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Journal of Chromatography A, 1057 (2004) 101–106
JOURNAL OF CHROMATOGRAPHY A
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Protein separation with surfactant-coated octadecylsilyl silica involving Cibacron blue 3GA-conjugated nonionic surfactant

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Received 17 May 2004; received in revised form 14 September 2004; accepted 21 September 2004

Abstract

A novel medium for protein separation, namely affinity admicelle, was prepared by mixing of octadecylsilyl (ODS) silica gels, a polyoxyethylene-type nonionic surfactant (Triton X-100), and a surfactant-conjugated substrate (affinity ligand) in an aqueous solution. The ligand was synthesized by mixing a triazine dye (Cibacron Blue 3GA, CB) and a polyethylene glycol monooleyl ether ($C_{18}EO_7$, $C_{18}EO_{10}$, or $C_{18}EO_{20}$) having different length of polyoxyethylene moiety in weakly alkaline solutions. The amount of Triton X-100 sorbed on 1 g of ODS silica was 0.2 mmol. Affinity ligands having highly hydrophobic oleyl group were predominantly sorbed on ODS silica. The losses of Triton X-100 and affinity ligand were within 0.3% and negligible by washing the admicelles were with a 25-fold volume of 1 mM Tris–HCl solution (pH 7.4). The coating ODS silica with Triton X-100 was effective to prevent the irreversible sorption of albumin (bovine, serum). An NADH-dependent enzyme, alcohol dehydrogenase (ADH, yeast), was successfully collected on the admicelles involving CB-conjugated ligands (CB-$C_{18}EO_{20}$). The maximum collection of ADH to 90 mg/ml of affinity admicelles was $68 \pm 4\%$. However, CB-$C_{18}EO_7$ and CB-$C_{18}EO_{10}$ having shorter polyoxyethylene unit were not available, suggesting the requirement of the spacer moiety in the affinity ligand. The recovery and purification factor based on the ratio of activity (unit)/protein (mg) from Whatman DE52-treated yeast extract was 27% and 12, respectively.

Keywords: ODS; Silica; Affinity ligand; Cibacron blue; Nonionic surfactant; Micelle; Alcohol dehydrogenase; Protein purification

1. Introduction

Surfactant molecules can be cooperatively sorbed to form aggregates (namely admicelles or hemimicelles) on the surfaces of different kinds of solid materials by hydrophobic or electrostatic interactions [1–3]. Since the aggregates provide hydrophobic media, a wide range of hydrophobic compounds are efficiently incorporated into them. Since admicelles were prepared by just mixing appropriate combination of surfactant and solid materials, their potential as the media for the collection of traces of hydrophobic substances in water has been extensively studied [1–5].

The admicelles were found to be useful media for the rapid and efficient concentration of trace hydrophobic compounds prior to several instrumental analyses [6–13]. When appropriate chelating agents were incorporated into the system, passing a sample through the admicelle-filled column also resulted in the collection of heavy metal ions in water [10,14]. These results suggest that any specific interaction for a certain compound can be introduced by impregnating selective hydrophobic ligand molecules into the admicelles.

We have studied the potential to use admicelles as novel media for protein separation [15]. A surfactant-conjugated artificial dye substrate (affinity ligand) was successfully sorbed on surfactant-coated porous polystyrene just by mixing the ligand, surfactant, and polystyrene in the aqueous buffer solution. When an artificial dye, Cibacron blue 3GA (CB), being

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doi:10.1016/j.chroma.2004.09.070

accepted as an analogue of NADH, was used as a substrate, albumin, alcohol dehydrogenase (ADH), and lysozyme were collected on the surfactant-coated polystyrene particles involving the ligand, while ovalbumin having no binding ability to CB was negligibly recovered. Particularly, lysozyme was well collected and successfully purified from chicken egg white. Just mixing support materials, surfactants, and ligand molecules can prepare the separation media. One can prepare affinity ligands for the desired biomolecules. However, the recovery of an NADH-dependent ADH was insufficient. The specific binding of ADH to CB has been widely utilized in affinity chromatography or aqueous two-phase extraction. Weak sorption or unfavorable orientation of affinity ligands may be the possible reason of the low extractability.

In the present study, octadecylsilyl (ODS) silica gel was used as a support material for achieving the stable sorption of surfactants or affinity ligands and their favorable orientation for protein interaction. A polyoxyethylene-type nonionic surfactant, Triton X-100, was employed because it had been accepted as a mild surfactant for wide range of proteins. The bio-mimetic affinity ligand (CB-$C_{18}EO_n$) was synthesized by conjugating CB with nonionic surfactants, polyethylene glycol monooleyl ethers ($C_{18}EO_n$), having different length of polyoxyethylene moieties. The length of polyoxyethylene moiety and the concentrations of the surfactant and affinity ligand were optimized.

2. Experimental

2.1. Reagents

Octadecylsilyl silica gel (ODS silica, Wakogel 50C18, 38–63 μm, for column chromatography) was obtained from Wako (Tokyo, Japan). After washing with ethanol, the ODS silica was equilibrated with 1 mM Tris–HCl buffer solution (pH 7.4). A nonionic surfactant, Triton X-100 (polyethylene glycol mono-*p*-isooctylphenyl ether, for biochemistry), was obtained from Nakarai Tesque (Kyoto, Japan). Polyethylene glycol monooleyl ethers [$C_{18}EO_n$ ($n = 7$, 10, 20)] were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Albumin (bovine serum, BSA), ovalbumin (chicken egg white), alcohol dehydrogenase (yeast), and Cibacron blue 3GA (CB), were purchased from Sigma (St. Louis, MO, USA). A buffer component, 2-amino-2-hydroxymethyl-1,3-propanediol (Tris, for biochemistry), and other reagents were obtained from Wako. Water used was prepared with a Milli-Q reagent water system (Millipore, St. Louis, USA).

2.2. Preparation of affinity ligand

A bio-mimetic affinity ligand (CB-$C_{18}EO_n$) was prepared by the method for synthesizing CB-conjugated polyethylene glycol [16] or CB-conjugated Triton X-114 [17] with the minor modification. To 50 ml of aqueous mixture containing 1 g of $C_{18}EO_n$, 5 g of CB, and 3.5 g of sodium chloride, was added 1 ml each of 1-M lithium hydroxide at three intervals for 180 min. After the mixture was heated at 80 °C for 60 min, it was neutralized with acetic acid. The resulting solution was dialyzed against Milli-Q water. It was applied to Sephadex G-25 gel chromatographic column (inner diameter: 20 mm; length: 200 mm) and subsequently to DE52 cellulose (Whatman, Kent, UK) ion-exchange chromatographic column (inner diameter: 20 mm; length: 50 mm) for removing free CB and surfactant.

2.3. Preparation of affinity admicelles

To 1.0 ml of aqueous suspension containing 90 mg of ODS silica was added the aqueous mixture containing prescribed amounts of CB-$C_{18}EO_n$ and Triton X-100. After the suspension was gently mixed for 2 h, the ODS silica involving Triton X-100 and CB-$C_{18}EO_n$ (affinity admicelles) were washed with 1 ml of 1 mM Tris–HCl buffer solution (pH 7.4 at 4 °C) six times. The sorption yields of CB-$C_{18}EO_n$ and Triton X-100 were estimated on the basis of their spectrophotometric determination in the supernatant. The molar absorptivities [l (mol cm)$^{-1}$] of CB-$C_{18}EO_n$, being approximated by those of CB, were 6.82×10^3 at 603 nm and 1.58×10^4 at 288 nm, while those of Triton X-100 was 0 at 603 nm and 4.30×10^2 at 288 nm [15].

2.4. Protein sorption

A 1.0-ml portion of the aqueous buffer solution (1 or 10 mM Tris–HCl, pH 7.4) containing 0.1 mg/ml of an interested protein was poured into a 1.5 ml centrifuge tube in which affinity admicelles prepared from 90 mg of ODS silica gel were precedently placed. After the tube was gently mixed for 30 min, it was centrifuged at 1500 rpm for 10 s. All procedures were performed at 4 °C. The yields of total protein sorption were calculated from the concentration of the protein in the supernatant determined by Bradford method [18]. ADH activity was measured based on the hydrolysis of 4-nitrophenylphosphate [19,20]. A 100-μl portion of the sample solution was added to 2 ml of 50 mM Tris–HCl (pH 8.8) containing 1 mM NAD^+ and 0.5 M ethanol. The absorbance at 340 nm was measured for monitoring the generation of NADH or the consumption of NAD^+. One unit of the activity was defined by the consumption of 1 μmol NAD^+ per 1 min.

2.5. Purification of ADH from yeast

A complex medium (MY) was prepared by adding 1.5 g of yeast extract, 1.5 g of malt extract, 2.5 g of polypeptone, and 5 g of glucose into 500 ml of water and, then, was autoclaved at 121 °C for 20 min. Yeast (bakers' dry yeast, Oriental Yeast, Tokyo, Japan) was cultivated in 25 ml of MY medium at 30 °C for 2 h. After the yeast suspension was centrifuging at 3000 rpm for 10 min, the precipitate was triplicate washed with 800 μl of 5 mM Tris–HCl solution (pH 8.0) containing 1 mM (±)-dithiothreitol and 10 mM EDTA. Then, the sus-

pension was homogenized with an ultrasonic homogenizer (MST UH-50, 50 W, 20 kHz) at 0 °C for 2 min. The yeast extract used was the supernatant obtained by centrifuging the homogenate at 15,000 rpm for 10 min.

Next, the yeast extract was passed through the column filled with 1 ml of Whatman DE52 cellulose to remove lipid components. Then, protein components sorbed on DE52 were eluted with 1 mM Tris–HCl buffer (pH 7.4) containing 3 M sodium chloride. After the eluate was diluted with five-fold volume of water, 1 ml of the resulting solution was added into a centrifuge tube in which 90 mg of affinity admicelles were precedently placed. The conditions for the collection were the same as described above. After the supernatant was removed, the admicelles were triplicate washed with 1 mM Tris–HCl (pH 7.4). ADH sorbed on the admicelles was eluted by gentle mixing with 1 ml of 50 mM phosphate buffer (pH 8.5) containing 30% (v/v) glycerol. All procedures after the homogenization were performed at 4 °C for minimizing protein degeneration.

3. Results and discussion

3.1. Formation of affinity admicelles

As shown in Fig. 1, Triton X-100 in the aqueous solution was well sorbed onto ODS silica because of the hydrophobic interaction between ODS and Triton X-100. The strong sorption of polyethoxylated alkylphenols onto ODS silica has been extensively used for their concentration in water analysis [21–23]. The time for their equilibrium sorption was within 2 h. Maximum sorption of Triton X-100 on 1 g of ODS silica gel was ca. 0.2 mmol. On the other hand, CB-$C_{18}EO_{20}$ added in the solution was quantitatively (>99%) sorbed on Triton X-100-coated ODS silica. The ligand having a highly hydrophobic oleyl-group preferentially sorbs onto ODS silica.

The loss of Triton X-100 from 90 mg of ODS silica-based admicelles was within 2% even after six-times washing with 1 ml of 1 mM Tris–HCl (pH 7.4). The stability seems to be sufficient for treating a few ml of solution. Additionally, negligible loss of CB-$C_{18}EO_{20}$ was observed on the basis of the measurement of the absorption ($A < 0.001$) at 610 nm. Very stable sorption of the affinity ligand is advantageous for collecting the objective protein without the loss of the ligand.

3.2. Collection of proteins to affinity admicelles

In the selective protein purification, nonspecific binding of proteins to the separation media has to be eliminated. BSA was used for studying the effect of Triton X-100 coverage on the elimination of nonspecific binding of protein to the admicelles. BSA is accepted as a protein that can be strongly sorbed on hydrophobic surfaces and, hence, is utilized as a blocking agent for preventing nonspecific binding of biomolecules to surfaces [24,25] or as a reagent for evaluating nonspecific binding [26,27]. In the absence of Triton X-100, BSA sorbed to the ODS silica gels (Fig. 2). Since ODS silica is a strong protein sorbent [28,29], BSA once sorbed on it hardly be eluted with any eluents. On the other hand, the collection of BSA decreased with increasing the sorption of Triton X-100 and, then, became negligible above 4.8 μmol of Triton X-100 (Fig. 2). Triton X-100 coated on the ODS silica surfaces can prevent the irreversible sorption of BSA.

Next, an affinity ligand, CB-$C_{18}EO_{20}$, was accompanied to the Triton X-100-coated ODS silica for providing specific interaction to ADH. As shown in Fig. 3, the collection of ADH increased with increasing the fraction of CB-$C_{18}EO_{20}$. In contrast, ovalbumin (chicken egg white) having no specific interaction to CB was negligibly collected (Table 1). CB specifically interacts with a series of NAD^+-dependent enzymes including alcohol dehydrogenase and extensively employed as a bio-mimetic dye-affinity ligand for separating these proteins in column chromatography or aqueous two-phase separation system [30–32]. The results in the present

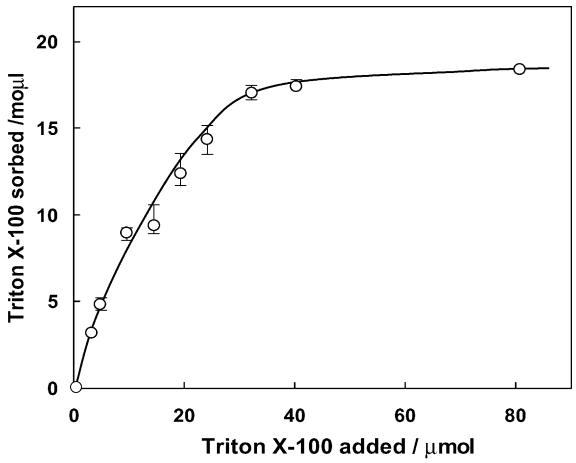

Fig. 1. Sorption of Triton X-100 onto 90 mg of ODS silica gels in 1 ml of 1 mM Tris–HCl (pH 7.4), 4 °C.

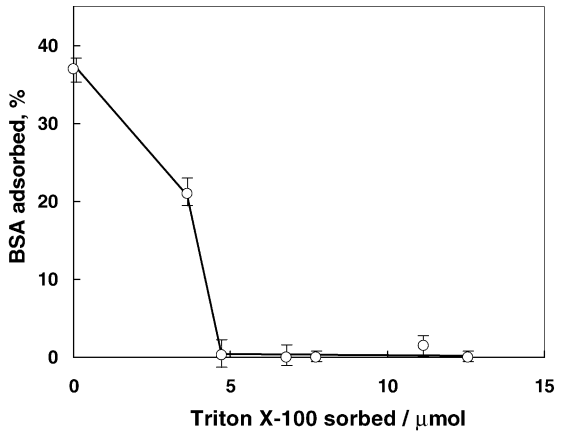

Fig. 2. Sorption of BSA onto Trition X-100-coated ODS silica gel. Solution volume: 1 ml, BSA: 0.1 mg, ODS silica gel: 90 mg, pH 7.4 (10 mM Tris–HCl), 4 °C.

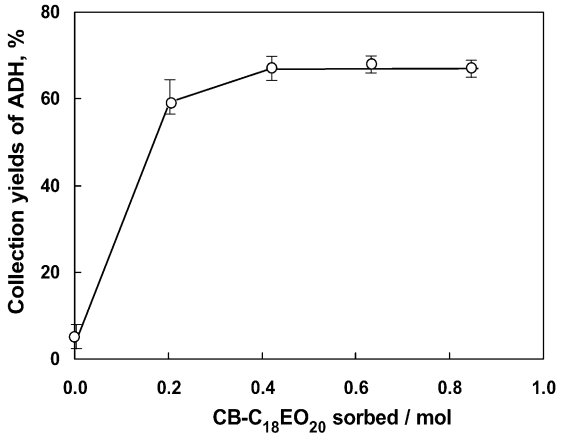

Fig. 3. Collection yields of ADH onto the affinity admicelles. Solution volume: 1 ml, ADH: 0.1 mg, ODS silica gel: 90 mg, Triton X-100: 4.8 μmol, pH 7.4 (1 mM Tris–HCl), 4 °C.

study indicate that CB-$C_{18}EO_{20}$ most likely acts as an affinity ligand.

In order to clarify the effect of spacer moiety of the ligand, CB-$C_{18}EO_n$s having different length of polyethoxy group were tested. When CB-$C_{18}EO_7$ or CB-$C_{18}EO_{10}$ having shorter polyethoxy chain was used, the collections of BSA and ADH were negligible (Table 2). These results are largely in contrast to the good protein recoveries in the use of the admicelles involving CB-$C_{18}EO_{20}$. In the conventional chemically bound affinity ligands, the spacer moiety that separates the substrate from the support was necessary for the binding of the substrate to protein [33,34]. Since Triton X-100 has 9.5 average EO length, the CB portion of CB-$C_{18}EO_7$ or CB-$C_{18}EO_{10}$ can be immersed by Triton X-100 molecules and, therefore, hardly interact with ADH. In contrast, 20 units of ethylene oxide of CB-$C_{18}EO_{20}$ seem to provide sufficient distance between CB and ODS silica surfaces coated by Triton X-100 having 9.5 ethoxy units (EO). This result strongly suggests the requirement of spacer moiety of the affinity ligand. The difference in the length of the polyethoxy moiety is longer than diaminopropanol (8′-azo-linked [35] or S^6-linked [35,36]) which has been utilized for the spacer arm.

Table 2
Effect of the length of ethoxy units of ligand on protein collection

Number of EO units	BSA collection (%)[a]	ADH collection (%)[b]
7	Negligible	Negligible
10	Negligible	Negligible
20	43 ± 3	68 ± 4

Average of three experiments; sample volume: 1.0 ml, ODS silica: 90 mg, Triton X-100: 4.8 μmol, affinity ligand: 0.78 μmol, protein: 0.1 mg.
[a] 10 mM Tris–HCl (pH 7.4).
[b] 1 mM Tris–HCl (pH 7.4).

The recovery of ADH (ca. 68%) was significant improvement on comparing with that in the polystyrene-based affinity admicelles (ca. 20%) [15]. In the case of polystyrene support, the use of CB-conjugated polyethylene glycol decyltetradecyl ether, having 20 ethoxy units, also was not effective for improving ADH recovery [37]. The study of electrokinetic characterization indicates that nonionic surfactants having long polyethoxy group form inhomogeneous flat bilayer on polystyrene surface [38]. If considerable portion of polyethoxy moiety is immersed by the bilayer, the orientation of CB for protein-ligand binding may be quite restricted. In contrast, surfactants and affinity ligands likely form monolayer-like organization on ODS silica existing linear alkyl chains. Such structure is necessary for working polyethoxy moiety as the spacer of affinity ligand. Thus, ODS silica seems to be superior for the support of affinity admicelle.

Next, the effect of surfactant coverage on ADH collection was also investigated. In contrast to the good protein recoveries in the use of affinity admicelles involving 4.8 μmol of Trition X-100, the recoveries significantly diminished by the surface coating with the further amount of Triton X-100, even in the presence of the larger amount of affinity ligand (Table 3). As already shown in Fig. 2, 4.8 μmol of Triton X-100 covering the surfaces of 90 mg ODS silica throughout, was enough for preventing nonspecific binding of proteins. Further amount of Triton X-100 possibly forms bulky aggregates that hinder the ligand-protein binding. In the present study, the admicelles composing of 4.8 μmol Triton X-100 and 0.78 μmol of CB-$C_{18}EO_{20}$ were used for protein collection.

Table 1
Collection of proteins to ODS silica, Triton X-100-coated ODS silica, and affinity admicelles

Protein	Recovery (%)		
	ODS silica[a]	Triton X-100-coated ODS silica[b]	Affinity admicelles[c]
BSA[d]	39 ± 6	0 ± 1	43 ± 5
ADH[e]	68 ± 3	9 ± 2	68 ± 4
Ovalbumin[d]	28 ± 4	0 ± 1	0 ± 1

Average of three experiments; solution volume: 1.0 ml, protein: 0.1 mg.
[a] ODS silica: 90 mg.
[b] ODS silica: 90 mg, Triton X-100: 4.8 μmol.
[c] ODS silica: 90 mg, Triton X-100: 4.8 μmol, CB-$C_{18}EO_{20}$: 0.78 μmol.
[d] 10 mM Tris–HCl (pH 7.4).
[e] 1 mM Tris–HCl (pH 7.4).

Table 3
Effect of the amount of Triton X-100 on protein collection

Triton X-100 added (μmol)	Triton X-100 sorbed (μmol)	BSA collection (%)[a]	ADH collection (%)[b]
4.84[c]	4.81 ± 0.01	43 ± 4	68 ± 4
9.68[d]	8.96 ± 0.39	7 ± 3	7 ± 5
14.52[d]	9.38 ± 0.28	Negligible	Negligible

Average of three experiments; solution volume: 1 ml, ODS silica: 90 mg, protein: 0.1 mg.
[a] 10 mM Tris–HCl (pH 7.4).
[b] 1 mM Tris–HCl (pH 7.4).
[c] Amount of CB-$C_{18}EO_{20}$: 0.78 μmol.
[d] Amount of CB-$C_{18}EO_{20}$: 1.6 μmol.

Table 4
Purification of ADH from yeast extract

	Total protein (mg/ml)	Activity (units/ml)	Specific activity (units/mg protein)
Yeast extract	1.3	0.88	0.70
DE52	0.89	1.1	1.8
Five-fold dilution	0.178	0.22	–
Affinity admicelles[a]	0.0074 ± 0.010	0.060 ± 0.003	8.3 ± 1.1

[a] Average of five experiments.

3.3. ADH purification from yeast extract

Finally, the affinity admicelles prepared in the present study were used for the purification of ADH from yeast extract. However, the recovery of ADH was quite low, when the yeast extract was directly applied. This may be ascribed to the interference of the lipid components from cell membranes. They tend to incorporate the admicelles and, thus, can significantly affect their property. The formation of vesicles or micelle-like aggregates may be another reason for the low recovery. An anion exchanger, Whatman DE52 cellulose, was used for removing the lipid components, because of the strong binding ability of the anion exchanger to the lipids [39,40].

Table 4 summarizes the results of purification of ADH from yeast extract. In this table, DE52 means the collection to a column filled with 2.5 ml of Whatman DE52 cellulose anion exchanger by loading 10 ml of yeast extract and the subsequent elution with 5 ml of eluate (1 mM Tris–HCl (pH 7.4) + 3 M NaCl). The concentration of total proteins decreased by the sorption to DE52, while the activity of ADH increased by the decrease of the solution volume. Admicelles indicates the purification with the affinity admicelles that had been prepared as described above.

In the ADH sorption to the admicelles, the concentration of buffer components significantly influenced the collection yields. Almost same yields (68 ± 4, $n = 6$) as the result of model experiment were obtained. A buffer component, Tris–HCl, significantly influenced the collection of ADH. In the presence of 10 mM Tris–HCl, the collection diminished to ca. 25%. On the other hand, salts and buffer components were available to induce the elution of ADH. Among the salts or buffer components, 50 mM phosphate buffer (pH 8.5) containing 30% (v/v) glycerol was the most effective for eluting ADH. However, the recovery of ADH in the purification step with the affinity admicelles was ca. 40%. Hydroxyl moieties of Triton X-100 molecules may bind to ADH and, therefore, hinder its elution. Further studies about the kind or the concentration of buffer components and additive will be necessary to improve the efficiency of the affinity admicelle-mediated protein purification.

The affinity admicelles may be comparable to ligand-conjugated polymer-coated matrix, in which the ligand can be introduced by coating alumina or iron oxide particles with ligand-conjugated polymers [41,42]. Both the methods provide simple way to introduce the plural ligand components with the quite easy manner. The development of the simple way of the ligand introduction would be important for efficient design of affinity separation media.

4. Conclusion

A novel separation medium, affinity admicelle, was prepared just by mixing ODS silica gels, surfactant (Triton X-100), and surfactant-conjugated artificial substrate (Cibacron blue 3GA). Collection of alcohol dehydrogenase based on the specific interaction between the protein and the substrate was successfully achieved. As a result of the optimization of the spacer moiety of the affinity ligand and of the surface coverage of the surfactant, ADH was effectively collected on the affinity admicelles. The studies about the combination of solid support, surfactant, and affinity ligand would extend the possibility of the application of the present method to the purification of a wide range of proteins.

Acknowledgment

This work was supported by the 21st Century COE Program “Nature-Guided Materials Processing” of the Ministry of Education, Japan.

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