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Novel buffer systems for macromolecular crystallization

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In protein crystallization, screening is initially performed to obtain an indication of the conditions under which a macromolecule might crystallize. These preliminary conditions are then optimized to produce (in a perfect world) well diffracting crystals; this process of optimization often involves fine grid screening around the initial conditions. An issue in optimization is to find factors which are independent, so as to simplify the analysis of the results of optimization trials. This is necessarily difficult with buffers, as a buffer and its pH range tend to be very highly correlated. Multi-buffer systems for pH modulation are presented which enable a broad pH range to be sampled without changing the chemical composition of the buffering component.

1. Introduction

A buffer is a very standard addition to a protein crystallization experiment: in the Jancarik and Kim screen (Jancarik & Kim, 1991) 39 of the 50 conditions contain a specific buffering chemical. The buffer component is often found at relatively high concentrations, most likely at 100 m*M*. The buffer can act either by modulating the pH of the protein solution during crystallization (as is suggested by 'buffer') or it can act as a chemical in the crystallization cocktail. There are around 100 structures in the Protein Data Bank (PDB; Berman *et al.*, 2000) that contain ordered MES, over 50 that contain ordered HEPES and more than 100 that contain ordered Tris, showing that it is not unusual to have specific interactions between buffers and protein molecules. Table 1 shows a more extensive list of buffers found in structures deposited in the PDB. Thus, a dilemma arises during optimization of how to decouple the chemical nature and the buffering properties of the 'buffer' component of a crystallization cocktail.

Traditionally, a crystallization laboratory would be cluttered with all the common buffers, where stocks of each buffer would be found at many pH values within the buffer's useful buffering range. This allows for optimization of any given starting condition very rapidly. However, there is an increasing use of automation in crystallization, not only to set up crystallization droplets, but also to prepare the arrays of crystallization cocktails used in the crystallization trials. Most of the liquid-handling robots that can be used to prepare crystallization cocktails have a limited number of stock solutions that can be used at any one time. So if the liquid-handling robot is to be capable of making up any given optimization condition, then all buffers at any given pH should be available. Assume that there are ten crystallization buffers: to have all ten buffers at five different pHs would require, traditionally, 50 stocks. By using only two stocks for each buffer, one set above the pK_a and one below, and by using the Henderson–Hasselbach approximation, one could generate any valid pH for the ten buffers from 20 stock solutions. If one had a stock of each buffer at its pK_a and concentrated acid and base stocks, then one could cover the same buffer space with 12 stock solutions (assuming that all the problems with concentrated acids and bases were resolved[1]).

This paper describes seven buffer systems suitable for protein crystallization which can be used to circumvent the problems described above. The idea of using extended-range buffer systems is not new: chromatofocusing ion-exchange chromatography uses broad-range buffer systems that are multicomponent (that is, they are made up of a number of different buffers) and these systems can yield a consistent buffering capacity over a wide pH range (Bates *et al.*, 2000; *Chromatofocusing with Polybuffer and PBE*, APBiotech Handbook 18-1009-07, Amersham Biosciences). However, commercial chromotofocusing buffers (*e.g.* Polybuffer 74, Polybuffer 96; APBiotech) are not obvious choices for macromolecular crystallization for two reasons: the subcomponents of the polybuffers are not well documented and the range over which they buffer is not broad enough.

[1] For example, concentrated bases can etch glass, which makes their use on systems with valves difficult. Concentrated acids tend to be volatile, as well as corrosive.

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Table 1
Buffers found in the systems described in this paper and their frequency in the PDB (December, 2003).

These numbers were obtained by using the HIC-Up (Kleywegt & Jones, 1998) site to identify a PDB code and then searching the PDB for instances of files containing those codes. In some cases the PDB was searched directly, using the 'Ligands and Prosthetic Groups' field of the SearchFields customisable form.

Buffer	PDB code	Count
Succinic acid	SIN	27
Phosphate	PHO, PO4	643
Glycine	GLY	16
Citrate	CIT	100
HEPES	EPE	69
CHES	NHE	8
Malonate	MLI	1
Imidazole	IMD	51
Borate	BO3	4
Propionate	PPI	5
Cacodylate	CAC	52
Acetate	ACY	128
ADA	MHA	1
Bicine	BCN	5
Malate	MLT	10
MES	MES	106
Tris	TMN, TRS	117
Tartrate	TLA, TAR	24
Bis-Tris	BTB	2

Each buffer system described here covers the pH range 4–9 (4–10 in some cases), so that a broad range of pH space is available without necessitating a change in the buffering chemical. Additionally, only two stocks, one at pH 4 and one at 9 (or 10) are needed to span that entire region of pH space. Furthermore, the ratios of the components within the buffer system have been selected so as to produce a reasonably linear response to pH, so that if the low pH stock is at pH 4 and the high pH stock is at pH 10, then a 3:1 mix of these two stocks would yield a solution of pH 5.5, a 1:1 mix would result in a pH 7 and a 1:3 ratio would give a solution of pH 8.5.

2. Materials and methods

21 crystallization buffers were selected that had different chemistries and well separated pK_a values. Three buffers were chosen for each broad-range system, choosing these for distinct pK_a values, as well as different chemistries. For the initial experiments, 100 m*M* stocks were made up of the three component buffers and these were mixed in the ratio 1:1:1. Half of this mix was set to pH 4 and the other set to a pH of 9, 9.5 or 10 (depending on the pK_a values of the components) using 1 *M* HCl or NaOH as appropriate. Ratios ranging from 10:0 to 0:10 (11 steps) of these two stocks were mixed and the pH was measured. The resulting pH curve was plotted on a graph and compared with an ideal (*i.e.* linear) curve. If the pH curve was significantly different from the ideal, then the three components were mixed in a different ratio and the process repeated. See Fig. 1 for a graphical representation of this process.

Table 2
Recipes for making up 10× high-pH and low-pH stock solutions for the buffer systems.

For the succinic acid/sodium phosphate/glycine system, one would dissolve 1.48 g of succinic acid, 6.04 g of NaH_2PO_4 and 3.28 g of glycine in 80 ml of water. This would be set to pH 4 using (approximately) 0.4 ml of 10 *M* NaOH and the final volume adjusted to 100 ml. The same amounts of the chemicals would be weighed out again and again dissolved in 80 ml water. This stock would be set to pH 10 using (approximately) 10.3 ml 10 *M* NaOH and the final volume adjusted to 100 ml. The other systems would be made in the same way, noting that the final pH of the high-pH stock is given in parentheses after the approximate volume of base in column 'high pH'. The system that contains ADA as one of the components is made up slightly differently, as only a 0.5 *M* stock of ADA (rather than the solid) was available.

Ratio	Chemical name and source	MW (Da)	g per 100 ml	Final volume (ml)	High pH	Low pH
2	Succinic acid (Sigma S3674)	118.1	1.48		10.3 ml	0.4 ml
7	Sodium dihydrogen phosphate monohydrate (Merck 1.06346)	137.99	6.04	100	10 *M* NaOH (pH 10.0)	10 *M* NaOH (pH 4.0)
7	Glycine (Merck 1.04201)	75.07	3.28			
2	Citric acid, anhydrous (Fluka 27487)	192.43	4.28		13.5 ml	2.0 ml
3	HEPES (Merck 1.10110)	238.3	7.94	100	10 *M* NaOH	10 *M* NaOH
4	CHES (Sigma C2885)	207.3	9.21		(pH 10.0)	(pH 4.0)
2	Malonic acid (Sigma M1750)	104.1	2.6		6.25 ml	1.3 ml
3	Imidazole (Fluka 56750)	68.08	2.55	100	10 *M* NaOH	10 *M* HCl
3	Boric acid (Sigma B9645)	61.83	2.32		(pH 10.0)	(pH 4.0)
2	Sodium propionate (Sigma P1880)	96.06	3.84		1.0 ml	12.9 ml
1	Sodium cacodylate trihydrate (Fluka 20838)	214.03	4.28	100	10 *M* HCl	10 *M* HCl
2	Bis-Tris propane (Sigma B6755)	282.3	11.29		(pH 9.5)	(pH 4.0)
1	Sodium acetate (Merck 1.06268)	82.03	2.74	100, of which	5.7 ml	2.95 ml
1	ADA (Hampton Research HR2-507, 0.5 *M* stock)	190.2	6.34	66.7 are 0.5 *M* ADA	10 *M* NaOH (pH 9.0)	10 *M* HCl (pH 4.0)
1	Bicine (Fluka 14872)	163.18	5.44			
1	L-Malic acid (Sigma M1000)	134.1	2.68	100	6.7 ml	2.1 ml
2	MES (Sigma M8250)	195.2	7.81		10 *M* NaOH	10 *M* HCl
2	Tris (trizma base, Sigma T1503)	121.1	5.22		(pH 9.0)	(pH 4.0)
3	Sodium tartrate dihydrate (Sigma S4797)	230.1	9.86		2.4 ml	5.1 ml
2	Bis-Tris (Aldrich 15,666-3)	209.24	5.98	100	10 *M* NaOH	10 *M* HCl
2	Glycylglycine (Sigma G1002)	132.1	3.77		(pH 9.0)	(pH 4.0)

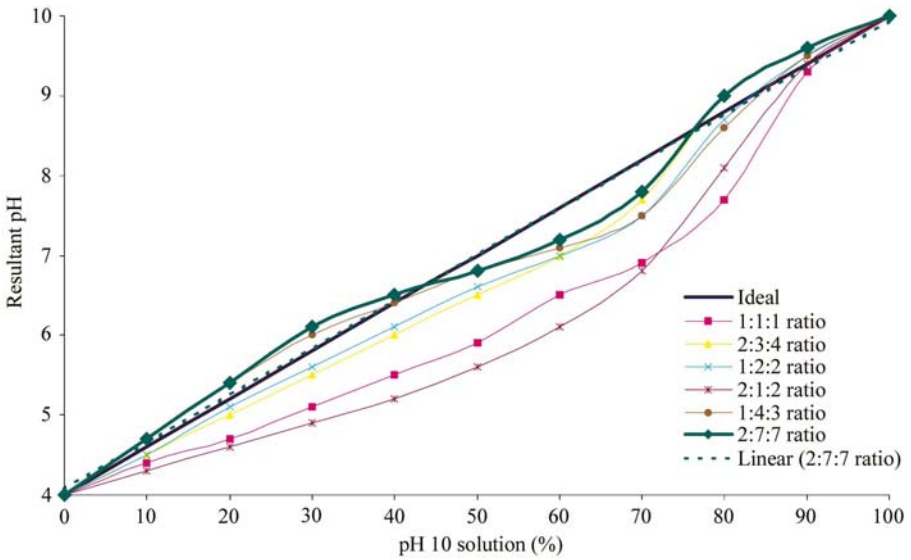

Figure 1
pH curves from six different ratios of the buffer-system components 100 m*M* succinic acid, 100 m*M* sodium phosphate and 100 m*M* glycine. Ideality (linearity) is shown by the dark blue line. The pH curve which approaches ideality most closely consists of succinic acid, sodium phosphate and glycine in a 2:7:7 ratio – this curve is shown in teal. The line of best fit through the 2:7:7 ratio curve (dashed teal line) has a correlation coefficient $R^2 = 0.985$.

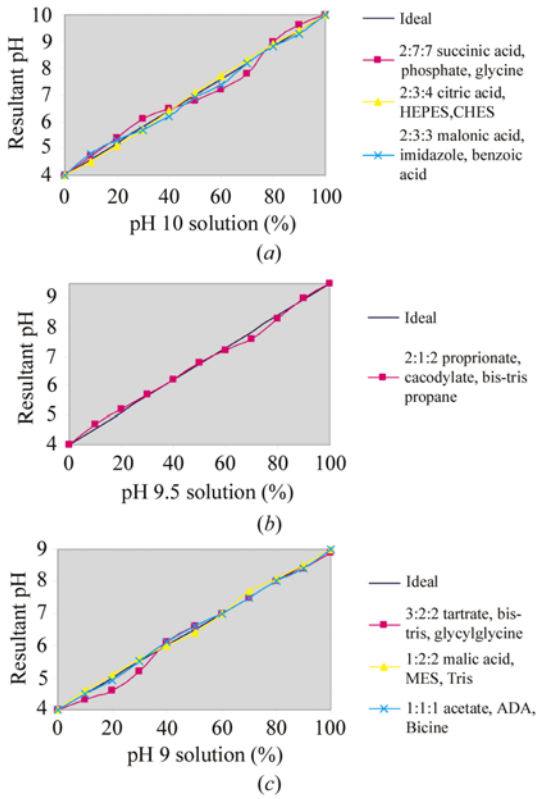

Figure 2
(*a*) The three buffer systems that span the pH range 4.0–10.0, (*b*) the buffer system that covers the pH range 4.0–9.5 and (*c*) the buffer systems that covers the pH range 4.0–9.0. The correlation coefficients of the lines of best fit range from 0.985 (succinic acid, phosphate, glycine) to 0.999 (citric acid, HEPES, CHES).

The ratio determinations were performed using 100 m*M* stocks of the component buffers, so that the final mixes would be equivalent to the concentration of the buffer within a crystallization experiment. It would be more useful to have a 10× stock of each of the high- and low-pH components, where the acid or base used to set the pH does not dilute the buffer as in the initial experiments. Table 2 shows the chemicals used in the system and describes how to make the undiluted low- and high-pH 10× stock for each system. The 10× stocks are thus 1 *M* in buffer, so that the final concentration would be 100 m*M*.

3. Results

There are three buffer systems that span the pH range 4–10, one that spans the pH range 4–9.5 and three which cover the range pH 4–9 (see Fig. 2). These buffer systems allow the normal range of pH space encountered in protein crystallization to be accessed without changing the buffer system. Moreover, if one were to replace the buffer in a screen with the buffer system containing that buffer, then one will have increased the chemical space sampled by that screen, as two further chemicals will have been sampled. As an initial test, these buffers were incorporated into a screen of 96 conditions, which is a derivative of the Hampton Crystal Screens I and II (Hampton Research), by replacing the buffer chemical in each condition with the buffer system containing that chemical and setting the buffer system to a similar pH. In cases where the condition also contained divalent metals, the buffer systems containing phosphate or borate were not used. This buffer-system screen was not tested in tandem with the screen containing single buffers, nor has it been extensively tested against many proteins. However, the buffer-system screen (in which the traditional buffer was swapped out for the buffer system) has produced at least two crystals of human proteins relevant to the pharmaceutical industry from proteins that had not crystallized under any of the commercially available screens, suggesting that it is suitable for further consideration and testing.

4. Conclusions

A series of seven broad-range buffer systems have been described which allow the decoupling of pH from buffer in protein crystallization experiments. Each broad-range system consists of three component buffers chosen to have a variety of chemistries within each buffer system. This allows one to increase both the pH range of the component buffers and the chemical variability of any screen or optimization strategy that incorporates these buffer systems. The seven systems are ideal for applications where the number of stock solutions is limiting, as is the case with most liquid-handling robots, as 21 unique buffers are available at any pH from 4 to 9 (or 10) using only 14 stock solutions. Preliminary testing of the buffer systems suggests that these systems are compatible with other common protein crystallization reagents.

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References

Bates, R. C., Kang, X. & Frey, D. D. (2000). *J. Chromatogr. A*, **890**, 25–36.
Berman, H. M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T. N., Weissig, H., Shindyalov, I. N. & Bourne, P. E. (2000). *Nucleic Acids Res.* **28**, 235–242.
Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–411.
Kleywegt, G. J. & Jones, T. A. (1998). *Acta Cryst.* D**54**, 1119–1131.