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PURIFICATION AND EFFECTIVENESS OF VACCINES AND ANTIVIRAL COMPOUNDS

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To my family; Aytug, Adriana and Jose, I would not have made it without your encouragement and love

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Preface

Chapters 3, 4 and 6 of this dissertation have previously been published in peer-reviewed journals, and are reproduced here with the permission of their respective publishers. I was the first author in all of the articles, I have done most of the experimental work presented in Chapter 3 and 4, and half of the experimental work presented in Chapter 6.

In Chapter 3, the majority of the experimental work, the collection of data and the writing of the paper were performed by myself. The immunohistochemistry experiments and analysis were performed by K. Saagar Vijayaragavan. Dr. Caryn Heldt, my advisor guided me through the process of performing the experimental work and in writing the publication. The experimental work presented in Chapter 4 was done by myself under the supervision of my advisor. I wrote the paper with the feedback of my advisor. The work presented in Chapter 6 was a collaborative research project with Dr. Dipendu Saha at Widener University and Dr. Jihua Chen and Dale K. Hensley at Oak Ridge National Laboratory (ORNL). Dr. Saha's undergrad students synthesized the soft-templated mesopoporous carbon materials used in this work, and the ORNL group, Dr. Chen and Mr. Hensley characterized the materials. I performed all the biocompability studies that are reported in this Chapter, under the supervision of my advisor. Dr. Saha wrote the majority of the publication, but Dr. Heldt and myself wrote the sections related to the biocompability studies. Chapter 5 is in preparation for submission as a communication paper. I have done all the experimental work and the data analysis in this chapter, under the supervision of my advisor. I wrote all the contents of Chapter 5, thanks to the corrections and suggestions of my advisor.

Proofs of permission from the publishers of the articles reproduced in these chapters, and figures reproduced in Chapter 2 can be found in Appendix A.

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List of abbreviations

Viruses

B19.	Human parvovirus B19
CMV.	Cytomegalovirus
DENV.	Dengue virus
EBOV.	Ebola virus
EV71.	Enterovirus 71
FCV.	Feline calicivirus
HAV.	Hepatitis A virus
HBV.	Hepatitis B virus
HCMV.	Human cytomegalovirus (HCMV)
HCV.	Hepatitis C virus
HIV.	Human immunodeficiency virus
HSV.	Herpes simplex virus
HSV-1.	Herpes simplex virus type 1
HSV-2.	Herpes simplex virus type 2
IFV-A.	Influenza virus type A
IFV-B.	Influenza virus type B
MNV.	Murine norovirus
NDV-LS.	Newcastle disease virus, strain LaSota
PoV.	Poliovirus
PPV.	Porcine parvovirus
SVHR.	Sindbis virus, heat resistant strain
RHV.	Rhinovirus
RSV.	Respiratory syncytial virus
VZV.	Varicella zoster virus
YFV.	Yellow fever virus

Others

A. SUL.	Ammonium sulfate
AC.	Affinity chromatography
AIDS.	Acquired immune deficiency syndrome
ALA.	D-alanine
ARG.	D-arginine
ATP.	Adenosine triphosphate
BET.	Betaine (Chapter 4 & 5)
BET.	Brunauer–Emmett–Teller (Chapter 6)
BHK-21.	Baby hamster kidney cell line
BSA.	Bovine serum albumin
Caco-2.	Human colon carcinoma cell line
CNTs.	Carbon nanotubes
CPE.	Cytopathic effect
DAPI.	4′,6-diamidino-2-phenylindole dihydrochloride
DLC.	Diamond-like carbon
DNA.	Deoxyribonucleic acid
DSP.	Downstream processes
EDXS.	Energy-dispersive X-ray spectroscopy
EGCG.	Epigallocatechin gallate
EPA.	Environmental Protection Agency
F36.	5,7,4'-trihydroxy-8-methoxyflavone
FE-SEM.	Cold-field emission scanning electron microscope

FBS.	Fetal bovine serum
FDA.	Food and Drug Administration
FIB.	Fibrinogen
GLY.	Glycine
H_2O.	Water
HCl.	Hydrochloric acid
HBsAg.	Hepatitis B virus surface antigen
HCP.	Host cell protein
HeLa.	Cervical cancer cell line
HIC.	Hydrophobic-interaction chromatography
HMDS.	1,1,1,3,3,3-Hexamethyldisilazane
HAS.	Human serum albumin
I.	Ionic strength
IC50.	The half maximal inhibitory concentration
IEC.	Ion exchange chromatography
IgG.	Immunoglobulin G
ISCOMs.	Immune-stimulating complexes
IRB.	Internal Review Board
KB.	Nasopharyngeal epiclermal carcinoma cell line
LRV.	Log reduction value
M. CHL.	Magnesium chloride
M. SUL.	Magnesium sulfate
MAN.	D-mannitol
MEM.	Minimum essential medium
MERS.	Middle East respiratory syndrome
MOFs.	Metal-organic frameworks
MTT.	Thiazolyl blue tetrazolium bromide
MTT_{50}.	The 50% infectious dose
MW.	Molecular weight
MWCO.	Molecular weight cut-off
NaCl.	Sodium chloride (in text)
NaOH.	Sodium hydroxide
NLDFT.	Nonlocal density functional theory
NNRTIs.	Non-nucleoside reverse transcriptase inhibitors
NRTIs.	Nucleoside reverse transcriptase inhibitors
NtRTIs.	Nucleotide reverse transcriptase inhibitors
PBS.	Phosphate-buffered saline
PEG.	Poly(ethylene) glycol
Pen/Strep.	Penicillin/streptomycin
pI.	Isoelectric point
PK-13.	Porcine kidney cell line
PRO.	L-proline
RAF.	D-(+)-raffinose pentahydrate
RBC.	Red blood cell
RNA.	Ribonucleic acid
ROS.	Reactive oxygen species
RT.	Reverse transcriptase
RV.	Resveratrol
SARS.	Severe acute respiratory syndrome
S. CHL.	Sodium chloride (in figure)
SEC.	Size exclusion chromatography
SDS.	Sodium dodecyl sulfate
SEM.	Scanning electron microscope
SER.	L-serine
SSA.	Specific surface area

SUC.	Sucrose
TEM.	Transmission electron microscopic
TF-3.	Theaflavin-3,3-digallate
TPB.	Tryptose phosphate broth
TFF.	Tangential flow filtration
TMAO.	Trimethylamine N-oxide
TRE.	(+)-Trehalose dehydrate
TRIS.	Tris(hydroxymethyl)aminomethane hydrochloride
USP.	Upstream processes
VLPs.	Virus-like particles
WHO.	World Health Organization

Abstract

Viral infections account for over 13 millions deaths per year. Antiviral drugs and vaccines are the most effective method to treat viral diseases. Antiviral compounds have revolutionized the treatment of AIDS, and reduced the mortality rate. However, this disease still causes a large number of deaths in developing countries that lack these types of drugs. Vaccination is the most effective method to treat viral disease, vaccines prevent around 2.5 millions deaths per year. Vaccines are not able to offer full coverage due to high operational costs in the manufacturing processes. Although vaccines have saved millions of lives, conventional vaccines often offer reactogenic effects. New technologies have been created to eliminate the undesired side effects. However, new vaccines are less immunogenic and adjuvants such as vaccine delivery vehicles are required.

This work focuses on the discovery of new natural antivirals that can reduce the high cost and side effects of synthetic drugs. We discovered that two osmolytes, trimethylamine N-oxide (TMAO) and glycine reduce the infectivity of a model virus, porcine parvovirus (PPV), by 4 LRV (99.99%), likely by disruption of capsid assembly. These osmolytes have the potential to be used as drugs, since they showed antiviral activity after 20 h. We have also focused on improving current vaccine manufacturing processes that will allow fast, effective and economical vaccines to be produced worldwide. We propose virus flocculation in osmolytes followed by microfiltration as an economical alternative for vaccine manufacturing. Osmolytes are able to specifically flocculate hydrophobic virus particles by depleting a hydration layer around the particles and subsequently cause virus aggregation. The osmolyte mannitol was able to flocculate virus particles, and demonstrate a high virus removal, 81% for PPV and 98.1% for Sindbis virus (SVHR). Virus flocculation with mannitol, followed by microfiltration could be used as a platform process for virus purification. Finally, we perform biocompatibility studies on soft-templated mesoporous carbon materials with the aim of using these materials as vaccine delivery vehicles. We discovered that these materials are biocompatible, and the degree of biocompatibility is within the range of other biomaterials currently employed in biomedical applications.

Chapter 1

Introduction and overview

1.1. Introduction

Over the last century, viral diseases such as acquired immune deficiency syndrome (AIDS), influenza, poliomyelitis (polio), and smallpox have affected millions of people. The most effective methods to combat viral infections are with antiviral drugs or vaccines. Synthetic antiviral drugs (i.e antiretrovirals) for AIDS have reduced the mortality rate by 40% from 1998 to 2013 [1]. However, the disease still causes a large number of deaths in nations that lack of these types of drugs [2]. The World Heath Organization (WHO) projects an increase in mortality of 7% from 2015 to 2030 [3]. The discovery of new, novel and natural antiviral compounds could be used to treat several viral diseases and reduce the high cost and severe side effects of synthetic antiviral compounds. In this study, our aim was to use natural antiviral compounds to reduce the infectivity of viruses.

Vaccines are the most successful method to control and eradicate viral diseases. Smallpox was one the most devastating disease to humans during the past century [4] with 300-500 millions deaths in the 20th century [5]. The virus was eradicated in 1980 following a global immunization campaign led by the WHO [6]. Polio is a crippling and potentially fatal infectious disease that does not have a cure and has taken the lives of millions of people [7]. The polio vaccine was discovered in 1955 [8], and thanks to its wide spread acceptance, the WHO declared the virus eradicated from the Americas and Europe in 1996 [9]. However, with 293 cases of polio being reported in 2013 in Pakistan, Afghanistan, Nigeria and Somalia [10], there is still more work to be done to duplicate the results that vaccination had on smallpox. Eradication of polio will require less expensive vaccines and a concerted effort for distribution. We have focused on improving current vaccine manufacturing processes that will allow fast, effective and economical vaccines to be produced for countries around the world. Developing and emerging economy countries are starting to developed their own vaccines [11], inexpensive manufacturing processes will allow all nations to have access to vaccines. In this work, our aim was to use virus flocculation followed by microfiltration as an economical alternative for vaccine manufacturing.

Vaccines not only need to induce a specific immune response, they also need to be safe

[12]. Conventional vaccines often have reactogenic effects. New technologies are safer and less reactogenic than conventional vaccines, but they are also less immunogenic [13-16]. In order to improve the immune responses in new technologies, vaccines require boosting agents called adjuvants. Adjuvants such as vaccine delivery vehicles can be used to encapsulate or surround the vaccine formulation [13, 17, 18]. These vehicles are able to target the immune cells and incorporate an adequate dose, so that booster doses are not necessary [17, 19]. In this work, we performed biocompatibility studies on soft-templated mesoporous carbon materials with the aim of using these materials as vaccine delivery vehicles.

1.2. Overview

In the studies covered in this dissertation, we worked with osmolytes as antiviral compounds and virus flocculants for vaccine manufacturing. We also worked with soft-templated mesoporous carbon materials, as future candidates for vaccine delivery vehicles. Osmolytes are natural compounds found in cells, and their main function is to stabilize intracellular proteins against environmental stress and maintain cell volume. They achieve these functions by preferential interactions with water molecules. In this study we proposed to use osmolytes as antiviral compound and as virus flocculants.

Soft-templated mesoporous carbon material is a new type of biomaterial. They are made through a soft template method, which includes the self-assembly of surfactant micelles into a carbon precursor matrix. Soft-templated-mesoporous carbon material are of great interest in different applications, such as separations, catalysis and energy storage, due to their large pore accessibility and the tunability of the pore structure. In this work, we proposed that soft-templated mesoporous carbon materials can be used for another application, future vehicles for vaccine delivery.

This dissertation begins with **Chapter 2,** where we will discuss the current knowledge of the interaction of osmolytes and soft-templated mesoporous carbons with biological materials. In the first part of the chapter, we will discuss the differences and advantages of natural antiviral compounds over synthetic compounds. In the second part of the chapter, we will discuss the

upstream processes (USP) and downstream processes (DSP) of the vaccine manufacturing processes. We focus on the DSP, since high costs of production are connected to the main units operations that are used in this stage. We will discuss the limitation of current unit operations and propose alternative unit operations for virus purification. Towards the end of the chapter we will discuss the benefits of vaccine delivery vehicles and the different types of vehicles, including soft-templated mesoporous carbons.

In **Chapter 3**, we demonstrate that two osmolytes, glycine and trimethylamine N-oxide (TMAO) have the ability to disrupt the virus infection cycle of a model virus, porcine parvovirus (PPV). Glycine and TMAO reduce the infectivity of PPV by 4 logs (99.99%). We believe that these two osmolytes disrupt the assembly of the PPV capsid by stabilizing capsid proteins and preventing them from assembling. The advantage of these osmolytes is that they can be applied post-infection, since they disrupt late stages in the virus infection cycle. This work has been published in the Journal of Antiviral Research.

We also use osmolytes with another purpose, as flocculants for virus purification. We took advantage of the fact that osmolytes change the water structure near proteins to induce a preferential flocculation. In **Chapters 4 & 5,** a variety of different types of osmolytes, including sugars, sugar alcohols and amino acids, were found to flocculate the non-enveloped virus PPV and the enveloped Sindbis virus (SVHR), and demonstrate a high percent removal with a 0.2 μm filter. One of our best osmolytes, mannitol at 0.3 M, was able to achieve 98% for SVHR and 81% for PPV. Microfiltration (0.1 to 10 μm), which is usually for bacterial removal; will very likely reduce costs as compared to typical ultrafiltration (0.01 to 0.1 μm) used for virus removal since a large pore-size filter would increase the flux and decrease the transmembrane pressure, as well as the high costs of membrane materials. Virus flocculation followed by microfiltration could be used as a global method for virus purification for enveloped and non-enveloped viruses, and could replace current expensive unit operations. The findings on PPV flocculation have been published in the Journal of Biotechnology and the discoveries on SHRV flocculation are being prepared for publication.

Chapter 6 focuses on the biocompatibility studies of soft-template mesoporous carbon

materials. *In vitro* studies showed that soft-templated mesoporous carbon materials are not toxic, are biocompatible with mammalian cells, and the degree of biocompatibility is within the range or higher than other biomaterials currently employed in biomedical applications. These tests are the first step in material biocompatibility before advancing to animal testing and clinical trials. Based on our biocompatibility results, we propose that soft-templated mesoporous carbon materials could be used as vaccine delivery vehicles. The material in this chapter has been published in the Journal of ACS Applied Materials & Interfaces.

Finally, **Chapter 7** outlines the conclusions of the research presented in this dissertation and discusses the future direction of the research.

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Chapter 2

Literature review

2.1. Literature review

Since the beginning of humankind viral infections have taken the life of millions of people. The number of people who have died from viral diseases exceed the number of deaths caused by war and all other diseases combined [1]. In an effort to understand and cure viral diseases, scientists have studied the structure and function of different viruses. The knowledge that we have gained in medical virology has led to the discovery of treatments to combat viral infections: antiviral drugs and vaccines. Antiviral drugs, such as Zovirax ® (acyclovir), which inhibit the infectivity of the herpes simplex virus type-1 (HSV-1) and type-2 (HSV-2), Epivir ® (lamivudine), which reduce the infectivity of hepatitis B virus (HBV), and Norvir ® (ritonavir), which interfere with the replication of the human immunodeficiency virus (HIV), have improved the quality of life of millions of people affected with these viral infections [2]. In addition to antiviral drugs, viral vaccines have been used to control yellow fever, measles, and mumps and have even eradicated smallpox and polio [3]. However, some viral diseases, such as dengue fever, and ebola hemorrhagic fever lack a vaccine or drug against infection. Treatment of these diseases is primarily focused on relieving the symptoms [4, 5]. The lack of effective vaccines can be attributed to the inability of vaccine candidates to evoke an appropriate immune response and to the high costs involved in the manufacturing process [6, 7]. An improvement in current manufacturing process and vaccine delivery systems is needed in order to achieve a highly pure product and to induce a strong immune response against the viral antigen.

2.2. Viruses and vaccines

Virus particles are composed of genetic material (either DNA or RNA) and surrounded by multiple copies of identical protein subunits, which form the capsid of the virus. The subunits can be arranged in two types of capsid structure. One type shows helical symmetry and the other type shows icosahedral symmetry [8]. Some viruses possess a lipid bilayer that surrounds the capsid, and they are known as enveloped viruses. The lipid bilayer is obtained after the virus is released through the cellular membrane. Other viruses, which are known as non-enveloped viruses, do not possess this lipid bilayer; the capsid proteins assemble into a strong shell that

protects the genetic material [9]. Diseases caused by enveloped viruses include AIDS (caused by HIV) and ebola hemorrhagic fever (Ebola virus (EBOV)). There is no cure or vaccine available for these types of virus infections, and contraction of them can be deadly. Diseases caused by non-enveloped viruses include the common cold and acute gastroenteritis, most commonly caused by the rhinovirus (RHV) and the norovirus, respectively. These viruses are rarely deadly, as is common for non-enveloped viruses. However, these types of virus infections can be fatal to young children and immunocompromised patients [10-12]. Consequently there is still a need for the discovery of antiviral compounds and vaccines that can fight a variety of virus infections, which include enveloped and non-enveloped viruses.

Viral vaccines are the most effective method to prevent viral infections [13]. Currently, vaccines prevent around 2.5 millions deaths per year [14]. Edward Jenner created the first vaccine to become available in the 1790s. He found that the infection of cowpox protected humans from the smallpox infection [15, 16]. After the smallpox vaccine, several other vaccines became available, including the rabies vaccine in 1885, the yellow fever vaccine in 1935 and the polio vaccine in 1962 [17]. Although effective vaccines have been developed for several viral diseases, there are some viral infections that have shown to be resilient to vaccine therapy, such as HIV, and hepatitis C virus (HCV) [18-20]. Worldwide, infectious diseases account for over 13 million deaths every year [13]. At the end of 2013, 35 million people had HIV, 2.1 million people became infected and 1.5 million died for AIDS-related causes, according to the World Health Organization (WHO) [21]. Other viral pathogens, like adenovirus, rhinovirus, human herpesviruses, and all the haemorrhagic fever viruses also lack any type of vaccines [18]. Additionally, new viruses are emerging and causing outbreaks around the word, such as severe acute respiratory syndrome (SARS) in 2004, influenza A H1N1 virus in 2009, and the Middle East respiratory syndrome (MERS) in 2012. Millions of more lives could be saved with improved access to current vaccines and the creations of new vaccines.

There are three types of vaccines licensed for use in humans: live-attenuated, inactivated or killed, and subunit vaccines [17, 22-24]. First-generation antiviral vaccines were made through live animals, like cow or rabbits [16]. In the middle of the twentieth century, virus growth was

adapted to cell culture. Virologists learnt that attenuation could be achieved by several culture passages [16, 17, 25]. Viral vaccines, such as the oral polio vaccine in 1963, the measles vaccine in 1963, the mumps vaccine in 1967, and the varicella vaccine (i.e. chicken pox) in 1995 were all made through attenuation of viruses in cell culture [26-29]. Another great discovery in the 20th century was that immunogenicity could be obtained if the pathogen was inactivated either by heat or chemical treatment [17, 25]. Vaccines, like influenza in 1936 and hepatitis A (HAV) in 1996, contain whole inactivated virus [30, 31]. Finally, subunit vaccines, such as HBV [32] and the human papilloma [33], are generated by expressing viral capsid proteins [17, 25]. Today, several inactivated and live-attenuated vaccines are propagated through cell culture. Although, cell culture-derived vaccines has helped to fulfill the demand of pandemics viral diseases and the increment of vaccines supplies, there are still viral infections, like EBOV and HIV without any type of vaccines offered, likely due to the fact that viral vaccines are difficult and expensive to develop. In this study, we propose to use virus flocculation with osmolytes, followed by microfiltration as an economical purification step in order to reduce the costs of current unit operations used in vaccine manufacturing. At the same time we found out that osmolytes can interfere with the replication cycle of infection virus particles and can be used as potential candidates for antiviral compounds.

2.3. Osmolytes

Osmolytes are natural, organic compounds that are found in the cells of many organisms [34-36] and their main function is to stabilize intracellular proteins against environmental stresses, such as extreme temperatures or high osmotic pressure by changing the water content of the cells [37]. There are two types of osmolytes, protecting and denaturing osmolytes. Protecting osmolytes fold proteins by structuring water and changing the water content around the protein backbone. Denaturing osmolytes have the opposite effect; they unfold the proteins by binding directly to the protein backbone (**Figure 2.1**) [38]. A balance between protecting and denaturing osmolytes assists in the delicate equilibrium needed for protein stabilization in nature [39].

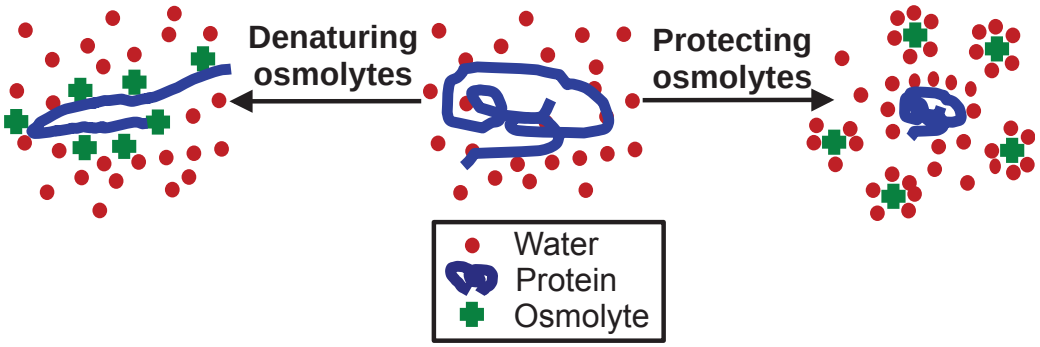

Figure 2.1. Protecting and denaturing osmolytes. Protecting osmolytes have the ability to fold proteins, by structuring water. Denaturing osmolytes have the ability to bind to the backbone of proteins, leading to protein unfolding.

The behavior of protecting and denaturing osmolytes with regard to proteins in solution can be explained with the preferential interaction theory. Protecting osmolytes have attractive interactions ($-\Delta G$) with water molecules, but unfavorable interactions with the protein backbone ($+\Delta G$). The preferential interaction between the protein backbone and water molecules is known as the preferential exclusion of solute. The preferential exclusion of solute orders water molecules in a thermodynamically unfavorable way, which causes protein folding in order to reduce the increase in entropy [40]. The interaction between denaturing osmolytes and protein backbone is known as a favorable preferential interaction [37]. Denaturing osmolytes appear to bind to the protein backbone by hydrogen bonds and they have repulsive interactions with water molecules [40].

The phenomena of protecting and denaturing osmolytes in solution with proteins have also been explained with a solvophobic/solvophilic effect. This theory suggests that the preferential exclusion of the protecting osmolyte can be explained as a solvophobia effect of the protein surface for the hydrated osmolyte as compared to water [37]. On the other hand, the favorable preferential interaction of denaturing osmolytes with water molecules can be explained as a solvophilic effect, since interactions between the denaturing osmolyte and proteins are more favorable than interactions between water and proteins [37].

Scientists have also measured the transfer free energy ($\Delta G_{transfer}$) in order to understand the interactions between the protein backbone and osmolytes. Street and coworkers measured

the free energy change of the backbone of the protein from water to 1 M osmolyte solutions [38]. They showed that protecting osmolytes have an increased change in the Gibbs free energy. This is likely because protecting osmolytes interact unfavorably with the protein, resulting in a preferential depletion from the protein backbone. On the other hand, denaturing osmolytes decreased the change in Gibbs free energy by interacting favorably with the unfolded state of the proteins, resulting in preferential binding of the osmolyte to the protein backbone [38]. It is likely that the protein backbone transfer free energy primarily determines if an osmolyte stabilizes or destabilizes a protein in solution.

We would like to take advantage of the behavior of protecting osmolytes in protein solutions and also of the fact that virus capsids are made of proteins. We propose to use osmolytes as antiviral compounds against viruses (**Chapter 3**), and as flocculants in order to improve virus purification techniques for vaccine production (**Chapter 4 & 5**). Protecting osmolytes have the ability to rearrange water molecules near the surface of the protein and to be excluded from the protein surface. They can fold and consequently stabilize the protein. Protecting osmolytes are potential candidates for antiviral compounds, since they could potentially stabilize viral capsid proteins and prevent them from assembling into infectious virus particles. On the other hand, protecting osmolytes can be used as flocculants. The osmolyte flocculants are able to deplete a hydration layer around the hydrophobic virus particles and thus cause virus aggregation. As osmolyte flocculant concentration is increased, more water molecules are removed from the capsid virus proteins, causing virus aggregation and flocculation. Osmolytes flocculation has many advantages. Osmolytes are often used as excipients to stabilize protein final formulations, and therefore this method only adds compounds that are already known to be safe for injection. Flocculation allows for larger pore-sized membranes to be used during purification, which decreases costs by lowering transmembrane pressures and reducing fouling.

2.4. Antiviral Compounds

Antiviral compounds have revolutionized the treatment of some viral infections that

cannot be controlled with vaccines, such as, HCV HIV, and HSV 1 & 2 [41, 42]. The first antiviral compound, acyclovir, was released in the 1970s. Scientists found that the compound could inhibit DNA replication of HSV-1 & 2 at concentrations that did not affect cellular DNA synthesis [43, 44]. During the last two decades, many antiviral compounds have been approved; most of them are antiretrovirals that are used to treat HIV infections [2, 45, 46], allowing people to live longer with the virus. Antiviral compounds are classified either into synthetic or natural. The aim in the development of effective antiviral compounds is to find new antiviral compounds, which can target the virus or its replication, without interfering with any metabolic cellular process [19]. Molecular biology has greatly accelerated our knowledge in understanding the virus replication cycle and will continue to aid in the discovery of new biological targets for antiviral compounds.

2.4.1. Synthetic antiviral compounds

Many synthetic antiviral drugs are currently approved for clinical use in the treatment of viral infections caused by HIV, HBV, HCV, HSV 1 & 2, and influenza virus type A & B (IFV-A & B) [41]. Viruses share similar stages in their replication cycle (see **Figure 2.2**), starting with adsorption and penetration into the host cells. Then, the genetic material is uncoated inside the cell. Small viruses use the host cell's replication machinery to produce nucleic acids and viral proteins, whereas larger viruses have these proteins encoded in their genome. Then virus particles are assembled and the virus progeny are released from the cell [19, 47].

Synthetic compounds that have been approved have been found to inhibit virus infection by different mechanisms of actions (**Table 2.1 & Figure 2.2**). The first stage in the virus infection cycle involves attachment of virus to host cells. After adsorption, virus particles can enter the cells either by fusion or endocytosis. Enfuvirtide and palivizumaba have been approved as virus-cell fusion inhibitor against HIV and respiratory syncytial virus (RSV) infections, respectively [48, 49]. Uncoating is an important step in the virus replication cycle, since the genetic material of the virus is release inside of the cells. Antiviral compounds that interfere with this stage have been found against the IFV-A virus [50].

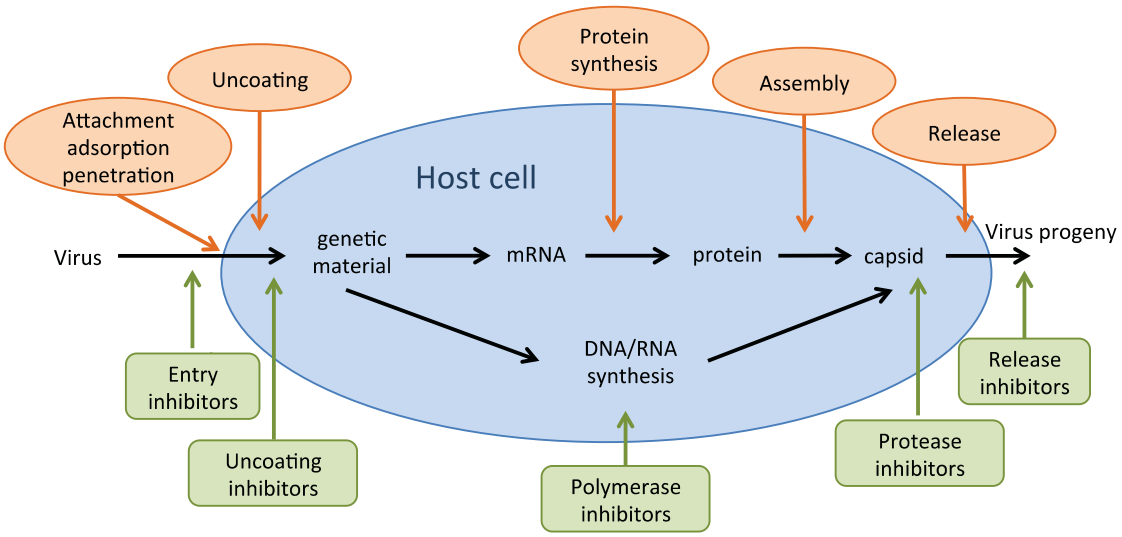

Figure 2.2. Virus infection cycle. The virus infection cycle starts with virus attachment to susceptible cells, followed by penetration and adsorption. In the uncoating step, the genetic material is released inside of the cells. Small viruses use the host cell's replication machinery to produce nucleic acids and viral proteins, whereas larger viruses have these proteins encoded in their genome. The virus particles are then assembled and the new virions are released out of the cell. Image adapted from [51].

Numerous viruses synthesize their own viral enzymes, such as DNA polymerase, RNA polymerase and retroviruses require reverse transcriptase (RT) to synthesize their nucleic acids [2]. Antiviral compounds that target these enzymes are of great interest, since these polymerases are required for the replication of the virus and are unique to viruses [52]. Nucleoside reverse transcriptase inhibitors (NRTIs), nucleotide reverse transcriptase inhibitors (NtRTIs), and non-nucleoside reverse transcriptase inhibitors (NNRTIs) are viral polymerase inhibitors approved for HIV infections [46]. NRTIs and NtRTIs are known as competitive subtract inhibitors. They block the RT activity, by acting as chain terminators and preventing completion of viral DNA synthesis [41]. NNRTIs have a different mode of action, they bind to an allosteric pocket in the p66 subunit of the RT and prevent DNA synthesis by a non-competitive mechanism of action [2]. HIV viral polymerase inhibitors are used in different combinations and doses to suppress the HIV infection and stop the progression of the disease. Protease inhibitors, such as ritonavir against HIV and telaprevir against HCV are drugs used to block viral protease activity [2, 18, 41, 46, 53].

Table 2.1. Synthetic antiviral compounds approved in the market

Antiviral drug	Target virus	Capsid	Mechanism of action	Reference
Enfuvirtide	HIV	E	Entry inhibitor	[2, 41, 48]
Maraviroc	HIV	E	Entry inhibitor	[2, 54]
Palivizumaba	RSV	E	Entry inhibitor	[2, 49]
Amantadine	IFV-A	E	Uncoating inhibitors	[2, 41, 50]
Rimantadine	IFV-A	E	Uncoating inhibitors	[2, 41, 50]
Acyclovir	HSV-1 & 2	E	DNA polymerase inhibitors	[2, 18, 41, 55]
Famciclovir	HSV-1 & 2	E	DNA polymerase inhibitors	[2, 18, 41, 55]
Valaciclovir	HSV-1 & 2	E	DNA polymerase inhibitors	[2, 18, 41, 55]
Ganciclovir	CMV	E	DNA polymerase inhibitors	[2, 18, 41]
Valganciclovir	CMV	E	DNA polymerase inhibitors	[2, 18, 41]
Brivudine	HSV-1 & 2	E	DNA polymerase inhibitors	[2, 18, 41]
Foscarnet	HSV-1 & 2	E	DNA polymerase inhibitors	[2, 18, 41]
Penciclovir	HSV-1 & 2	E	DNA polymerase inhibitors	[18, 41]
Cidofovir	CMV	E	DNA polymerase inhibitors	[2, 18, 41]
Trifluridine	HSV-1 & 2	E	Disrupt DNA replication	[41]
Idoxuridine	HSV-1 & 2	E	Disrupt DNA replication	[41]
Fomivirsen	CMV	E	Block translation of mRNA	[41]
Abacavir	HIV	E	NRTIs	[2, 18, 41, 46]
Didanosine	HIV	E	NRTIs	[2, 18, 41, 46]
Emtricitabine	HIV	E	NRTIs	[2, 41, 46]
Stavudine	HIV	E	NRTIs	[2, 18, 41, 46]
Zalcitabine	HIV	E	NRTIs	[2, 18, 41, 46]
Zidovudine	HIV	E	NRTIs	[2, 18, 41, 46]
Lamivudine	HIV & HBV	E	NRTIs	[2, 18, 41, 46]
Entecavir	HBV	E	NRTIs	[2]
Telbivudine	HBV	E	NRTIs	[2]
Adefovir	HBV	E	NRTIs	[2]
Tenofovir	HIV & HBV	E	NRTIs	[2, 18, 41, 46]
Efavirenz	HIV	E	NNRTIs	[2, 18, 41, 46]
Etravirine	HIV	E	NNRTIs	[2, 46]
Delavirdine	HIV	E	NNRTIs	[2, 18, 41, 46]
Nevirapine	HIV	E	NNRTIs	[2, 18, 41, 46]
Rilpivirine	HIV	E	NNRTIs	[2, 46]
Ribavirin	HCV & RSV	E	RNA inhibitor	[2, 18, 41]
Raltegravir	HIV	E	Integrase inhibitor	[2, 46]
Amprenavir	HIV	E	Protease inhibitors	[2, 18, 41, 46]
Atazanavir	HIV	E	Protease inhibitors	[2, 41, 46]
Fosamprenavir	HIV	E	Protease inhibitors	[2, 46]
Indinavir	HIV	E	Protease inhibitors	[2, 18, 41, 46]
Lopinavir	HIV	E	Protease inhibitors	[2, 18, 41, 46]
Nelfinavir	HIV	E	Protease inhibitors	[2, 18, 41, 46]
Ritonavir	HIV	E	Protease inhibitors	[2, 18, 41, 46]
Saquinavir	HIV	E	Protease inhibitors	[2, 18, 41, 46]
Darunavir	HIV	E	Protease inhibitors	[2, 46]
Tipranavir	HIV	E	Protease inhibitors	[2, 46]
Boceprevir	HCV	E	Protease inhibitors	[2, 56]
Telaprevir	HCV	E	Protease inhibitors	[2, 53]
Oseltamivir	IFV-A & B	E	Neuraminidase inhibitors	[2, 18, 41, 57]
Peramivir	IFV-A & B	E	Neuraminidase inhibitors	[2]
Zanamivir	IFV-A & B	E	Neuraminidase inhibitors	[2, 18, 41, 58]

Viral proteases are important in the replication of the virus, since they catalyze the process of viral polyproteins production and in the processing of making precapsids [2]. The last step in the virus infection cycle is release of virus progeny out of the cells. Some compounds

have been found to inhibit this stage, by acting on the viral proteins involved in this step. Oseltamivir and Zanamivir inhibit the influenza neuraminidase activity, protein that enables the virus to be released from the cells [41]. Both of them have been approved for the treatment of IFV-A & B infection [57, 58].

Although synthetic antiviral compounds have been shown to treat viral diseases, some of these antivirals have been shown to present severe side effects. Acyclovir, which is one of the most commonly used medications to treat HSV 1 & 2, causes confusion and ataxia [59] and also kidney failure [60]. The main side effect of ribavirin, a drug used to treat HCV, is haemolytic anemia [61]. NRTIs have revolutionized the treatment of AIDS, however, high active doses coincide with mitochondrial toxicity, which can lead to serious sides effects such as hepatic failure and lactic acidosis [62]. The ineffectiveness of current treatments for several viral diseases and the fast grow of new drug-resistant viral strains have pressed researchers for the discovery of new, natural antiviral compounds [20]. Researchers are now interested in natural products, which can often ameliorate the severe side effects of synthetic drugs [19]. Such natural products can be obtained more easily [63]; consequently, these products are inexpensive as compared to synthetic compounds [64]. Moreover, viruses are becoming resistant to current synthetic drugs, and new treatments are required [19]. Natural products have been shown great promise in medicine, approximately 40% of the most commonly used medicines contains compounds derived from natural sources, using either the isolated substance or a synthetic version [64].

2.4.2. Natural antiviral compounds

The complexity of many synthetic compounds and the lack of accessibility in developing countries have created a massive interest by the academia and public organizations in natural antiviral compounds [65]. Since the beginning of humankind, plants extracts and essential oils have been widely used in traditional medicine to treat different types of diseases [66-69]. Many plants have been reported to have antiviral activity [19, 68, 70, 71] and some of them have been used to treat viral diseases in animals and humans [72, 73]. However there are innumerable compounds from different plants waiting to be evaluated and exploited for the therapeutic effect

against different types of viruses [19]. Natural products are promising candidates for novel antiviral compounds. Because they come from natural sources, they offer structural diversity and biochemical specificity. Natural antiviral products can be classified either as plant extracts or as isolated substances. The extracts are unpurified samples where the antiviral compounds are not known and the isolated compounds are purified compounds where we know the exact activity of that compound. Isolated compounds can then be made from purification of the plant extract or synthetically to become a commercial product.

2.4.2.1. Natural antiviral extracts

Recent *in vitro* studies on antiviral compounds have shown that plant extracts and essential oils exhibit antiviral activity against certain types of viruses (**Table 2.2**). Several antiviral essential oils alter initial stages of the virus infection cycle (**Figure 2.2**). Inactivation of herpes simplex virus (HSV) particles prior to attachment was seen with essential oil from star anise which reduced the infectivity of HSV-1 by 99% [74], artemisa oil, which reduced the infectivity of HSV-1 & 2 by 80% [75], the essential oils derived from ginger, thyme, hyssop, and sandalwood that reduced the titers of HSV-1 by 99% [76], manuka essential oil that reduced the infectivity of HSV-1 & 2 by 99.5% and 98.9%, respectively [77], the Australian tea tree oil (TTO) reduced the infectivity of HSV-1 & 2 by 98.2% and 93.0% respectively [78]. Essential oils such as clove oil, oregano, and sataria, were able to inhibit the adsorption of nororovirus surrogates, such as feline calicivirus (FCV) and murine norovirus (MNV) by inactivating the virus capsid [79, 80]. Oregano and clove oils essential oils were also able to lyse the lipid envelope of HSV-1 and Newcastle disease virus, strain LaSota (NDV-LS) [81]. Essential oils were able to prevent virus attachment and adsorption, probably by disruption of virus capsids and viral envelopes.

Natural extracts of phyllanthus orbicularis, quillaja saponaria and chamomile were able to inactivate HSV particles and inhibit virus attachment [82-85]. Extracts of ribes nigrum L, and quillaja saponaria, were able to prevent virus adsorption of other types of enveloped viruses, such as IFV-A & B and HIV-1 & 2, respectively [83, 86]. Plant extracts have also been able to inhibit late stages in the virus infection cycle of enveloped viruses. Extracts of scutellaria baicalensis,

eugenia caryophyllus, lavender and sage were found to disrupt virus replication of dengue virus (DENV), HSV-1 & 2, HSV-1 and HSV-2, respectively [84, 87, 88]. Non-enveloped viruses were also affected by natural extracts. Extracts from raoulia australis and quillaja saponaria, blocked virus attachment of RHV and rotavirus, respectively, probably by interacting with the virus particle [89, 90]. Fruits extracts or juices (strawberry, grape, apple, and cranberries) inhibit virus adsorption of non-enveloped viruses [91-100]. Extracts for different types of plants were able to inhibit the infectivity of enveloped and non-enveloped at different stages in the virus infection cycle. However, further studies are required with plant extracts and essential oils since the identification and isolation of the active compound is required for the production of the antiviral drug.

Table 2.2. Essential oil and plant extracts as antiviral compounds

Essential oils	**Virus**	**C**	**Mechanism**	**Reference**
Star anise	HSV-1	E	Entry inhibitor (attachment)	[74]
Artemisia	HSV-1 & 2	E	Entry inhibitor (attachment)	[75]
Ginger	HSV-1	E	Entry inhibitor (attachment)	[76]
Thyme	HSV-1	E	Entry inhibitor (attachment)	[76]
Hyssop	HSV-1	E	Entry inhibitor (attachment)	[76]
Sandalwood	HSV-1	E	Entry inhibitor (attachment)	[76]
Manuka	HSV-1 & 2	E	Entry inhibitor (attachment)	[77]
TTO	HSV-1 & 2	E	Entry inhibitor (attachment)	[78]
Clove	FCV, MNV	N	Entry inhibitor (attachment)	[79]
Oregano	FCV, MNV	N	Entry inhibitor (attachment)	[79]
Zataria	FCV, MNV	N	Entry inhibitor (attachment)	[79]
Oregano	MNV	N	Entry inhibitor (attachment)	[80]
Oregano & clove	HSV-1 & NDV-LS	E	Entry inhibitor (attachment)	[81]
Plant extracts	**Virus**	**C**	**Mechanism**	**Reference**
Ph. orbicularis	HSV-1 & 2	E	Entry inhibitor (attachment)	[82]
Quillaja saponaria	HIV-1 & 2	E	Entry inhibitor (attachment)	[83]
Chamomile	HSV-1	E	Entry inhibitor (adsorption)	[84]
Melissa officinalis	HSV-1	E	Entry inhibitor (adsorption)	[85]
Ribes nigrum L	IFV-A & B	E	Entry inhibitor (adsorption)	[86]
Quillaja saponaria	HIV-1 & 2	E	Entry inhibitor (attachment)	[83]
S. baicalensis	DENV	E	Replication inhibitor	[87]
E. caryophyllus	HSV-1 & 2	E	Replication inhibitor	[88]
Lavender	HSV-1	E	Replication inhibitor	[84]
Sage	HSV-2	E	Replication inhibitor	[84]
Raoulia australis	RHV	N	Entry inhibitor (attachment)	[89]
Quillaja saponaria	Rotavirus	N	Entry inhibitor (attachment)	[90]
Straberry extract	PoV	N	Entry inhibitor (attachment)	[94]
Cranberry extracts	Reovirus	N	Entry inhibitor (attachment)	[97]
Cranberry juice	Rotavirus	N	Entry inhibitor (attachment)	[91, 92]
Grape seed extract	FCV, MNV	N	Entry inhibitor (attachment)	[98]
Cranberry juice	FCV, MNV	N	Entry inhibitor (attachment)	[99]
Pomegranate juice	FCV, MNV	N	Entry inhibitor (attachment)	[93]
Grape juice	PoV	N	Entry inhibitor (attachment)	[95, 96]

2.4.2.2. Isolated natural antiviral compounds

The main aim in the development of any antiviral drugs is the identification and isolation of the active ingredient. Specific biochemical substances such as polyphenols, polysaccharides, peptides, proteins and other found in natural sources have been identified and found to reduce the infectivity of certain types of viruses (**Table 2.3**). Catechins from black and green tea have been shown to have antiviral activity against enveloped and non-enveloped viruses. Epigallocatechin gallate (EGCG) is the most abundant catechin and has shown antiviral activity against several viruses. EGCG prevents attachment of HCV, disrupts the envelope of HSV-1 & 2, disrupts replication of IFV-A & B, inhibits HBV DNA synthesis, and suppresses replication of the enterovirus 71 (EV71) [101-107]. Theaflavin-3,3-digallate (TF-3) derivate from black tea was found to inactivate the viral envelope of HSV-1 & 2 and IFV-A & B [108-110]. Several tea polyphenols have been found to inhibit IFV-A & B adsorption [111-113]. Polyphenols found in plant and fruits have also shown antiviral effects. The flavonoid, 5,7,4'-trihydroxy-8-methoxyflavone (F36) inhibit the fusion of viral envelopes with the endosome/lysosome membrane, which occurs at the early stages of the virus infection cycle [114, 115]. Resveratrol (RV) has been shown to inhibit virus replication of enveloped viruses, such as varicella zoster virus (VZV) and IFV-A [116, 117]. Polyphenols in pomegranate, such as ellagic acid, caffeic acid, luteolin, and punicalagin block replication of virus RNA of IFV-A [118]. In general, polyphenols work by disruption of the early stages in the virus infection cycle of enveloped viruses, with only few exceptions.

Polysaccharides isolated from fungi and plants have shown antiviral activity. Polysaccharides from agaricus brasiliensis a species of mushrooms inhibit the attachment of poliovirus (PoV) into the host cells [119]. Yellow fever virus (YFV) envelope was disrupted by polysaccharides found in the lichen, parmelia perlata [120]. Inhibition of HSV-1 & 2 adsorption was achieved with polysaccharides found in the plant, sargassum patens [121, 122]. Isolated polysaccharides from rhizophora mucronata were found to bind to HIV particles and inhibit virus attachment [123]. Finally polysaccharides found in nothogenia fastigiata have shown inhibition of

human cytomegalovirus (HCMV) adsorption [124]. Polysaccharides were found to disrupt early stages in the virus infection cycle of enveloped viruses and non-enveloped virus.

Other natural compounds such as peptides and osmolytes have shown antiviral properties against non-enveloped viruses. A peptide isolated from Enterococcus mundtii was found to disrupt the virus capsid of PoV [125], while osmolytes were able to inhibit the assembly of the porcine parvovirus (PPV) virus capsid [126]. The advantage of natural compounds that disrupt virus replication is that they can be applied post-infection, which increase their potential therapeutic drugs. The majority of the antiviral compounds found in the literature present antiviral activity against enveloped viruses, due the fact the envelope capsid are easier to inactivate. The main advantage of antiviral compounds is that they can reduce the high costs of synthetic antiviral compounds. Several compounds have been isolated from natural sources and they have shown antiviral activity against different types of viruses, demonstrating that natural antiviral compounds could be used as new treatments for viral infections. All the studies presented in this section have been done *in vitro*, if people are treated with these substances, large quantities are needed and more studies needs to be done in order to determine the side effects.

Table 2.3. Polyphenols, polysaccharides and other compounds as antivirals

Polyphenols	**Virus**	**Capsid**	**Mechanism**	**Reference**
EGCG	HCV	E	Entry inhibitor (attachment)	[101, 102]
	HSV-1 & 2	E	Entry inhibitor (attachment)	[103]
	IFV-A & B	E	Replication inhibitor	[104, 105]
	HBV	E	Replication inhibitor	[107]
	EV71	N	Replication inhibitor	[106]
	IFV-A & B	E	Entry inhibitor (adsorption)	[113]
TF-3	HSV-1 & 2	E	Entry inhibitor (attachment)	[108, 109]
	IFV-A & B	E	Entry inhibitor (attachment)	[110]
	IFV-A & B	E	Entry inhibitor (adsorption)	[111, 112]
F36	IFV-A & B	E	Entry inhibitor (attachment)	[114, 115]
RV	VZV	E	Replication inhibitor	[117]
	IFV-A	E	Replication inhibitor	[116]
Ellagic acid	IFV-A	E	Replication inhibitor	[118]
Polysacharides from	**Virus**	**Capsid**	**Mechanism**	**Reference**
Agaricus brasiliensis	PoV	N	Entry inhibitor (attachment)	[119]
Parmelia perlata	YFV	E	Entry inhibitor (attachment)	[120]
Sargassum patens	HSV-1 & 2	E	Entry inhibitor (adsorption)	[121, 122]
Rhizophora mucronata	HIV	E	Entry inhibitor (attachment)	[123]
Nothogenia fastigiata	HCMV	E	Entry inhibitor (adsorption)	[124]
Others	**Virus**	**Capsid**	**Mechanism**	**Reference**
Peptide from Enterococcus mundtii	PoV	N	Replication inhibitor	[125]
Osmolytes	PPV	N	Replication inhibitor	[126]

2.5. Vaccine manufacturing

The aim of the vaccine market is to produce and manufacture safe and effective vaccines. The vaccine market presents several challenges, such as complexity in the production process, insufficient supply, high costs, and stringent regulatory requirements [127]. Vaccine production requires the use of living cells and the grow of the immunogenic agent, followed by a multifaceted purification process, in order to obtained the desired product [7]. The supply of vaccines is insufficient for industrialized, developing, and third-world countries, due the fact that there are too few manufacturers [17]. The biggest challenge in the vaccine industry is to reduce the cost of vaccine production. Currently, pharmaceutical companies avoid the vaccine industry because it is economically expensive, and involves many regulatory barriers [7, 24]. The cost of a new vaccine is around $300 to $800 million, and pharmaceutical companies need to provide the cost of research and development [17]. Additionally, pharmaceutical companies do not feel attracted to the market since the financial investment return tends to be low and unpredictable as compared to other types of drugs in the areas of oncology, cardiovascular and neurodegenerative diseases [24, 128]. The regulatory considerations are extremely high in the vaccine market, since vaccines are molecules that are more difficult to characterize than any other small pharmaceuticals [127]. Moreover, there is not a standard platform process for vaccine manufacturing similar to the antibody manufacturing process, due to the fact that vaccines are unique molecules that require different routes of production [129]. Despite all the limitations in the vaccine market, the World Health Organization (WHO) reported in 2010 that the vaccine market growth rate was 9-15% per year, higher than the growth rate of other pharmaceuticals at 5-7% per year [130]. The annual sales of the vaccine market were reported to be $21.7 and $25.3 billion in 2009 and 2010, respectively, and are projected to reach $39.5 billion in 2015 [131]. Expansion of the medical market and reduction in costs in current process is needed in order to increase the access to vaccines.

The vaccine production process is divided into upstream processes (USP) and downstream processes (DSP) (**Figure 2.3**). The USP includes cell and virus propagation, while

the DSP include a variety of steps for the recovery and purification of the desired vaccine. Different types of DSP have been developed to purify viruses and most of them follow similar unit operations. After cell culture and virus harvesting, cell lysis and clarification needs to be done in order to remove cells debris. The virus is clarified by low speed centrifugation, and the supernatant is recovered and filtered. Then the virus solution can be purified with centrifugation and filtration, or filtration and chromatography. The final step in the DSP is the polishing step, which is usually done with chromatography or filtration [132-135]. The increase in USP production yields and harvest volumes is creating a strain on current DSP to handle the increased product load [23]. Moreover, the DSP account for 70% of the overall cost of any biotherapeutic production process [136, 137]. To satisfy the current and future market demand, an improvement in current DSP is needed.

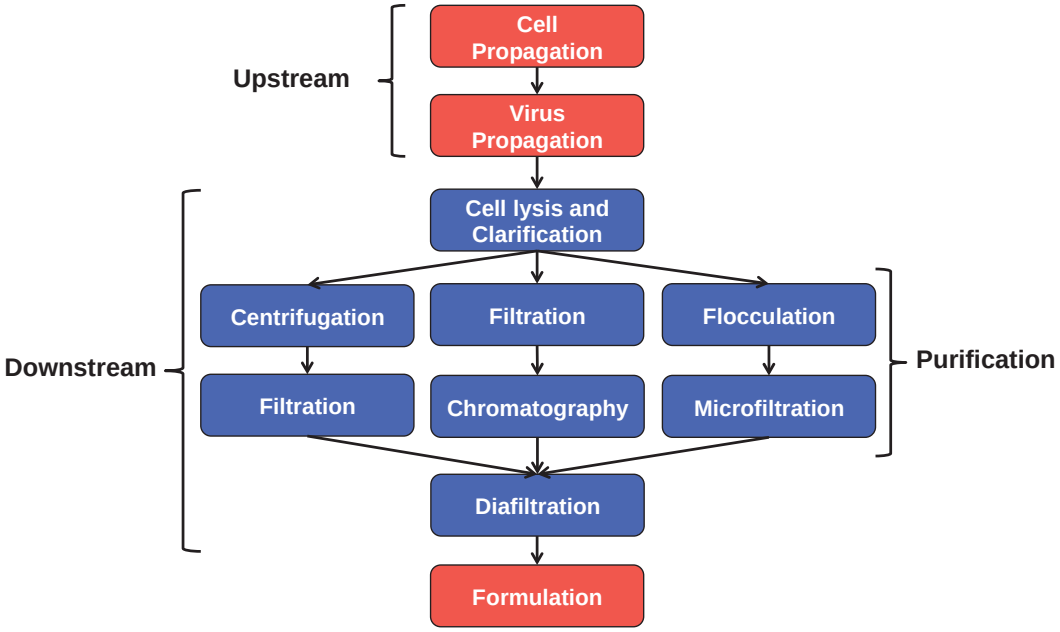

Figure 2.3. Upstream and downstream processing in vaccine manufacturing.

2.5.1. Upstream processes

In the first stages of the vaccine manufacturing process, the virus is generated. Viruses are grown either in primary cells (chicken eggs) or in established cell lines. Recombinant-vaccines can be made with an expression system, such as yeast, bacteria, or mammalian cells

[138]. The egg-based technology presents scale-up challenges, which include highly manual, bacteria contamination, slow production times, low yields and a high quantity of eggs needed [138, 139]. Manufacturing companies prefer cell-based vaccine manufacturing processes, since they offer easy scale-up, faster processing time and low risk of contamination [138, 140]. The parameters most relevant in USP are the design of new bioreactors or modifying exiting ones, optimization of current processes, and manipulations of the cell culture media [141].

2.5.2. Downstream processes

The DSP of biological products is affected by the impurities and contaminants, like host cell protein (HCP), cell debris, and host cell DNA that are generated in the USP [142, 143]. Followed by the production of the virus particles a series of steps in the DSP are required in order to recovery and purify the virus from contaminants. The first steps in the DSP are cell lysis and clarification. At a manufacturing level, cells are lysed by microfluidization, tangential flow filtration or osmotic shock [144]. Clarification is the step used to remove cell debris, particulates and insoluble matter after cell lysis. This step is usually done with centrifugation, filtration, or a combination of both of them, either in batch or continuous mode [135, 139, 144]. After the virus particles have been recovered, further virus purification steps are required in order to obtain the final product.

Different types of methods have been proposed for virus purification. The first methods used for virus purification at small-scale were ultracentrifugation and density gradient centrifugation [135, 145]. However, due to the size of the equipment, these methods are difficult to maintain, scale-up restricted, cost-ineffective, labor-intensive, require long processing times, and often several impurities remain at the end of the process [144, 146]. Moreover, ultracentrifugation can lead to low recovery yields due to virus degradation and loss of infectivity [145]. Chromatography used for protein purification has been adapted to virus purification to overcome the limitations of ultracentrifugation.

Chromatography is the most prevalent technology for large-scale purification of viral particles, since it is fast, scalable and reproducible [144, 147]. Additionally, chromatography

offers many advantages compared with density gradient ultracentrifugation, which include removal of HCPs and contaminated DNA, high yields, and faster processing times [138]. However, purification of virus particles is a challenge due the large particle sizes, low diffusion and complex molecular surfaces structures [148, 149]. There is not a universal chromatography process that can be applied to all types of viruses. The stationary phases used in chromatography are packed beds, monoliths and membranes [150]. Packed beds are conventional chromatographic supports that were designed for the purification of proteins and are not suitable for large biomolecules, such as virus particles [151]. The main disadvantage of conventional chromatography is that virus particles present limited diffusion into the pores of the resins. Virus particles have difficulties accessing the high internal surface area of the resins [152, 153] and as a consequence the recovery of the viral particles is low, the pressure drop is high due to poor pore diffusion through the bed and the time processing rates are slow [23, 132, 135]. Monoliths and membranes allow rapid convective mass transfer, however, it is difficult to keep uniform properties through the membranes and the capacity of the membrane can be limited [153]. All of the typical chromatography modes have been utilized for virus particle purification, including gel filtration or size-exclusion chromatography (SEC), ion exchange chromatography (IEC), affinity chromatography (AC), and hydrophobic-interaction chromatography (HIC) (**Figure 2.4**) [23], and all of them present limitations.

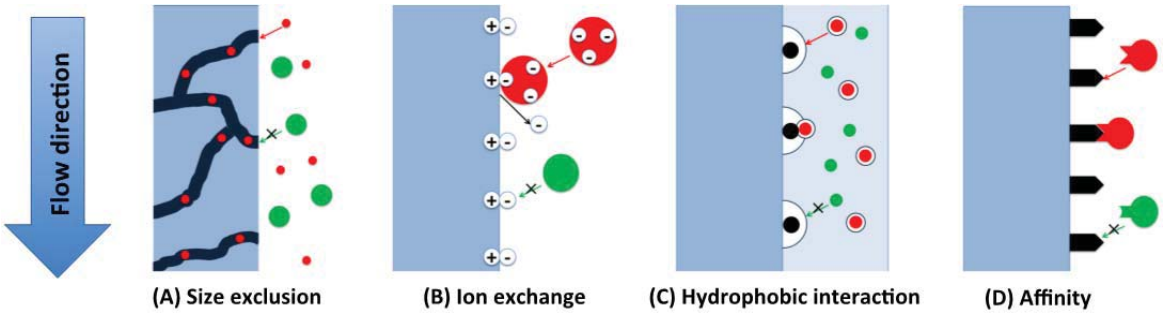

Figure 2.4. Chromatography modes. A) Size exclusion, B) Ion exchange, C) Hydrophobic interaction, D) Affinity.

SEC could be used with several strains of viruses, since virus binding does not occur during the chromatography run [135]. However, SEC cannot remove large contaminants [153],

presents low capacity and the matrix has poor pressure resistance [23]. IEC is the most widely chromatography technique used for virus purification [153]. The principle of IEC is charge-charge interaction between the virus and chemical groups on the matrix. The selection of the right matrix depends of the isoelectric point (pI) of the virus. This method offers high selectivity and is easy to scale-up [135, 153]. However, IEC cannot remove empty capsids [153], presents low specificity and co-elution of contaminants [23]. AC is based on highly specific interactions between the target molecule and ligands bound on the matrix. AC offers high selectivity, reduction of contaminants, and the possibility of reducing purification steps [135, 154]. Nevertheless, AC is not recommended for large-scale process [23], since the ligands are expensive. HIC is based on interactions between the virus and hydrophobic ligands on the surface of the stationary phase. The main disadvantages of HIC are low recovery and viral degradation due to the high concentrations of salts [144]. All the modes used in chromatography columns have presented several limitations that could increase the operational cost of the DSP.

The large size differences between virus particles and typical protein contaminants suggest the use of filtration as an alternative for virus purification [153]. Membranes filtration processes are usually combined with chromatography. Membrane-based tangential flow filtration (TFF) techniques, which are based on size differences, including ultrafiltration and nanofiltration, have shown high viral titers and high recoveries [148, 155, 156]. Additionally, membrane processes are very advantageous techniques due to the easy scalability and high throughput [148, 157]. However, the capacity of the membranes can be affected by fouling, which can occur by pore blocking, pore constriction, caking or a combination of all of them [158]. Fouling can lead to longer filtration time, high transmembrane pressure, and low flux through the membrane [23, 136]. All of these drawbacks could increase the cost of production of DSP.

Precipitation and flocculation followed by centrifugation or filtration has been used for virus purification [136]. Commonly, the additives are compounds that promote aggregation, like irons, salts or polymeric agents, such as poly(ethylene) glycol (PEG) [159]. Chemical flocculation with salts and irons, followed by microfiltration and/or ultrafiltration have been used to remove viruses, such as bacteriophages and ocean viruses from protein-free conditions [160-162]. Salts

and iron flocculants provides high virus removal of the MS2 phage, >7.4 logs and >4.0 logs, respectively [160-161]. However, it is likely that high salt concentrations may precipitate more than one protein, if the virus is in a solution containing other proteins. PEG is a well-known additive, which is used to precipitate all proteins [163], although specific conditions have been used to selectively precipitate IgG4 from cell culture [164] and viruses bacteriophages from DNA and protein contaminants [165]. Appropriate processing conditions for PEG precipitation might lead to effective primary purification of bioproducts in large-scale process. However, PEG precipitation present some disadvantages. PEG precipitation requires extended periods of incubation time [166] and polymer additives can interact with virus particles and form complexes that are difficult to dissociated [167, 168]. The aim in virus precipitation/flocculation is to improve process robustness; a selective, global, nontoxic, and economical precipitant/flocculants is desired [23]. Membrane processes and chromatography processes do not offer a general platform that can be used for all types of viruses [148, 169]. We propose to use virus flocculation with osmolytes, followed by microfiltration, as an alternative to the current manufacturing system [170]. The osmolyte flocculants are able to deplete a hydration layer around the virus particles and subsequently cause virus aggregation [170]. We hypothesize that osmolytes are better than salts or polymer flocculants, since they are often used as an excipient to stabilize the final formulation of drugs or biotherapeutics [171, 172].

The final steps in the DSP are polishing and formulation. The polishing step is done in order to remove traces amounts of contaminants. Usually, the polishing step is done with either size exclusion or TFF [139]. After polishing, the product goes to final formulation. The biggest challenge in the DSP is to keep pace with the increase in the upstream yields [139]. Virus flocculation with osmolytes for virus purification could be uses as a novel and economical alternative.

2.6. Vaccine delivery systems

Vaccine development requires that the vaccine is effective and can give a specific immune response [173]. Conventional vaccines, such as live-attenuated vaccines and whole

inactivated vaccines have been very successful at inducing immune responses against several virus infections. However, these types of vaccines are reactogenic and often create unwanted side effects. Moreover, conventional vaccines offer poor immune response in immunocompromised patients. Additionally some pathogens are very difficult to grow in cell culture (e.g. HCV & HAV) [174]. To ameliorate these limitations, scientists have focused on new technologies, such as recombinant proteins subunit, synthetic peptides, protein-polysacharrides conjugates and plasmid DNA vaccines. These types of vaccines are safer and less reactogenic than conventional vaccines, but they are also less immunogenic [174-177]. In order to induce an effective immune response in new technologies, vaccines require boosting agents, which are called adjuvants. Adjuvants need to be capable of stimulating a protective immune response and be safe for humans [173, 178]. Adjuvants can be classified into immunostimulatory adjuvants and vaccine delivery systems [174]. Immunostimulatory adjuvants are derived from pathogens, and are known to stimulate immunity as they are identified by pattern recognition receptors, which are known to activate the cells of the immune system. Vaccine delivery systems or vehicles are gaining attention in the medical field. The vaccine antigen is either encapsulated or attached onto the surface of the vehicle [6, 174, 179]. The advantages of vaccine delivery systems include, specifically targeting the immune cells and incorporation of an adequate dose, so that booster doses are not necessary [179, 180]. Adjuvants are able to boost, maintain and potentiate an immune response against the antigen, and consequently they could lead to the improvements of vaccine products, vaccination coverage, and reduction of costs in the manufacturing processes [181]. Thus, they have the potential to be useful against viral diseases for which there are no effective vaccines yet. The same systems used in drug delivery are used in vaccine delivery. In 2008, the demand for drug/vaccine delivery system was predict to increase by more than 10% a year [182] and is predict to reach $160 billions by 2016 [183].

A drug/vaccine delivery system can be classified based on the route of administration, either topical (skin or mucous), enteral (oral), or parenteral (injection) [182]. Oral and mucosal delivery vehicles could help to efficiently transport the virus antigen to the immune system and improve the therapeutic outcome of conventional vaccines that are administered by the parenteral

route, such as measles, and chickenpox (varicella) [184, 185]. Vaccines that are administered through this route present several limitations, such as risk of transmission of diseases, poor solubility, tissue damage, rapid breakdown of the therapeutic *in vivo*, poor distribution and lack of target selectivity [184]. A controlled drug/vaccine delivery systems is designed to control the rate and period of a specific type of therapeutic in a specific target area of the body [186]. A drug/vaccine delivery system can also be classified by the delivery mechanism into active or passive [182]. An active mechanism permits the controlled initiation of release of a therapeutic by using a chemical feauture of the vehicle. A passive mechanism allows the controlled release of the therapeutic by molecular diffusion, and the release mechanism usually takes advantage of human physiology [182]. The main goal of a vaccine/drug delivery system is to transport the therapeutic product to the patient by using the optimum route and the best mechanism [187]. Vaccine delivery systems are in an early stage of development. The discovery of efficient vaccine delivery vehicles may allow the development of more effective vaccines.

Scientists have classified vaccine/drug delivery vehicles into virus-like particles (VLPs), liposomes, immune-stimulating complexes (ISCOMs), polymer-based nanoparticles, and non-degradable nanoparticles [6, 173, 187]. VLPs are self assembly viral envelope without genetic material [188] (**Figure 2.5A**). They are the great interest, because they can stimulate strong immune responses [189, 190]. Vaccines based on VLPs, such as the hepatitis B virus surface antigen (HBsAg) [191] and the human papilloma virus capsid protein L1 [192] are been used as commercial vaccines. The baculovirus expression system is the most commonly used for the generation of VLPs. The main disadvantage of VLPs production is the inability of this expression system to produce mammalian glycoproteins [6]. Liposomes are biodegradable molecules that can consist of a phospholipid bilayer shell with an aqueous interior [193] (**Figure 2.5B**). They can improve the immunogenicity of weak proteins antigens by simulating the structure of natural lipid bilayer membranes and entering cells through endocytosis [193-195]. Some of these delivery systems are in the market or under clinical trials, and are used for the treatment of IFV-A & B and HAV infections [196]. The biggest limitations of liposomes are low stability, low encapsulation efficiency, rapid leakage of water-soluble drugs, poor storage stability, laborious manufaturing

process and poor quality assurance [177, 197, 198]. ISCOMs are open, cage-like complexes that are made with saponin, cholesterol, and phospholipids [6, 173, 199] (**Figure 2.5C**). The antigen can be attached into the membrane of the ISCOMs or encanpsulated inside the ISCOMs [6, 177]. ISCOMs are able to boost the imune system by promoting high levels of antibody and strong T-cells responses [179]. ISCOMs incorporating recombinant hepatitis B surface antigen have shown immune response throught intranasal administration [200]. The two main problems with ISCOMs are stability and toxicity [177].

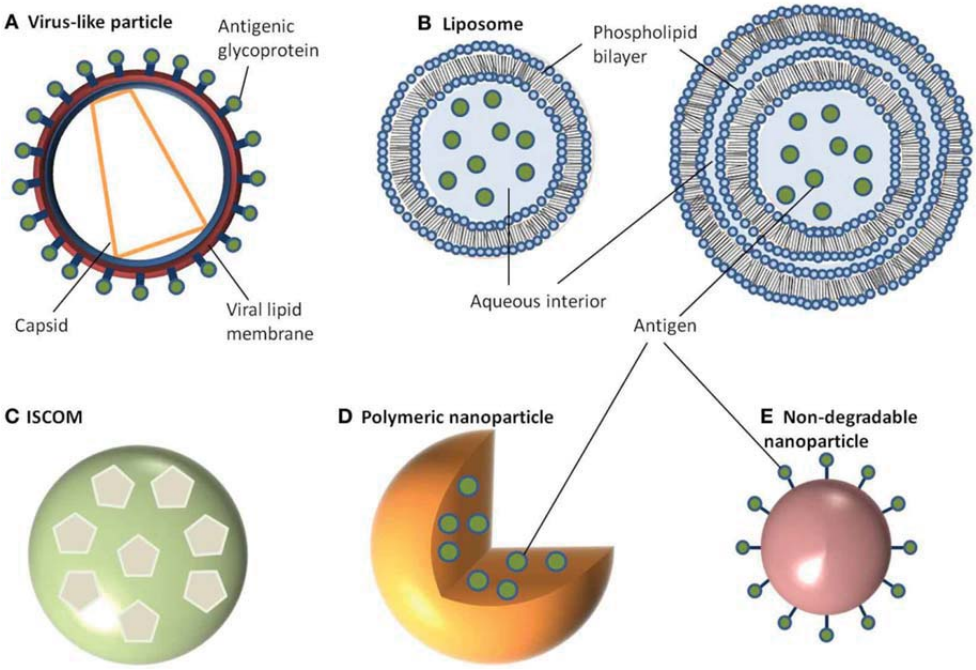

Figure 2.5. Vaccine delivery systems. A) Virus-like particle, B) Liposome, C) ISCOM, D) Polymeric nanoparticle, E) Non-degradable nanoparticle [6].[1]

Polymer nanoparticles are the great interest since they are biodegradable molecules [201], which can be used to improve antigen stability and target delivery at slow release rates [183] (**Figure 2.5D**). The have been used for nasal and oral delivery [179]. They can be divided into nanocapsules and nanospheres. In nanocapsules, the antigen is surronded by a polymer membrane, and in nanospheres, the drug is uniformly dispersed throughout the polymeric matrix

[1] Reproduced from [6] Gregory AE, Titball R, Williamson D. Vaccine delivery using nanoparticles. Frontiers in cellular and infection microbiology. 2013;3. with permission of Frontiers.

[173, 181, 197]. The biggest disadvantage of polymer nanoparticles is the high cost involved in the production. Non-degradables nanoparticles, are commonly made of gold, silica or carbon [6] (**Figure 2.5E**). They have a shell where the vaccine antigen is encapsulated or attached [6]. Gold nanoparticles are monodispers and uniforme in shape, which allow consisintency of mainting vaccine loading between batches [6]. Silica-based materials are prepared with surfactants, such as polymers and phospholipids using a hydrolysis and condensation technique (**Figure 2.6**). The materials can be arranged into different morphologies, such as microsshpheres and monoliths [202]. They resulting particles possess a mesopores within the surface, which are used to pack and protect the vaccine antigen.

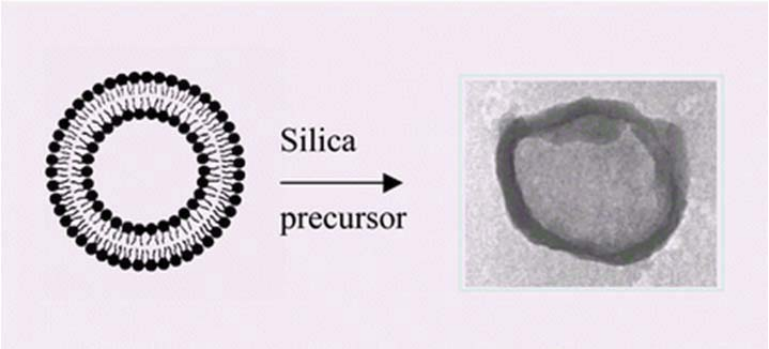

Figure 2.6. Silica-based material using unilamellar liposomes with a high sol–gel temperature transition phase as a template [202].[2]

Mesoporous materials are potential vaccine delivery vehicles due to all the features they offer, such as an ordered pore network that allow load and release of the vaccine; high pore volume, which allow to hold the required amount of vaccine; and a high surface area that is required for vaccine delivery [186]. The first mesoporous silica-based material MCM-41, a highly ordered mesoporous material was synthetized in 1991. Researchers were atracted to this material due to their potential to be used in technological applications, such as catalysis, lasers, sensors, and solar cells [187]. In 2001, the MCM-41 was proposed as a drug delivery vehicle and

[2] Reproduced from [202] Begu S, Durand R, Lerner DA, Charnay C, Tourne-Peteilh C, Devoisselle JM. Preparation and characterization of siliceous material using liposomes as template. Chem. Commun. 2003:640-1. with permission of The Royal Society of Chemistry. **DOI:** 10.1039/B210927A

was used to transport ibuproben, an antiinflammatory drug [203]. After MCM-41, SBA-15 and MCM-48 have been shown to transport and release several types of drugs such catopril, erythromycin and gentamicin [186]. Althought silica-based mesoporous materials have been succesful employed for the delivery of several types of drugs, they have been reported to weaken the immune system [204, 205]. Researches have foccused on carbon-based mesoporus materials as an altertanive to silica-based materials.

Mesoporous carbon is a new, synthetic, non-nano, and porous type of material that could be used as a smart delivery vehicle. They are of great interest in many applications besides vaccines delivery, such as separation, catalysis and energy conversion and storage [206]. The advantages of this type of material include large pore accessibility and tunability of the pore structure. The synthesis of this type of material can be made by hard template or soft template methods [206]. The conventional hard template method use silica as a template [207]. The method is complex, since the scaffolds need to be prepared, carbonization conditions are harsh, chemicals are corrosive, and finally the structures tend to collapse [208]. The soft-template method involves the self-assembly of surfactant micelles in the carbon precursor matrix (**Figure 2.7**) [209]. In the soft-template method, the mesoporous carbon is synthetized by crosslinking an organic resin with surfactant, usually amphiphilic block copolymers. The process is followed by pyrolysis, where the surfactants are removed and carbonization of the matrix [209]. The advantages of the soft-template method compared to hard-template include high microporosity, high surface area and a relatively large volume of mesopores [210]. Mesoporous carbon materials are projected to be potential candidates for biological applications such as drug delivery vehicles [186]. Mesoporous carbon materials are known to have a high surface area and an ordered pore system, which could assist with the adsorption and release process of multiple types of therapeutics, including vaccines

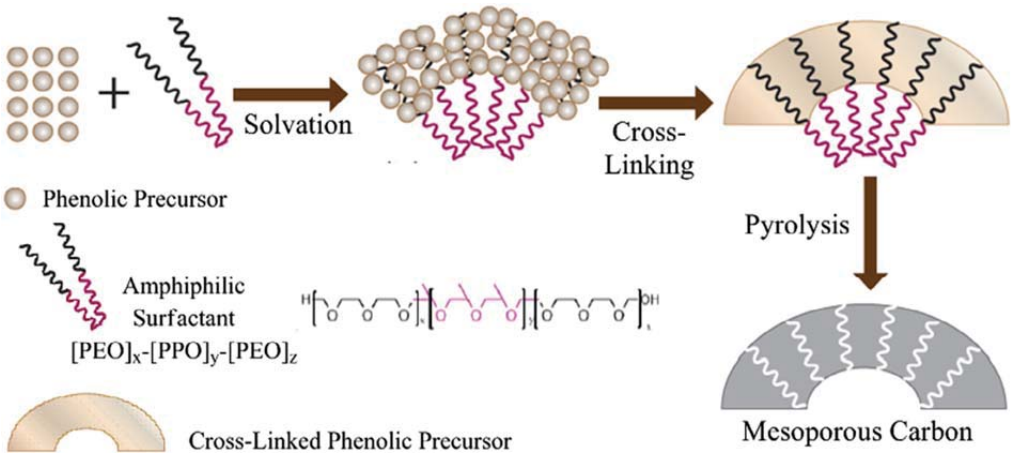

Figure 2.7. Soft-template technique. Self-assembly of mesoporous carbon by using a carbon precursor and a surfactant [209].[3]

Soft-templated mesoporous materials possess an ordered pore network, which permit controlled drug/vaccine release, the high surface area and large pore volume allow the uptake and transport of a large amount of drug/vaccine [186]. Other advantages include low bulk density, which allows the material to float in the gastrointestinal system, when used in oral delivery, and the ability to adhere to the gastrointestinal system [182]. Recently studies have reported that soft-template mesoporous materials have successfully shown controlled release of model drugs, including antypirine, captopril, furosemide, and ranitidine hydrochloride [209, 211]. In order to approve any type of biomaterial as a drug delivery vehicle, *in vitro* biocompability and toxicity tests are required prior to *in vivo* animal models and clinical trials [212, 213]. We propose that soft-templated mesoporous carbon materials have the potential to be used as drug delivery carriers, since they have shown low toxicity and high biocompatibility with model mammalian cells [214]. We would like to take advantage of these features and use these materials as potential vaccine/drug delivery vehicles (**Chapter 6**).

[3]Reprinted from Carbon, Vol 71, Saha D, Warren KE, Naskar AK, Soft-templated mesoporous carbons as potential materials for oral drug delivery, Pages 47-57, Copyright 2014, with permission from Elsevier.

2.7. Conclusions

Natural antiviral compounds, improvements in current unit operation in the vaccine manufacturing process and the creation of effective vaccine delivery vehicles will help to reduce the mortality and the morbidity cause by viral diseases. In this study, we propose to use natural products as new antiviral compounds because they are less expensive than synthetic drugs. In the area of vaccine manufacturing, we propose virus flocculation in osmolytes, followed by microfiltration as an economical alternative. Osmolytes have several advantages; they are able to preferentially flocculate hydrophobic virus, while leaving contaminants in solutions, and have been used as an excipient to stabilize therapeutics. Therefore, they could be integrated into the process without adding new compounds that may require toxicity testing and additional removal processes. Vaccine delivery vehicles are of great interest these days, since they can increase the immune response, without the use of large doses. In this study we propose soft-templated mesoporous carbon materials as vaccine delivery vehicles. The biggest advantage of this material is that they can control the released of the vaccine in the specific target area.

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Chapter 3

Osmolytes as antiviral compounds against porcine parvovirus[1]

[1] The material contained in this chapter was previously published in Antiviral Research. Reprinted from Antiviral Research, vol. 99, Tafur, M. F., Vijayaragavan, S. V., Heldt, C. L. Reduction of porcine parvovirus infectivity in the presence of protecting osmolytes, Pages 27-33, Copyright 2013, with permission from Elsevier.

3.1. Introduction

Many diseases are caused by pathogenic virus infection. In recent decades, scientists have defined the structure and function of many different viruses. This has aided in the creation of specific antiviral compounds. Compounds that inactivate certain viruses have changed the treatment of many diseases, including respiratory syncytial virus (RSV) [1] and herpes simplex virus type-1 (HSV-1) and type-2 (HSV-2) [2]. The AIDS epidemic has been treated with HIV protease inhibitors that now allow people to live decades with the virus [3]. However, there is still a need for the continued discovery of antiviral compounds.

Many researchers are now studying natural products as a source of antiviral compounds, since viruses are starting to become resistant to current drugs [4]. In an effort to find natural compounds that have antiviral activity, we screened the antiviral activity of a panel of osmolytes and a salt against the non-enveloped virus porcine parvovirus (PPV). Osmolytes are small organic compounds that are found in the cells of many organisms and they have the ability of stabilize intracellular proteins against environmental stress, such as extreme temperature or high osmotic pressure [5]. A balance between protecting and denaturing osmolytes assist in the delicate equilibrium needed for protein stabilization [6]. Protecting osmolytes fold proteins by structuring the water around themselves and changing the interaction between water and the protein backbone. Denaturing osmolytes bind directly to the protein backbone, causing the protein to unfold [7].

Parvoviruses, from the family *Parvoviridae*, are small, non-enveloped, icosahedral, single-stranded DNA viruses that infect vertebrates and arthropods [8]. PPV infects the intestines of pigs and is the most frequent cause of swine reproductive failure [9]. This virus is often used as a model for the human parvovirus B19. Although different natural compounds have been studied in recent decades, osmolytes have not been previously shown to have antiviral activity. This study describes the reduction of PPV infectivity in the presence of the protecting osmolytes TMAO and glycine.

3.2. Materials and methods

3.2.1. Materials

The osmolytes trimethylamine N-oxide (TMAO) dihydrate, glycine, betaine, D-alanine, D-arginine, sucrose, trehalose dihydrate, urea, and the salt ammonium sulfate were purchased from Sigma-Aldrich (St. Louis, MO) at a minimum purity of ≥98.0%. Poly-L-lysine, 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), agarose type I, low EEO, neutral red solution (0.33%), and sodium dodecyl sulfate (SDS) were purchased from Sigma-Aldrich (St. Louis, MO). Phosphate-buffered saline (PBS, pH 7.2) and 0.25% trypsin/EDTA for cell propagation were purchased from Life Technologies (Grand Island, NY). 12.1 M hydrochloric acid (HCl) and 3.7% formaldehyde in water were purchased from VWR (Radnor, PA). The monoclonal mouse anti-PPV antibody was purchased from VMRD (Cat no. 3C9D11H11, Pullman, WA) and the polyclonal Alexa fluor 546-conjugated rabbit anti-mouse antibody was purchased from Life Technologies (Cat no. A11060, Grand Island, NY). All solutions were made with NanoPure water (Thermo Scientific, Waltham, MA, resistance >18 MΩ) and filtered with either a 0.2 µm syringe filter (Nalgene, Rochester, NY) or a Millipore 0.2 µm bottle top filter (Billerica, MA) prior to use.

3.2.2. Cell propagation

Porcine kidney (PK-13) cells were a gift from Dr. Ruben Carbonell at North Carolina State University and were propagated as described previously [10]. Briefly, the cells were grown in minimum essential medium (MEM) (Life Technologies, Grand Island, NY), supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Oakwood, GA) and 1% penicillin/streptomycin (Life Technologies, Grand Island, NY).

3.2.3. Virus production and titration

PPV strain NADL-2 was a gift from Dr. Ruben Carbonell at North Carolina State University and were propagated in PK-13 cells, as described previously [10]. Briefly, cells were infected with 10^3 MTT_{50} of PPV, and 1.5 h later 9 ml of supplemented media were added. After 4-

6 days, the flasks were placed at -20°C. The flasks were thawed, and the monolayer was scraped. The scraped cells and media were centrifuged at 5000 rpm in a Sorvall ST16R Centrifuge (Thermo Scientific, Pittsburgh, PA) at 4°C for 15 min to remove the cell debris. The supernatant was stored at -80°C.

PPV was titrated with a colorimetric cell viability assay, the MTT Assay [10]. The reduction of the MTT tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) inside the mitochondria of metabolically active cells produces formazan crystals [11]. Upon dissolving the crystals, the cell viability can be quantified by measuring the absorbance of the solution at 550 nm. This has been shown to be linearly comparable to a $TCID_{50}$ for PPV on PK-13 cells [10]. Cells were seeded in 96-well plates, as described earlier [10]. The cells were infected with PPV in quadruplicate and 5-fold serial dilutions were made across the plate. After five days, 10 µl/well of 5 mg/ml of MTT in PBS was added. Four hours later, 100 µl/well of solubilizing agent (0.01M HCl and 10% SDS) were added. Plates were read on a Synergy Mx microplate reader (BioTek, Winooski, VT) at 550 nm between 18-24 h after addition of the solubilizing agent. The 50% infectious dose (MTT_{50}) value was determined to be the virus dilution that yielded 50% of the uninfected cell absorbance. The value was converted to a per milliliter basis and stated as the MTT_{50}/ml titer [10].

3.2.4. Cytotoxicity assay

Antiviral activity was determined in a similar way to the virus titration described in **Section 3.2.3**. After virus was added to the cells, 25 µl of osmolyte or salt at various concentrations were added to the infected cells.

To determine the effect of osmolyte concentration on antiviral activity, 25 µl of either TMAO or glycine with a final concentration ranging from 0.00 to 0.30 M was added to the infected cells. To determine the effect of the time between infection and osmolyte addition on antiviral activity, 0.20 M of either TMAO or glycine was added at various times post-infection. MTT reagent addition was performed after five days, as described in **Section 3.2.3**. Calculation of the log reduction is shown in **Eq 3.1**.

$$\log reduction = -\log\left(\frac{virus\ titer\ with\ osmolyte}{virus\ titer\ of\ control}\right) \quad (\mathbf{3.1})$$

3.2.5. Osmolyte toxicity

Cell viability was assessed with an MTT assay and was used to determine the toxicity of TMAO and glycine to PK-13 cells. Cells were seeded as described in **Section 3.2.3** in 100 µl of media. Osmolytes diluted to a final concentration ranging from 0.00 to 0.60 M in NanoPure water were added to the cells after 24 h at a volume of 25 µl. MTT reagent addition was conducted after five days, as described **Section 3.2.3**. Calculation of the % survival of cells is shown in **Eq 3.2**.

$$\%\ survival = \left(\frac{absorbance\ with\ osmolyte}{absorbance\ of\ control}\right) \quad (\mathbf{3.2})$$

3.2.6. Pre-infection treatment

Pre-infection treatment was measured with an MTT assay. PK-13 cells were seeded as described in the **Section 3.2.3**. PPV virus was incubated with the osmolytes diluted to a final concentration ranging from 0.06 – 0.60 M in water for two hour before adding to the cells in five-fold dilutions. MTT reagent addition and the calculation of the virus titer were done as described in **Section 3.2.3**.

3.2.7. Plaque reduction assay

Plaque assays were performed as described previously [10]. Briefly, PK-13 cells were seeded into 25 cm^2 tissue culture flasks with a final concentration of 4×10^5 cells per flask and incubated at 37°C and 5% CO_2 until 70% confluent. Ten-fold serial dilutions of 10^8 MTT_{50}/ml were made in either PBS with 3% FBS or PBS with 3% FBS containing 0.20 M TMAO or 0.20 M glycine. Cells were infected with 200 µl of different sample dilutions. After 1 h of incubation, virus inoculum was removed and infected cells were overlaid with 1:1 of 2% agarose in Nanopure water and 2 x supplemented media. Overlay media for osmolytes samples also contained 0.20 M glycine or 0.20 M TMAO. Flasks were stained with 2 ml of 4% neutral red at 3.3 g/L in the overlay media after 4 days of incubation. Plaques were counted 4-8 h after staining.

3.2.8. Yield reduction assay

Intracellular and extracellular viable virus particles were measured. PK-13 cells were seeded in 6-well plates at the same cell density as the MTT Assay described in **Section 3.2.3**. Cells were infected with 2 x 10^3 MTT_{50} of PPV. After 1 h, the virus inoculum was removed and media added. Osmolytes were added either at this time (Treatment 1) or 5 min prior to sample collection (Treatment 2). Virus supernatant was removed at various times to measure extracellular viable virus particles. To assay intracellular viable virus particles, cells were detached by the addition of trypsin/EDTA and equal volume of media was added to deactivate the trypsin. All samples were frozen at -20°C for 24 h, thawed at room temperature, and centrifuged at 5000 rpm in a Sorvall ST16R Centrifuge (Thermo Scientific, Pittsburgh, PA) for 15 min at 4°C. The supernatants were removed and titrated as described in **Section 3.2.3**.

3.2.9. Immunohistochemical detection of virus capsid protein production

Intracellular virus capsid protein production was assessed through immunohistochemistry of PPV-infected cells with and without osmolytes. Glass slides (25 x 75mm) from VWR (Radnor, PA) were soaked in 2 M HCl for 1 h to etch and remove any grease. Then, 200 µl of poly-L-lysine was added on the area of cell growth. After five minutes, the slides were washed with water and dried for 2 h under UV light. Cells were seeded on the slides at a density of 5 x 10^4 cells/slide with a total volume of 50 µl/slide. Cells were incubated for 6 min at 37°C, and 5% CO_2. Ten ml of fresh media was added to the petri dish that contained the slides. After 48 h of incubation, the media was removed and cells were washed with PBS. Each slide was infected with 50 µl/slide of either PPV (10^8 MTT_{50}/ml), PPV containing 0.20 M TMAO or glycine (10^8 MTT_{50}/ml), PBS, or media containing 0.20 M TMAO or glycine. After 30 min, the cells were washed twice with PBS to remove any unattached PPV, and 10 ml of fresh media with or without osmolytes were added. The cells were placed at 37°C, 100% humidity, and 5% CO_2 for different times. The media was removed and the cells were washed once with PBS. The infected cells were fixed with 200 µl of 3.7% formaldehyde for 20 minutes at room temperature, and then washed twice with PBS. The cells were blocked with 200 µl of 0.3% low-fat milk in PBS. After 1 h, 50 µl of 1:100 v/v anti-PPV

antibody were added and incubated for 1 h at 37°C followed by two PBS washes. Then, 50 µl of Alexa fluor 546-conjugated rabbit anti-mouse IgG (1:500 v/v) were added and incubated for 1 h at 37°C followed by two PBS washes. Slides were washed again with PBS. To fix the antibodies, 200 µl of 3.7% formaldehyde were added for 20 min at room temperature, and then the cells were washed twice with PBS. Finally, 50 µl of 150 µM DAPI were added for 5 minutes at room temperature and washed with PBS. Images of the cells were taken with an Olympus IX51 microscope with a DP72 camera (Olympus, Center Valley, PA). Fluorescence per cell was analyzed with ImageJ (NIH). Image study was conducted on 2-3 slides with 10 images per slide.

All images were procured from Olympus CellSens imaging software. The ImageJ analysis was performed by gray scaling 100 ms exposure PPV images and 5 ms exposure DAPI images. The obtained multi-color images were converted to single color images by converting to 16 bit or a gray scale image (**Figure. 3.1**). The threshold was adjusted and consistent values were maintained to analyze the entire set of images. A binary version of the image was created with pixel intensity of 0 (white) and 255 (black). Any noise levels less or equal to 2 pixel density was removed and each image was analyzed for particle count and area. The PPV/DAPI ratio was determined by taking the area of the binary PPV image and dividing by the number of nuclei found in the DAPI binary image (see **Figure 3.1**).

3.3. Results

3.3.1. Virus cytotoxicity in the presence of osmolytes and a salt

Antiviral activity of a group of osmolytes and a salt was screened. These compounds were the protecting osmolytes TMAO, betaine, sucrose, glycine, alanine and trehalose, the denaturing osmolyte urea, and the salt ammonium sulfate. Two to three different concentrations of each osmolyte and salt were tested for their ability to reduce the infectivity of PPV (**Figure 3.2**). Cell viability was measured with the MTT assay. Reduction of cell viability can be caused by either the compound toxicity or the virus cytotoxicity. The MTT assay has been compared to a $TCID_{50}$ (i.e. visual inspection of cell death) for PPV cytotoxicity on PK-13 cells, and shown to be linearly correlated [10].

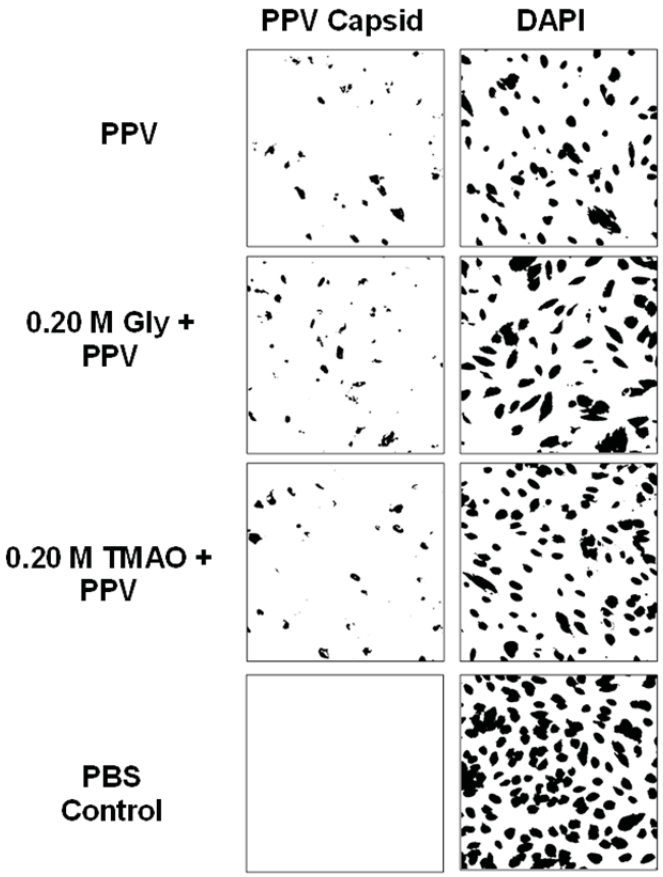

Figure 3.1. Binary images produced in ImageJ for immunohistochemistry analysis.

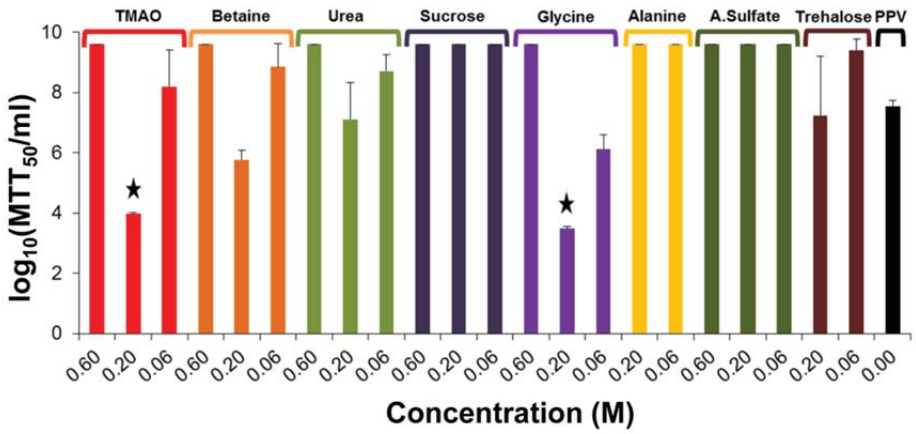

Figure 3.2. Antiviral activity of a panel of osmolytes and a salt. 7 $\log_{10}(MTT_{50}/ml)$ of PPV was used to infect cells in media with different osmolyte concentrations for five days. This was followed by the evaluation of virus cytotoxicity with the MTT assay. The stars represent the osmolytes with the greatest antiviral activity. The maximum detection limit of the assay was 9.6 $\log_{10}(MTT_{50}/ml)$. All data points are the average of three separate experiments and the error bars represent the standard deviation.

A value of 9.6 $\log_{10}(MTT_{50}/ml)$ indicates that none of the cells were viable. At all osmolyte concentrations tested, sucrose, alanine and ammonium sulfate were found to be toxic to the cells, and therefore no viable cells were detected (**Figure 3.2**). All other compounds tested, except for trehalose, demonstated compound toxicity at the highest concentrations tested. TMAO, betaine, urea, and glycine were all toxic at 0.60 M. They demonstrated antiviral activitiy at 0.20 M and then returned to near PPV control values at 0.06 M. For most compounds, this return to PPV control values is due to the compound being present at too low of a concentration to have antiviral activity. In the case of betaine and trehalose, the lowest concentration of osmolyte increased the virus titer above the control. It is possible that at low concentrations, the compounds increased and enhanced the virus' ability to infect the cells. This has been seen for other compounds [12].

TMAO and glycine at a concentration of 0.20 M showed a four log reduction, which is equal to 99.99% of infectious virus reduction. These two osmolytes had the greatest antiviral activity and were the focus of all subsequent work.

3.3.2. Osmolyte toxicity

Osmolyte toxicity was measured in PK-13 cells. As **Figure 3.3A** shows, the cells had a high rate of survival at low doses of osmolytes. Increasing the osmolyte concentration resulted in a decrease in cell survival, likely due to hypotonic lysis of the cells. TMAO exhibited a sigmoidal dose response with a calculated 50% inhibitory concentration (IC_{50}) of 0.37 ± 0.01 M. Glycine did not display a clear sigmoidal curve, but an IC_{50} value of 0.42 ± 0.02 M was calculated

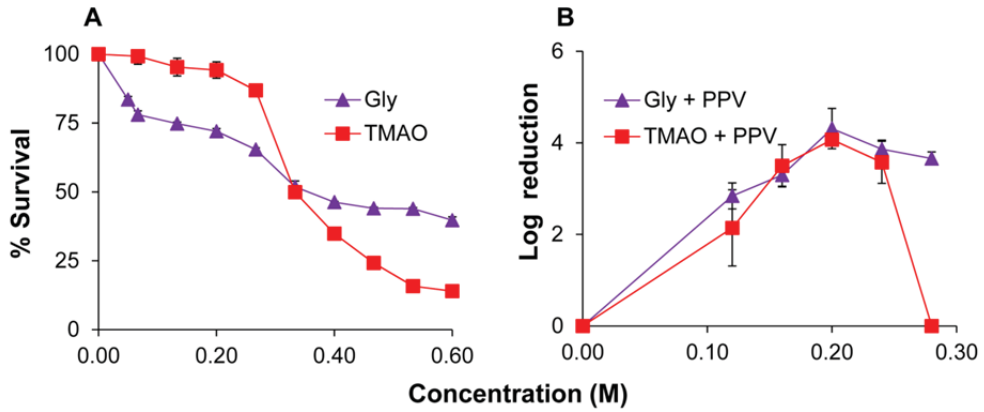

Figure 3.3. Osmolyte toxicity and reduction of virus cytotoxicity with different concentrations of TMAO and glycine. (A) Osmolyte toxicity on PK-13 cells, a susceptible host for PPV in the presence of glycine and TMAO, and (B) log reduction of PPV infectivity in the presence of various concentrations of glycine and TMAO. Cells and infected cells were exposed to osmolytes for five days, followed by evaluation of cytotoxicity with the MTT assay. The log reduction is defined in Eq. 3.1. and the % survival is defined in Eq. 3.2. All data points are the average of three separate experiments and the error bars represent the standard deviation.

3.3.3. Reduction of virus cytotoxicity with TMAO and glycine

The antiviral activities of various concentrations of TMAO and glycine were studied. Glycine and TMAO showed the highest log reduction, four logs (99.99%), at 0.20 M (22.29 mg/mL of glycine and 15.01 mg/mL of TMAO) (**Figure 3.3B**). The MTT Assay measures cell viability and this can be caused by compound toxicity or virus infectivity. At high or low osmolyte concentration, cell viability was reduced probably due to compound toxicity or virus infectivity.

Many antiviral compounds work by interrupting virus entry. However, some antiviral compounds work by interrupting replication. To determine if disruption of virus entry was the antiviral mechanism of the osmolytes, the time between virus infection and osmolyte addition was varied. When 0.20 M of TMAO or glycine was added at the initial stages of virus infection, a four log reduction in PPV infectivity was observed for both osmolytes (**Figure 3.4**). As the time between virus infection and osmolyte addition increased, the reduction in infectivity slowly decreased over time and approached one log reduction (90%) at 20 h (**Figure 3.4)**.

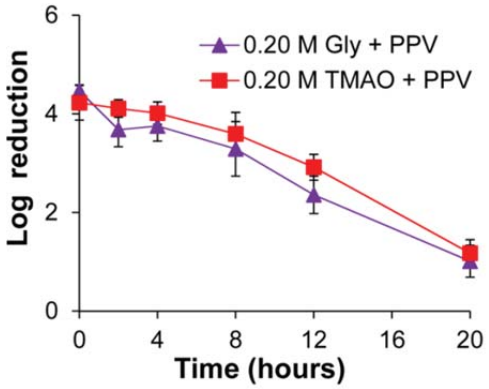

Figure 3.4. Reduction of virus cytotoxicity in the presence of osmolytes added post-virus infection. 0.20 M glycine and 0.20 M TMAO were added at different times post-virus infection and after five days, followed by evaluation of virus cytotoxicity with the MTT assay. The log reduction is defined in Eq. 3.1. All data points are the average of three separate experiments and the error bars represent the standard deviation.

Osmolytes are not likely disrupting virus entry because they were still effective when added 20 h post-virus infection. Treatment of the virus with osmolytes prior to infection did not reduce virus infectivity, shown in **Figure 3.5**. This demonstrates that the osmolytes are likely not disrupting virus attachment or entry.

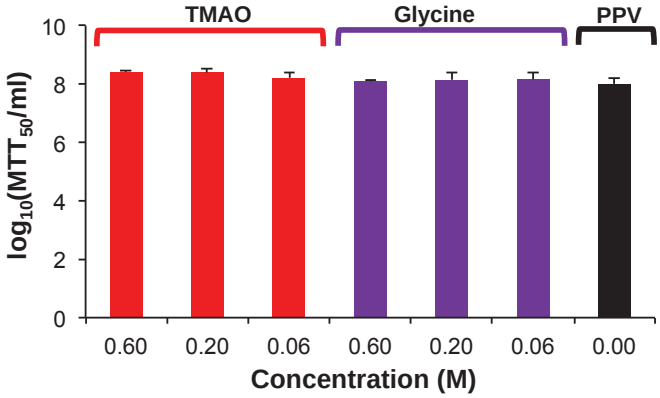

Figure 3.5. Pre-infection treatment of PPV with osmolytes. The virus and osmolytes were incubated for two hours prior to cell infection. The virus was incubated with cells for five days prior to evaluation with the MTT Assay.

3.3.4. Plaque reduction assay

A plaque reduction assay was performed to corroborate data from the cytotoxicity assay. A plaque assay was performed for 0.20 M TMAO and 0.20 M glycine and a >7 log reduction was

found. No plaques were formed in the presence of 0.20 M TMAO and glycine (**Table 3.1**).

Table 3.1. PPV plaque assay

Sample	Titer (pfu/ml)
PPV	$5.5 \times 10^7 \pm 1.1 \times 10^7$
0.2 M glycine + PPV	ND*
0.2 M TMAO + PPV	ND*

*not detected

3.3.5. Impact of osmolytes on infectious virus yield

The presence of intracellular and extracellular viable virus particles was examined. This was studied under two different osmolyte treatments. In Treatment 1, the osmolytes were added at the beginning of the infection cycle and in Treatment 2, they were added five minutes before sample collection. Treatment 2 was done in order to determine if the addition of osmolyte after infection would affect the MTT assay. As shown in **Figure 3.6**, there was no difference between Treatment 2 and the control (i.e. virus infection with no osmolytes addition).

With Treatment 1, there appeared to be little infectious virus extracellularly (**Figure 3.6A**) or intracellularly (**Figure 3.6B**), even after 32 h. Without osmolyte addition, infectious virus began to appear extracellularly after 15 h and intracellularly after 10 h. This is consistent with detection of an increase in DNA at about 8 h post infection [9]. After 32 h, 0.2 M TMAO and glycine had a 3.5 ± 0.5 log and 4.2 ± 0.5 log reduction extracellularly, respectively, as compared to the PPV control, and a 2.9 ± 0.2 log and a 3.0 ± 0.5 log reduction intracellularly, respectively. This is consistent with a >4 log reduction after five days incubation, as shown in **Figure 3.4.**

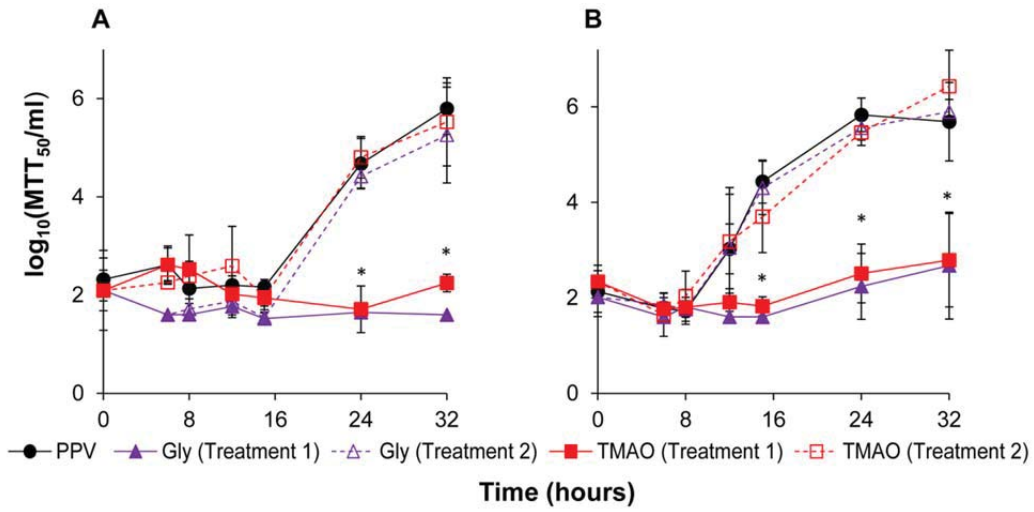

Figure 3.6. Impact of osmolytes on infectious virus yield. (A) Extracellular, and (B) intracellular infectious virus yield. Osmolytes were added at the time of infection (Treatment 1) or 5 min before sample collection (Treatment 2). The yield of infectious virus particles was determined with the MTT Assay. The minimum limit of detection of the assay was 1.6 log10(MTT50/ml). All data points are the average of three separate tests and the error bars represent the standard deviation. Student's t-test was used to evaluate the statistical significance between PPV infected cells containing osmolytes for Treatment 1 and PPV infected cells without osmolytes. *p value of <0.05.

3.3.6. Virus capsid protein production with the addition of osmolytes

It was explored with immunohistochemistry if the virus capsid proteins were being produced within the cells. PPV capsid proteins were found in all samples infected with PPV, including those containing 0.20 M TMAO and 0.20 M glycine (**Figure 3.7A**). PBS without virus was used as a negative control, and did not show any virus capsid protein formation. TMAO and glycine were also tested without virus present and no virus capsid protein formation was detected (data not shown). In **Figure 3.7B**, fluorescence per cell started to increase after 8 h, and this agrees with virus production data (**Figure 3.6B**) and DNA production data [9], although we did not specifically test if PPV DNA was produced. Similar trends were observed with PPV and osmolytes (**Figure 3.7B**). These results show that viral capsid proteins are produced (**Figure 3.7B**), but viable virus particles are not produced (**Figure 3.6**).

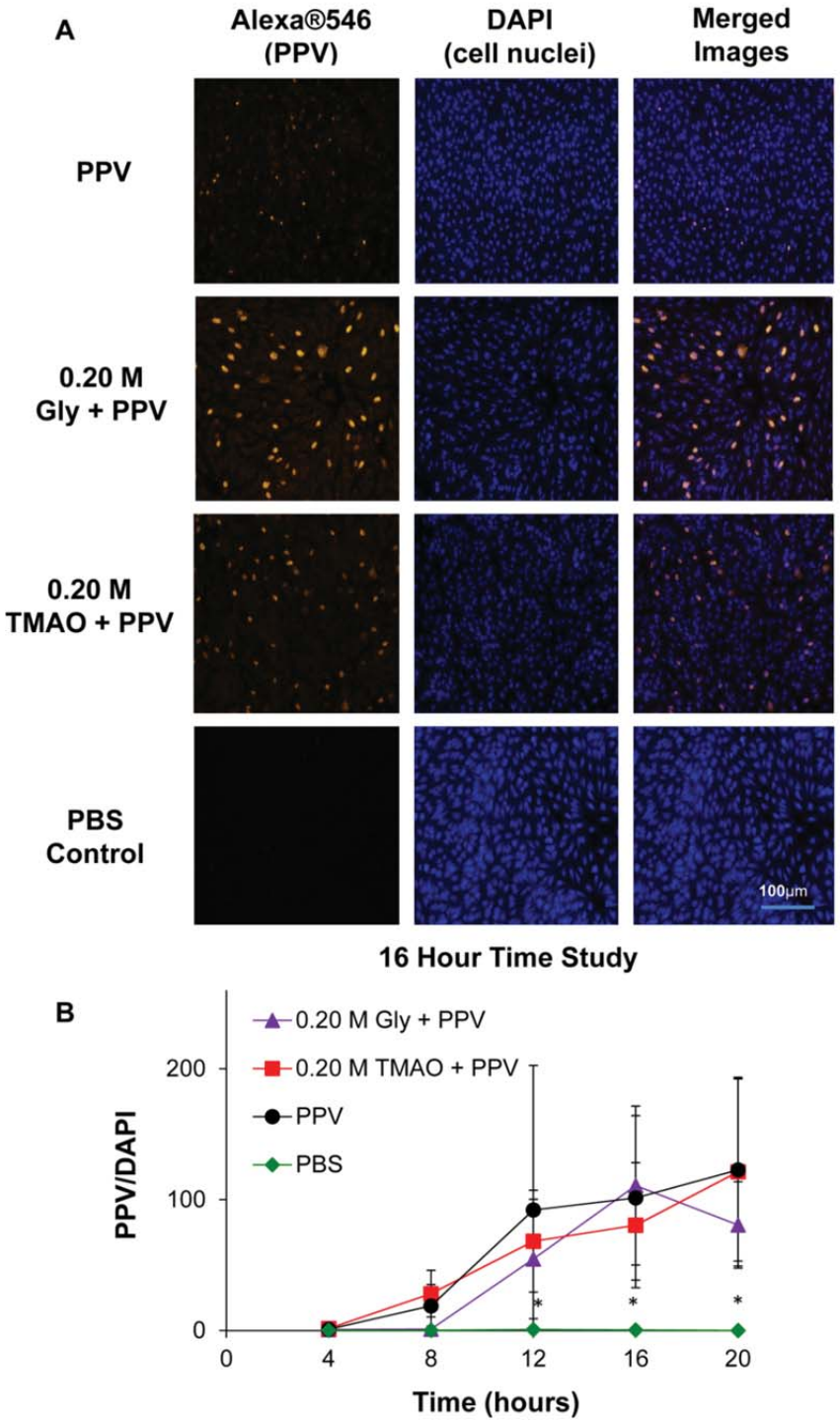

Figure 3.7. Impact of osmolytes on virus capsid protein formation. (A) Images of PPV and PBS mock infected cells at 16 h, and (B) the ratio of the fluorescence of PPV capsid proteins to the count of DAPI. All data points are the average of three independent slides with 10 images per slide and the error bars represent the standard deviation. TMAO and glycine were tested in order to determine osmolytes cross-reactivity with antibodies and no virus capsids were found (data not shown). Student's t-test was used to evaluate the statistical significance between cells infected with PPV (with or without osmolyte) and the PBS negative control. *p value of <0.05

3.4. Discussion

Antiviral compounds are known to disrupt virus entry, replication, assembly of virus particles, or a combination of these. Antiviral compounds that destroy the enveloped capsid of a virus are required in small quantities [13]. Other compounds are able to alter the virus-host interactions and are required in higher quantities [14]. The advantage of natural compounds that alter the virus-host interactions is that they can be applied post-infection. In this study, two osmolytes, TMAO and glycine, demonstrated this behavior and are therefore promising candidates for therapeutic drugs. TMAO and glycine are both small (molecular weight of 75 g/mol), zwitterionic species. More work will need to be done to determine why these osmolytes, compared to the many that were tested (**Figure 3.2**) demonstrated antiviral activity.

The fact that virus capsid proteins were produced in the presence of osmolytes (**Figure 3.7**) strongly suggests that osmolytes work post-virus infection (**Figure 3.4 & 3.6**). We hypothesize that osmolytes are disrupting capsid assembly by stabilizing viral capsid proteins and preventing the assembly process. Osmolytes stabilize proteins by causing a preferential hydration around proteins [5]. TMAO has also been shown to preferentially order water molecules around α-chymotrypsin and to stabilize the enzymatic activity of the protein [15]. This preferential hydration causes proteins to adapt a compact configuration [16]. It is likely that this compact configuration of the VP2 protein in PPV is not able to self-assemble into a virus capsid. The protein capsids of other parvoviruses have been studied and their assembly is likely to be kinetically stable, but not thermodynamically stable [17]. This may demonstrate that the osmolytes thermodynamically stabilize the capsid proteins, therefore reducing their propensity to kinetically assemble. The other possibility is that the high osmolyte concentration in the cells reduces the activity of the assembly machinery that guides the DNA inside the virus capsid. However, enzymes are usually stabilized and still have activity in a solution of osmolytes [15], therefore, we hypothesize that the osmolytes are disrupting the ability of the capsid proteins to self-assemble.

3.5. Conclusions

A variety of osmolytes were screened to find antiviral compounds against PPV, a small, non-enveloped, single-stranded DNA virus [18]. We have discovered that two protecting osmolytes, TMAO and glycine, at 0.20 M, reduce the infectivity of PPV by four logs (99.99%). These results are consistent with the literature, since high quantities of antiviral compounds are often required for the inactivation of non-enveloped viruses. Both osmolytes showed antiviral activity after being added 20 h post-infection. In the presence of TMAO or glycine, infected cells produce virus capsid proteins, but not infectious viable virus particles. We propose that the osmolytes TMAO and glycine interfere with the virus capsid formation and are potential candidates for therapeutic drugs.

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Chapter 4

Purification of porcine parvovirus with osmolytes flocculation, followed by microfiltration[1]

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4.1. Introduction

More than 90% of all human diseases are caused by viral infections [19]. However, viruses do not only cause disease; they can be modified into viral vaccines or viral vectors to prevent or treat diseases [20]. Many viral diseases, like yellow fever and polio, are under control due to the development of effective vaccines [21]. Treatment of a wide variety of diseases, such as cancer and Alzheimer's disease, will benefit from future viral gene therapy vectors [22-24]. The World Health Organization (WHO) reported in 2010 that the vaccine market growth rate is 10-15% per year, higher than the growth rate of other pharmaceuticals at 5-7% per year [25]. Since there is a large market for viral vaccines, a more efficient method to manufacture and purify viral particles is needed. Lyddiatt and Sullivan estimated that between 10^{11} and 10^{14} viral vectors will be required for a single dose of gene therapy vectors [26]. Due this large dosage, an efficient, cost-effective, and quick process will be required to satisfy the viral vector market demand.

Conventional virus purification techniques, like ultracentrifugation and density gradient centrifugation, have been used to isolate viruses at the laboratory scale [27, 28]. However, due to the size of the equipment, the difficult scale-up, long processing times, and low purity, ultracentrifugation remains cost-ineffective for large-scale vaccine production [29-31].

Alternatives such as filtration and chromatography have been used to overcome the disadvantages of ultracentrifugation. Tangential flow filtration (TFF) has effectively recovered and purified virus particles [32, 33]. TFF is successful due to the large size difference of virus particles and typical protein contaminants. The main advantages of TFF are easy scalability, high throughput, high viral titer and high viral recoveries [34-36]. However, the capacity of the membranes can be affected by fouling and this can lead to longer filtration times, low flux through the membrane and high transmembrane pressures [37, 38]. Additionally, a platform approach for virus filtration does not exist, since the success of current processes depend on the properties of individual viral therapeutics [39].

Chromatography is the predominant technique for large-scale virus purification [27, 40].

All of the typical chromatography modes have been utilized for virus particle purification [41]. The more successful chromatographic purification processes for viruses are combined with size-based separation methods [27, 38]. However, a universal chromatography process cannot be applied to all the viruses, since the operating conditions depend on the charge, size, and specificity of the target virus [35, 42]. Moreover, conventional chromatography resins are designed for the purification of proteins, but not for large biomolecules, such as virus particles. Virus particles have difficulties accessing the high internal surface area of the resins [43, 44], making the resins highly inefficient. Membrane chromatography has shown promise to overcome many of the disadvantages of pore accessibility in resin chromatography [45, 46]. However, there still remains the difficulty of optimizing the binding and elution conditions for each viral product without causing virus inactivation.

Precipitation and flocculation followed by separation using centrifugation or filtration have been used as an alternative for virus purification [38]. Commonly, the additives are compounds that promote aggregation, like salts or polymeric agents [30]. Salts and polymers often precipitate all proteins in solution, although there have been reports of selective precipitation with these agents. Ammonium sulfate at specific conditions has been shown to selectively precipitate immunoglobulin G (IgG) over bovine serum albumin (BSA) [47] likely due to the hydrophobicity difference of the two proteins [48]. Salts have been used to precipitate viruses from protein-free solutions [49-51]. Polyethylene glycol (PEG) is well-known to precipitate all proteins [52], although specific conditions have been found to selectively precipitate IgG4 from cell culture [53] and viruses from DNA and protein contaminants [54] using PEG. However, PEG precipitation requires extended periods of incubation time [55] and polymer additives may interact with virus particles and form complexes that are difficult to dissociate [56, 57].

The aim in virus flocculation is to improve process robustness; a selective, global, nontoxic, and economical flocculant is desired [41]. In this study we propose to use osmolytes to overcome these challenges. Osmolytes are found in the cells of many organisms, and their main goal is to stabilize intracellular proteins against environmental stresses, such as extreme temperatures or high osmotic pressures, by changing the water content of the cells [58-60].

There are two types of osmolytes, protecting and denaturing. Protecting osmolytes have the ability to fold proteins by binding to the water molecules and changing the water contents around the protein backbone. Denaturing osmolytes have the ability to unfold the proteins, by binding directly to the protein backbone [61]. One advantage of osmolytes, such as glycine, alanine and mannitol as compared to salt or polymer flocculants, is that they are often used as an excipient to stabilize the final formulation of biotherapeutics [62, 63], making them an ideal addition to a biotherapeutic manufacturing process.

Porcine parvovirus (PPV), a non-enveloped, single-strand DNA, icosahedral virus, with a diameter of 18 - 26 nm [8, 19] was used to demonstrate the ability of osmolytes to preferentially flocculate virus particles. We propose that osmolytes bind to water, thus leading to the reduction of a hydration shell around the virus and causing PPV particle aggregation. Using high-throughput screening methods, we have discovered that osmolytes flocculate PPV and demonstrate a >80% removal with a 0.2 μm filter with <5% removal of model host cell proteins. This micropore filter is usually used to retain bacteria, and therefore this is a unique application of microfiltration for small virus particle removal and future purification.

4.2. Materials and methods

4.2.1. Materials

The osmolytes, trimethylamine N-oxide (TMAO) dihydrate, glycine, betaine, D-alanine, D-arginine, L-proline, L-serine, D-mannitol, sucrose, D-(+)-trehalose dihydrate, D-(+)-raffinose pentahydrate, and urea, and the salts, ammonium sulfate, sodium chloride (NaCl), magnesium sulfate, and magnesium chloride were purchased from Sigma-Aldrich (St. Louis, MO) at a minimum purity of 98%. Sodium hydroxide (NaOH) and PEG, with a molecular weight (MW) of 12,000 Da, and albumin from bovine serum (BSA) were also purchased from Sigma-Aldrich (St. Louis, MO). Tris (hydroxymethyl) aminomethane hydrochloride (Tris) was purchased from Thermo Scientific (Waltham, MA). MTT Assay reagents, thiazolyl blue tetrazolium bromide (MTT), sodium dodecyl sulfate (SDS) and hydrochloric acid (HCl) were purchased from VWR (Radnor, PA). Cell culture reagents, phosphate-buffered saline (PBS, pH 7.2), 0.25%

trypsin/EDTA, 1% penicillin/streptomycin (pen/strep), and minimum essential medium (MEM) were purchased from Life Technologies (Carlsbad, CA). Lysozyme, chicken egg white was purchased from EMD Millipore (Billerica, MA). All the solutions were prepared with NanoPure water (Thermo Scientific, Waltham, MA) to a resistance >18 MΩ, or PBS and filtered with either a 0.2 µm syringe filter (Nalgene, Rochester, NY) or a Millipore 0.2 µm bottle top filter (Billerica, MA) prior to use.

4.2.2. Cell propagation, virus production and titration

Porcine kidney (PK-13) cells were a gift from Dr. Ruben Carbonell at North Carolina State University. PK-13 cells were grown at 37°C, 5% CO_2 and 100% humidity in MEM supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA) and 1% pen/strep, as described earlier [10].

PPV strain NADL-2 was a gift from Dr. Ruben Carbonell at North Carolina State University and was propagated as described previously [10]. Briefly, PK-13 cells were seeded 24 h prior to inoculation with 1 ml of PPV at 10^3 MTT/ml diluted in PBS, 3% FBS and 1x pen/strep. After 1.5 h, 9 ml of fresh media was added and the infected cells were incubated for 5 days. Flasks were frozen, scraped, and clarified. The clarified virus solution was stored at -80°C.

PPV was titrated with a colorimetric cell viability assay, MTT assay, which has been described previously [64]. The MTT assay measures enzymatic activity in active mitochondria and can be used to quantify cytopathic effect of PPV in PK-13 cells. Briefly, PK-13 cells were seeded in 96-well plates. After 24 h, each plate was infected with PPV in quadruplicate and 5-fold serial dilutions. After five days, the MTT solution was added, followed by solubilizing agent 4 h later. After 18 - 24 h, plates were read on a Synergy Mx microplate reader (BioTek, Winooski, VT) at 550 nm. The 50% infectious dose (MTT_{50}) value was defined as the virus dilution that achieved 50% of the uninfected cell absorbance. The value was determined on a per milliliter basis and stated as the MTT_{50}/ml titer [10].

4.2.3. Virus flocculation and filtration

To flocculate PPV, the clarified virus was diluted 1:100 in PBS to a final concentration of 10^6MTT/ml, then 30 µl was mixed with720 µl of osmolytes solutions in NanoPure water for 2 h at room temperature, in triplicate. As a control, NanoPure water was incubated with PPV. Typically, the pH was not controlled. However, it was checked to make sure that the pH remained consistent for each osmolyte over multiple trials. For the pH studies, the pH was adjusted by addition of HCl or NaOH. Flocculated virus particles were filtered in a high throughput manner with either a 0.2 µm 96-well filter plate (Millipore, Billerica, MA) or a 300 kDa filterplate (Seahorse Bio, North Billerica, MA) at 200 × *g* and 4°C for 20 min (40 min for the 300 kDa plate) in a Sorvall ST16R Centrifuge (Thermo Scientific, Pittsburgh, PA). Virus samples before and after filtration were titrated as described in **Section 4.2.2**. Log reduction value (LRV) and % removal were calculated with **Eq. 4.1** and **4.2**, respectively,

$$LRV = -log\left(\frac{c_f}{c_i}\right) \quad \textbf{(4.1)}$$

$$\%\ Removal = \left[1 - \left(\frac{10^{c_f}}{10^{c_i}}\right)\right] \times 100 \quad \textbf{(4.2)}$$

where c_f is the concentration of infectious virus after filtration and c_i is the concentration of infectious virus before filtration.

4.2.4. Shear stress effect

Shear stress was generated on PPV aggregates at different glycine concentrations. Low shear effect was generated with manual rotation for 2 h, medium and high shear effect were produced with a vortex, at low and high speed, respectively, for 2 h. High-low shear effect was generated with 2 h at the vortex followed by manual rotation for 2 h. Virus samples were flocculated and filtered as described in **Section 4.2.3**. Samples before and after filtration were titrated as described in **Section 4.2.2** and % removal was calculated with **Eq. 4.2**.

4.2.5. Ionic strength effect

The ionic strength effect on PPV flocculation was tested with the addition of NaCl to alanine and mannitol solutions. The ionic strength (I) was calculated with **Eq. 4.3**,

$$I = \frac{1}{2} \times \sum_{i=1}^{n} c_i z_i^2 \quad \textbf{(4.3)}$$

where c_i is the concentration of ion i, z_i is the charge of ion i. Alanine is a zwitterionic molecule, while mannitol is a neutral molecule. For alanine, two different values of ionic strength were calculated. In both cases the experiment was the same, 1.0 M alanine had the same concentrations of NaCl, ranging from 0.2 M to 0.6 M, added to it. However, different calculations of ionic strength were applied. In one case, we assumed that alanine did not contribute to the ionic strength and in the other case, we assumed that alanine did contribute. Mannitol was considered to not contribute to ionic strength. For mannitol two sets of experiments were carried out, 0.2-0.6 M NaCl and 1.2-1.6 M NaCl was added to the 1 M mannitol solution. The lower NaCl concentrations in mannitol were compared to alanine assuming it did not contribute to ionic strength and the higher NaCl concentrations in mannitol were compared to alanine assuming it did contribute. Virus samples were flocculated and filtered as described in **Section 4.2.3**. Samples before and after filtration were titrated as described in **Section 4.2.2** and % removal was calculated with **Eq. 4.2**.

4.2.6. Protein flocculation and filtration

BSA and lysozyme were diluted in PBS to a final concentration of 20 mg/ml. 30 µl of protein solutions were mixed with 720 µl of 1 M of mannitol or 1 M of alanine. After 2 h of incubation at room temperature, flocculated proteins were filtered as described in **Section 4.2.3**. Protein absorbance before and after filtration was measured on a Synergy Mx microplate reader at 280 nm. % Protein removal was calculated with **Eq. 4.4**,

$$\%Protein\ removal = \left(\frac{P_i - P_f}{P_i}\right) \times 100 \quad \textbf{(4.4)}$$

where P_f is the concentration of protein solution after filtration, and P_i is the concentration of protein solution before filtration.

4.3. Results and discussion

4.3.1. PPV flocculation in the presence of osmolytes, salts, and PEG

A variety of osmolytes were screened for their ability to flocculate PPV **(Figure 4.1)**. In

addition, salts and PEG were used as positive controls and Tris buffer, PBS, and water were used as negative controls, since they have not been shown to flocculate proteins or virus particles. Osmolytes were chosen because amino acids, a type of osmolyte, have been shown to flocculate the parvovirus B19 [65]. Salts were considered a positive control because salts have been used to flocculate virus particles such as enteroviruses and retroviral vectors [66, 67]. PEG has been used as a flocculent to recover virus particles [10, 68] and was also considered a positive control.

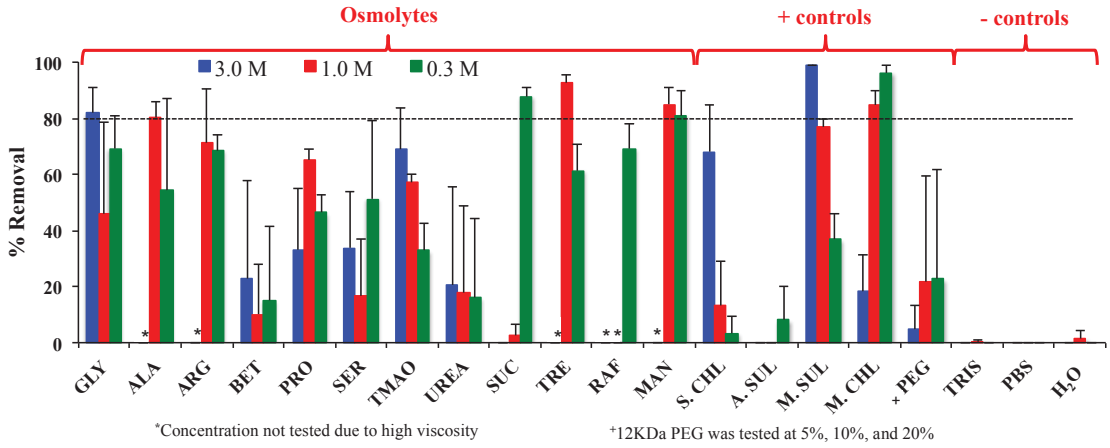

Figure 4.1. High-throughput screening of virus flocculants. A variety of osmolytes were compared to the positive control salts and PEG and the negative controls, Tris, PBS, and water for flocculation of PPV using a 0.2 µm micropore filter. 80% removal was used as the cut-off to pursue testing. % removal is defined in Eq. 4.2. All data points are the average of three separate experiments and the error bars represent the standard deviation.

The protecting osmolytes glycine, alanine, sucrose, trehalose and mannitol flocculated PPV, and demonstrated a >80% removal with a 0.2 µm filter (**Figure 4.1**). This large micropore filter is usually used for bacterial removal, and not for small viruses [69], such as PPV with a diameter of 18 - 26 nm [19]. Salts such as magnesium sulfate and magnesium chloride presented a high virus removal (>80%) (**Figure 4.1**) as compared to ammonium sulfate and sodium chloride. This is likely due to the fact that Mg^{2+} is a strongly hydrated cation, which decreases the protein and virus solubility and promotes aggregation. Whereas, the NH^{4+} and Na^{+} ions are weakly hydrated cations, which are known to increase protein solubility and destabilize aggregates. In PEG samples, virus removal was low compared to other positive controls and

many osmolytes (**Figure 4.1**), likely because PEG precipitation requires longer incubation times and lower temperatures than those tested here [55].

We confirmed that the reduction in virus titer was due to virus filtration and not virus inactivation in the osmolyte and control solutions tested (**Table 4.1)**.

Table 4.1. LRV compared to water control

Flocculant	$\log_{10}(MTT_{50}/ml)$	LRV[a]
3.0 M GLY	4.72 +/- 0.43	0.00
1.0 M GLY	4.96 +/- 0.16	0.00
0.3 M GLY	5.49 +/- 0.17	0.00
1.0 M ALA	5.00 +/- 0.61	0.00
0.3 M ALA	5.73 +/- 0.17	0.00
1.0 M ARG	5.13 +/- 0.44	0.00
0.3 M ARG	5.56 +/- 0.87	0.00
3.0 M BET	4.75 +/- 0.30	0.00
1.0 M BET	4.67 +/- 0.30	0.00
0.3 M BET	4.48 +/- 0.15	0.17
3.0 M PRO	4.65 +/- 0.58	0.00
1.0 M PRO	4.77 +/- 0.04	0.00
0.3 M PRO	5.00 +/- 0.45	0.00
3.0 M SER	4.41 +/- 0.06	0.23
1.0 M SER	4.52 +/- 0.22	0.12
0.3 M SER	4.55 +/- 0.03	0.09
3.0 M TMAO	5.34 +/- 0.11	0.00
1.0 M TMAO	5.21 +/- 0.33	0.00
0.3 M TMAO	5.11 +/- 0.46	0.00
3.0 M UREA	4.46 +/- 0.73	0.19
1.0 M UREA	4.08 +/- 0.12	0.57
0.3 M UREA	3.35 +/- 0.45	**1.30**[b]
1.0 M SUC	4.47 +/- 0.13	0.18
0.3 M SUC	4.67 +/- 0.13	0.00
1.0 M TRE	4.60 +/- 0.27	0.05
0.3 M TRE	4.62 +/- 0.34	0.02
0.3 M RAF	4.77 +/- 0.18	0.00
1.0 M MAN	4.62 +/- 0.39	0.02
0.3 M MAN	5.38 +/- 0.47	0.00
3.0 M S. CHL	4.69 +/- 0.35	0.00
1.0 M S. CHL	4.52 +/- 0.16	0.12
0.3 M S. CHL	4.46 +/- 0.21	0.18
3.0 M A. SUL	3.42 +/- 0.04	**1.22**[b]
1.0 M A. SUL	3.10 +/- 0.10	**1.55**[b]
0.3 M A. SUL	2.46 +/- 0.04	**2.18**[b]
3.0 M M. SUL	3.92 +/- 0.10	0.72
1.0 M M. SUL	5.13 +/- 0.31	0.00
0.3 M M. SUL	4.46 +/- 0.56	0.19
3.0 M M. CHL	4.51 +/- 0.76	0.14
1.0 M M. CHL	4.94 +/- 0.34	0.00
0.3 M M. CHL	4.65 +/- 0.20	0.00
20% PEG	4.20 +/- 0.45	0.45
10% PEG	4.56 +/- 0.38	0.08
5% PEG	4.78 +/- 0.24	0.00
TRIS	4.58 +/- 0.28	0.07
PBS	4.11 +/- 0.59	0.53
H_2O	4.65 +/- 0.24	0.00

[a]LRV was calculated with Eq. 4.1, where c_f is the concentration of infectious virus in the presence of the flocculant and c_i is the concentration of infectious virus in the presence of water. [b]Flocculants that reduced the virus titer by > 1 LRV

This was done by testing the virus titer of each flocculating solution prior to filtration and comparing to the virus titer of the water control solution. All tested concentrations of ammonium sulfate and 0.3 M urea reduced the virus titer by > 1 LRV, inactivating the virus prior to filtration (see **Table 4.1**). None of the other osmolytes or salts tested inactivated the virus. With evidence that virus flocculation with osmolytes, followed by microfiltration could be a potentially new virus purification process we pursued the evaluation of osmolytes to a greater extent.

The osmolytes that were able to remove PPV with a 0.2 µm filter were also tested with a nanopore filter, 300 kDa MWCO, which has an approximately 0.03 µm pore size. The LRV was higher at 1.0 M for the all the osmolytes tested, except for glycine and sucrose with both filters (**Figure 4.2A & B**).

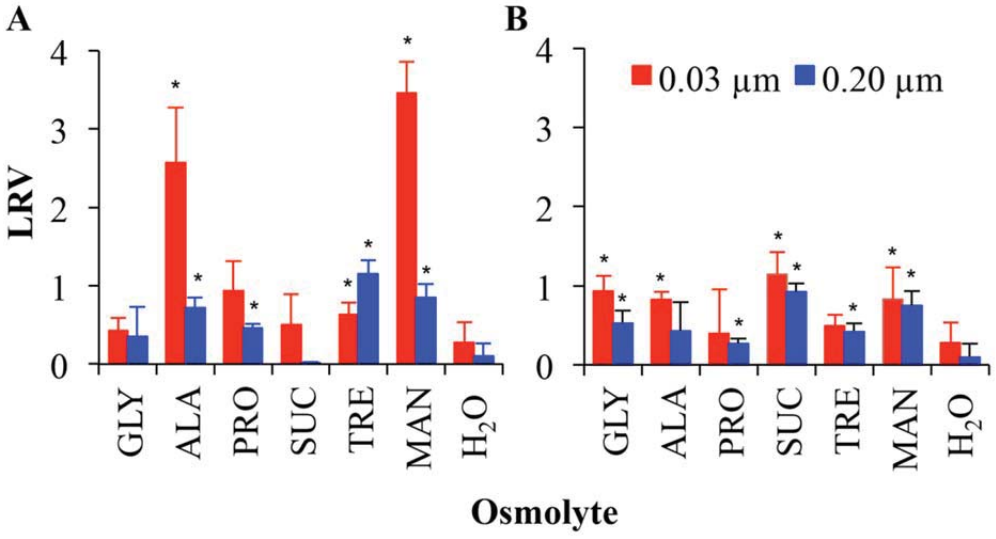

Figure 4.2. Effect of pore size and osmolyte concentration on log reduction value (LRV). Osmolyte concentrations of (A) 1.0 M and (B) 0.3 M. LRV is defined in Eq. 4.1. All data points are the average of three separate experiments and the error bars represent the standard deviation. **p* value of <0.05 as compared to the water control using Student's t-test.

These results indicate that the concentration of osmolytes required to achieve high percent of virus removal may differ from osmolyte to osmolyte. Alanine and mannitol filtered with a 300 kDa filter at a concentration of 1.0 M showed a 2.6 LRV (99.75% removal) and 3.5 LRV (99.97% removal), respectively (**Figure 4.2A**). A 3.5 LRV is close to the 4 LRV minimum requirement by the Food and Drug Administration (FDA) [70] and the Environmental Protection

Agency (EPA) [71] for any virus removal step process. This indicates that additional optimizations of this system may determine that mannitol addition could allow larger pore sized filters to remove parvoviruses from FDA regulated manufacturing processes.

4.3.2. Qualitative shear stress effect on PPV flocculation

The effect of shear stress on PPV aggregates was tested at different glycine concentrations. Low, medium and high shear stress were applied to the formation of aggregates. As shown in **Figure 4.3**, virus removal under high shear stress was very low (<12%) at all the concentrations tested, indicating that the virus aggregates are fragile and can be broken by shear stress. Due the fact that the aggregates are fragile, it is likely that tangential flow filtration could break the aggregates. Therefore, dead-end microfiltration will need to be used after the virus flocculation step. As shear stress decreased, virus removal increased.

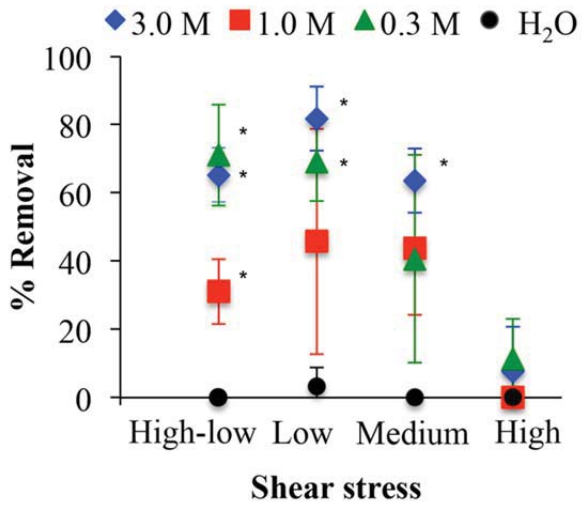

Figure 4.3. Qualitative effect of shear stress on PPV flocculation. PPV was flocculated with glycine. % removal is defined in Eq. 4.2. All data points are the average of three separate experiments and the error bars represent the standard deviation. *p value of <0.05 as compared to the water control using Student's t-test.

The highest virus removal (82%) was achieved at low shear stress with 3.0 M of glycine (**Figure 4.3**). We found that PPV aggregates can be recovered after high shear stress followed by low shear stress, as shown by a 66% removal by 3.0 M glycine (**Figure 4.3**). Glycine at 3.0 M and 0.3 M showed higher virus removal than 1.0 M of glycine, indicating that virus flocculation

with glycine may work as a salting in-salting out process, where optimal conditions for aggregation are found at low and high concentration of the osmolyte.

4.3.3. pH effect on PPV flocculation

Alanine, glycine, mannitol and trehalose were used to explore the pH effect on PPV flocculation. Since PPV has been found to be stable in the pH range 3 - 9 [72], the pH was explored between 5 - 8 (**Figure 4.4**). Osmolyte pH was changed in order to change the charge of the virus and increase virus aggregation. The isoelectric point (pI) of parvovirus capsid has been tested and found to be ~5 [73]. When the pH of the zwitterionic amino acids solutions was close to the pI of the virus, virus removal was improved (**Figure 4.4A**). In the presence of zwitterionic osmolytes, the neutrally charged virus prefers to aggregate. As the virus becomes negatively charged above its pI, the virus surface experiences electrostatic repulsion. The electrostatic repulsion between the charged virus particles likely decreases virus aggregation. The increase in virus charge leads to greater interactions with water molecules, which tend to increase solubility. The neutral compounds, mannitol and trehalose, did not present a pH dependence behavior (**Figure 4.4B**), as shown for the zwitterionic amino acids.

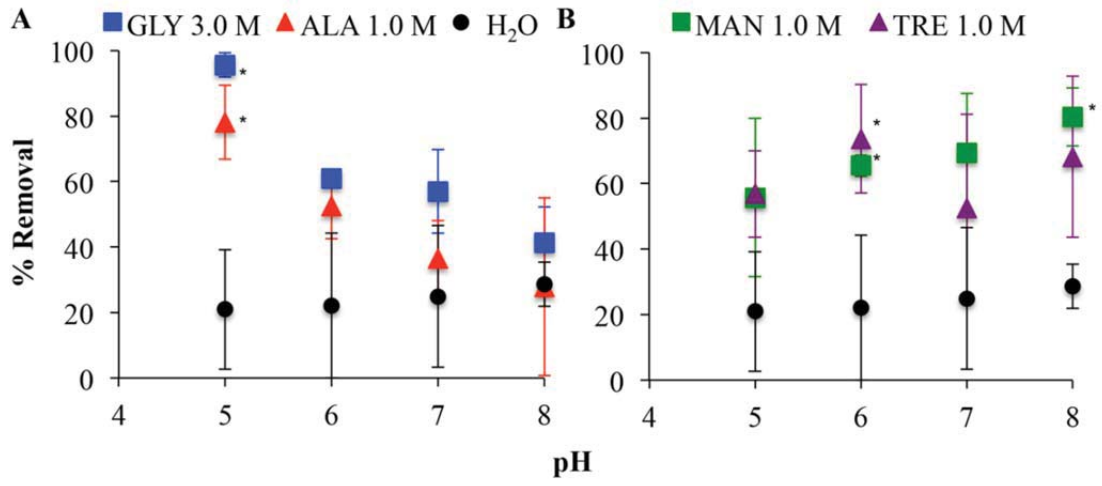

Figure 4.4. pH effect on PPV flocculation with a micropore filter. % removal is defined in Eq. 4.2. All data points are the average of three separate experiments and the error bars represent the standard deviation. **p* value of <0.05 as compared to the water control using Student's t-test.

Virus aggregation can be affected by different interactions of the virus with water and with the osmolytes. These interactions include charge-charge, hydrogen-bonding, and hydrophobic interactions. It has been found that sugars are able to fold proteins by a preferential exclusion model [74]. Sugars are excluded from the neighboring areas close to the proteins, leading to a preferential hydration shell around proteins and a strong inclination for hydrogen-bonding [75]. It is likely that the changes in pH in the zwitterionic osmolyte solutions increase the charge-charge repulsion of the virus particles and altered the water structure around the virus. However, the strong hydrogen-bonding interactions created by the addition of sugars and sugars alcohol cannot be affected by the changes in pH.

4.3.4. Ionic strength effect on PPV flocculation

Alanine and mannitol were used to study the ionic strength effect on PPV flocculation. These two osmolytes were chosen to test the difference between a zwitterionic molecule and a neutral molecule. There is not clear evidence in the literature determining if zwitterionic molecules contributed to ionic strength [76-79], therefore we compared alanine and mannitol assuming alanine did and did not contribute to ionic strength. For alanine, the same set of data was plotted twice (**Figure 4.5**), once assuming alanine did not contribute to the ionic strength and once assuming alanine did contribute to the ionic strength. For alanine, virus removal decreased as ionic strength increased. For mannitol, virus removal from 0.2 to 0.6 M NaCl did not display a clear trend. In the range of 1.2–1.6 M of NaCl, virus removal decreased in a dose dependent manner (**Figure 4.5**). The osmolytes, alanine and mannitol were viscous solutions at the concentration tested (1.0 M), due to the strong interactions between the solute and the solvent, leading to high solute particle movement resistance [80]. Disaccharides and monosaccharides have a tendency to increase the viscosity of protein solutions; however, the viscosity can be reduced with the addition of salts [75]. We also show that a small amount of NaCl added to a 1.0 M mannitol solution greatly decreased the virus removal and aggregation. The decrease in viscosity could play a role in this aggregation tendency (**Figure 4.5**).

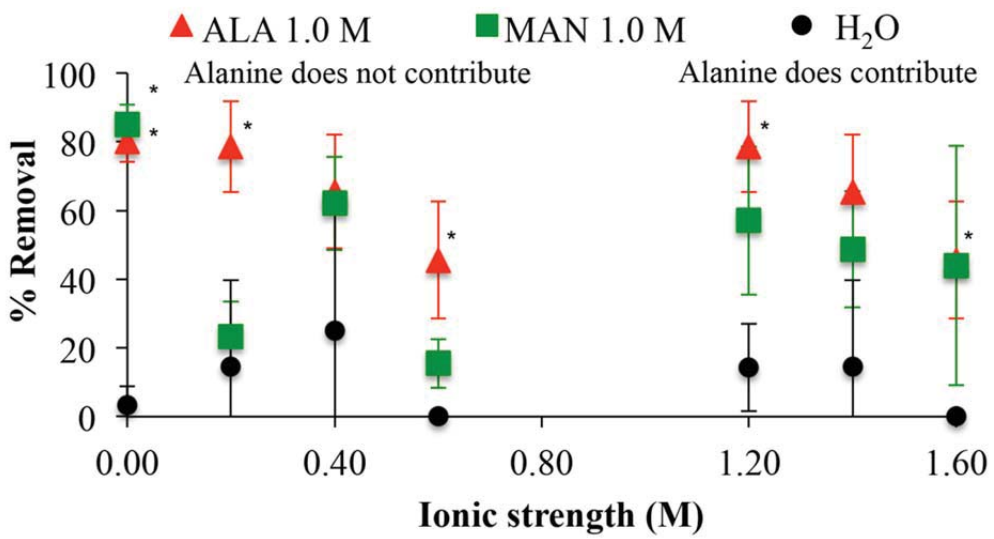

Figure 4.5. Ionic strength effect on PPV flocculation with a micropore filter. Two assumptions were used to test the ionic strength effect of zwitteric alanine. Ionic strength range of 0.0 - 0.6 M shows 1 M mannitol with the addition of 0 – 0.6 M NaCl, and alanine with the addition of 0 - 0.6 M NaCl and the assumption that alanine did not contribute to ionic strength. Ionic strength range of 1.2 - 1.6 M shows 1 M mannitol with the addition of 1.2 – 1.6 M NaCl, and alanine with the addition of 0 - 0.6 M NaCl and the assumption that alanine did contribute to ionic strength. All data points are the average of three separate experiments and the error bars represent the standard deviation. *p value of <0.05 as compared to the water control using Student's t-test.

Stellwagen and coworkers gave experimental evidence that zwitterions do not contribute to ionic strength. They studied the effect of the zwitterion tricine in capillary electrophoresis. The mobility of double-stranded DNA was expected to decrease with the square root of ionic strength, however, the mobility of the DNA was independent of the concentration added of the zwitterion tricine [78]. The study suggested that tricine does not contribute to the ionic strength of a solution. However, other groups have looked at the charge distribution of zwitterions and assumed that zwitterions contribute to the ionic strength without experimental evidence [79]. Comparing the two sets of data, mannitol had the same trend as alanine when alanine was assumed to contribute to ionic strength (**Figure 4.5**). This is weak evidence that alanine does contribute to the ionic strength since it took a higher salt concentration for mannitol to have a similar trend as alanine. The evidence is weak because the virus removal with a change in pH has different trends for alanine and mannitol (see **Figure 4.4**), so we do not have a basis to propose that the trends for ionic strength should be the same. Further studies will be done to

clarify if alanine contributes to ionic strength of a virus solution. It has been proposed that interactions between salts and amino acids in aqueous systems are affected by the nature of the salt and the length of the hydrocarbon chain of the amino acids [81]. It is probable that the distance between the two charges in alanine is far enough apart that they may contribute to the ionic strength of a solution, whereas the charges in tricine do not contribute [78].

4.3.5. Preferential PPV flocculation

PPV was preferentially flocculated in the presence of osmolytes, whereas model proteins, BSA and lysozyme, were not, as shown in **Figure 4.6**. BSA and lysozyme were chosen because they represent different protein sizes and charges. Removal of proteins was very low in the presence of 1 M of mannitol and 1 M alanine (<5%). PPV removal was high with osmolytes, 80.2% with alanine, and 85.1%with mannitol. Magnesium sulfate was used as a control flocculant to demonstrate that salts flocculated both PPV (98.6%) and other proteins, BSA (53.2%), and lysozyme (86.4%). Osmolytes were not able to flocculate model host cells proteins, demonstrating that they are specific to our model virus. Virus flocculation with osmolytes is a potential method to purify the viral products from host cell proteins.

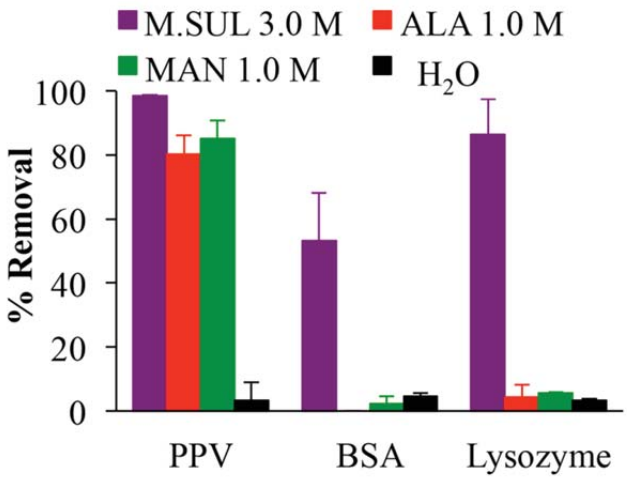

Figure 4.6. Protein and PPV removal with a micropore filter. All data points are the average of three separate experiments and the error bars represent the standard deviation.

In order to use preferential flocculation as a virus purification method, the virus needs to be recovered from the membrane surface. We have recovered infectious virus from the membrane surface, but the recovery is currently low (~2%). We continue to explore methods to recover the virus by changes in eluent composition, membrane material of construction, and backflushing.

4.4. Conclusions

Viral particles play an important role in reducing the incidence of infectious diseases and they show promise in decreasing the incidence of many other deadly diseases. To overcome limitations of current purification methods for viral vectors and vaccines, like chromatography and filtration, we propose to use virus flocculation with osmolytes, followed by microfiltration. A variety of osmolytes were screened in a high throughput manner to find compounds that promote flocculation of PPV. We have discovered that glycine, alanine, sucrose, trehalose, and mannitol have the ability to remove >80% of PPV with a 0.2 μm microfiltration membrane, which is usually used to remove bacteria, not small viruses such as PPV. The use of a micro-filter would increase the flux and decrease the transmembrane pressure of typical virus filters.

Virus removal was improved by reducing the shear stress on the aggregates, by adjusting the pH close to the pI of the virus, and having a low ionic strength. Our top removal was 96% of PPV in 3.0 M glycine at low shear stress and a pH of 5, without addition of NaCl. PPV aggregates formed by the addition of osmolytes are fragile and can be broken with moderate shear stress. Adjusting the pH of the solution to the pI of PPV improved virus removal in zwitterionic amino acid solutions. It is likely that virus aggregation increased when the net charge of individual virus particles is neutral. The neutrally charged sugar and sugar alcohol tested did not change their aggregation propensity with a change in pH. As total ionic strength increased, virus removal decreased. This is likely due to the weakly hydrating sodium cation that is known to increase protein solubility.

We demonstrated that osmolyte flocculation is specific to virus particles as compared to two model host cell proteins. We propose that the specificity is due to high virus hydrophobicity.

Our past work on virus binding peptides concluded that both hydrophobicity and charge play a major role in the binding of porcine parvovirus [82-84]. Osmolytes are able to change how water molecules align around the virus. We hypothesized that osmolytes are able to preferentially flocculate hydrophobic virus particles by strongly binding to water molecules, consequently depleting the hydration layer around the virus particles and stimulation of virus aggregation. Highly hydrophobic viruses flocculate in lower concentrations of osmolytes than the less hydrophobic proteins, providing for selective flocculation and purification.

The aim of virus flocculation with osmolytes followed by microfiltration is to replace current unit operations used in virus purification, such as chromatography and nanofiltration. If process integration is needed, a buffer exchange step before any chromatography column will be required, due to the high osmolytes concentration. Further studies will need to be done in filter capacity and pressure-flux profiles for successfully commercialization of virus flocculation in osmolytes, along with recycling of the osmolyte solution.

A major advantage of using osmolytes for virus flocculation, rather than salts or PEG, is that osmolytes have been used as excipients in the final formulation of some biotherapeutics. We propose that virus flocculation with some osmolytes, including sugars, sugar alcohols and amino acids, followed by microfiltration could be used as an alternative process for virus purification. Our goal is to find an osmolyte that could be used as a platform purification for viral products.

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Chapter 5

Purification of Sindbis virus with osmolytes flocculation, followed by microfiltration[1]

[1] The material contained in this chapter is in preparation for submission

5.1. Introduction

Viral diseases account for over 13 millions deaths per year. Viral vaccines are the most effective method to prevent and control viral infections [1]. Currently, vaccines prevent around 2.5 millions deaths per year [2]. There are two main reasons why vaccines are not able to offer a complete coverage: emerge of operational limitations on the supply side, and negative advertisements on the effects of vaccines on the consumption side [1]. As scientists, we can improve the operational limitations in the manufacturing process, but as society we should increase the awareness of the importance of vaccination. Scientists are working together to develop safe and effective vaccines at a lab scale. However, once the vaccine is ready, the speed of the manufacturing process of the vaccine needs to increase and the high costs involved in the manufacturing process need to decrease in order to have sufficient supplies for every nation [3, 4]. The improvement of current manufacturing processes could lead to the fast development of effective and economical vaccines.

Vaccine manufacturing process is divided into upstream processes (USP) and downstream processes (DSP). The vaccine antigen is produced in the USP. The DSPs account for 70% of the overall cost of the production process [5, 6] and consist of the recovery and purification of the desired vaccine. The elevated costs involved in the DSP stages are related to column chromatography and nanofiltration, each of which are highly used in the purification stages [7]. Chromatography is the predominant unit operation for virus purification. However, it is not suitable for all types of viruses. Conventional chromatography resins present limited diffusion for large virus particles, due the fact that virus particles have difficulties accessing the high internal surface area of these resins [8, 9]. Ultrafiltration has been used to purify virus particles as an alternative to chromatography. However, the capacity of the membranes can be affected by fouling [10], leading to longer filtration time, high transmembrane pressure, and low flux through the membrane [5, 11]. Due to all the limitations of chromatography and ultrafiltration, we propose to use virus flocculation with osmolytes, followed by microfiltration as an alternative to current unit operations. A micro-filter, typically used to retain bacteria and not viruses, would

increase the flux and decrease the transmembrane pressure as compared to ultra-filters.

Osmolytes, which we propose as flocculants, are natural compounds found in the cells of many organisms, and their main function is to stabilize intracellular proteins against environmental stresses and maintain cell volume [12]. There are two types of osmolytes, protecting and denaturing. Protecting osmolytes have the ability to fold proteins by structuring water and rearranging the water content around the protein. Denaturing osmolytes have the opposite effect, they unfold the proteins by binding directly to the protein backbone [13]. In our previous studies, we have shown that protecting osmolytes, such as sugar alcohols and amino acids, preferentially flocculate a non-enveloped virus, porcine parvovirus (PPV), and our top osmolyte demonstrated a 96% removal in 3.0 M glycine (pH of 5) with a 0.2 μm filter while leaving model proteins in solution [14]. In this study we propose to use protecting osmolytes to flocculate an enveloped virus, in our quest to create a platform approach to virus and vaccine purification for non-enveloped and enveloped viruses. Both types of viruses possess a capsid made of multiple copies of identical protein subunits that enclose the genetic material. The difference between enveloped and non-enveloped viruses is that the capsids of enveloped viruses are surrounded by a lipid bilayer, into which glycoproteins are incorporated. We propose that the osmolyte flocculants are able to specifically flocculate hydrophobic non-enveloped and enveloped virus particles by depleting a hydration layer around the particles and subsequently cause virus aggregation. We are attempting to establish that osmolytes can could be used as a platform purification for viral products.

5.2. Materials and methods

5.2.1. Materials

The osmolytes, trimethylamine N-oxide (TMAO) dihydrate, glycine, betaine, D-alanine, D-arginine, L-proline, L-serine, D-mannitol, sucrose, D-(+)-trehalose dihydrate, D-(+)-raffinose pentahydrate, and urea; and the salts, ammonium sulfate, sodium chloride (NaCl), magnesium sulfate, and magnesium chloride, at a purity of >98% were purchased from Sigma-Aldrich (St. Louis, MO). Polyethylene glycol (PEG) (MW=12,000 Da), and albumin from bovine serum (BSA)

were also purchased from Sigma-Aldrich. Cell culture reagents, phosphate-buffered saline (PBS, pH 7.2), 0.25% trypsin/EDTA, gentamicin reagent solution, and minimum essential medium (MEM) were purchased from Life Technologies (Carlsbad, CA). MTT Assay reagents, thiazolyl blue tetrazolium bromide (MTT), sodium dodecyl sulfate (SDS) and hydrochloric acid (HCl) were purchased from VWR (Radnor, PA). Tryptose phosphate broth (TPB) for cell culture was also obtained from VWR. Lysozyme from chicken egg white was purchased from EMD Millipore (Billerica, MA). All solutions were prepared with NanoPure water (Thermo Scientific, Waltham, MA, resistance >18 MΩ), and then filtered with a 0.2 µm syringe filter (Nalgene, Rochester, NY).

5.2.2. Cell propagation, virus production and titration

Baby hamster kidney (BHK-21) cells and Sindbis virus heat resistant strain (SVHR) were a gift from Dr. Raquel Hernandez at North Carolina State University. BHK-21 cells were grown at 37°C, 5% CO_2 and 100% humidity in MEM supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA), 5% TPB, and 1% gentamicin, as described earlier [15]. BHK-21 cells were propagated every day and split 1:3.

SVHR was propagated as described previously [15]. Briefly, BHK-21 cells were incubated for 24 hours, and then infected with 1 ml of SVHR at a MOI between 10-50 pfu/cell diluted in PBS. After 1.5 hours, 9 ml of media was added, and the infected cells were incubated until cytopathic effects were observed. Virus was clarified, and the supernatant virus was stored in 10% glycerol at -80°C.

The cytopathic effect (CPE) of SVHR in BHK-21 cells was quantified with either a cell viability assay, the MTT assay or a $TCID_{50}$ virus titration assay. The MTT assay is a colorimetric assay that measures enzymatic activity in active mitochondria and can be used to measure CPE. BHK-21 cells were seeded in 96-well plates at a density of 1.1 x 10^5 cell/ml (MTT assay) or 5.0 x 10^5 cell/ml ($TCID_{50}$ assay). After 24 hours, cells were infected with BHK-21 in quadruplicate and 5-fold serial dilutions were done across the plate. For the MTT assay, the MTT solution was added after 2 days, followed by the solubilizing agent after 4 hours. Plates were read on a Synergy Mx microplate reader (BioTek, Winooski, VT) at 550 nm, between 18-24 hours later.

The 50% infectious dose (MTT_{50}) was stated as the virus dilution that achieved 50% of the uninfected cell absorbance. The value was defined on a per milliliter basis (MTT_{50}/ml) titer. For the $TCID_{50}$ assay, plates were read manually after 2 days. Wells were considered to have CPE when there was a loss of contact between the cells. Wells were scored by the Karber method [16].

5.2.3. Flocculation and filtration

Virus and protein flocculation was conducted as described previously [14]. Briefly, 30 µl of SVHR at 10^6 $TCID_{50}$/ml (MTT_{50}/ml) or 20 mg/ml of BSA or lysozyme in PBS were incubated with 720 µl of an osmolyte solution for 2 h at room temperature. As a control, NanoPure water was incubated with SVHR at 10^6 $TCID_{50}$/ml (MTT_{50}/ml) or 20 mg/ml of BSA or lysozyme in PBS. Flocculated virus particles or protein particles were filtered with a 0.2 µm, 96-well filter plate (Millipore, Billerica, MA) at 200 xg, 4°C for 20 minutes in a Sorvall ST16R Centrifuge (Thermo Scientific, Pittsburgh, PA). Virus samples before and after filtration were titrated as described in **Section 2.2**. Protein sample absorbance before and after filtration was measured on a Synergy Mx microplate reader at 280 nm. Percent of protein or virus removal was calculated with **Eq. 5.1,**

$$\%\ Removal = \left[1 - \left(\frac{C_f}{C_i}\right)\right] \times 100 \qquad (\mathbf{5.1})$$

where c_f is the concentration of infectious virus or protein after filtration, c_i is the concentration of infectious virus or protein before filtration.

5.2.4. Effect of pH and ionic strength

The osmolytes betaine, proline, and mannitol at 0.3 M were tested for their ability to aggregate and flocculate SVHR particles at different pH levels. The pH of the osmolyte solutions was adjusted by the addition of HCl or NaOH. The osmolytes proline and mannitol, both of them at 0.3 M, were chosen to study the ionic strength effect. For both osmolytes, 0.2 M - 0.6 M of NaCl was added to the osmolyte solution. The ionic strength was calculated with **Eq. 5.2**,

$$\mathrm{I} = \frac{1}{2} \times \sum_{\mathrm{i}=1}^{\mathrm{n}} \mathrm{c_i}\, \mathrm{z_i^2} \qquad (\mathbf{5.2})$$

where c_i is the concentration of ion *i*, and z_i is the charge of ion *i*.

Virus samples were flocculated and filtered as described in **Section 5.2.3**. Samples before and after filtration were titrated as described in **Section 5.2.2** and % virus removal was calculated with **Eq. 5.1.**

5.3. Results and discussion

Sindbis, our enveloped model virus, is a virus from the *Togaviridae* family. It is one of the smallest enveloped viruses, with a diameter of between 60-70 nm, is a single-stranded, RNA virus, with an icosahedral capsid [17]. It is a model virus for eastern and western equine encephalitis viruses [18] and hepatitis C virus. Purification and removal of Sindbis virus has been done with a combination of centrifugation and chromatography unit operations [19-21]. However, these methods have shown to inactivate the virus by 2 logs due to acidic wash buffers [20]. In this study, we propose virus flocculation in osmolytes as a new purification method. The goal is to find an osmolyte that will not inactivate the virus and can be used as a global flocculant.

5.3.1. Flocculation of SVHR with osmolytes, PEG and salts

A variety of osmolytes were screened to demonstrate their ability to flocculate SVHR (**Figure 5.1**). The salts magnesium sulfate, magnesium chloride and PEG were used as positive controls. Osmolytes were selected in part from our results of a previous study on osmolyte flocculation of a non-enveloped virus [14]. Salts were selected as positive controls since they have been used to flocculate enveloped viruses, like the vaccinia virus and the cowpox virus; and non-enveloped viruses, like bacteriophages [22, 23], enteroviruses [22], and poliovirus [24, 25]. PEG was also chosen as a positive control, since it has been used as a flocculent to purify virus particles, such as bacteriophages [26], adenovirus [27], and hepatitis A [28]. Many osmolytes, such as the amino acid proline, the N-oxide betaine, and the neutral molecules sucrose, trehalose, raffinose, and mannitol were able to flocculate SVHR and demonstrate a high % of virus removal (>80%) at all the concentrations tested.

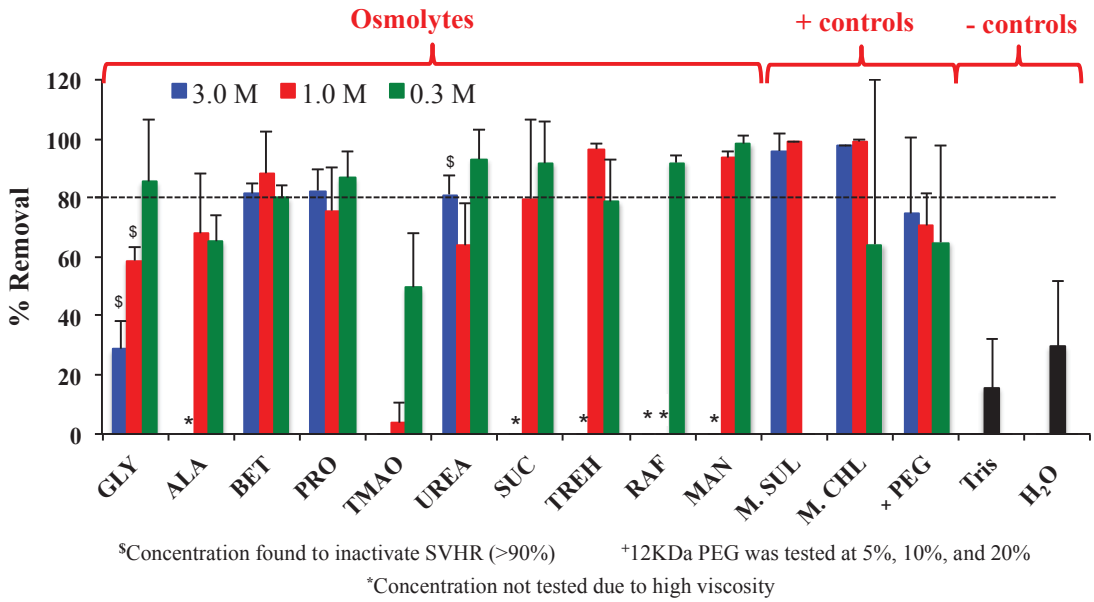

Figure 5.1. High-throughput screening of osmolytes as virus flocculants. A variety of osmolytes were compared to the positive control salts and PEG and the negative controls, Tris, and water for flocculation of SVHR using a 0.2 µm micropore filter. 80% removal was used as the cut-off to pursue testing. % removal is defined in Eq. 5.1. All data points are the average of three separate experiments and the error bars represent the standard deviation.

The osmolytes urea and glycine, showed a >80% virus removal at low concentrations, but at high concentrations, they inactivated SVHR particles (**Table 5.1)**. We confirmed virus inactivation by testing the virus titer of each flocculating solution prior to filtration and comparing to the virus titer of the water control solution. Glycine at 3.0 M and 1.0 M; and urea at 3.0 M reduced the virus titer by > 1 LRV, inactivating the virus prior to filtration (see **Table 5.1**). Osmolytes, such as arginine and serine and the salts sodium chloride and ammonium sulfate were also tested for virus flocculation and they were found to inactivated SVHR (> 1 LRV) at all the concentrations tested (see **Table 5.1)**.

Urea at high concentrations has been shown to inactivate enveloped viruses, such as hepatitis B and influenza [29, 30], likely due to the fact that urea destabilizes viral proteins. Arginine showed SVHR inactivation of ~2 logs. This osmolyte has been shown to inactivate other enveloped viruses, such as influenza virus and the herpes simplex virus [31, 32]. Arginine has been proposed to replace the low pH step that can damage antibody products during viral clearance processes [32]. The mechanism of inactivation is not clear yet, but it has been

suggested arginine binds to proteins without denaturing them [31, 32]. In this study, we focused on flocculants that show high percent removal and did not inactivate SVHR.

Table 5.1. LRV compared to water control

Flocculant	$log_{10}(MTT_{50}/ml)$	LRV[a]
3.0 M GLY	2.97 +/- 0.05	**1.22**[b]
1.0 M GLY	2.62 +/- 0.05	**1.57**[b]
0.3 M GLY	3.46 +/- 0.68	0.74
1.0 M ALA	3.62 +/- 0.07	0.58
0.3 M ALA	4.09 +/- 0.13	0.11
1.0 M ARG	2.22 +/- 0.09	**1.97**[b]
0.3 M ARG	2.62 +/- 0.06	**1.57**[b]
3.0 M BET	4.88 +/- 0.19	0.00
1.0 M BET	4.99 +/- 0.38	0.00
0.3 M BET	4.92 +/- 0.09	0.00
3.0 M PRO	4.50 +/- 0.24	0.00
1.0 M PRO	4.85 +/- 0.32	0.00
0.3 M PRO	5.32 +/- 0.10	0.00
3.0 M SER	2.23 +/- 0.28	**1.96**[b]
1.0 M SER	2.25 +/- 0.19	**1.95**[b]
0.3 M SER	2.49 +/- 0.32	**1.71**[b]
3.0 M TMAO	3.75 +/- 0.17	0.44
1.0 M TMAO	4.24 +/- 0.15	0.00
0.3 M TMAO	4.47 +/- 0.15	0.00
3.0 M UREA	2.87 +/- 0.17	**1.33**[b]
1.0 M UREA	3.97 +/- 0.14	0.22
0.3 M UREA	4.94 +/- 0.11	0.00
1.0 M SUC	5.08 +/- 0.21	0.00
0.3 M SUC	4.82 +/- 0.44	0.00
1.0 M TRE	4.44 +/- 0.05	0.00
0.3 M TRE	4.79 +/- 0.15	0.00
0.3 M RAF	4.39 +/- 0.07	0.00
1.0 M MAN	5.05 +/- 0.18	0.00
0.3 M MAN	5.50 +/- 0.23	0.00
3.0 M S. CHL	2.03 +/- 0.23	**2.16**[b]
1.0 M S. CHL	1.83 +/- 0.40	**2.36**[b]
0.3 M S. CHL	3.13 +/- 0.56	**1.06**[b]
3.0 M A. SUL	3.07 +/- 0.02	**1.12**[b]
1.0 M A. SUL	2.40 +/- 0.12	**1.79**[b]
0.3 M A. SUL	1.71 +/- 0.09	**2.49**[b]
3.0 M M. SUL	3.96 +/- 0.81	0.23
1.0 M M. SUL	4.60 +/- 0.26	0.00
0.3 M M. SUL	3.10 +/- 0.11	1.00
3.0 M M. CHL	3.95 +/- 0.10	0.24
1.0 M M. CHL	4.36 +/- 0.14	0.00
0.3 M M. CHL	4.38 +/- 0.32	0.00
20% PEG	3.76 +/- 0.23	0.43
10% PEG	3.88 +/- 0.10	0.32
5% PEG	3.81 +/- 0.18	0.38
TRIS	4.68 +/- 1.74	0.00
H_2O	4.20 +/- 0.68	0.00

[a]LRV was calculated with Eq. 5.1, where c_f is the concentration of infectious virus in the presence of the flocculant and c_i is the concentration of infectious virus in the presence of water. [b]Flocculants that reduced the virus titer by > 1 LRV

The advantage of osmolyte over salt flocculation is that high concentrations of salts (3 M & 1 M) were required to flocculate virus particles, whereas low concentrations of osmolytes (0.3 M) were enough to flocculate SVHR particles (**Figure 5.1**). The positive control, PEG, did not reach a high percent removal (>80%), likely due to the fact that PEG flocculation requires low

temperatures and long incubation times [27, 28]. In our previous studies with the non-enveloped virus PPV, we report higher virus removal with the amino acid alanine than with the amino acid proline [14]. In this work, proline at all the concentrations tested was able to aggregate and flocculate the enveloped virus, SVHR. This is likely due the fact that proline is more soluble in water than alanine and has the ability to structure more water molecules around SVHR that alanine. The osmolytes that achieved the highest percent removal were tested for their ability to aggregate and flocculate virus particles at different pH values and ionic strengths.

5.3.2. pH effect on SVRH flocculation

The osmolytes betaine, mannitol and proline were used to study the pH effect on SVHR flocculation. Osmolyte solution pH was adjusted in order to change the overall charge of the virus. SVHR has been show to be stable in the pH range of 5-8 [33], so a pH range of 5.5 to 8.5 was selected to study the pH effect on SVHR flocculation (**Figure 5.2**). The highest percent removal occured near the isoelectric point (pI) of Sindbis virus, which has been tested and found to be ~ 4.2 [34]. At pH values close to the pI of the virus, the overall charge became neutral and virus aggregation increased. On the other hand, as the pH value was increased, the negative charge on the virus increased, and virus removal decreased, likely due to electrostatic repulsion between the virus particles.

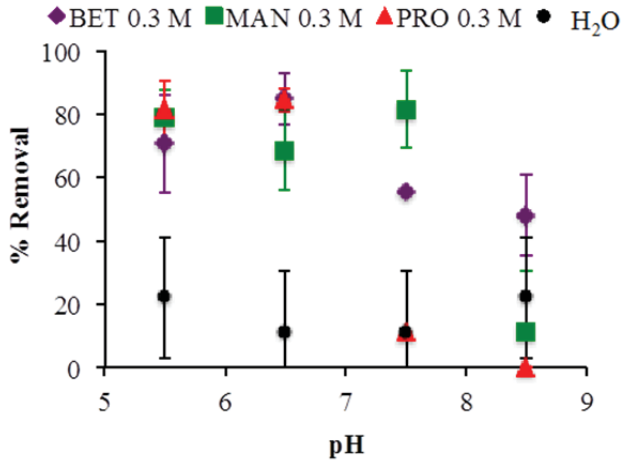

Figure 5.2. pH effect on SVHR flocculation with a micropore filter. % removal is defined in Eq. 5.1. All data points are the average of three separate experiments and the error bars represent the standard deviation.

The neutral molecules mannitol and betaine showed a higher removal than the zwitterion, proline, at pH 7.5 and 8.5, probably due the fact that the increase of charge on SVHR particles was further enhanced by the charged proline molecule, thus increasing the charge repulsion of the SVHR particles. In our previous study, we showed that changes in pH in the zwitterionic osmolytes glycine and alanine can increase the charge-charge repulsion of PPV particles. This behavior was not seen with the sugar and sugar alcohol osmolytes, likely because neutral molecules are able to promote virus flocculation by strong hydrogen-bonding interactions; and not charge-charge interactions [14]. Zwitterion and neutral osmolytes likely have the same manner of function with pH changes on virus aggregation with non-enveloped and enveloped viruses.

5.3.3. Ionic strength effect on SVHR flocculation

The zwitterion, proline and the neutral molecule, mannitol were used to investigate the ionic strength effect on SVHR flocculation. Some researchers have suggested that zwitterions do contribute to the ionic strength of a solution and other groups have demonstrated the opposite [35-37]. Stellwagen *et al*. have shown with experimental data that zwitterions do not contribute to the ionic strength of a solution containing DNA [37]. They used capillary electrophoresis to determine if the zwitterion tricine contributed to the ionic strength of a solution. DNA mobility was expected to decreased, as the square root of the ionic strength decreased. However, there was not a dependency on the mobility of the DNA with respect to the concentration of the zwitterion tricine [37]. This study suggested that the zwitterion tricine does not contribute to the ionic strength of a solution. Other research groups without experimental evidence have argued that charges on zwitterionic molecules are separated with enough distance that zwitterionic molecules do contribute to the ionic strength of a solution [35, 36]. In this study, we assumed that the zwitterionic proline does not contribute to the ionic strength of a solution. SVHR removal did not change with an increase in ionic strength for the osmolytes, proline and mannitol (**Figure 5.3**). We suggested that besides hydrophobic interactions, charge-charge and hydrogen bonding interactions also affect virus aggregation. We propose that charge-charge interactions are predominant with zwitterionic osmolytes, and hydrogen-bonding interactions are predominant with

neutral osmolytes. It is likely that the strength of electrostatic interactions and hydrogen bonding interactions are not appreciably affected by low concentration of the weak chaotropic agent, NaCl. For our water control, virus removal increased as we increased the concentration of salt, likely due to a salting-in and salting-out mechanism. As we increased salt concentration, we decreased virus solubility, and consequently we increased virus aggregation.

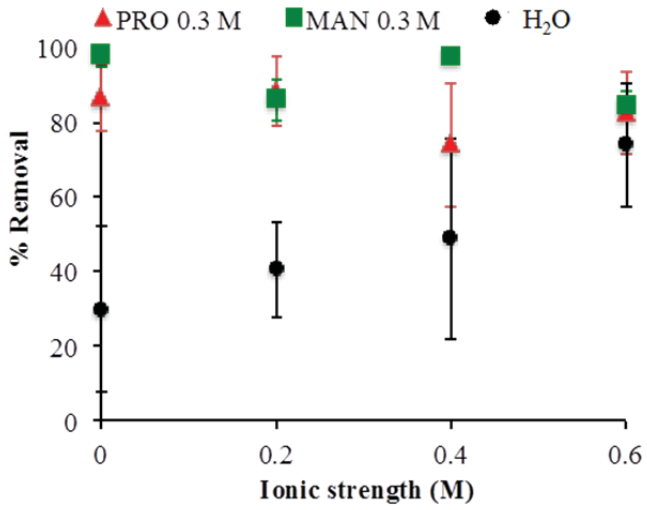

Figure 5.3. Ionic strength effect on SVHR flocculation with a micropore filter. % removal is defined in Eq. 5.1. All data points are the average of three separate experiments and the error bars represent the standard deviation.

In our previous studies, we showed that PPV flocculation, and consequently PPV removal, was lower with the neutral molecule mannitol than with the zwitterion alanine, at low concentrations of ionic strength (0 to 0.6 M), where we assumed that alanine did not contribute to ionic strength [14]. It is likely that NaCl affected the hydrogen bonding interactions between mannitol and water molecules with PPV flocculation and not SVHR. Probably, the hydrophobic proteins in the enveloped virus promote strong hydrogen bonding between mannitol and water molecules, which are not easy to disrupt. More studies need to be done assuming that zwitterion proline does contribute to ionic strength with the enveloped virus, so that both assumptions can be compared for the two model viruses, PPV and SVHR. We have shown both assumption with the zwittherions molecules in our PPV flocculation studies [14] and the same methodology will be followed for our SVHR flocculation future studies.

5.3.4. SVHR preferential flocculation with osmolytes

The model proteins bovine BSA and lysozyme were used to demonstrate the effect of preferential flocculation of osmolytes with virus particles (**Figure 5.4**). These two proteins were selected due to the difference in their physical properties, such MW, stokes radius, and isoelectric point (See **Table 5.2**).

Table 5.2. Physical properties of BSA and lysozyme

Protein	MW (kDa)	Stokes radius (nm)	Isoelectric point	Reference
BSA	66.4	3.5	4.7	[38-40]
Lysozyme	14.3	2.1	11.3	[41, 42]

BSA and lysozyme removal was very low in the presence of osmolytes (< 23%) as compared to the salt, magnesium sulfate (54% for BSA and 87% for lysozyme), indicating that salts, and not osmolytes, enhance protein flocculation. The osmolytes mannitol and proline at 0.3 M and the salt magnesium sulfate at 3.0 M were able to flocculate SVHR particles. High concentrations of salts compared to osmolytes were required to achieved virus flocculation and consequently high virus removal (**Figure 5.1 & 5.4**). SVHR was preferentially flocculated in the presence of low concentration of osmolytes, whereas BSA and lysozyme were not. Osmolyte flocculation is specific to virus particles, making this a method to purify the virus particles from protein contaminants.

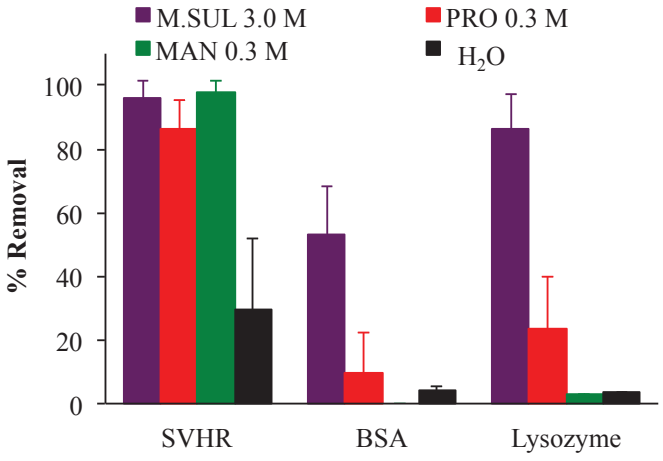

Figure 5.4. SVHR and protein removal with a micropore filter. All data points are the average of three separate experiments and the error bars represent the standard deviation.

5.4. Conclusions

Virus purification is essential in vaccine manufacturing. Current unit operations, such as chromatography and nanofiltration present several drawbacks. In this study, we have shown that protecting osmolytes, like proline and mannitol were able to aggregate and flocculate the enveloped virus, SVHR and demonstrate >80% virus removal with a 0.2 μm filter. Our top removal was 98.1% of SVHR in 0.3 M mannitol. In our previous studies, mannitol at the same concentration was able to aggregate and flocculated a non-enveloped virus, PPV (81% removal) [14]. This osmolyte flocculant structures water molecules around the hydrophobic enveloped or non-enveloped virus, leading to a decreased hydration layer around the virus and increasing hydrophobic interactions between virus particles. Virus flocculation with mannitol, followed by microfiltation could be used as a global process for virus purification.

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Chapter 6

Biocompatibility studies of soft-template mesoporous carbons[1]

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6.1. Introduction

Carbon-based materials have attracted great attention in biomedical and biological fields owing to their stability, chemical inertness, mechanical strength and high surface area [1]. Morphologically, carbon-based materials can be classified into two distinct categories, nano carbons and non-nano carbons. Within the nano carbon variety, carbon nanotubes (CNTs) were considered to be an excellent material and examined several times for their characteristic biocompatibility [2, 3]. Although the results from different research groups appeared to be conflicting and counter-intuitive [4], it has been almost universally accepted that CNTs are toxic and their toxicity can be related to an adverse dermal [5], respiratory [6, 7], pulmonary [8, 9], or cellular [10] response. It was also suggested that toxicity of CNTs vary widely with their degree of agglomeration, functionalization and catalyst contents [2-4]. Besides CNTs, fullerenes also demonstrated characteristic toxicity [11]. On the other hand, non-nano carbons appeared to be much more benign. Diamond-like carbon (DLC) was employed as an attractive candidate for implants purposes, and it did not show significant toxic behavior [12-14]. Activated carbon is another type of non-nano carbon and has long been used for drug overdose and accidental toxin ingestion without any sign of toxicity [15, 16]. Plenty of evidence has come to light that any nano-sized particle may bear potential health hazards [17], an approach towards building a non-nano carbon-based drug carrier might avoid any potential health risk in the first place.

Soft-templated mesoporous carbon is a relatively newer variety of synthetic, non-nano and porous carbon that finds its key distinct features in controlled large pore accessibility and tunability of pore textures. Although mesoporous carbon has already established its role in the field of environmental applications, gas separation and storage, and energy harvesting, biological applications of this material are a relatively new and growing field. Synthesis of this material can employ different classes of amphiphilic surfactants for templating purposes, the role of which is very similar to that of silica in the case of hard-templating (i.e., to dictate mesoporosity). Typically, the crosslinked phenolic carbon precursors are held together by the micelles of amphiphilic surfactants through hydrogen bonding and translate the mesoporosity to the carbon

matrix upon pyrolysis. **Figure 6.1** shows the general schematic of fabricating an ideal mesoporous carbon from soft templates. Over time, both synthetic and natural carbon precursors were employed to fabricate mesoporous carbons by soft-templating.

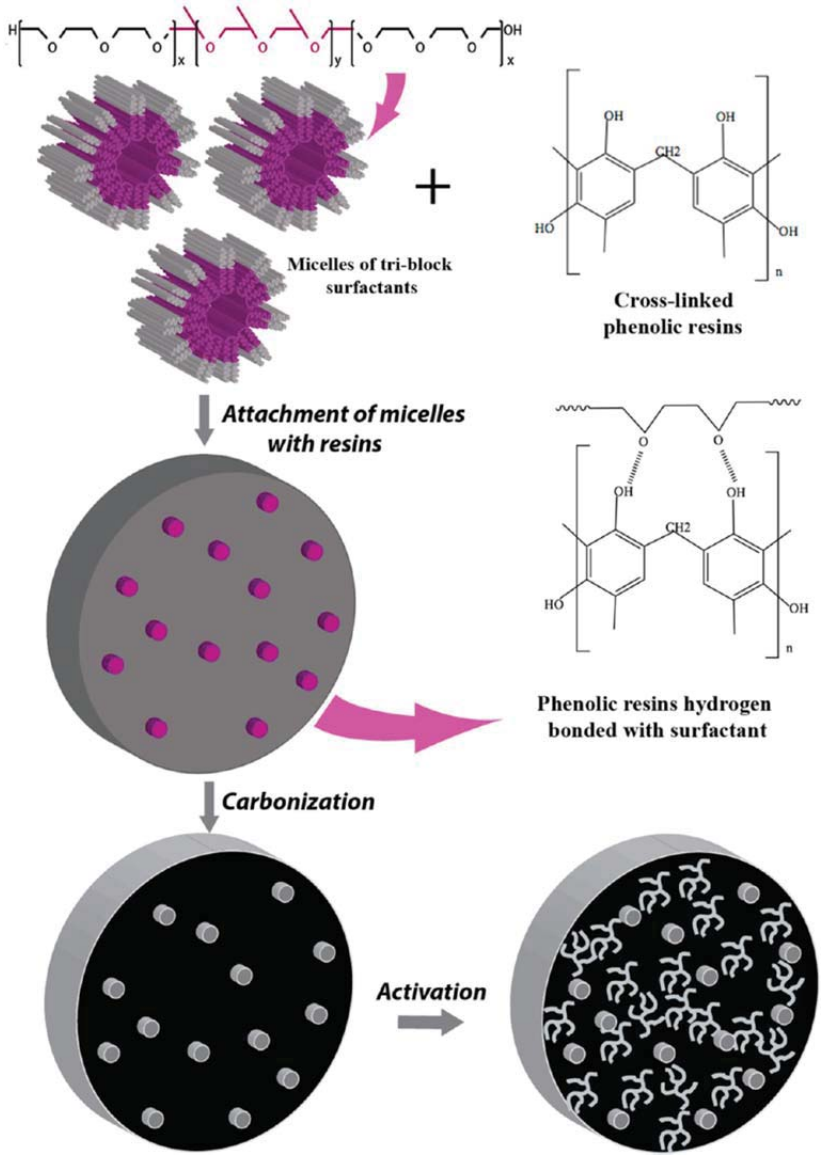

Figure 6.1. Generalized correlation of synthesizing an ideal mesoporous carbon from phenolic precursor via soft-templating. In this schematic, we have employed resorcinol-formaldehyde cross-linked resin as carbon precursor and a tri-block copolymer, [PEO]x-[PPO]y-[PEO]z (PEO: Polyethylene oxide; PPO: Polypropylene oxide) as soft-template. For better visualization of micelles within phenolic resin and transformation of micellar regions onto mesopores, we have incorporated an imaginary circular sliced section of polymer composite and the resultant carbon upon carbonization and activation.

Recently published literature by part of our group and other researchers demonstrated that mesoporous carbon could be employed as a unique and controlled drug delivery vessel. Saha et al. reported successful controlled release of four model drugs, captopril [18, 19], ranitidine hydrochloride [19], furosemide [19], and antipyrine [20] from mesoporous carbons, aimed towards oral drug delivery. Ibuprofen [21, 22], indomethacin [22], and lovastatin [23] were three other drugs that were employed to examine the performance of mesoporous carbon as drug delivery vehicles. Besides oral drug delivery, nanosized or thin film mesoporous carbons were also successfully employed for controlled and targeted release of anticancer drugs, namely, doxorubicin [24], camptothecin [25], and mitoxantrone [26], aimed towards blood plasma or transmembrane delivery.

Owing to the disadvantages of mesoporous silica or metal-organic frameworks (MOFs) as drug delivery vehicles [19, 27-31], mesoporous carbon could be a better choice as a porous-media-based drug delivery system, in addition to its high material tunability. Although there are several reports on mesoporous carbons as drug delivery vehicles, biocompatibility studies of this material are quite handful and not universal. Karavasili et al.[22] performed toxicity and cellular uptake studies of mesoporous carbons with human colon carcinoma (Caco-2) cells that revealed no significant toxicity or abnormal change in cell morphology in contact with mesoporous carbons. Zhao et al.[23] examined the cytotoxicity of uniform mesoporous carbon spheres with the same type of cells (Caco-2) and revealed no cytotoxicity. Zhu et al.[24] and Kim et al.[32] confirmed the null toxicity of nanosized mesoporous carbon particles on cervical cancer (HeLa) cells. Fang et al.[33] reported similar evidence of zero toxicity of mesoporous carbon with human nasopharyngeal epiclermal carcinoma (KB) cells. Gu et al.[25] reported on the in vitro cytotoxic behavior of camptothecin loaded mesoporous carbon, but they did not report the toxicity studies of pristine carbons. Although the past literature confirmed zero to minimal cytotoxicity of mesoporous carbons, these studies did not verify other parameters of biocompatibility, including the role of pore textural properties on the degree of biocompatibility.

In this collaborative research, we have synthesized soft-templated mesoporous carbon from two phenolic precursors, resorcinol and phloroglucinol and two triblock copolymers, Pluronic

F127 and 17R4, as soft templates along with post-synthetic activation to improve the pore textural properties. We have incorporated these materials with varying porosity in the studies of cell toxicity with HeLa cells, cell growth with fibroblast cells, blood protein adhesion and hemolysis. To the best of our knowledge, this is the first report on the detailed biocompatibility studies of mesoporous carbons or any nanoporous carbon in general. These mesoporous carbons were fabricated by using varying synthesis conditions and contain different degrees of porosity so that the results of the study can be analyzed and interpreted in a broad platform.

6.2. Materials and methods

6.2.1. Materials synthesis

First, 50 g resorcinol and 40 g Pluronic F127 were dissolved in a mixture of 400 ml of water/20 ml of ethanol with 60 ml of HCl (6 M) for 1 h 30 min. After that, 48 ml of formaldehyde as a cross-linking agent was added and stirred for 2 h until the polymer layer settled to the bottom with the solvent on top. The polymer layer was separated and carbonized in a tube furnace in a N_2 flow from room temperature to 400 °C at a rate of 1 °C/min, and from 400 °C to 1000 °C at a rate of 2 °C/min; the final temperature was maintained for 15 min, followed by cooling to room temperature in the same N2 flow. To perform the activation, we mixed this material with solid KOH in a 1:3 ratio, heated the mixture in the tube furnace in a N_2 flow from room temperature to 1000 °C at a rate of 10 °C/min, and cooled the mixture in the same N_2 flow. The activated and inactivated are termed *MC-1* and *MC-2*, respectively. To synthesize another material, we mixed 100 g phloroglucinol and 140 g Pluronic 17R4 in a mixture of 320 ml of water/480 ml of ethanol in the presence of 60 ml HCl (6 M), followed by addition of 96 ml of formaldehyde. The polymer was collected in a similar fashion and carbonized with the same protocol. This material was named *MC-3*.

6.2.2. Material characterization

MC-1, *MC-2* and *MC-3* were characterized with pore textural properties in a Quntachrome Autosorb iQ (Boynton Beach, FL) by N_2 adsorption-desorption at liquid N_2

temperature (77 K) and CO_2 adsorption-desorption at 273 K. Brunauer–Emmett–Teller (BET) specific surface area and pore size distribution by nonlocal density functional theory (NLDFT) were calculated by using the instrument's built-in software. High-resolution transmission electron microscopic (TEM) images were obtained in a Carl Zeiss Libra 120 TEM (Thornwood, NY) operating at 120 kV. The samples were dispersed in ethanol at about 0.5 wt % concentration and ultrasonicated for 5 min before being drop-casted onto an amorphous carbon (~20 nm in thickness) coated TEM grid (Ted Pella). Scanning electron microscope (SEM) images of pure mesoporous carbons samples were obtained in a Carl Zeiss Merlin SEM operating at 30 kV. No additional samples preparation protocol was employed for SEM images; the as-received samples were directly inserted into the sample holder for image capturing. The energy-dispersive X-ray spectroscopy (EDXS) results were obtained with a system from Bruker Nano GmbH using a XFlash detector 5030. The analysis was conducted with Bruker's Quantax Esprit Hypermap mode. The elements were selected using the automatic and find modes. Oncea map was obtained, a 25 μm area of interest was selected for the map data results so an average could be obtained. The interactive standards were set during the quantify method. The SEM high voltage was set to 30 kV, and the stage was tilted 30° to help alleviate the problem of absorption of X-rays by the rough surface. Sample thickness varied from 10 to 100 μm. The SEM stage holder and tweezers were cleaned with isopropyl alcohol and clean wipes. Fresh carbon tape was employed to mount the samples.

6.2.3. Cytotoxicity

HeLa cells (H1HeLa, CRL1958) were purchased from ATCC (Manassas, VA) and propagated in HeLa media, which consisted of minimum essential medium (MEM) (Life Technologies, Carlsbad, CA) supplemented with 5 % fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA) and 1 % penicillin/streptomycin (pen/strep) (Life Technologies, Carlsbad, CA). The cells were propagated at 37 °C, 5 % CO_2 and 100 % humidity. HeLa cells were seeded in a 96-well plate at a density of $8x10^4$ cell/ml and 100 μL cells per well. Stock solutions were prepared with 500 μg/ml of mesoporous carbon samples in HeLa media and

sonicated for 20 min in a Misonix XL-2000 Ultrasonic Probe Sonicator (Sonics & Materials, Newtown, CT). Samples ranging from 50-450 µg/ml were prepared from the stock solution. All the samples, including the stocks were sonicated in an Ultrasonic Water Bath (VWR, West Chester, PA) for 20 min. Samples were added to the cells after 24 h at a volume of 25 µL. Cell activity was measured after 5 days by the conversion of the MTT tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; VWR, Radnor, PA) to its formazan form and was performed according to previous studies [34]. Percent survival was calculated with **Eq. 6.1:**

$$\%\ \text{Survival} = \left(\frac{A_{mc}}{A_{mock}}\right) \times 100 \qquad \textbf{(6.1)}$$

where A_{mc}, and A_{mock} were the absorbance of the mesoporous carbon (mc) and the mock media (mock), respectively.

6.2.4. Cell viability

Fibroblast (NIH/3T3, CRL1658), purchased from ATCC were propagated in fibroblast media, which consisted of Dulbecco's modified eagle medium (DMEM) (Life Technologies, Carlsbad, CA) supplemented with 5 % FBS and 1 % pen/strep. Fibroblast cells were grown at 37 °C, 5 % CO_2 and 100 % humidity. Mesoporous carbon samples at 100 µg/ml in fibroblast media were sonicated for 20 min in a Misonix XL-2000 Ultrasonic Probe Sonicator. Samples were added to 24-well plates at a volume of 100 µl/well. After the samples were added, fibroblast cells were seeded on the mesoporous carbon samples at a density of $2.5x10^5$ cells/ml and 500 µl per well. Cells were incubated with the mesoporous carbon samples from 1 to 4 days. Cell viability was measured with a trypan blue exclusion assay. After the media were removed, 200 µl of 0.25 % trypsin/EDTA (Life Technologies Carlsbad, CA) was added to detach cells from the well and the mesoporous carbon. Then 200 µl of fibroblast media was added to inactivate the trypsin. Next, 40 µl of trypan blue stain (0.4 %) (Life Technologies, Carlsbad, CA) was added to the cells and the viable and non-viable cells were counted in a hemocytometer. Trypan blue is excluded from cells with an intact cell membrane, and these cells are assumed to be viable. Percent viability was calculated with **Eq. 6.2**:

$$\% \text{ Viability} = \left[1 - \left(\frac{N_{dc}}{N_{tc}}\right)\right] \times 100 \quad \textbf{(6.2)}$$

where N_{dc} and N_{tc} were the number of dyed cells (dc) and total cells (tc), respectively.

6.2.5. Hemolysis test

Human blood samples for the hemolysis test were obtained from a voluntary donor at the Portage Health Clinic at Michigan Technological University. All work was performed in a certified Biosafety Level 2 laboratory with approval from the Internal Review Board (IRB). The hemolysis test was conducted as stated by Fan et al.[35]. Briefly, the blood sample was collected in tubes containing EDTA and diluted 10-fold in a saline solution (0.9% NaCl). The diluted blood was centrifuged at 1500 rpm for 10 min in an Accu SpinTM 400 centrifuge (Thermo Fisher Scientific, Waltham, MA). The upper phase was removed, and the packed erythrocytes were washed three times with saline solution. Packed erythrocytes were diluted 2 v/v % in saline solution. Stock mesoporous carbon samples of 500 µg/ml were prepared in saline solution and sonicated for 20 min in a Misonix XL-2000 Ultrasonic Probe Sonicator. Four samples ranging from 100-500 µg/ml were prepared from the stock solution and sonicated in an ultrasonic water bath for 20 min. After sonication, 0.45 ml of diluted erythrocytes were added to 0.45 ml of carbon sample and equilibrated at 37 °C. Diluted erythrocytes were also added to NanoPure water (Thermo Scientific, Waltham, MA, resistance >18 MΩ; positive control) and to saline solution (negative control). After 1 h, samples were centrifuged at 1500 rpm for 10 min. The supernatant was removed and the absorbance was measured on a Synergy Mx microplate reader (BioTek, Winooski, VT) at 545 nm. Percent hemolysis was calculated with **Eq. 6.3**:

$$\% \text{ Hemolysis} = \left(\frac{A_{mc} - A_{neg}}{A_{pos} - A_{mc}}\right) \times 100 \quad \textbf{(6.3)}$$

where A_{mc}, A_{neg}, A_{pos}, were the absorbance of the mesoporous carbon (mc), the negative control (neg) and the positive control (pos), respectively.

6.2.6. Protein adsorption

Albumin from bovine serum (BSA) and fibrinogen from bovine plasma, purchased from (Sigma-Aldrich, St. Louis, MO), were diluted in phosphate-buffered saline (PBS, pH 7.2) (Life

Technologies, Carlsbad, CA) to a final concentration of 1 mg/ml. The protein solutions were incubated with 0.0025 g of mesoporous carbon samples for 2 h at 37 °C. Protein absorbance before and after contact with mesoporous carbon samples was measured on a Synergy Mx microplate reader at 280 nm. The difference between the concentration before and after incubation with the carbon samples was determined as the concentration adsorbed on the mesoporous carbon samples.

6.2.7. Imaging of cell growth of mesoporous carbon materials

Cell growth on mesoporous carbon samples was done in a similar way as Correa-Duarte et al.[36]. Fibroblast cells were seeded on mesoporous carbon samples as described in **Section 6.2.4**. After 3 days, the cells attached to mesoporous carbon samples were scrapped out of the plates and placed into centrifuge tubes. Samples were centrifuged at 1500 rpm for 10 min in a Sorvall ST16R Centrifuge (Thermo Fisher Scientific, Waltham, MA) and washed 3 times with 500 µl of PBS. Samples were fixed with 500 µl of 2.5 % glutardehyde (Sigma-Aldrich, St. Louis, MO) in 0.1 M sodium cacodylate (Sigma-Aldrich, St. Louis, MO), pH 7.4, for 2 h at 4 °C. After several washes with 0.1 M sodium cacodylate, samples were dehydrated with ethyl alcohol (Pharmco-Aaper, Brookfield, CT) in series (25%, 50%, 75%, 95%, 100%, 100%) for 10 min at each step. Samples were chemically dried in 1,1,1,3,3,3-Hexamethyldisilazane (HMDS) (Thermo Fisher Scientific, Waltham, MA), in series at 50% and 100% for 10 min at each step and placed overnight in a fume hood at 22 °C. Samples were mounted and coated with 2.5 nm of platinum/palladium (Hummer Sputtering System, Union City, CA) and imaged with a Hitachi S-4700 cold-field emission scanning electron microscope (FE-SEM; Tustin, CA) with an accelerating voltage of 5 kV.

6.3. Results and discussion

6.3.1. Materials characterization

The BET specific surface area (SSA) of *MC-1* and *MC-2* are 1221 and 560 m^2/g, respectively. *MC-3* represents the lowest BET surface area of 315 m^2/g. Pore size distributions

of three materials calculated by NLDFT are shown in **Figure 6.2**. The pore textural characteristics, including BET SSA, external SSA, and total pore volume are provided in **Table 6.1**. *MC-1* and *MC-3* possess the median mesopore width of 36 and 70 Å with a total pore volume of around 0.9 and 0.55 ml/g, respectively. To visualize the presence of micropore distributions present in these samples, we employed CO_2 adsorption isotherms, and the pore size distribution plot is inserted as an inset in **Figure 6.2.**

Table 6.1. Pore textural properties of mesoporous carbons samples

Carbon species	BET SSA (m^2/g)	External SSA (m^2/g)[a]	Total Pore Volume (cm^3/g)[b]
MC-1	1221	399	0.90
MC-2	560	322	0.60
MC-3	315	244	0.55

[a] Calculated by statistical thickness (*t*-plot) method. [b] Calculated by NLDFT method.

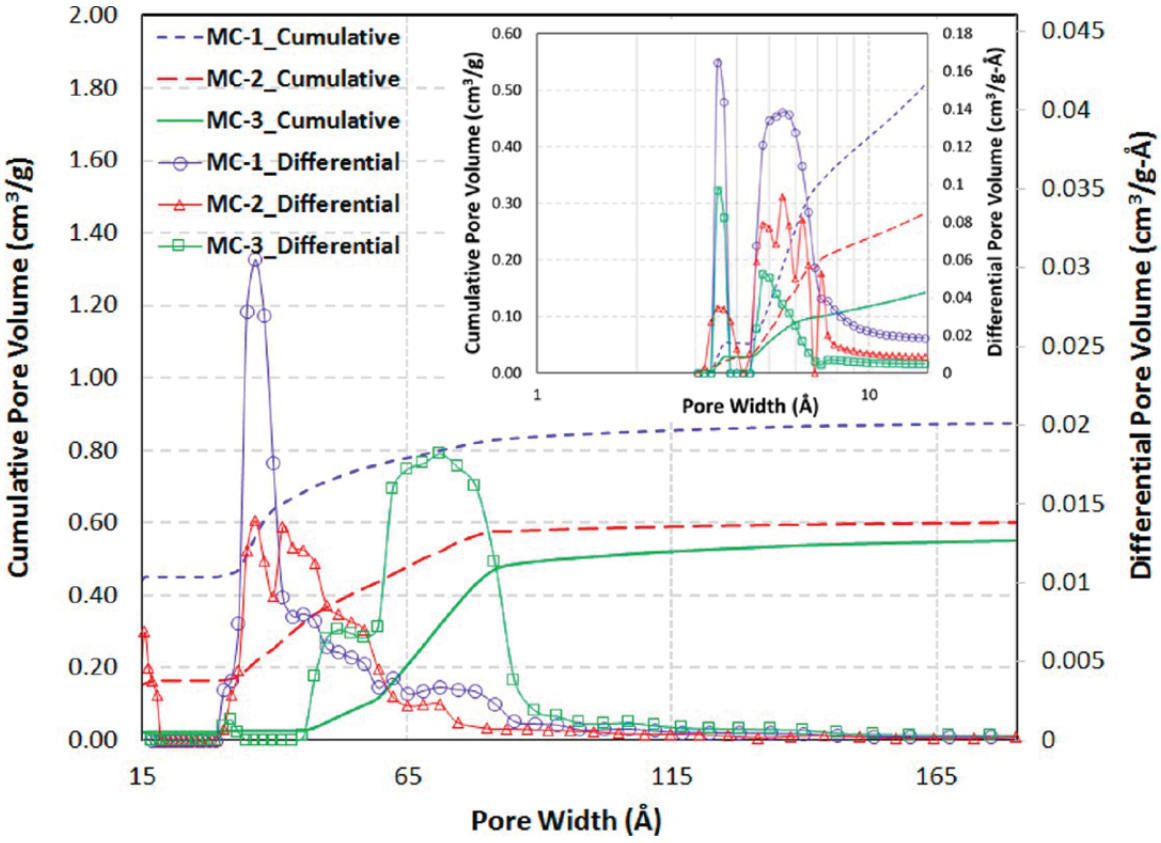

Figure 6.2. Pore size distributions of *MC-1*, *MC-2*, and *MC-3*. (Inset) Micropore distribution of the mesoporous carbon samples calculated from CO_2 adsorption isotherm at 273 K; pore width is in log scale.

MC-3 shows a higher mesopore width at 70 Å with a lower mesopore volume of 0.55 ml/g. TEM images (**Figure 6.3 A, C, E**) of these mesoporous carbons did not reveal a geometrical order of pores; instead it demonstrated a 'worm-like' porous entity in the carbon matrix. SEM images (**Figure 6.3 B, D, F**) showed that all the mesoporous carbon particles are highly irregular in external morphology.

We performed EDXS studies to get the elemental analysis of the carbon samples, and the results are shown in **Table 6.2**. The carbon content varies within 80-87 % along with a large proportion of oxygen, 12-19 %. The origin of oxygen can be rooted to the hydroxyl groups in the carbon precursors that might have converted to other carbon containing functional groups during chemical reaction and carbonization. Slightly lower oxygen content in *MC-1* can most likely be attributed to the reheating of the carbon sample during activation, which might have caused cleavage of oxygen-containing functional groups. Presence of oxygen in metallic oxides, possibly arrived from porcelain boats in the course of carbonization, may also contribute to total oxygen content, although such contribution is much smaller compared to the surface functionality.

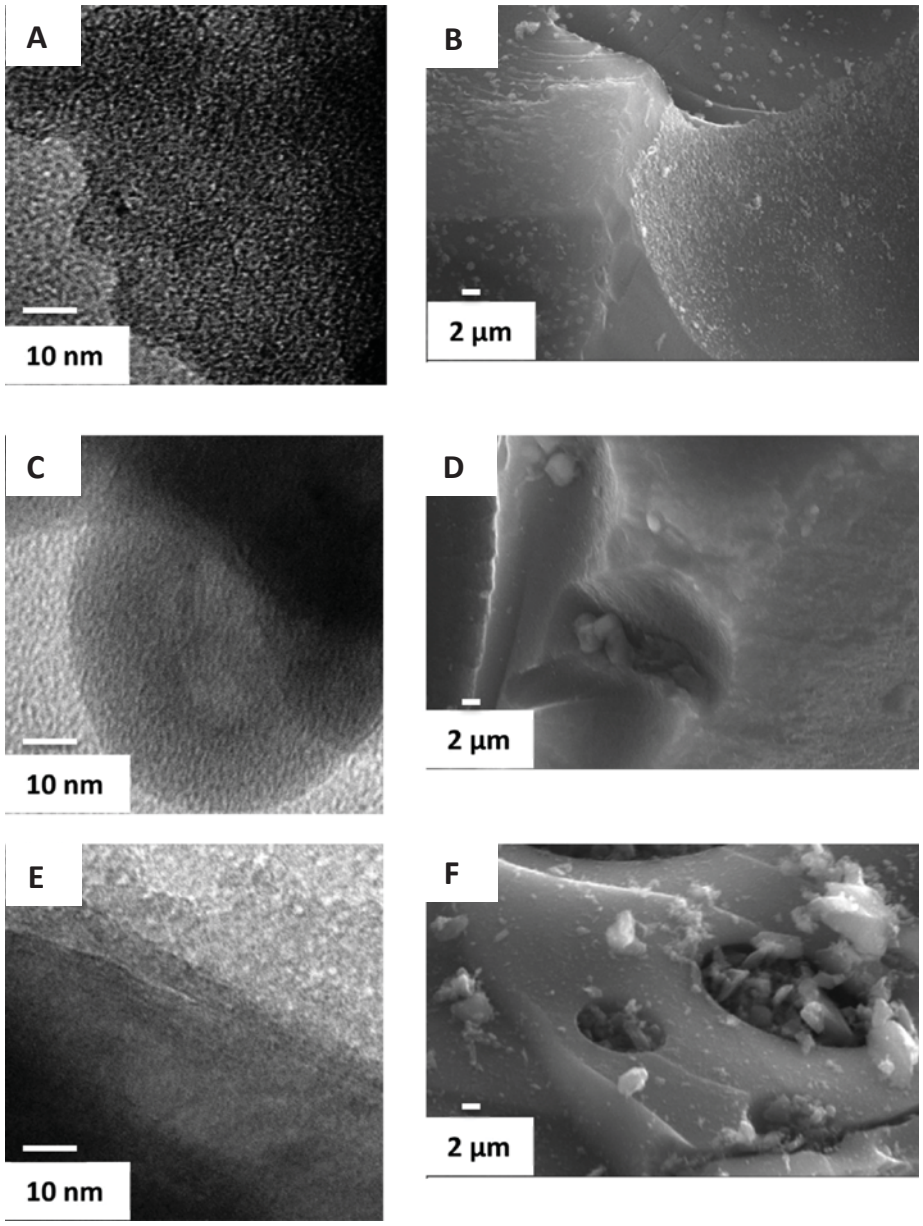

Figure 6.3. Electron microscopic images of mesoporous carbon samples. (A) TEM and (B) SEM images of *MC-1*; (C) TEM and (D) SEM images of *MC-2*; (E) TEM and (F) SEM images of *MC-3*.

Table 6.2. Elemental analysis of mesoporous carbons samples

Carbon species	Carbon (%)	Oxygen (%)	Potassium (%)	Others (%)
MC-1	87.06	12.04	0.76	0.14
MC-2	79.59	19.77	0.03	0.61
MC-3	80.76	18.76	0.01	0.47

MC-1 also has a higher percentage of potassium (0.76%) compared to the rest of the carbon samples, and that can definitely be attributed to the activation with KOH. All the mesoporous carbon samples contain a very small fraction of nitrogen and trace elements (0.14-0.47 % aluminium, zirconium, calcium, and magnesium) that might have originated from the porcelain boat in the course of carbonization or from an impurity in the precursor chemicals.

6.3.2. In vitro cell interactions

In vitro biocompability and toxicity tests are the prerequisites for any biomaterials prior to in vivo animal model evaluation and clinical trials [2, 12]. Mesoporous carbon toxicity was examined in HeLa cells with an MTT cell viability assay. The MTT assay is a common method employed to measure the biochemical activity of cells seeded on carbon materials [37, 38]. **Figure 6.4** shows the results of cytotoxicity studies of the mesoporous carbons; none of the carbon samples demonstrated acute cytotoxicity. *MC-2* and *MC-3* demonstrated negligible toxicity in the range of 50 µg/ml to 500 µg/ml (survival >90%). *MC-1* showed slight toxicity with cell survival decreasing with an increase in concentration and demonstrated around 70% survival at the highest concentration tested (500 µg/ml). The concentration-dependent cell survival of the mesoporous carbon materials are higher than the nanosized mesoporous carbon reported by Fang et al.[33] in which cell survival was not more than 60% at a much lower carbon concentration of 100 µg/ml with a shorter incubation period of 24 h. A closer inspection of our result can reveal a definite pattern of cytotoxicity, that is, it increases with the increase in surface area of the carbon materials tested (*MC-3*<*MC-2*<*MC-1*) at all concentrations. In fact, such dependence of cytotoxicity is quite prevalent not only in the case of porous carbon materials [23], but also for mesoporous silicas [39, 40].

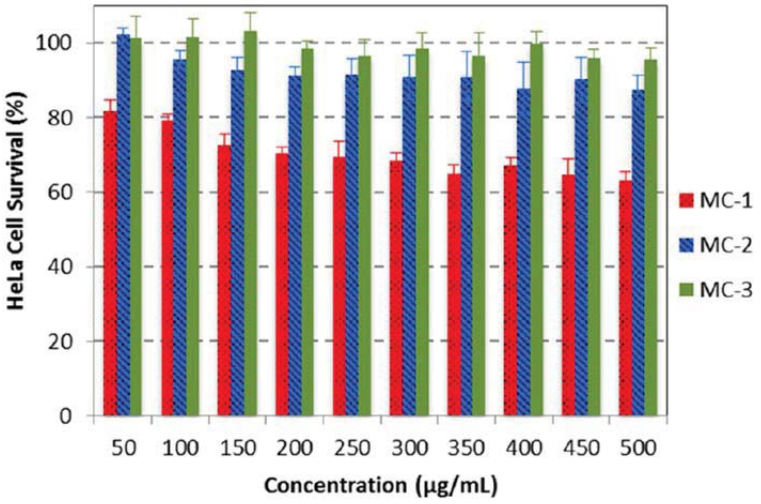

Figure 6.4. Cytotoxicity of mesoporous carbon samples in HeLa cells. Cells were incubated with mesoporous carbon materials for 5 days, followed by evaluation of cell viability with an MTT Assay. Percent survival is defined in Eq. 6.1. All data points are the average of three separate experiments and the error bars represent the standard deviation.

The so-called cytotoxicity at the higher carbon concentration may be attributed to the simple reason of physical hindrance to the cell proliferation [22]; however, the patterned toxicity may need different sets of explanations. Unlike nanosized carbons, the cellular uptake of the carbon matrix can definitely be ruled out for any of our samples. Although external morphologies, including shapes and size of porous matrix, were counted as plausible credential for porous silica towards cytoxocity [39], the mesoporous carbon samples tested in this study are highly irregular in shape and, most likely, a shape factor did not contribute towards toxicity. A closer inspection suggests the size of *MC-1* and *MC-2* are in the order of 20-50 µm in size whereas *MC-3* particles are larger than 100 µm. Smaller particles may interact better with the HeLa cell (size ~ 14 µm) providing a stronger obstruction towards their proliferation. It is also noteworthy that the external surface areas are in the exact order of cytotoxicity (*MC-1*>*MC-2*>*MC-3*) and the higher external surface area also possesses a greater chance of more intimate interactions with cellular bodies.

Surface chemistry is another significant factor that may corroborate the patterned toxicity. Although *MC-1* has a higher concentration of potassium, the literature does not provide any evidence that potassium causes cytotoxicity. *MC-1* has the lowest concentration of oxygen (**Table 6.2**), which essentially means it has lower percent of oxygen-containing functional groups

and possesses the highest carbon content. As the oxygen-containing functional groups may have greater change for providing higher degree of hydrophilicity on the hydrophobic carbon surface, *MC-1* could be more hydrophobic than rest of the samples. It is mentioned in the literature [41] that a hydrophilic surface can interact with a protein surface through an intermediate layer of water molecules, whereas a hydrophobic surface possesses a higher chance of directly interacting with such proteins. This direct interaction could cause denaturation and conformational changes of the proteins. One known effect of hydrophobic surface interaction with cells has been shown to lead to the adverse effects of adenosine triphosphate (ATP) depletion and triggering of cell apoptosis [42]. It is also suggested that a hydrophobic surface itself may provide a higher risk of cell apoptosis [43]. Although the higher toxicity of *MC-1* can be attributed to its hydrophobic nature, such interactions may not be supportive to distinguish the cytotoxic behavior between *MC-2* and *MC-3,* as both of them possess similar oxygen content and apparently the same hydrophobic/hydrophilic nature.

Earlier it was suggested that a material with high external surface area could be less favorable as a biomaterial owing to its higher cytotoxicity [44]. Our results demonstrated that the internal or the BET surface area could be the most intriguing factor towards explaining the cytotoxicity pattern. Earlier, it was reported that silica could be responsible for generating the reactive oxygen species (ROS) radicals that cause cell damage [45]. Yet, the precise role of surface area as a catalytic agent in such phenomena was neither properly understood nor thoroughly investigated. We hypothesize that another indirect phenomena may also be responsible towards this effect. We suggest that a higher surface area material can inherently adsorb larger amount of nutrients within its porous moiety from the proximity or in contact with the cellular bodies, and therefore, the cells may die because of lack of nutrients to survive. Such phenomena, if true, can support the patterned cytotoxic behavior. However, more rigorous experiments with varying conditions, which are not within the scope of this work, are required to experimentally validate this hypothesis. Nevertheless, the overall experimental work suggests that mesoporous carbon samples demonstrated minimal cytotoxic nature and are benign, similar to other *non-nano* carbons [46].

To investigate cell viability, we incubated the mesoporous carbon samples were with fibroblast cells from 1-4 days, along with the mock media as a control substrate for comparison. The concentration of carbons samples employed was 100 µg/ml. The results of this study indicate that cell viability was very high and almost constant (>98%) from day 1 to day 3 without any significant difference with the mock media (**Figure 6.5A**). After the third day, cell viability decreased (~82%) for all the mesoporous carbon samples tested, including the mock media. The number of fibroblast cells also decreased after the third day for all the samples and the mock media (**Figure 6.5B**).

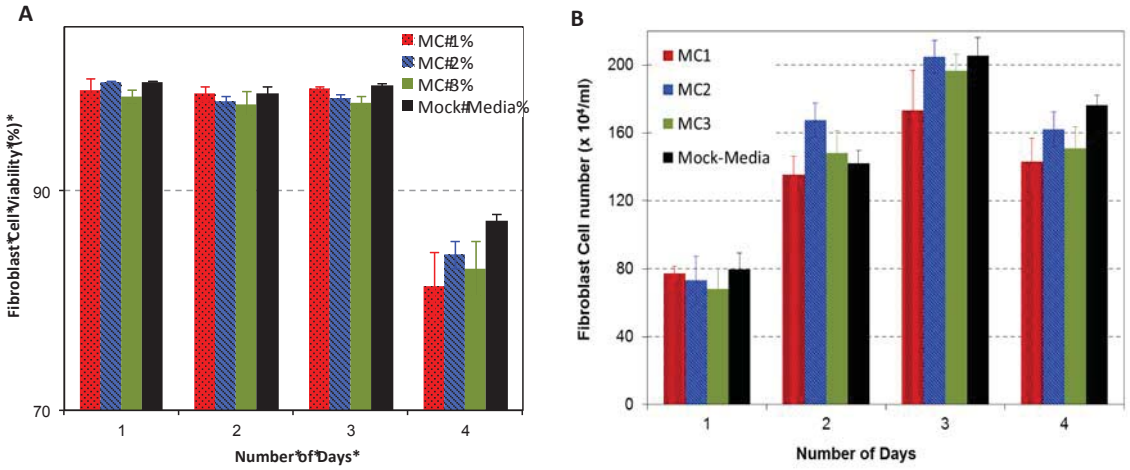

Figure 6.5. Biocompatibility of mesoporous carbon samples in fibroblast cells. (A) Percent viability and (B) cell number. Cells were incubated with mesoporous carbon materials from 1 to 4 days. Cell viability was measured with a trypan blue exclusion assay. Percent viability is defined in Eq. 6.2. All data points are the average of three separate experiments; error bars represent the standard deviation.

The cause of the decreasing trend in cell populations can certainly be ascribed to the lack of nutrients in the culture medium without any possible influence of the mesoporous carbons. The cells likely needed fresh media with nutrients after the third day of culture, but it was not possible to replenish with fresh nutrients in the course of experiments owing to the atypical nature of dispersed mesoporous carbons in culture media. It is also noteworthy to mention that the cell viability assay did not reveal a patterned behavior with mesoporous carbons, and it essentially confirms that the cell viability is independent of porosity, size and shape of such materials.

We have analyzed the growth of fibroblast cells on mesoporous carbon samples with FE-SEM for visual inspection purposes (**Figure 6.6**). All the mesoporous carbon samples were incubated with fibroblast cells for 3 days. We found that the fibroblast cells surrounded the carbon particles, suggesting that the carbon surface did not offer adverse effects to the proliferation of cells.

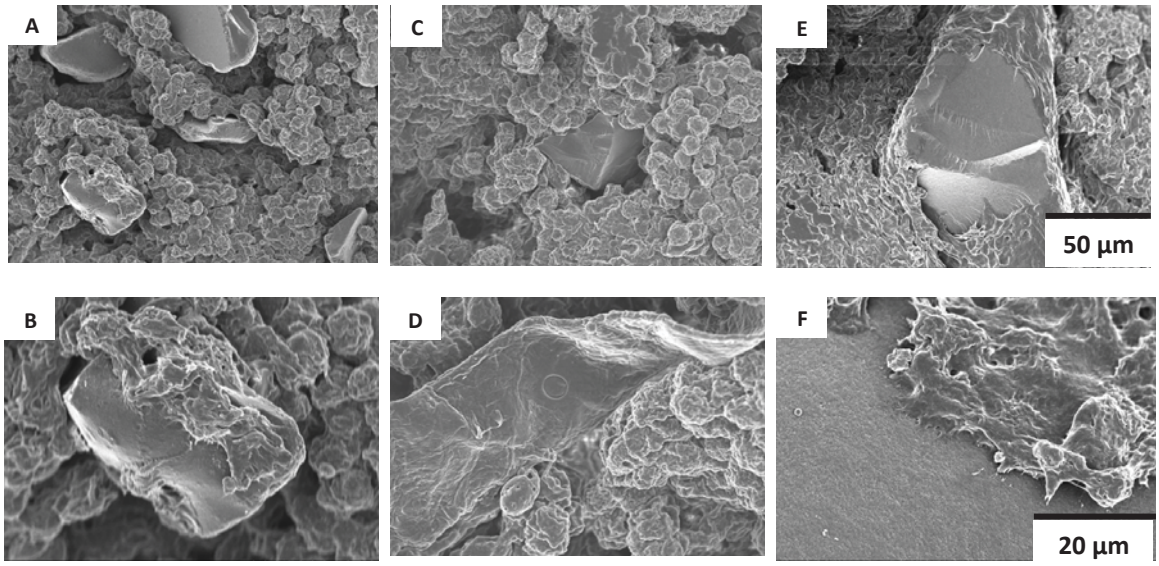

Figure 6.6. Fibroblast cell growth on mesoporous carbon samples. (A and B) *MC*-1, (C and D) *MC-2*, and (E and F) *MC-3*. Cells were incubated with carbon samples for 3 days. Scale bars are (top) 50 and (bottom) 20 µm.

Although all three varieties of carbon provided good platforms for fibroblast cell growth, *MC-3* appeared to demonstrate a better contact and adhesion surface for the cells, as shown in **Figure 6.6 E&F.** Apparently, better cell compatibility of *MC-3* is in agreement with its highest cell survival with HeLa cells, but it did not provide a similar trend with fibroblast cell viability and cell number (**Figure 6.5 A&B**), suggesting that cell contact has a minimal influence in the viability assay. The better adhesion properties of *MC-3* may be attributed to the lower porosity of the material providing better "anchoring", higher hydrophilic surface, or better transport of nutrients through the larger pores of the *MC-3* material.

6.3.3. Hemocompatibility of mesoporous carbon materials

The hemocomapatibility of a drug carrier is the prerequisite for intravascular drug delivery. In our study, we employed two hemocomapatibility tests: hemolysis and adsorption of blood plasma proteins. Hemolysis studies determine the stability of red blood cell (RBC) in contact with a foreign body. Four concentrations of mesoporous carbons ranging from 100-500 µg/ml were employed to examine the hemolysis. The results are shown in **Table 6.3**.

Table 6.3. Hemolysis (%) caused by mesoporous carbon samples[a]

Carbon species	100 µg/ml (%)	200 µg/ml (%)	300 µg/ml (%)	500 µg/ml (%)
MC-1	0.18 ± 0.32	0.36 ± 0.29	0.79 ± 0.35	0.71 ± 0.24
MC-2	0.13 ± 0.04	0.33 ± 0.12	0.43 ± 0.12	0.63 ± 0.04
MC-3	0.25 ± 0.04	0.15 ± 0.20	0.30 ± 0.27	0.76 ± 0.66

[a] The standard deviation is calculated from three samples

Primary observations suggest that the hemolysis is minimum (< 1%) for all samples at all concentrations. Although the majority of the data represented demonstrated slight enhancement of hemolysis with an increase in carbon concentration from 100 to 500 µg/ml, we did not find ubiquity of such behavior. Additionally, the relationship of hemolysis with different samples (i.e., surface area and particle size) did not reveal any patterned behavior. Such behavior is completely in disagreement with previous studies with mesoporous and nanosized silica particle, where hemolysis was proved to be a strong function of porosity, shape and size [47-49]. Comparison of hemolysis data with mesoporous silica suggests that silica can cause hemolysis as high as 20-80 %[47, 48] at the highest concentration of study (500 µg/ml), these results are orders of magnitude higher than our results. Although the mesoporous carbon employed in our study was not nanosized and it may appear that the size effect was not investigated, Zhao et al.[49] confirmed that the smaller particles, in fact, can potentially be safe towards RBC as they get adsorbed on the RBC surface without disturbing the cell membrane or morphology. Based on this finding, we can draw a hypothesis that nanosized mesoporous carbon may possess an even lesser threat towards intravascular drug delivery. This suggests that mesoporous carbon material can serve as a better choice over mesoporous silica for intravascular drug delivery.

The amount of plasma protein adsorption onto biomaterials is an important parameter towards its biocompatibility in terms of implants, intravascular delivery and tissue engineering. When a foreign body comes in contact with the bloodstream, the surface could be rapidly covered with plasma protein, often termed the protein corona [50, 51]. Although in rare occasions where fibronectin adsorption has facilitated cell attachment [52, 53], nonspecific adsorption of proteins onto the biomaterial surface is mostly undesirable as it may trigger adverse effects, such as localized inflammation, hyperactive immune response, or conformational changes of protein structure leading to loss of activity[54] and thrombolysis [55, 56]. Here, we have studied the nonspecific adsorption of two plasma proteins: bovine serum albumin (BSA) and bovine serum fibrinogen (FIB). **Table 6.4** shows the results of protein adsorption.

Table 6.4. Protein adsorption on mesoporous carbon samples[a]

Carbon species	BSA adsorption (g of protein/m^2 of carbon) x 10^{-4}	FIB adsorption (g of protein/m^2 of carbon) x 10^{-4}
MC-1	2.21 ± 0.31	25.5 ± 3.48
MC-2	3.01 ± 0.03	38.1 ± 0.93
MC-3	6.08 ± 1.72	48.8 ± 1.82

[a] The standard deviation is calculated from three samples

As BET SSA encompasses a large portion of narrow micropore surfaces that may not take part in larger protein adsorption, we employed external SSA (**Table 6.1**) for calculating protein-binding capacity. We found that BSA adsorption was < 1 mg/m^2 of carbon surface, whereas fibrinogen adsorption was 1 order of magnitude higher, 2-4 mg/m^2.

The BSA adsorption capacity was in line with the overall protein adsorption onto other porous biomaterials, like hydroxyapatite, zirconia and alumina [55]. A comparison of BSA adsorption on mesoporous carbon samples (1-2 nmol protein/mg carbon) with human serum albumin (HSA) adsorption onto mesoporous silica nanoparticles (MSN [56]; 3-7 nmol protein/mg MSN) suggests that the carbon surface provides less affinity towards albumin. Although fibrinogen adsorption was higher (more than 3 nmol protein/mg carbon), we could not compare its adsorption with other porous biomaterials owing to the lack of reported data. Slightly higher affinity towards fibrinogen may result in somewhat higher risk of thrombolysis, but as suggested

in the literature, functionalization or covering the surface with biocompatible PEG molecules will reduce such risk [57].

To estimate the percent monolayer coverage, we employed a closed packing hard-sphere model of adsorbed proteins. The monolayer model suggests a surface density of 4.8 and 8.5 mg/m^2 for BSA and FIB, respectively. On the basis of these surface densities, we estimated that BSA covered 4.5, 6.1 and 12.4 % of *MC-1*, *MC-2* and *MC-3*, respectively, whereas FIB possesses the higher surface coverage of 26.5, 43.2 and 57.9% for *MC-1*, *MC-2* and *MC-3,* respectively. Although the ideal hard sphere model can be deviated in terms of *(1)* uncoiling or flattening of the protein molecule in proximity to adsorption surfaces [58, 59] and *(2)* nonspecific and undesirable locations of calculated external surfaced area, the coverage percent can provide an approximation of surface occupancies by protein molecules.

A clear pattern of protein adsorption onto mesoporous carbons can be deduced. For both types of proteins, the adsorption capacity increases in the order of *MC-1*<*MC-2*<*MC-3*. It is observed that fibrinogen adsorption is 1 order of magnitude higher for all three types of carbons. Although the higher protein adsorption to *MC-2* and *MC-3* can be related to the higher oxygen content resulting in a greater number of hydrogen bonds, such an explanation does not fully support the trend in loading amounts. The explanations for higher fibrinogen binding and patterned relation between binding capacity and materials properties definitely require much detailed research and understanding of carbon surface, including precise functionality characterization, hydrophobicity/hydrophilicity and Z-potential determination, which are beyond the scope of this work. Nonetheless, the protein adsorption data suggests that the carbon surface is mostly biocompatible and similar to the other biomaterials.

6.4. Conclusions

In this work, we have studied the in vitro biocompatibility of soft-templated mesoporous carbons by cytotoxicity experiments with HeLa cells, cell viability with fibroblast cells and blood compatibility with hemolysis and protein adsorption. *MC-2* and *MC-3* samples showed minimal cytotoxicity while *MC-1* demonstrated only slight toxicity within the concentrations of 50-500

μg/ml. Cell growth assays with fibroblast cells demonstrated a constant viability at a concentration of 100 μg/ml of mesoporous carbons and a clear visual observation of cell-carbon contact was confirmed. None of the carbon samples demonstrated hemolysis (<1 %). Protein adsorption with bovine serum albumin (BSA) and fibrinogen demonstrated lower protein binding with a decreasing trend with an increase in carbon surface area. All the results suggested that the mesoporous carbon materials are biocompatible and, the degree of biocompatibility is within the range or higher than other biomaterials currently employed in biomedical applications.

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Chapter 7

Conclusions and future work

7.1. Conclusions

In this dissertation, we discovered that osmolytes function as antiviral compounds against viruses and as flocculants in order to improve virus purification methods for vaccine production. We also demonstrated that soft-templated mesoporous carbons are biocompatible and not toxic for mammalian cells. Future work on these materials will determine if they can be used as vehicles for vaccine delivery.

We have discovered that two protecting osmolytes, trimethylamine N-oxide (TMAO) and glycine at 0.2 M reduce the infectivity of porcine parvovirus (PPV), a non-enveloped virus, by 4 LRV (99.99%), likely by disruption of capsid assembly [1]. These results are of great interest, since to our knowledge, the majority of synthetic and natural compounds that have been found are for enveloped viruses, and not for non-enveloped viruses. The majority of these antiviral compounds found in the literature inhibit virus attachment, and they are able to do this by inactivating the virus envelope (see **Chapter 2**). TMAO and glycine showed antiviral activity after being added 20 h post-infection, indicating that they could be used after the virus has infected the cells, indicating that they could be used after the virus has infected the cells. This increases their potential to be used as therapeutic drugs.

We also found that osmolytes could be used as flocculants for virus purification, and overcomes many of the limitations of current purification methods, such as chromatography and nanofiltration. Osmolytes are able to specifically flocculate hydrophobic virus particles by depleting a hydration layer around the particles and subsequently cause virus aggregation. The best flocculants for PPV, were found to be glycine, alanine, sucrose, trehalose and mannitol [2]; and the best flocculants for the enveloped Sindbis virus (SVHR) were proline, betaine, sucrose, trehalose, raffinose, and mannitol. These osmolytes demonstrate a high removal (>80%) of virus with a 0.2 μm filter, which is usually used to remove bacteria, not small viruses such as PPV and SVHR. A micro-filter increases the flux and decreases the transmembrane pressure of typical virus filters. We were able to preferentially flocculate PPV and SVHR while leaving model proteins in solution. Protein removal was very low with osmolytes (< 20%) as compared to the

salts (55% for BSA and 87% for lysozyme). Salts, and not osmolytes were able to enhance protein flocculation, whereas both were able to flocculate virus particles. The aim in this project was to find an osmolyte that could be used as platform purification for viral products. Mannitol at 0.3 M was able to demonstrate a high virus removal, 81% for PPV [2] and 98.1% for SVHR. Virus flocculation with mannitol, followed by microfiltation could be used as a platform process for virus purification.

One of the biggest challenges in vaccine technology is to develop vaccines that are safe and offer a strong immune response to patients [3]. Conventional vaccines offer effective immune response but they offer reactogenic effects. New vaccine technologies have been able to mitigate these effects, but often with the cost of reduced effective immune response. Vaccine delivery vehicles, which can target the immune cells with adequate doses, have been suggested for improving the immune response. In this study, we proposed soft-templated mesoporous carbon as a new type of vaccine delivery vehicle. In this study, soft-templated mesoporous carbon materials showed minimal toxicity and high cell viability with mammalian cells. Hemocompatibility studies show a <1% of hemolysis and low adsorption of blood proteins to mesoporous carbon materials. The results showed that our soft-templated mesoporous carbon materials are biocompatible, and the degree of biocompatibility is within the range or higher than other biomaterials currently used in biomedical applications [4].

7.2. Future work

For antiviral compounds, we would like to explore the antiviral properties of fruits rich in polyphenols. Many studies have demonstrated antiviral properties of different types of fruits against non-enveloped viruses [5-14]. However, the active compounds have not been isolated yet. Tea and other plant polyphenols have demonstrated antiviral activity with enveloped viruses [15-21]. We believe we can find antiviral properties of specific polyphenols found in fruits against non-enveloped viruses, such as the rhinovirus, which cause the common cold in humans, and hepatitis A virus. The aim in this research area is to find natural compounds that can inhibit virus infectivity without causing severe effects in the cells. Fruits are known to be healthy and are used

for consumption, so we believe they are of great interest. With the knowledge that our lab has in virology assays, we hope to find new therapeutic antiviral compounds for non-enveloped viruses that affect humans.

The aim of virus flocculation with osmolytes followed by microfiltration is to replace current unit operations used in virus purification, such as chromatography and nanofiltration. In order to validate this method for virus purification, the virus needs to be recovered from the membrane surface. We have recovered infectious virus particles from the membrane surface, but the recovery is currently low (~2%). We are exploring new methods to recovery the virus, such as the possibility of using a diafiltration setup. Diafiltration is a technique that has been used before to concentrate virus particles and has led to high recoveries of virus [22, 23]. It can enhance the product yield, along with recycling the osmolyte solution. Further studies will need to be done in order to determine the best filtration system, the filter capacity and the pressure-flux profiles for successfully commercialization of virus flocculation in osmolytes.

We are also interested in trying virus flocculation in osmolytes with different types of membrane materials in our filtration step. In our lab, we have been able to created functionalized nanofibers, which have shown 2-4 logs reduction of PPV and SVHR [24, 25]. We would like to make our own membranes based on functionalized nanofibers and used these membranes after virus flocculation in osmolytes in order to enhance the virus removal. We would also like to try a double effect of flocculants. We can try different combinations of our top osmolytes to enhance virus removal. With the best combination of osmolytes flocculants, we could try different times of flocculation and reduce our current incubation time, which is 2 hours. Kinetics studies could help us to understand how fast is the aggregation process and how different conditions (i.e. temperature, flocculants, pH) could affect the speed of the aggregation process.

For our collaborative research project, we are planning to perform other types of cell viability and cytotoxicity assay to make our hypothesis stronger. We have assessed cell viability with a trypan blue assay, where trypan blue stains dead cells, which can be counted using a hemocytometer under a microscope. However, trypan blue is a cell exclusion assay. Cells that are alive, but have a reduced function, are counted equally as cells that are alive and healthy. At

the same time, it can stain cells whose membranes are temporarily permeable, wrongly identifying them as dead [26]. The cytotoxicity assay that we use, the MTT Assay, is a well-known cell-viability assay that measures mitochondrial activity in living cells. This MTT Assay only indicates if cells are dead or alive. Since we are more interested in cell metabolism functions, we are planning to measure the concentration dependent adenosine triphosphate (ATP) depletion of cell and the concentration dependent reactive oxygen species (ROS) of new soft-templated mesoporous carbon materials. ATP is the energy currency of the cells, is in charge of transporting energy within the cells for several metabolic functions. ATP depletion is associated with toxicity, and can cause cell damage and apoptosis [27]. ROS are reacting molecules containing oxygen, they are produced during normal metabolism and are involved in several cellular functions, such as cell proliferation, differentiation and apoptosis [28, 29]. ROS are produced inside of the human body and removed by antioxidants [30], since high concentrations of ROS can be toxic to the cells [29]. The positive results that we expect to get from the ATP and ROS studies will make our biocompatibility studies stronger. With the hope of finding positives results in our *in-vitro* assays, we hope to move to *in-vivo* assays.

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