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Desiccation of nucleated mammalian cells in nanoliter droplets

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A B S T R A C T

This paper reports on the development and testing of two techniques for nebulizing cell solutions into aerosols that contain living cells in nanoliter droplets: one pneumatic technique and one ultrasonic technique. NIH 3T3 fibroblasts were tested for viability and the ability to attach and grow post-spraying. Viabilities for the two techniques ranged from 50 to 70% and virtually all cells that maintained membrane integrity post-spraying were able to attach and divide. The cellular aerosols were used to study the effect of drying rates and drying temperature on the post-rehydration survival of fibroblasts that were loaded with trehalose. Nanoliter droplets were developed for this study so that non-uniformities in moisture content and temperature within the samples would be minimized, and thus the drying rates experienced by the cells could be inferred. The main findings of this study are that the relative humidity of the drying environment does have a strong effect on the post-rehydration viability of desiccated cells, but that effect is entirely explained by differences in the residual moisture content experienced by the cells dried at different humidities. The post-rehydration viability of the cells was found to be dependent only on the residual moisture content achieved during drying and on whether or not trehalose was loaded inside the cell, and was not dependent on the drying rate or on the temperature at which drying occurred. A comparison of viabilities for cells dried in nanoliter droplets and cells dried in more commonly used 10 μL drops suggested that cells in larger drops were not experiencing the average water content for the drop as a whole during critical phases of the drying process. The apparent non-uniformity of moisture inside larger drops may contribute to difficulties in understanding and establishing successful long-term storage techniques in a dried state.

Keywords: Desiccation; Nebulization; Anhydrobiosis; Drying rate

1. Introduction

Preservation of mammalian cells in a dried state has been the focus of numerous research efforts over the last decade (Acker et al., 2004; Crowe et al., 2004). The most notable successes in this area have been the preservation of erythrocytes and platelets using freeze drying techniques (Goodrich et al., 1992; Tang et al., 2006). Sperm of various types have been successfully stored in partially dehydrated states and they have retained their ability to fertilize eggs using intracytosolic injection (ICSI) (Wakayama and Yanagimachi, 1998; Bhowmick et al., 2003). Attempts to dry other mammalian cell types have led to some limited successes. Numerous cell types have been shown to maintain membrane integrity when rehydrated after substantial dehydration (Guo et al., 2000; Chen et al., 2001); but very few nucleated cell types have retained the ability to grow, divide or function after experiencing dehydration. Acker et al. (2002) reported full recovery of murine fibroblasts from a dehydrated state. The cells were able to attach and divide if they were rehydrated immediately after desiccation; but even short periods of storage in a dried state led to cell death. Nucleated cells, such as fibroblasts have not yet been successfully stored in a dried state. Even short periods of dehydrated storage, on the order of hours, are not yet possible for nucleated cells.

The reasons why mammalian nucleated cells do not remain stable under storage in the dried state is unknown. The

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Nomenclature

M	molarity
Osm	osmolarity
RH	relative humidity
RMC	residual moisture content (mass of water/mass of sample after baking)

physio-chemical theory that suggests dried storage should be possible is fairly straightforward. Trehalose is believed to provide two forms of protection for cells experiencing desiccation: potential formation of a stable glass at a relatively high temperature and direct stabilization of biomolecules and cellular membranes (Crowe et al., 2004). Survival of cells during desiccation depends on both of these effects, but long-term storage usually requires formation of a glassy state (Roos, 1995). Mammalian cells that are stabilized by the addition of sugars on both sides of the membrane should enter a glassy state when dried. Once the glass state is achieved, all molecular motion is arrested, or at least slowed to insignificant levels, and the cell should remain stable more or less indefinitely. That is, if the cells can survive dehydration and rehydration in and out of the glassy state, they ought to remain alive, or recoverable, for a very long time when they are dry. In practice, however, this appears not to be true. A number of mammalian cells can survive high levels of dehydration for short periods of time, but they do not retain viability when stored in this state (Kanias and Acker, 2006).

One of the major complications to studying and improving cellular desiccation protocols is that the drying is always performed in glass forming liquids (sugar solutions) which leads to non-uniformities in the sample and a subsequent lack of information about the state of the cell or its microenvironment. It is has been well established in the field of polymer science that the drying of glass forming liquids can lead to large non-uniformities in the moisture content of the solution (Romdhane et al., 2001). This behavior during the drying of the trehalose and sucrose solutions that are used in cellular desiccation has recently been observed numerically and experimentally (Elliott et al., 2003; Aksan et al., 2006; Chen et al., 2006). The formation of a glassy layer at the air–liquid interface, in particular, leads to a dried surface layer that impedes drying of the sample's interior. Moisture contents for dried cellular samples are always reported as averages over the entire sample, but it is almost certain that this average moisture content is not what the cells actually experience. This uncertainty in moisture content not only makes it impossible to know the state of the cell during storage, but it also makes it impossible to study the effect of drying and rehydration rates on cellular survival. We can know the rate at which the entire sample loses moisture, but we do not know how well this reflects the drying that occurs near the cell. Moreover, membrane water transport, whose understanding has proven to be critical in the study of cryopreservation (Mazur, 2004), has not been studied in the context of dehydration at all. Progress in the dried preservation of nucleated mammalian cells seems unlikely unless methods can be developed that allow for greater knowledge of the moisture contents inside and outside the cell during and after drying.

This study is aimed at studying the effect of drying rate on the survival of mammalian cells that undergo desiccation. In order to know and control the drying rate experienced by the cells, the cells were suspended inside nanoliter droplets. The small size of the droplets reduces the non-uniformity of the moisture content within each drop and also allows us to achieve high rates of drying. This paper reports on the development of the techniques for placing cells in nanoliter droplets, the viability of cells as a function of drying rate and final moisture content and a comparison of the apparent drying kinetics for cells in nanoliter droplets relative to those of cells dried in the more commonly used 5 μL or 10 μL drops.

2. Methods and materials

Two different techniques were employed for nebulization of media containing cells in order to suspend cells in nanoliter droplets: ultra-sonic and pneumatic nebulization. Pneumatic nebulization has been used in a number of recent studies in order to spray cells onto surfaces or wounds (Navarro et al., 2000) or for tissue engineering applications (Hafez et al., 2003; Nahmias et al., 2005; Veazey et al., 2005; Roberts et al., 2005). The most common technique uses an air-gun paint sprayer to create the aerosol. This technique is reported to result in very high yields in terms of cell viability (greater than 90%), but the size of the droplets is relatively large, highly non-uniform and poorly characterized (Navarro et al., 2000; Roberts et al., 2005). The lack of characterization and non-uniformity do not pose problems for studies whose aim is to deposit cellular aerosols onto wounds or scaffolds, but for our studies we desired cells that were suspended into droplets that were as small as possible in order to reduce non-uniformities in the moisture content and increase drying rates as much as possible.

2.1. *Ultra-sonic nebulizer*

Our first nebulizer was based on a piezo-electric device that operated at 125 kHz and is used in chemical vapor deposition. This nebulizer creates droplets with a diameter of approximately 20 μm (theoretically large enough to just barely contain a fibroblast). Repeated attempts at nebulization of media containing fibroblasts, however, demonstrated that no cells survived the nebulization process. All cells were completely destroyed.

In order to increase the likelihood of cell survival following nebulization a lower frequency transducer was selected. Droplet diameters are inversely proportional to the 2/3 power of the vibrational frequency according to the Kelvin equation (Tsai et al., 1996; Lozano et al., 2003). We selected a piezo-electric lead zirconate titanate crystal PZT (Sonics, Model CV188, 20 kHz) which is predicted to give a median droplet diameter of 90 μm when nebulizing water. Droplet diameters depend on surface tension, so the nebulization of culture media containing mammalian cells might result in different droplet diameters. A wide dispersing atomization probe was used (Sonics, TI-6A1-4V, 20 kHz) and the system was powered by a Sonics VCX 130 PB power supply. The system is capable of continuously generating a nebulized mist with droplets traveling at very low velocity.

2.2. *Pneumatic nebulizer*

Standard pneumatic nebulizers, such as those used for drug delivery in respiratory inhalers produce droplets that are too small for the suspension of living cells: 1–5 μm (Hess, 2000). In order to produce droplets in the 100 μm diameter range we