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Interaction between anionic molecule and colored copolypeptide microspheres

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Summary

Tryptophan (Trp) residues within copoly(L-glutamic acid-*co*-L-Trp) (GAT) form a tricyclic structure when treatment with trifluoroacetic acid (TFA) and show reversible color change from yellow (above pH 8.0) to red (below pH 7.0) when the nitrogen atom in an indole ring dissociates positively in the neutral pH region. The interaction between TFA-treated GAT (GAT-T) and an anionic molecule such as warfarin was investigated using VIS and fluorescence spectrophotometry. Trp residues within GAT-T were found to interact with warfarin electrostatically besides hydrophobic interaction resulting in a color change from red to yellow. Release behavior of warfarin from GAT-T microspheres (MS) was also investigated. Binding amount and retention time of warfarin in GAT-T MS were found to be superior to those in untreated GAT MS.

Introduction

The single Trp residue within human serum albumin (HSA) exhibits high affinity for bulky heterocyclic molecules with a negative charge (1)-(4), and these molecules quench fluorescence originating in Trp (5)-(7) and function as specific markers for specific drug-binding site of HSA (8) (9).

In our previous studies (10)(11), Trp derivative treated with TFA was found to form a tricyclic structure and show reversible, pH-dependent color change from yellow (above pH 5.5) to red (below pH 4.0) when a nitrogen atom in the indole ring dissociates positively (scheme 1). Moreover, it was found that colored hydrogel can be prepared by treating a copoly(*N*-hydroxyethyl L-glutamin-*co*-Trp) with TFA. Dissociation of the indole ring caused drastic variations in the physical properties of the membrane, the degree of swelling, and solute permeability.

The aim of the present study is to prepare a novel material capable of monitoring the interaction with Trp through a color change (e.g., the material that show color change by adsorption or desorption of drug and capable judging the efficacy of the drug by color).

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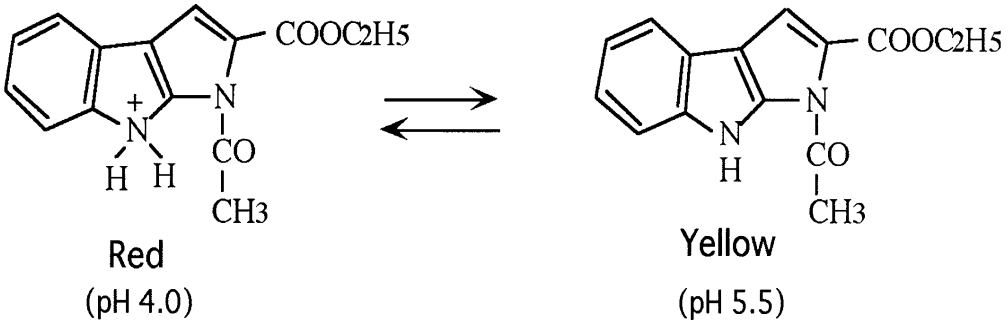
Moreover, the trait of this material is to excel in bio-specificity and bio-compatibility compared with the material that uses a conventional dye through it uses a natural amino acid as a chromophore.



Scheme 1 Dissociation form of *N*-acetyl Trp ethylester treated with trifluoroacetic acid.

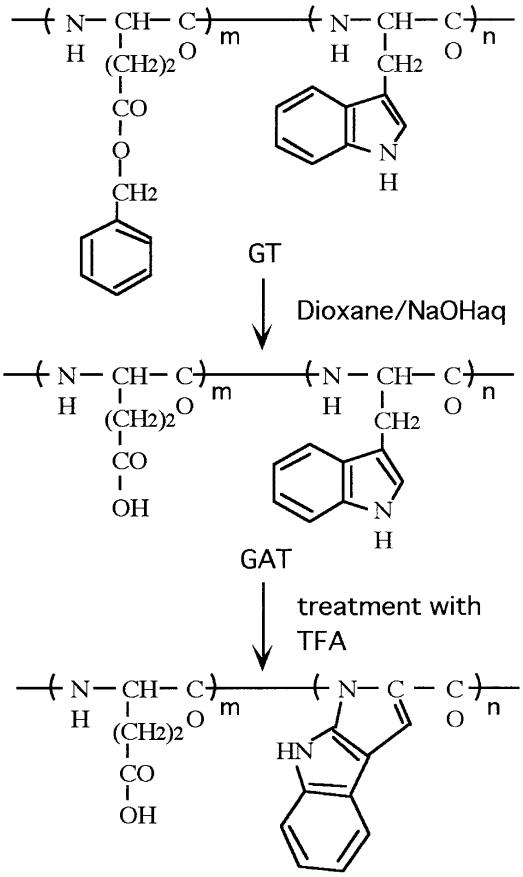
In this paper, we will describe our spectroscopic examination of the interaction between Trp containing copolypeptide treated with TFA and an anionic molecule such as warfarin, and the release behavior of warfarin from colored copolypeptide MS treated with TFA.

Experimental

Materials

The starting copolymer composed of γ-benzyl L-glutamate and Trp (GT) was synthesized by *N*-carboxyanhydride (NCA) method, as described in our previous papers (10) (11). The viscosity-average molecular weight and Trp content of GT were determined to be 376,000 and 20 mol%, respectively. All the solvents used in the synthesis were distilled twice.

To induce the dissociation of Trp residues treated with TFA by introduction of opposite charges, Trp containing copolypeptide treated with TFA (GAT-T) was prepared by dissolving copoly(L-glutamic acid-*co*-Trp) (GAT), obtained by the saponification of GT in dioxane with NaOH aq, in TFA under irradiating UV light (wavelength 365 nm, intensity 300 μw/cm^2) for 72 hr. Debenzylation of GT was confirmed by the disappearance of absorption due to the presence of an ester group at 1740 cm^{-1} in the IR spectrum. Conversion of Trp residue into tricyclic

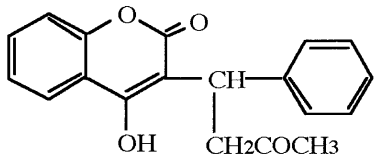


Scheme 2 Schematic diagram of the preparation of GAT-T.

structure was determined to be 20% in previous paper (11). Though several compounds are also formed except for the compound with the tricyclic structure, only the compound with the tricyclic structure showed the color change (11). GAT-T was obtained through dialysis and then lyophilized. Scheme 2 is a schematic diagram of the preparation of GAT-T.

Red-colored, GAT-T MS were prepared by following method. GAT-T aqueous solution (0.32 g/l ml) was dispersed to 25 wt% PMMA toluene/chloroform mixed solution and then 1 ml of 20 % (v/v) hexamethylene diisocyanate/toluene solution was added to the solution as a crosslinking agent. The reaction was carried out at room temperature for 21 hr. GAT-T MS were washed with acetone to remove remaining PMMA and dried in vacuo. The diameter of MS prepared by this method was in the range of 10-100 μm as determined by optical microscopy.

Warfarin-bound GAT-T MS were obtained by adding GAT-T MS (50 mg) to warfarin aqueous solution (1.2 mg/40 ml) and stirring at room temperature for 24 hr followed by centrifugation. The amount of warfarin bound to the GAT-T MS was determined by UV measurement of the absorption intensity at 305 nm resulting from warfarin remaining in the supernatant. Using a prepared standard concentration curve, the absorption intensity was converted into the concentration (mol/l).



Scheme 3 Structure of warfarin.

Warfarin (scheme 3) purchased from Nacalai Tesque, Inc. was extra pure grade reagent, and it was used in the present study without further purification. Warfarin is analogous to vitamin K which is necessary for the formation of blood coagulation factor and acts as anticoagulant.

Measurement

To investigate the interaction between GAT-T and warfarin VIS and fluorescence spectra were measured.

VIS spectra of GAT-T in the presence and absence of warfarin were measured using a Jasco V-520 UV/ VIS spectrophotometer with a quartz cell having a path length of 10 mm. The concentrations of GAT-T and warfarin were $1x10^{-2}$ mol/l. The pH was adjusted with 5N HCl and NaOH to assure that changes in concentration were minimized.

Fluorescence spectra of GAT-T in the presence or absence of warfarin were measured by Shimazu RF-

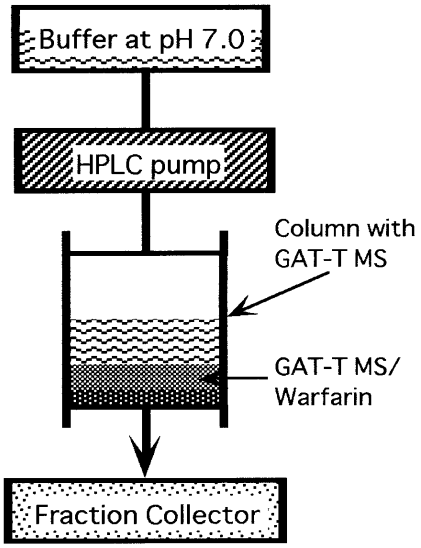


Fig. 1 Schematic diagam of dynamic flow column.

540 spectrophotofluorometer. The concentration of GAT-T was $1x10^{-5}$ mol/l. An excitation wavelength of 292 nm was used for the measurements, since Trp residues within GAT-T exhibit fluorescence maxima at this wavelength.

Release measurement of warfarin was performed using a dynamic flow column as shown in Figure 1. Buffer solution at pH 7 was pumped through the column at 0.4 ml/min with HPLC pump. Fractions were collected every 30 min for 20 hr. Each fraction was analyzed at 305 nm by UV measurement to determine the warfarin concentration eluted from GAT-T MS.

To compare the strength of interaction between warfarin and MS, apparent diffusion coefficient (Dapp) was calculated from following equation,

$$Mt / C0 = (4Dap \cdot t/\pi)^{1/2}$$

where t is release time (sec), Mt is the amount of warfarin eluted from MS after t (mol), C0 is the total amount of warfarin bound by Ms (mol), Dapp is apparent diffusion coefficient (cm^2/sec). Dapp is given as the slop in plot of $4Dapp \cdot t/\pi$ versus t.

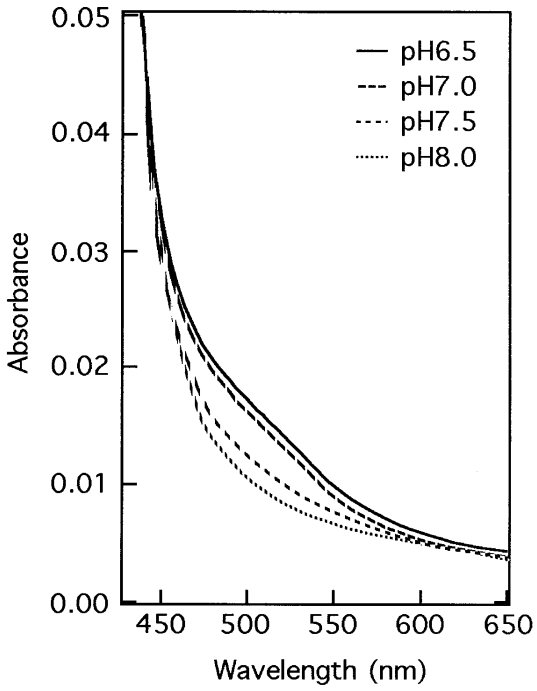


Fig. 2 pH dependence of VIS spectra of GAT-T.

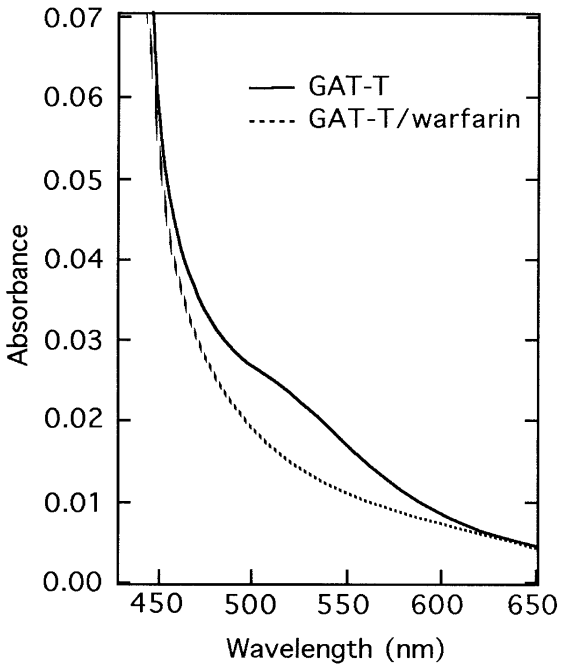


Fig. 3 VIS spectra of GAT-T at pH 7.0 in the presence and absence warfarin.

Results and Discussion

Interaction between GAT-T and warfarin

Figure 2 shows pH dependence of VIS spectra of GAT-T. GAT-T turned red below pH 7.0 as it absorbed the wavelength (around 500 nm) that appears as blue, and became yellow above pH 8.0 as it absorbed the wavelength (around 400 nm) that appears as bluish-purple to purple. GAT-T showed color change in the pH region of 7.0 to 8.0 through nitrogen atom in an indole ring dissociates positively (10)(11). Moreover, the dissociation pH of Trp residues within GAT-T could be

shifted to neutral pH by introduction of carboxyl groups in comparison with that of copoly(*N*-hydroxyethyl L-glutamin-*co*-L-Trp) treated with TFA (EGT-T) which has so far been used (10)(11).

Figure 3 shows VIS spectra at pH 7.0 in the presence and absence of warfarin. The absorption intensity of 500 nm of GAT-T was reduced and the color of the solution changed from red to yellow when warfarin was added. This result suggests that Trp residues treated with TFA interact strongly with bulky anionic drug such as warfarin. Figure 4 shows the variation of fluorescence intensity of GAT and GAT-T at pH 7.0 as a function of warfarin/Trp ratio. GAT and GAT-T exhibit their fluorescence maximum at 354 nm and 384 nm, respectively, when excited at 292 nm. Fluorescence due to GAT was slightly quenched by the addition of warfarin because of weak hydrophobic interaction between Trp residues and warfarin. On the other hand, the fluorescence intensity of GAT-T was reduced as the warfarin/Trp ratio increased. Moreover, since the fluorescence of GAT-T was quenched to a greater extent than that of untreated GAT, it is regarded that Trp residues within GAT-T interact with warfarin electrostatically besides hydrophobic interaction through the pKa value of warfarin is 4.8. Figure 5 exhibits pH dependence of fluorescence intensity of GAT-T in the presence of warfarin. When warfarin was added to the GAT-T solution, fluorescence intensity of GAT-T was reduced greatly as pH value decrease in comparison with the case of GAT-T alone. This result suggests that Trp residues treated with TFA come close to

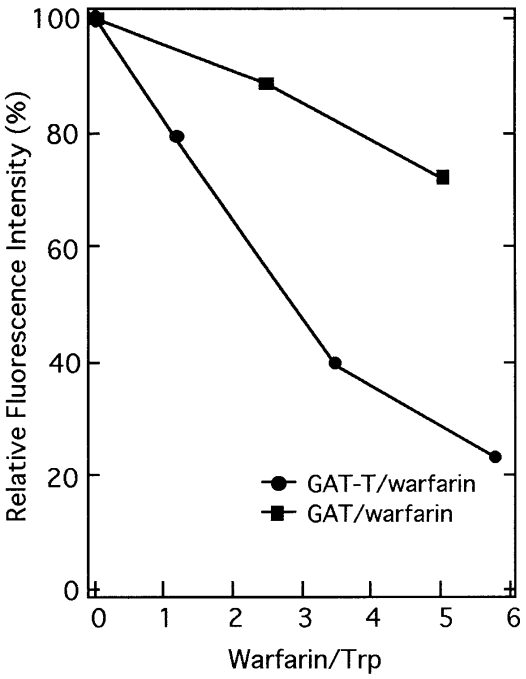


Fig. 4 Variation of fluorescence intensity as a function of warfarin/Trp ratio, ●: GAT-T/warfarin, ■: GAT/warfarin.

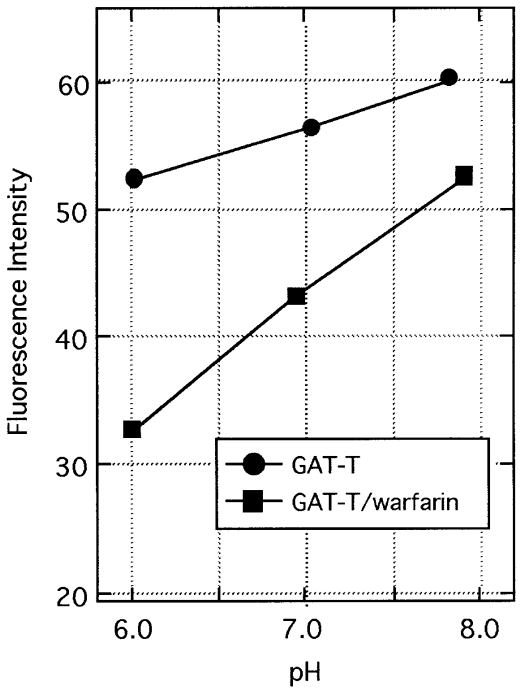


Fig. 5 pH dependence of fluorescence intensity of GAT-T in the presence and absence of warfarin.
GAT-T=warfarin=1×10^{-5} mol/l.

warfarin by electrostatic interaction besides hydrophobic interaction as pH decreases. From these results, it is confirmed that the electrostatic and hydrophobic interaction between Trp treated with TFA and warfarin causes the color change of GAT-T.

Release behavior of warfarin from copolypeptide MS

Release behavior of warfarin from copolypeptide MS at pH 7.0 was investigated using dynamic flow column. Figure 6 shows the results of release measurement of warfarin from untreated GAT and GAT-T MS. The amount of warfarin bound by untreated GAT MS was only 1.27×10^{-7} mol /40 mg through nitrogen atom of indole ring can not dissociate at pH 7.0.

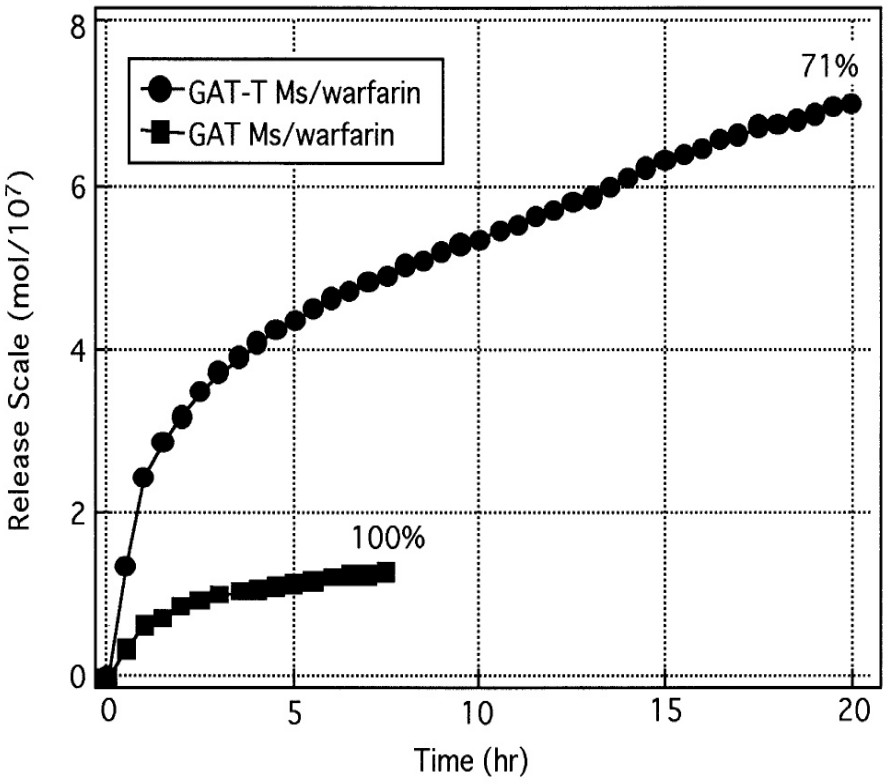


Fig. 6 Results of release mesurement of warfarin from GATand GAT-T Ms,
●: GAT-T MS, ■: GAT MS.

On the other hand, the amount of warfarin bound by GAT-T MS increased markedly (9.89×10^{-7} mol /40 mg) owing to the dissociation of Trp residues treated with TFA, and was about eight times that bound by untreated GAT MS. The retention time of warfarin in MS was also improved together with the binding amount: the amount of warfarin eluted from GAT-T MS was suppressed in 71 % even after 20 hr versus 100 % of warfarin that was eluted from untreated GAT MS within 7.5 hr. The calculated Dapp of warfarin from GAT-T MS was about 1/6 (4.48×10^{-6} cm^2/sec) that from untreated GAT MS (2.89×10^{-5} cm^2/sec) and this supports that Trp residues treated with TFA interact strongly with warfarin compared with untreated Trp residues. Furthermore, the color of GAT-T MS changed from yellow to red after all of warfarin had been eluted. Based on the results of release measurements, it was

confirmed that binding amount and retention time of warfarin was improved in GAT-T MS through Trp residues that have positive charge due to the treatment with TFA and that interact with warfarin electrostatically besides hydrophobic interaction, and the color change in GAT-T MS was taken place due to absorption or desorption by bulky anionic drug such as warfarin.

As previously mentioned, Trp residues within GAT-T interact with warfarin electrostatically besides hydrophobic interaction by the treatment with TFA and the color change of GAT-T was caused by interaction with warfarin. Thus, a novel material that can monitor the interaction with Trp by color change can be prepared and it is expected to be applicable as a transdermal drug delivery system with accompanying color change.

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