig. 1B shows the simultaneous UV and fluorescence detection of a sample. It is seen that the sensitivity of UV detection is lower than the fluorescence detection (see the baseline irregularities compared to peak height). FITC appears also as more than one peak in UV, although fewer components can be detected than with fluorescence. The electro osmotic flow (EOF) was only detectable by UV (see the system peak at ca. 4 min in Fig. 1B) and this allowed the calculation of the effective mobilities. At pH 8.3, transferrin has a negative net

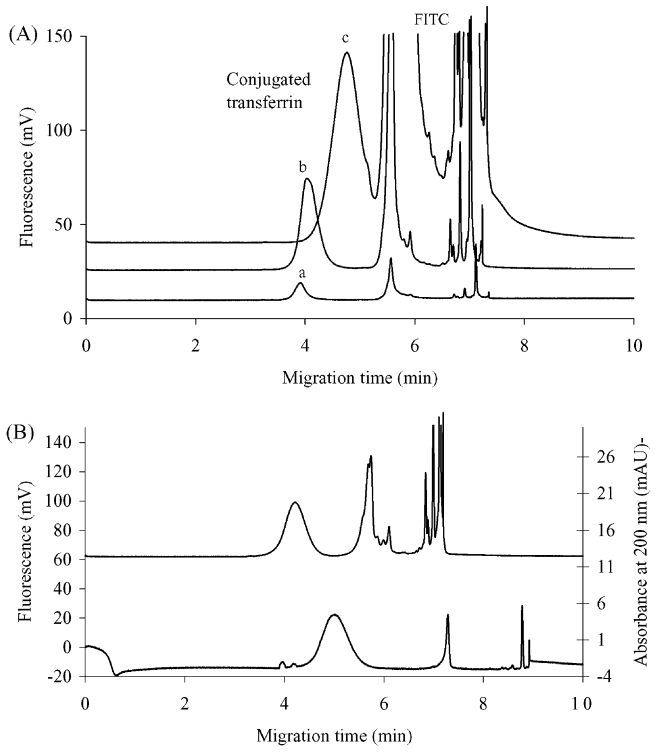

Fig. 1. (A) Zone electrophoresis patterns of FITC-labeled transferrin samples by fluorescence detection. The unbound dye (providing a main peak and several minor ones) was not removed from the samples. Experimental conditions: background electrolyte, 100 mM borate buffer, pH 8.3; voltage, 20 kV; capillary 59 cm (effective length 41 cm) × 75 μm i.d.; injection of samples 100 mbar × s; 20 °C; detection with fluorescence detector (240–400 nm, broadband excitation filter and a 495 nm cut off emission filter). The reaction was left to continue for 20 h, and the reaction mixtures contained 13 μM (1mg/ml) Tf and (a) 0.01 mM FITC, (b) 0.1 mM FITC, and (c) 1 mM FITC. (B) Zone electrophoresis patterns of a FITC-labeled transferrin sample by simultaneous fluorescence (upper trace, left axis), and UV detection (lower trace, right axis). The unbound dye shows several peaks with both detections. Experimental conditions: background electrolyte, 100 mM borate buffer, pH 8.3; voltage 20 kV; capillary 59 cm (effective length: fluorescence 41 cm, UV 50.5 cm) × 75 μm i.d.; injection of samples 100 mbar × s; 20 °C; detection with fluorescence detector (240–400 nm, broadband excitation filter and a 495 nm cut off emission filter). The reaction was left to continue for 20 h, and the reaction mixture contained 6.5 μM (0.5 mg/ml) Tf and 0.1 mM FITC.

surface charge (isoelectic point, p*I* is around 6 [21]) and its apparent mobility was of 57 × 10^{-9} m^2 V^{-1} s^{-1}. FITC provides several peaks but one main peak in zone electrophoresis. The main component of free FITC had a mobility of 37 × 10^{-9} m^2 V^{-1} s^{-1}.

The increase of the amount (molar ratio) of FITC results in an increase both in mobility and peak area of the conjugated-transferrin zone. The more dye molecules bind to the protein the bigger is the transferrin peak area and the closer is the mobility of the labeled transferrin molecules to that of the unbound fluorescent dye (Fig. 2). Although, transferrin has several isoforms those cannot be resolved in such conditions (a relatively broad peak shape appears). Following the time course of the conjugation, a saturation curve was obtained (Fig. 3). The time necessary for complete reaction (to reach

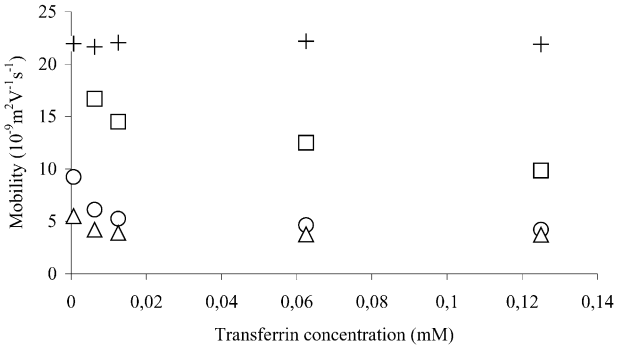

Fig. 2. Calculated effective mobility values of unbound FITC (plus sign) and transferrin-FITC conjugates. The initial concentration of transferrin in the reaction mixture changed between 0 and 0.13 mM while the initial concentration of FITC was 1 mM (square), 0.1 mM (circle) and 0.01 mM (triangle). Experimental conditions: 100 mM borate buffer, pH 8.3; voltage, 20 kV; capillary 59 cm (effective length 50.5 cm) × 75 μm i.d.; UV detection, 200 nm.

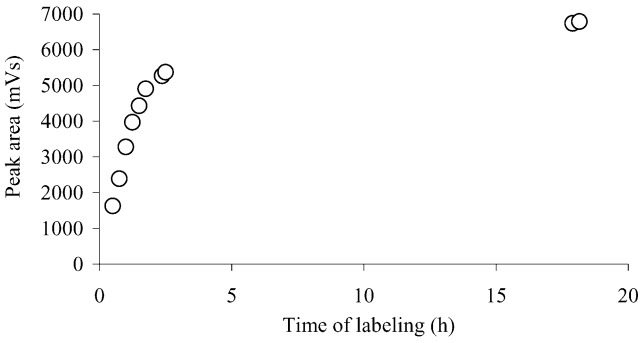

Fig. 3. Time course of peak area of conjugated iron-free transferrin in zone electrophoresis upon labeling with FITC. The reaction mixture contained 13 μM (1 mg/ml) transferrin and 1 mM FITC. Experimental conditions are given at Fig. 1.

the plateau) is depending on the relative and absolute dye to protein ratios, but a 20-h labeling was always enough for complete reaction. The peak area values of the conjugated-transferrin zones showed linearity depending on the transferrin concentration in the reaction mixture (Fig. 4). The number

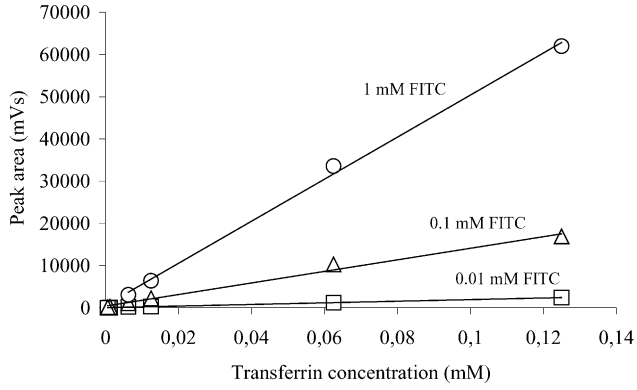

Fig. 4. Calculated peak area values of FITC-labeled transferrin zones in CZE experiments of conjugation mixtures of FITC and transferrin. The initial FITC concentrations varied from 0.01 mM to 1 mM. Experimental conditions are given in Fig. 1.

Table 1
FITC to transferrin ratio in the conjugated transferrin samples determined by CZE

Transferrin concentration	1 mM FITC	0.1 mM FITC	0.01 mM FITC
0.13 mM (10 mg/ml)	2.5 ± 0.5	0.8 ± 0.1	0.11 ± 0.05
0.066 mM (5 mg/ml)	3 ± 0.5	0.9 ± 0.1	0.13 ± 0.05
13 μM (1 mg/ml)	3.5 ± 0.5	1.1 ± 0.1	0.25 ± 0.05
6.6 μM (0.5 mg/ml)	n.s.[a]	1.2 ± 0.1	0.4 ± 0.05
1.3 μM (0.1 mg/ml)	n.s.[a]	2.2 ± 0.1	1.4 ± 0.05

n (number of experiments) = 5.
[a] n.s.: no baseline separation of the conjugated protein molecules from unbound FITC in CZE of the reaction mixture.

of the FITC molecules attached to the protein molecules were determined by two methods. When the method recommended by the vendor of FITC was applied (see Section 2) uneven results were obtained. The dye to protein ratios were found to be of 3.4, 4.1, and 2.1 when 1 mM FITC reacted with 0.13 mM (10 mg/ml), 6.6 μM (5 mg/ml), and 13 μM (1 mg/ml) transferrin (final concentrations), respectively. Similarly, a "maximum curve", i.e., the dye to protein ratios were found to be of 0.44, 0.63, and 0.60 when 0.1 mM FITC was reacting with 13.2 mM, 6.6 mM, and 1.32 mM transferrin, respectively. Since, the method can be applied only for concentrated samples we used another approach to estimate the dye to protein ratio of the conjugates. The labeled transferrin is baseline separated from the unreacted fluorophore in CZE runs, hence, no purification step was necessary for this determination. Assuming that the fluorescent intensity of FITC does not suffer from significant quenching upon binding to transferrin a calibration curve of the peak areas in the electrophoretic pattern of freshly dissolved dye can be used to estimate the amount of FITC in the FITC-transferrin zone. Table 1 shows the dye to protein ratios obtained with this estimation. Fig. 5 shows a plot of the mobility values of the conjugated FITC-transferrin molecules versus the molar ratio of FITC/transferrin obtained. A "limit of detection" of the fluorescent protein sample was determined by LIF detection in two different ways. The LOD of a highly derivatized sample was ca. 10 ng/ml = 0.13 nM FITC-transferrin (signal to noise ratio was higher than one to three). The lowest protein concentration used in the conjugation giving satisfactory staining with good signal, was ca. 10 μg/ml = 0.13 μm.

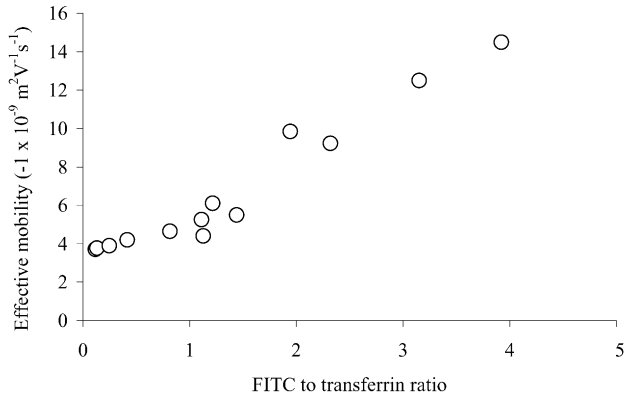

Fig. 5. Plotting effective mobility values of FITC-transferrin conjugates vs. degree of labeling (molar ratio of FITC to protein). Experimental conditions of the CZE runs are given in the legend to Fig. 1.

4. Discussion

Human serum transferrin is a good candidate for drug targeting of cancer cells. To follow the uptake of transferrin by cells can be easily done with fluorescent-labeled protein molecules. Since, such labeling might result in heterogeneous conjugates and also because the transferrin molecules occur in blood as various isoforms of the same protein it is advisable to follow the labeling reaction. Capillary electrophoresis proved to be a good tool to monitor the labeling of transferrin with FITC. The labeling produced conjugates but in the electrophoretic pattern unbound FITC molecules were also seen. Fortunately, the unbound FITC zones do not overlap with the labeled transferrin only at high degree of labeling. At the pH recommended for derivatization (around 9), the ε-amino groups of lysines are positively charged, whereas FITC is negative [22]. When the dye molecules are bound to the protein surface, positive charges of lysine residues will be replaced by negative charges of FITC, which causes mobility change of the protein. Mobilities of conjugates will approach to the ones of unbound FITC in zone electrophoresis (Fig. 1). Although, transferrin is a heterogeneous protein (isoforms can be resolved even by capillary zone electrophoresis in coated capillary [21]) it is difficult to resolve the isoforms with CZE runs in uncoated capillaries. Conjugation of different number of FITC molecules to proteins may result in separable components, as it was observed in the case of myoglobin where the staining with different number of dyes provided well resolved peaks [23]. In our case, however, only a broad zone was observed. The calculated degree of labeling, therefore, should be considered as an average. Transferrin has several (54) lysines, mostly at well-exposed positions. Since, we found that, generally, not more than 3.5 FITC molecules were bound to the protein it needs further studies to determine where the conjugation occurs on the protein surface. Such a degree of labeling will not decrease the biologic activity of this protein [5]. The simultaneous detection of fluorescence and UV absorbance of the conjugated protein molecules allowed a simple and fast estimation of the molar ratio of FITC bound to transferrin and the determination of the effective mobilities. It was observed, that the FITC-transferrin conjugates show a quite stable fluorescence in time. However, it was reported that the quantum yield of fluorescent dyes could change due to environmental influences. This includes coupling to other molecules, such as proteins [24,25]. It is also shown, that the fluorescence intensity of conjugates can be decreased if there are too many fluorescent molecules attached to the protein due to self-quenching [5]. The method recommended for determination of the degree of labeling with purification of the stained protein can be used only with samples, which are concentrated enough both for protein and dye. The simultaneous fluorescence and UV detection proved

to be a rapid and suitable method for monitoring the labeling in case of reaction mixtures with various compositions.

Acknowledgements

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