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Viewpoint

Inversely Finding Peculiar Reaction Conditions toward Microfluidic Droplet Synthesis

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Abstract: With the development of microfluidics, there are increasing reports of syntheses using not only conventional laminar flow at the microscale, but also the dissociation and aggregation of microdroplets. It is known, to some extent, that the microfluidics scale differs from normal scales in terms of the specific surface area, mass diffusion, and heat conduction; these are opposite to those in scale-up in-plant chemical engineering. However, it is not easy to determine what changes when the microdroplet flows through the channel. In this context, the author would like to clarify how the behavior of chemical species, which is expected to appear unique at the nanoscale, contributes to chemical reactions. What do we need in order to develop a completely new theory of chemical reactions? The characteristics of chemical reactions on the nanoscale are clarified via the encountering of solutions by the microfluidic device itself, or the chemical reaction of nanoscale droplets generated by the microfluidic device. Specifically, in recent years, experimental reports have accumulated that are expected to develop a fluidic device that can stably generate nanodroplets, and complex reactions of different reactivity are expected to occur that are specific to the nanoscale. In this short article, microfluidic devices, nanoscale droplets, experimental synthetic examples, and findings that may provide solutions are described.

Keywords: microfluidics; microdroplet; metal complexes; preparation; reaction condition

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1. Introduction

The author would like to create protein crystals containing metal complexes with aldehydes with hydrophobic moieties, such as photo-functionality (azobenzene) and Schiff bases, which are N-terminal-condensed water-soluble proteins, such as lysozyme, as ligands, which are usually difficult, and to stably synthesize useful multi-functional materials, such as SOD–active-metal complex catalysts (artificial metalloproteins), that work in water-soluble environments [1,2]. Therefore, the purpose of *the hypothesis* of this research is to obtain knowledge that will be useful in the future for "conditions for accelerating the droplet reaction of complex-containing protein crystals" by taking into account the accumulation and discussion of negative data "due to the inverse scale effect", aiming to make a contribution to the establishment of elemental technology for droplet synthesis in microfluidic devices (discovering the reactions and processes in which the scale effect is effective).

In recent years, the development and spread of droplet synthesis in microfluidic devices have made big leaps forward, such as promoting chemical reactions and carrying out processes that are difficult to synthesize, using chemical reactivity unique to the nanoscale space [3,4]. Research using microdroplets in chemistry has been conducted along with microfabrication technology for drains, which can be called reaction vessels, and has become more and more complex, starting with applications in micro-instrumental analysis in biochemistry [5], biochemical reactions with small sample volumes [6–8], organic chemical reactions whose activation energy must be controlled [9], electrochemical reactions including diffusion-controlled processes [10], relationships with surface wettability [11],

and the emergence of thermodynamically non-equilibrium [12,13] and irreversible open-system microdroplets [14,15].

The peculiarity of chemical reactions in the nanoscale space is treated as a "scale effect", but there is still little knowledge about the establishment of a reaction theory [16] that considers the reaction system and spatial factors and clearly shows their effects, and it is necessary to continue research on the basics (dissolution [17] and mixing at the interface [17,18], diffusion, surface tension [14], activation energy, solvent viscosity, the effects of electric and magnetic fields [19], self-assembling intermolecular interactions to form colloids [20,21], etc.), while working toward application through the creation of useful functional materials. In recent years, it has been pointed out that the proton-transfer reaction is subject to the quantum-tunneling effect. The metal–organic frameworks (MOFs), which play an important role in the field of metal coordination chemistry, often produce products in which metal ion/organic ligand units repeat infinitely three-dimensionally in crystals due to the self-assembly process according to conventional synthetic methods. As well as manipulating interfacial reactions to obtain two-dimensionality, microdroplets have been reported to be useful for constraining repetition to dimensionality and finite size [22–25].

Therefore, the author decided to focus on molecular assemblies that do not follow the "usual thermodynamic stability". Moreover, in the field of coordination chemistry, crystalline polymorphs exhibiting structural phase transitions and self-assembled MOFs with kinetically stable aggregation structures are already widely known. However, the (unstable) docking of chiral metal complexes with proteins under the conditions expected to be non-equilibrium open systems aims to minimize, not minimize, the energy, so to speak.

In particular, with the aim of contributing to the establishment of elemental technology for droplet synthesis in microfluidic devices (discovering reactions and processes in which the scale effect is effective), the accumulation and discussion of negative data are positively regarded as being "due to the inverse scale effect", and the purpose is to obtain knowledge that will be useful in the future for "conditions for promoting the droplet reaction of complex-containing protein crystals".

2. Microdroplets and Conventional Methods for Artificial Metalloproteins

Experiments have shown that a significant number of synthetic steps benefit from the use of microfluidics [26–28]. The synthesis of azobenzene-containing amino-acid-Schiff-base–copper(II) complexes consists of an azo-coupling reaction (keeping the pH acidic and reacting at a low temperature), an imine synthesis reaction (an acid or base catalyst, a difference in solubility due to amino acid), and a complex formation reaction (the abstraction of phenolic proton by a weak base, the equilibrium of coordination bond, the coordination protection of the amino acid functional group). Microfluidic devices are known to dramatically reduce reaction rates without the need to control the temperature and pH conditions. However, when specific amino acids are used, synthesis is not always successful, and microdroplets are expected.

Therefore, using the negative data of amino acids that are difficult to synthesize, the author will examine the possibility of promoting a reaction by determining the presence or absence of the "reverse scale effect" among factors such as a temperature decrease, concentration decrease, and solvent amount increase.

The author has been studying Schiff-base–metal complexes and conjugation to proteins for several years. It goes without saying that the development of a methodology for such microdroplet synthesis itself has significance as advanced research. However, aside from the MEMS (micro-electromechanical systems) technology, which is a methodology, a reaction theory that considers the reaction system and spatial factors and clearly shows their effects has not been established regarding the base chemistry. Papers that serve as conventional methods and technologies have already been published [2], and there is also an accumulation of negative data. In particular, in recent years, the author and his colleagues have established the synthesis conditions [29] and calculation results [30] for

amino-acid-Schiff-base–metal complexes in order to apply them to microwave synthesis [31] (the realization of spatial and electromagnetic temperature distribution) and microfluidic device synthesis (the realization of nanoscale-specific kinetics and thermodynamics). This experience and knowledge provide support for the research proposal on the "reverse scale effect". In the research focusing on the functionality of the amino-acid-Schiff-base–metal complex shown in the scheme below, the need to increase the hydrophobicity of the organic ligand arose. However, if the metal source, the ligand raw material, and the resulting complex are soluble in the polar solvent methanol, then they are easy to handle.

The "scale effect" in microdroplet synthesis is an immature field. For this reason, we adopted a division-of-labor system in which we performed complex synthesis chemistry and water-soluble protein crystallization on a normal scale, and proposed, to specialized researchers, processes that seem to be effective on the microscale. In this work, using this approach, the author proposed a method to determine the possibility of promoting reactions in microdroplets from reactions that are not necessarily successful on a normal scale. In other words, in the process via which the negative data were obtained, a research method was adopted in which the expression of the "scale effect" was investigated via reversing the reaction conditions advantageous to microdroplet synthesis.

The synthesis of azobenzene-containing amino-acid-Schiff-base–copper(II) complexes (Figure 1) consists of an azo-coupling reaction (keeping the pH acidic and reacting at a low temperature), an imine synthesis reaction (an acid or base catalyst, a solubility difference due to the amino acid), and a complex formation reaction (the abstraction of the phenolic proton by a weak base, coordination bond equilibrium, the coordination protection of the amino acid functional group). Microfluidic devices are known to dramatically reduce reaction rates without the need to control the temperature and pH conditions. However, when specific amino acids are used, synthesis is not always successful, and microdroplets are expected. Therefore, using the negative data for amino acids that are difficult to synthesize, the author and his colleagues will examine the possibility of promoting the reaction by determining the presence or absence of the "reverse scale effect" from factors such as a temperature decrease, concentration decrease, and solvent amount increase.

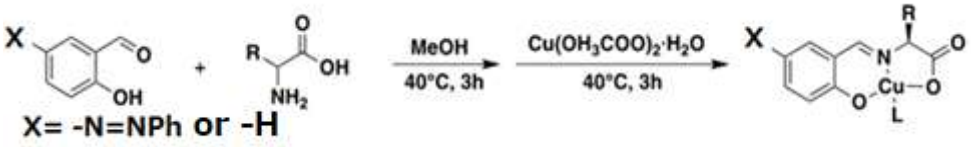


Figure 1. General synthetic scheme for the (azobenzene-containing) amino-acid-Schiff-base–copper(II) complex.

There are two main methods for introducing an azobenzene-containing amino-acid-Schiff-base–copper(II) complex into a single crystal of lysozyme, which is a typical water-soluble protein and is relatively easy to handle (Figure 2). One is the co-crystallization method (the complex solution is injected into the protein solution for hanging-drop crystallization, and the suspended drop is left in steam at a low temperature), and the other is the immersion method (the pH-adjusted saturated solution of the complex is immersed in the protein single crystal, which has already been crystallized by the hanging drop, at a low temperature). However, proteins denature at low temperatures. There is a discrepancy in optimal solvents, with metal complexes being soluble in alcohol, and lysozymes being soluble in water (buffer solutions). Salt concentration and pH adjustment are necessary for protein crystallization but, as a result, there is the problem that copper(II) ions are easily dissociated from the complex. Therefore, from the negative data of protein crystallization, chemical thermodynamic conditions such as the vapor pressure, concentration, temperature, etc., which can cause a "reverse scale effect", are established, and the possibility of promoting crystallization is examined.

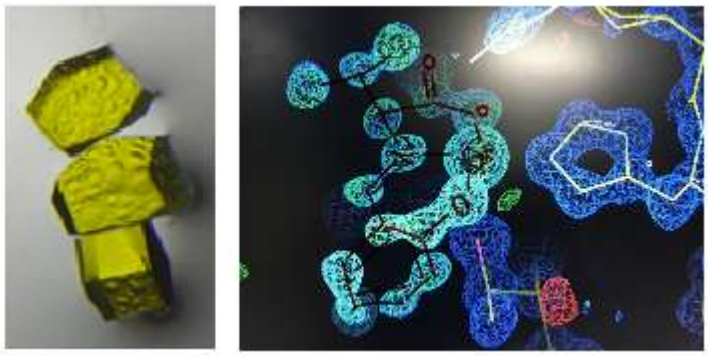


Figure 2. (**Left**) crystals of lysozyme containing copper(II) complexes (without azobenzene), (**right**) the analysis of the crystal structures.

In the study of the threonine-derivative–metal complex [1], for which only protein crystal structure analysis had been possible so far, the theoretical calculation result demonstrated that the hydroxyl group in the ligand that remains after binding to the lysozyme is important to the reaction mechanism for SOD activity. Furthermore, the author and his colleague have already performed theoretical calculations and preliminary experiments using amino-acid (valine, serine)-Schiff-base–copper(II) model complexes with experimentally changed ligand hydroxyl groups and lysozyme-bound copper complexes (Figure 2). A coordination bond between a central metal atom in the copper(II) complex and histidine residues in the lysozyme was suggested. Furthermore, the serine complex with a hydroxyl group showed better activity than the valine complex without a hydroxyl group, suggesting that hydrogen bonding has a large effect on SOD activity. In addition, through photoisomerizing azobenzene via light irradiation, it is possible to control the degree of hydrophobicity and hydrophilicity, and it is expected that we should consider the environment of hydrogen bonding and supporting water molecules. In this way, the reactions in the target system include cases where the proton-transfer reaction is subject to the quantum-tunneling effect, which is said to exhibit the scale effect. Therefore, due to the difference in the experimental results for the SOD activity, the "reverse scale effect" occurs, and the conditions are established to examine the possibility of further promoting the enzymatic/catalytic chemical reaction.

3. The Accumulation of Negative Data for the "Reverse Scale Effect"

There are several things that are usually considered difficult or disadvantageous in the synthesis of the above system; temperature control in azo coupling (a side reaction due to a temperature increase). These include the solubility of the Schiff base (the condensation of the imine formation) for synthesis with amino acids with azobenzene; the encounter between an organic ligand with increased hydrophobicity and copper acetate, a metal source soluble in polar solvents and the dissociation conditions for phenolic protons; and, in many cases, the combination or crystallization of a water-unnecessary metal complex with a fragile protein that is soluble in a buffer solution (an aqueous solvent).

Corresponding with the advantages of microfluidic synthesis, a large specific surface area is advantageous for heat dissipation. If a few solutions (droplets) can mix at a 'narrow' interface, then solvent compatibility conflicts can be reduced, leading to diffusion-enhanced reactions. However, if we ventured to extend the usual synthesis conditions "reversely", then the advantages that would be offered by microfluidic synthesis could be highlighted.

The author and his colleagues have been studying Schiff-base–metal complexes and conjugation to proteins for a long time. Studies of elemental technologies have already been published, and there is also an accumulation of negative data. In particular, in recent years, our research group members have organized the synthesis conditions for amino-acid-Schiff-base–metal complexes in order to apply them to microwave synthesis (the realization of spatial and electromagnetic temperature distribution) and microfluidic device synthesis

(the realization of nanoscale-specific kinetics and thermodynamics). This type of experience and knowledge forms the basis for this study of the "reverse scale effect".

Artificial metalloproteins use metal complexes or abiotic metal ions as cofactors to catalyze chemistry that cannot be catalyzed by natural proteins. In recent years, they have been actively researched in anticipation of their potential to exhibit chemical functions and a reactivity that surpass those in nature. The main approaches to creating artificial metalloproteins include (1) the diversion of natural metals (coordination bonding), (2) the substitution of metals in metalloenzymes with other metals, (3) the reconstruction of protein chemical reaction fields (the docking of metal complex ligands via non-covalent bonding), and (4) metal introduction by immobilizing ligands (covalent bonding). Of these, (1) and (2) are designed for metal ion units, and (3) can use metal complexes, but has the disadvantage of weak bonds. If the hydrophobic substituents of the complex are fortunate enough to interact with the hydrophobic pocket on the protein surface, the calculated thermodynamic value will allow docking. Therefore, (4) is essential for stable creation.

The peculiarity of chemical reactions in the nanoscale space is treated as a "scale effect", but there are many mysteries regarding specific factors. It is necessary to continue verification while facing concrete examples, such as dissolution and mixing at the interface, diffusion, surface tension, activation energy, solvent viscosity, and the effects of electric and magnetic fields.

Examples of failure to synthesize azobenzene-salicylaldehyde (a precursor of the ligand) are given below (Figure 3). Points to be considered in solution-mixing (the interface/diffusion/time) and pH adjustment (a reduction in the activation energy of the proton reaction), which are advantages in droplet synthesis, are described. It is speculated that reversing these conditions will result in a successful synthesis reaction.

- Students added sodium nitrite too quickly.
- An insufficient amount of sodium hydroxide was added.

  → The color was still reddish brown. It was considered to contain impurities, but it resulted in a large yield.

  They obtained too much.
- Too much sodium hydroxide was added, resulting in a high pH.

  → It turned dark brown and separated into a transparent liquid and a solid.
- A short stirring time, a slow stirring speed.

  → The amount obtained was small.

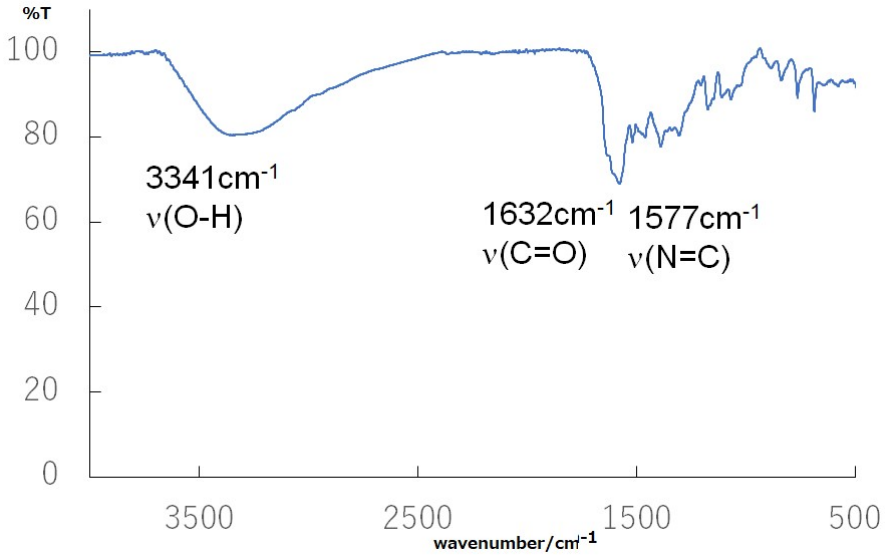
Examples of imine synthesis failures are given below (Figure 4). The experimental NMR spectra did not agree with the expected ones (not shown). Possibly due to an insufficient low-temperature condition control, the yield of the main product was low, and other by-products were also produced, resulting in an unclear spectrum. Points for reflection on temperature control, solubility, and solution viscosity are listed. These points also appear frequently as features and advantages of droplet synthesis. Therefore, it is presumed that improvements in the synthetic reaction can be expected via the adjustment of these condition settings in an appropriate direction or via a method that can be widely applied.

- Two equivalents of amino acids were not synthesized successfully.

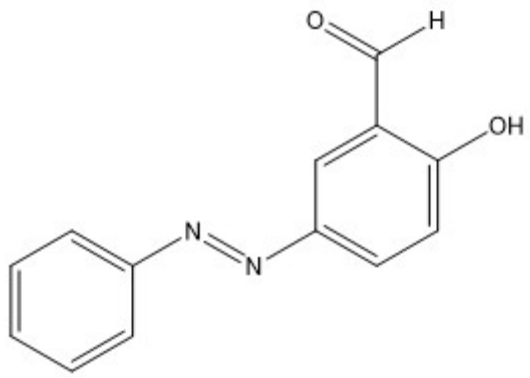
  → Undissolved amino acids occurred. They did not melt even at high temperatures under reflux.
- In order to dissolve the amino acid, it was dissolved in water and then added to an ethanol solution of azobenzene, but it could not be synthesized.

  → Precipitation occurred after addition to the ethanol solution.
- When an equivalent amount of KOH was added to the precipitated ethanol solution, it dissolved and became red and transparent.

→ A viscous compound was obtained. NMR measurements were performed, but the results were not convincing.



(**a**)



(**b**)

Figure 3. (**a**) The IR spectrum of failed azobenzene-salicylaldehyde. (**b**) The expected structure of azobenzene-salicylaldehyde.

The "scale effect" in microdroplet synthesis is an immature field. Using reactions that are not necessarily successful on a normal scale, the author will search for factors that determine the possibility of promoting reactions with microdroplets. Adopting a unique research method that focuses on the "reverse scale effect" by examining whether to reverse the reaction conditions that are advantageous for microdroplet synthesis in the process for which negative data were obtained. At least for complexes with tridentate ligands and vacant coordination sites, various linking methods are known, as shown in the figure below, and the microdroplets' effect of limiting the spatial extent of MOF linking, which has already been reported, can be expected.

An example of a failed imidazole-attached copper(II) complex (L = imidazole in Figure 1) is given below. As before, we can consider the encounters of reagents, pH adjustment, and the properties of solutions. The thermodynamic processes (evaporation and mixing) of solvents for crystallization as metal complexes are mentioned and comprise a new aspect to consider. These are also expected to change the conditions from those obtained via classical methods in microdroplet synthesis. However, empirically speaking, it is necessary to be careful because there are some aspects that do not always work well if we "solely" set the conditions that do not work.

- Copper acetate and ligand were added simultaneously but failed. This was done under basic conditions.
  → Is it worth trying under acidic conditions?
- The resulting filtrate was allowed to stand in a fume hood.
  → The filtrate volatilized, but the solid obtained was quite viscous.
- An attempt was made to crystallize the filtrate via the solvent diffusion method.
  → Nothing changed.
- We attempted to recrystallize the filtrate in the refrigerator.
  → Nothing changed.

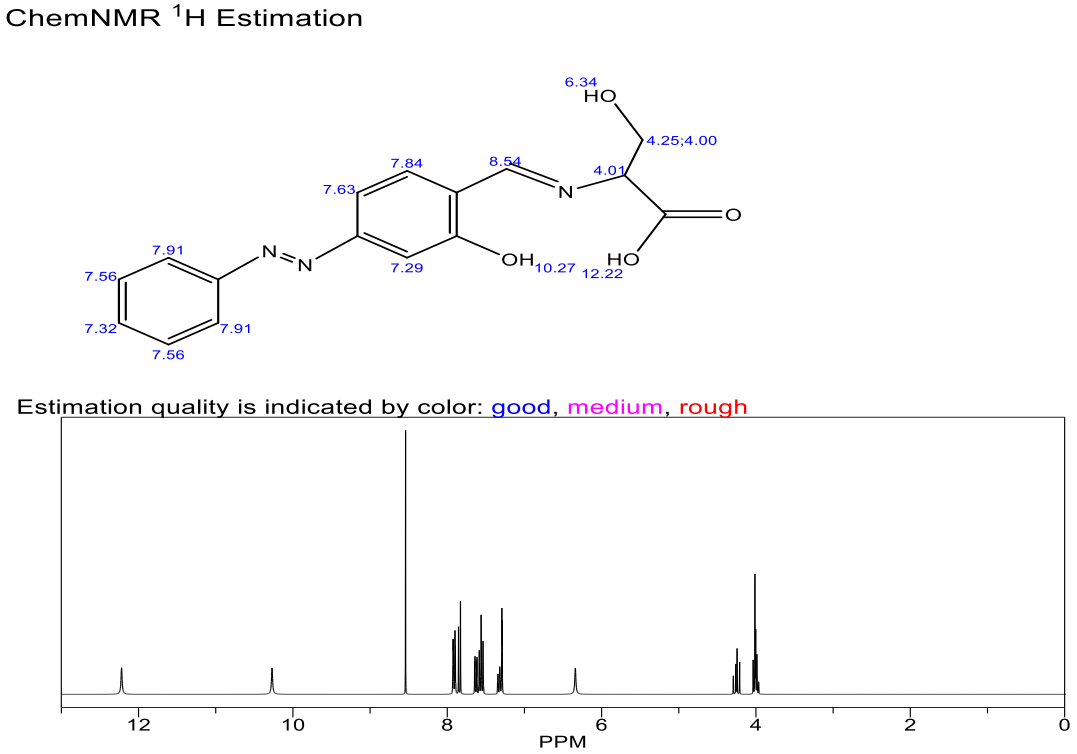


Figure 4. The expected ^{1}H-NMR spectra of a failed Schiff base ligand.

Inducing thermodynamically unstable docking under special conditions, artificial metalloproteins are expected to impart new catalytic ability and improve reactivity to water-soluble proteins, which are difficult aims to achieve with normal reaction-inactive stable structure docking. Pursuing the principle of the state-of-the-art method leading to this creation seems to presently be bringing about a significant breakthrough. In addition, from the viewpoint of unstable structures caused by proteins, it is also connected to the open-system chemical thermodynamics of "dissipative structures", which has the potential to explore new aspects of chemical reactions.

There are clear demands on the hydrophobicity/hydrophilicity and biomolecular compatibility of the complexes. However, in some cases, it is serious that synthesis based on ordinary solvent-mixing becomes difficult. In principle, for example in [31,32] and related systems, the copper(II) complex, which is obtained via the reaction formula, comprising covalently bonded amino acids and aldehydes, as a ligand, has been the subject of research focused on various functions, such as its use a photocatalyst for heavy metal reduction in an aqueous solvent via the introduction of a hydrophilic group, as an electron mediator between an oxygen reductase and an electrode via introducing a hydrophobic group, and as having an antibacterial effect against Gram-positive and -negative bacteria

whose hydrophilic–hydrophobic balance (water/octanol partition coefficient) differs for each target.

Furthermore, the introduction of a hydrophobic azobenzene group poses a problem regarding compatibility with biomolecules (which generally tend to be hydrophilic), in addition to its merits. If the synthesis method for this research can be established, then the introduction of Schiff-base–metal complexes in water-soluble proteins (lysozyme, human serum albumin, laccase, etc.) and water (buffer solution) via chemical bonding instead of docking will be realized and, as they are artificial metalloproteins, functional composite catalyst systems with a high reactivity, and functional materials using biomolecules, can be created. In terms of compatibility with proteins, there are other factors. These include asymmetric molecular recognition between enantiomers; and external field controls, such as ultraviolet polarization (isomerization) and magnetic fields (orientation change) using azobenzene and metal ion sites are also expected. Furthermore, the author is considering extending synthesis under thermodynamically non-equilibrium conditions (in microfluidic devices and droplets) and, with a view to two-solvent system microdroplet synthesis in the future, the author and his collaborators believe that the ripple effect of artificial metalloproteins and metal complexes, which cannot be synthesized under normal synthesis conditions, or are extremely difficult to synthesize, is by no means small.

4. Conclusions

The peculiarities of chemical reactions in the nanoscale space are treated as "scale effects", but there are many mysteries regarding specific factors, such as dissolution and mixing, diffusion, surface tension, activation energy, solvent viscosity, and the effects of electric and magnetic fields at interfaces. A study of such conditions may seem rudimental at first glance, but it can be positioned as having a key significance when we review it from the perspective of it leading to cutting-edge research.

Only a small number of extreme failure cases are presented here, to demonstrate the hypothesis. Under proper conditions, these compounds can be synthesized in ordinary beakers and flasks. Also, although there are examples of microfluid synthesis [26], microdroplet synthesis is a work in progress. At the beginning of the research stage, the purpose was to improve the efficiency of the synthesis time and conditions via micronization, but this purpose has gradually shifted to presenting results that are impossible with normal synthesis, but proving or exploring "what is impossible to prepare" is actually difficult.

As an application, research on artificial metalloproteins has attracted worldwide attention. The mainstream focus of research is the incorporation of catalyst complexes using the spatially defined, hydrophobic, and asymmetric reaction fields inside proteins. From the viewpoint of the asymmetric and anisotropic structural properties of chiral complexes and photoreactions, the principal investigators started research on artificial metal complexes from original metal complexes and representative proteins. For example, mediator complexes were investigated to improve the electron transfer efficiency between oxygen reductase and electrodes. The electron transfer efficiency is not always determined by static (asymmetric) molecular recognition, that is, the score function of thermodynamic docking calculations. On the other hand, in the artificial metalloprotein of lysozyme and an SOD active complex catalyst reported this year, the molecular design was carried out based on the elucidation of the reaction mechanism and the concept of multiple complex functions and an external field control.

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