Deciphering the Mechanisms of Action in the Release of Molecules from Biological Samples Using Pressure Cycling Technology (PCT)

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Abstract

Boston Biomedica, Inc. has developed a Sample Preparation System based on Pressure Cycling Technology (PCT SPS). The PCT SPS is a rapid, reproducible and closed system for the extraction of molecules from cells and tissues. The system consists of a Barocycler™ instrument and single-use, sample processing containers - PULSE™ Tubes. To extract biomolecules, PULSE Tubes with samples and buffers are placed in fluid-filled reaction chambers of a Barocycler and exposed to rapid cycles of pressure between ambient and high levels (typically 35 kpsi or 235 MPa). During the process, cellular components including small molecules, nucleic acids, and proteins are released into buffers; at completion, a crude lysate is obtained for downstream purification or direct analysis. During extraction, temperature can be controlled between –30 and +37°C. PCT is compatible with the common downstream applications that are usually accomplished by more conventional extraction methods, such as mortar-pestle. The PCT SPS has successfully extracted DNA, RNA, proteins, and small molecules from a wide variety sample types, including "hard-to-lyse" microbes, plant and animal materials. Here, hypothetical mechanisms of action of PCT process are presented (Table 1). It may be possible to develop more specific and efficient PCT extraction procedures by determining which of these mechanisms causes releases of biomolecules from cells and tissues.

Table 1. Hypothetical Mechanisms of Action of PCT Extraction

	MECHANISM OF ACTION	APPARENT EFFECT
I	Microbubble collapse during pressure cycles	Localized cavitation, which causes cell and tissue damage
II	Rapid freeze-thaw under subzero temperatures and cyclic pressure conditions	Formation of ice crystals, which causes cell and tissue damage
III	Thermodynamic effects of low temperature and high pressure	Protein denaturation and disruption of biomolecular interactions, e.g. disassociation of multimeric proteins and biomolecular complexes
IV	Compression by ram and lysis disk	Physical shearing and squeezing; multiple rounds of solvent exchange
V	Enhance dissolution power of extraction buffers	Increases in solubility of both hydrophilic and hydrophobic molecules

Extraction Using Pressure Cycling Technology

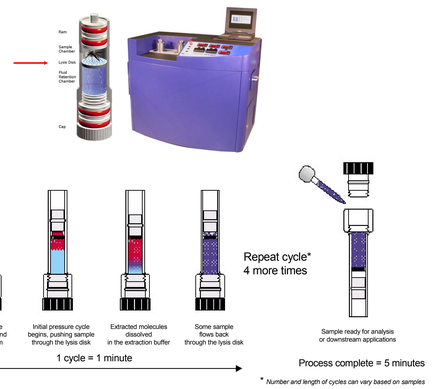

Fig. 1. The PCT Sample Preparation System includes an instrument, the Barocycler NEP2017, and specially designed PULSE Tubes. An illustration of the PCT extraction process is shown. → points to the lysis disk.

The Barocycler NEP2017 can raise pressure from ambient to 35 kpsi (235 MPa) in less than 5 seconds and ramp down pressure from its maximum to ambient in a fraction of a second. Up to six PULSE Tubes can be processed simultaneously. The process temperature can be controlled using an external circulating water bath, allowing experiments to be performed between -30 and 37°C.

PULSE Tubes FT500 can hold 20 – 500 mg of solid sample or 1.2 – 1.5 mL of liquid sample. The sealed PULSE Tubes are immersed in pressurization fluid in the two reaction chambers of the Barocycler NEP2017 and are then subjected to cycled pressure. The recovered volume of PCT prepared samples is between 0.8 and 1.4 mL.

Applications of PCT Extraction in Genomics and Proteomics

Using the PCT SPS, high quality total RNA and proteins were extracted from 25–500 mg of animal tissue including those known to contain high amounts of RNase or that are high in lipid content, such as rat liver, brain, spleen, and tail.

Example 1: RNA Expression Profile of Rat Brain Analyzed on a Microarray

- 200 mg fresh frozen rat brain was extracted in 1.1 mL TRI Reagent (MRC) using standard PCT conditions, i.e. 35 kpsi or 235 MPa, five 1 min cycles at 4°C. A positive control sample was obtained using mortar-pestle grinding on dry ice.
- Crude lysate was clarified by centrifugation. Total RNA was purified using the TRI Reagent protocol, examined by formaldehyde agarose gel and quantified by OD_{260}.
- 1 µg of each purified total RNA was examined using the SuperArray microarray GEArray™ Q-series protocol on a MM-013N membrane (Neuroscience-1 Ion Channel & Transporter series) (SuperArray Bioscience Corp, Frederick, MD). Chemiluminescent signals were detected by CCD camera.

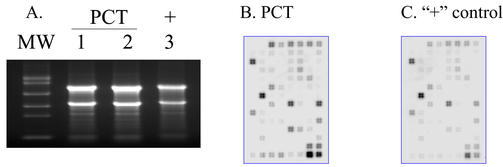

Fig. 2. RNA extracted from rat brain examined by formaldehyde agarose gel (A), and DNA microarray analysis using PCT SPS (B) and mortar-pestle (C) produced samples. The RNA expression patterns obtained from samples processed using PCT or mortar-pestle grinding methods were similar.

Example 2: Total Protein Extracted from Rat Liver Analyzed by 1D and 2D Gel Electrophoresis

- 200 mg fresh frozen rat liver was extracted in three different buffers (1D gel) and 100 mM Tris, 0.15M KCl, 1 nM PMSF, 1 mM DTT, Sigma protease inhibitor cocktail (Cat. No. P8340) pH 8.0 (2D gel) using the standard PCT procedure. A positive control sample was obtained using mortar-pestle grinding on dry ice.

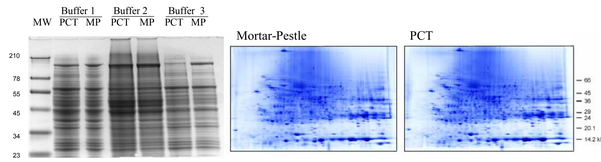

Fig. 3. Proteins extracted from rat liver examined by 1D SDS PAGE and 2DGE. Similar protein patterns were obtained by both the PCT and mortar pestle extraction methods.

Table 2. Biological samples from which nucleic acids and proteins have been successfully extracted using the PCT Sample Preparation System and a list of applications for which these molecules were used following the extraction (1).

Sample Group	Examples	Molecules Extracted[1,2]	Applications
Animal tissues	Soft: liver, brain, pancreas, kidney, lung	DNA, RNA, Proteins	PCR, RT-PCR, ELISA, SDS-PAGE, Western blot
	Hard: heart, skeletal muscle, breast tumor, aorta	DNA, RNA	PCR, RT-PCR
Plant tissues	Soft: corn sprout, leaves (corn, tomato, juniper, wheat), grape skin	DNA, RNA, Proteins	PCR, RT-PCR, SDS-PAGE
	Hard: stem, pine needle, grape seed	DNA	PCR
Insects, small organisms	Mosquito, *C. elegans*	DNA, RNA, protein	PCR, RT-PCR, SDS-PAGE
Blood	Blood, blood spot	DNA	PCR
Microbes	Yeast	DNA, RNA, Proteins	PCR, RT-PCR, SDS-PAGE
	Mycobacteria sp., bacteria/spores, soil	DNA, RNA	PCR

[1]Not every example has been tested for RNA or proteins with RT-PCR, WB or ELISA.
[2]Comparable yields were generally achieved in these experiments by comparing to either one or two conventional methods.

Mechanisms of Action in PCT Extraction

Hypothesis 1. Microbubble Collapse Model

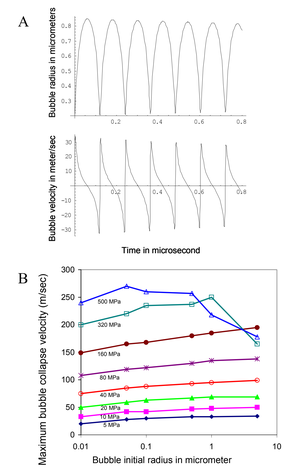

Fig. 4. Using simple bubble dynamics models, we established a quantitative relationship between the cycled pressure and the bubble oscillation dynamic characteristics. A single spherical bubble was modeled by the Rayleigh-Plesset equation (2,3):

$$R\ddot{R} + \frac{3}{2}\dot{R}^2 + \frac{4\mu}{\rho R}\dot{R} = \frac{1}{\rho}[P_v(T) - P_\infty + P_g - \frac{2\sigma}{R}]$$

A typical bubble dynamics response is shown in Fig. 4A. The applied pressure compresses the bubble radius to a fraction of its equilibrium radius. Upon the release of the applied pressure, the bubble tries to relax to its equilibrium radius. Because of the inertia effect, the process is oscillatory. The viscous dissipation in the liquid will eventually damp out the vibration. Because of its non-linearity, the bubble vibration is not harmonic. It results in very large velocity and velocity change when the bubble radius is near its minimum. At different levels of pressure, each bubble radius and the maximum velocities of the bubble wall during the contracting phase are obtained (Fig. 4B).

Hypothesis 2. Freeze-thaw Driven by Pressure Cycling

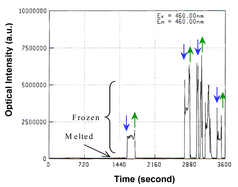

Fig. 5. Rapid phase change of Solution D containing 2 M guanidinium thiocyanate monitored by light scattering.
Pressure: ambient to 270 MPa or 40 kpsi
P rise time: < 60 sec; *P release time*: < 60 sec.
Temperature: - 10 °C

Solution D froze when pressure was released (↓) and melted when pressure was applied (↑). Instant phase change response was observed by changing pressure, e.g. between ambient and 35MPa or 5 kpsi.

Hypothesis 3. Denaturation of Proteins: Damage of Membrane Structure or Protein Complex

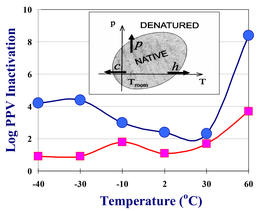

Fig. 6. A temperature profile of porcine parvo virus (PPV) inactivation. *Pressure*: 537 MPa or 80 kpsi. *P rise time*: < 10 sec. *P release time*: < 3 sec. *Number of pressure cycles*: 60 (0.5' ↑, 1'↓, •) or 1 (30' ↑, ■)
Medium: a therapeutic protein solution

The temperature profile of the inactivation is close to a typical protein p-T phase diagram. The hypothetical mechanism of action is the denaturation of the viral particle protein.

Hypothesis 4. Role the Lysis Disk

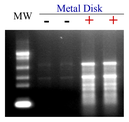

Fig. 7. Extraction efficiency may be improved by alternative designs of the lysis disk or ram that help to increase the surface area of tissue exposed to PCT. The agarose gel shows total RNA from pieces of rat skin extracted by PCT with or without an additional lysis disk made of steel. RNA in the crude extracts were purified using QIAGEN RNeasy method. It is demonstrated here that an additional lysis disk can significantly improved extraction.

Hypothesis 5. Pressure Effect on Buffer Activity and Molecular Solubility

It is known that high pressure can change the dynamic properties of lipid bilayers, ionization volume of charged groups in certain molecules, denaturant activity, and acceleration in enzymatic activity of some lytic enzymes (data not shown).

Discussion

We have proposed several mechanisms of action of PCT to account for the release of DNA, RNA, proteins and small molecules form cells and tissues. We suggest that the hypothetical microbubble collapse has the most profound effect in the process. However, it is possible and likely that two or more mechanisms are acting in concert. By deciphering these mechanisms of action, we hope to discover process conditions that release greater amount high quality biomolecules. Furthermore, the PCT process may even be used in "fine-tuned" releases of intact complex cellular components, such as protein complexes or organelles. In Table 3, we list potential experimental designs based on our hypotheses of the mechanism of PCT that may lead to improved release of biomolecules and potentially new discoveries in genomics and proteomics.

Table 3. Considerations in Experimental Design to Improve Extraction by PCT

A. Basic Considerations	
1. Characterize the sample	• Liquid or solid, hard or soft • Basic structure of cell and tissue • How the sample was collected and stored
2. Targets of extraction and expected concentration	• Nucleic acids, proteins, small molecules, molecular complexes, or sub-cellular complexes • Preservation requirement
3. Select extraction buffer system and concentrations of each component	• Aqueous, organic, detergent, denaturant, salt, reducing agent, destructive enzyme inhibitor, competitive inhibitor chelator, and lytic enzymes
4. Determine initial PCT process condition	• Consider to begin the test with the standard 5 × 1' cycle protocol, decide temperature of process
B. Enhancement or Optimization based on the Mechanisms of Action	
1. Microbubble collapse	• Examine pressure cycle profiles • Add bubble nucleation center and/or agent
2. Pressure driven freeze thaw	• Select appropriate buffer: rapid freeze-thaw • Lower temperature to freezing point
3. Protein denaturation	• Determine temperature profile at constant pressure cycle conditions • Use maximum pressure and additional # of cycles
4. Compression of ram and lysis disk	• Choose and use metal disk accessory • Employ additional number of pressure cycles
5. Pressure enhanced lytic buffer	• Choose the most lytic buffer appropriate for the sample • Pressure responsive buffers (under investigation)
C. Additional Comments	
1. To maximize the PCT extraction of target molecules	• Be aware of the inherited variability in the biological samples themselves • Enhancement or optimization may require in depth analysis of the sample and the PCT process • Multi-factorial analysis in optimization experimental design • Expert experimental design package is to be developed
2. Compatibility with downstream process	• This could mean the use of less than efficient lysis buffer in the process. In this case, optimization of the physical conditions may be helpful.
3. Take advantage of the other benefits of PCT extraction process	• The PULSE Tube may be used as the sample collection, storage and post-processing storage device • The PCT system may be sheared by a few laboratories, even those who process very different kinds of samples

Summary

The PCT Sample Preparation System (PCT SPS):

- employs a new method for the extraction of biomolecules from cells and tissues
- functions by a different mechanism than other available systems to release biomolecules
- is rapid, safe and reproducible
- is fully contained to minimize cross contamination
- is capable of releasing small molecules, nucleic acids, and proteins into buffers suitable for downstream purification or direct analysis
- is capable of releasing biomolecules from a wide variety sample types, including "hard-to-lyse" microbe, plant and animal materials (Table 2)
- is amenable to further optimization based on an understanding of its mechanisms of action (Table 3)
- may lead to new discoveries in genomics and proteomics due to its novel mechanisms of action.

References

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