Deciphering Mechanisms for Infectious Agent Inactivation Using Cyclic High Pressure or Pressure Cycling Technology (PCT)

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Abstract

Infectious agent inactivation is a critical step in biopharmaceutical preparation, blood fractionation, and vaccine production. Inactivation of viruses and bacteria is currently performed using several different methods, including the use of UV or gamma irradiation, or with chemicals, such as the solvent-detergent method. However, most current methods exhibit limitations in inactivating all types of infectious agents or in preserving important proteins. In addition, some methods can be cumbersome, time consuming, expensive, or hazardous. To overcome these problems, we performed studies to evaluate a novel high pressure-based technology for infectious agent inactivation. **Pressure Cycling Technology (PCT) is a physical process that uses cycles of ultra high and low hydrostatic pressures with controlled temperature.**

A number of viruses and bacteria were used as representative organisms to determine pressure and temperature conditions that can effectively inactivate these organisms while preserving the structure and function of several important proteins, such as coagulation Factor VIII and the immunoglobulins. Data show that 5 to 60 cycles of pressure, ranging between ambient to 80 kpsi for 0.5 – 2 min per cycle at -40 °C to 20 °C, can effectively inactivate 4 to 8 logs of most of the representative viruses or bacteria in either growth media or plasma. Our data also indicate that PCT may be used in combination with other techniques, such as the addition of chemicals or nucleases, for the effective inactivation of a broad spectrum of viruses and bacteria. The PCT process can be adapted to accommodate batch- or flow-type process designs.

In summary, PCT may offer the biopharmaceutical industry an important new method of viral inactivation that is safer, more versatile, and potentially more cost effective than conventional methods. PCT can be also an interesting research tool in the study of structure, stability, function of microorganisms, proteins and biomolecular assemblies.

Current Inactivation Technologies

Table 1. List of current viral inactivation technologies (1,2).

	Methods	Benefits	Challenges
Inactivation	Pasterurization	Env. Or non-env., HAV	temperature, requirement of stabilizer, limited effects on heat stable microorganisms
	Terminal Dry Heat	Env. Or non-env., HAV	Freeze cycle, lyophilization, temperature homogeneity, residual moisture
	Vapor Heat	Env. Or non-env., HAV	Freeze cycle, lyophilization, temperature homogeneity, residual moisture
	Solvent/Detergent (SD)	enveloped viruses; preserve proteins	uneffective on non-enveloped viruses; SD removal; reagent concentration;temperature
	Acid pH	enveloped viruses	limited on non-enveloped viruses, elevated temperature, pH, duration
Removal	Precipitation	purified proteins; potential for removal of all viruses	Difficult to model; selection of precipitation agents; protein performance; temperature; contamination
	Chromatography	purified proteins; potential for removal of all viruses	risen selection, buffer, resin santitization between runs, elution profile, resin lifespan
	Nanofiltration	Env. Or non-env., HAV, parvo B19	pore size, small viruses may not totally removed, filter integrity/defect, back pressure, protein concentration, surface area

Other alternative microbial inactivation technologies (3,4): microwave, RF irradiation, pulsed electrical field, high voltage arc discharge, pulsed light, oscillating magnetic fields, UV light, ultrasound, X-rays, supercritical fluid, and γ-irradiation

Novel Method in Infectious Agent Inactivation using PCT

Experimental Method:

- Test samples were packaged in either in polypropylene tubes sealed with Parafilm, or EVA plastic bags and thermally sealed.
- Pressure treatments were conducted with Barocycler V2.2, V2.4 or its modified versions shown below (BBI Source, Garden Grove, CA). Temperature of the reaction chamber (blue cylinder in the picture) is controlled with a circulating water bath or chiller.
- After the pressure treatment, titers of the live virus or bacterium were determined and compared with positive controls, i.e. samples that were processed in parallel, but without cyclic pressure treatment.

Fig. 1. Barocycler V.2.4.
Pressure: ambient to 537 MPa or 80 kpsi
Pressure rise time: < 10 sec.
Pressure release time: < 3 sec.
Temperature: between - 40 and 70 °C
Sample volume: up to 5 mL

Table 2. Representative data from PCT inactivation studies.

	MICROBES	TITER REDUCTION (LOGS)	CONDITIONS			
			Pressure (MPa, kpsi)	Temp (°C)	Number of Cycles	Medium
Viruses	Lambda Phage	**7**	345, 52	-5	20 (1'↑, 1'↓)	1:10 stock:human plasma
	HIV-1 (B subtype)	**6**	336, 50	-10	5 (1'↑, 1'↓)	Tissue culture media
	Herpes Simplex Virus (HSV)	**6**	336, 50	-10	5 (1'↑, 1'↓)	Human plasma
	MS2 (ATCC 15597-B1)	**5**	537, 80	-20	30 (0.5'↑, 0.5'↓)	Anticoagulated human plasma
	Porcine Parvo Virus (PPV, ATCC VR-742)	**4.5**	537, 80	-30	50 (0.5'↑, 1'↓)	Plasma; therapeutic protein solution; growth media
Bacteria	*Bacillus cereus* (ATCC 14579)	**5**	270, 40	-5	10 (1'↑, 1'↓)	Human plasma
	Enterococcus faecium (ATCC 49032)	**6**	336, 50	-5	10 (1'↑, 1'↓)	Human plasma
	Escherichia coli(ATCC 43894)	**7**	336, 50	-5	10 (1'↑, 1'↓)	Human plasma
	Pseudomonas aerug. (ATCC 14502)	**8**	270, 40	-5	10 (1'↑, 1'↓)	Human plasma
	Staphylococcus aureus (ATCC 9144)	**6**	336, 50	-5	10 (1'↑, 1'↓)	Human plasma

Table 3. Representative plasma component activities after PCT(5).

PROTEIN	ACTIVITY	CONDITIONS		
		Pressure (MPa, kpsi)	Temp (°C)	Number of Cycles
Immunoglobulins				
Anti CMV IgG	>95%	345, 52	-5	10 (1'↑, 1'↓)
Anti CMV IgM	90%			
Coagulation Factors				
Factor X	90%	345, 52	-5	10 (1'↑, 1'↓)
Factor VIII	70%			
Enzymes				
Lipase	>95%			
Alkaline Phosphatase	>95%			
Total amylase (T-AMY)	>95%	275, 41	-5	10 (1'↑, 1'↓)
Alanine aminotransferase*	>80%			
Aspartate aminotransferase*	<10%			
Lactate dehydrogenase*	<10%			
Albumin	>90%	345, 52	-5	10 (1'↑, 1'↓)
Blood Chemistries	100%	345, 52	-5	10 (1'↑, 1'↓)

* Multimeric proteins.

Mechanisms of PCT Inactivation:

Table 4. Hypothetical mechanisms of PCT inactivation for discussion.

	CAUSE	HYPOTHETICAL EFFECT
I	Solution under subzero temperature, cyclic pressure conditions	Rapid freeze-thaw; formation of ice crystals and cause cell damages, e.g. in bacteria
II	Thermodynamic effects of low temperature, and high pressure	Protein denaturation and disruption of biomolecular interactions, e.g. disassociation of multimeric proteins and viral particles
III	Rapid pressure change	microbubble collapse to cause localized cavitation and viral or cell damages
IV	Changes in permeability of cell membrane or viral particle	Transient or permanent exposure of proteins or nucleic acids to extracellular enzymes or inhibitors
V	Pressure cycling and dynamic enhancement	Pressure cycling has beneficial effects on both the rate of inactivation and recovery of protein activity

Test of the Hypotheses and Discussion

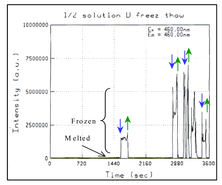

Fig. 2. Rapid phase change of Solution D containing 4 M guanidinium thiocyanate monitored using light scattering.
Pressure: ambient to 270 MPa or 40 kpsi
Pressure rise time: < 60 sec.
Pressure release time: < 60 sec.
Temperature: - 10 °C

Discussion: Solution D froze when pressure was applied (↑) and thawed when pressure was released (↓). Instant phase change response was observed. Only small pressure changes, e.g. 35MPa, 5 kpsi, were needed. This is a piece of evidence for plausible Hypothesis I.

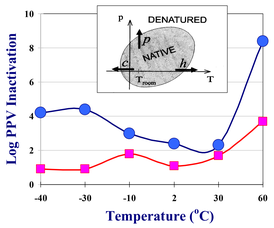

Fig. 3. A temperature profile of porcine parvo virus (PPV) inactivation.
Pressure: 537 MPa or 80 kpsi
Pressure rise time: < 10 sec.
Pressure release time: < 3 sec.
Number of pressure cycles:
60 (0.5'↑, 1'↓, ●) or 1 (30'↑, ■)
Medium:
a therapeutic protein solution

Discussion: The viral inactivation temperature profile is coincidently similar to protein stability phase diagram (insert), i.e., virus is less stable in both low or high temperature ranges. Also data shown in Table 2 and reference 7, support the idea that pressure can have profound impact on protein stability and function either by protein denaturation or dissociation of biomolecular complexes, in support of Hypothesis II.

Discussion: Hypothesis IV has been tested by conducting PCT viral inactivation in the presence of high concentration nucleases or chemical inhibitors. Data showed small differences in the inactivation of a number of viruses. Further studies are being conducted to increase permeability of doped nucleases and inhibitors in viruses or bacteria.

Table 5. 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 (6).

Sample Group	Examples	Materials Extracted	Applications
Animal tissues	Soft: liver, brain, pancreas, spleen, kidney, lung	DNA, RNA[1], Proteins[2]	PCR, RT-PCR, ELISA, SDS-PAGE, Western Blot
	Hard: tail, heart, intestine, skeletal muscle, breast tumor, aorta	DNA, RNA[1]	PCR, RT-PCR
Plant tissues	Soft: corn sprout, leafs (corn, tomato, Juniper, wheat), grape skin	DNA, RNA[1], Proteins[2]	PCR, RT-PCR, SDS-PAGE
	Hard: stem, pine needle, grape seed	DNA	PCR
Insects, small organisms	mosquito, fruit fly, mealworm, tick	DNA, RNA[1]	PCR, RT-PCR
Blood	Blood, blood spot	DNA	PCR
Microbes	Yeast, *C. elegans*	DNA, RNA, Proteins	PCR, RT-PCR, SDS-PAGE
	mycobacteria, bacteria/spores, soil	DNA, RNA[1]	PCR

[1, 2] Not every example has been tested for RNA or proteins and subsequent RT-PCR or WB, ELISA.

Discussion: PCT has been in used in extraction or releasing of biomolecules from a wide variety of biological samples. One plausible hypothesis is that rapid pressure pulses creates microbubbles. These air bubbles can expand and collapse to cause cavitation in cells and tissues, similar to sonication. The main difference between the two methods is that PCT introduces high activation state of air bubbles by compressing the system using ultrahigh hydraulic pressure. The entire system is uniformly energized using PCT, in contrast to sonication which energy is distributed at the tip of a ultrasonic probe. Although it has not been experimentally studied in depth about the bubble collapse hypothesis (Hypothesis III), computational models of the process are being established.

Discussion: Experiments were also performed on viral inactivation at different pressure (pressure profile), rate of pressurization or de-pressurization, and pressure/ambient pressure incubation periods (pressure cycle profiles). It shows that there are significant differences in inactivation as well as protein or enzyme preservation. Dynamic properties in pressure profiles of PCT inactivation have shown profound impact (Hypothesis V). Detailed dynamic and thermodynamic analysis will provide further information.

In Summary, infectious agent inactivation can be a very complex process. We found that different viruses and therapeutic products exhibit different response to cyclic high pressure. To develop a practical application, mechanisms of PCT inactivation including thermodynamic properties need to be better understood. A combination use of PCT and other inactivation techniques needs to be studied, that may offer additional advantages of PCT in blood fractionation, biopharmaceutical preparation, vaccine production, sterilization of food or clinical reagents and products.

Reference

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