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*Research Article*

# Basic Principles of MLC

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Micellar liquid chromatography (MLC) is an efficient alternative to conventional reversed-phase liquid chromatography with hydro-organic mobile phases. Almost three decades of experience have resulted in an increasing production of analytical applications. Current concern about the environment also reveals MLC as an interesting technique for "green" chemistry because it uses mobile phases containing 90% or more water. These micellar mobile phases have a low toxicity and are not producing hazardous wastes. The stationary phase is modified with an approximately constant amount of surfactant monomers, and the solubilising capability of the mobile phase is altered by the presence of micelles, giving rise to a great variety of interactions (hydrophobic, ionic, and steric) with major implications in retention and selectivity. From its beginnings in 1980, the technique has evolved up to becoming in a real alternative in some instances (and a complement in others) to classical RPLC with aqueous-organic mixtures, owing to its peculiar features and unique advantages. The addition of an organic solvent to the mobile phase was, however, soon suggested in order to enhance the low efficiencies and weak elution strength associated with the mobile phases that contained only micelles.

## 1. Introduction

Micellar liquid chromatography (MLC), which uses mobile phases containing a surfactant above its critical micellar concentration (CMC), is an alternative to conventional reversed-phase liquid chromatography and provides a solution to the direct injection of physiological or food samples by solubilising proteins (that are eluted together or shortly after the solvent front) [1–3]. The possibility of directly injecting samples into the chromatograph simplifies and expedites treatment, which confers analytical procedures greater accuracy and a lower cost.

The versatility of MLC is due to the wide variety of interactions that are established among the eluted solutes, the stationary phase, the aqueous phase and micelles. Their eluent characteristics allow the analysis of compounds with a wide range of polarities.

The presence of a surfactant not only modifies the interactions established inside the column but also reduces the necessary amount of organic solvent in the mobile phase, which can be recycled due to low evaporation. These characteristics are genuinely interesting given current concerns about reducing organic contaminant residues in laboratories.

MLC shares the basic components of reversed-phase liquid chromatographic (RPLC) systems, that is, a non-polar stationary phase and a polar aqueous mobile phase. However, hydro-organic mobile phases in conventional RPLC are homogeneous, whereas micellar solutions are microscopically heterogeneous, being composed of two distinct media: the amphiphilic micellar aggregates (micellar pseudophase) and the surrounding bulk water or aqueous-organic solvent that contains surfactant monomers in a concentration approximately equal to the CMC. On the other hand, the stationary phase is modified by the adsorption of surfactant monomers, creating a structure similar to an open micelle, and reducing silanophilic interactions. With nonionic surfactants, only the polarity of the stationary phase changes, whereas with ionic surfactants, a net charge (positive or negative) appears on its surface with major implications.

On the other hand, a new micellar chromatographic mode has been recently described: high submicellar chromatography [4, 5], where the surfactant forms micelles and

organic solvent content is high. This mode opens up a range of possibilities to new applications in this chromatographic technique and complements low submicellar chromatography (also known as ion pair chromatography) where the number of free molecules of the surfactant in the mobile phase is insignificant, but sufficient to cover the stationary phase.

It should be highlighted that the fundamental studies into MLC have served to develop the technique and to establish its theoretical basis, without which its later use in diverse applications would be impossible.

## 2. Particularities of the Micellar Mobile Phase

Micelles provide hydrophobic and electrostatic (for ionic surfactants) sites of interaction. In the micelles, three sites of solubilisation can be identified: the core (hydrophobic), the surface (hydrophilic), and the palisade layer (the region between the surfactant head groups and the core). Solutes associated to micelles experience a microenvironment that is different from that of bulk solvent [6].

Although pure micellar mobile phases are sometimes used, most separations in MLC are performed with hybrid micellar mobile phases in a buffered medium that contains micelles, surfactant monomers, molecules of organic solvent, and water. The organic solvent decreases the polarity of the aqueous solution and alters the micelle structure. Although the separation mode is still predominantly micellar in nature, the micelle is perturbed by the organic solvent. This can change micellar parameters, such as the CMC and surfactant aggregation number. A high percentage of organic solvent can disrupt the micelle structure. The maximal allowable concentration depends on the type of organic solvent and surfactant.

*2.1. Critical Micellar Concentration.* A suitable surfactant for MLC should have a low CMC. A high CMC would imply operating at high surfactant concentration, which would result in viscous solutions, giving undesirable high system pressure and background noise in UV detectors. The selection is often limited to the following surfactants: the anionic sodium dodecyl sulphate (SDS), the cationic cetyltrimethylammonium bromide (CTAB), and the nonionic Brij-35, whose main characteristics are summarized in Table 1. The CMC values of these surfactants in pure water are low enough for MLC. It should also be taken into account that the CMC is strongly affected by the presence of an organic solvent. The changes are related to the modification of the structure of the micelle, which also induces, at least partially, the reduced retention in MLC [7]. Recently, some novel ionic liquid-based surfactants like 1-hexadecyl-3-butylimidazolium bromide have been used in MLC [8, 9].

*2.2. Krafft Point.* The Krafft point is defined for ionic surfactants as the temperature at which the solubility of a surfactant monomer becomes equal to the CMC [12]. Below the Krafft point temperature, the solubility is quite low and the solution appears to contain no micelles. Chromatographic work in MLC should be conducted above this temperature to avoid surfactant precipitation. This means that the Krafft point should be well below room temperature. The Krafft point for SDS and CTAB is around 15°C and 20–25°C, respectively [13, 14].

Table 1: Characteristics of the most common surfactants in MLC[a].

| Surfactant | Molecular weight (g/mol) | CMC (mol/L) | R[b] (nm) | V[c] (L/mol) |
|---|---|---|---|---|
| SDS | 288.4 | $8.2 \times 10^{-3}$ | 2.5 | 0.246 |
| CTAB | 364.5 | $9 \times 10^{-4}$ | 3.2 | 0.364 |
| Brij-35 | 1198 (avg.) | $9 \times 10^{-5}$ | | |

[a]References [10] and [11]; [b]Micellar radius; [c]Molar volume.

Non-ionic surfactants also have a specific temperature, that if exceeded, phase separation occurs, which is called the cloud point [11, 15]. Chromatographic work with these surfactants should be conducted below this temperature (e.g., Brij-35, is ca. 100°C for aqueous 1–6% solutions, whereas for Triton X–100 this value is 64°C).

*2.3. pH of the Mobile Phase.* MLC employs the same packing materials as classical RPLC, which for conventional columns have a limited working pH range of 2.5–7.5. Appropriate pH values depend on the nature of the analytes and the surfactant selected. The pH of the micellar mobile phase is commonly fixed with phosphoric or citric acid buffers [2, 3]. For mobile phases containing SDS, potassium salts are not recommended as potassium dodecyl sulphate presents a high Krafft point and precipitates from aqueous solutions at room temperature [2].

*2.4. Organic Solvents: Types and Concentration.* The selection of the appropriate organic solvent modifier in MLC should consider the polarities of the analytes. For polar compounds, sufficiently short retention times (below 20 min) are obtained with 1-propanol, 2-propanol, or acetonitrile. For nonpolar compounds or compounds with high affinity for the surfactant adsorbed on the stationary phase, stronger solvents as 1-butanol or 1-pentanol are needed [16]. However, it should be noted that the two latter alcohols give rise to microemulsion formation at sufficiently high concentration [17]. In practice, the amount of organic solvent that can be added is limited by its solubility. It should be noted that at high organic solvent concentration, the micelles disaggregate and the mobile phase contains only free surfactant molecules. The organic solvent contents that preserve the integrity of micelles are below 15% for propanol and acetonitrile, 10% for butanol, and 6% for pentanol [18]. These contents are low in comparison with those needed in classical RPLC. The lower organic solvent consumption results in reduced cost and toxicity, which may become prominent for "green chemistry". Also, the stabilization of the organic solvent in the micellar media decreases the risk of evaporation. This means that micellar mobile phases can be preserved in the laboratory for a long time without significant changes in their composition.

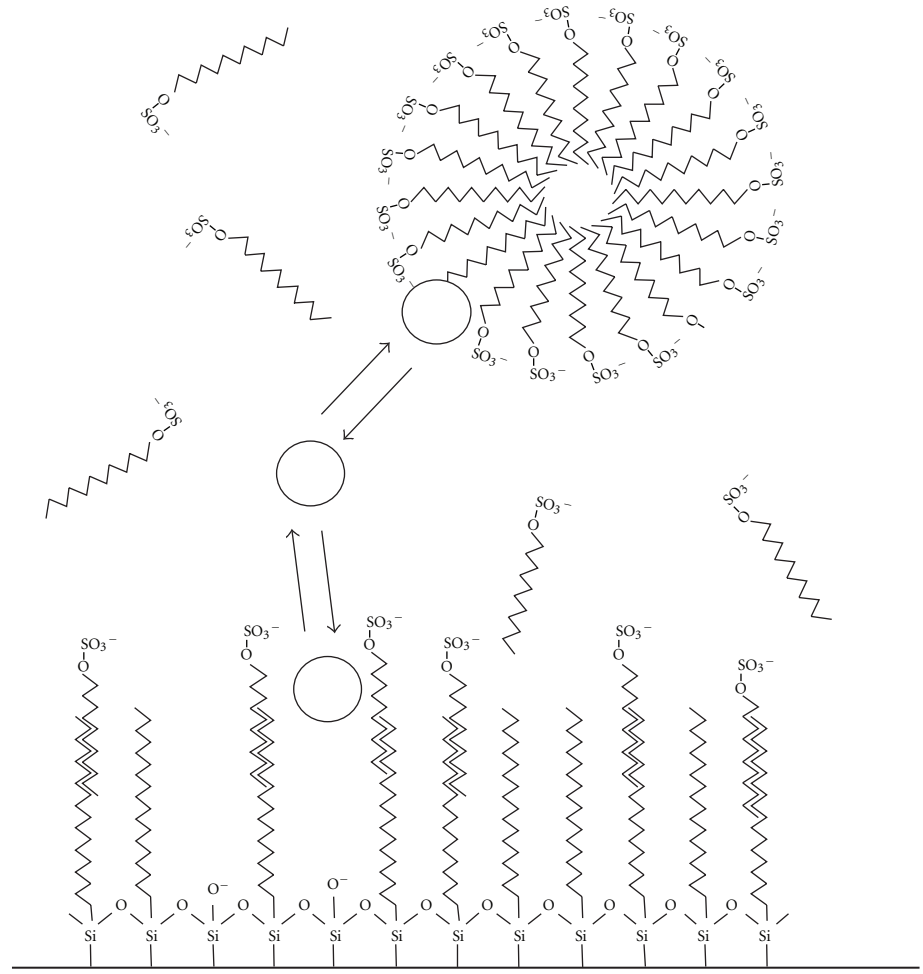

Figure 1: Solute environment in a chromatographic system using octadecyl-bonded phase, and mobile phase containing the anionic SDS. Equilibria between bulk solvent, micelle, and surfactant-modified stationary phase are depicted (reproduced with permission from [22].)

## 3. Modified Stationary Phase

*3.1. Surfactant Adsorption.* The alkyl-bonded C18 is the stationary phase most widely used in MLC, but other columns can be selected (e.g., C8 and cyanopropyl). Alkyl-bonded phase columns are strongly modified when SDS, CTAB, or Brij-35 is incorporated into the mobile phase.

Surfactant adsorption on the porous RPLC packing affects drastically the chromatographic retention, owing to the change of diverse surface properties of the stationary phase (e.g., polarity, structure, pore volume, and surface area). Surfactant molecules coat the stationary phase pores, reducing appreciably their volume [19].

Ionic compounds are frequently added to micellar mobile phases for pH buffering and, eventually, ionic strength adjustment. Salt addition may change the amount of adsorbed ionic surfactant due to the reduction of both electrostatic repulsion and surfactant CMC, and the enhancement of hydrophobic interactions [20].

Surfactant coating masks the bonded-stationary phase. This means that a full similar coating would render the stationary phases all similar. Solid-state nuclear magnetic resonance studies for the most common used surfactant, SDS, reveal that the hydrophobic tail was found to be associated with the C18 alkyl-chain bonded to the silica stationary phase, the sulphate head group oriented away from the surface (Figure 1) [21]. This creates a negatively charged hydrophilic layer affecting the penetration depth of solutes into the bonded phase.

*3.2. Presence of an Organic Solvent in the Mobile Phase.* Organic solvents are added to micellar mobile phases to improve peak efficiencies and reduce retention times, giving rise to the so-called hybrid micellar mobile phases. Competition between alcohols and surfactant molecules for adsorption sites on the stationary phase explains the linear reduction in the amount of adsorbed surfactant with increasing concentration of alcohol in the mobile phase. Mobile phases rich in organic solvent can sweep completely the adsorbed surfactant molecules from the bonded phase.

## 4. Care of the Chromatographic System in MLC

*4.1. Mobile Phase Saturation.* Pure and hybrid micellar solutions contain high amounts of water (usually more than 90% v/v) and are able to dissolve small amounts of silica, which could produce serious column damage. This is especially critical at 30°C and/or pH 6. For this reason, a saturating short column packed with 10 $\mu$m bare silica, or alternatively, the same packing as the analytical column, should be placed after the pump and before the injection valve to reduce pressure build-up.

*4.2. Column Conditioning.* A column for MLC is generally stored in 100% methanol. Before starting column conditioning, the solvent should be replaced by 100% water. For this operation, a low flow rate ($\leq$0.5 mL/min) should be selected at the beginning because of the high viscosity of the methanol-water mixture. Once the pressure decreases, the flow-rate may be raised. At least 30 column volumes of water are required to assure complete organic solvent removing. Now, the system is ready to be flushed with the micellar mobile phase. Different studies of column coating through surfactant breakthrough patterns have revealed that most surfactant adsorbs in less than one hour on the bonded-stationary phase [8, 20].

*4.3. Mobile Phase Flushing.* The micellar mobile phase should be continuously flushed through the system. If the chromatographic system is stopped during several hours, the micellar solution should not stay in contact with the bonded-silica-based stationary phase to avoid surfactant precipitation. A static micellar mobile phase can also produce crystals around the pump plungers and seals. Such crystals may obstruct the system producing plugged connecting tubing and frits, seal failure, or scratched pistons. A micellar mobile phase can be kept inside the chromatographic system overnight if the pump is not off. This avoids daily cleaning and re-equilibration. To reduce the cost, the mobile phase can be recycled, reducing the flow-rate to a minimal value (often 0.1–0.25 mL/min). However, it should be noted that in case of energy supply failure, column damage can occur. Mobile phase recycling is possible because of the low evaporation risk of organic solvents in hybrid micellar eluents. For the same reason, the micellar mobile phase can be recycled during the analysis, as long as a low number of injections are made.

*4.4. Column Cleaning.* In general, regeneration can be appropriately performed with methanol, where most surfactants are highly soluble [23]. The cleaning protocol comprises a two-step procedure that takes about half an hour.

(i) First, the micellar mobile phase should be replaced by 100% pure water, by rinsing the chromatographic system with 10 to 20 column volumes of pure water. This step is necessary to avoid salt crystallization provoked by a brutal change from a buffered micellar mobile phase to 100% methanol.

(ii) Next, water will be replaced by 100% methanol to remove the adsorbed surfactant on the stationary phase. The same caution commented under "column conditioning" about the initial use of a low flow-rate should be followed. To assure complete surfactant desorption, at least 10 column volumes of methanol should be passed through the column.

## 5. Solute-Micelle and Solute-Stationary Phase Interactions

The unique capabilities of micellar mobile phases are attributed to the ability of micelles to selectively compartmentalise and organise solutes at the molecular level. However, the association of the surfactant monomers to the bonded phase has deep implications with regard to retention and selectivity. The chromatographic behaviour in an RPLC system of a solute eluted with a mobile phase containing a surfactant above the CMC can be explained by considering three phases: stationary phase, bulk solvent, and micellar pseudophase. Figure 1 illustrates the three-phase model. Solutes are separated on the basis of their differential partitioning between bulk solvent and micelles in the mobile phase or surfactant-coated stationary phase. For water-insoluble species, partitioning can also occur via direct transfer of solutes between the micellar pseudophase and the modified stationary phase (Figure 2).

The partitioning equilibria in MLC can be described by three coefficients: $P_{WS}$ (partition between aqueous solvent and stationary phase), $P_{WM}$ (between aqueous solvent and micelles), and $P_{MS}$ (between micelles and stationary phase). The coefficients $P_{WS}$ and $P_{WM}$ account for the solute affinity to the stationary phase and micelles, respectively, and have opposite effects on solute retention: as $P_{WS}$ increases, the retention increases, whereas as $P_{WM}$ increases, the retention is reduced due to the stronger association to micelles.

The retention behaviour depends on the interactions established by the solute with the surfactant-modified stationary phase and micelles. Neutral solutes eluted with non-ionic and ionic surfactants and charged solutes eluted with non-ionic surfactants will only be affected by nonpolar, dipole-dipole, and proton donor-acceptor interactions [24]. Besides these interactions, charged solutes will interact electrostatically with ionic surfactants (i.e., with the charged surfactant layer on the stationary phase and the charged outer layer of micelles). In any case, the steric factor can also be important.

With ionic surfactants, two situations are possible according to the charges of solute and surfactant: repulsion or attraction (by both surfactant-modified stationary phase and micelles). In the case of electrostatic repulsion, charged solutes cannot be retained by the stationary phase and elute at the dead volume, unless significant hydrophobic interaction with the modified bonded layer exists. In contrast, combined electrostatic attraction and hydrophobic interactions with the modified stationary phase may give rise to strong retention in MLC. Mixtures of polar and nonpolar solutes can be resolved, provided that an appropriate surfactant is chosen.

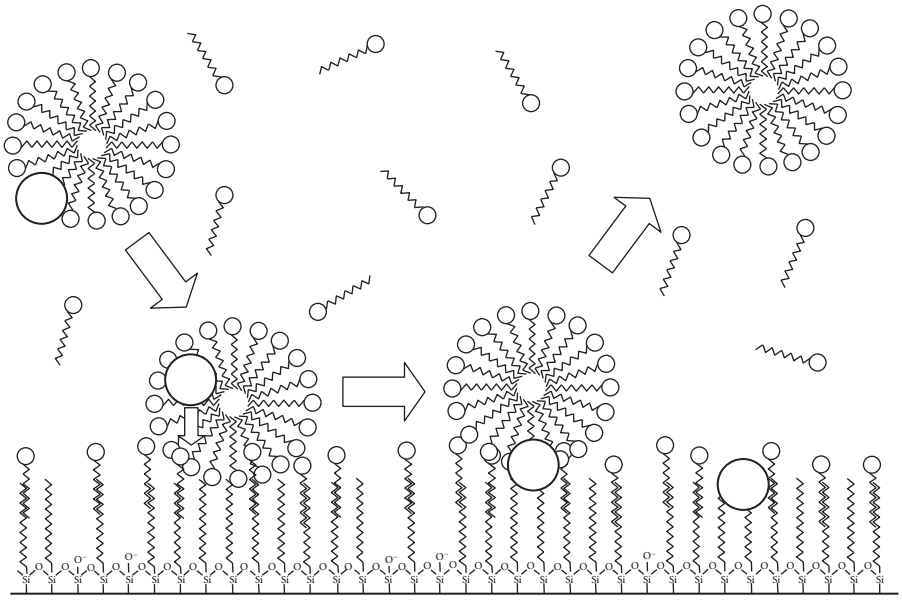

Figure 2: Direct transfer of highly hydrophobic solutes between micelle and surfactant-modified stationary phase (reproduced with permission from [22].)

## 6. Submicellar Liquid Chromatography: A New Mode in MLC

Depending on the concentration level of surfactant and organic solvent, two micellar chromatographic modes have been recently described [4, 5, 25], each one with particular characteristics.

*6.1. Low Submicellar Chromatography.* In this chromatographic mode, the stationary phase is coated with SDS, acquiring thus a negative charge. Cationic solutes can interact hydrophobically with the uncovered alkyl-bonded layer, or with the adsorbed surfactant monomers through electrostatic attraction. As long as the adsorbed amount of surfactant does not reach the maximal capacity of the column, surfactant coating on the stationary phase increases with its concentration in the mobile phase. This derives in larger retention times.

Under submicellar conditions at low surfactant concentration, the amount of free surfactant molecules in the mobile phase is negligible. This indicates that an ion-exchange retention mechanism is dominant and ion-pair formation with the surfactant in the mobile phase is practically inexistent. The addition of an organic solvent to the mobile phase increases the elution strength as a result of the decreased mobile phase polarity, and the competition between organic solvent and surfactant molecules for adsorption sites, which reduces the amount of surfactant adsorbed on the stationary phase.

*6.2. High Submicellar Chromatography.* The surfactant is at a concentration where micelles are formed in water, and the organic solvent content is high. This prevents the formation of micelles. Consequently, only surfactant monomers exist in the mobile phase, which are dissolved in the hydro-organic medium. The retention mechanism dominant in this region depends on the amount of surfactant that has been swept off the alkyl-bonded phase by the organic solvent and the existence of micelles. As long as a certain amount of surfactant remains adsorbed, and micelles exist, the retention mechanism will be the typical of the micellar mode. When micelle disaggregation occurs, a submicellar situation is achieved where ion-pair interactions with surfactant monomers in the bulk mobile phase will replace those with micelles.

## 7. Conclusions

The addition of a surfactant to the mobile phase in RPLC changes the chromatographic behaviour with aqueous-organic mixtures. In MLC, neutral and charged surfactants are used, and the surfactant concentration exceeds the CMC, which has major implications in both stationary and mobile phases. The stationary phase is modified, but now the adsorption reaches saturation or shows relatively small changes with mobile phase composition. In this way, a stable stationary phase is obtained (in a reversible process) with features remarkably different from those of the underlying bonded phase. This has a deep impact on solute interactions. Not less important is the fact that above the CMC, surfactant monomers aggregate to form micelles, which show particular solubilising properties, remarkably different from those of aqueous-organic mixtures. Highly hydrophobic solutes are removed effectively from the stationary phase transported by the micelles. The presence of surfactant associated to either stationary phase or mobile phase in RPLC implies a change in retention mechanisms, which affects the retention and selectivity.

The variety of interactions found in MLC does not exist in any homogeneous aqueousorganic mobile phase. Owing to the amphiphilic nature of surfactants, solutes can associate with both micelles and the surfactant-coated stationary phase through a combination of electrostatic, hydrophobic, and steric interactions. For this reason, micellar mobile phases are compatible with a wide range of solutes (ionic to water-insoluble). The main strength of MLC lies precisely in the capability of performing and controlling the separation of mixtures of cationic, anionic, and uncharged polar and nonpolar solutes, with isocratic elution.

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