Osmolyte Enhanced Aqueous Two-Phase System for Virus Purification

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Abstract

Due to the high variation in viral surface properties, a platform method for virus purification is still lacking, A potential alternative to the high-cost conventional methods is aqueous two-phase systems (ATPSs). However, optimizing virus ATPS purification requires a large experimental design space, and the optimized systems are generally found to operate at high ATPS component concentrations. The high concentrations capitalized on hydrophobic and electrostatic interactions to obtain high viral particle yields. This study investigated using osmolytes as driving force enhancers to reduce the high concentration of ATPS components while maintaining high yields. The partitioning behavior of porcine parvovirus (PPV), a non-enveloped mammalian virus, and human immunodeficiency virus-like particle (HIV-VLP), a yeast-expressed enveloped VLP, were studied in a PEG 12kDa-citrate system. The partitioning of the virus modalities was enhanced by osmoprotectants glycine and betaine, while TMAO was ineffective for PPV. The increased partitioning to the PEG-rich phase pertained only to viruses, resulting in high virus purification. Recoveries were 100% for infectious PPV and 92% for the HIV-VLP, with a high removal of the contaminant proteins and >60% DNA removal when glycine was added. The osmolyte induced ATPS demonstrated a versatile method for virus purification, irrespective of the expression system.

1. Introduction

Vaccine design has been evolving from using attenuated and inactivated viruses to modular designs of vectors and recombinant virus like particles (VLPs). These viral modalities utilize various cell platforms, resulting in different contaminant profiles of host cell proteins and DNA. The complex physicochemical profile of target viral product and contaminants and the increased upstream yields has challenged the downstream processing to maintain yield, purity, and robust processability. Up to this point, virus downstream processing has heavily relied on filtration, chromatography, and ultracentrifugation. Filtration technologies are generally used in the clarification step, to remove cell debris and large aggregates, or in the virus concentration step. However, two crucial challenges in implementing filtration techniques for clarification are virus adsorption onto the membranes and loss of viral structural integrity due to shear effects { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. The traditional method of virus purification in the past has been ultracentrifugation. The poor scale-up, labor-intense, and time-consumption attributes of ultracentrifugation led to a shift towards chromatography. However, conventional chromatography has low binding capacity for larger sized virus particles as compared to proteins, longer contact times, higher energy consumption, higher instrumental footprint, and higher environmental impact { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. In addition, a future goal is continuous production of viral products. Advances to make chromatography continuous are being made, but the process is only currently semi-continuous. Thus, alternative methods need to be explored to address the viral vaccine and vector downstream processing challenges while improving the production throughput at lower costs.

One solution to tackle the downstream hurdles is to purify viral particles with aqueous two-phase systems (ATPSs). Viral purification using ATPS has demonstrated high yields and

continuous operation capability in bioprocessing { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. However, this technique still requires extensive study to understand viral behavior in these types of biphasic systems. Traditionally, viral partitioning has been studied in a polyethylene glycol (PEG)-dextran system { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. Recent studies have majorly focused on PEG-salt systems { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. The previous studies emphasized that with the appropriate optimization strategies, ATPS can yield high recoveries of the target viral products. However, there are more opportunities to upgrade and modulate the two-phase characteristics to achieve better product throughput. Recent studies have demonstrated a new class of biofriendly ATPSs using ionic liquids and deep eutectic solvents { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. However, these novel phase forming components are yet to be evaluated by the FDA for use in therapeutic grade bioprocessing. This study explores a PEG-salt system that enhances virus partitioning to the PEG-rich phase for increased recovery and purity by addition of a third component. The third component has been limited to molecules that are currently approved for use in biotherapeutic manufacturing.

Numerous studies have tried to understand the characteristics of ATPSs that lead to biomolecule partitioning. The partitioning of biomolecules is synergistically influenced by the excluded volume theory, electrostatic interaction, and hydrophobic interaction { ADDIN EN.CITE

<EndNote><Cite><Author>Grilo</Author><Year>2014</Year><RecNum>206</RecNum><DisplayText>(Grilo<style face="italic"> et al.</style>, 2014)</DisplayText><record><rec-number>206</rec-number><foreign-keys><key app="EN" db-id="a0addzpwcr5x5ee55tyvxpprx05efws59sex" timestamp="1531663422">206</key><key

app="ENWeb" db-id="">0</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Grilo, António Lima</author><author>Raquel Aires-Barros, M.</author><author>Azevedo, Ana M.</author></authors></contributors><titles><title>Partitioning in Aqueous Two-Phase Systems: Fundamentals, Applications and Trends</title><secondary-title>Separation & Purification Reviews</secondary-title></titles><periodical><full-title>Separation & Purification Reviews</full-title></periodical><pages>68-80</pages><volume>45</volume><number>1</number><dates><year>2014</year></dates><isbn>1542-2119
1542-2127</isbn><urls></urls><electronic-resource-num>10.1080/15422119.2014.983128</electronic-resource-num></record></Cite></EndNote>}. However, recent reports have demonstrated critical contributions from the electrostatic and hydrophobic interactions in a PEG-salt system for virus purification { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}, with a contradictory contribution of the excluded volume theory. The virus prefers the PEG-rich phase once it is salted out of the salt-rich phase. This is accomplished by employing a strong kosmotropic salt, such as citrate, for salting out, and a higher molecular weight PEG to provide hydrophobic interaction. One could also operate the ATPS at higher system compositions, which will create a higher ionic strength of the salt-rich phase and an increase in relative hydrophobicity of the PEG-rich phase, thus promoting virus separation { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. However, harsh, high ionic strength environments may cause functional deterioration in vaccines and biotherapeutics. Thus, there is a need for driving force enhancers that will preferentially increase the virus partitioning at milder system compositions.

Promising driving force enhancers are osmolytes. Osmolytes are a class of molecules that occur naturally and are used by cells to compensate for changes in osmotic stress { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. Osmolytes have been divided into two groups based on the nature of their interaction with the protein backbone: 1) osmoprotectants or protecting osmolytes and 2) denaturing osmolytes. Osmoprotectants, such as betaine and trimethylamine N-oxide (TMAO), help in stabilizing protein structures { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. Denaturing osmolytes, such as urea and guanidinium chloride, have been shown to destabilize protein structures { ADDIN EN.CITE <EndNote><Cite><Author>Street</Author><Year>2006</Year><RecNum>650</RecNum><DisplayText>(Bennion & Daggett, 2003; Street<style face="italic"> et al.</style>, 2006)</DisplayText><record><rec-number>650</rec-number><foreign-keys><key app="EN" db-id="a0addzpwcr5x5ee55tyvxpprx05efws59sex" timestamp="1586699882">650</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Street, Timothy O.</author><author>Bolen, D. Wayne</author><author>Rose, George D.</author></authors></contributors><titles><title>A molecular mechanism for osmolyte-induced protein stability</title><secondary-title>Proceedings of the National Academy of Sciences</secondary-title></titles><periodical><full-title>Proceedings of the National Academy of Sciences</full-title></periodical><pages>13997-14002</pages><volume>103</volume><number>38</number><dates><year>2006</year></dates><publisher>National Acad Sciences</publisher><isbn>0027-8424</isbn><urls></urls></record></Cite><Cite><Author>Bennion</Author><Year>2003</Year><RecNum>661</RecNum><record><rec-number>661</rec-number><foreign-keys><key

app="EN" db-id="a0addzpwcr5x5ee55tyvxpprx05efws59sex" timestamp="1588095267">661</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Bennion, Brian J.</author><author>Daggett, Valerie</author></authors></contributors><titles><title>The molecular basis for the chemical denaturation of proteins by urea</title><secondary-title>Proceedings of the National Academy of Sciences</secondary-title></titles><periodical><full-title>Proceedings of the National Academy of Sciences</full-title></periodical><pages>5142</pages><volume>100</volume><number>9</number><dates><year>2003</year></dates><urls><related-urls><url>http://www.pnas.org/content/100/9/5142.abstract</url></related-urls></urls><electronic-resource-num>10.1073/pnas.0930122100</electronic-resource-num></record></Cite></EndNote>}. Osmolytes function as water structure modulators, either to maintain osmotic balance { ADDIN EN.CITE <EndNote><Cite><Author>Sleator</Author><Year>2002</Year><RecNum>683</RecNum><DisplayText>(Kinne, 1993; Sleator & Hill, 2002)</DisplayText><record><rec-number>683</rec-number><foreign-keys><key app="EN" db-id="a0addzpwcr5x5ee55tyvxpprx05efws59sex" timestamp="1588337563">683</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Sleator, Roy D.</author><author>Hill, Colin</author></authors></contributors><titles><title>Bacterial osmoadaptation: the role of osmolytes in bacterial stress and virulence</title><secondary-title>FEMS microbiology reviews</secondary-title></titles><periodical><full-title>FEMS microbiology reviews</full-title></periodical><pages>49-71</pages><volume>26</volume><number>1</number><dates><year>2002</year></dates><

publisher>Blackwell Publishing Ltd Oxford, UK</publisher><isbn>1574-6976</isbn><urls></urls></record></Cite><Cite><Author>Kinne</Author><Year>1993</Year><RecNum>684</RecNum><record><rec-number>684</rec-number><foreign-keys><key app="EN" db-id="a0addzpwcr5x5ee55tyvxpprx05efws59sex" timestamp="1588337669">684</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Kinne, Rolf K. H.</author></authors></contributors><titles><title>The role of organic osmolytes in osmoregulation: from bacteria to mammals</title><secondary-title>Journal of Experimental Zoology</secondary-title></titles><periodical><full-title>Journal of Experimental Zoology</full-title></periodical><pages>346-355</pages><volume>265</volume><number>4</number><dates><year>1993</year></dates><publisher>Wiley Online Library</publisher><isbn>0022-104X</isbn><urls></urls></record></Cite></EndNote>} or induce interactions that stabilize proteins during osmotic stress { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. Osmoprotectants have been utilized to reduce intermolecular interactions, thereby inducing intramolecular interactions to stabilize protein biologics in final formulations { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. Conversely, osmolytes have been shown to induce intermolecular interactions to flocculate viruses { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}, which aided in the development of filtration-based methods for virus purification. The exact mechanism of osmolyte function in protein stabilization or viral flocculation, however, is not clearly understood.

Osmolytes have been shown to modify ATPS partitioning. However, most osmolytes have been reported to effect the partitioning of small organic molecules or proteins in polymer-

polymer systems { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. This study explores the effect of osmolytes on a non-enveloped virus and an enveloped virus like particle (VLP) in a PEG 12kDa-citrate system. Our previous study showed that negatively-charged, non-enveloped viral particles partition to the PEG-rich phase only at high system compositions { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. In this work, we added osmolytes to explore the partitioning at lower system compositions. This was likely by improving the hydrophobic interaction of virus and VLP with the PEG-rich phase. Moreover, the efficiency of osmolyte type to improve virus recover in the hydrophobic PEG-rich phase has led to theories on the mechanism of osmolyte action. Understanding the mechanism driving partitioning has direct correlation not only in viral purification, but also in understanding the differential action of osmolyte towards biomolecules.

2. Materials and Methods

2.1 Materials

Eagle's minimum essential media (EMEM), sodium bicarbonate, phosphate buffered saline (PBS, pH 7.2), penicillin-streptomycin (pen-strep) (10,000 U/ml), fetal bovine serum (FBS, USDA approved) and trypsin/EDTA for cell propagation were purchased from Life Technologies (Carlsbad, CA). 2-(3,5-diphenyltetrazol-2-ium-2-yl)-4,5-dimethyl-1,3thiazole bromide (MTT, 98%) was purchased from Alfa Aesar^{TM} (Haverhill, MA) and sodium dodecyl sulfate (SDS, BioReagent, ≥98.5%) was purchased from Sigma Aldrich (Radnor, PA). Polyethylene glycol with an average molecular weight of 12,000 Da (PEG 12 kDa), trisodium citrate dihydrate (ACS reagent grade, ≥99%), and citric acid monohydrate (ACS reagent, ≥99%) were a generous gift from MilliporeSigma (Burlington, MA). Osmolytes glycine (BioUltra, ≥99.0%), betaine (BioUltra, ≥99.0%), trimethylamine N-oxide (TMAO) dihydrate, and urea

were purchased from Sigma-Aldrich (St. Louis, MO). Stable isotopes glycine (1-13C, 99%), betaine (D11, 98%), TMAO (D9, 98%), and urea (13C, 99%; 15N2, >98%) were purchased from Cambridge Isotope Laboratory (Tewksbury, MA). All solutions were made with Nanopure water (Thermo Scientific, Waltham, MA) at a resistance of ≥18 MΩ and filtered with a 0.2 μm Nalgene (Thermo Scientific) bottle top filter or 0.22 μm cellulose acetate syringe filters (VWR, Radnor, PA) prior to use.

HIV-VLP crude stock was a generous gift from Esperovax (Plymouth, MI). HIV-VLP with genetically infused green fluorescent protein (GFP) were expressed in *Saccharomyces cerevisiae* with a proprietary method.

2.2 Methods

Cell maintenance, virus propagation and virus titration

Porcine kidney cells (PK-13, CRL-6489™) purchased from ATCC were cultured in EMEM as previously described { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. Porcine parvovirus (PPV) strain NADL-2, gifted by Dr. Ruben Carbonell (North Carolina State University, Raleigh, NC), was propagated in PK-13 cells as described previously { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. PPV titration was performed by a colorimetric cell viability assay using MTT salt as described previously { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}.

Generation of binodal curve and tie lines

Binodal curves were generated using the turbidity method { ADDIN EN.CITE { ADDIN EN.CITE.DATA }} with 50 w/w% PEG and 35 w/w% citrate, and 0.5 M osmolyte stocks. Titration was done using 0.5 M osmolyte solutions to maintain the osmolyte concentration in the systems.

The tie lines were determined using the conductivity measurements of the citrate-rich phases as described previously { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. The conductivity contribution of osmolytes was measured and determined to be negligible as compared to the conductivity of citrate. The tie line length (TLL) and tie line ratios (TLR) were calculated as described previously { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}.

Osmolyte detection and quantification

Osmolyte in the citrate-rich phases were quantified using LC-MS. A Thermo Scientific™ UltiMate™ 3000 HPLC (Thermo Fisher Scientific, Waltham, MA) was coupled with an Orbitrap Elite mass spectrometer (Thermo Fisher Scientific) utilizing the electrospray ionization method. Separate LC-MS/MS methods were developed for detection and quantification of glycine, betaine, TMAO, and urea. For each method, a corresponding isotope was added to the calibration solutions and samples at the same concentration. The samples were separated on an Ascentis Express HILIC 15 cm x 2.1 mm, 2.7 micron (Sigma Aldrich). Eluent A was acetonitrile (HPLC grade, Fisher Scientific) and eluent B was 0.1 v/v% formic acid (98-100%, Sigma Aldrich) in HPLC grade H_2O (Fisher Scientific). The LC-MS/MS conditions used for each osmolyte are listed in **Table S1** in the Supplementary Information. For quantifications, the selected ion monitoring (SIM) of the parent ion $[M+H]^+$, or the selected reaction monitoring (SRM) of the suitable fragmentation ion were used. For SRM, the transitions used for parent ion and fragment ion used for each osmolyte are reported in **Table S1**.

Virus and virus-like particle quantification

ATPSs were prepared from 50 w/w% PEG and 35 w/w% citrate stocks. Appropriate stock concentration of each osmolyte was used to maintain a concentration of 0.5 M in the total system. Appropriate amounts of stock solutions with 0.1 g crude virus were added to a

microcentrifuge tube to total a 1g system. The remaining procedure was similar to our study reported earlier { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. The virus and VLP recoveries and partition coefficient (K) were calculated using equations 1 and 2, respectively.

$$\% \, Recovery = \frac{V_{P/C} * T_{P/C}}{V_i * T_i} * 100 \quad (1)$$

$$K = \frac{V_P * T_P}{V_C * T_C} \quad (2)$$

where *V* refers to the volume of either PEG-rich phase (*P*), citrate-rich phase (*C*), or initial stock (*i*) and *T* refers to the titer of the virus or VLP in either phases or initial stock. PPV titer was expressed as MTT_{50}/mL. HIV-VLP titers were expressed as relative fluorescent unit (RFU)/0.1 mL, as measured with a Synergy Mx microplate reader (BioTek, Winoski, VT) at an excitation wavelength of 485 ± 9 nm and emission wavelength 509 ± 9 nm with 80% sensitivity and 10 mm probe offset in black 96-well plates (VWR).

Protein partitioning qualification by SDS-PAGE

The contaminant protein partitioning was detected using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 4–12% Bis-Tris NuPage gel and NuPage MOPS running buffer (Life Technologies, Carlsbad, CA). SeeBlue Plus2 pre-stained protein ladder (molecular weight range: 14–191 kDa, Life Technologies) was used as the marker. Samples were reduced with 7 v/v% β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO) and heated to 90 °C for 10 minutes prior to loading onto the gel. The SDS-PAGE was run for 55 min at 200 V. The gel was loaded at constant volume and was stained with the SimplyBlue Safe stain (Life Technologies, Carlsbad, CA) as per the manufacturer's protocol.

DNA Quantification

Host cell dsDNA content was measured using the Quant-iT™ PicoGreen® dsDNA kit (ThermoFisher Scientific, Waltham, MA) as per the manufacturer's protocol. The PEG-rich

phase and citrate-rich phase were diluted 10-fold in water to reduce the interference with the assay. The fluorescence (excitation: 480 nm and emission: 520 nm) of the samples were measured using a Synergy™ Mx microplate reader. The DNA recoveries were calculated using equation 3

$$\%\ DNA\ Recovery = \frac{V_{P/C} * DNA\ concentration_{P/C} * DF}{V_i * DNA\ concentration_{P/C} * DF} * 100 \quad (3)$$

where *V* denotes volume of respective phases and *DF* denotes respective dilution factor.

Statistical analysis

Statistical analysis was performed using an unpaired, two tailed Student's t test. An asterisk (*) denotes $p < 0.05$ between samples. The outliers for the PPV recoveries were calculated using 1.5*Interquartile Range method and were not used in the analysis.

3 Results

3.1 Effect of osmolytes on two-phase behavior

Both protecting and denaturing osmolytes were used in this study to modulate the partitioning of viruses in ATPS. Protecting osmolytes change the water structure in the solution due to their strong solvation ability, denoted by the hydration number. The classification and characteristic hydration number of osmolytes in the concentration range used in this study is listed in **Table 1**. The osmolytes affected the two-phase systems by changing the threshold compositions of immiscibility and/or altering the equilibrium phase compositions.

Binodal curves generated with the addition of 0.5 M osmolyte in the PEG-citrate system changed the two-phase formation. The addition of osmoprotectants shifted the binodal curve towards the PEG axis, whereas the denaturant urea did not show considerable change in the two-phase region (**Figure 1A**). The incompatibility of the phases is driven by the salting-out ability of the salt-rich phase towards the ethylene oxide groups in the PEG chains { ADDIN EN.CITE

<EndNote><Cite><Author>Hey</Author><Year>2005</Year><RecNum>240</RecNum><DisplayText>(Hey<style face="italic"> et al.</style>, 2005)</DisplayText><record><rec-number>240</rec-number><foreign-keys><key app="EN" db-id="a0addzpwcr5x5ee55tyvxpprx05efws59sex" timestamp="1531663505">240</key><key app="ENWeb" db-id="">0</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Hey, Michael J.</author><author>Jackson, Daniel P.</author><author>Yan, Hong</author></authors></contributors><titles><title>The salting-out effect and phase separation in aqueous solutions of electrolytes and poly(ethylene glycol)</title><secondary-title>Polymer</secondary-title></titles><periodical><full-title>Polymer</full-title></periodical><pages>2567-2572</pages><volume>46</volume><number>8</number><section>2567</section><dates><year>2005</year></dates><isbn>00323861</isbn><urls></urls><electronic-resource-num>10.1016/j.polymer.2005.02.019</electronic-resource-num></record></Cite></EndNote>. The binodal shift caused by the osmoprotectants likely indicated an improved salting-out ability of the citrate-rich phase. The lack of binodal shift for urea-containing system might be due to the low urea concentration used in this study to permit significant changes in the phase behavior { ADDIN EN.CITE <EndNote><Cite><Author>Rämsch</Author><Year>1999</Year><RecNum>627</RecNum><DisplayText>(Rämsch<style face="italic"> et al.</style>, 1999)</DisplayText><record><rec-number>627</rec-number><foreign-keys><key app="EN" db-id="a0addzpwcr5x5ee55tyvxpprx05efws59sex" timestamp="1582654989">627</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Rämsch, Christian</author><author>Kleinelanghorst,

Lutz B.</author><author>Knieps, Esther A.</author><author>Thömmes, Jörg</author><author>Kula, Maria-Regina</author></authors></contributors><titles><title>Aqueous Two-Phase Systems Containing Urea: Influence on Phase Separation and Stabilization of Protein Conformation by Phase Components</title><secondary-title>Biotechnology Progress</secondary-title></titles><periodical><full-title>Biotechnology Progress</full-title></periodical><pages>493-499</pages><volume>15</volume><number>3</number><dates><year>1999</year><pub-dates><date>1999/01/01</date></pub-dates></dates><publisher>American Chemical Society (ACS)</publisher><isbn>8756-7938</isbn><urls><related-urls><url>https://doi.org/10.1021/bp990030+</url></related-urls></urls><electronic-resource-num>10.1021/bp990030+</electronic-resource-num><access-date>2020/02/25</access-date></record></Cite></EndNote>}. Overall, glycine showed a maximum change in the binodal curve followed by similar changes by betaine and TMAO (**Figure 1E**).

The other important characteristic of two-phase systems are tie-lines, representing the set of systems at thermodynamic equilibrium with identical final phase compositions. The slope of a tie-line connects the equilibrium global system compositions. The change in the TL slopes due to addition of osmolytes is compared to the osmolyte-free system in **Table 2** and shown in **Figures 1 B-D**. Osmoprotectants betaine and TMAO showed a negative change in the TL slope, while glycine showed an insignificant change as compared to the osmolyte-free system. The denaturant urea demonstrated a positive change in the slope, but the changes in the final phase compositions were insignificant. Overall, TMAO showed a maximum change in the TL slope followed by betaine, glycine, and urea (**Figure 1E**).

The three osmoprotectants demonstrated a prominent change in the PEG-rich concentration, but minimal changes in the citrate-rich concentrations (**Figure 1B-D**). To understand the effect of osmolytes on the phase composition modulation and the interaction of viruses and proteins with the phases, the osmolytes were quantified in the citate-rich phase by LC-MS. The LC-MS profiles for each osmolyte and their isotopes are shown in **Figure S1 and S2**. Osmoprotectants preferred the citrate-rich phase, whereas the urea highly partitioned to the PEG-rich phase (**Figure 2A**). More than 90% of glycine was recovered in the citrate-rich phase at all TLLs. Glycine partitioning to the citrate-rich phase shifted the binodal curve towards the PEG axis and increased the length of tie-lines, without affecting the tie-line slope, as compared to the osmolyte-free system (**Figure 1**). ~60% recovery was observed for both betaine and TMAO in the citrate-rich phase at the lowest TLL and increased to 80% recovery at the higher TLLs (**Figure 2B**). Only 40% of urea was recovered in the citrate-rich phase at all TLLs. Overall, the degree of osmolyte partitioning to the citrate-rich phase corresponded to the shift of binodal curve away from the osmolyte-free system.

3.2 Virus and VLP

Two viruses - porcine parvovirus (PPV) and human immunodeficiency virus-group specific antigens virus-like particles (HIV-VLP) were used to determine the effect of osmolytes on the partitioning within ATPS. The structural and physiochemical characteristics of PPV and HIV-VLPs are compiled in **Table 3**. PPV is a non-enveloped, infectious virus that is comprised of three capsomeres VP1, VP2, and VP3, assembled in an icosahedral geometry { ADDIN EN.CITE <EndNote><Cite><Author>Simpson</Author><Year>2002</Year><RecNum>228</RecNum><DisplayText>(Simpson<style face="italic"> et al.</style>, 2002)</DisplayText><record><rec-

number>228</rec-number><foreign-keys><key app="EN" db-id="a0addzpwcr5x5ee55tyvxpprx05efws59sex" timestamp="1531663478">228</key><key app="ENWeb" db-id="">0</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Simpson, A. A.</author><author>Hebert, B.</author><author>Sullivan, G. M.</author><author>Parrish, C. R.</author><author>Zadori, Z.</author><author>Tijssen, P.</author><author>Rossmann, M. G.</author></authors></contributors><auth-address>Department of Biological Sciences, Lilly Hall of Life Sciences, Purdue University, West Lafayette, IN 47907-1392, USA.</auth-address><titles><title>The structure of porcine parvovirus: comparison with related viruses</title><secondary-title>J Mol Biol</secondary-title></titles><periodical><full-title>J Mol Biol</full-title></periodical><pages>1189-98</pages><volume>315</volume><number>5</number><edition>2002/02/06</edition><keywords><keyword>Amino Acid Sequence</keyword><keyword>Capsid/*chemistry</keyword><keyword>Crystallography, X-Ray</keyword><keyword>Feline Panleukopenia Virus/chemistry</keyword><keyword>Minute Virus of Mice/chemistry</keyword><keyword>Models, Molecular</keyword><keyword>Molecular Sequence Data</keyword><keyword>Parvovirus, Porcine/*chemistry</keyword><keyword>Phylogeny</keyword><keyword>Protein Structure, Quaternary</keyword><keyword>Protein Subunits</keyword><keyword>Sequence Alignment</keyword></keywords><dates><year>2002</year><pub-dates><date>Feb 1</date></pub-dates></dates><isbn>0022-2836 (Print)
0022-2836 (Linking)</isbn><accession-num>11827486</accession-num><urls><related-urls><url>https://www.ncbi.nlm.nih.gov/pubmed/11827486</url></related-

urls></urls><electronic-resource-num>10.1006/jmbi.2001.5319</electronic-resource-num></record></Cite></EndNote>}. HIV-VLP is an enveloped, non-infectious, VLP comprised of gag polyproteins with green fluorescent protein (GFP) embedded and enclosed in the envelope derived from *S. cerevisiae* cell membrane { ADDIN EN.CITE <EndNote><Cite><Author>Sakuragi</Author><Year>2002</Year><RecNum>623</RecNum><DisplayText>(Sakuragi<style face="italic"> et al.</style>, 2002)</DisplayText><record><rec-number>623</rec-number><foreign-keys><key app="EN" db-id="a0addzpwcr5x5ee55tyvxpprx05efws59sex" timestamp="1582043522">623</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Sakuragi, Sayuri</author><author>Goto, Toshiyuki</author><author>Sano, Kouichi</author><author>Morikawa, Yuko</author></authors></contributors><titles><title><style face="normal" font="default" size="100%">HIV type 1 Gag virus-like particle budding from spheroplasts of </style><style face="italic" font="default" size="100%">Saccharomyces cerevisiae</style></title><secondary-title>Proceedings of the National Academy of Sciences</secondary-title></titles><periodical><full-title>Proceedings of the National Academy of Sciences</full-title></periodical><pages>7956</pages><volume>99</volume><number>12</number><dates><year>2002</year></dates><urls><related-urls><url>http://www.pnas.org/content/99/12/7956.abstract</url></related-urls></urls><electronic-resource-num>10.1073/pnas.082281199</electronic-resource-num></record></Cite></EndNote>}. Enveloped viruses are prone to structural loses in the bioprocessing due to the presence of a fragile and semi-fluid lipid bilayer. These two models

were used to determine the osmolyte action to modulate the partitioning of both non-enveloped and enveloped virus/VLP in the PEG-citrate system.

3.3 Influence of osmolyte class on virus partitioning at similar global composition

Initially, three global compositions having similar osmolyte concentrations were studied to understand the efficacy of different osmolyte classes to modulate virus partitioning in the PEG-citrate system at pH 7. Glycine had most significantly altered the binodal curve as compared to the other osmolytes used (**Figure 1A**), indicating elevated driving forces for phase separation. The improved driving forces in the glycine system drove PPV to the PEG-rich phase (**Figure 3**). Glycine-containing systems were able to enhance the PPV partitioning at the lowest composition studied, yielding 96 ± 106 % PPV recovery in the PEG-rich phase as compared to 0 ± 0% with the osmolyte-free systems (**Figure S3A**). Note: the recovery error is often high when expressed in a linear system due to the titration assay being measured on a log scale. The two higher glycine-containing systems compositions both yielded >100% recovery of PPV and showed a significant increase in partition coefficient. On the other hand, betaine, which had a similar binodal shift as glycine, showed a similar partitioning trend as glycine (**Figure 3A**). However, the PPV recovery in the PEG-rich phase was low at the lowest TLL (**Figure S3A)**. The partitioning and recovery improved at higher TLLs (**Figure 3A** and **Figure S3A)**. TMAO, which partitioned to the citrate-rich phase, and urea that partitioned to the PEG-rich phase, had a small to no binodal shift (**Figure 1 and 2**) and both had similar PPV partitioning as the control system (**Figure 3**). Since all of the osmolytes, except urea, partitioned to the salt-rich phase, the osmolyte addition would not affect subsequent downstream processing of the virus from the PEG-rich phase.

A similar study was performed to determine the effect of osmolytes on the enveloped HIV-VLP. At the lowest system composition, the glycine-containing system marginally increased the partition coefficient and betaine and TMAO reduced the partition coefficient as compared to the control (**Figure 3B**). The partition coefficient elevated at the mid-system composition containing glycine, betaine, and TMAO (**Figure 3B and Figure S3C**). The highest system composition containing glycine demonstrated an increase in the partition coefficient as compared to the osmolyte-free system but yielded only an 83 ± 7% recovery (**Figure 3B and Figure S3C**). On the other hand, betaine and TMAO-containing systems did not show any significant differences in the partition coefficients (**Figure 3B**).

3.4 Influence of osmolyte class on protein and HC-DNA partitioning

As glycine and betaine had demonstrated the most improvement in the virus partitioning, host cell proteins (HCP) and host cell DNA (HC-DNA) were tracked only in these systems. The HCP were only detected in the citrate-rich phase, except at the highest TLL in which one band at 51 kDa appeared in the PEG-rich phase (**Figure 4A & B**). Also, all the contaminant proteins from HIV-VLP crude partitioned to the citrate-rich phase (data not shown).

The PEG-rich phase excluded most of HC-DNA when osmolytes were added (**Figure 4C&D**). Glycine-containing systems achieved 87 ± 10 % and 67 ± 2% DNA removal at the lowest two TLLs, whereas betaine containing systems achieved 91 ± 1% and 89 ± 1% removal, respectively. At the highest TLL, glycine enhanced the HC-DNA recovery in the PEG-rich phase (**Figure 4C**), but 61 ± 3% removal was still achieved. Betaine-containing system achieved a 83 ± 11% DNA removal at the highest TLL. Most of the DNA was recovered in the interface for all the systems suggesting the negatively charged DNA was salted-out but was not uptaken in the PEG-rich phase. Overall, the lower TLLs with lower PEG and citrate compositions achieved a

high yield and purification of the viruses in the PEG-rich phases when glycine or betaine were used as additives.

3.5 Effect of tie-line framework on virus partitioning

Tie line ratios guide the volume ratio of the phases and may change the partitioning mechanism. At high TL ratios, the PEG phase disperses in the bulk salt phase, causing PEG droplets to come into contact with the bulk salt phase during mixing of the phases and transport of the viral particles from one phase to another. However, the system characteristics change at the phase inversion point, where the dispersed phase changes from the salt-rich phase to PEG-rich phase as the TL ratio decreases { ADDIN EN.CITE <EndNote><Cite><Author>Merchuk</Author><Year>1998</Year><RecNum>99</RecNum><DisplayText>(Merchuk<style face="italic"> et al.</style>, 1998)</DisplayText><record><rec-number>99</rec-number><foreign-keys><key app="EN" db-id="a0addzpwcr5x5ee55tyvxpprx05efws59sex" timestamp="1531662868">99</key><key app="ENWeb" db-id="">0</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Merchuk, J. C.</author><author>Andrews, B. A.</author><author>Asenjo, J. A.</author></authors></contributors><titles><title>Aquoeus two phase system for protein separation studies on phase inversion</title><secondary-title>Journal of Chromatography B</secondary-title></titles><periodical><full-title>Journal of Chromatography B</full-title></periodical><pages>285-293</pages><volume>711</volume><number>1-2</number><dates><year>1998</year></dates><urls></urls></record></Cite></EndNote>}. The phase inversion dictates if the viral particles directly partition to the bulk PEG-rich phase or co-accumulates with the dispersed PEG-rich phase. We studied the effect of PPV partitioning at

different TL ratios to evaluate the phase inversion effect. The partition coefficient of PPV increased with the increasing TL ratio at lower TLLs and remained constant at the highest TLL (**Figure 5,** recoveries are shown in **Figure S4**). This likely indicates that at the lower TL ratio, where the citrate-rich phase is the dispersed phase, there is no salting out of the viral particles. The increase in the TL ratio, where the PEG-rich phase is the dispersed phase, resulted in a comparatively higher partition coefficient. PPV still partitioned to the citrate-rich phase at the lowest TLL. However, more PPV was in the PEG-rich phase than the citrate-rich phase at the mid-TLL, which likely indicates that the salting-out effect caused the viral particles to partition at the interface and coalition of the PEG-rich phase droplets collected the viral particles from the interface. At the highest TLL studied, PPV likely exhibited hydrophobic interaction with the PEG-rich phase as the partition coefficients were high. The PPV partitioning was high in the glycine-containing and betaine-containing systems as compared to the osmolyte-free systems at all TLLs (**Figure 5B&C**). However, the PPV partitioned to the citrate-rich phase at lower TLL in the betaine-containing systems. The addition of TMAO did not help the PPV partitioning (**Figure 5D**). Interestingly, urea containing systems demonstrated similar trend as osmoprotectants (**Figure 5E**). However, the partition coefficients were lower than glycine and betaine, but close to TMAO (**Figure 5B-E**). The overall trend of PPV partition coefficients implies that the osmolytes provide consistent partitioning of viral particles across the tie line, which is contrary to the osmolyte-free systems.

The effect of osmolytes on the interaction of PPV and phases is too complex to just be explained by the partition coefficients. Glycine containing systems yielded high PPV titers in the PEG-rich phase at all the TLL and TL ratio yielding high recoveries (**Table 4 and Figure S4**). However, the titers in the citrate-rich phase dropped with the increasing TLL. A similar trend

was observed for systems with betaine, except the PPV titers in the PEG-rich phase were lower than with glycine. On the other hand, both TMAO and urea-containing systems demonstrated an increasing PPV titer with increasing TLLs, but the titers were much lower than with glycine and betaine.

3.6 Effect of pH on virus partitioning

Surface charge of the viruses plays a vital role in the paritioning between the PEG-rich and citrate-rich phases. Negatively charged viruses experience repulsion effects by citrate ions, causing the salting-out effect. Similar repulsion effect is observed on the DNA by the citrate-rich phase (**Figure 4C&D**). The repulsion decreases at the isoelectric point of the virus, causing the majority of viruses to partition to the citrate-rich phase { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. PPV has a pI near 5, thus it has a net neutral charge at that pH { ADDIN EN.CITE

<EndNote><Cite><Author>Mi</Author><Year>2020</Year><RecNum>628</RecNum><DisplayText>(Mi<style face="italic"> et al.</style>, 2020)</DisplayText><record><rec-number>628</rec-number><foreign-keys><key app="EN" db-id="a0addzpwcr5x5ee55tyvxpprx05efws59sex" timestamp="1579829082">628</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Mi, Xue</author><author>Bromley, Emily K.</author><author>Joshi, Pratik U.</author><author>Long, Fei</author><author>Heldt, Caryn L.</author></authors></contributors><titles><title>Virus Isoelectric Point Determination Using Single-Particle Chemical Force Microscopy</title><secondary-title>Langmuir</secondary-title></titles><periodical><full-title>Langmuir</full-title></periodical><pages>370-378</pages><volume>36</volume><number>1</number><dates><year>2020</year><pub-

dates><date>2020/01/14</date></pub-dates></dates><publisher>American Chemical Society</publisher><isbn>0743-7463</isbn><urls><related-urls><url>https://doi.org/10.1021/acs.langmuir.9b03070</url></related-urls></urls><electronic-resource-num>10.1021/acs.langmuir.9b03070</electronic-resource-num></record></Cite></EndNote>}. The systems containing the osmolytes glycine, betaine, or TMAO at pH 5 seem to have no effect on the interaction between PPV and the PEG-rich phase, as the systems infused with either of these osmolytes resulted in similar partitioning (**Figure 6**). However, an increase in the TLL increased the PPV partitioning, which is likely due to an increase in the ionic strength of the citrate-rich phase. This is a preliminary, yet significant observation, and more studies need to be performed to fully understand the effect of osmolytes on biomolecules at their pI.

4 Discussion

Viral vaccines and biotherapeutics have a variety of molecular designs and vary greatly in physicochemical properties. One of the most significant differences is the presence or absence of a lipid bilayer envelope. Enveloped viruses are more prone to structural disintegration in harsh processing conditions than are non-enveloped viruses. Our ATPS processing strategy using osmolytes worked for both an infectious, non-enveloped virus and an enveloped VLP. The addition of osmolytes lowered the system compositions required to salt-out viruses in the studied ATPS and increased the partitioning to the PEG-rich phase, thereby alleviating the high salt concentration dependency to transport viruses from the citrate-rich phase to the PEG-rich phase (**Figure S3**). Osmoprotectants boosted the favorable partitioning of virus at lower ionic strength system compositions. This avoided high ionic strength conditions, which are known to be a factor that can cause envelope disruption. The osmolyte addition could help in bridging the

processing gaps of enveloped and non-enveloped viruses' purification without affecting the structural integrity of enveloped viruses.

The partitioning of biomolecules in the PEG-salt systems is suggested to be highly affected by the balance of electrostatic and/or hydrophobic interactions { ADDIN EN.CITE <EndNote><Cite><Author>Asenjo</Author><Year>2011</Year><RecNum>77</RecNum><DisplayText>(Asenjo & Andrews, 2011)</DisplayText><record><rec-number>77</rec-number><foreign-keys><key app="EN" db-id="a0addzpwcr5x5ee55tyvxpprx05efws59sex" timestamp="1531661784">77</key><key app="ENWeb" db-id="">0</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Asenjo, J. A.</author><author>Andrews, B. A.</author></authors></contributors><auth-address>Centre for Biochemical Engineering and Biotechnology, Department of Chemical Engineering and Biotechnology, Institute for Cell Dynamics and Biotechnology: A Centre for Systems Biology, University of Chile, Beauchef 850, Santiago, Chile. juasenjo@ing.uchile.cl</auth-address><titles><title>Aqueous two-phase systems for protein separation: a perspective</title><secondary-title>J Chromatogr A</secondary-title></titles><periodical><full-title>J Chromatogr A</full-title></periodical><pages>8826-35</pages><volume>1218</volume><number>49</number><keywords><keyword>Chemical Fractionation/*methods</keyword><keyword>Hydrophobic and Hydrophilic Interactions</keyword><keyword>Polyethylene Glycols</keyword><keyword>Proteins/chemistry/*isolation & purification</keyword><keyword>Salts</keyword><keyword>Water</keyword></keywords><dates><year>2011</year><pub-dates><date>Dec 09</date></pub-dates></dates><isbn>1873-3778 (Electronic)
0021-9673 (Linking)</isbn><accession-num>21752387</accession-

num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/21752387</url></related-urls></urls><electronic-resource-num>10.1016/j.chroma.2011.06.051</electronic-resource-num></record></Cite></EndNote>}. The goal of this study was to improve the virus recovery in the PEG-rich phase at lower TLL, thus lower ATPS compositions, where high recoveries were not achieved without additives. Osmolytes are known to modulate solvent properties and induce hydrophobic interactions in aqueous solutions { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. Changes in electrostatic and hydrophobic interactions can result in either intraparticle or interparticle interactions. The flexible smaller proteins are known to undergo conformational changes (intraparticle interactions) in the presence of osmoprotectants { ADDIN EN.CITE { ADDIN EN.CITE.DATA }} and comparatively rigid viruses have been shown to flocculate (interparticle interactions) { ADDIN EN.CITE { ADDIN EN.CITE.DATA }} in the presence of similar osmolytes. In this study, the osmolytes induced interfacial interactions between virus particles and the PEG-rich phase to improve virus recovery. However, the addition of osmolytes affected only the viruses and not the contaminating proteins, thus providing a highly selective separation. It is likely that the osmolytes only affected the conformation of proteins, and not the partitioning.

The mechanism of protecting osmolytes glycine, betaine, and TMAO to stabilize the proteins structures and flocculate viruses is still unclear. In one study, the stabilizing effect of glycine and betaine disrupted the hydrogen bond network of elastin-like polypeptide with water { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. The changes in the water structure were caused by osmolytes partitioning away from the polypeptide. On the other hand, TMAO was observed to favorably interact with the backbone of a collapsed protein and a hydrophobic homopolymer { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. Among the three osmoprotectants, TMAO was

least recovered in the citrate-rich phase and this might indicate an interaction with the globular PEG molecules in the PEG-rich phase. Moreover, in this study, glycine and betaine (in that order) likely induced hydrophobic interactions between the virus models and the PEG-rich phase to improve the partition coefficient (**Figure 3**). TMAO did not seem to improve the hydrophobic interaction, as previously shown { ADDIN EN.CITE <EndNote><Cite><Author>Athawale</Author><Year>2005</Year><RecNum>631</RecNum><DisplayText>(Athawale<style face="italic"> et al.</style>, 2005)</DisplayText><record><rec-number>631</rec-number><foreign-keys><key app="EN" db-id="a0addzpwcr5x5ee55tyvxpprx05efws59sex" timestamp="1583368747">631</key><key app="ENWeb" db-id="">0</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Athawale, M. V.</author><author>Dordick, J. S.</author><author>Garde, S.</author></authors></contributors><auth-address>The Howard P. Isermann Department of Chemical & Biological Engineering, Rensselaer Polytechnic Institute, Troy, New York 12180, USA.</auth-address><titles><title>Osmolyte trimethylamine-N-oxide does not affect the strength of hydrophobic interactions: origin of osmolyte compatibility</title><secondary-title>Biophys J</secondary-title></titles><periodical><full-title>Biophys J</full-title></periodical><pages>858-66</pages><volume>89</volume><number>2</number><edition>2005/05/17</edition><keywords><keyword>Computer Simulation</keyword><keyword>Hydrophobic and Hydrophilic Interactions</keyword><keyword>Methylamines/*chemistry</keyword><keyword>*Models, Chemical</keyword><keyword>*Models, Molecular</keyword><keyword>Molecular Conformation</keyword><keyword>Osmosis</keyword><keyword>Polymers/*chemistry</keyword><keyword>Solubility</keyword><keyword>Solutions</keyword><keyword>Solvents/ch

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5 Conclusion

This study reports the use of osmolytes to modify the partitioning behavior of PPV and HIV-VLP. Glycine followed by betaine, among the zwitterionic osmolytes, demonstrated an increase in the salting-out ability of the citrate-rich phase and an increase in the phase hydrophobicity of the PEG-rich phase. The elevated driving forces improved the recoveries of viruses at lower TLLs, where the systems without osmolytes are insufficient to induce preferential virus partitioning. TMAO increased the ability of two-phase formation at lower system compositions, but it did not improve the virus recovery. ~100% of PPV and 90% of HIV-VLP were recovered using glycine at the lowest TLL studies. The order of efficiency of osmolytes to enhance the driving forces was determined to be glycine > betaine > TMAO > urea.

High recovery and purity of viral modalities in the PEG-rich phase implies that osmolyte addition enhances the interfacial interactions for comparatively hydrophobic and rigid viruses as compared to the intramolecular interactions of flexible proteins. The HC-DNA partitioned to the citrate-rich phase at low TLL but increased towards the PEG-rich at higher TLL in presence of glycine and betaine. This study provides evidence that osmolyte addition improves the viral recovery irrespective of the expression system. Moreover, this study showed that the effect of

osmolytes on virus is drastically different than protein, which is essential to develop a robust and versatile downstream process for viral particle manufacturing.

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Conflict of Interest

D.O'H. and S.N. have financial interest in Esperovax. The other authors declare no conflict of interest.

Author Contributions

PUJ: Conceptualization, Data Curation, Formal Analysis, Methodology, Investigation, Validation, Visualization, Writing – Original Draft, Writing – Review & Editing, DGT: Investigation, MS: Investigation, BJ: Investigation, AL: Investigation, SK: Investigation, MK: Formal Analysis, Methodology, Investigation, DOH: Resources, SN: Resources, CLH: Conceptualization, Funding Acquisition, Project Administration, Supervision, Writing – Review & Editing

Data Availability

Data available upon request

References

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Table 1. Osmolytes and their characteristics

Osmolyte	Class	Molecular mass (g/mol)	Hydration number (Concentration, mol/L)*	References
Glycine	Amino acid	75..07	7	{ ADDIN EN.CITE <EndNote><Cite><Author>Parsons</Author><Year>2005</Year><RecNum>1116</RecNum><DisplayText>(Parsons & Koga, 2005)</DisplayText><record><rec-number>1116</rec-number><foreign-keys><key app="EN" db-id="a0addzpwcr5x5ee55tyvxpprx05efws59sex" timestamp="1612822295">1116</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Parsons, Matthew T.</author><author>Koga, Yoshikata</author></authors></contributors><titles><title>Hydration number of glycine in aqueous solution: an experimental estimate</title><secondary-title>The Journal of chemical physics</secondary-title></titles><periodical><full-title>The Journal of chemical physics</full-title></periodical><pages>234504</pages><volume>123</volume><number>23</n

Betaine	Modified amino acid	117.15	31 (0.1 - 1)	umber><dates><year>2005</year></dates><publisher>American Institute of Physics</publisher><isbn>0021-9606</isbn><urls></urls></record></Cite></EndNote> } { ADDIN EN.CITE <EndNote><Cite><Author>Shikata</Author><Year>2002</Year><RecNum>1117</RecNum><DisplayText>(Shikata, 2002)</DisplayText><record><rec-number>1117</rec-number><foreign-keys><key app="EN" db-id="a0addzpwcr5x5ee55tyvxpprx05efws59sex" timestamp="1612822393">1117</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Shikata, Toshiyuki</author></authors></contributors><titles><title>Dielectric relaxation behavior of glycine betaine in aqueous solution</title><secondary-title>The Journal of Physical Chemistry A</secondary-title></titles><periodical><full-title>The Journal of Physical Chemistry A</full-title></periodical><pages>7664-7670</pages><volume>106</volume><number>34</number><dates><year>2002</year></dates><publisher>ACS Publications</publisher><is

TMAO	Amine oxide	75.11	22.6 - 23 (0.9 - 0.46)	bn>1089-5639</isbn><urls></urls></record></Cite></EndNote> } { ADDIN EN.CITE <EndNote><Cite><Author>Fedotova</Author><Year>2017</Year><RecNum>1118</RecNum><DisplayText>(Fedotova<style face="italic"> et al.</style>, 2017)</DisplayText><record><rec-number>1118</rec-number><foreign-keys><key app="EN" db-id="a0addzpwcr5x5ee55tyvxpprx05efws59sex" timestamp="1612822649">1118</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Fedotova, Marina V.</author><author>Kruchinin, Sergey E.</author><author>Chuev, Gennady N.</author></authors></contributors><titles><title>Hydration structure of osmolyte TMAO: concentration/pressure-induced response</title><secondary-title>New Journal of Chemistry</secondary-title></titles><periodical><full-title>New Journal of Chemistry</full-title></periodical><pages>1219-1228</pages><volume>41</volume><number>3</number><dates><year>2017</year></dates><publisher>Ro

Urea	Carbamide	60.06	~1 - 1.8 (0.5 - 1)	yal Society of Chemistry</publisher><urls></urls></record></Cite></EndNote>} { ADDIN EN.CITE <EndNote><Cite><Author>Agieienko</Author><Year>2016</Year><RecNum>1119</RecNum><DisplayText>(Agieienko & Buchner, 2016)</DisplayText><record><rec-number>1119</rec-number><foreign-keys><key app="EN" db-id="a0addzpwcr5x5ee55tyvxpprx05efws59sex" timestamp="1612822738">1119</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Agieienko, Vira</author><author>Buchner, Richard</author></authors></contributors><titles><title>Urea hydration from dielectric relaxation spectroscopy: old findings confirmed, new insights gained</title><secondary-title>Physical Chemistry Chemical Physics</secondary-title></titles><periodical><full-title>Physical Chemistry Chemical Physics</full-title></periodical><pages>2597-2607</pages><volume>18</volume><number>4</number><dates><year>2016</year></dates><publisher>Royal Society of

Chemistry</publisher><urls
></urls></record></Cite></
EndNote>}

*Values in parenthesis denote the range of concentrations for which the hydration numbers were determined

Table 2. Changes in the TLL and TL slope with addition of osmolytes

Osmolyte	Δ TLL	Average TL Slope	Δ TL Slope	t-test values of Δ TL Slope
	(w/w%)	Deg (rad)	Deg (rad)	*p*
-		-1.48 ± 0.05		
Glycine	10.4	-1.54 ± 0.12	-0.06	0.4688
Betaine	9.5	-1.95 ± 0.18	-0.47	0.0121
TMAO	7.8	-2.23 ± 0.11	-0.75	0.0004
Urea	2.3	-1.33 ± 0.07	+0.16	0.0392

Δ values are respect to the osmolyte-free systems

Table 3. Biophysical and biochemical characteristics of the study virus/VLP

Model	Species	family	Parent cell type	pI	Size (nm)	genome	Envelope	Reference
Porcine parvovirus	Virus	Parvoviridae	Mammalian	5	18-26	ssDNA	No	{ ADDIN EN.CITE { ADDIN EN.CITE .DATA }}
HIV- VLP	Virus-like particles	Retroviridae (Original virus)	Yeast	N.A.	80-120	-	Yes	

Table 4. Average of PPV titers across TL ratio and TLL in osmolyte-containing ATPS. All titer values are represented as $\log_{10}(MTT_{50}/mL)$ measured using MTT assay.

Glycine		
TLL	**PEG-rich Phase**	**Cit-rich Phase**
Low	7.6 ± 0.5	6.8 ± 0.4
Mid	7.9 ± 0.3	5.7 ± 0.4
High	8.1 ± 0.2	4.2 ± 1.0

Betaine		
TLL	**PEG-rich Phase**	**Cit-rich Phase**
Low	7.1 ± 0.5	7.8 ± 0.4
Mid	7.7 ± 0.3	5.7 ± 0.5
High	7.6± 0.2	4.8 ± 0.3

TMAO		
TLL	**PEG-rich Phase**	**Cit-rich Phase**
Low	5.0 ± 0.4	7.4 ± 0.2
Mid	6.8 ± 0.4	5.6 ± 0.5
High	6.9 ± 0.3	4.9 ± 0.3

Urea		
TLL	**PEG-rich Phase**	**Cit-rich Phase**
Low	5.4 ± 0.9	7.3 ± 0.5
Mid	6.3 ± 0.7	5.1 ± 0.5
High	6.6 ± 0.4	3.5 ± 0.9

Figure Legends

Figure 1. (A) Binodal curves and (B, C, D) tie lines for PEG 12kDa – citrate systems in presence of various 0.5 M osmolytes. Denaturing osmolyte – (C) urea and protecting osmolytes – (C) TMAO, (D) betaine, and glycine. (E) Schematic changes induced by osmolytes in the binodal curve and tie lines as compared to the osmolyte-free systems.

Figure 2. Partitioning of the osmolytes at common compositions of ATPS with increasing TLL represented as (A) ln (K) and (B) % recovery in the corresponding citrate-rich phases.

Figure 3. Effect of different osmolytes on (A) PPV and (B) HIV-gag VLP partitioning. Biomolecule partitioning upon addition of protecting (glycine, betaine, and TMAO) and non-protecting (urea) osmolytes are compared to osmolyte-free (control) system. Experiments were done in triplicate and the error bars represent the standard deviation. p-value was determined using Student's t test at 0.95 confidence interval. * denotes a $p < 0.05$ as compared to the control at that composition.

Figure 4. Contaminant protein (A & B) and HC-DNA (C & D) recoveries from the crude PPV stock in the two-phases at varying TLL in presence of 0.5 M glycine or betaine. Contaminant protein partitioning were visualized on Coomassie stained SDS-PAGE and HC-DNA were quantified using Quant-iTTM PicoGreen® dsDNA assay. The error bars represent standard deviations from the experiments done in triplicate.

Figure 5. Partition coefficient of PPV at different TL and TLR in (A) osmolyte-free system compared with systems added with osmolytes (B) 0.5 M glycine (C) 0.5 M betaine (D) 0.5 M TMAO (E) 0.5 M urea. The averages represent results from three to six experiments and the error bars represent the standard deviation from the respective experiments.

Figure 6. PPV partitioning at varying TLL in presence of (A) 0.5 M glycine, (B) 0.5 M betaine, and (C) TMAO maintained at pH 5 and 7. The error bars represents standard deviation of experiments done in triplicate.

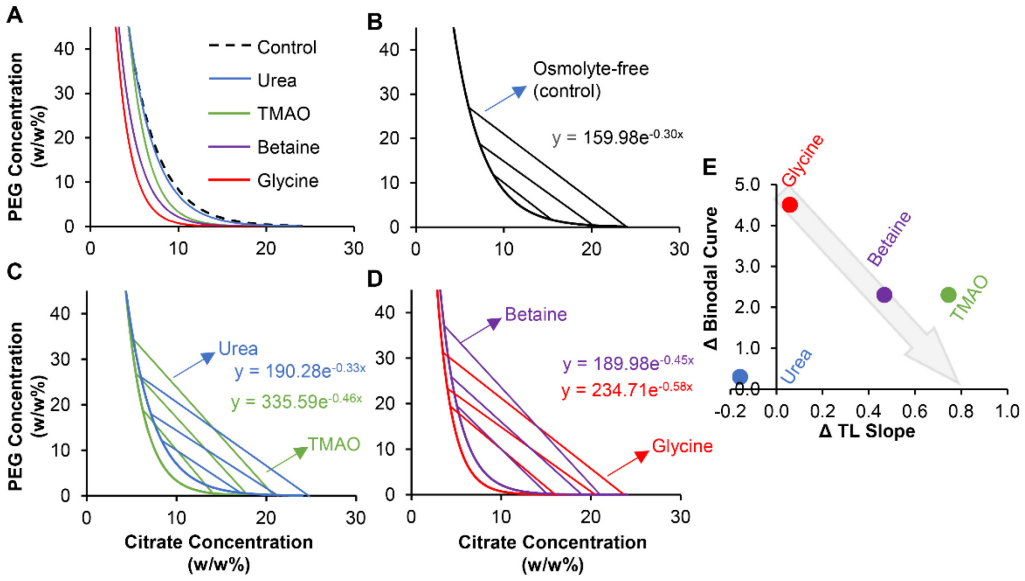
A
PEG Concentration (w/w%)
Control
Urea
TMAO
Betaine
Glycine
B
Osmolyte-free (control)
y = 159.98e-0.30x
C
Urea
y = 190.28e-0.33x
y = 335.59e-0.46x
TMAO
Citrate Concentration (w/w%)
D
Betaine
y = 189.98e-0.45x
y = 234.71e-0.58x
Glycine
Citrate Concentration (w/w%)
E
Glycine
Betaine
TMAO
Urea
Δ Binodal Curve
Δ TL Slope

Figure 1

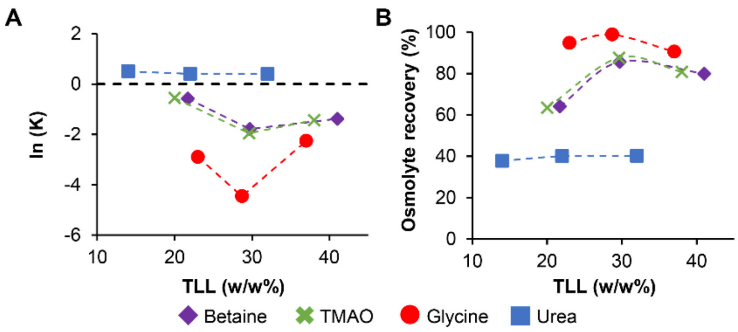
A
B
ln (K)
Osmolyte recovery (%)
TLL (w/w%)
TLL (w/w%)
Betaine
TMAO
Glycine
Urea

Figure 2

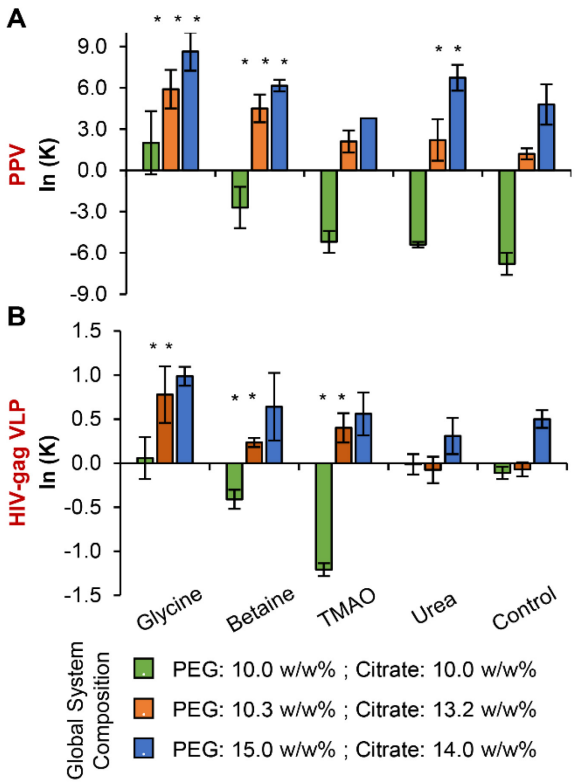
A
PPV
ln (K)
9.0
6.0
3.0
0.0
-3.0
-6.0
-9.0
B
HIV-gag VLP
ln (K)
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
Glycine
Betaine
TMAO
Urea
Control
Global System Composition
PEG: 10.0 w/w% ; Citrate: 10.0 w/w%
PEG: 10.3 w/w% ; Citrate: 13.2 w/w%
PEG: 15.0 w/w% ; Citrate: 14.0 w/w%

Figure 3

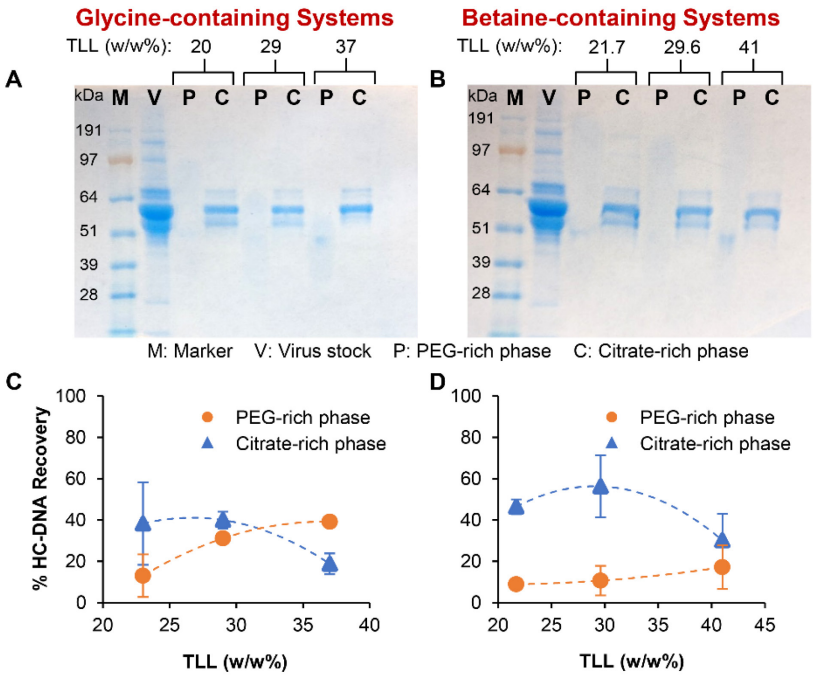
Glycine-containing Systems
Betaine-containing Systems
A
B
TLL (w/w%): 20 29 37
TLL (w/w%): 21.7 29.6 41
kDa M V P C P C P C
191
97
64
51
39
28
M: Marker V: Virus stock P: PEG-rich phase C: Citrate-rich phase
C
D
% HC-DNA Recovery
PEG-rich phase
Citrate-rich phase
TLL (w/w%)

Figure 4

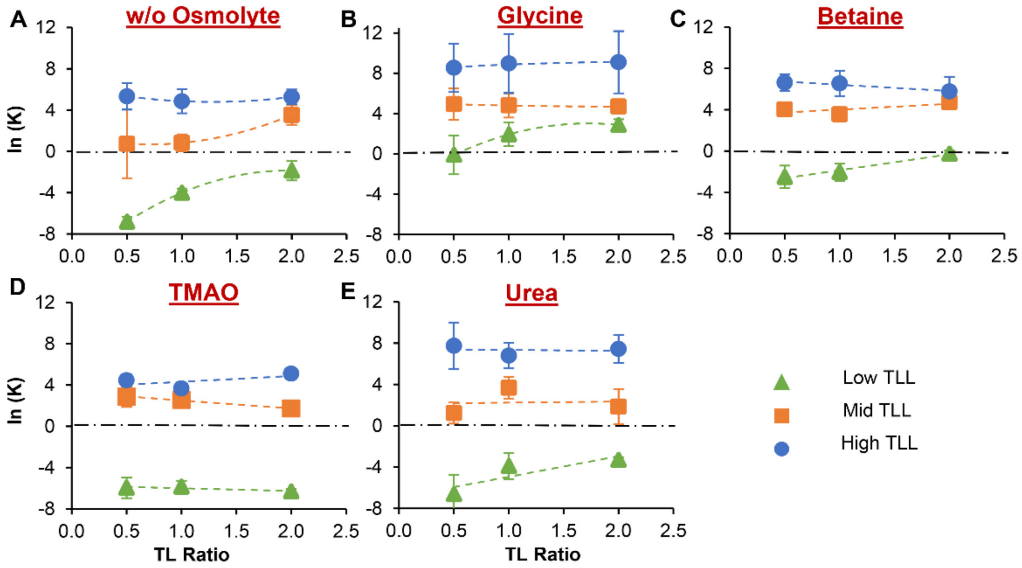
A
w/o Osmolyte
B
Glycine
C
Betaine
D
TMAO
E
Urea
ln (K)
TL Ratio
Low TLL
Mid TLL
High TLL

Figure 5

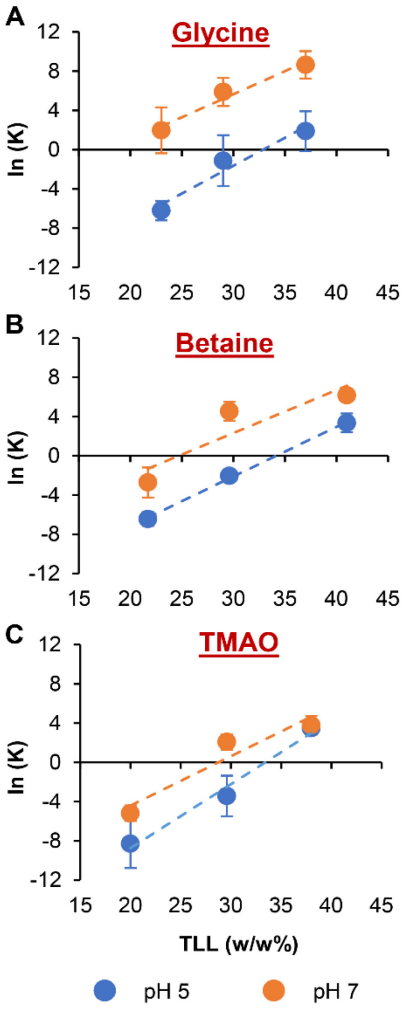
A
Glycine
B
Betaine
C
TMAO
ln (K)
12
8
4
0
-4
-8
-12
15
20
25
30
35
40
45
TLL (w/w%)
pH 5
pH 7

Figure 6