Invited Lectures 2

Studies on Reversed Micellar Membranes for Biotechnology in Japan

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Introduction

Reversed micelles (RMs) are nanometer-scale molecular assemblies in organic media. Their surface films (membranes) are composed of surfactant molecules normally holding two hydrocarbon hydrophobic chains. Di-2-ethylhexyl sulfosuccinate (AOT), which is a negatively charged molecule, is most popular surfactant utilized to form reversed micelles. For some cases, positively charged surfactants such as trioctyl methyl ammonium chloride (TOMAC) or cetyl trimethyl ammonium bromide (CTAB) are used. The outside of the RMs is an organic solvent but aqueous water pools are included inside the membranes.

The size of reversed micelles is several nanometers, about the same as the size of proteins. So, RMs can dissolve macromolecules like proteins in its aqueous inner pool. Since the size is much smaller than the wavelength of visible light, RMs are not visible by human eyes.

In this lecture, I would like to introduce recent development of the RM research that I have worked with Professor M. Goto of Kyushu University and some more developments achieved by researchers in Japan.

1. Extraction of biological substances

Since RMs prepare the water pool in its droplets, they can dissolve proteins, peptides and amino acids. Most of studies on application of RMs were on extraction of these substances. Generally, extraction is driven by the size exclusion and the electrostatic interaction between the surfactant and the extracted substances. When AOT is used as the surfactant, positively charged substances are extracted. There is a certain AOT concentration in the organic phase to achieve the 100 % forward extraction. It is called as minimal AOT. (Ichikawa *et al.*, 1992; Naoe *et al.*, 1996) Flexibly-structured lysozyme was found to be extracted easily (Naoe *et al.*, 1999).

Amino acids and oligopeptides are also extractable by RMs, and further we found

that DNA could be extracted by RMs using TOMAC (Goto *et al.*, 1999).

2. Protein refolding by reversed micelles

Many proteins are produced by recombinant microorganisms in a form of inclusion bodies. The proteins of the inclusion body should be refolded to become native/active proteins. In industrial operations the dilution method is applied for this purpose. However, large amount of buffer solution and sometimes the redox agent such as glutathione are necessary for the dilution method. By applying RMs for the media of refolding, high dilution ratio can be avoided and the refolding process will become more economical.

Nibonuclease A (RNase A) was chosen as the model protein for refolding (Goto *et al.*, 2000). First it was produced by recombinant *Escherichia coli* in a form of inclusion bodies. The inclusion body was mixed with an organic solution containing RMs. Then, it was kept in a thermostated vessel for several hours. The RM method was found to refold RNase A almost completely although the dilution method revealed 50 % recovery of the native protein. (Sakono *et al.*, submitted for publication)

3. Detection of DNA mutation by reversed micelles

RMs can be used as media to detect mutation of DNA. In general DNA hybridization proceeds in a few seconds in aqueous media. However, in organic media with RMs it proceeds very slow and finishes in the order of minutes or even hours. Thus, the time course of DNA hybridization can be monitored in the RM media. Single strand oligonucleotide has the UV absorbance at 260 nm, but the duplex DNA does not. Therefore, by measuring the absorbance of 260 nm using spectrophotometer we can follow the course of hybridization. Now, when mismatching occurs to base groups caused by mutation, the rate of hybridization is slower than the case without mismatch. Comparing the UV absorption, it is possible to evaluate the degree of the DNA mutation.

Experiment was performed using a 20 mer oligonucleotide (DNA) fragment from exon 8 of p53 gene (Maruyama *et al.*, 2004). The organic solvent was isooctane and the surfactant was AOT. The original oligonucleotide was probe to hybridize with sample oligopeptides (targets). The hybridization process between the probe and the target was monitored with a UV spectrophotometer. The hybridization in organic media proceeded for a few hours.

From the experiment, it was found that the hybridization rate was slower when mutation occurred at the middle part of DNA than at the end of the DNA chain. Also, it was slower when mutation of plural bases occurred. Thus, the detection of the DNA mutation was found possible. Fluorescent probes are unnecessary in this method.

This RM method was further applied to a gene related with B-type hepatitis. A 40-mer DNA fragment was chosen as the model sample. In this case, the mutation was in the middle region of the fragment. The hybridization rate changed depending sensitively on the kinds of mutation. Thus, careful observation of the hybridization rate is found to enable the detection of DNA mutation.

4. Other topics of reversed micelles

Yamazaki *et al.* (2004) studied on hydrolysis of phospholipids by phospholipase A_2 in RM. They found that the reactivity increased by adding butanol to the organic reaction media. The enzyme stays in the aqueous inner water pool while the substrate phospholipid is located in the interface of RM. In this case other surfactants were not used, *i.e.* the substrate phospholipid served as the surfactant as well. By adding butanol the mobility of the membrane was said to be increased, which helped the reaction proceed.

Ichikawa *et al.* studied biocompatible RMs as well. Phosphatidylcholine was used as the surfactant with oleic acid. Ethyl oleate was the organic phase. The structure was characterized by small angle X-ray scattering (SAXS). The radius of gyration was 3 - 5 nm. (Sugiura *et al.*, (2001)) Also, extraction of proteins using affinity interaction between Cibacron Blue and bovine serum albumin (BSA) was attempted. (Sun et al., 1998)

Nagayama *et al.* studied enzymatic esterifications and interesterifications using reversed micelles. Their recent work (2002) was concerned with gelatin-containing lecithin microemulsion-based organogels that were used as carriers for esterificaiton of lauric acid by *Candida rugosa* lipase. They say that the activity increased during repeated use of the immobilized enzyme.

Noritomi *et al.* (1999) proposed recovery (back extraction) of cytochrome c and RNase A from the RM phase in the form of gas hydrate. The hydrate formed by 1,1,1,2-tetrafluoroethane was found to be most effective for recovery of proteins.

Summary

Reversed micelles can be applied to various purposes in biotechnology not only for separation but also for other purposes such as protein refolding and detection of the DNA mutation. Biocompatible RMs are preferred in some cases and the use of phospholipids to prepare RMs is possible and promising for future developments of application to biotechnology. We can find many useful researches on RMs carried out in Japan, which are briefly introduced in this lecture.

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