Hypothesis

Phase separation in cytoplasm, due to macromolecular crowding, is the basis for microcompartmentation

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Abstract The macromolecular diversity and concentrations in the fluid phase of cytoplasm constitute conditions necessary and sufficient for aqueous phase separation. Consequences of phase separation in cytoplasm, including its 'compartmentation', are inferred from analogies with the physicochemical properties of aqueous two-phase systems and with the partitioning behavior of biomaterials in them.

Key words: Aqueous phase system; Cytoplasm; Macromolecular crowding; Microcompartmentation; Phase separation

1. Introduction

Mixtures of aqueous solutions of structurally distinct macromolecules, above certain concentrations, undergo phase separation [1–3]. The phases formed are generally solutions which differ in concentration and physical properties. Cytoplasm contains high concentrations of proteins [4–7] and nucleic acids [8]. Can these mixtures exist without undergoing phase separation? If multiple phases do form, their properties may be the basis for many cytoplasmic phenomena ascribed to 'microcompartmentation' [9,10]. Here we explore this possibility, drawing parallels between the well-studied properties of two-phase aqueous mixtures of macromolecules and observations on the behavior of cytoplasm.

2. Phase separation in aqueous solutions of macromolecules

The types of phase separation relevant to the present discussion are of two kinds, depending on whether the interacting macromolecules are compatible or incompatible. If two macromolecular species attract each other, complex coacervation can occur [11]. In this type of phase separation, usually observed with mixtures of molecules of opposite net charge, one phase is enriched in both species while the second phase, often of much larger volume, contains low concentrations of both species. The extreme concentrating effect and creation of a distinct solution environment which can result was proposed by Oparin as the mechanism of formation of primordial cells in his theory of the origin of life [12].

The second more common type of phase separation occurs when two macromolecular species repel each other in solution, leading to phases each of which is enriched with respect to one of the species. Such phases may result from mixtures of synthetic polymers [2], polysaccharides [2], proteins [13], or combinations of these materials with themselves [14] or with nucleic acids [11]. Multiple, mutually incompatible components can lead to multiple phases in equilibrium with one another, each containing unique combinations of concentrations of the materials involved. A related separation into two phases has been observed below 15°C in isolated cytoplasm from cells of the eye lens [3,15].

The interface between two aqueous phases is characterized by a small but measurable interfacial tension which is strong enough to cause immobilization in the interface of particles of dimensions greater than a few hundred Ångströms [1]. Any change which increases the difference in composition across the interface, such as increases in macromolecule concentration or partition of salts in favor of one phase, will increase the magnitude of the interfacial tension. The interface can also present a barrier to transport processes, the diffusion of a macromolecule depending both on its diffusion constant in each phase and on its equilibrium partition coefficient. The magnitude of the interfacial tension and the differential affinity of each phase for a solid surface determines the physical form of a phase. For phase volumes which are too small to be affected significantly by gravity, the phase which has the highest affinity for any surface with which it is in contact will tend to coat that surface. The second phase will then take the form with the lowest possible surface area within the constraints of the geometry imposed by the bounding surfaces. Chromatographic column materials have been synthesized based on this principle in which one phase is completely rejected from the gel bead and limited to the mobile phase, the gel being occupied by the non-rejected phase [3].

Salts and small charged solutes in two-phase systems distribute largely on the basis of electrostatic properties. In phases containing concentrated polyelectrolytes, such as proteins and nucleic acids, differences in concentration of the components will often lead to differences in total numbers of charges in each phase, producing a Donnan potential difference across the interface which will affect the distribution of smaller charged species [16].

The most widely studied aqueous two-phase systems are derived from mixtures of two polymers, particularly dextran and poly(ethylene glycol). Partitioning in these systems is an established method for the separation and characterization of biomaterials [1–3]. The partition behavior of salts, small solutes,

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macromolecules and particulates in such systems, and the physical properties of the phases, provide many analogies to microcompartmentation within cytoplasm. Their consideration leads to a model of a pervasive, yet dynamic, infrastructure for the fluid phase of cytoplasm.

3. Is phase separation likely in cytoplasm?

In mixtures of neutral polymers, phase separation typically occurs at concentrations greater than a few weight percent of each species [1–3]. These concentrations are relatively low because unstructured polymers can interact over their whole chain length, maximizing the effect of incompatibility which leads to phase separation. The concentration of protein in cytoplasm is in the range 17 to 35% w/w [4]. In *E. coli* the nucleic acid concentration is about 7.5% w/v and the total macrosolute concentration 27.5% w/v [8]. Because proteins and nucleic acids are more structured and compact, higher concentrations are required to induce phase separation of the type observed in incompatible polymer mixtures. Protein–protein two-phase systems generally do not form until the concentration of each reaches 7–10% [13], concentrations seldom achieved by single macromolecular species in cytoplasm. Single proteins, therefore, would not be expected to be present in high enough concentration to form phases if separation occurred as it does in mixtures of two purified proteins.

In the high concentration environment of the cytoplasm another factor comes into play, however. It is known as 'macromolecular crowding' and is the result of the reduced volume available to any single macromolecule due to the volume from which it is excluded by surrounding macromolecules in solution [7,17]. Relatively long-lived macromolecular structures, such as fibrillar elements, also restrict the effective volume available, although they are expected to have a smaller effect on solution reactions than dissolved macromolecules [18]. The effects of macromolecular crowding on any reaction which proteins or nucleic acids undergo in the cytoplasm can be very large, the thermodynamic activity of these species being predicted to increase by factors of several fold, up to orders of magnitude, depending on the molecular weights and geometry of the reacting species and the background concentration of the crowding species [7,17]. The dependence is so strong that prediction of chemical activity in the cytoplasm based on relatively dilute solution measurements in purified systems may be extremely misleading.

In the presence of macromolecular crowding, phase separation is expected to occur at much lower concentrations of the species involved than is predicted from phase diagrams determined in the absence of crowding [7]. Hence, any two incompatible macromolecular species or two species which have a net attraction for each other could, in principle, drive phase separation and produce small, localized phases of distinct composition bounded by an interface with an interfacial tension. While in bulk solution such phases would stratify with the less dense phase on top of the more dense one, given the small dimensions of a cell this would be unlikely to occur and the phases would distribute on the basis of their wetting properties for the fibrils and surfaces with which they were in contact. Because of the large number of protein species present, multiple phases could well occur, each localized by interfacial tension and/or nearby solid bounding surfaces with which it is in contact. Hence, the presence in cell cytoplasm of numerous liquid phases distributed in contact with the solid-like fibrils and organelle surfaces is not unreasonable. In the remainder of this paper we relate some consequences of this model.

4. Molecular and particulate partitioning in aqueous two-phase systems

The feature of the two-phase aqueous systems which has led to their detailed study is the preferential partitioning behavior exhibited by added molecules and particles, used for isolation and characterization of the partitioned species [1–3]. We are not aware of any systematic studies on partitioning between phases formed by proteins or proteins and nucleic acids, most of the work having been done with systems containing neutral polymers. Based on observations in such systems and general thermodynamic principles, a number of similar dependences would be expected to hold in two-phase systems composed of proteins or proteins and nucleic acids and therefore are relevant for consideration in terms of our model for microcompartmentation of cytoplasm. Partitioning of small solutes between phases would most likely be unequal only due to relatively large electrostatic effects; small neutral molecules would be expected to partition roughly equally between the phases. If phase separation resulted in separation of proteins or nucleic acids of different charge densities the distribution of salts and small charged molecules would mirror this difference so as to produce electroneutrality in each phase. Significant salt concentration differences could result.

The partitioning behavior of proteins and other macromolecules not responsible for phase separation would be more extreme and depend on a number of factors, including their molecular weight, shape, charge and hydrophobicity. Higher molecular weight materials, such as oligomers, will always partition more in favor of one phase than equivalent lower molecular species, such as monomers, due to the greater area available for intermolecular interaction with the phase-forming species. If a solute has even a weak tendency to associate with one of the phase-forming species the solute will concentrate in the phase in which that species predominates (affinity partition). For example, the more hydrophobic of a phase-forming pair would tend to collect hydrophobic solutes. The molecular weight of the phase-forming species also plays a role, causing increased partitioning into phases in which the molecular weight of the predominant species is reduced. This is due to an increase in the entropy of mixing.

In vitro, protein separations by affinity partitioning have been effected using gene manipulation to add to a target protein a particular peptide that has an affinity for one of the phase-forming polymers [3]. A similar strategy could have evolved for localization of proteins in the phases under consideration here.

Studies on nucleic acid partitioning indicate a strong dependence on ionic environment and electrostatic effects that would be expected to be mirrored in partitioning in phase-separated protein systems. Double- and single-stranded DNA differ greatly in partitioning behavior as do supercoiled and linear DNA [2]. Base- and sequence-specific macroligands attached to one of the phase-forming macromolecules have permitted extraction of nucleic acids on these grounds [1–3].

While partitioning of soluble materials occurs between the two bulk phases, partitioning of particulates takes place, as

their size increases, among both the bulk phases and the interface and, finally, between one bulk phase and the interface [1–3]. Besides size, chemical properties of the particle surface and its relative affinity for the phases determine the degree to which one or the other phase wets the surface and thus the particle's location in the system.

5. Consequences of phase separation in cytoplasm

'Microcompartmentation' of cell cytoplasm and its major biological implications and ramifications have been the subject of innumerable papers and many books (e.g. [9,10]). Initially used to designate the volume occupied by a metabolite-coupled enzyme complex the term is now more generally applied to non-homogeneous distribution of biomaterials (solute and some particulate) in spaces not demarcated by membranes. The physical events most likely responsible for such distributions are interactions of biomaterials with cytoskeletal elements and, as considered here, the materials' localization in compartments resulting from phase separation.

Cytoplasmic regional differences in ion concentrations (as reported, e.g. for H^+ [9], Na^+ [19], K^+ [20], Cl^- [19]) can result from the necessity to neutralize phase-forming or partitioned polyelectrolytes in microphases. Such differential partitioning of solutes among adjoining phases can result in the formation of gradients (e.g. H^+, ATP [9], Ca^{2+} [9]). Since different ion concentrations in adjoining aqueous phase compartments result in electrostatic potential differences between them [1,2], the known concentration differences of some ions within cytoplasm (see above) would yield Donnan potential differences. The magnitudes of these would, however, not be expected to be large based on measurements reported to date [1].

Protein–ion [9], protein–small solute (e.g. coenzyme) [21] and protein–protein (e.g. enzyme clusters as in the glycolytic complex) [9,22,23] binding have all been observed 'compartmentalized' in cytoplasm. Glycogen, a branched polysaccharide, could well produce phase separation with some proteins, providing a microphase in which the degradative reactions of glycolysis and the synthesis of glycogen occur.

The preferential partitioning of a biomaterial into a given phase results in the material's immobilization in that phase without the need or use of solid supports. The preferential partitioning of enzyme complexes into a particular phase has been observed in aqueous two-polymer phases. Weak heterogeneous enzyme–enzyme interactions (e.g. of enzymes in a specific metabolic sequence) result in complexes which partition differently from the individual components [24], providing a localization mechanism. Thus, compartmentation of metabolic sequences [6,22] in cytoplasm (and the channeling of intermediates [25]) can result from partitioning into a specific microphase. Reaction products of the polymer phase–'immobilized' enzyme complexes, being smaller, generally partition more equally, reducing thereby biofeedback effects [3].

The partitioning of particulates (e.g. membranes, organelles) in two-polymer aqueous phase systems depends most sensitively on their surface properties (e.g. charge, hydrophobicity) [1–3]. The heterogeneous distribution of organelles and other particles (e.g. mitochondria [6,9,26]; ribosomes [26]) within the cytoplasm of mammalian cells may be due to their differential partitioning among cytoplasmic phase compartments. Since partitioning into the interface occurs with an increase in the size of the biomaterials [1,2] the latter would also be involved in partitioning phenomena occurring within cytoplasm. Particles adsorbed at an interface would exhibit reduced Brownian motion, as has been observed [6], due to the elastic properties of the phase boundary. Part of the immense (calculated) intracellular surface areas [6,27] may be ascribable to interfaces.

Diffusion in cytoplasm of water [28], small solutes [29,30] and macromolecules [5,7,27,31–33] as well as particulates (e.g. ribosomes and polysomes [34]) proceeds in a non-ideal, slow manner. This has been attributed to the viscosity of cytoplasm, macromolecular crowding and reversible binding of the diffusing biomaterial to cytoskeletal elements [7,21,27,31]. Cytoplasmic phase separation requires diffusion of biomaterials across interfaces. The diffusion of proteins in aqueous polymer systems depends not only on the diffusion constant but also on the protein's partition coefficient [2]. Thus while cytoplasmic crowding and viscosity must affect the rate of diffusion, the interface can pose, depending on the protein's partition coefficient, a unidirectional retarding barrier. Consistent with certain observations in cytoplasm (e.g. [31]), interface barriers do not mandate a proportional relationship between molecular size and diffusion. The invocation of reversible interactions of all diffusing materials with cytoskeletal elements may also not be necessary.

Phase separation of concentrated polymer solutions, whether resulting in phases enriched with different polymers or in phases that are polymer-rich and water-rich, usually results in phases having different bulk viscosities [1,2]. Regional differences in bulk viscosities are thus an anticipated consequence of cytoplasmic phase separation.

Affinity partitioning in aqueous two-phase systems, i.e. the selective extraction of a biomaterial into a particular phase, occurs when a specific ligand (e.g. coenzyme) for the material of interest is bound, covalently or non-covalently, to one of the phase-forming polymers [1–3,35]. Alternatively, the surface of the biomaterial can be modified (e.g. by attaching chemically or by genetic engineering a 'leader sequence' to it with desired partitioning characteristics [3]). 'Sorting labels' which direct intracellular traffic of biomaterials [36,37] may analogously have characteristics required to effect appropriate partitioning to a desired destination.

The specific or non-specific adherence of certain cytoplasmic proteins [7,32] to cytoskeletal elements can result in the orientation within the fluid phase of cytoplasm of even those phases not in contact with solid supports. If a protein is either a phase-forming species or partitions strongly into a phase, its attachment to the cytoskeleton or any other surface can cause the phase with which it is compatible to wet that surface. Other phases will not be able to contact the wetted surface and will be forced into spaces adjacent to it.

6. Concluding remarks and summary

While aqueous phase separation has been alluded to in the literature, either directly (e.g. [38–40]) or peripherally, as a possibility in cytoplasm, a detailed consideration of the nature and variety of physicochemical phenomena it could effect has not been provided previously. Here we have culled from the literature some observations on the behavior of biomaterials in the fluid phase of cytoplasm that have analogies with those in aqueous two-phase systems (Table 1).

Table 1
Some physical events in aqueous two-phase systems and analogous observations in cytoplasm*

Aqueous phase systems	Cytoplasm
Dif. partitioning across multiphases [2].	pH and some other (e.g. ATP, Ca^{2+}) gradients [9].
Donnan potential between phases containing certain salts [1,2].	Donnan potential: a likely occurrence due to unequal distribution of ions.
Exclusion of macromolecular solutes from a phase [3].	Macromolecular crowding [7,8].
Affinity partitioning: binding of ligands to a phase-forming polymer [1–3,35].	Protein–ion [3,9]; protein–small solute interactions [21].
Protein–protein interactions [1–3,24].	Protein–protein interactions [9,22,23].
Altered protein partitioning due to reaction with small molecule and conformational change [3].	Altered protein distribution due to reaction with small molecule and conformational change [9].
'Steered' partitioning by attachment of 'leader sequences' with desired partitioning properties [3].	Destination determined by attached 'sorting labels' [36].
Fermentation in one phase and partitioning of product to other phase [3].	Compartmentation of metabolism [6,22] and channeling of intermediate [25].
Diffusion across interfaces [2].	Non-ideal, slow diffusion of micro- [29,30] and macromolecules [5,7,27,31–33] and particulates [34].
Preferential wetting by a phase of selected coated surfaces/ repulsion of one phase by a surface causing another phase to adhere to that surface [3].	Preferential adherence of certain proteins to cytoskeletal elements [6,32].
Preferential partitioning of particulates [1–3].	Compartmentation of organelles (e.g. mitochondria) [6,9,26].
Interface between phases [1–3].	Intracellular surface areas are, by calculation, immense [6,27]: could be due to interfaces.

*For discussion see text.

Our model of the fluid phase of cytoplasm is one of compartmentalization produced by aqueous multi-phase separations resulting from compatible or incompatible interactions among cytoplasmic macromolecules magnified by the effects of macromolecular crowding. This would provide an organized, yet dynamic, structure. The initial organization would depend on the interaction of components of some of the phases with intracellular structures (e.g. cytoskeletal elements) – either because of their affinity (biospecific, charge, hydrophobic) for the structure or because of the exclusion of an adjacent phase – which causes the components' adherence, providing thereby the three-dimensional positioning within the fluid phase of cytoplasm even of phases not in contact with solid supports. The dynamic aspects of the structure rest on the compositional differences of the various phases, themselves subject to change, which cause biomaterials to partition between and among them. Differences in ionic composition and concentration, due to the necessity to maintain each phase electrically neutral, will result in gradients which can have significant impact on biological activities and on partitioning behavior.

Phase separation occurs only above certain macromolecular concentrations. Thus changes in such concentrations can cause adjacent phases to become homogeneous solutions, or homogeneous solutions to phase separate. Such reversible effects can alternately mix and segregate phase components. The biosynthesis as well as the partitioning of biomaterials will alter the macromolecular composition and concentration of phases and affect the latter's physical properties.

We see cytoplasmic phase separation as an inevitable consequence of macromolecular synthesis that produces an evolutionary pressure on developing cells. The process, being a physical phenomenon, is not under direct genetic control. Its evolutionary effects may be visualized as being similar to those associated with lipid synthesis which results in spontaneous membrane formation. The absence of cytoplasmic phases would indicate that the evolutionary pressure they represent was sufficiently negative to result in very low concentrations of unbound macromolecular species in cytoplasm.

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