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Review

Catalysis in abiotic structured media: an approach to selective synthesis of biopolymers

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Abstract. Micro- and nanoenvironments formed by amphiphile self-assembled structures, water-ice lattices and minerals have well-defined, repeating, chemical and physical properties that can be used for selective synthesis of biopolymers, such as RNAs and proteins. The advances made in the development of polymerization supported by these micro- and nanosystems are reviewed here. In particular, it is shown that these systems promote non-enzymatic biopolymerization, yielding long polymers whose sequence composition is determined by the interactions between monomers and the supporting environment. When used to compartmentalize enzymatic biopolymerization, micro- and nanostructures allow the implementation of molecular selection and evolution schemes, which are difficult in homogeneous medium, yielding very active molecules. Thus, micro- and nanoenvironment approaches to the synthesis and selection of biopolymers could be developed into a new biotechnological tool for the production of biopolymers with novel functions.

Key words. Biocatalysis; micro- and nanoenvironment; compartmentalization; amphiphile structures; eutectic phase in water ice; minerals; synthesis and selection of RNA and proteins.

Introduction

Recent studies of micro- and nanostructures have broadened our understanding of interactions between these structures and solute molecules dissolved in the surrounding, aqueous media. This knowledge can now be applied to the design of biopolymer synthesis supported by micro- and nanoenvironments (also called here structured media or environments).

Abiotic structured environments, e.g., formed by self-assembling molecules or mineral surfaces, can perform two major functions which can enhance biopolymer synthesis: They can physically confine or compartmentalize molecular reactions. They can also promote reactions due to chemical and physical properties that differ from those of bulk aqueous media, permitting the assembly of reagents in normally unstable configurations.

Micro- and nanoenvironment-supported biopolymer synthesis has been studied using two general types of reaction: non-enzymatic polymerization from chemically activated monomers [1] and enzymatic polymerization with natural substrates [2]. The former type of synthesis stems from the need of origin of life researchers to find abiotic pathways by which the first catalytic RNA could have appeared and could later self-replicate on the early Earth [3]. It was recognized early that any polymerization in a homogenous aqueous solution would be relatively inefficient [4] because of the high water activity that prevents formation of monomer assemblies conducive to polymerization and favors hydrolysis of both the activated monomers and polymeric products, the potential templates for self-replication [5]. Processes such as adsorption on surfaces and dehydration, which could allow a shift in chemical equilibria toward efficient polymeriza-

tion, were therefore investigated, introducing the idea of structured-media-supported biopolymerization. On the other hand, enzymatic polymerization encapsulated in structured environments, such as liposomes, was developed at first because these systems could be used as compartments to investigate cellular activities [6]. Systems could also be designed to create bioreactors, model systems for protocells [7] and possibly artificial cells with applications in biotechnology [8].

A selective, structured-media-supported synthesis is still a challenging proposition. Its realization, however, could create a powerful tool for the synthesis of biopolymers with novel functions. Recent developments in this research area [9–11] have clearly demonstrated that in vitro-selected biopolymers composed of natural monomeric units can perform an array of non-biological functions with potential applications in biotechnology, diagnostics and therapeutics. Chemical modifications of the monomers can further improve these novel biopolymers [12, 13] by increasing their stability in biological fluids, permitting stronger non-covalent binding with target molecules, or imparting new catalytic activities to the biomolecules. However, whereas monomers can be easily modified, their insertion into polymers depends on their compatibility with biosynthetic enzymes [14]. Compared to enzyme engineering [15], non-enzymatic polymerization supported by structured environments could offer a general solution to compatibility issues because monomer recognition would be based on the presence of interacting chemical groups rather than on a specific molecular shape, as is the case with enzymes. It is also likely to yield a pool of random modified polymers that is different from that obtainable by solid phase synthesis, e.g., based on phosphorimidate chemistry, because aqueous structured media allow interactions between monomers, nascent polymer chains and structured environment that are absent in homogeneous organic media. These interactions should result in a selection of sequences during polymer synthesis, which make them more suited for this or similar environments (with respect to solubility, stability, aggregation and presumably functionality properties), a proposition difficult to achieve by solid-phase synthesis.

Micro- and nanoenvironments, as biomimetic compartments, can also improve in vitro selection methodologies, such as SELEX (Systematic Evolution of Ligand by EXponential enrichment) [9, 16] or mRNA display [11, 17] developed in homogeneous media. These techniques are based on the screening of up to 10^{16} different biopolymers (phenotype) synthesized from large libraries of random nucleic acid templates (genotype). In order to apply selection directly to such a population of molecules and amplify active molecules, the genotype-phenotype link must be preserved. In homogeneous media, this usually is achieved by covalent, or strong non-covalent linkages, whereas nature does it by compartmentalization within cells. Similarly, abiotic self-assembled compartments can be used to encapsulate the transcription and translation of each template and spatially maintain the genotype-phenotype link without requiring chemical linkages, as selections in water-in-oil (w/o) emulsions have shown [18–21].

This review deals with advances in the synthesis of RNA and peptides in structured environments with emphasis on intrinsic properties that enhance biopolymer synthesis and activity compared to homogeneous-media reactions. Possible ways of using these properties to direct a selective synthesis will then be highlighted.

Micro- and nanostructured environments

We will first examine the basic properties of micro- and nanostructured environments that have been used for biopolymer synthesis (see table 1). Structured media create localized environments whose intrinsic chemical and physical properties (e.g., altered water activity, superficial interaction potential) differ from their surrounding bulk medium, giving them the capacity to concentrate and assemble substrate molecules into unique configurations so that they can enter into reactions.

Amphiphile structures

Structured media can be formed by self-assembling amphiphiles (see fig. 1) yielding vesicles, monolayers and bilayers in the crystalline state, reverse micelles in emulsions and micelles. The type, size and electrostatic properties of the structure will depend on the amphiphile type, its concentration (there is always a critical aggregate concentration above which structures form and remain stable), and on medium conditions such as pH [22] and ionic strength [23–26].

Amphiphile vesicles or liposomes (fig. 1a) are composed of an aqueous volume surrounded by shells that are composed of closed molecular bilayers. The non-polar moieties of the amphiphile molecules form the hydrophobic interior of the bilayer, and the hydrophilic parts (the headgroups) are in contact with the aqueous phases. They can be uni- or multilamellar, the latter being a structure in which the internal water core is surrounded by several concentric amphiphile bilayers. Their size ranges between 20 nm and tens of micrometers in diameter [27]. By using mixtures of amphiphiles, e.g., zwitterionic phosphatidylcholines (PCs) and didodecyldimethylammonium bromide (a cationic amphiphile), the liposome surface charge can be precisely tuned to optimize interactions with charged solutes in the aqueous phase.

Reverse micelles or w/o emulsion compartments (fig. 1b) are built by mixing an aqueous solution and an apolar, or-

Table 1. Examples of the micro- and nanostructured environments used for biopolymer synthesis.

Type of structured environment	Molecular composition	Function	Biopolymer(s)	Usage(s)	Reference(s)
Liposomes	Amphiphiles: Lipids, fatty acids	Confine	RNA, DNA and peptides	Synthesis, expression	[97–99, 101, 105, 115]
			Enzymes	Activity modulation	[106, 107]
		Promote reaction	Amino acids	Polymerization	[68–71, 74]
Emulsions (reverse-micelles)	Amphiphiles: Ionic and nonionic surfactants	Confine	RNA, enzymes	Synthesis, selection, activity modulation	[18–21, 112]
Micelles	Amphiphiles: Single-chain surfactants	Promote reaction	RNA	Activity modulation	[116]
		Promote reaction	Amino acids	Polymerization	[46]
Mineral/Clays	Montmorillonite, Illite, Hydroxylapatite, Pyrite	Promote reaction	RNA Amino acids	Polymerization	[47, 64, 117] [35, 39, 67]
Eutectic phase in water-ice	Water crystals	Promote reaction	RNA	Polymerization	[26, 29, 30]
				Activity modulation	[79]
			Amino acid	Polymerization	[28]

ganic solvent or mineral oil containing amphiphile molecules. These molecules rearrange themselves so that their headgroups face the aqueous phase and their hydrophobic chains the apolar phase, forming an interface between the two phases, which stabilizes the structure. Their size, several microns in macroemulsions and several nanometers in microemulsions, depends on the ratio of water to surfactant, the mechanical energy injected in the system (e.g., by stirring) and the temperature of the system.

When amphiphiles in an aqueous phase cannot support the formation of bilayers because of repulsive electrostatic interactions between headgroups, they will adopt a nanoscale tubular or spherical organization, the micelle (fig. 1c). It minimizes the exposure of their hydrophobic chains to water and reduces the repulsive interactions between headgroups.

In the vicinity of amphiphile assemblies, a localized environment exists with physical properties that depend on the amphiphile headgroups and ionic salts present in the aqueous phase (red areas in figure 1a–c). This environment is similar to that found at the polar and apolar interfaces in vivo.

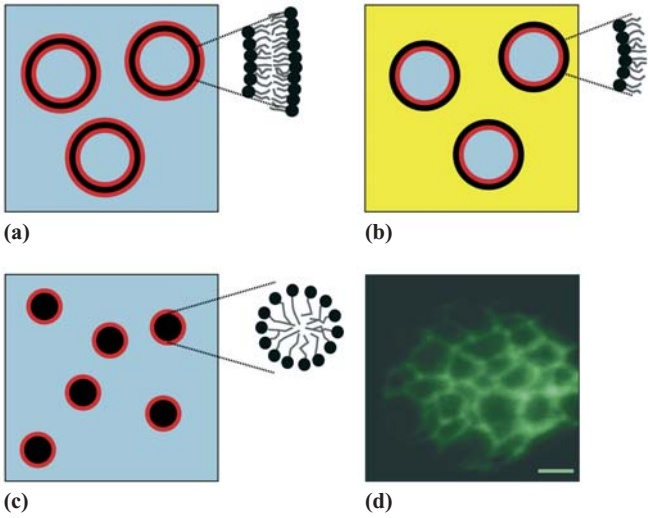


Figure 1. Micro- and nanostructured environments. (*a–c*) Amphiphile self-assembled structures: (*a*) liposomes, (*b*) reverse-micelles and emulsion compartments, and (*c*) micelles. Amphiphile self-assembled structures are in black. The media are the aqueous phases in light blue and the external apolar phase in yellow. (Red) The area where water behavior is strongly influenced by the amphiphile headgroups. (*d*) Water-ice lattices: A sample with 100 µg/ml acridine orange [29] was frozen within 5 min by cooling samples from room temperature to –18 °C. On the epifluorescence micrograph (1000-fold magnification), the concentrated dye (light green outline) is clearly visible around the ice crystals (dark masses). The bar represents 26.7 µm.

Eutectic phase in water-ice lattices

Another type of structured medium (see fig. 1d) used in biopolymer synthesis is formed by freezing an aqueous solution [26, 28–30]. When an aqueous solution is slowly frozen at a temperature above the eutectic-point tempera-

ture, solutes are excluded from the ice crystals and remain within the liquid fraction (eutectic phase), resulting in the concentration of reaction mixtures. The final system characteristics, such as the volume of the eutectic phase, will depend on the rate of cooling, final temperature and initial solute concentration. Dilute or concentrated starting solutions always reach the same molal concentration with respect to an individual solute, which is determined by the final incubation temperature [31]. An initially dilute solution will therefore form a larger amount of ice than a concentrated one. Because of this colligative character, even solutes that are not involved in a reaction and do not influence reaction rates in an unfrozen sample will contribute to the decrease of the frozen phase, thereby influencing molecular reactions [26]. Furthermore, the presence of additives, e.g., antifreeze proteins that bind to particular crystal planes [32, 33], can modify crystal growth.

Minerals and clays

Dispersions of minerals provide nanostructured environments [34] at particle charged surfaces, whose surface charge distribution depend on the mineral lattice and its processing. Various inorganic minerals, such as hydroxylapatite [$Ca_5(PO_4)_3OH$], pyrite (FeS_2) [35] and clays, such as montmorillonites and illite (a clay mica), have been used to promote biopolymer synthesis. Clays, especially homoionic montmorillonites [36], were investigated in detail [37–40] because of their capacity as natural ion exchangers that permit charge density alteration (by titration with a given metal chloride, or etching) while keeping the underlying lattice intact. Their structure is composed of alternating tetrahedral/octahedral layers of silicates and silicates partially substituted by alkali metal or alkaline earth metal and Al ions. These cations can readily be exchanged for divalent cations such as magnesium [41, 42], which is particularly well suited for nucleic acid polymerization.

The aqueous content of the micro- and nanostructures will define the reaction type they can support: low aqueous-content environments (crystalline lipid bilayer, water-ice matrices and clay dispersions) directly promote molecular reactions by relying on intrinsic interactions with the reacting species. On the other hand, aqueous environments (liposomes, emulsions) will usually serve as confinement and compartmentalization tools for enzyme-mediated reactions and allow the speciation of an entrapped reaction.

Non-enzymatic biopolymerization

The insertion of chemically modified monoribonucleotides and amino acids into biopolymers by enzymes is often limited by substrate specificity. Therefore, it would be quite valuable to achieve the selective insertion of these monomers by non-enzymatic means. A selective polymerization must rely on some recognition process, like the base pairing in nucleic acid chemistry, based either on intrinsic properties of the structured environment or on complexes composed of oligomeric templates and monomers whose formation is promoted by the medium. A molecular recognition that is only mediated by the presence of a chemical function on the monomer rather than by its shape would permit polymerization of a large number of chemically modified monomers. Such a synthetic approach would allow modified oligomers to be produced and amplified, creating libraries of medium-size molecules (15- to 30-mer) that could be tested for functions (e.g., binding or catalytic activity).

Homogeneous aqueous media

Peptide and nucleic acid oligomers capable of catalysis, molecular recognition or genetic regulation tend to be composed of at least 15–20 units because of their ability to form not only secondary structures but also tertiary structural motifs. For example, miRNAs (micro RNAs), siRNAs (small interfering RNAs) and small ribozymes (catalytic RNAs), are between 20 and 30 nucleotides in length [43, 44], with a large nucleobase fraction involved in the formation of structural motifs. Such an oligomer length cannot be reached by non-enzymatic synthesis in homogeneous aqueous solution via the general polymerization scheme depicted in figure 2a [4]. This reaction type uses either condensing agents, such as carbonyldiimidazole (CDI), with amino acids or activated monoribonucleotides, such as phosphoimidazolide-activated monomers (see structures in fig. 2b), with divalent metal ion catalysts as chemical energy sources. Even using high concentrations of activated monomers and metal ion catalysts [45] or of monomers and condensing agents [46], reactions in bulk aqueous medium usually yield low mass products. This is due in part to the hydrolysis of the active species [5, 29] and to the incapacity of the hydrophilic reactive molecules (e.g., uracil derivatives) to form structures, such as monomer stacks, that would facilitate polymerization. Oligomer elongation either by monomer addition or by ligation of short oligomers is equally unsuccessful [35, 47].

Base pairing on nucleic acid templates does not overcome the poor capacity of pyrimidine monomers to self-assemble. Reasonable yields are only achieved by the templated polymerization of activated G on poly(C) templates. C-rich mixed templates without consecutive adenines in their sequence also permit a templated polymerization of short oligomers [48–51], but pyrimidine polymerization on a complementary template is generally poor [48, 52–56]. Using hexitol nucleic acids (a DNA analog with

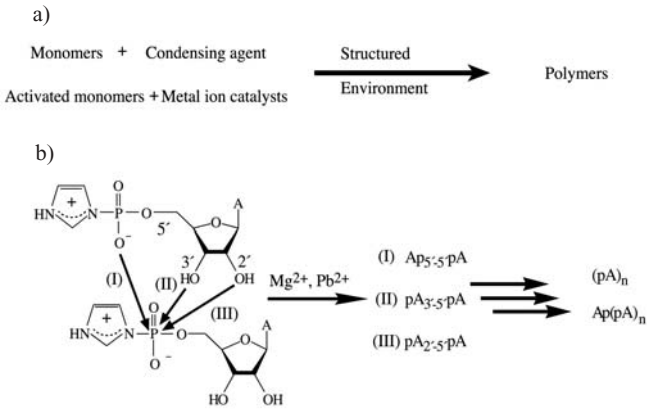


Figure 2. General scheme for non-enzymatic polymerization reactions from monomers in micro- and nanostructured media. (*a*) In the absence of polymerizing enzymes, monomers must be activated chemically either with condensing agents or with a leaving group in conjunction with metal ion catalysts. However, both activations are ineffective in homogeneous aqueous solution. Only in the presence of micro- and nanostructures can efficient polymerization be observed. (*b*) Polymerization of an activated monoribonucleotide, ImpA (adenosine 5′-monophosphate imidazolide). These derivatives were shown to be better building blocks than the corresponding unactivated nucleotides in the presence of condensing agents [1]. Imidazole-activated monomers can form 5′-5′ (I), 3′-5′ (II) and 2′-5′ (III) linkages. In bulk aqueous medium, the frequency of formed linkages follows the sequence I⩾III>II and III≥II⩾I in ice-water lattices and on clays.

a six-membered sugar backbone) as templates to partially preorganize the template/activated-monomer complexes in favorable A-type nucleic helical structure [57, 58] does not solve the poor pyrimidine assembly problem [59]. This limitation severely reduces the applicability of templated RNA polymerization in bulk aqueous solution.

Finally, non-enzymatic polymerization of biopolymers from unprotected activated monomers presents an additional challenge: the regioselectivity of the linkages (see fig. 2b). For example, because the 2′ hydroxyl group on the ribose is more reactive, activated monoribonucleotides in homogeneous aqueous medium tend to predominantly form 2′–5′ linkages [60, 61] with the notable exception of the poly(C) templated oligo-G polymerization. Amino acids with carboxylate side chains can form branched products.

Structured media

All micro- and nanoenvironments used in non-enzymatic biopolymerizations have to a certain extent the capacity to concentrate the active species by adsorption (clays, amphiphile structures) or physical dehydration (water-ice lattices), effectively reducing water activity and shifting reaction equilibrium. Simultaneously, they promote reagent molecular self-assemblies in configurations that facilitate polymerization and prevent unwanted side reactions. The nature of the monomers plays an important role in the interactions with the supporting environments. Natural nucleic acid monomers all have similar chemical properties, possessing a charged moiety (the phosphate group) and an aromatic moiety (the nucleobase itself). In contrast, amino acids possess a broad range of chemical properties: their side chains that mediate interactions with structured environments can be hydrophobic, hydrophilic and negatively or positively charged. This fact will shape reaction outcomes.

Minerals and Clays

Minerals used in non-enzymatic polymerization of RNA and peptides are all inorganic structures with charged surfaces. This surface net charge results in the capacity of minerals to interact with charged solutes by selective adsorption. The interaction strength depends on the adsorption and desorption rates of a given solute, i.e., its adsorption propensity and residency half-time. As expected from cohesive interactions, polyionic solutes tend to adsorb better than monomers [4, 42, 62]. The adsorption of linear single-stranded or double-stranded DNA or RNA [42] on montmorillonite occurs at lower concentrations of Mg(II) ions than that of monomers and is more extensive. Each monoribonucleotide seems to interact differently, even though their adsorption is thought to be mediated by a magnesium bridge between their phosphate and the montmorillonite [42]. In fact, Ferris and co-workers have clearly demonstrated that the nucleobase itself and even the activating group will contribute to the adsorption, and thus affect the polymerization. At the same monomer and clay concentrations, 53% of imidazole activated adenosine 5′-monophosphate (ImpA), but only 9% of ImpC and ImpU is bound to the clays after a 2-h equilibration [63], whereas 1-methyl-adenine-activated purines and pyrimidines both adsorb equally well, as their polymerization suggests.

The polymerization of ImpA by extension of a primer $(pdA)_9pA$ acting as an anchor yielded up to a length of 50-mer RNAs [47] when activated monomers were regularly added to replenish the monomer stocks and prevent the growth-limiting effect of monomer hydrolysis. However, a primer extension by ImpU only reached length of 30-mer (i.e., only 20 ImpU were incorporated) [64]. With 1-methyladenine-activated U and A, copolymers as long as 30- to 40-mers could be polymerized from monomers alone [37].

Montmorillonite-supported RNA polymerization is selective both at the level of the product nucleobase sequence and the regiochemistry of the linkages. While all possible dimers are formed in ImpA-ImpC mixtures, only a small number of all longer oligomer isomers predicted by the random synthesis are formed [40]. The 3′-terminal nucleobase (purine or pyrimidine) of the nascent oligomer and the regiospecifity of its phosphodiester bond (2′–5′ or 3′–5′ linkage) determine the reactivity for

elongation. Because of the higher reactivity of a 3′–5′-linked terminal nucleotide, the ratio of 2′–5′ or 3′–5′ linkages observed on montmorillonite is modified in favor of the natural isomers (3′–5′) compared to that in homogeneous medium. Furthermore, since the first nucleotide at the 5′-oligomer end is predominantly a purine, and a 3′-terminal purine has a higher intrinsic reactivity, the formation of purine-rich oligomers is favored [40].

Templated RNA polymerization (ImpA on polyU and ImpG on polyC) can be mediated by hydroxylapatite [65, 66], ferric oxide, and attapulgite, a clay [66]. One may reasonably assume that a templated polymerization of recently synthesized 1-methyladenine-activated pyrimidines on montmorillonite will soon be possible. Their intrinsically stronger adsorption should prolong the half-life time of the purine template/monomer complexes on the clay, allowing a more effective templated polymerization.

Modulation of the interactions between amino acids and the mineral surfaces by amino acid side chains results in stronger adsorption specificity compared to nucleic acids, influencing product composition [35]. For example, homooligomers of aspartic acid, $(Asp)_{10}$, adsorb efficiently on hydroxylapatite and illite but not on kaolinite, another clay, whereas those composed of arginine $(Arg)_{10}$ adsorb on kaolinite and illite only. Illite is a promising mineral surface that interacts with negatively and positively charged amino acids.

The polymerization of α-, β-amino acids or chemically modified derivatives, e.g., O-phospho-L-serine, is enhanced in the presence of minerals: homopolymers up to 45 monomers in length can be obtained with multiple additions of fresh monomers and condensing agents [47, 67]. Even though some minerals, such as illite, can promote a slightly enhanced polymerization of both positively and negatively charged amino acids (at least 10–12-mers instead of 5–7-mers without the mineral), the simultaneous incorporation of all amino acids, especially those with hydrophobic or apolar side chains, has not been achieved yet. Short peptides (≤7-mer) from amino acids with hydrophobic side chains alone are only formed when these amino acids are dried on montmorillonites [39]. However, their incorporation could be achieved by using relatively short oligomers mainly composed of charged amino acids, which would act as anchor on which the elongation by amino acids with apolar side chains could occur.

Amphiphile structures

Three structures formed by amphiphiles have been investigated for their potential in promoting peptide formation: bilayers of liposomes [68–71], micelles [46] and monolayer lattices [72, 73]. As for the mineral-supported reactions, substrate monomers can be adsorbed on the amphiphile-structure surface that can easily be functionalized by mixing zwitterionic and charged amphiphiles to yield various surface charge potentials. In addition, amphiphile structures offer the possibility of hydrophobic interactions within the bilayer or micelle hydrophobic core itself.

Luisi and co-workers studied the polymerization of amino acids and dipeptides in the presence of liposomes, using CDI and the more lipophilic 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) as condensing agents [68–71]. Liposomes formed by zwitterionic 1-palmitoyl, 2-oleyl-*sn*-glycero-3-phosphocholine (POPC) clearly increase yields in the polymerization of tryptophan (Trp) compared to a reaction in homogeneous aqueous solution: the total monomer incorporation into oligomers was over 80%, and a length of at least 13-mer was obtained. Product length could be increased to over 25-mer with multiple monomer additions, whereas reactions in homogeneous solutions reached only 7-mer, possibly because of a solubility issue [68, 69]. In contrast, the polymerization of negatively charged glutamic acid (Glu) only yielded short oligomers. However, when cationic liposomes [a mixture of POPC and didodecyldimethylammonium bromide (DDAB)] were used as matrix, co-polymerization of Trp and Glu occurred yielding products up to 10 amino acids in length, with a 1:1 amino acid ratio. The effect of cationic amphiphile headgroups on the polymerization of Glu, Asp and O-phosphoserine was also observed with another nanostructure type, the micelle [46]. Similarly, dipeptides containing a Trp linked to arginine or histidine were polymerized on negatively charged liposomes (a mixture of POPC and dioleyl-*sn*-glycero-3-phosphate). This interaction behavior seems to fit the electrostatic and hydrophobic nature of the adsorption.

Amphiphile self-assembly can also promote polymerization of amino acids covalently modified by an amphiphile moiety, acting as activating group [72, 73], or of Gly at high pressures and temperatures [74]. In the former case, the amphiphile bioconjugate moiety is driving the formation of monolayers at the water/air interface on which an enantioselective polymerization can be initiated. When bioconjugate enantiomers were mixed, racemic and enantiomorphous domains were formed in the monolayer and increased relative abundance of homochiral oligopeptides [72].

Eutectic phase in water-ice lattices

Contrary to the two other classes of micro- and nanoenvironments, the mechanism to concentrate monomers in the eutectic phase in water-ice is probably not based on a direct adsorption on the ice-crystal surface (mediated by electrostatic or hydrophobic interactions), but rather on a physical dehydration of the monomer solutions. The exact processes involved are not yet elucidated. But, in all polymerizations supported by this structured environ-

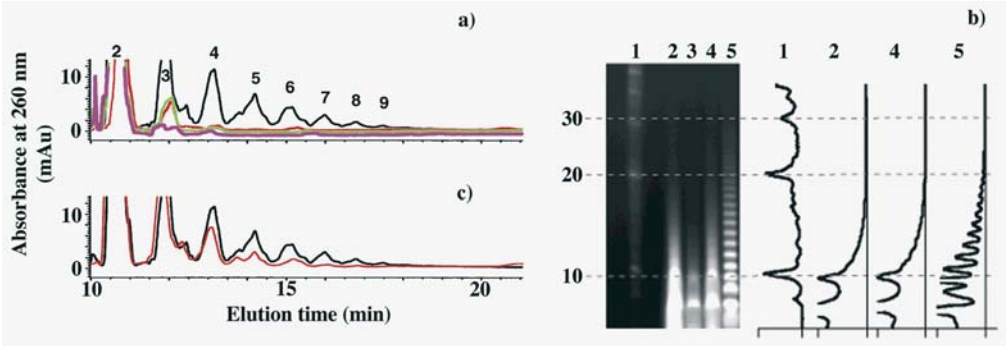


Figure 3. Polymerization of RNA from activated monomers in eutectic phase in water-ice. (*a*) ImpU oligomerization products formed at various temperatures after 4.8-day incubation. The black profile corresponds to a frozen sample at –18.4 °C, the red, green and purple profiles to liquid samples at –14.0, –6.0 and +22.0 °C, respectively, analyzed by ion-exchange high-pressure liquid chromatography. The numbers indicate the oligomer length by comparison with hydrolysis products of a synthetic polyU. (*b*) Length analysis of mixed RNA products by gel electrophoresis. (left 20% polyacrylamide gel, right normalized trace analysis; the numbers identify the samples). Lane 1: RNA molecular markers of 100, 90, 80, 70, 60, 50, 40, 30, 20 and 10 nucleotides. Lanes 2, 3 and 4: Samples containing equimolar four-nucleobase mixtures were incubated at –18.4 °C for 24 days. (2) 5 mM ImpN with 5.2 mM Mg(II)/0.6 mM Pb(II). (3) 10 mM ImpN with 5.2 mM Mg(II)/0.6 mM Pb(II). (4) 10 mM ImpN with 10.4 mM Mg(II)/1.2 mM Pb(II). (5) A control with 5 mM ImpU and 5.2 mM Mg(II)/0.6 mM Pb(II). (c) Monomer concentration effect. A 5-mM ImpU (black line) and 0.65-mM ImpU (red line) with the same catalyst concentration.

ment, the essential aspect for successful reactions is the ice nucleation, supercooled solutions are usually not conducive to any efficient reaction (see fig. 3a). This approach should have a larger application potential because it is not extensively dependent on monomer activation [29] or structure [30]. When mixtures containing all four monoribonucleotides are incubated, quasi-equimolar incorporation of all monomers into medium-length mixed oligomers (15- to 30-mer) is observed (fig. 3b).

During freezing, a large increase in solute concentration occurs which allows submicromolar activated monomer concentrations (radiolabeled products) to polymerize as efficiently as millimolar concentrations. For example, an 8-fold decrease of the initial monomer concentration did not influence either the incorporation yields (≥80% of monomers) or the length of the longest oligomer (see fig. 3c). During freezing, complex monomer-catalyst structures that facilitate the polymerization are formed. Contrary to reactions on clays or homogeneous solutions where the metal ions are usually equal to or in excess over the monomer concentration, the polymerization was successful at ratios of catalyst to monomers as low as 1:20. Typically, mixtures of Mg(II)/Pb(II) are used, but Pb(II) ions are the catalysts, and reactions without Mg(II) yield the same monomer incorporation into oligomers of a slightly shorter length. At a ratio of 1:8 (considering the catalyst only), polymerization yielded more than 80% monomer incorporation [30], a fact that seems to indicate that the molecular structures in the ice-water are dynamic assemblies. Product regiospecificity is also improved compared to homogeneous aqueous reactions: in mixtures of three and four activated nucleobases, approximately half of the oligomers (≥3-mer) contain at least one 3′–5′ linkage [30].

As part of its ability to promote polymerization, eutectic phase in water-ice has the advantage of reduced hydrolysis rates. In the polymerization of ImpU [29], the hydrolysis of the activated monomer was low, 6% of its initial concentration, whereas it is accelerated by metal ions proportionally to monomer concentration in homogeneous aqueous solutions [75] as well as on montmorillonite up to 600-fold [62]. This could be essential for the generation and selection of long oligomers with ribozyme activity.

Eutectic phase in water-ice seems to be conducive to the template-directed polymerization of ribonucleotides. Miller and co-workers established that template-directed oligomerization of ImpG can occur in the eutectic phase in water ice [76], a result confirmed by our observations [P.-A. Monnard and J. W. Szostak, unpublished results]. Short uridine oligomers (3–5-mer) were also ligated on poly(A) at –25 °C [77]. The sequence specificity in the templated reaction may be lowered in the ice nanostructures compared to that in homogeneous solutions, as experiments with hairpin ribozymes that catalyze ligation preferentially in a frozen medium [78, 79] show. Indeed, whereas the ribozyme ligation at room temperature requires 10 base pairing nucleotides between substrate oligomers (two single-stranded RNAs) and ribozyme, the ligation at –10 °C requires only 4 base-pairing nucleotides. However, the potentially lower base-pair specificity of a template-directed polymerization could prove to be advantageous for evolutionary processes. Note that this ligase also demonstrates the potential of the water-ice system as a selection tool for a new ribozyme.

Eutectic phase in water-ice was also reported to enhance polymerization of β-amino acids [28] and L/DL-leucine mixtures [80]. In the latter case, a short incubation results in enhancement of enantiomeric excess.

Summary

Micro- and nanoenvironments can support enhanced non-enzymatic polymerization of activated monomers, improving yields, polymer length, and product regio- or enantiospecificity compared to the same reaction in homogeneous solution. These environments create conditions that allow the reacting species to be concentrated and promote the formation of molecular arrangements conducive to polymer synthesis. Each environment has a slight selectivity toward some monomers due to intrinsic interactions that could be used for a selective synthesis of particular polymers. However, compared to enzymatic substrate specificity, the substrate specificity seems weaker, e.g., α- and β-amino acids and O-phosphoserine can be polymerized on the same mineral. Provided a chemically modified monomer possesses the essential chemical group (e.g., a negative side chain, the phosphate group on O-phosphoserine) to interact with the supporting environment, it should be inserted as well as natural monomers. Thus, the micro- and nanoenvironment could be further developed to prepare libraries of biopolymers or bio-copolymers (e.g., bioconjugates of RNA and peptides) composed of natural and modified monomers that could be evaluated for binding and catalytic activity.

Finally, at this time, a template-specific non-enzymatic polymerization at the fidelity and processivity levels obtained in enzymatic polymer replication has not yet been achieved. However, template-directed RNA polymerization supported by structured environments is possible, and RNA products are probably long enough to function as templates for their own replication [81] within micro- and nanostructures. Thus, structured environments could offer a path for sequence-specific amplification of modified biopolymers and their subsequent molecular evolution.

Enzymatic biopolymerization and molecular selection

The applications of micro- and nanoenvironments for biopolymer synthesis go beyond the promotion of non-enzymatic polymerization. Non-communicating amphiphile biomimetic compartments can also entrap enzymatic biopolymer synthesis, allowing the observation of biopolymer activity at the single-molecule level [82] and biopolymer selection from large template libraries. Liposomes and w/o emulsion compartments with encapsulated enzymatic systems have been investigated as biomimetic micro- and nanoreactors for a few decades, and a large number of reviews on the matter already exist (see, e.g., [6, 83] about liposomes and [19, 84–86] about reverse-micelles and w/o emulsion). Here, we will review mainly their application in biopolymer synthesis and selection.

Homogeneous aqueous media

In an in vitro selection experiment in homogenous medium, such as SELEX [9] or messenger RNA (mRNA) display [11], biopolymers with novel functions are selected out of up to 10^{16} different biopolymers (phenotype), enzymatically synthesized from libraries of random nucleic acids templates (genotype). To recover active molecules, genotype and phenotype must be linked. Singled-stranded RNAs and DNAs represent both genotype and phenotype, a fact which enables the direct selection of molecules binding a given ligand. To select for catalytic activity, nucleic acids must either self-modify or be tethered to a single substrate molecule [9]. This results in an "intramolecular single-turnover" selection. In mRNA display in homogeneous medium, proteins (phenotype) must be covalently linked to their template or mRNA (genotype) to preserve the genotype-phenotype link. Thus, only one protein will usually be synthesized per template. Although these methodologies allow the selection of single molecules, even with very low activity, they neglect essential aspects of an efficient catalyst such as substrate-association and product-release rates or the capacity for multiple catalytic turnovers. Often, a selected ribozyme will perform poorly under multiple-turnover conditions [19]. A spatial genotype-phenotype link (co-localization) achieved by compartmentalizing each biopolymer synthesis represents a substantial improvement: selections with multiple biopolymer copies based on catalytic parameters, such as turnover number, become possible.

Structured media

In a compartmentalized selection, the general scheme would call for the individual encapsulation of a single DNA template with all the necessary transcription/translation machinery in each compartment, much like in living cells. During synthesis, more than one copy of the active molecule can be obtained for each template and remain co-localized within the same compartment. Free substrate molecules required for turnover catalysis could then be added. Ideally, intact compartments with an encapsulated active biopolymer can be recovered individually. This step could be achieved using fluorogenic substrates and sorting fluorescent compartments by fluorescence-activated cell sorting (FACS), but this method

imposes some limitations in terms of compartment size (≥0.5 μm) and number of compartments sorted per second [87].

Suspension of liposomes and amphiphile vesicles

Properties of liposomal systems

Numerically speaking, compartmentalization of a selection library of 10^{15} molecules seems feasible in liposomes. For instance, in 1 ml of large unilamellar liposomes (LUVs) at 135 mM POPC, approximately 10^{15} individual noncommunicating compartments can be formed with a diameter of 0.1 μm. That is, each template could theoretically be entrapped into a separate reaction vessel. However, such a calculation assumes that all lipid molecules participate in the formation of unilamellar liposomes with an exact 0.1-μm diameter. Moreover, 100% encapsulation efficiency for the templates and high conservation of the transcription/translation machinery activity upon encapsulation are required, though the total internal volume with this ideal liposome suspension would only account for 41.7% of the total aqueous volume. Note that the term 'encapsulation' relates only to lipid mixtures that form spherical liposomes with encapsulated DNAs and not to those yielding DNAs coated by pure cationic bilayers, which are unlikely to act as templates.

Preparative techniques, such as freeze/thaw [88], dialysis [89], dehydration/rehydration [90] or organic solvent removal [91] followed by size homogenization by extrusion [92], have been developed to increase biopolymer encapsulation within zwitterionic liposomes. The encapsulation of nucleic acids can further be improved by using cationic bilayers: e.g., a 61% encapsulation of 369-bp DNA fragments (37 μg/ml) in 120-nm liposomes with 2.5 mol% positively charged amphiphiles (160 mM lipid during encapsulation) was reported [93]. That is, 5.6×10^{13} molecules were entrapped within 1.6×10^{15} of these cationic compartments. Under the same conditions, zwitterionic liposomes only encapsulated 25% of the DNA fragments. Positive charge density should be kept low to minimize interactions between solutes and charged bilayers, which can influence enzymatic activity [94].

The template encapsulation per se is not enough, and one wonders whether such a small container (120 nm in diameter) can harbor an efficient biopolymer synthesis. A small volume should increase reaction rates in a coupled transcription/translation by permitting rapid accumulation of intermediate products, mRNAs [95], but it could also profoundly influence the same reaction because substrate molecules are more likely to collide/interact with the vesicle boundaries than with the enzymes [96].

Liposome-entrapped biopolymerization

Oberholzer et al. showed that polymerase chain reaction (PCR) amplification or mRNA translation could be carried out compartmentalized in liposomes as small as 100 nm in diameter [97, 98]. Our recent results have shown that DNA-template-mediated RNA transcription by T7 RNA polymerase works within liposomes extruded with 200-nm pore size filters, though yields increased with 400-nm and 800-nm extruded liposomes [P.-A. Monnard and D. W. Deamer, unpublished results]. In general, yields were low. A coupled transcription/translation required for protein selection was so far only achieved in giant unilamellar liposomes, GUVs (diameter of more than 1 μm) [95, 99, 100]. In most of these investigations, all the components of the polymerization machinery were encapsulated along with their substrates. Although these systems lack one essential characteristic of selection compartments in that they do not truly interact with their surrounding medium (reactions will proceed only for a limited period of time determined by the substrate supply), they are enlightening in several ways. First and foremost, functional coupled transcription/translation machinery could be entrapped simultaneously within liposomal compartments, such as GUVs (5.1×10^{-16} to 4.2×10^{-12} l internal volume of 1–10 μm liposomes), while a DNA amplification or mRNA translation were entrapped in a single LUV internal volume as small as 4.2×10^{-18} l (100-nm extruded liposomes). Second, compartmentalization permits accumulation of mRNA products up to concentrations which induce an accelerated translation of active protein compared to that in homogeneous medium [95]. Third, translated proteins are active [99]. Fourth, once encapsulated, the integrity of small compartments is preserved even under quite extreme conditions, such as high temperature cycling of a PCR reaction [97] and in the presence of degradative agents. For example, digestive enzymes in the external aqueous phase cannot interfere with the internalized biopolymer synthesis [93, 99, 101].

The drawback of such stability is that PC bilayers represent an effective barrier to free diffusion of polar and ionic solutes, the level of permeability depending on solute and bilayer characteristics [101–103]. To achieve a steady supply of substrate molecules across the compartment boundaries, PC bilayers must be altered so that they become selectively more permeable to substrates while preventing the release of components essential for the catalytic activity or of reaction products, especially if those are used to monitor biopolymer activity. Different liposome preparations require different strategies to modulate their permeability by either physical alteration of the bilayers or introduction of mediated transport. Transient packing defects in the bilayers, more frequent at the lipid phase transition temperature (the temperature at which lipid molecules in the bilayer gradually pass from

a gel state to a liquid-crystalline state) [102, 104], can allow the passive diffusion of ionic solutes, such as NDPs (nucleotide diphosphates) [101, 105] and NTPs (nucleotide triphosphates) [102], which serve as substrates for RNA polymerases. The addition of detergents, which permeabilize PC bilayers at sublytic concentrations [106], can be used to supply encapsulated enzymes [106, 107]. This method is rather difficult to implement because the threshold concentration of detergent which allows small solute to permeate but prevents protein crossing (e.g., DNase I [106]) is not well defined. Oberholzer and colleagues used lipid-layer interdigitation by ethanol [2], but this effect only applies to GUVs because of their low curvature stress. Protein and synthetic channels with openings that discriminate against large solutes have also been inserted within vesicle bilayers [108]. Varying the number of channels within the bilayers even permits entrapped enzymatic activity comparable to that of a free enzyme [109], but usually requires GUVs whose surface areas can support the stress induced by multiple channel insertions.

Recovery of biopolymers from liposomes

The recovery of active biopolymers requires a liposomal system permitting an unequivocal distinction between the active and inactive species within the liposomes. As proposed, fluorescent tags, products of internalized activity could be used provided that the activity, level and liposome size are suitable for detection with a FACS unit. Light-microscopic investigations of liposomes have shown that this distinction is possible if the compartment size is at least 1 μm: in figure 4a, empty liposomes can be easily distinguished from those with encapsulated DNA after fluorescent staining. Furthermore, the synthesis of a mutant green fluorescent protein by coupled transcription/translation in GUVs with a average size of 1–5 μm clearly demonstrates that FACS can be used to monitor reactions [99], though the detection level in a selection, especially in early selection rounds when the biopolymer activity level is low, may be more challenging.

The polymerization of biopolymers (RNA or proteins) in liposomes has been achieved even by supplying substrates in the external medium. Liposomal compartments with entrapped fluorescent biopolymer can be recovered, e.g., using FACS. However, a selection of biopolymers synthesized within a liposomal compartment has yet to be performed. The challenge is to integrate all aspects of a selection compartment into a single system and ensure their simultaneous, coupled function in a controlled manner. This integration will lead to systems capable of a wide range of tasks and adaptation to a variety of environments.

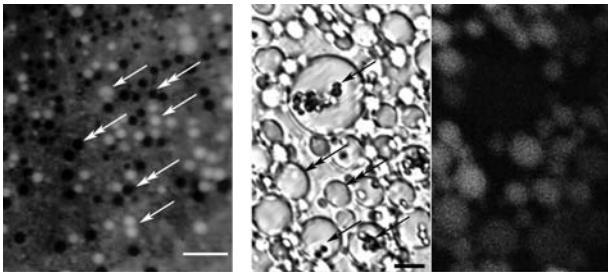
Compartments in emulsions

Properties of emulsion systems

Compared to liposomes, w/o micro- and macroemulsions seem relatively simple to prepare: an aqueous phase and an apolar, oil phase containing the amphiphile (also named emulsifier) are mixed, and a complete compartmentalization of the aqueous phase is obtained. However, number, size and stability of amphiphile compartments are influenced by the amphiphile used and three parameters: the ratio of water to amphiphiles, the ratio of water to oil, and the mechanical energy (stirring) injected in the system. Microemulsions (average compartment size of less than 1 μm), which could contain up to 10^{15} compartments per milliliter, are usually obtained using charged amphiphiles, such as di(ethylhexyl)sulfo-succinate (AOT) and an organic solvent. These small compartments can have a more extensive inhibitory effect on entrapped enzymatic reactions than observed with liposomes. They tend to prevent the accumulation of RNA products by excluding them into the organic phases where they precipitate [105], and supercoiling of DNA can be induced in microemulsions [110]. In fact, at low ratios of water to amphiphiles at which small compartments are formed, most water molecules are bound to the amphiphile interface and have the same properties as immobilized water (a low dielectric constant and a suppressed freezing point); as this ratio increases, water starts to behave as in homogeneous aqueous medium [85].

For their first protein selection [18], Tawfik and Griffiths designed macroemulsion compartments composed of non-ionic amphiphile mixtures (Span, Tween or Triton) in mineral oil to overcome the microemulsion limitations. Most emulsion-based protein selections at this time use a slightly modified version of this original macroemulsion recipe, even though these large compartments are thermodynamically metastable [111].

By varying the ratios of water to amphiphile and of water to oil, compartments can be obtained whose size is comparable to that of a cell and whose stability is such as to permit PCR reactions, while compartments smaller than 1.0 μm would separate into separate oil and water phases during temperature cycling. Along with the transcription/translation machinery, these compartments can entrap large solutes, such as micrometer-size beads, or bacterial cells, providing an effective way to visually check the compartmentalization of a single DNA template per emulsion compartment. Under the right conditions, a single template can either be transfected into a bacterial cell or individually attached to a microbead, both of which can be observed by light microscopy (see fig. 4b) to determine the ratio of bead/bacteria per compartment which directly correlates with that of template per compartment. To obtain a homogeneous solute distribution (i.e., compartments with one entrapped bacterium represent the



(a) (b)

Figure 4. Light micrographs of liposome- and emulsion-entrapped biopolymers. (*a*) Epifluorescence micrographs of liposomes produced from amphiphile mixtures with encapsulated salmon testis DNA (approximately 600 bp in length). A dilute solution of acridine orange dye was added to stain the DNA. Liposomes that contain DNA become fluorescent under ultraviolett-illumination (single-head arrows). Empty vesicles do not take up dye, and therefore do not fluoresce (double-head arrows). Bar, 20 μm. (*b*) Compartmentalization in w/o emulsion (Courtesy of A. Luptak and J. W. Szostak). (Left, phase contrast image) Streptavidin-coated beads (1-μm polystyrene beads) with biotinylated DNA templates (approximately one template per bead) were entrapped without homogenization, and some compartments contain bead aggregates (single-head arrows), some are empty (double-head arrows). The compartment size distribution without homogenization is also polydisperse. (Right, epifluorescence image) The fluorescence of Oregon Green 488 also present in the aqueous compartment can be used to visualize the compartments. Bar, 5 μm.

main compartment fraction), the rate of stirring is the determining factor [20].

The ratio of total aqueous volume to organic solvent or mineral oil is also critical because it will determine whether compartments are exchanging contents (by means of collision-fusion-fission or partition in the apolar phase and diffusion process) or stay noncommunicating [20, 85]. The number of exchange events is also influenced by the thermal energy (i.e., the temperature relative to the surface tension) in the system. By diluting the compartments in oil, exchanges of NTPs can be reduced to undetectable levels [20]. Thus, in the selection for Taq polymerases with higher thermal stability [20], one polymerase mutant was expressed within a particular compartment, and upon PCR cycling it only amplified its own gene to the exclusion of those in other compartments.

Biopolymerization entrapped in emulsion compartments

Large compartments have a drawback insofar as the possible number of individual transcription/translation reactions: the selections conducted in macroemulsions screened a maximal template number of 10^6–10^9, about seven orders of magnitude less than in homogeneous medium. But these investigations established some important proofs of principle. It is clear from these experiments that one particular active molecule can be isolated relatively rapidly from a 10^7 excess of non-active molecule [18] or from a template library of same size [20, 21]. In ideal systems [18], it is not unusual to observe up to a 1000-fold enrichment per selection round. However, in actual selections from partially randomized libraries [20, 21], a 50- to 100-fold enrichment is observed. Compartmentalization can also allow reactions at substrate concentrations comparable to those in cells which would not be possible in homogeneous media [18]. Coevolution of both the genotype and phenotype can be achieved as the silent-mutation rates on the Taq polymerase templates demonstrate [20]. Finally, the selection for extremely fast phosphotriesterases [112] demonstrates that selections in w/o emulsions can be based on catalytic turnover, a proposition difficult to implement in homogeneous medium.

Recovery of biopolymers from emulsions

The selective recovery of an active biopolymer synthesized in a w/o emulsion can be achieved by different methods: direct detection of the products, FACS sorting of microbeads on which product molecules are adsorbed during selections or direct separation of the emulsion compartments. The two first methods are reminiscent of those applied in homogenous media in that they require breaking the emulsion and therefore do not take advantage of the individual compartmentalization for sorting. For example, in the case of Taq polymerase selection, direct product detection is warranted because the activity of polymerase in one compartment will lead to the sole accumulation of its encoding gene [20]. One-micron beads that act as supports for the template and as adsorption surface for product molecules are large enough to be directly sorted by FACS [112, 113], provided that product molecules are fluorescent or can be fluorescently labeled. With these microbeads, the selection can be based on multiple-turnover rates by varying the fluorescence threshold used to sort them.

Tawfik and co-workers also demonstrated that, in principle, emulsion compartments can be directly sorted by re-emulsifying a w/o emulsion, i.e., forming water-oil-water (w/o/w) compartments, which can be sorted on a FACS machine [114]. If two emulsions, one containing a positive reaction with fluorescently labeled proteins, the other with the negative reaction without labeled protein (in this model, the fluorescence is not the product of the reaction), are mixed at ratio of 1:100 and re-emulsified, compartments with the labeled proteins can be enriched up to 38.5 times after two sorting rounds. Moreover, no fluorophore exchanges between the two compartment types occurred with these large reporter molecules. However, reemulsification often results in w/o/w compartments containing both positive and negative w/o compartments, decreasing the final enrichment factor.

Water-in-oil emulsions were prepared to compartmentalize in vitro selection systems with only a single template

per reaction compartment, thereby yielding multiple transcripts or proteins. These systems allow for selection based on catalytic characteristics (e.g., turnover rates) not available in bulk media, but at this time, only small libraries (10^6–10^9 random templates) can be used.

Summary

Liposomes and emulsions seem suitable for biopolymer syntheses and selections. Both compartment types can spatially link genotype and phenotype, a requirement for biopolymer evolution and selection, and could harbor selections under multiple-turnover conditions. They are also complementary: small liposomes are very stable and could be used in RNA selections from large template libraries with solute exchanges between their internal aqueous volume and the external medium as a potential selection-pressure tool. Compartments of w/o emulsions allow the selection of proteins because an efficient coupled transcription/translation is more easily achieved. However, some problems have to be solved before the micro- and nanostructure-supported selection methodology can be broadly applied. Liposome encapsulation efficiency and the stability of present w/o emulsions in a narrow range of composition, i.e., their lack of adaptability, remain critical issues.

Conclusion

Although some aspects of truly efficient single-biopolymer selection in micro- and nanostructured media have yet to be elucidated, this review shows that micro- and nanostructures allow more efficient non-enzymatic polymerization of biopolymers from natural monomers than homogeneous media. When forming noncommunicating compartments, they also permit linking of genotype and phenotype, which is crucial for in vitro selection and evolution of biopolymers with novel functions.

As our knowledge of micro- and nanostructured media characteristics increases and existing detection technologies such as FACS are improved (higher sorting rates, higher sensitivity to small structures) or new technologies emerge, we may be able to promote the polymerization of novel biopolymers with modified monomers that are incompatible with biosynthetic enzymes, and to improve in vitro selection of biopolymers with novel functions by using micro- and nanoenvironment supported polymerization.

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