laboratory notes

The lipid cubic phase or *in meso* method for crystallizing proteins. Bushings for better manual dispensing

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The lipid cubic phase or *in meso* method can be used to set up crystallization trials of soluble and membrane proteins. The cubic phase is noted for being a sticky and viscous mesophase. Dispensing the protein-laden mesophase by hand into wells on crystallization plates has been facilitated by the use of an inexpensive repeat dispenser. However, the assembled dispensing device is prone to damage. Specifically, the delicate plunger used to dispense the viscous mesophase by positive displacement can be bent and scarred when the locking nut that fixes the plunger to the ratchet-driven dispensing mechanism is inadvertently overtightened. A defective plunger can render the device useless as a dispensing tool. More importantly, it can lead to catastrophic loss of valuable protein and lipid due to leakage when the dispensing syringe is being recharged with fresh mesophase. This note describes two types of bushings designed to protect the plunger from mechanical damage, which provide facile and reliable dispenser performance. One is a split bushing in brass and is a highly durable solution. The other is a small sleeve made from readily available plastic tubing. While it lacks durability, the plastic sleeve is simple yet highly effective, and can be replaced as the need arises.

1. Introduction

The lipid cubic phase (LCP) or *in meso* method for crystallizing membrane proteins has generated some of the highest profile structures in recent memory. Included on the list of successes is the β_2-adrenergic receptor-Gs protein complex which figured in the 2012 Nobel Prize in Chemistry (Rasmussen *et al.*, 2011; Benovic, 2012). To date, the method has been responsible for 189 recorded entries in the Protein Data Bank (Berman *et al.*, 2003), 55 of which refer to G protein-coupled receptors. The latter are of great physiological importance and are major targets for drug discovery (Katritch *et al.*, 2013). The cubic phase method also works with soluble proteins (Landau *et al.*, 1997; Caffrey, 2000; Aherne *et al.*, 2012).

The *in meso* method employs a viscous, lyotropic liquid crystal or mesophase, the cubic phase, as the medium in which the target protein is first reconstituted and subsequently grows crystals (Caffrey & Cherezov, 2009; Caffrey *et al.*, 2012). Trials are set up manually for low-throughput screening and optimization as well as for ligand and heavy-atom soaking and labeling applications (Cherezov & Caffrey, 2003, 2005; Caffrey & Porter, 2010). Robots have been developed for high-throughput screening (Cherezov *et al.*, 2004; Caffrey & Cherezov, 2009; Li *et al.*, 2012).

The mesophase is prepared by thoroughly mixing together lipid and an aqueous solution of the soluble or membrane protein. A coupled syringe mixing device, which operates usually with 50 or
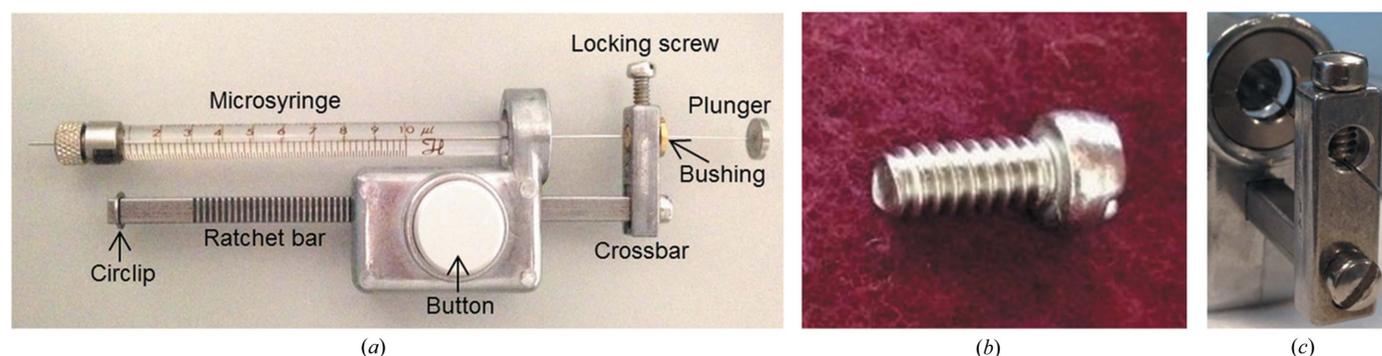

Figure 1
Microsyringe repeat dispensing device with split brass bushing. Fully assembled dispenser complete with 10 µl syringe and brass bushing (*a*). Expanded view of locking screw (*b*), revealing the sharp end (left) that cuts into and damages the plunger when overtightened (*c*).

100 µl gas-tight Hamilton syringes, was developed for this purpose (Cheng *et al.*, 1998; Caffrey & Cherezov, 2009; Caffrey & Porter, 2010). The lipid is placed in one syringe and the protein solution in the other. The two syringes are connected by a narrow bore coupler, and the molten lipid and protein solution are passed back and forth from one syringe to the other through the coupler to effect mixing, homogenization and cubic mesophase formation. In the process, either the protein gets reconstituted into the lipid membrane or it remains in the aqueous channels of the cubic mesophase. The protein-laden mesophase, which is quite viscous and sticky, is now ready for dispensing into the crystallization wells. A precipitant solution is placed over the mesophase, and the plate is sealed and placed in an incubator for crystal growth.

Dispensing the viscous protein-laden mesophase is not usually done from the large-volume syringes in which the original material was prepared. Typically, a 10 µl syringe, to which the pre-formed mesophase has been transferred, is used. The latter is mounted in a repeat dispenser (Hamilton) equipped with a 50-tooth ratchet arm (Fig. 1*a*) (Cherezov & Caffrey, 2005). Each activation (press) of the dispensing button on the device dispenses one-fiftieth of the volume of the syringe, which in the case of a 10 µl syringe amounts to a 200 nl mesophase bolus. For the device to work properly, the ratchet bar of the dispenser must be securely fixed to the syringe plunger. This is realized by means of a locking screw mounted in a crossbar that connects the plunger and the ratchet bar (Fig. 1*a*). Tightening the screw on the plunger secures it in place. Thus, for every activation of the dispensing button the ratchet arm and the plunger move together, ideally, parallel to one another.

2. The problem

We have encountered a weakness in this device that can result in catastrophic loss of valuable protein and lipid. This Laboratory Note describes two solutions to the problem that have been implemented successfully in the Membrane Structural and Functional Biology Group. The problem with the device arises as a result of needing to secure a tight grip on the plunger. The locking screw cannot be turned by hand with enough force to provide for a secure grip. A screwdriver is used instead, but invariably too much torque is applied and the tip of the nut with its sharp edges (Fig. 1*b*) cuts into and deforms the plunger (Fig. 1*c*). This creates problems at two stages when crystallization trials are being set up. First, the damaged plunger may fail to enter the syringe barrel to properly dispense the mesophase into the

well of the crystallization plate. Second, at the stage where the 10 µl syringe is being loaded with pre-formed mesophase by way of the narrow bore coupler (Cheng *et al.*, 1998; Cherezov & Caffrey, 2005; Caffrey & Cherezov, 2009; Caffrey & Porter, 2010), leakage is bound to occur when a damaged plunger is used in the receiving 10 µl syringe. Unless the plunger moves smoothly and with ease in the barrel, back pressure builds and mesophase will leak, usually at the coupler where the two syringes are joined. This can best be described as a disaster. The setup needs to be abandoned with loss of valuable protein, lipid and time. Further, the device must be disassembled and parts thoroughly washed, cleaned and dried in preparation for another attempt.

A solution to the problem is to avoid direct contact between the lock nut and the plunger. Here, we describe two ways of doing this. The first is a more permanent fix that requires machining two small bushing parts in brass but that should provide for a long-term solution. The second is just as effective but less durable. However, since the material required for option two is cheap and ubiquitous, replacing the worn part is trivial.

3. Solutions

3.1. Brass split bushing

The first solution takes the form of a split bushing made of brass. The bushing consists of a quarter and a three-quarter circle part (Figs. 2 and 3). The two fit together to form a full circle split bushing that fits into the plunger slot of the dispenser crossbar. The plunger passes through a hole in the center of the bushing where the two bushing parts meet. Because the inside radius in the quarter circle is undersized slightly with respect to the three-quarter circle part, when the former is pressed on by the locking screw its sharper edge tightens on the plunger. In this way, the force is distributed along the length of the plunger, minimizing damage but providing a grip that is reliably secure. Further, our experience is that with the split bushing in place the locking screw can be tightened by hand. As a result of not needing to use a screwdriver, the device is much less cumbersome to handle and is more easily and rapidly assembled and disassembled.

In use, we find it most convenient to remove the two bushing parts during the syringe loading process. This ensures that the plunger will move freely in the barrel, thereby facilitating mesophase transfer that is leak free. When loading is complete, the two bushings are slotted into place around the plunger as it passes through the crossbar, the three-quarter circle part first (Fig. 2*c*). The quarter circle part is aligned with the locking screw, which can be loosened or tightened by hand conveniently, as noted (Figs. 2*d* and 2*e*). We recommend removing the quarter circle part when the crossbar and ratchet arm are being repositioned along the plunger for repeat dispensing to ensure that the plunger is completely disengaged for recharging. The rim on the quarter circle part is chamfered to facilitate its removal from the crossbar by levering it out by fingernail (Figs. 2*b* and 3).
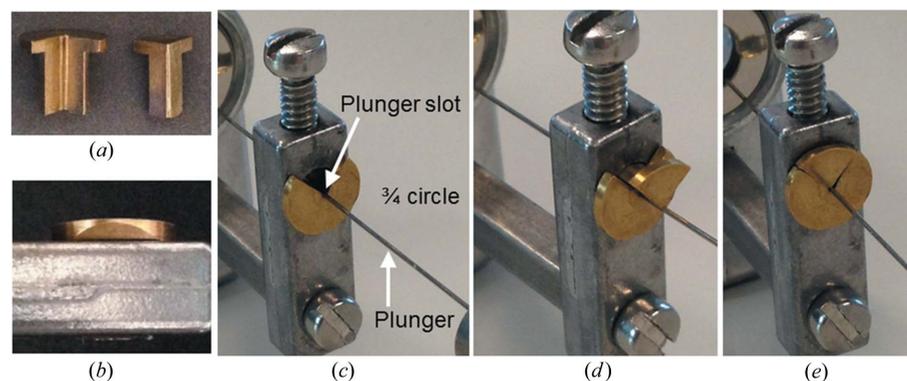
3.2. Plastic tubing

The second solution takes the form of a piece of soft plastic tubing. The tubing fits along the length of the plunger (Figs. 4a–4*f*). The locking screw, when tightened on the
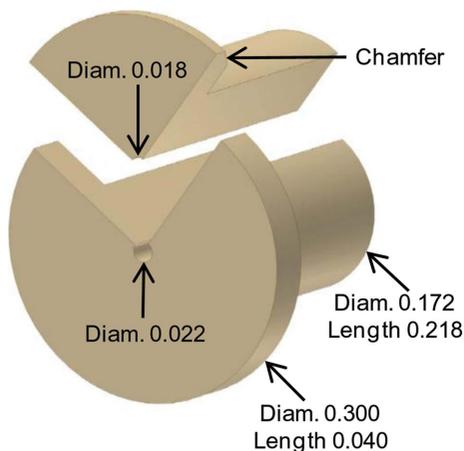

Figure 2
Microsyringe repeat dispensing device with close-up views of the brass bushing parts in and out of the device. The bushing consists of quarter and three-quarter circle parts (*a*). The rim on the quarter circle is chamfered for easy removal (*b*). The plunger is shown seated in the three-quarter circle bushing piece in the plunger slot (*c*). The quarter circle part is shown inserted over the syringe plunger into the plunger slot and is aligned with the locking screw (*d*), (*e*).

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Figure 3
Specifications for the split bushing. The bushing is made in two pieces of brass, a three-quarter circle piece (bottom) and a quarter circle piece (top), the upper rim of which is chamfered slightly to assist removal from the plunger slot in the crossbar of the dispenser. The outside diameters and lengths of the two pieces are the same. The inside diameters of the two pieces are slightly different, as indicated. Dimensions shown are in inches (1 inch = 25.4 mm). The method for making the two bushing sections involves machining two identical full bushings, with the exception that one has a 0.022 inch inside diameter hole and the other has a 0.018 inch inside diameter hole. Both parts are machined extra-long so that they can be held extending from an indexing head. The quarter circle section is milled out of the piece with the 0.018 inch inner diameter hole. The three-quarter circle section is milled out of the piece with the 0.022 inch inner diameter hole.

tubing, can deform it, thereby gripping without damaging the plunger. If the tubing is sufficiently soft, the screw can be tightened or loosened by hand. We have found tubing with an internal diameter of 1.5 mm and a wall thickness of 0.8 mm to be ideal. The tubing can be cut from stock and a section 6 mm long suffices. After several uses, the tubing may become permanently deformed and perhaps even cut by the locking screw, in which case it should be replaced.

Split plastic tubing can be used too (Fig. 4*f*). Split tubing is easy to place on and remove from the plunger while it is mounted in the assembled device. However, we have found that such split tubing deforms quite readily, requiring that it be replaced more frequently.

4. Concluding remarks

While the *in meso* or LCP method was developed for crystallizing membrane proteins, it also works with soluble proteins (Landau *et al.*, 1997; Cherezov *et al.*, 2004; Tanaka *et al.*, 2004; Aherne *et al.*, 2012). Soluble proteins that have yielded to the method include lysozyme, α-lactalbumin, insulin and thaumatin. Indeed, a protocol has been published that describes the growth of diffraction quality lysozyme crystals *in meso* within an hour (Aherne *et al.*, 2012). It is proving to be an extremely useful instructional tool and has been employed successfully for several years now in crystallization workshops and crystallography schools conducted by the corresponding author at sites worldwide. The benefits of the bushings described in this Laboratory Note were very obvious in these training courses, where not a single plunger was damaged. Because the crystallization of soluble proteins *in meso* has been likened to convection-free crystallization under conditions of microgravity or in a gel where the zone of depletion is maintained, the method is likely to find increasing application with soluble targets where higher-resolution structures are required. Regardless of whether the method is used with
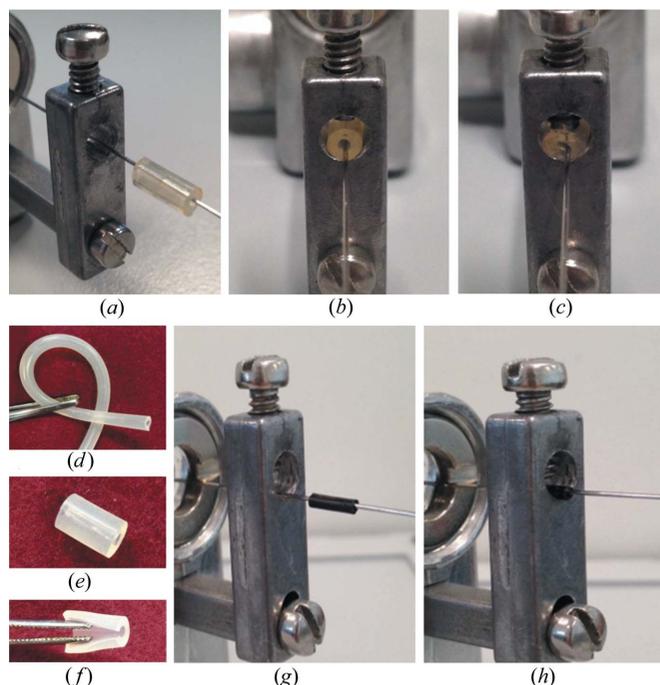

Figure 4
Microsyringe repeat dispensing device with plastic bushings. Assorted views of the soft plastic bushing at various stages of use in the device (*a*)–(*c*). Soft plastic tubing (*d*) used to make sections of bushing (*e*), (*f*). A section of split tubing is shown in (*f*). A different type of tubing that can be used as bushing material is shown in (*g*), (*h*).

membrane or soluble proteins, this Laboratory Note describes two highly effective approaches for leak-free setting up of *in meso* crystallization trials and thus for more efficient use of time and valuable materials.

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