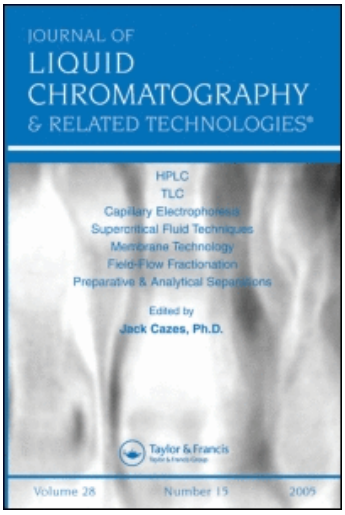

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PERFORMANCE OF ZWITTERIONIC AND CATIONIC FLUOROSURFACTANTS AS BUFFER ADDITIVES FOR CAPILLARY ELECTROPHORESIS OF PROTEINS

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ABSTRACT

In this work, a study has been made of the performance of a zwitterionic fluorosurfactant and mixtures of a zwitterionic and a cationic fluorosurfactant, when used as buffer additives in capillary electrophoresis. Thus, it showed to be possible to change the direction of the electroosmotic flow by changing the pH of the buffer solution. Possible mechanisms for the behaviour of the electroosmotic flow at different pH and when using different surfactant combinations are suggested.

High efficiency separations of some model proteins can be obtained, when a mixture of a cationic and a zwitterionic fluorosurfactant is added to the running buffer solution. By changing concentration proportions between the surfactants, a change in separation selectivity is obtained. This procedure provides an alternative way for selectivity tuning in protein separations by capillary electrophoresis.

INTRODUCTION

Capillary electrophoresis has been recognized as a highly promising tool for separation of biomolecules, and a considerable effort has been made to improve the performance of this technique. In particular, separation of proteins has been a target object of several research groups (*e.g.*1). One of the most important problems recognized in this context has been the tendency of proteins to adhere onto the capillary inner wall. This leads to a severe degradation of the separation performance, and in some cases, even an irreversible adsorption of solutes can occur.

Several methods for suppression of protein adsorption have been proposed. A variety of permanent wall coatings, with the purpose of shielding the silanol groups of the silica surface have been presented (*e.g.* 2-10). However, the drawbacks of permanent wall coatings include time consuming and labourious procedures, sensitivity towards buffer pH extremes, and limited column life time.

An alternative to permanent coatings is the use of a running buffer with a high salt concentration, where the increased ionic strength leads to a competitive interaction with the negative sites of the wall (11). Other means to decrease adsorption problems are to use either a low buffer pH, resulting in a more neutral wall charge, as the silanols are protonized (12), or a high buffer pH, leading to a negative charge of both the wall and the solutes, which counteracts wall interactions by electrostatic repulsion (13). However, the use of extreme buffer pH values or buffers with a high ionic strength can cause either protein denaturation, or excessive electric currents and joule heating.

Previously, we have presented a concept where a cationic fluorosurfactant was employed as a buffer additive. The fluorosurfactant

forms an admicellar bilayer. This leads to a charge reversal and an electrostatic repulsion of positively charged proteins from the wall. A considerable improvement in protein bandshapes, separation efficiencies and reproducibility was obtained. (14, 15).

An interesting possibility to decrease adsorbtion, is by using of zwitterions as additives to the running buffer. Such compounds have a similar effect as adding salts, however without causing the drawback of an increased electrical conductivity (16-18). Zwitterions have also been incorporated in polyacrylamide layers of wall coated capillaries (19). The unique characteristics of zwitterions in addition to our encouraging earlier results obtained with fluorosurfactants, initiated the present study, where the behaviour of a zwitterionic fluorosurfactant as well as combinations of fluorosurfactants as a buffer additive was investigated.

EXPERIMENTAL

A custom made CZE apparatus, which is described elsewhere (14), was used. A UV-detector (Model 200, Linear Instruments, Reno, NV, USA), including a capillary cell (model 9550-0155, Linear Instruments) was employed for on-column detection. Injections were carried out by timer-controlled electromigration. Prior to use, new capillaries (fused silica, 50 μm ID, *ca.* 60 cm total length, and 50 cm length from the injection point to the detection window) were flushed with 0.4M NaOH for 30 min - 1 hour and then to neutrality with water, and finally with running buffer for 20 minutes. When a change between buffers of different compositions was made, the capillary was rinsed for 20 minutes with the new buffer. In some cases, a stabilization of the surface conditions was obtained by applying a voltage (20kV) across the capillary for 20 minutes. The cationic surfactant FC134 -

($C_8F_{17}O_2NH(CH_2)_3N^+(CH_3)_3I^-$) (3M Company, St. Paul, MN, USA) and the zwitterionic fluorosurfactant - ($F(CF_2CF_2)_{3-8}CH_2CH(OCOCH_3)CH_2N^+(CH_3)_2CH_2CO_2^-$) (Dupont, Wilmington, DE, USA) were added to the running buffer, either as individual additives or in different combinations, at concentrations ranging between 10 and 400 μm/ml. The model proteins myoglobin, ribonuclease, trypsinogen and lysozyme (Sigma, St. Louis, MO, USA) were used in concentrations between 0.02 mg/ml and 0.04 mg/ml each. The total protein concentration of the samples was kept below 0.2 mg/ml.

RESULTS AND DISCUSSION

The proposed mechanism, causing the effective reduction of protein adsorption, when a cationic fluorosurfactant (FC134) is added to the running buffer has been described earlier (14, 15). One of the important characteristics of this additive is the formation of an admicellar bilayer at the capillary wall surface. This leads to a positively charged wall , which will repell positively charged solutes. The use of a zwitterionic fluorosurfactant presents opportunities to change the magnitude and sign of the charge on the wall, by changing the buffer pH. This drastically influences the electroosmotic flow (EOF) and associated separation characteristics, as will be shown below.

To determine the pI of the zwitterionic fluorosurfactant, the electrophoretic migration time of a neutral compound (mesityl oxide) was determined in a series of experiments, where buffers with different pH values, containing the surfactant, were used. The pI showed to be around 8 (figure 1). When a negative potential is applied at the inlet side of the capillary, and the pH is kept below 8, the EOF is in the direction from the

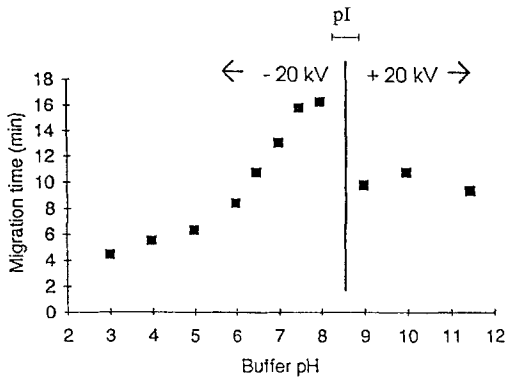

FIGURE 1. Migration time of mesityloxide *vs.* buffer pH.
200 µg/ml zwitterionic fluorosurfactant added to the running buffer.
Applied voltage: -20 kV (pH3-8), +20 kV (pH 9-11.5).

cathode to the anode. The EOF decreases with increased pH, since the positive charge of the zwitterion is decreasing. Above pH 8 , the zwitterion changes sign of charge, and the compound will then behave as an anionic additive. At this point the electroendosmotic flow reverses towards the direction of the cathode.

Recently, we observed that combinations of different fluorosurfactants added to the running buffer can be used to influence the separation selectivity between different proteins (20). This gave raise to the question how different mixtures of the zwitterionic and the cationic fluorosurfactant, used at different buffer pH, would influence the electroendosmosis, the separation selectivity and protein peak shapes.

In a first study, the migration time for mesityl oxide was measured as a function of the buffer pH, where a mixture of the cationic and the zwitterionic fluorosurfactant had been added. The results are shown in

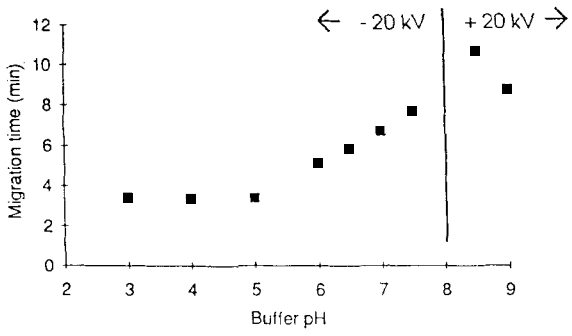

FIGURE 2. Migration time of mesityloxide *vs.* buffer pH.
100 µg/ml zwitterionic fluorosurfactant and 50 µg/ml cationic fluorosurfactant (FC 134) added to the running buffer.
Applied voltage: -20 kV (pH 3-8), +20 kV (pH 8.5-9).

figure 2, and it can be seen that the turnover pH for flow reversal is the same as in the case where only the zwitterion was added (*c.f.* fig. 1). However, in the range between pH 3 and pH 8, the use of the surfactant mixture resulted in a more constant and also higher electroosmotic flow rate (as can be observed from the migration data in fig. 2). It is fairly obvious that these results are related to the behaviour of the surfactant bilayer on the capillary wall. An increased charge density is obtained when the cationic compound is added to the zwitterion. This explains the differences in migration rates between the results shown in fig. 1 and 2. Also, the zwitterion rapidly loses charge, as the pH is raised from 3 to 8. In the case where the cationic surfactant is also present, the charge at the wall will be more dense and stable, until a pH is reached, where negative charges from the zwitterion begin to interact with the quarternary ammonium group of the cationic surfactant.

Figure 3 shows a proposed model for the appearance of the bilayers at different pH conditions.

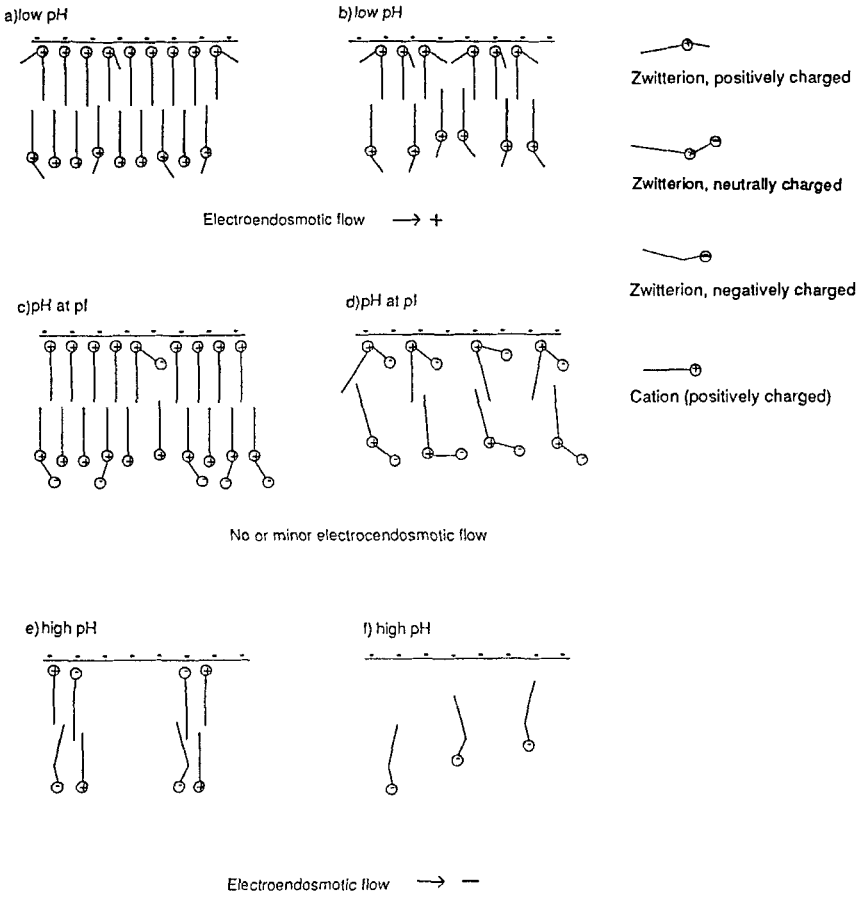

FIGURE 3. Proposed models for adhered layers of fluorosurfactants on a silica wall.
a, c and e) : buffer solution containing a cationic and a zwitterionic fluorosurfactant. b, d and f) : buffer solution where only a zwitterionic fluorosurfactant is added. a and b) : The pH of the buffer is below the isoelectric point of the zwitterion.
c and d: The pH of the buffer is at or near the isoelectric point of the zwitterion. e and f: The pH of the buffer is above the isoelectric point of the zwitterion.

In subsequent investigations, the influence of having different proportions of the two fluorosurfactants on the magnitude of the EOF was examined. In all experiments, a mixture of the two surfactants was employed, where the concentration of either one of the two compounds was varied, while the concentration of the other fluorosurfactant was kept constant. The migration speed of mesityl oxide was measured at pH 7, (where both surfactants are positively charged). At this pH value, an increased concentration of zwitterion surfactant resulted in longer migration times (a decrease of the EOF). On the other hand, a constant concentration of the zwitterion and a increase of the cationic surfactant FC134 resulted in shorter migration times. The results are shown in table 1. The obtained results can be explained in the following way: At pH 7, the cationic surfactant FC134 has a higher net positive charge then the zwitterion, which has lost a large part of its positive charge at this pH value. If the relative amount of cationic fluorosurfactant is increased, the positive charge of the bilayer at the wall will increase, which leads to an increased electroendosmotic flow.

At pH 9, the two surfactants are oppositely charged, which results in a more complex behaviour of the mixture. It has been suggested that cationic surfactants will co-adsorb together with anionic surfactants from such mixtures and form ion pairs onto a negatively charged surface (21-23, figure 3 e). Thus, when adding the cationic surfactant to a solution, containing the zwitterionic surfactant, a form of " charge-buffering" action is obtained. This suggestion corresponds well with the results obtained from the experiments (table 2 - upper part). A progressive increase of cationic surfactant did not influence the EOF to an appreciable extent, as long as a sufficient excess of the zwitterion was present.

When the concentration of the cationic surfactant was kept constant, and the concentration of the zwitterion was changed, the results obtained were

TABLE 1

Migration Times for Mesityl Oxide Using Different Concentrations of Fluorosurfactant Buffer Additives.

Concentration of cationic fluorosurfactant (μg/ml)	Migration time for mesityl oxide (min)
10	8.7
25	7.4
50	6.2
Concentration of zwitterionic fluorosurfactant (μg/ml)	**Migration time for mesityl oxide (min)**
25	5.6
100	6.2
400	7.0

Buffer pH = 7

Upper part of the table: Different cationic fluorosurfactant concentrations, while the zwitterionic fluorosurfactant concentration was 100 μg/ml in all experiments.

Lower part of the table: Different zwitterionic fluorosurfactant concentrations, while the cationic fluorosurfactant concentration was 50 μg/ml.

The applied voltage was- 20 kV.

significantly different (table 2 - lower part). At present, we have only a partial explanation for this behaviour. It is known that a mixture of oppositely charged fluorosurfactants behaves in a non-ideal way, which makes it difficult to predict the influences of surfactant concentration changes on the EOF. It is known for example (21) that the critical micellar concentration (CMC) for such mixtures is considerably lower than the average value, calculated from the CMC values of the individual components. Also, there can be a difference in composition between the micelles, the bilayer at the wall, and the free monomer solution (24-29).

TABLE 2
Migration Times for Mesityl Oxide When Different Concentrations of Fluorosurfactant were Added to the Running Buffer.

Concentration of cationic fluorosurfactant (μg/ml)	Migration time for mesityl oxide (min)
10	7.6
25	7.5
50	7.9
Concentration of zwitterionic fluorosurfactant (μg/ml)	**Migration time for mesityl oxide (min)**
25	9.2
100	7.9
400	10.2

Buffer pH=9
Upper part of the table: Different cationic fluorosurfactant concentrations, while the zwitterionic fluorosurfactant concentration was 100 μg/ml in all experiments.
Lower part of the table: Different zwitterionic fluorosurfactant concentrations, while the cationic fluorosurfactant concentration was 50 μg/ml.
The applied voltage was + 20 kV.

These differences in distribution of the surfactants can change, as the proportions between the individual surfactants are altered.

In view of the results obtained, it was of interest to investigate, how the presence of the fluorosurfactants would influence the CE separation behaviour of proteins. Thus, evaluations, using some model proteins (table 3) were carried out in presence of either the zwitterionic fluorosurfactant, or a combination of the cationic and the zwitterionic surfactant.

Figure 4a, b and c show the electropherograms recorded at pH 3, 4 and 5 respectively in presence of the zwitterionic fluorosurfactant. Significant differences in selectivity can be noted. However, the separation efficiency

TABLE 3
Characteristics of the Four Model Proteins.

Protein	Isoelectric point (pI)	Molecular weight (Mw)
Myoglobin	7.4	17 000
Trypsinogen	9	24 000
Ribonuclease	9.6	13 700
Lysozyme	11	14 300

does not exceed 60.000 theoretical plates, which is considerably below the efficiencies, that were obtained in an earlier study (14), where we used the cationic fluorosurfactant as an additive. We attribute the inferior efficiency to the lower density and charge of the admicellar bilayer of zwitterions. As the pH increases, the charge and bilayer density will be further reduced, and wall interactions become more pronounced. This can also be part of the cause for the decrease of the peak height for the most basic protein, lysozyme, at pH 5. (fig. 4 c)

Considerably improved results can be obtained when combinations of the zwitterionic and the cationic surfactant FC 134 are added to the running buffer. However, a concentration of not less then ca 50 µg/ml FC 134 is needed to obtain a sufficiently dense admicellar bilayer. An initial trial with a low FC 134 concentration (10 µg/ml) did not give satisfactory results (figure 5 a). The separation between the trypsinogen and ribonuclease was poor, and the separation efficiency was moderate. When the FC 134 concentration was increased to 50 µg/ml, a distinct improvement of both the selectivity and the efficiency was obtained (ca 200 000 theoretical plates). By changing the concentration of the zwitterion, the selectivity of the separation can be influenced, while the pH of the buffer is kept at a constant value (figure 5 b - d).

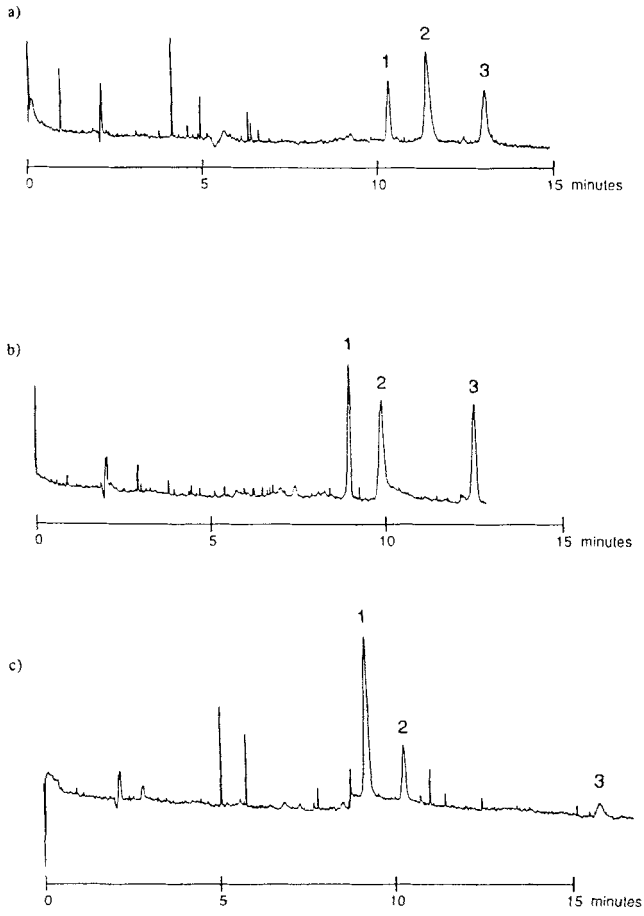

FIGURE 4. Electropherograms of model proteins in presence of the zwitterionic fluorosurfactant at different buffer pH. a) pH 3, b) pH 4, c) pH 5. Zwitterion concentration: 200 µg/ml. Running buffer: 0.02 M phosphate. Applied voltage: -20 kV. Injection: Electromigration at 10 kV for 10 sec. Total protein concentration: 0.2 mg/ml. Wavelength: 210 nm.Compounds: 1. Myoglobin. 2. Ribonuclease. 3. Lysozyme.

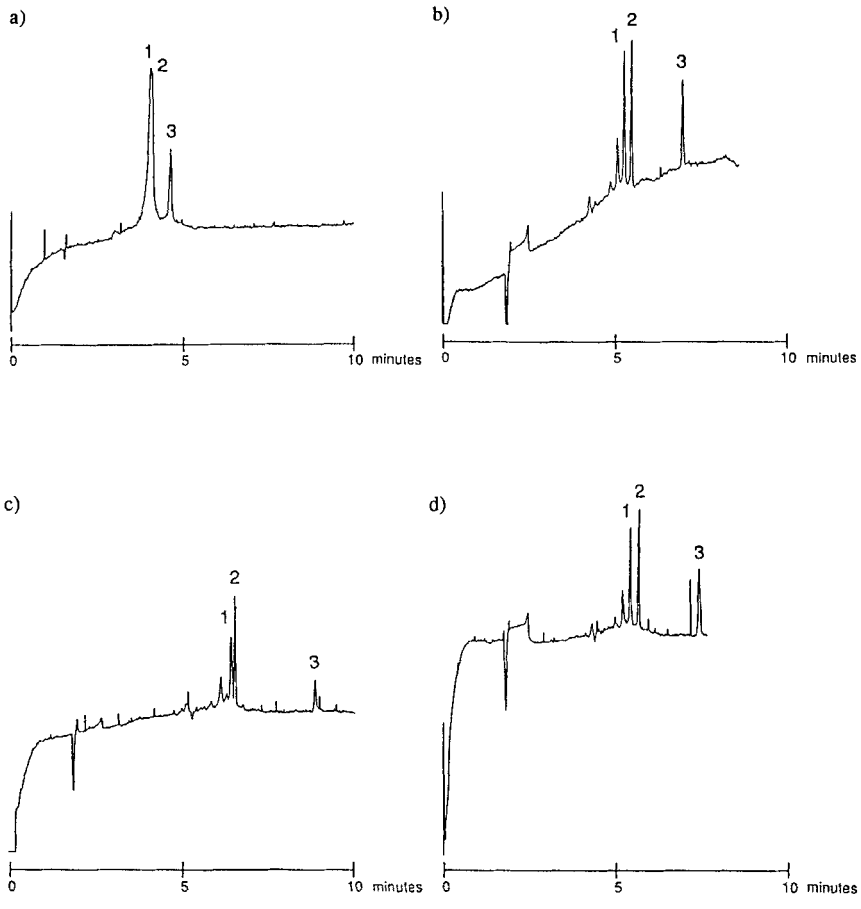

FIGURE 5. Electropherograms of model proteins in presence of a zwitterionic and a cationic fluorosurfactant
a) Concentration of the zwitterion: 100 μg/ml, Concentration of the cation: 10μg/ml. b) Concentration of the zwitterion: 25 μg/ml, Concentration of the cation: 50μg/ml. c) Concentration of the zwitterion: 100 μg/ml, Concentration of the cation: 50μg/ml. d) Concentration of the zwitterion: 400 μg/ml. Concentration of the cation: 50μg/ml. Running buffer: 0.01 M phosphate buffer at pH 4. Other conditions as in fig. 4. Compounds: 1. Trypsinogen. 2. Ribonuclease. 3. Lysozyme.

When comparing with results from our earlier work (14), where only the cationic fluorosurfactant FC 134 was added to the buffer, we found that the reproducibility of the migration times was lower, when surfactant mixtures were utilized. The migration time reproducibility of the separations in the present work are ca 2 - 3 % RSD. One of the reasons for this may be that the mixed systems are more sensitive to small pH changes. It should also be pointed out that the employed electrophoretic system did not have possibilities for temperature control or automated injection. It is likely, that the reproducibility can be improved by utilizing such facilities.

In conclusion, it showed to be possible to change the direction of the electroosmotic flow by a change of buffer pH, if a zwitterionic fluorosurfactant is added to the running buffer. This can also be accomplished when a mixture of a zwitterionic and a cationic fluorosurfactant are utilized.

A high separation efficiency and short migration times can be obtained for some basic proteins, when mixtures of the fluorosurfactants are employed as buffer additives zwitterionic fluorosurfactant. In this context, a number of issues, like quantitative aspects, interaction of the fluorosurfactants with the proteins etc. still need to be further investigated. However, we have shown that the separation selectivity can be tuned at a particular pH, by changing the individual concentrations of the additives. This charge-tuning method should provide new possibilities for optimization of protein separations.

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