CELL guidance systems

User Guide

PODS™

Growth Factors

Version 2.0

Contents

Introduction to PODS™

POlyhedrin Delivery System (PODS™) provides an effective solution to the inherent instability of proteins. PODS™ technology exploits the natural properties of the polyhedrin protein which, when expressed, forms crystals within a cell. PODS™ co-crystals are formed when a tagged protein of interest (the active protein) is co-expressed with the *Bombyx mori* cypovirus polyhedrin protein. The polyhedrin protein forms regular, cubic crystals within which the active, protein specifically binds via a short protein tag. PODS™ crystals provide a slow-release depot formulation for the active protein.

Storage

Upon receipt, store at 4°C.

PODS™ crystals are stable for at least a year at 4°C.

Reconstitution

PODS™ crystals may be reconstituted at $200x10^6$ crystals/ml in water. Alternatively, 20% glucose has a buoyant density closer to PODS™ crystals and can be useful for aliquoting. PODS™ crystals are highly stable when stored in aqueous solution (pH range 6-8) at 4°C and have been shown to maintain stability for at least 6 months.

Physical Characteristics

Size and Shape

PODS™ crystals are cubic and typically 1-15 microns in size with a modal size of 3-4 microns. During the manufacturing process, some PODS™ crystals may fracture resulting in smaller crystal fragments.

Buoyant Density

PODS™ crystals are heavier than water and will settle on the surface of a culture vessel. Care should be taken when aliquoting, since PODS™ crystals will sink to the bottom of a tube within a few minutes. The majority of PODS™ crystals will remain in suspension for up to 60 min in a 20% glucose solution (or a solution of similar density).

Physical factors affecting stability

PODS™ crystals are highly stable when stored in solutions between pH 6-8. PODS™ crystals are also stable at 37°C for extended periods of time (> 10 days). Above pH 10, PODS™ crystals lose their stability and can dissolve within a few hours, particularly at elevated temperatures.

Elution characteristics

Since PODS™ crystals are protein structures, they are broken down in solutions that contain proteases. **PODS™ do not readily degrade or release the active protein in simple aqueous buffers.** Proteases may be derived from components of the solution (e.g. serum) or secreted by cells. Consequently, the culture system affects the amount of growth factor available in solution. In contrast to gel-encapsulated proteins (made using hydrogels such as PLGA), PODS™ crystals do not produce an initial burst release of active proteins. Elution characteristics have been determined for PODS™ LIF crystals in cell culture, for which peak release has been shown to occur at day 2, then gradually diminishes (See figure below).

Modifying the release profile

Release of active proteins into cell culture occurs over a period of 2-3 weeks. This release period can be extended to several months if the PODS™ crystals are combined with a scaffold, such as collagen.

Other characteristics

PODS™ crystals will adhere to most plastics. For aliquoting, we recommend using low adherence plastics, such as low protein binding centrifuge tubes made by Nunc. PODS™ crystals are stable in high temperatures in neutral solutions and remain intact after boiling for 1 hour.

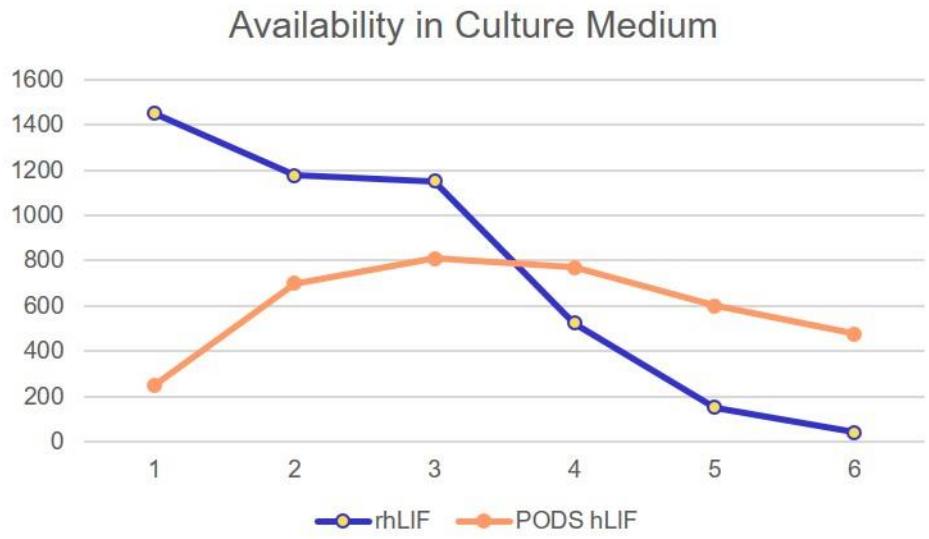

Figure 1. The concentration (pg/ml) of a recombinant growth factor or a PODS™ growth factor in solution was measured using an ELISA assay over a period of 6 days. At the start of the experiment, on day 1 recombinant growth factor (rhLIF) was maximal but was completely depleted by day 6. In contrast >50% of peak levels were still present for PODS™ LIF proteins on day 6. (Nishishta et al 2011).

Quantification of PODS™

During production of PODS™ crystals, active proteins are immediately incorporated into the polyhedrin crystal within the insect cell. As a result, it is not possible to obtain a direct measurement of the amount of active protein that is present in a sample. When PODS™ crystals are produced, both polyhedrin proteins and active proteins are expressed from within a single vector and both utilize individual copies of the same

polyhedrin promoter. Therefore, the ratio of active to polyhedrin protein will be constant. The size distribution of PODS™ crystals is also constant. Consequently, the number of crystals has been adopted as the unit of quantification for PODS™.

How many PODS™ crystals should be used?

PODS™ crystals provide a depot of active proteins which are steadily secreted. In the above experiment, approximately 50% of peak levels of LIF were still available in the culture system after 6 days of culture. The amount of PODS™ crystals that is optimal for a particular experiment should be determined empirically. Assuming 50 million PODS™ crystals has equivalence to 3.3 µg of standard growth factor is a good starting point.

How often should media and PODS™ crystals be replaced?

The required frequency of media change depends on (a) the speed with which nutrients are exhausted or degraded and (b) the speed with which toxic metabolites accumulate. In most cell culture systems, stability of growth factors is an overriding issue which drives media replenishment. However other factors will become important once the growth factor stability issue has been addressed. Changing half the media is likely to be more effective than complete media changes. As with any protocol, optimization is important.

Equivalence to standard growth factors

The stability of standard recombinant growth factors varies significantly. The most labile, such as FGF-1, have a half-life measured in minutes (Zakrzewska et al) limiting utility. The stability of PODS™ growth factors that are encased in PODS™ crystals is much longer, but once released will be the same as their standard recombinant counterparts.

The amount of available growth factor in a culture system is a function of the speed of release from the crystal and their subsequent stability. In the first few hours in a culture dish containing only newly added PODS™ crystals, there is little growth factor protein available for the cells. Significant amounts of growth factor protein are available after one day. The initial lag of protein availability may be corrected, if necessary, by adding a small amount of standard recombinant growth factor.

Refractivity

PODS™ crystals refract light. A large amount of PODS™ crystals may interfere with the use of imaging techniques such as phase-contrast microscopy and will also interfere with measurements based on absorbance. When using a colorimetric assay, the PODS™ crystals should excluded from the assay chamber. This may be achieved by removing the cell culture media into a separate plate for measurement. Alternatively, adding alkaline buffer to dissolve the PODS™ crystals may be effective (see the section "Western Blotting").

Physical impact on cell behavior

Physical features on a culture surface may impact the behavior of certain cells. If this is a concern, PODS™ crystals may be incorporated into a hydrogel surface coating. Custom

made Matrigen™ Softwell plates (flat, 2D hydrogels of defined elasticity) containing PODS™ crystals are available as a custom order from Cell Guidance Systems. Many tissue culture plasticware suppliers including Corning, Falcon, Nunc and Sarstedt offer sterile permeable hanging inserts for tissue culture plates which separate the components of culture, although we have not yet tested these ourselves.

Biological Characteristics

Post-translational modification

PODS™ crystals are made in insect cells. PODS™ active proteins therefore contain most of the post-translational modifications that are found in mammalian cells. For example, PODS™ Wnt-3a, which requires palmitoylation and glycosylation for biological activity, is highly functional.

Immunogenicity

The polyhedrin protein has been tested in-vivo in several mammalian species (Matsumoto et al (2012) and unpublished observations) and an inflammatory response has not been apparent. Lack of an inflammatory response for foreign proteins is not unusual and many foreign proteins are in routine clinical use: silk fibroin protein from the silk worm *Bombyx mori* is commonly used for surgical stitching and Botulinum toxin is widely used in cosmetic procedures.

Characterization of the active protein

Ultimately, the utility of PODS™ proteins depends on the biological activity of the active protein. The active protein may also be analyzed using standard characterization techniques. PODS™ crystals first require breaking down, either by proteolytic or alkaline degradation, to release the active protein.

In our experience, most antibodies that detect standard recombinant proteins will also detect the same proteins released from PODS™ crystals. However, there are some cases where the tag used to attach the protein to the polyhedrin crystal may modify or obscure the epitope.

ELISA Assays

ELISA assays have been successfully used to monitor the amount of free available active protein released into cell culture media. See for example Nishishita et al (2011).

Western Blots

PODS™ crystals may be dissolved by incubation in alkali. Prepare a buffer containing PBS (pH 11) and 1% SDS. Incubate at 65°C for one hour. Add 4 µl of protein loading buffer for each 1 µl of dissolved PODS™ crystals.

Immunoaffinity

PODS™ crystals can be readily attached to plastic surfaces. The presence of the active protein can be confirmed by performing an ELISA-like assay directly using a detection antibody against intact PODS™ crystals and using Empty PODS™ crystals (available from Cell Guidance Systems) as a control.

Applications of PODS™

Localized deposition

PODS™ proteins crystals can be readily attached to surfaces and provide a depot for growth factors which are secreted into surrounding media at physiologically relevant concentrations. PODS™ crystals may be localized by spotting using a pipette. A 0.5 µl aliquot forms a disc around 2 mm across. Alternatively, PODS™ crystals may be combined with a hydrogel. This reduces spreading and slows release. As PODS™ crystals are heavier than water, they will accumulate in the base of V well dishes or hanging drops, widely used for embryoid body formation. There are a range of printing techniques which may be applicable to PODS™ crystals.

Generation of gradients

Where PODS™ crystals have been localized on a surface, it is possible to generate a biologically effective gradient simply by eliminating any agitation during the period of culture. Such gradients can be maintained for weeks allowing the modelling of complex developmental programs.

In-vivo delivery

PODS™ crystals provide excellent devices for the long-term, localized delivery of growth factors and other proteins with potential medically utility. PODS™ growth factors are sold for research use only. However, if you are considering the development of a protein therapeutic, please contact us for support.

References

1. Matsumoto et al, Bone regeneration by polyhedral microcrystals from silkworm virus Scientific Reports (2012)
2. Zakrzewska et al, Increased Protein Stability of FGF1 Can Compensate for Its Reduced Affinity for Heparin, JBC (2004)
3. Nishishita et al, The use of leukemia inhibitory factor immobilized on virus-derived polyhedra to support the proliferation of mouse embryonic and induced pluripotent stem cells. Biomaterials (2011)

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