Low-Transition-Temperature Mixtures (LTTMs) for Dissolving Proteins and for Drug Formulation

Low-transition-temperature mixtures (LTTMs) for dissolving proteins and for drug formulation

Erzheng Su[a,b] and Alexander M. Klibanov*[a]

[a] *Departments of Chemistry and Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139, U.S.A. Tel.: 617-253-3556; E-mail: klibanov@mit.edu*

[b] *Enzyme and Fermentation Technology Laboratory, College of Light Industry Science and Engineering, Nanjing Forestry University, Nanjing 210037, China. Tel.: 86 25 85428906; E-mail: ezhsu@njfu.edu.cn*

Abstract

Several diverse proteins are found to readily dissolve in neat low-transition-temperature mixtures (LTTMs). They undergo no irreversible denaturation in such unusual solvents, and the resistance of hen egg-white lysozyme against thermoinactivation in LTTMs is greater than in aqueous solution at extreme pHs. Separately, the water-sensitive drug aspirin is found to form concentrated transparent LTTMs, where it is some 10-fold more stable against cleavage than in water.

Keywords: Low-transition-temperature mixtures, protein solubility, water-sensitive drugs, drug formulation

Dissolving proteins in media other than their natural aqueous milieu may offer new opportunities for bioprocessing, delivery of pharmaceutical proteins, and biocatalysis [1, 2]. Therein, it is mechanistically important to distinguish proteins suspended in organic solvents [3] or solubilized in them when aided by detergents (via reversed-micelle formation) [4] from scenarios where proteins dissolve (i.e., form molecular dispersions) by themselves.

Even though common proteins are insoluble in most neat organic solvents, they do dissolve at relatively high concentrations (>10 mg/mL) in a few such solvents that are protic, polar, and very hydrophilic [3, 5]. Furthermore, recently proteins also have been found to dissolve, albeit at low concentrations, in neat ionic liquids [6].

Over the last decade, a novel class of organic liquids has emerged, the low-transition-temperature mixtures (LTTMs), prepared by mixing solid compounds that become liquid at room temperature upon interacting with each other due to hydrogen bonding [7]. LTTMs are exemplified by pioneering studies of Abbott *et al.* [8] who demonstrated that heating a mixture of choline chloride and urea (hydrogen-bond acceptor and donor, respectively) resulted in a liquid which did not solidify even upon subsequent cooling. Subsequently, LTTMs have been employed as solvents for a variety of applications [7, 9, 10]. In this work, we explored LTTMs as potential solvents for proteins and indeed found many of them, both previously described and new ones, to dissolve common proteins with no irreversible denaturation.

LTTMs for protein dissolution were prepared by selecting seven organic quaternary ammonium salts and one inorganic salt as hydrogen-bond acceptors and fifteen organic acids, alcohols, sugars, and urea as hydrogen-bond donors. In a typical procedure, a binary mixture of a hydrogen-bond donor and acceptor was heated until a liquid formed (usually at about 100^{o}C), followed by cooling. Out of those combinations, 28 liquid LTTMs stable at room temperature were obtained (Table S1). Six of them, heretofore unreported [7, 9, 10], originated from tetrabutylammonium bromide or tetraethylammonium chloride (previously found by us to lower the viscosity of concentrated aqueous protein solutions [11]) as hydrogen-bond acceptors. We selected half of those 28 LTTMs exhibiting lower viscosities for further investigations.

Bovine serum albumin (BSA), bovine pancreatic α-chymotrypsinogen A (CTgen), subtilisin Carlsberg from *Bacillus licheniformis*, and hen egg-white lysozyme were chosen as models for this study. The solubility of these well-characterized proteins, common workhorses of biophysical investigations, in neat LTTMs was typically measured by placing 30 mg of a solid protein into a 2-mL screw-cap scintillation vial, followed by addition of 1.0 g of a LTTM. The resultant mixture was shaken at 37°C and 600 rpm for 16 h and then centrifuged at 16,000 *g* for 2 h. An aliquot of the supernatant was withdrawn, diluted 10-fold with distilled water, and its protein content was determined using the Bradford assay [12]. As seen in Table 1, all 14 neat LTTMs dissolved measurable concentrations of the four proteins, sometimes at multiple mg/mL levels. The solubility was found to be both protein- and LTTM- dependent. Some of the protein solubilities in the LTTMs (Table 1) far exceed those previously observed for ionic liquids [6, 13, 14] and approach those in protic, hydrophilic, and polar organic solvents [5].

Next, using the enzyme lysozyme, we examined whether a protein dissolved in a LTTM undergoes irreversible denaturation. To this end, 0.5 mg/mL lysozyme solutions in all the LTTMs were diluted 10-fold with an aqueous buffer (66 mM phosphate, pH 6.2), and the enzymatic activities of the resultant lysozyme solutions were measured based on the rate of lysis of dried *Micrococcus lysodeikticus* cells [15] and compared to those of the native enzyme at the same

concentration. As seen in Table 2, nearly all enzymatic activity was recovered upon aqueous dilution. Hence lysozyme dissolved in LTTMs undergoes no appreciable irreversible inactivation, which is in contrast to previous reports involving protic organic solvents or ionic liquids [14, 16].

Organic liquids can be characterized by several macroscopic and microscopic properties, including the liquid range, density, pH, viscosity, polarity, and ability to form hydrogen bonds [17]. We attempted to correlate some of such properties of LTTMs with the latters' propensity to dissolve proteins. However, as indicated by Table 1, no discernible correlation was observed between any of these LTTM properties and protein solubility (see Fig. 1S, showing the results obtained with lysozyme as an example), attesting to the complexity of the underlying molecular interactions.

A number of degradation reactions can take place in aqueous solutions of proteins at high temperatures, especially at acidic and alkaline pH values [18]. Since suspending enzymes in some organic solvents or ionic liquids can improve their stability [3, 19], we examined the thermal stability of lysozyme dissolved in LTTMs at two extreme pHs, namely 1.5 and 9.9, and compared it with that in aqueous buffer. As seen in Fig. 1, at both pH values, the resistance of lysozyme to inactivation at 100^{o}C in LTTMs exceeded that in the corresponding aqueous solution.

Encouraged by this enhanced protein stability, we decided to switch gears and explore whether increases in stability also could be observed with some low-molecular-weight drugs that suffer from insufficient stability in aqueous solution. To this end, we selected aspirin which due to the presence of an ester bond has not been formulated commercially as an aqueous solution because even at an optimal pH value its half-life is under two weeks [20], i.e., far less than at least a 2-year shelf-life required for most commercial pharmaceuticals. Furthermore, to raise the dissolved aspirin concentration, we attempted to form LTTMs with aspirin as a hydrogen-bond donor, as opposed to using the drug merely as a solute. As shown in Table S2, 50 binary or ternary LTTMs containing aspirin were attempted, and 12 of them were found to result in stable, transparent, colorless liquids at room temperature. (Another common commercial drug, acetaminophen, also was found to form LTTMs under comparable conditions.) One of them, choline chloride:aspirin (1:2 molar ratio) (Entry 2, Table S2), was chosen to investigate the stability of aspirin in a LTTM. Note that the aspirin concentration in this LTTM, 72% (w/w) is over 200 fold greater than the solubility in water under the same conditions (0.32%). As seen in Fig. 2, the cleavage of aspirin to salicylic and acetic acids in the LTTM was some 10 times slower than in a buffered aqueous solution at pH 2.5 (optimal for aspirin's stability[20]): the calculated half-lives of 7.0 h *vs.* 41 min, respectively.

In summary, herein we have demonstrated for the first time that LTTMs can (i) dissolve mg/mL concentrations of common proteins without irreversibly denaturing them and (ii) also be used to formulate water-sensitive or/and poorly soluble drugs.

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Table 1. Solubility of different proteins in various neat LTTMs and the latters' physicochemical parameters

LTTM[a]	Protein solubility (mg/mL)[b]				Viscosity (cP)[c]	pH[c]	E_{NR}[c]	β[c]	π*[c]
	BSA	Chymotrypsinogen	Subtilisin	Lysozyme					
ChCl:LA	0.879±0.022	2.00±0.08	0.199±0.005	5.18±0.043	117	2.19	49.2	0.575	1.00
ChCl:Gly	0.552±0.005	0.326±0.005	7.58±0.08	0.977±0.026	194	5.78	49.6	0.408	1.24
ChCl:EG	0.233±0.009	3.23±0.06	0.206±0.008	2.29±0.02	33.5	7.00	50.5	0.480	1.19
ChCl:urea	0.271±0.007	0.757±0.021	0.574±0.008	0.998±0.014	386	9.93	49.7	0.351	1.31
ChCl:PAA	2.55±0.04	0.329±0.005	0.162±0.005	1.13±0.01	196	1.47	48.4	0.723	0.908
ChCl:MA	0.315±0.011	0.758±0.019	0.122±0.003	1.64±0.02	1110	0.280	47.7	0.527	1.01
BE:LA	16.5±0.1	3.92±0.05	2.87±0.06	6.50±0.08	451	4.68	51.1	0.485	0.984
TEAC:EG	1.69±0.04	2.44±0.08	0.285±0.009	8.45±0.07	34.3	6.79	50.4	0.589	1.18
TEAC:LA	8.42±0.07	0.851±0.016	1.37±0.03	1.18±0.04	85.6	3.62	50.8	0.664	1.04
TBAB:EG	0.304±0.009	1.31±0.01	0.118±0.005	2.98±0.03	114	8.72	50.6	0.674	1.12
TBAB:LA	0.586±0.011	0.468±0.008	0.588±0.019	0.599±0.025	245	1.47	50.3	0.754	0.984
TBAB:AOE	11.0±0.2	3.81±0.06	0.196±0.009	3.76±0.04	121	12.4	51.1	0.744	1.13
$ZnCl_2$:EG	7.61±0.11	3.43±0.05	0.216±0.006	7.07±0.07	231	3.12	46.2	0.238	1.29
$ZnCl_2$:PPD	1.93±0.02	4.20±0.07	0.533±0.015	2.88±0.03	750	3.18	46.9	0.304	1.20

[a]Abbreviations: ChCl, choline chloride; LA, levulinic acid; Gly, glycerol; EG, ethylene glycol; PAA, phenylacetic acid; MA, malonic acid; BE, betaine; TEAC, tetraethylammonium chloride; TBAB, tetrabutylammonium bromide; AOE, 2-(2-aminoethoxy)ethanol; PPD, 1,2-propanediol. [b]The protein solubility was measured according to section 1.3 in the ESI. [c]The physicochemical parameters (viscosity, pH, E_{NR}, β, and π*) were determined according to section 1.5 in the ESI.

Table 2. Activity recovery upon dilution of lysozyme dissolved in LTTMs

LTTM[a]	Activity recovered after dilution (%)[b]	LTTM[a]	Activity recovered after dilution (%)[b]
ChCl:LA	93.9±1.7	TEAC:EG	97.1±0.8
ChCl:Gly	100±1.4	TEAC:LA	92.3±1.2
ChCl:EG	101.3±1.9	TBAB:EG	98.9±1.4
ChCl:urea	99.2±1.6	TBAB:LA	96.5±1.3
ChCl:PAA	[c]	TBAB:AOE	101.3±1.5
ChCl:MA	96.8±1.2	$ZnCl_2$:EG	[d]
BE:LA	86.4±2.3	$ZnCl_2$:PPD	[d]

[a]For abbreviations, see Table 1. [b]0.2 mL of lysozyme solution in LTTM was diluted with 1.8 mL of 66 mM phosphate buffer (pH 6.2), the solution was stirred at 4°C for 1 h, and the lysozyme activity was determined according to section 1.4 in the ESI. [c]After dilution, the solution turned turbid. [d]The $ZnCl_2$:EG and $ZnCl_2$:PPD LTTMs flocculated the *M. lysodeikticus* cells, thus interfering with lysozyme activity assay.

Figure captions

Figure 1. The time course of thermal inactivation of lysozyme dissolved in LTTMs and in aqueous buffers with the pH of 1.5 (a) or 9.9 (b) at 100^{o}C.

Figure 2. The time course of the cleavage of aspirin to salicylic and acetic acids in a ChCl:aspirin LTTM and in an aqueous buffer at 100^{o}C.

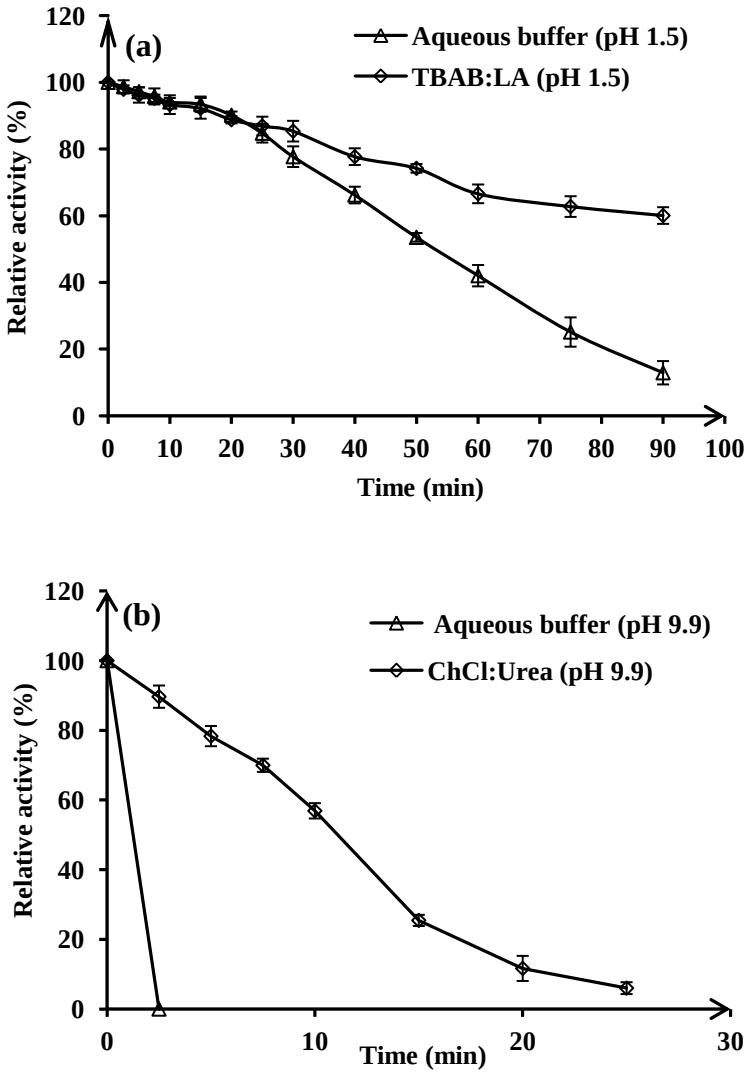
(a)
Aqueous buffer (pH 1.5)
TBAB:LA (pH 1.5)
Relative activity (%)
Time (min)
0
20
40
60
80
100
120
0
10
20
30
40
50
60
70
80
90
100
(b)
Aqueous buffer (pH 9.9)
ChCl:Urea (pH 9.9)
Relative activity (%)
Time (min)
0
20
40
60
80
100
120
0
10
20
30

Figure 1

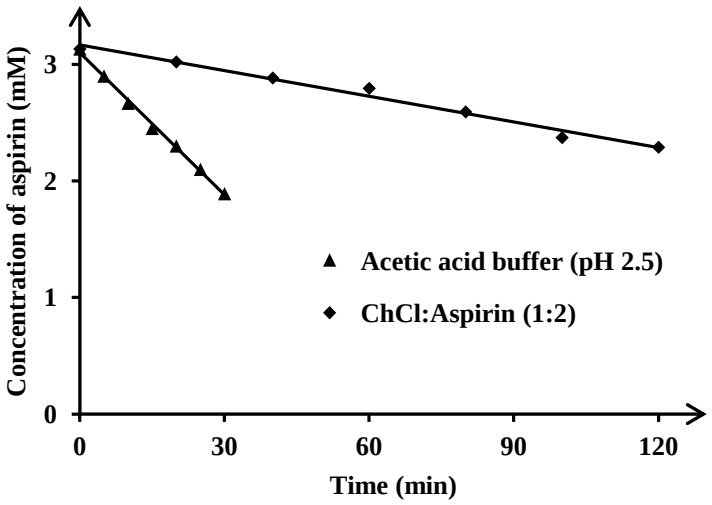
Concentration of aspirin (mM)
3
2
1
0
0
30
60
90
120
Time (min)
Acetic acid buffer (pH 2.5)
ChCl:Aspirin (1:2)

Figure 2