NOVA SCHOOL OF SCIENCE & TECHNOLOGY

DEPARTAMENT OF CHEMISTRY

ESTABLISHMENT OF AN INSECT CELL-BASED PLATFORM FOR PRODUCTION OF ADENOVIRUS-LIKE PARTICLES (ADDomer PARTICLES) AS A SNAKEBITE THERAPY

Cláudia Cristina Sobreiro Paiva

Bachelor in Biotechnology

MASTER IN BIOTECNHOLOGY

NOVA University Lisbon
October, 2022

NOVA SCHOOL OF SCIENCE & TECHNOLOGY

DEPARTAMENT OF CHEMISTRY

Cláudia Cristina Sobreiro Paiva

Bachelor in Biotechnology

ESTABLISHMENT OF AN INSECT CELL-BASED PLATFORM FOR PRODUCTION OF ADENOVIRUS-LIKE PARTICLES (ADDomer PARTICLES) AS A SNAKEBITE THERAPY

MASTER IN BIOTECNHOLOGY

NOVA University Lisbon
October, 2022

DEPARTAMENT OF
CHEMISTRY

ESTABLISHMENT OF AN INSECT CELL-BASED PLATFORM FOR PRODUCTION OF ADENOVIRUS-LIKE PARTICLES (ADDomer PARTICLES) AS A SNAKEBITE THERAPY

Cláudia Cristina Sobreiro Paiva

Bachelor in Biotechnology

Adviser: António Manuel Missionário Roldão
Head of Cell-based Vaccines Development Lab, Instituto de Biologia Experimental e Tecnológica

Co-advisers: Cristina Maria da Costa Peixoto Lisboa
Head of Downstream Processes Lab, Instituto de Biologia Experimental e Tecnológica

Examination Committee:

Chairperson: Ana Rita Cruz Duarte
Associate Professor, NOVA School of Science and Technology
University of Lisbon

Opponent: Luís Joaquim Pina da Fonseca
Associate Professor, Instituto Superior Técnico
University of Lisbon

Supervisor: António Manuel Missionário Roldão
Head of Cell-based Vaccines Development Lab, Instituto de Biologia Experimental e Tecnológica

MASTER IN BIOTECHNOLOGY

NOVA University Lisbon
October, 2022

Establishment of an insect cell-based platform for production of Adenovirus-like particles (ADDomer particles) as a snakebite therapy

Copyright © Cláudia Cristina Sobreiro Paiva, NOVA School of Science and Technology, NOVA University Lisbon.

The NOVA School of Science and Technology and the NOVA University Lisbon have the right, perpetual and without geographical boundaries, to file and publish this dissertation through printed copies reproduced on paper or on digital form, or by any other means known or that may be invented, and to disseminate through scientific repositories and admit its copying and distribution for non-commercial, educational or research purposes, as long as credit is given to the author and editor.

This document was created with Microsoft Word text processor and based on NOVAthesis Word template.

PUBLICATIONS

This work was supported by Horizon 2020 European-funded project "ADDovenom" (grant number: 899670), aiming to develop a novel snakebite therapy platform of unparalleled efficacy, safety, and affordability.

The results described in this work were presented at ADDovenom project scientific meetings:

Garcia R., Paiva C., Moleirinho M., Peixoto C., Roldão A., Alves PM a "Scalable bioprocess for ADDomer particles production", ADDovenom Spring Meeting, April 2022, *online*.

Garcia R., Paiva C., Moleirinho M., Peixoto C., Roldão A., Alves PM a "Scalable bioprocess for ADDomer particles production", ADDovenom 2nd annual Meeting, September 2022, Marseille (France).

À minha família

ACKNOWLEGMENTS

I would like to acknowledge all the people directly and indirectly involved to this work.

To Professor Paula Alves and Professor Manuel Carrondo for promoting the quality and excellence of work at the Animal Cell Technology Unit (ITQB/iBET), for offering all the resources available, and for projecting Portugal as a point of reference in bioprocessing viral-based therapies.

To my supervisors, Dr. António Roldão and Dr. Cristina Peixoto, for given me the opportunity to do my dissertation in this innovative and passionate project, for the kindness with they welcomed me into their teams, for trusting and believing in me, and for challenging me to do and want more.

To Raquel Garcia and Mafalda Moleirinho for the guidance, support, knowledge and dedication, for trusting in me and for giving me the freedom to make mistakes, learn and try again. Also, to Bárbara Fernandes and Sofia Moreira for the knowledge, advices and patience. A special acknowledgment to Ricardo Correia for always being available to help me and for being an example of excellence and kindness in the scientific community.

To Dr. Tiago Bandeiras for giving me the opportunity to develop part of my laboratorial work at Biology for Drug Discovery Unit of Merck Healthcare KGaA Satellite Lab (ITQB/iBET), and to Micael Freitas and Ana Rita Lemos for the guidance and knowledge.

To my colleagues from the insect team and from the downstream processing team for always being available to help me. A special acknowledgment to Nikolaus Virgonili, Marco Silvano, Ines Muzaferovic, Albert Carli, Taja Zotler, Marta Silva, Inês Macieira and João Mendes for supporting and encouraging me. Also, to Ana Raposo for always being so kind and affectionate.

À minha família pelo apoio incondicional, amor, afeto, atenção, devoção e educação. Aos meus pais, Joaquim Paiva e Vírgina Paiva, por me transmitirem os seus valores morais, por me inspirarem e suportarem em todas as minhas conquistas, por acreditarem em mim, por me impulsionarem a abraçar novos desafios independentemente das contrariedades e por me estimularem todos os dias a ser uma pessoa melhor, a fazer a diferença. À minha querida irmã Rita Paiva que sempre foi e será o meu *role model*, pelo acompanhamento, carinho, partilha e ajuda. À minha querida avó Maria Fernanda Santos que é uma força da Natureza, pelos sábios ensinamentos, perspicácia, acompanhamento, carinho e alegria que acrescenta à minha vida. Ao Ricardo Cruz pelo apoio incondicional, carinho e por acreditar em mim nos momentos em que eu própria não acredito.

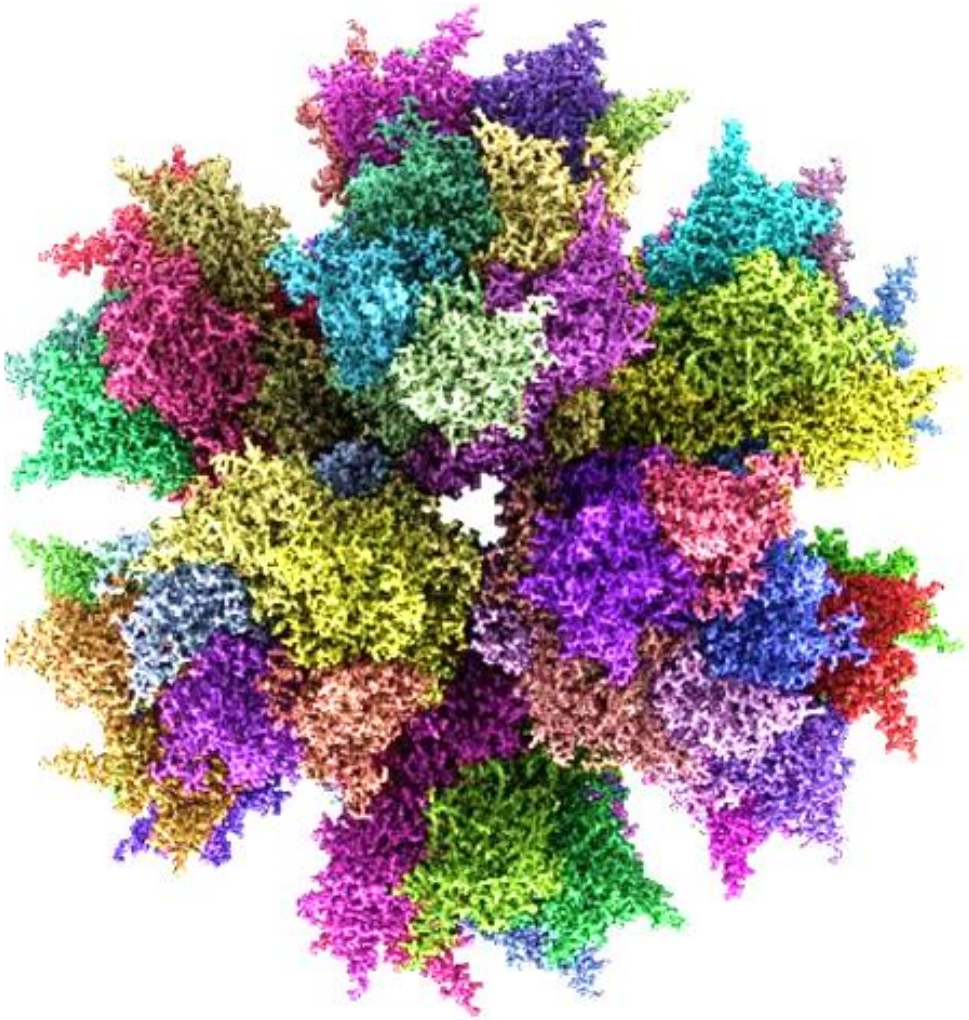
Às minhas amigas Sara Cardoso, Filipa Almeida, Ana Rita Gonçalves, Débora Gomes, Inês Chaves e Komal Khalil por estarem sempre comigo, me apoiarem e nunca se esquecerem de mim nos momentos de ausência.

Ao professor Rui Palma e professor Jorge Silvestre por me ensinarem a força do poder da mente, a ser perseverante, a ultrapassar os meus medos e a lutar pelos meus objetivos.

"Where there is a will there is a way"

George Herbert,

adapted version of New Monthly Magazine (1822)



ABSTRACT

Snakebite envenoming is a neglected tropical disease which causes up to 138 thousand deaths per year. Nowadays, snake antivenom is the only available treatment in the market, originated from horse serum, with limited efficiency and non-reproducible production.

This study aimed at implementing and improving ADDomer particles production and purification processes towards the development of a next regeneration ADDomer-based antivenom therapy designed to recognize, interact, and remove snake venom toxics from human body. ADDomer particles were produced intracellularly in insect High Five cells using a baculovirus expression vector system and purified using a three-step purification process (incl. freeze-thaw cycles, centrifugation, size-exclusion and anion exchange chromatography) according to baseline bioprocess. Purified ADDomer particles have a size and morphology similar to those previously reported with a production yield of 19 mg ADDomer particles per L of culture, a purity > 99% and a melting point of ~ 54℃ (as assessed by thermal shift assay). To improve ADDomer particles production, different insect cell lines and culture medium were explored, with High Five cells and *Insect Xpress*^{TM} medium being the most promising combination. To improve ADDomer particles purification, an integrated cell lysis and clarification procedure was devised using 0.2% deviron surfactant (for cell lysis) and a 0.005 m^2 BioOptimal^{TM} MF-SL hollow fibber (for concentration and clarification), replacing the freeze-thaw cycles and centrifugation techniques applied in baseline bioprocess.

Overall, this work demonstrates the potential of an insect cells-based platform for ADDomer particles production and represents the basis for the development of an ADDomer-based snakebite therapy.

Keywords: *ADDomer particles, Baculovirus, Insect cell lines, Process development, Snake Antivenom.*

RESUMO

O envenenamento por mordida de cobra é uma doença tropical negligenciada que por ano causa até 138 mil mortes. Atualmente, o antídoto de cobra é o único tratamento existente no mercado produzido a partir do plasma de cavalos com uma eficiência limitada e produção não reprodutível.

Este estudo tem como objetivo implementar e melhorar o processo de produção e de purificação das partículas ADDomer para o desenvolvimento de uma terapia de segunda geração de um antídoto de mordida de cobra baseada na tecnologia ADDomer, desenhada para reconhecer, interagir e remover tóxicas de veneno de cobra de pacientes humanos. As partículas de ADDomer foram produzidas intracelularmente em células de inseto *High Five* usando o sistema vetorial de expressão de baculovírus e purificadas usando um processo de purificação de 3 etapas (incl. ciclos de congelação/descongelação, centrifugação, cromatografia de exclusão molecular e troca aniónica) de acordo com o bioprocesso base. As partículas ADDomer purificadas têm um tamanho e morfologia semelhante ao reportado com rendimento de produção de 19 mg de partículas ADDomer por L de cultura, grau de pureza > 99% e temperatura de fusão ~ 54℃ (avaliado por ensaio de deslocamento térmico). Para melhorar a produção das partículas ADDomer, diferentes linhas celulares de insetos e meios de cultura foram explorados, com as células *High Five* e o meio *Insect Xpress*™ a serem a combinação mais promissora. Para melhorar a purificação das partículas ADDomer, um procedimento integrado de lise celular e clarificação foi concebido usando 0.2% de surfactante deviron (para a lise celular) e o dispositivo de fibras ocas *BioOptimal™ MF-SL* de 0.005 m^2 (para concentrar e clarificar), substituindo os ciclos de congelação/descongelação e a técnica de centrifugação aplicada no bioprocesso base.

No geral, este trabalho demonstra a potência da plataforma baseada em células de inseto para produzir as partículas ADDomer e representa a base para o desenvolvimento de uma terapia para a mordida de cobra baseada na tecnologia ADDomer.

Palavras-chave: *Antídoto para a mordida de cobra, Baculovírus, Desenvolvimento de processo, Linhas celulares de insetos, Partículas ADDomer.*

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF EQUATIONS

LIST OF ABBREVIATIONS

AcMNPV	*Autographa californica* multicapsid nucleopolyhedrovirus
AEX	Anionic exchange chromatography
AVs	Snake Antivenom
BCA	Bicinchoninic acid
CAS	Cassette
CCI	Cell concentration at point of infection
cfu	Colony-forming unit
CHES	N-Cyclohexyl-2-aminoethanesulfonic acid
CPP	Critical Process Parameters
CT	Chemically-competent
CTLs	C-types Lectins
DF	Diafiltration
DLS	Dynamic light scattering
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
*ds*DNA	Double-stranded DNA
ECHA	European Chemicals Agency
E. coli	*Escherichia coli*
EMA	European Medicines Agency
ET	Electro-competent
FDA	Food and Drug Administration
FNU	Formazin nephelometric units
High Five	Cell line derived from *Trichoplusia ni* ovarian cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HF	Hollow fibber
HLPC-SEC	Size exclusion-high-performance liquid chromatography
hpi	hours post-infection
hpt	hours post-transfection
iBET	Instituto de Biologia Experimental e Tecnológica
IC-BEVS	Insect cells-Baculovirus expression vector system
LB	Luria-Bertani

MES	2-(N-morpholino) ethanesulfonic acid
MF	Microfiltration
MOI	Multiplicity of infection
MOPS	3-(N-morpholino) propanesulfonic acid
MVS	Master virus stock
NPVAC	*Autographa californica nuclear polyhedrosis virus* specie
P_0	Parental generation of baculoviruses
P_1	Passage 1 of generation of baculoviruses
P_2	Passage 2 of generation of baculoviruses
pACeBac1_addomer	Plasmid shuttle vector with genes that codes for ADDomer particles
PCR	Polymerase chain reaction
PBS	Phosphate-buffered saline RNase-free at pH 7.4
Pt-Dd	Penton base protein
pUC-19	Plasmid cloning vector
rBAC	Recombinant baculovirus stock
RNA	Ribonucleic acid
SARS-CoV-2	*Severe acute respiratory syndrome coronavirus* 2
SDS-Page	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
Sf-9	Cloned cell line derived from *Spodoptera frugiperda* ovarian cells
superSf-9-2	Transgenic cell line derived from *Sf*-9 cells
SPG	Sucrose phosphate glutamate
SNP	Supernatant
TFF	Tangential flow filtration
T_m	Melting temperature
TEM	Transmission electron microscopy
TOH	Time-of-harvest
T.ni	*Trichoplusia ni* specie
TSA	Thermal shift assay
UF	Ultrafiltration
UoB	University of Bristol
VLP	Virus-like particles
WB	Western blot
WHO	World Health Organization
YFP	Yellow fluorescent protein

LIST OF SYMBOLS

$	American dollar
€	Euro
µg	Microgram
%	Percentage
L	Litre
mM	Millimolar
mg	Milligram
mL	Millilitre
ng	Nanogram
nm	Nanometre
pH	Hydrogen potential
U	Unit

1 INTRODUCTION

1.1. Snakes

Snakes are cold-blooded animal whose hallmarks include an elongated body and tail, continuous and often disconcerting stare, sharp tongue, and coloured scales. It's thought that snakes have evolved from terrestrial lizards during the Middle Jurassic Epoch (from 174.1 million to 163.5 million years ago), having lost the movable eyelids, the external ear openings, the urinary bladder, and the left lung due to natural selection. The selective traits have allowed snakes to develop a tracheal lung in the neck region, a skin highly sensitive to temperature variation, and specialized salivary glands which elaborate a potent venom and a venom-conducting system for internally injecting the venom into the prey [1]. These anatomical specifications make them highly competent predators with advantage in many ecological niches [2]. Snakes belong to order Squamata, composed of 3434 species and 500 genera, well distributed around the world [3].

1.1.1. Venomous Snakes

Venomous snakes use venom to subdue their prey and to survive in their habitat. Evolutionary pressure has led to the development of selected venom toxins targeting animal tissues [2]. The snakes' venoms might contain more than 100 different compounds: toxic and non-toxic proteins and enzymes, peptides, carbohydrates, lipids, amines, and other small molecules [2,4]. Derived from its heterogeneity, snake venoms are the most complex of all natural venoms and poisons [2].

Viperidae, Elapidae, Colubridae and Lamprophiidae are the families of venomous snakes that have been reported to cause human envenomation through snakebite [4,5]. Specifically, Viperidae and Elapidae families include the deadliest snakes ever reported worldwide such as *Echis pyramidum leakeyi* and *Dendroaspis viridis* [6]. These snakes' venoms can kill a person within 2 to 10 hours post snakebite, depending on the amount of venom injected and on victim's weight [6].

1.1.2. Snakebite Envenomation

Snakebite envenomation is a clinical condition developed upon snakebite and venom injection [7]. It's a neglected and rather devastating occurring disease in many developed and developing countries that has been largely ignored by the medical community. The World Health Organization (WHO) estimated 1.8 to 2.7 million cases of snakebite envenomation, causing 81 to 138 thousand deaths and resulting in 400 thousand physical long-lasting disabilities and

psychological sequalae annually [5,6,7,8,9]. The highest incidence occurs in tropical and subtropical countries, with the vast majority (95%) being reported in Africa and the Middle East, and Asia [5,7,10] (**Figure 1.1)**. The real number of cases is much higher than those provided by official health statistics, owing to limitations in data acquisition by health authorities, poor attendance of affected people at health centres, limited access to healthcare facilities, absence of post venom-control consultation, and lack of knowledge of health staff trained to identify snakebites and recognise their clinical signs and treatments [5,6,7,8].

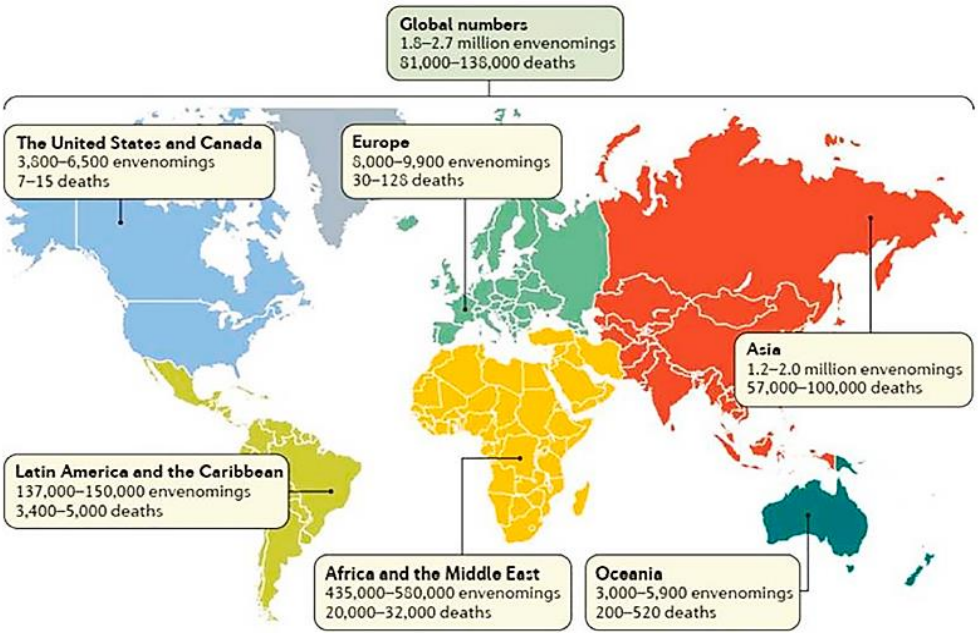


Figure 1.1 *Estimated worldwide geographical distribution of snakebite envenomation incidence and mortality rate. Taken from Alangode et al., (2020) [9].*

Following its recognition as a neglected tropical disease by WHO in 2009 [2], many studies have been done to prevent and control snakebite envenomation. In 2019, the WHO launched a global strategy to reduce by 50% the number of fatalities and disabilities caused by the disease until 2030 [6,7]. The first goal is to increase confidence in therapeutic agents, the second is to improve access to it, and the third is to correctly inform the population and healthcare staff for envenomation [6]. In spite of efforts made so far, snake antivenom (AVs) is still the only scientifically validated therapy to treat snakebite envenomation [9,10].

1.1.3. Snakes Antivenom

AVs is a clinical formulations composed of specific antibodies that directly neutralize snakes venom toxins [9,10]. Albert Calmette, in 1895, developed the first AVs to target an Indian Snake venom [11]. Conventionally, these are produced from a fractionation of plasma of immunized animals, usually horses, that have been repeatedly injected with sublethal doses of snake-extracted venom [8,9,10]; this process is illustrated in ***Figure 1.2***. AVs can be composed of polyclonal (when generated against the venom of different species of snakes) or monoclonal antibodies

(when raised against venom of one snake species), depending on their production specifications [8,9].

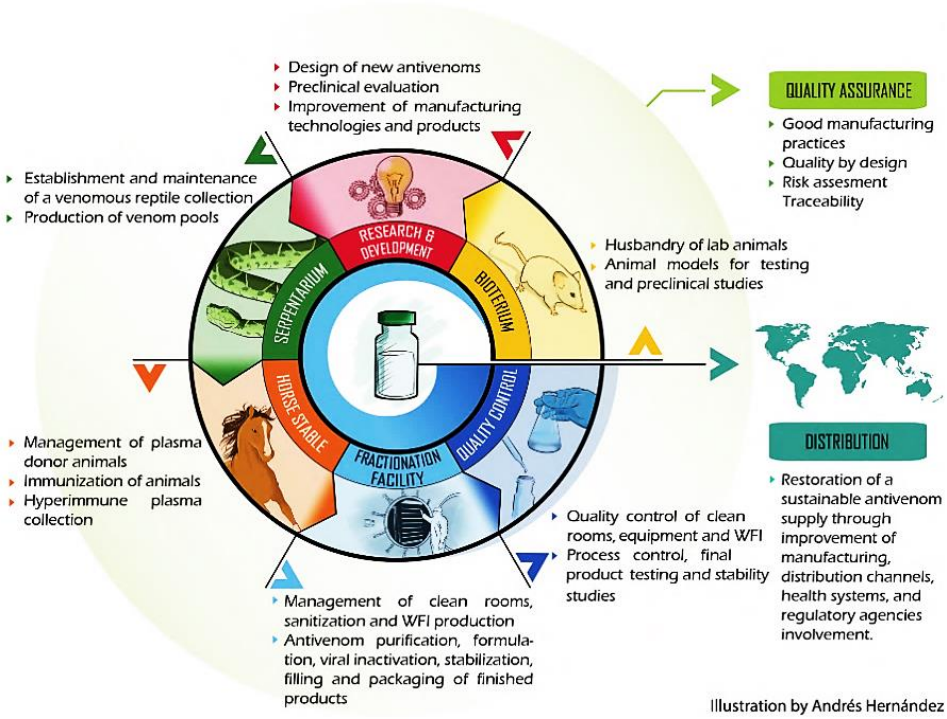


Figure 1.2 *Schematic overview of the manufacturing processes of conventional plasma-derived snakes antivenom. Taken from Vargas et al., (2021) [10].*

The efficacy of AVs has been questionable over time. It was verified that, depending on age, sex and geographic habitat of snakes, the extracted venom exhibits different toxicity and activity, inducing batch variability during AVs production. At a molecular level, such variations can result from minor changes either on structure or on chemistry surface of the toxins [9]. Moreover, upon AVs administration, hypersensitivity have been shown to occur in 10% of patients. Based on their large size, AVs are slowly absorbed by the lymphatic system, which results in low bioavailability in human body, resulting in only 1.4 to 6% of injected AVs reaching the snakebite zone when these are intramuscularly administered. Contrastingly, 85% reach the target bite area when being intravenously administered [8]. Nevertheless, upon formation of the venom-AVs complex, this is rapidly eliminated from the body [6].

AVs prices are generally high [6], accounting for 79% of the snakebite envenomation total treatment cost [7]. During the 2017 snakebite program, the Nigerian government spent US $980 per million population and this amount was only enough to treat 4% of patients. *FavAfrique* produced by *Sanofi Pasteur* and *SAIMR* produced by *SAVP* are the most famous AVs in the African market, and cost US $140 per vial and US $315 per vial, respectively [12]. The fact that only one vial is not sufficient to treat a patient [6], and the low public healthcare budget in most countries, emphasizes that AVs is not an affordable treatment to everyone [12]. Efforts have been

made to substantially reduce AVs prices, increase its volumetric productivity, and widen its distribution. In the last three decades, innovative technologies have been developed to produce next generation AVs, e.g. non-serum-based AVs and non-antibody-based venom-neutralizing agents [9,11,13], that are more sustainable (by overcoming the hurdle of antivenom production in animals such as horses), safe, and cost-effective.

1.2. ADDomer technology

ADDomer particles are self-assembling protein-based nanoparticles highly attractive for a range of biomedical applications [14]. They are easy-to-manufacture (production compatible with industrial processes already implemented), heat-stable (maintain its effectiveness without refrigeration), amenable for lyophilisation, safe when administered in low-doses and do not promote allergic reaction in humans [16,17]. This innovation, created by the University of Bristol (*UoB*) biotech start-up *Imophoron*, has recently been used as a vaccine-platform to fight SARS-CoV-2 pandemic [17]. Based on the same technology, an ADDomer-based AVs therapy is being developed [15]. Due to its highly efficient multiepitope surface, illustrated in ***Figure 1.3***, ADDomer particles can be pursued as a snakebite therapy through the modification on its surface display to be able to bind, neutralize and remove snake venom toxics from bloodstream of victims, and to annihilate their pathogenic effects without trigger an immune response [16]. Contrary to conventional AVs, the ADDomer-based AVs therapy is expected to be a low-cost treatment, clinically more efficient and independent of taxonomy or geography of snakes [16,17].

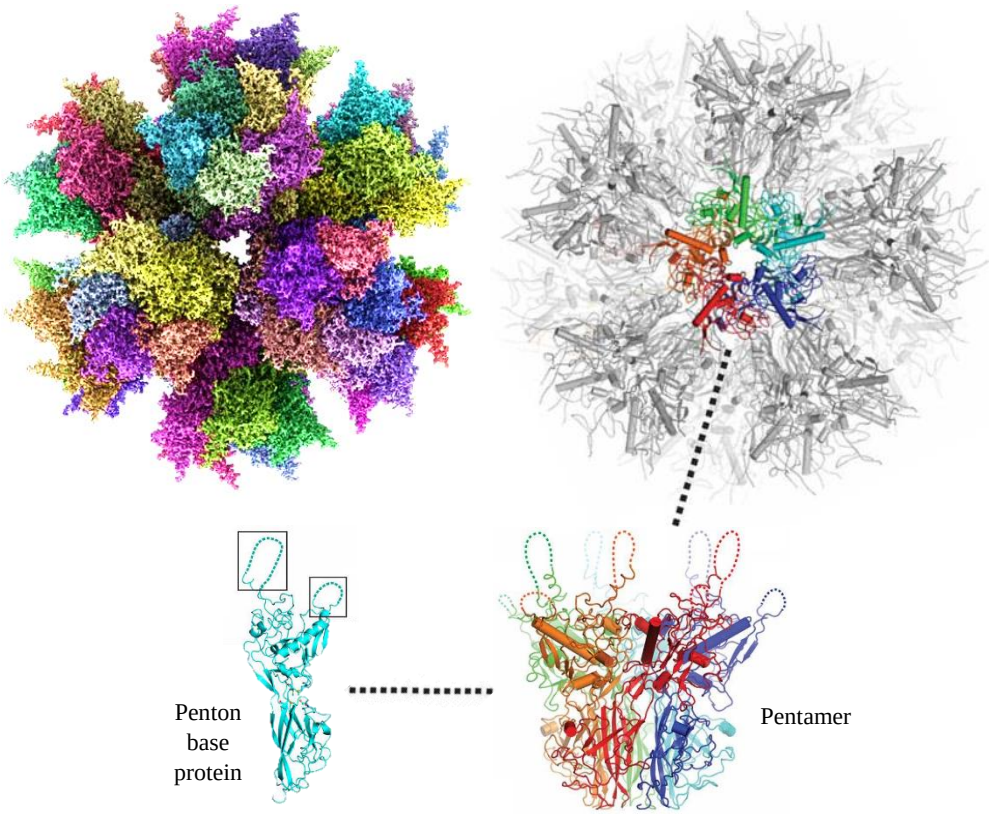


Figure 1.3 *Dodecahedron structure of ADDomer particles in different projections emphasizing the 60 protomers and the 12 pentons. Adapted from Vragniau et al., (2019)* [14].

1.2.1. Insect cells-baculovirus expression vector system

Baculoviruses are insect pathogenic lytic viruses. The *Autographa californica* multiple nucleopolyhedrosis virus (AcMNPV), belonging to α-baculoviruses genera, shows natural ability to infect insects of the *Lepidoptera* order, and thus has been employed to produce recombinant protein using the insect cells-baculovirus expression vector system (IC-BEVS). This archetype differs from the others by expressing GP64 fusion protein essential to mediate insect cell host infection [18]. Since the early 1980s, IC-BEVS has been widely used for manufacturing of viral particles and recombinant proteins [19]. The system is characterized as robust, versatile, safe, and scalable [19,20].

This transient expression platform is based on the transfection of insect cells with a baculovirus vector genetically modified to encode the genes coding for the product of interest. Designed for a range of applications, artificial AcMNPV genome, denominated as bacmid-DNA, is a single-copy bacterial artificial chromosome capable of replicating in bacteria. Therefore, bacmid-DNA has in its constitution essential baculovirus coding genes, prokaryotic and eucaryotic promotors [21]. Using *E.coli* cells as editing tools, bacmid-DNA can incorporate foreign genes through the genetic recombinant zone [22,23]. Modified bacmid-DNA is select according to the phenotype of transformed colony and it is further used to transfect insect cells. Upon transfection, selected bacmid-DNA translocate to the cell nucleus and shut off the transcription of cells genes for overexpression of heterologous viral genes [24]. After this, baculoviruses are produced and used to infect insect cells for expression of the product of interest. Upon infection, as it is described in ***Figure 1.4.***, baculoviruses enter into cells by endocytosis. They migrate to the nucleus expanding their virion envelope in the mediation of a nucleus pore, allowing the nucleocapsid enter. In the nucleus, nucleocapsid is remove, viral DNA replicates, and new nucleocapsids are assembled and packaged. Then, they migrate to the cytoplasm through the nuclear egress, and exit the cell acquiring the host membrane-derived virion envelope during the process [18]. The release of baculoviruses occurs at a very late stage of infection [24]. During infection, cells increase in size (swelling phenomena), stop duplicating, become granular in appearance and lose viability [24].

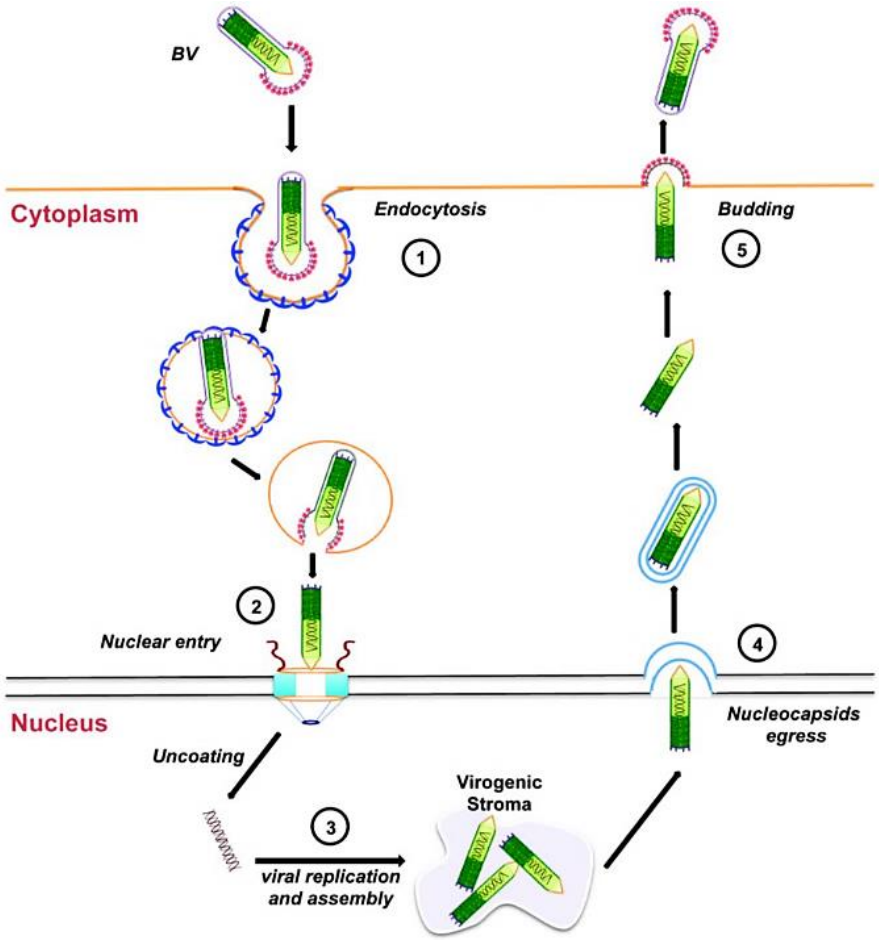


Figure 1.4 *Schematic representation of baculovirus-host cell infection divided in 5 phases: (1) Endocytosis, (2) Nucleus penetration, (3) Nucleocapsid production, (4) Nucleocapsids egress and (5) Budding. Taken from Au et al., (2013) [18].*

Sf-9, *Sf*-21 and High Five cells are the most widely used insect cell lines [19]. Despite allowing reduced glycosylation processing comparing to mammalian cells, insect cells are amenable to be adapted to suspension cultures, cell growth can be improved through medium formulations and additives, and they are able to grow in serum-free medium, thus facilitating further purification processes [19,20]. IC-BEVS derived products are commercially available for human and veterinary use, being Cervarix™ (against the human papillomavirus) and Novavax™ vaccines (against SARS-CoV-2) two examples [25].

Compared to other biomanufacturing platforms available, the IC-BEVS offers a high expression level of recombinant proteins and ability to generate self-assembling multimeric structures such as virus-like-particles (VLPs) [19,25]. VLPs consist of viral proteins that assemble into nanostructures resembling their virus counterparts, but are not infectious for being devoid of genetic material [20]. Like VLPs, ADDomer particles are self-assemble nanostructures composed of viral particles, and thus IC-BEVS is poised as a potent platform for their production. ADDomer particles were engineered from a single component of Human Adenovirus B serotype 3, the penton base protein (Pt-Dd) [17]. Pt-Dd units can spontaneously self-assemble into a symmetric

particle with 60 protomers, that assemble into 12 Pt-Dds and form a hollow dodecahedron [26]. Despite its size and conformation, Pt-Dd preserve adenovirus-like ability to penetrate in epithelial cells and originates non-enveloped particles less immunogenic [26]. These characteristics have been shown in Vragniau *et al.*, (2019) [14], where the 30 nm dodecahedron particle is described as soluble, thermostable (stable at temperature > 55℃), neutrally charged and safe.

1.2.2. Purification of viral particles

Purity and safety are essential requirements of biomedical products to be considered throughout for manufacturing, by following guidelines of regulatory agencies such as Food and Drug Administration (FDA) and European Medicines Agency (EMA) [25]. The use of IC-BEVS as a production platform poses some challenges as the co-production of recombinant baculovirus in the form of enveloped viruses (which need to be removed or inactivated) [27] and the release of proteases (can induce degradation of recombinant protein produced) as consequence of cell lysis [28].

Processes for viral particles purification should be as simple as possible, focus on robustness (consistent differentiation of recombinant protein from impurities), scalability (maintaining recovery yields over scale), purity (high removal of impurities), potency (higher titers or concentration), and cost-effectiveness [20,29].

Classical purification approaches include an initial step of cell lysis (if recombinant protein is intracellular) or supernatant collection (if recombinant protein is extracellular), followed by clarification, one or more operation units of purification, polishing, concentration and sterile filtration [20]. At each processing stage, a wide range of techniques have been applied over the years derived from their efficiency. A few examples are briefly described below.

1.2.2.1. Centrifugation

Centrifugation is widely used to purity viral proteins. Despite being a simpler separation technique to remove cell debris and clarify VLPs suspensions at small-scale, is time-consuming and requires higher investments [20,30]. Continuous centrifugation is available for processing large scales, however they require a considerable initial capital investment [30].

1.2.2.2. Tangential flow filtration

Tangential flow filtration (TFF) is an operation mode used in filtration procedure where the feed flows tangential to the membrane surface and the applied pressure causes one portion of the flow stream to pass through the membrane (permeate) while the remaining flow (retentate) is recirculated back to the feed reservoir. It is an attractive alternative to centrifugation approaches since a low shear can be use, limiting the impact on the structure of viral particles [30]. This method is scalable, reduces the processing volume and, consequently, the cost [20,29]. It is preferentially used in clarification operation unit with a microfiltration (MF) technique, and in

concentration operation unit coupled with ultrafiltration (UF) technique, by using different membrane devices such as hollow fibber (HF) and cassette (CAS) [31].

In TFF, the pressure applied on the membrane surface is monitored by measuring the transmembrane pressure (P_{TMP}). P_{TMP} is referred as the main driving force required to pump a solution through the membrane [32] and it is assessed by monitoring feed-inlet, retentate and permeate pressures. Thus, P_{TMP} can be determined by applying ***Equation 1.1***.

$$P_{TMP} = \frac{P_f + P_r}{2} - P_p \quad \textbf{(1.1)}$$

Equation 1.1 *Calculation of transmembrane pressure (P_{TMP}, bar) of a membrane filtration system, having in consideration feed-inlet pressure (P_f, bar), retentate pressure (P_r, bar) and permeate pressure (P_p, bar).*

1.2.2.2.1. Microfiltration

According to the pore size of membranes (normally ≥ 0.1 μm), MF is designed to retain larger components in suspension, such as cells and cells debris, while it permeates smaller components [31]. MF is often applied in clarification operation unit, and when in TFF mode it has a tendency for membrane fouling which reduces its performance [30]. As for infected insect cells, these are more sensitive to shear stress and break apart easily when high flow rates are applied [30]. To overcome this, HF devices operated in MF in TFF mode have been used to retain infected insect cells without compromising its integrity by decreasing the shear stress [33].

1.2.2.2.2. Ultrafiltration

UF coupled to diafiltration (DF) is used routinely to concentrate solutions by isolating components < 0.1 μm inside of membranes, allowing the removal of lower molecular weight impurities while buffer exchanging [20]. The UF/DF can be applied throughout the process as concentration operation units to reduce processing volume, being primarily used between polishing and sterile filtration operation units. The fouling events that usually occur on UF membrane reduce the permeate flux and, consequently, increase the processing time.

1.2.2.3. Anion-exchange chromatography

Anion-exchange chromatography (AEX) is a non-destructive technique in which negatively charged recombinant proteins are removed from solution by interacting more avidly with charged resin beads than positive charged impurities. For that reason, in gene therapy and vaccine manufacturing, AEX elution are often performed by increasing the ionic strength (salt concentration) of the buffer to maximize the product recovery and impurities removal [34]. AEX is a procedure largely used for intermediate purification steps [20].

1.2.2.4. Size-exclusion chromatography

Size-exclusion chromatography (SEC) is a non-destructive technique highly efficient to separate viral particles from impurities in solution, as well as incorrectly assembled recombinant proteins [20,35]. Based on hydrodynamic radius of proteins, larger components are eluted in the dead volume of column, while smaller components freely penetrate in SEC pores facing longer retention times and later elute according to their size [20,35]. SEC is widely employed in polishing operation unit. Despite being a high-resolution technique, SEC is always limited by the processing volume since it only can process 10% of column bed volume [20].

1.3. Main goals and thesis outline

The aim of the dissertation is to establish an insect cell-based platform for production and purification of ADDomer particles, as well as the implementation of analytical methods for ADDomer particles characterization. This thesis was performed within the scope of the Horizon 2020 European-funded project "ADDovenom" that aims at the development of a low-cost, heat-stable, and safe for administration ADDomer-based snakebite therapy.

To achieve the objectives proposed the following tasks were performed:

- Generation of baculovirus stocks: bacmid-DNA encoding the genes coding for the ADDomer particles was produced and used to transfect *Sf*-9 cells for generation of passage 0 (P_0) recombinant baculoviruses (rBAC). These were later amplified up to passage 2 (P_2) in order to generate a master virus stock (MVS) for use in all experiments scheduled in the master thesis proposal.
- Production and purification of ADDomer particles using baseline bioprocess: the MVS generated above was used to infect High Five cells according to pre-defined standard conditions. Produced ADDomer particles were then purified following a baseline purification process. In addition, a buffer screening study was performed to assess ADDomer particles melting point (T_m) stability.
- Improving ADDomer particles production and purification processes: ADDomer particles expression was evaluated in different insect cell lines and culture medium, and primary recovery operation units (cell lysis, clarification, and concentration) were designed/optimized to replace baseline purification process.
- Development and implementation of supporting analytical methods for ADDomer particles characterization.

2

MATERIAL AND METHODS

2.1. Bacmid-DNA production

2.1.1. Chemically competent acquisition

EmBacY cells, a modified strain of *E.coli* cells, were kindly provided by Prof. Dr. Christiane Berger-Schaffitzel (*UoB*, United Kingdom). *EmBacY* cell culture were spread on LB-agar plates (InvivoGen) supplemented with 50 µg/mL of kanamycin (sigma) and 10 µg/mL of tetracycline (sigma) and incubated overnight at 37℃. A single colony was chosen to be incubated overnight in 50 mL of LB liquid medium (InvivoGen) supplemented with 50 µg/mL of kanamycin (sigma) and 10 µg/mL of tetracycline (sigma) at 37℃ and 180 rpm (innova44). 15 mL of pre-inoculum was used to incubate 500 mL of LB liquid medium (InvivoGen) supplemented at 37℃ and 180 rpm (innova44), absorbance 600 nm was routinely measured in a BioPhotometer (Eppendorf) until it reached 0.5 to 0.6. Then, the culture was centrifuged at 5000 xg for 10 minutes at 4℃ and the pellet was slowly resuspended on 100 mL of sterile and cold 0.1M $CaCl_2$ (sigma) solution. It was incubated 15 minutes on ice and centrifuged at max-velocity (Eppendorf) for 10 minutes at 4℃. A cold and sterile 0.1M $CaCl_2$ (sigma) and 80% glycerol (Alfa Aesar) solution was used to resuspend cells, which were then aliquoted and stored at -80℃.

2.1.2. Preparation of electro-competent cells

From the aforementioned seed of *EmBacY* cells on LB-agar plates with 50 µg/mL of kanamycin (sigma) and 10 µg/mL of tetracycline (sigma), a single colony was chosen to incubate 5 mL of LB liquid medium (InvivoGen) with the same selection pressure, overnight at 37℃ and 180 rpm (innova44). The following day, 3 mL of pre-inoculum was used to incubate 200 mL of LB liquid medium (InvivoGen) supplemented at 37℃ and 180 rpm (innova44), and absorbance 600 nm weas measured in a BioPhotometer (Eppendorf) until it reached 0.5 to 0.7. The culture was incubated on ice for 30 minutes before. It was centrifuged at max-velocity (Eppendorf) for 15 minutes at 4℃, 4 times. The pellets were washed, in the first two washes were performed with cold and sterile miliQ water, whereas the last two were with a cold and sterile 10% glycerol solution. Afterwards, aliquots were made and stored at -80℃.

2.1.3. Thermal stock

pACEBac1_CHIMERA452Gly plasmid (at 508 ng/mL) produced by MultiBac^{TURBO} technology was generously provided by Prof. Dr. Cristiane Berger-Schaffitzel (*UoB*, United Kingdom). The vector contains genes expressing ADDomer particles, *mini-Tn7* recombinant binding sites and gentamycin resistance gene.

For this procedure, 100 µL of chemically competent *EmBacY* cell culture was transformed with 10 ng, 100 ng, and 1 µg of *pACEBac1_CHIMERA452Gly* plasmid [36]. After 20 minutes of incubation on ice, a thermal stock at 42℃ for 45 seconds was applied using a thermostatic batch (Bunsen), followed by and recovery on ice for 2 minutes. Then, added 900 µL of SOC medium (Invitrogen) were added and cells were incubated overnight at 37℃ and 180 rpm (innova44). In the next day, transformed culture were diluted in series ranging from 10^0 to 10^{-2} times and the remaining were centrifuged at 2000 xg (Eppendorf) at 4℃ for 10 minutes to recover the cell pellet. From these dilutions, 100 µL was spread on LB-agar plates (InvivoGen) supplemented with 50 µg/mL of kanamycin (sigma), 10 µg/mL of tetracycline (sigma), 10 µg/mL of gentamycin (gibco), 100 µg/mL of X-Gal (Thermoscientific) and 40 µg/mL of IPTG (Thermoscientific) and plates were incubated at 37℃.

2.1.4. Electroporation

50 µL of electrocompetent *EmBacY* cells were mixed with 10 ng, 100 ng or, 1 µg of *pACEBac1_CHIMERA452Gly* plasmid was added. The mix was transferred to a cold electroporation cuvette (Bio-Ren) and incubated 1 minute on ice. 1 pulse of 2.5 kV and 5.8 ms was applied using an electroporator (Ependorf). To each, 950 µL of SOC medium (Invitrogen) was added and incubated for 7 hours at 37℃ and 180 rpm (innova44). Transformed cultures were diluted in series ranging from 10^0 to 10^{-3} times and the remaining were centrifuged at 2000 xg (Eppendorf) at 4℃ for 10 minutes to recover the cell pellet. The pellet and diluted samples were treated as described in section **2.1.3.**

2.1.5. Competence estimation of *EmBacY* cells

Simultaneously to each transformation, 10 ng of *pUC-19* plasmid at 50 pg/mL (New England BioLabs) was used to transform each type of competent *EmBacY* cells, to function as a positive control to estimate the efficiency of transformation, having in consideration ***Equation 2.1***.

$$Efficiency\ (cfu/\mu g\ DNA) = \frac{colonies\ counted}{pg\ of\ DNA\ used} \times \frac{10^6\ pg}{1\ \mu g} \times \frac{volume\ transformed}{volume\ spread} \times dilution\ factor \quad \textbf{(2.1)}$$

Equation 2.1 *Determination of efficiency of transformation having in consideration the amount of DNA and cells used in each dilution.*

2.1.6. Screening and selection of transformed *EmBacY* cell culture

White colonies (transformation with *pACEBac1_CHIMERA452Gly* plasmid resulted in disruption of *lacZ* genes and consequently produced the bacmid-DNA of interest) were identified, collected with a loop, and spread on LB-agar plate (InvivoGen) supplemented to confirm the phenotype, and afterwards used to inoculated 3.5 mL of LB liquid medium (InvivoGen) supplemented with 50 µg/mL of kanamycin (sigma), 10 µg/mL of tetracycline (sigma), 10 µg/mL of gentamycin (gibco).

2.1.7. Bacmid extraction and quantification

To extract and purify the engineered bacmid-DNA from cell culture the buffers from GeneJET plasmid Miniprep Kit (Thermo Scientific) were used following a modified protocol hereby described. After an overnight incubation, 3 mL of each cell clone was centrifuged at 2900 xg (Eppendorf) for 10 minutes at 4℃. The pellet was resuspended in 250 µL of resuspension buffer and lyse with 250 µL of lysis buffer, inverted and incubated 4 minutes. Then, 350 µL of neutralization buffer was added and slowly mixed, precipitating cell debris. This was removed by centrifuging at max-velocity (Eppendorf) for 10 minutes, collecting the supernatant and re-centrifuging. To supernatant 800 µL of 100% isopropanol was added and incubated for 10 minutes on ice. This was centrifuged at max-velocity (Eppendorf) for 10 minutes and its supernatant was removed, together with RNA-white precipitates present. To the transparent pellet formed, 150 µL of 70% ethanol (Honeywell) was added following centrifugation at max-velocity (Eppendorf) for 5 minutes. After removing supernatant, DNA pellet was allowed to dry on the hood chamber for 10 minutes, and dissolved in 70 µL of ultrapure water (HyClone). Bacmid-DNA concentration was determined by NanoDrop™ Spectrophotometer ND-2000c (Thermo Scientific) at 280 and 260 nm absorbance.

PCR screening and agarose gel electrophoresis analysis were used to confirm the presence of the transformed insect in the extracted bacmids-DNA, in order to select the most promising bacmid-DNA designs.

2.1.8. Polymerase Chain Reaction

For PCR analysis, oligonucleotides *M13* forward and reverse primers (ThermoFischer Scientific) were used, annealing into *mini-Tn7* recombinant binding sites of engineering bacmid-DNA. On the other hand, *P1* and *P6* primers were designed using SnapGene software, custom-made by Integrated DNA Technologies and they hybridize within the ADDomer particles gene insert transformed in bacmid-DNA. More information described in ***Table 2.1***.

Table 2.1 *Oligonucleotides targeting engineered bacmid-DNA with ADDomer particles genes for PCR analyses. Annealing temperature (T_a,℃) used with Phusion DNA polymerase (Thermo Scientific) and the size of amplified DNA-fragments (L, bp) calculated by SnapGene Software are shown for each primer set.*

Primer pair	Sequence	T_a	L
***M13* forward**	5'-CCCAGTCACGACGTTGTAAAACG-3'	65	3986
***M13* reverse**	5'-AGCGGATAACAATTTCACACAGG-3'		
P1	5'-CGGTCCTAAGGTAGCGAGT-3'	59	2035
P6	5'-CTCTACAAATGTGGTATGGCTG-3'		
P1	5'-CGGTCCTAAGGTAGCGAGT-3'	64	2690
***M13* reverse**	5'-AGCGGATAACAATTTCACACAGG-3'		

Briefly, 1 times Phusion HF buffer (Thermo Scientific), was mixed with 200 μM dNTPs (Nzytech), 0.5 μM of each primer sets, 50 ng bacmid-DNA and 0.02 U/μL Phusion DNA polymerase (Thermo Scientific). Ultrapure water (HyClone) was added up to 25 μL per reaction. The PCR was run on a T3000 thermocycler (Biometra). After an initial denaturing step at 98℃ for 60 seconds, 30 cycles of denaturation at 98℃ for 15 seconds, annealing temperature for 20 seconds, and extension step at 72℃ (30 seconds per Kbp) were done, and finished with an extension step at 72℃ for 10 minutes. In each PCR run, a positive (adenovirus serotype 3 bacmid sample) and negative control (ultrapure water (HyClone)) were used to control the amplification process.

2.1.9. Agarose gel electrophoresis

To analyse PCR products, an agarose gel electrophoresis was performed. For that, a 1% agarose (Nzytech) gel was prepared in TAE running buffer (Promega) with 1 time gelred (intron Biotechnology). Meanwhile, 5 μL of amplified samples were mixed with 1 time DNA loading buffer (grisp), and loaded on the prepared agarose gel, together with NzyDNAladder III (Nzytech). The power pack P25T (Biometra) supplied the electric current for electrophoresis run. The gels were revealed by ChemiDoc™ XRS equipped with ImageLab software (Bio-Rad).

2.1.10. Purification of amplified PCR fragments

PCR products were purified using the GFX™ PCR DNA and gel band purification kit (Cytiva), according to manufacture' specifications. The purified bacmid DNA PCR-fragments amplified were sent to Eurofins Genomics to perform Sanguer sequencing, therefore confirming the sequence of the insert into bacmid produced.

2.2. Insect cell cultures

Suspension cultures of *Sf*-9 cells (Invitrogen, United Kingdom), *superSf*-9-2 (Oxford Expression Technologies, United Kingdom) and *T.ni* High Five cells (RedBiotech, Switzerland) were routinely cultured in 250mL shake flasks (25 mL of working volume) (Corning) at 27℃ and 100

rpm (innova44). *Sf-900 II*™ serum-free medium (Gibco) and *Insect Xpress*™ (Lonza) were used to maintain *Sf*-9 based cells and High Five cells, respectively. Cells were re-cultured every 2-3 days to maintain cell concentration < $4x10^6$ cells/mL (for *Sf*-9 and *superSf*-9-2 cells) and < $3x10^6$ cells/mL (for High Five cells). Cell maintenance was performed having in consideration ***Equation 2.2*** and ***Equation 2.3***.

$$C_1 \times V_1 = C_2 \times V_2 \quad \textbf{(2.2)}$$

Equation 2.2 *Solution preparation according to dilution factor. A starting solution 1 will be diluted to prepare the final solution 2, consistently to the culture concentration (C, cells/mL) and culture volume (V, mL) intended.*

$$\frac{dX}{dt} = \mu X \quad \textbf{(2.3)}$$

Equation 2.3 *Determination of insect cells specific growth rate (μ, h-1) according to measure of culture concentration (X, cells/mL) over time (t, hour).*

2.3. Baculovirus stocks

2.3.1. Transfection of *Sf*-9 cell culture

The generate of P_0 rBAC encoding the genes for expression of ADDomer particles, the bacmid-DNA selected was transfected with Cellfectin II reagent (Invitrogen) into *Sf*-9 cells. Briefly, 100 µL of Grace's insect medium (Gibco), 4 µL of Cellfectin II reagent (Invitrogen) and 2 µg of undiluted bacmid-DNA were mixture and incubated for 20 minutes at 20℃, and then added to 1 mL of *Sf*-9 cells previously plated at $1x10^6$ cells/mL in a 6-well plate (Falcon). One replicate of each transfection was performed and a positive (non-infected *Sf*-9 cells) and a negative (only *Sf900*™ II medium (Gibco)) controls were included. After incubating for 4 hours at 27℃, 1 mL per well of *Sf900*™ II medium (Gibco) was added and the plate was incubated for 5 days at 27℃.

Confluence was assessed every day. After 120 hours post-transfection (hpt), P_0 rBAC were collected by centrifuging rBAC-enriched supernatant at 500 xg (VWR) for 5 minutes at 4℃, followed by storage of clarified supernatant at 4℃. To screen for ADDomer particles presence, 1 mL of sterile dimethyl sulfoxide (sigma) was used to resuspend the adherent cell monolayer from each well. The recover infected cell pellet was collected by centrifugation at 500 xg (VWR) for 5 minutes and stored at -20℃ for further western blot analysis.

2.3.2. Virus amplification

Amplification of P_1 rBAC was performed by infecting Sf-9 cells at cell concentration at infection (CCI) of 1x106 cells/mL with P_0 rBAC at a dilution of 1:400 [37]. Infected *Sf*-9 cells in 500 mL shake flash (50 mL of working volume) (Corning) were incubated at 27℃ and 100 rpm (innova44). Cell concentration and viability were measured every 24 hours until cell viability of 80-70% was attained. At 96 hours post-infection (hpi), 1 mL of infect culture was centrifuged at

300 xg (VWR) for 10 minutes at 4℃ and the pellet was stored at -20℃ for further western blot analysis. The remaining culture bulk was centrifuged at 300 xg (VWR) for 10 minutes at 4℃ and supernatant was again centrifuged at 2000 xg (VWR). Clarified P_1 rBAC was titrated and stored at 4℃ until further use.

For production of P_2 rBAC, *Sf*-9 cells was cultured in 1L shake flask (200 mL of working volume) (Corning) at 27℃ and 100 rpm (innova44). They were infected with P_1 rBAC at $1x10^6$ cells/mL using a multiplicity of infection (MOI) of 0.1 pfu/mL according to ***Equation 2.4***. Culture was handled as described above.

$$V_{virus} = \frac{CCI \times V_c \times MOI}{T} \quad \textbf{(2.4)}$$

Equation 2.4 *Calculation of the amount of virus required to infect a culture of insect cells, having in consideration the concentration at point of infection (CCI, cells/mL) and volume (Vc, mL), as the multiplicity of infection (MOI, pfu/mL) and virus titer (T, pfu/mL).*

2.4. ADDomer particles production and purification: baseline bioprocess

2.4.1. Expression test

Insect High Five cells were infected with rBAC-ADDomer-P_2 at CCI of $1x10^6$ cells/mL and MOI of 1 pfu/cell using *Insect Xpress*™ (Lonza) culture medium and 250 mL shake flasks (25 mL working volume) (Corning). Cells were incubated as reported previously. Cell concentration and viability were measured every 24 hours to define optimal time-of-harvest (TOH) and pellets were stored at -20℃ for further screening by SDS-Page and western blot analysis.

2.4.2. Production of biomass

For further ADDomer particles purification a production was performed in 1 L shake flask scale (200 mL working volume) (Corning). High Five cells were infected at $1x10^6$ cells/mL and 1 pfu/mL with rBAC-ADDomer-P_2 and incubated at 27℃ and 100 rpm (innova44). After 72 hpi, when cell viability was ~ 80% viability, culture bulk was harvested and centrifuged at 2000 xg (VWR) for 10 minutes at 4℃. Pellets were stored overnight at -80℃ to proceed to baseline purification process.

2.4.3. Culture lysis

Frozen pellets were resuspended in 10 mL of 50 mM Tris (Merck Millipore) and 150 mM sodium chloride (Merck Millipore) solution at pH 7.5, supplemented with EDTA free protease inhibitor (cOmplete, Roche). Cell membranes were disrupted by 3 cycles of freeze-thaw, using a mixture of dry ice and 96% ethanol (Panreac) to freeze, and a warm water bath (Grant) to thaw cell suspension, to promote the release of ADDomer particles into suspension.

2.4.4. Clarification and nuclease digestion

To clarify the lysate cell culture, an UC was performed using an Optima XE-100 Ultracentrifuge (Beckman coulter, United States of America). Lysed cells were centrifuged at 40 000 xg for 40 minutes at 4℃. To digest host and viral nucleic acids in solution, 25 U/mL of benzonase (Merck) and 2 mM of magnesium chloride (Merck Millipore) were added to supernatant, which was incubated overnight at 20℃ with agitation. Clarified supernatant was centrifuged at 4 000 xg (Eppendorf) for 15 minutes at 4℃, and was filtrated with 0.45 µm filter (Sarstedt AG) to be further purified using SEC.

2.4.5. Intermediate purification

Filtrated supernatant enriched in ADDomer particles was purified at 4℃ by a SEC using a Work Beads 40/10 000 SEC resin (KN40350003, Bio-Works) packed in a XK 26/100 column (Cytiva), according to manufacturer's recommendation. Its compression factor, theoretical plate number (HETP) and peak asymmetry were further determined after packing. The column was connected to an AKTA system (Amersham Biosciences) equipped with ultraviolet lamp (UV-900), conductivity and pH sensors (pH/C-900). The system and the column were equilibrated with 50 mM Tris (Merck Millipore) and 150 mM sodium chloride (Merck Millipore) solution at pH 7. 5 mL of sample was loaded in the SEC column, at a flow velocity of 23 cm/h and fractions were collected for further analysis. Fractions were analysed by SDS-Page, and the ones corresponding to ADDomer particles peak were pooled and concentrated 3 times using an amicon of 50 kDa (Millipore). 2 mM of magnesium chloride (Merck Millipore) and 25 U/mL of benzonase (Merck) were added and sample was incubated overnight at 20℃ with agitation. Before the AEX, the sample was centrifuged at 4 000 xg (Eppendorf) for 15 minutes at 4℃, and it was filtrated with 0.45 µm filter (Sarstedt AG).

2.4.6. Polishing

ADDomer particles were further purified at 4℃ by AEX using an EconoFit Macro-Prep High Q column (12009268, Bio-Red) that was equilibrated with 50 mM Tris (Merck Millipore) and 150 mM sodium chloride (Merck Millipore) solution at pH 7.5. The column was connected to an AKTA system (Amersham Biosciences) equipped with ultraviolet lamp (UV-900), conductivity and pH sensors (pH/C-900). Using a 50 mL superloop (cytiva), 35 mL of sample was loaded into the column, and its molecular components were separated at a constant flow velocity of 30 cm/h. Elution step was performed with 50 mM Tris (Merck Millipore) and 1 M sodium chloride (Merck Millipore) solution at pH 7.5. Fractions were collected, analysed by SDS-Page, and the ones enriched in ADDomer particles were pooled and 2 times concentrated at 4℃, 2000 xg (Eppendorf) and 15 minutes, using an amicon of 50 kDa (Millipore). To finish the baseline purification, elution buffer was exchange to PBS pH 7.4 (Merck Millipore).

After baseline purification process, the yield of ADDomer particles produced was determined using ***Equation 2.5***.

$$Y_{ADDomer} = \frac{P \times V_e}{V_c} \quad \textbf{(2.5)}$$

Equation 2.5 *Estimation of production yield of ADDomer particles ($Y_{ADDomer}$, mg ADDomer particles/L culture), considering the amount of purified ADDomer particles (P, mg/mL), the final formulation volume (Ve, mL) and the volume of culture bulk (Vc, L).*

2.5. ADDomer particles production and purification: optimized process

2.5.1. Expression study

In order to assess any potential improvements in production of ADDomer particles, new culture medium and cell lines were tested and compared to the baseline production. First, ADDomer particles were produced using insect High Five cells cultured in *Sf-900 II*™ serum-free medium (Gibco) using the same critical process parameters (CPP) of the baseline bioprocess (i.e., CCI of 1x106 cells/mL, MOI of 1 pfu/cell and TOH at 72 hpi). Second, the insect *Sf*-9 and *20uper*-9 cell lines, also cultivated in *Sf-900 II*™ serum-free medium (Gibco), were tested at a combination of different CCI, MOI and TOH (summarized in ***Table 2.2***). Productions were performed in 250 mL shake flask (25 mL working volume) (Corning) and infected using rBAC-ADDomer-P_2. Cells were incubated at 27℃ and 100 rpm (innova44). Every 24 hours, cultures were monitored for cell concentration and viability, and pellets were stored at -20℃. The relative concentration of ADDomer particles was estimated by western blot conjugated with densitometry analysis of band intensity (Image J, United States of America). Purified ADDomer particles produced using the baseline bioprocess were used as standard. Biological duplicates were performed (n=2).

Table 2.2 *Conditions screened for optimization of ADDomer particles production in High Five, Sf-9 and superSf-9-2 cells. CCI: Cell concentration at point of infection (cells/mL), MOI: multiplicity of infection (pfu/cell), TOH: time of harvest (hours).*

Insect cell line	Culture medium	CCI	MOI	TOH
High Five	*Insect Xpress*™ *Sf-900 II*™ serum-free	1 x10^6	1	0 24 48 72
***Sf*-9** ***superSf*-9-2**	*Sf-900 II*™ serum-free	1 x10^6 2 x10^6	0.1 1	0 24 48 72 96

2.5.2. Lysis study

The effect of different surfactants were evaluated in a fresh culture bulk and in fresh cell pellet to assessed which has more disruption activity on cell membrane. Surfactant solutions of 1% (V/V) triton X-100 (sigma), 2% (V/V) deviron™ C16 (Merck), 20mM of zwittergent 3-14 (ChemCruz)

and 0.5% (V/V) tween 20 (Tween™) were prepared in 500 mM Tris (Merck Millipore) and 1.5 M sodium chlorite (Merck Millipore) at pH 7.5, were used for fresh culture bulk evaluation. Surfactant solutions of 0.1% (V/V) triton X-100 (sigma), 0.2% (V/V) deviron™ C16 (Merck), 2 mM of zwittergent 3-14 (ChemCruz) and 0.05% (V/V) tween 20 (Tween™) were prepared in 500 mM Tris (Merck Millipore) and 1.5 M sodium chlorite (Merck Millipore) at pH 7.5, were used for fresh cell pellet lysis. The released of intracellular protein to suspension was screened by InstantBlue Coomassie Protein Stain (abcam) coloration of SDS-Page gel (invitrogen). Negative control of cell lysis were considered.

A culture of High Five cells in 1 L shake flask (200 mL working volume) (Corning) in *Sf900*™ II medium (Gibco) was infected at $1x10^6$ cells/mL using rBAC-ADDOmer-P_2 at 1 pfu/mL. The cell culture was incubated at 27℃ and 100 rpm (innova44) for 72 hours, harvested at 80-70% viability and divided into 10 mL samples. Surfactants were tested in fresh culture bulk harvest, and on its fresh cell pellet centrifuged at 300 xg (VWR) for 10 minutes. Fresh cell pellet was further resuspended in 1 mL of 500 mM Tris (Merck Millipore) and 100 mM sodium chlorite (Merck Millipore) solution at pH 7.5. Incubation timepoints of 30 minutes and 1 hour were considered and 500 µL samples were collected and centrifuged at 200 xg (VWR) for 10 minutes. Frozen pellets at -80℃ were used as culture lysis positive control by performing 3 cycles of freeze-thaw to disrupted cell membranes. This assessment was performed in duplicates (n=2).

2.5.3. Microfiltration study

High Five cells cultured in 2 L and 3 L shake flask (400 mL and 1 L working volume) (Corning) in *Sf-900 II*™ serum-free medium (Gibco) were infected at $1x10^6$ cells/mL using rBAC-ADDomer-P_2 at 1 pfu/mL. Cultures were incubated at 27℃ and 100 rpm (innova44) for 72 hours and were harvested at 80-70% of cell viability. To clarify the infected cell culture, an HF was used for MF to concentrate and lyse the culture, and to remove the cell debris associated. For this, a BioOptimal™ MF-SL HF with a membrane area of $0.005m^2$ (AsahiKasei) was used. The system was operated in TFF at a constant feed rate of 160 mL/min and a shear rate of 2000 s^{-1}, using a tanden 1081 pump (sartorius stedim biotech) connected to the pressure transducers scipres sensors (SciLog) by silicone tubing number 16 (masterflex). Data pressure was recorded. At the start, the hollow fibber was equilibrated with 50 mM Tris (Merck Millipore) and 150 mM sodium chloride (Merck Millipore) solution at pH 7.5. Cell cultures were concentrated ~ 10 times and lysed using 0.2% (V/V) deviron™ C16 (Merck) in 50 mM Tris (Merck Millipore) and 150 mM sodium chloride (Merck Millipore) solution at pH 7.5, supplemented with EDTA free protease inhibitor (cOmplete, Roche) and 10 U/mL of benzonase (merck), in a proportion of ~ 1:7. The concentrated cell culture and lysis buffer mixture recirculates into HF system for 15 minutes and permeate-enriched in ADDomer particles collected afterwhile.

2.5.4. Ultrafiltration study

Pellicon XL CAS biomax 50 and 100 kDa with a membrane area of 0.005m^2 (Merck Millipore) were used to further purity the clarified ADDomer particles. The system operated in TFF was set up accordingly with the manufacturer's instructions. A constant feed rate of 40 mL/min was used and for that, a tanden 1081 pump (sartorius stedim biotech) was connected to pressure transducers scipres sensors (SciLog) by silicone tubing number 16 (masterflex). Data pressure was also recorded. Initially, the cassettes (CASs) were equilibrated with 50 mM Tris (Merck Millipore) and 150 mM sodium chloride (Merck Millipore) solution at pH 7.5. At this point, clarified ADDomer particles were concentrated 10 times and diafiltrated 4 times with 50 mM Tris (Merck Millipore) and 150 mM sodium chloride (Merck Millipore) solution at pH 7.5.

2.6. ADDomer particles stability study

The 96 buffer-system conditions incorporated into HT 96 RUBIC Buffer Screen Plate (MD1-96, Molecular Dimensions) were used to assess T_m in each buffer formulation. 6 µg of concentrated ADDomer particles were mixed in a microAmp™ enduraPlate™ optical 96-well clear reaction plates with barcode (Applied Biosystems) with protein thermal shift™ dye 1000 times (Applied Biosystems), diluted 1:500 in PBS pH 7.4 (Merch Millipore), and 16.8 µL/well of buffer-system conditions in test. After resuspension, plate was centrifuged at 1000 xg for 1 minute at 20℃ and ADDomer particles T_m were quantify by QuantStudio 7 flex real-time PCR system (Applied Biosystems). According to protein thermal shift™ dye specifications, excitation and emissions radiation were measured at 470 and 520 nm, using ROX reference at 580 nm and 623 nm, respectively. T_m was determined taking into count the integration of sigmoidal melting curve of fluoresce intensity with the increase of temperature. To the most promising conditions, the procedure was repeated in triplicates.

2.7. Analytics

2.7.1. Cell concentration and viability

Cell concentration and viability were measured using image-based cell analyser Cedex HiRes (Roche), diluting the sample 1:2 in 1 time DPBS (Corning) and 2% Plutoric (gibco) solution, or by manual count using haemocytometer (superior Marienfeld, profundity 0.200 mm and 0.065 mm^2). Both methods use the trypan blue exclusion dye (Roche) to estimate cell viability.

2.7.2. Virus titration using MTT assay

Baculovirus infectious particles were titrated using the microculture tetrazolium method described in Roldão *et al.,* (2009) [38]. Briefly, *Sf*-9 cells cultured at 0.5x10^6 cells/mL were seeded in 96-well plate (Falcon) and infected with serial dilutions of rBAC ranging from 10^{-1} to 10^{-10} times after 1 hour of incubation at 27℃. A positive (non-diluted rBAC) and negative (only virus-

free *Sf900*™ II medium (Gibco)) controls were included in each plate. After 6 days of incubation at 27℃, 0.5 mg/mL of 3-(4,5-dimethylthiazol-2-yl) (abcam) was added in 10 µL/well and plates were incubated for 4 hours at 27℃. Afterwards, supernatant was removed, and dimethyl sulfoxide (sigma-Aldrich) was added in 150 µL per well and incubated for 20 minutes shaking at 20℃. The 570 and 690 nm absorbances were measured on Infinite 200 Pro plate reader (Tecan, Switzerland) and the titers were estimated in a graphing solution software (GraphPad Prism 4, United Kingdom).

2.7.3. Viral titration using cell growth cessation assay

Baculovirus infectious particles were also titrated according to the evaluation of kinetic of infected *Sf*-9 cells, related to inhibition effect of rBAC into cell growth addressed in Roldão *et al.*, (2009) [38]. *Sf*-9 cells in 125 mL shake flask (12.5 mL of working volume) (Corning) at 27℃ and 100 rpm (innova44) were infected at $2x10^6$ cells/mL with rBAC serial dilutions ranging from 10^0 to 10^{-3} times. Non-infected *Sf*-9 cells were maintained as negative control. After 23 hours of incubation at 27℃ and 100 rpm (innova44), culture concentration and viability were measured, and titer was estimated based on sigmoidal inhibition curve and $TCID_{50}$ inference using the Solver function in Excel (Microsoft, United States of America).

2.7.4. Differential scanning fluorimetry

To assess the T_m of ADDomer particles, the purified protein was study in microAmp™ enduraPlate™ optical 96-well clear reaction plates with barcode (Applied Biosystems) using QuantStudio 7 flex real-time PCR system (Applied Biosystems). After a centrifugation of 15 minutes, 6 µg of ADDomer particles were mix with protein thermal shift™ dye 1000 times concentrate (Applied Biosystems) diluted 1:500 in PBS pH 7.4 (Merck Millipore). A constant heat rate of 0.016℃/s was applied to the samples between 20℃ and 90℃. As soon as ADDomer particles structure starts to be compromised, fluoresce intensity increases, and all data is collected at QuantStudio 7 flex software™ (ThermoFisher Scientific). Based on protein thermal shift™ dye specifications, excitation and emissions radiation were measured at 470 and 520 nm, using ROX reference at 580 nm and 623 nm, respectively. T_m was determined taking into count the integration of sigmoidal melting curve of fluoresce intensity with the increase of temperature.

2.7.5. Turbidity analysis

Turbidity measurements were performed using a Turbidimeter (2100 Qis Portable HACH, Colorado, USA). Samples were diluted 1:3 in resuspension buffer before measurement.

2.7.6. SDS-Page and western blot analysis

Thawed pellets were resuspended in 200 µL i-PER™ insect cell protein extraction reagent (ThermoFisher Scientific) and incubated on ice for 10 minutes. A sample of the lysed mixture (hereon named “total protein” sample) was immediately stored. The remaining lysed mixture was

centrifuged at max-velocity (VWR) for 15 minutes at 4℃ and a sample of the resulting supernatant (hereon named "soluble protein" sample) was stored. Afterwards, a master mix considering a proportion of 3.5:9 of 10 times Novex NuPAGE sample reducing agent (invitrogen) and 4 times LDS sample buffer (invitrogen) was added to the samples in the proportion of 2:1. To denature samples, these were heated at 70℃ for 10 minutes. 10 or 15 µL of sample were loaded on NuPage Novex 4-12% Bis-Tris electrophoresis gel 1.0 mm (Invitrogen). Proteins were separated by applying 200 V and 400 mA for 35 minutes using MES running buffer. 5 µL of SeeBlue™ plus2 prestained standard (Invitrogen) and Magic mark XP (Invitrogen) were the molecular weight markers used in analysis. In SDS-Page procedure, proteins were stained by InstantBlue Commassie Protein Stain (abcam). In western blot analysis, proteins were transferred to a nitrocellulose membrane using an iBlot2 Transfer (Invitrogen). The membranes were blocked for 1 hour at 20℃ with a solution of 5% skim milk (Merck) in tris buffered saline pH 8.0 (Sigma-Aldrich) and Tween 20 (Merck). After an incubation overnight at 20℃ with primary antibody anti-adenovirus serotype 5 (6982, abcam) diluted 1:4000, the membranes were washed 5 times with a solution of tris buffered saline pH 8.0 (Sigma-Aldrich) and Tween 20 (Merck), and then incubated 1 hour with secondary antibody goat anti-rabbit IgG-AP (SC-2007, Santa Cruz Biotechnology) diluted 1:5000. Afterwards, proteins were detected by stained the membranes with 1-Step™ NBT/BCIP (Thermo Scientific) and membranes were scanned in a benchtop scanner ChemiDoc™ Imaging System (Bio-Rad, Portugal).

2.7.6.1. Antibodies selection

Commercial antibodies that recognised penton-base protein of baculovirus serotype 3 are not commercially available. To select one that recognised ADDomer particles, a study among penton-base proteins of baculovirus was performed in *Uniprot* database against *Q2Y0H9* (*Uniprot I.D.* of ADDomer particles) [14]. Primary antibody anti-adenovirus serotype 5 (6982, abcam) was assessed with a purified sample and it showed to be able to recognize ADDomer particles. This analysis is reported in ***Appendix A.***

2.7.6.2. Densitometry analysis

For quantification of relative ADDomer particles concentration resulting from production using different conditions and/or cell lines, Image J software (National Institutes of Health, United States of America) was used. Relative intensity of bands corresponding to ADDomer particles was estimated, having as reference a purified sample and a pellet sample to normalize the data between gels/membranes. Therefore, blots were translated into intensity profile with vertical projection of bands based on its protein amount. After defining background baselines, peaks were integrated, and the resulting area defined as band intensity.

2.7.7. Total protein quantification

Protein concentration was quantified by BCA protein kit (ThermoFischer Scientific), according to manufacturer's recommendation. Serial dilution ranging from 10^0 to 10^{-8} times were performed in a 96 well plate (Falcon). Absorbance (562 nm) was measured on Infinite 200 Pro plate reader (Tecan, Switzerland). Bovine serum albumin protein was used to establish a standard curve ranging from 2 mg/mL to 25 µg/mL.

2.7.8. Dynamic light scattering

The purified ADDomer particles were analysed by Xtal Concepts GmbH (Merck) equipped with SpectroLight 610 to assess the size discrepancy of protein population. Firstly, the sample was centrifuged at 25 000 xg for 20 minutes at 4℃ to remove precipitate and 1 µL of it was added to a DI-vbatch plate (Jena Bioscience GmbH), filled with paraffin oil (Merck) to prevent samples from drying out. Measurement was carried out at 660 nm and 100 mW, performing 10 readings of 20 seconds each at 20°C.

2.7.9. Transmission electron microscopy

Transmission electron microscopy analysis was performed at Instituto Gulbenkian de Ciência (Portugal). Negative staining method was applied in transmission electron microscopy (TEM) to analyse the presence, integrity, and morphology of ADDomer particles samples. The samples were adsorbed onto a formvar 150 mesh copper grid (Veco) for 2 minutes, being washed 5 times with sterile water, soaked in 2% of uranyl acetate for 2 minutes and dried at 20℃. A Hitachi H-7650 120 Kv electron microscope (Hitachi high-technologies) was used to analyse the grids.

2.7.10. Total *ds*DNA impurities quantification

Total *ds*DNA impurities were quantified by Quant-iT™ Picogreen™ *ds*DNA Assay Kit (invitrogen), according to manufacturer's recommendation. Serial dilution ranging from 10^0 to 10^{-8} times were performed in a black 96 well plate (Thermo scientific). Fluorescence was measured on Infinite 200 Pro plate reader (Tecan, Switzerland) having an excitation at 480 nm and an emission at 520 nm. λDNA was used as standard ranging from 1 µg/mL to 7.8 pg/mL.

2.7.11. High performance liquid chromatography-size exclusion chromatography

Purified ADDomer particles were analysed in high performance liquid chromatography (HPLC) system equipped with ultraviolet and photodiode array detector (Thermo fischer scientific, United States of America). HPLC-SEC analysis was carried out at 0.7 mL/min on polymethacrylate normal face (30 cm x 7.8 mm I.D., 10 µm particle size, 100 nm pore size), in TSKgel size exclusion G5000PW_{XL} HPLC column (Tosoh Bioscience). Column temperature was maintained at 25℃. 5 µL of protein sample was injected into the column using PBS pH 7.5 as mobile face. The SEC separation was monitored at absorbance of 220 nm.

ADDomer particles calibration curve, described in ***Appendix B.***, was performed with purified ADDomer particles from baseline purification process using absorbance of 220 nm.

2.7.12. Mass spectrometry

Mass spectrometry analysis was performed in Mass Spectrometry Unit of iBET (Portugal).

2.7.12.1. Protein characterization analysis

Purified ADDomer particles was digested with trypsin and alkalized with iodoacetamide. Peptide m/z spectra acquisition was performed in a *micro*LC-MS, the TripleTOF 6600 mass spectrometer (Sciex, United States of America). Protein identification was processed using Protein Pilot Software v.5.0 (Sciex, United States of America), considering the UniProt (reviewed and unreviewed) protein sequence of *Trichoplusia ni* (AcMNPV) database plus the sponsor ADDomer particles protein sequence (*Uniprot I.D.: Q2Y0H9*). In proteins analysis was done considering a confidence > 95%, accepting sequences with significant homology scores at less equal to limit score for a significant identification (P < 1.3) with a minimum of 38 peptides matched and a protein sequence percentage of coverage of 95.

2.7.12.2. Mass intact analysis

Initially, purified ADDomer particles were treated in an ultrafiltration procedure using 10 kDa cutoff filtration units having a mobile face of 0.1% formic acid in water. Peptide m/z spectra acquisition was performed in LC-X500B QTOF mass spectrometer (Sciex) equipped with Turbo V ion source (Sciex) and connected to ExionLD™ AD UPLC system (Sciex), operated at positive TOF-MS mode. UPLC analysis was carried out at 200 µL/min on Acquity UPLC protein BEH C4 column (2.1 x 150 mm I.D., 1.7 µm particle size, 300 Å) (Water). Column temperature was maintained at 60℃. Peptides were eluted using 15-min gradients from 15 to 90% solvent B (solvent A: 0.1% formic acid in water; solvent B: 0.1% formic acid in acetonitrile). Linear ion trap was operated in a range of 600 to 3000 m/z with an accumulation time of 1 second. Peptide MS/MS data was acquired by Sciex OS software (version 1.7, Sciex) and evaluated with BioPharmaView software (version 3.0, Sciex), performing a search against the UniProt (reviewed and unreviewed) protein sequence of *Trichoplusia ni* (AcMNPV) database plus the sponsor ADDomer particles protein sequence (*Uniprot I.D.: Q2Y0H9*).

3

RESULTS AND DISCUSSION

3.1. Generation of baculovirus stocks

A recombinant baculovirus stock (rBAC) was generated for the production of ADDomer particles using IC-BEVS, mostly in line with Prof. Dr. Christiane Berger-Schaffitzel (*UoB*, United Kingdom) protocol. Bacmid-DNA-producer *EmBacY* cells was transformed with a plasmid containing the genes of interest, and the resulting bacmid-DNA was amplified, extracted, and purified. To screen the integrity of the bacmid-DNA produced, PCR analysis was implemented. After using the bacmid-DNA to transfect *Sf*-9 cells, the resulting baculoviruses were collected, and further amplified and titrated. During the amplification of baculovirus stocks controls implemented at iBET were used replacing YFP screening (*yfp* gene is incorporated in backbone of bacmid-DNA, allowing continuous expression of YFP even if ADDomer particles genes are not integrated in baculoviruses genome). This work's outline, summarized in ***Figure 3.1***, was divided in 3 parts: (1) development of engineered bacmid-DNA, (2) generation of P_0 rBAC and (3) amplification of baculovirus stock.

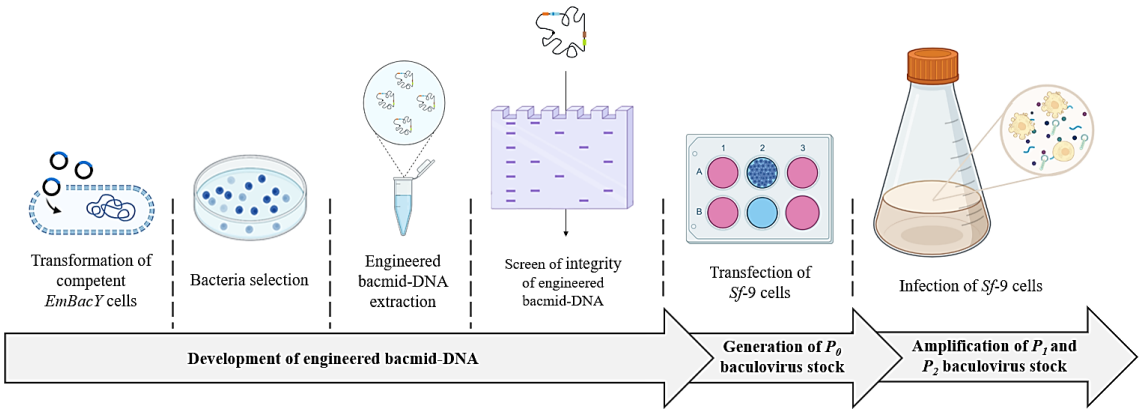


Figure 3.1 *Schematic representation of process implemented for production of baculovirus stocks for expression of ADDomer particles in insect cells.*

3.1.1. Development of engineered bacmid-DNA

The *Multi*Bac^{Turbo} expression system and the *EmBacY* cells developed at *UoB* [36] were used for the generation of bacmid-DNA with the coding genes for production of ADDomer particles. The bacmid genome present in these cells has further genome modifications such as the deletion of *chiA* and *v-cath* genes to prevent the presence of chitinase and cathepsin enzymes, and thereby delaying lysis of infected insect cells and minimizing the degradation of recombinant proteins

being produced [36,39]. The *pACeBac1_addomer* plasmid received was transformed into *EmBacY* cells. Transformation reaction, described in ***Figure 3.2***, is mediated by homologous recombination into *mini-att*Tn7 binding site, causing the disruption of *lacZα* genes, which consequently results in growth of bacteria colonies with a white phenotype when plated in agar supplemented with IPTG and X-Gal [36].

EmBacY cells need to acquire competence in order to be transformed. *EmBacY* cells chemically- and electro-competent were generated with competencies of $3.1x10^3$ cfu/µg DNA and $8.88x10^{10}$ cfu/µg DNA, respectively, when transformed with 250 pg *pUC*-19 control plasmid. In transformation with *pACeBac1_addomer* plasmid, thermal stock technique shown to be less efficient than electroporation technique, based on the number of white-transformed colonies counted and selected. These results are consistent with literature. Based on simplicity of technique, costs and shock intensity, thermal stock is considered the most convenient method to use to transform *E. coli*, while electroporation is described as the most efficient transformation method, especially for large plasmid (>100 kb) [37], which is the case of bacmid-DNA. Therefore, electroporation technique is more efficient to promote the transformation of inserts from donor plasmid into bacmid-DNA.

Upon transformation with *pACeBac1_addomer* plasmid, engineered bacmids-DNA were extracted from 4 chemically-competent (CT) and 15 electro-competent (ET) white-transformed colonies, and analysed by PCR to screen its integrity. The concentrations of bacmid-DNA extracted from each colony are summarized in (***Appendix C- Table C***). PCR-analyses of purified bacmid-DNA is described in ***Figure 3.3***. Analysis with commercial M13 primers (***Figure 3.3A***), and commercial M13rv and designed P1 primer (***Figure 3.3B***) were used to select the most promising bacmids-DNA, based on DNA concentration and amplification of more than one DNA product or the presence of empty bacmid-DNA (~ 300 bp band, the reference size to bacmids-DNA without any insert). Afterwards, the design and analysis of P1 and P6 primers was performed (***Figure 3.3D***) to implement a set of primers higher specific for the identification of coding genes for ADDomer particles.

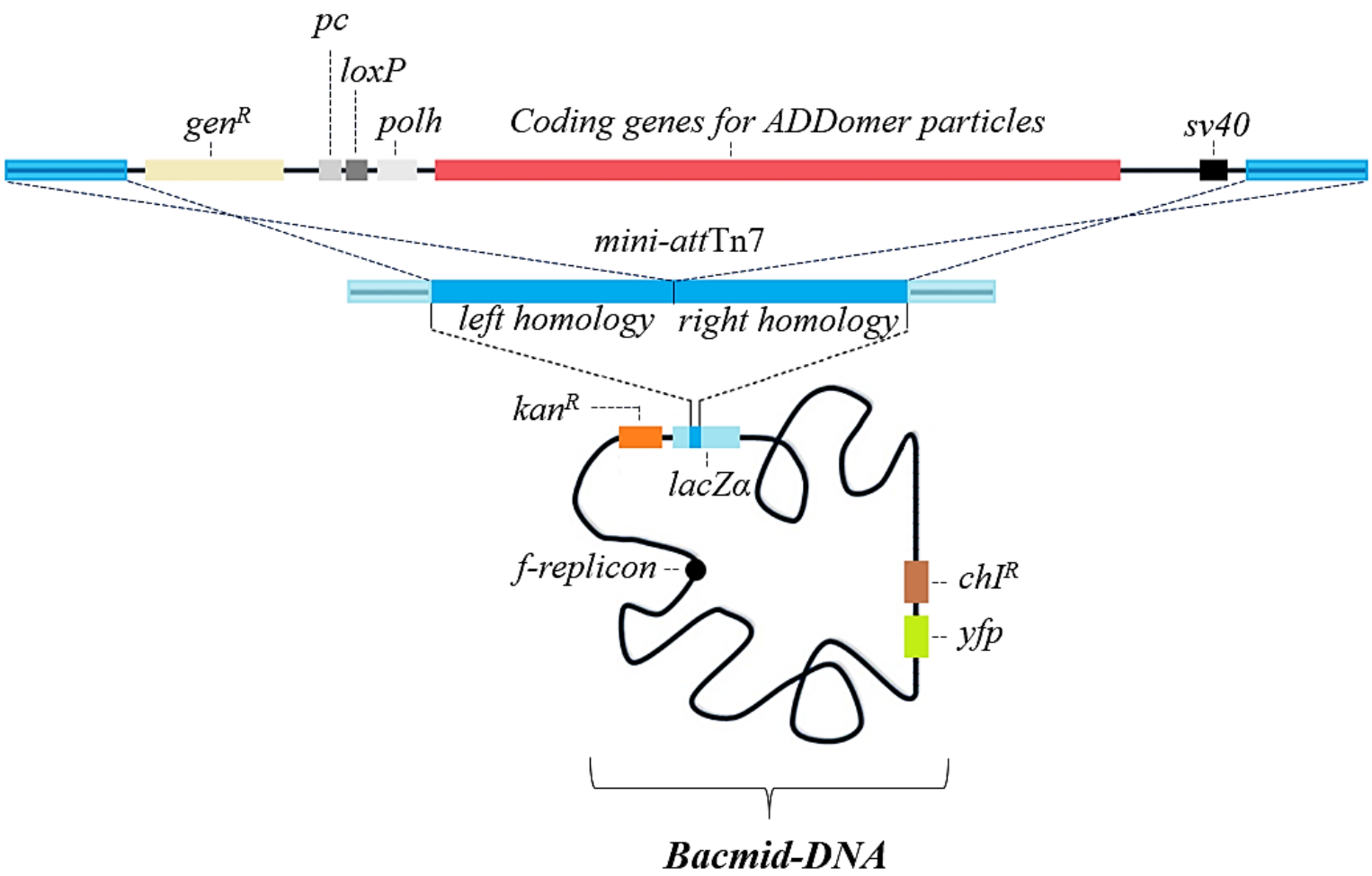


Figure 3.2 *Bacmid-DNA genome for expression of ADDomer particles (adapted from MultiBac^{Turbo} technology user guide* [36]*). Bacmid backbone genome of 134 kp has constitute kanamycin (kan^R) and tetracycline (tet^R) resistance marker genes, as well as coding genes for lacZα, yfp and disrupted v-cath and chiA non-represented. Conservative mini-attTn7 region emphasizing left and right homology region of 139 bp and 157 bp, respectively. Linear pACeBac1_ addomer of 3986 bp has coding genes for gentamicin (gen^R) resistance marker, promotors pc and polyhedrin (polh), non-functional loxP, ADDomer particles and terminator simian virus 40 polyA (sv40). The final recombined Bacmid construct has resistance to gentamycin, kanamycin, and tetracycline.*

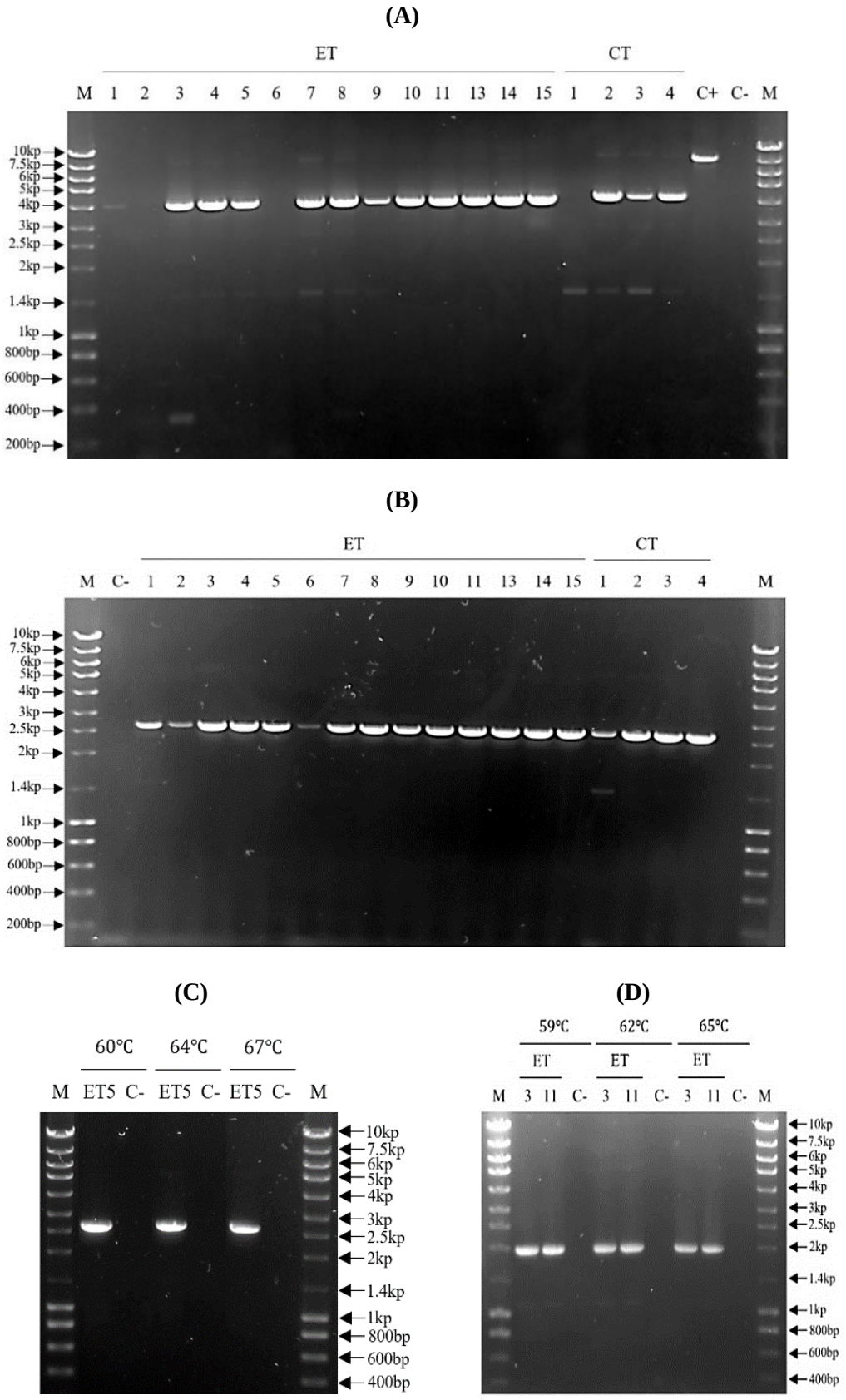


Figure 3.3 *Screening of engineered bacmid-DNA purified from selected white-transformed EmBacY colonies previously electrotransformated (ET) and chemitransformated (CT). PCR-reaction performed with Phusion DNA polymerase. A bacmid-DNA harbouring coding genes for a baculoviruses was used as a positive control (C+) over PCR-reaction and water as a negative control (C-).* ***(A)*** *Identification of DNA in PCR amplification product with commercial M13 primers, considering 65℃ as annealing temperature*

and 2 minutes of extension reaction. ***(B)*** *Identification of DNA in PCR amplification product with commercial M13rv and designed P1 primers, using 64*℃ *as annealing temperature and 90 seconds to extension reaction.* ***(C)*** *Screening of annealing temperature for commercial M13rv and designed P1 primers, having 90 seconds of extension reaction.* ***(D)*** *Screening of annealing temperature for designed P1 and P6 primers, having 1 minute of extension reaction. The annealing temperature was defined at 59*℃ *for further analyses.*

When performing PCR-analysis with commercial M13 primers, bacmids containing the insert with the coding genes for ADDomer particles should amplify a 3986 bp product illustrated in ***Figure 3.3A***. In here, it is possible to conclude that some of the selected colonies resulted in poor identification of bacmid-DNA (ET1), presence of empty bacmid-DNA (ET6) and non-specific primer interaction (ET3 to ET9, ET15 and CT1 to CT4). Non-specific amplification products can result of incorrect primer annealing or compromised integrity of ADDomer particles genes into bacmid-DNA. In PCR-analysis performed with commercial M13rv and designed P1 primers, amplified products of successfully transformed bacmids-DNA should be 2690 bp and are available in ***Figure 3.3B***. Unlike the first assay, this analysis resulted in more consistent band expression at the expected size for the majority of bacmids-DNA, exception CT1 (for having non-specific primer interaction), and ET2 and ET6 (for having a poor identification of bacmid-DNA with coding genes for ADDomer particles). These data show that performed PCR-analysis with commercial M13 primer increases the depth of the screening, derived from the fact that the use of these primers results in amplification of the *mini-att*Tn7 conservative binding site that flanks the region where coding genes for ADDomer particles are inserted. PCR-analysis performed with designed P1 and P6 primers using ET3 and ET10 bacmid-DNA positive controls (with and without non-specific primer interaction, respectively) is shown in ***Figure 3.3D***. PCR amplified products expected should have a 2035 bp, and again, this analysis demonstrates more consistent band expression at the expected size without detection of non-specific primer interaction.

ET10 and ET11 bacmids-DNA were selected for transfecting insect *Sf*-9 cells in order to generate P_0 rBAC. ET10 bacmid-DNA was produced by electroporation with 100 ng of *pACeBac1_addomer* and is at 481.2 ng bacmid-DNA/mL, while ET11 bacmid-DNA was produced by electroporation with 1 µg of *pACeBac1_addomer* and is at 266.8 ng bacmid-DNA/mL. To support ET10 and ET11 bacmid-DNA selection, Sanger sequencing results proof that both constructs have the coding genes for ADDomer particles, being able to confirm the presence of the whole region amplified by primers-walking.

3.1.2. Generation of P_0 baculovirus

The bacmids-DNA ET10 and ET11 were used to transfect insect *Sf*-9 cells for the generation of 10N and 11N P_0 rBAC that express ADDomer particles, respectively. Having in consideration the production of baculoviruses via transfection of *Sf*-9 cells, it has been described that a cell viability of 40-60% corresponds to the optimal harvest stage of baculoviruses, when they are being naturally released to extracellular environment [40]. Since transfection was performed in

adherent cultures, cell viability was not possible to assess without compromising the culture. Thus, cells confluency relative to non-infected control was considered as parameter to track the progression of transfection. 10N and 11N P_0 rBAC were harvest from the supernatant at 120 hpt. The monitored transfection process is summarized in ***Figure 3.4***.

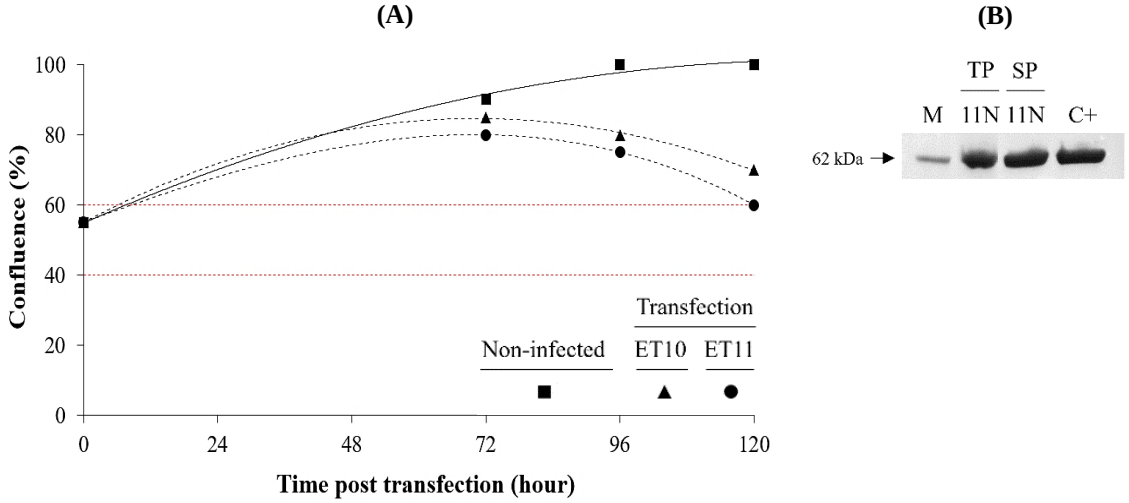


Figure 3.4 *Production of P_0 rBAC using bacmids-DNA ET10 and ET11.* ***(A)*** *Confluence profile of transfected and non-transfected insect Sf-9 cells. Optimal confluence range for harvesting is identified as being between the dashed red lines.* ***(B)*** *Western blot analysis of ADDomer particles at time of harvest showing the total protein (TP) and the soluble protein (SP) of adherent cell pellet. Purified ADDomer particle provided by UoB (United Kingdom) was used as a positive control (C+).*

Figure 3.4A shows the confluence of transfected *Sf*-9 cells relative to non-infected control during P_0 rBAC production. At 72 hpt, transfected *Sf*-9 cells demonstrated a growth behaviour similar to non-infected *Sf*-9 cells control. This could be related to the deletion of *chiA* and *v-cath* genes in bacmid backbone, which caused a delay on lysis of cells to promote a higher amplification of P_0 rBAC [39]. After 72 hpt, evidence of infection started to be noticed through decreased of confluence and increased swelling of transfected cells, contrasting with non-infected cells control. At 120 hpt, P_0 rBAC were harvest from supernatant and the transfection performed with bacmid-DNA ET11 was considered for further rBAC amplifications (hereon named rBAC-ADDomer-P_0). The decision was based on the lower confluence of transfection using bacmid-DNA ET11 which suggested a higher virus titer produced. The presence of ADDomer particles was confirmed by WB analysis in the harvest rBAC-ADDomer-P_0 infected cell pellets (***Figure 3.4B***).

3.1.3. Amplifications of baculovirus stock

Titer of P_0 rBAC produced by transfection of insect *Sf*-9 cells normally ranges from $1x10^6$ to $1x10^7$ pfu/mL[37]. The amplification of P_1 rBAC was performed by infecting *Sf*-9 cells at a MOI of ~ 0.1 pfu/mL. When cells are infected at such lower MOI, those not infected in a first round (as have 1 virus per 10 cells) continue to growth exponentially and are only, later in time, infected with the virus released from the first round [24]. For this to happen, cells should ideally be able

to duplicates in the early stage of infection. Growth profiles of infected and non-infected *Sf*-9 are described in ***Figure 3.5***.

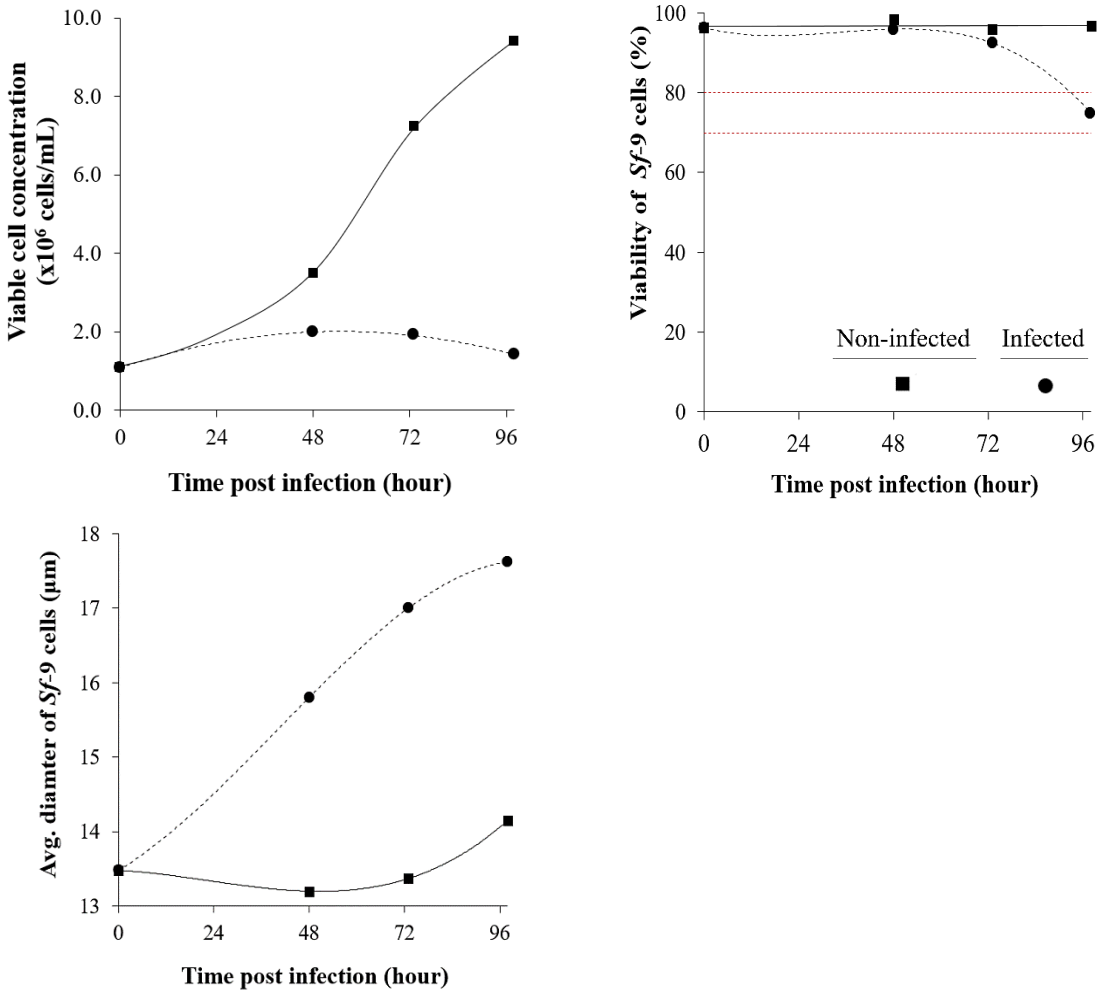


Figure 3.5 *Production of rBAC-ADDomer-P_1 using rBAC-ADDomer-P_0. Cell growth kinetics of infect and non-infected Sf-9 cells. Optimal viability range for harvesting is identified as being between the dashed red lines.*

P_1 rBAC amplification was produced simultaneosly with a non-infected *Sf*-9 cells control with a growth rate of 0.0258 h^{-1}. In ***Figure 3.5***, cell growth kinetics followed a typical profile of a low MOI infection process, characterized by an initial phase of cell growth followed by cell growth arrest around 48 hpi and decrease in cell viability. Even though the 24 hpi values are missing, it is percetible that infected *Sf*-9 cells were able to duplicate. When cell viability dropped below 80% at 96 hpi, P_1 rBAC was harvested (hereon named rABC-ADDomer- P_1). Harvesting baculovirus between 80-70% viability ensures that baculoviruses are naturally released to extracellular enviroment, while this is not overloaded with metabolites and other cell components (e.g. proteases) produced by the cells that can compromise the quality of the viruses.

The titers of P_1 rBAC amplified from P_0 rBAC usually range from $1x10^7$ to $1x10^8$ pfu/mL [37]. By performing MTT assay, a titer of $2.5x10^7$ pfu/mL was estimated for rBAC-ADDomer-P_1,

demonstrating that this virus titer was within the expected range (target titer range of $4x10^7$ pfu/mL).

P_2 rBAC was further produced by infecting insect *Sf*-9 cells with rBAC-ADDomer-P_1 at a MOI of 0.001 pfu/cell. The kinetics of cell growth of infected and non-infected *Sf*-9 cells are illustrated in **Figure 3.6**.

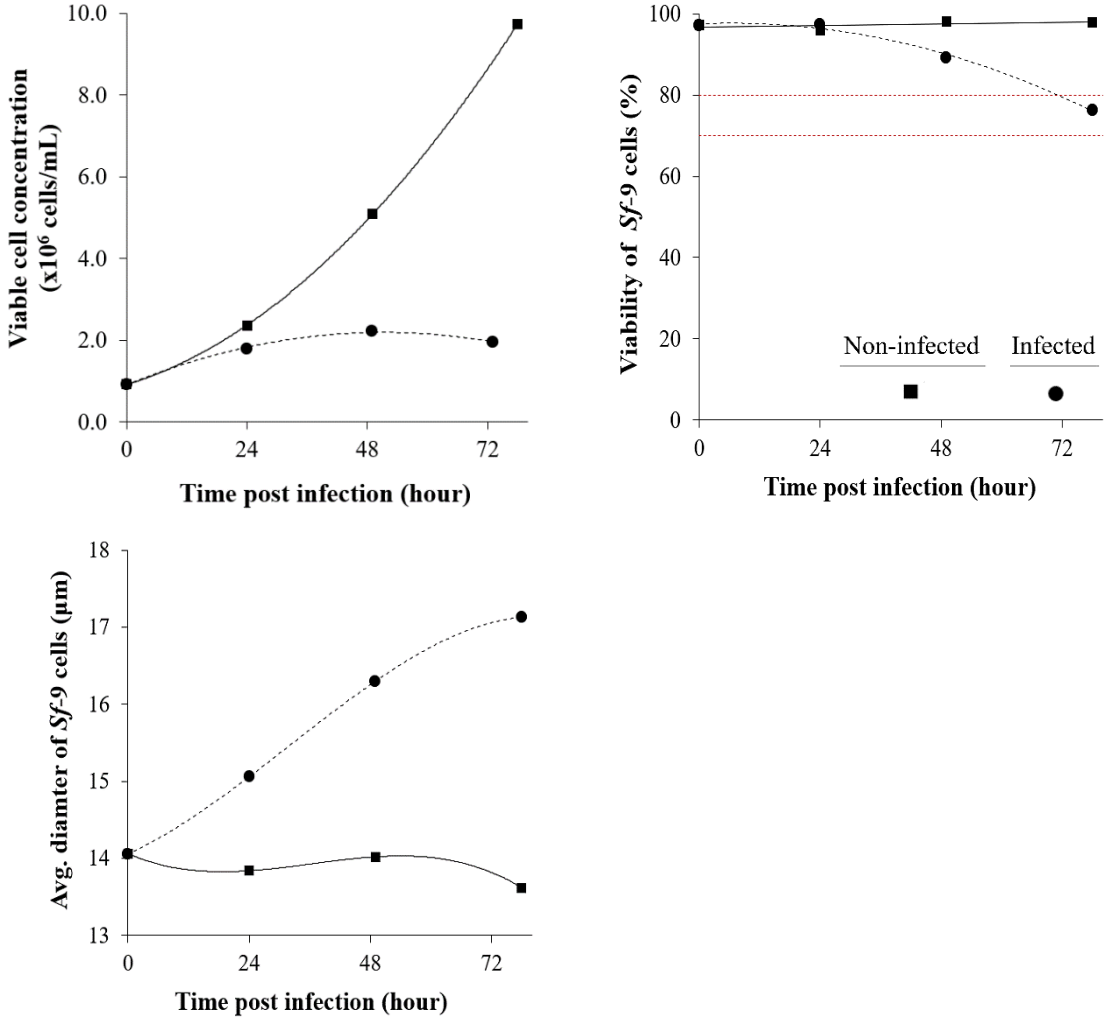


Figure 3.6 *Growth kinetic of the amplification of rBAC-ADDomer- P_2 using insect Sf-9 cells infected with rBAC-ADDomer-P_1. Optimal viability range for harvesting is identified as being between the dashed red lines.*

In P_2 rBAC amplification, a non-infected *Sf*-9 cells was used as control with a growth rate of 0.0262 h^{-1}. The kinetics of cell growth were similar to those of the previous amplification. In ***Figure 3.6***, is possible to verify that growth of *Sf*-9 cells is arrested around 48 hpi. When cell viability dropped below 80% at 72 hpi, P_2 rBAC was harvested (hereon named rBAC-ADDomer-P_2). The titer of rBAC-ADDomer-P_2 was estimated as being $3.0x10^7$ pfu/mL (assessed by MTT assay) and $1.2x10^9$ pfu/mL (assessed by growth cessation assay). The variations in titer between titration methods has been previously described by Roldão *et al.*, (2009) [38], with growth cessation assay being the method inducing more variations in titer. Therefore, the titer estimated

by MTT was used thereon for optimisation of ADDomer particles production and purification processes.

3.2. Production and purification of ADDomer particles: baseline bioprocess

ADDomer particles were produced and purified according to instructions provided by Prof. Dr. Christiane Berger-Schaffitzel (*UoB*, United Kingdom). The baseline production and purification processes, described in ***Figure 3.7***, were divided into 3 main parts: (1) production of ADDomer particles, (2) three-step purification process and (3) ADDomer particles characterization.

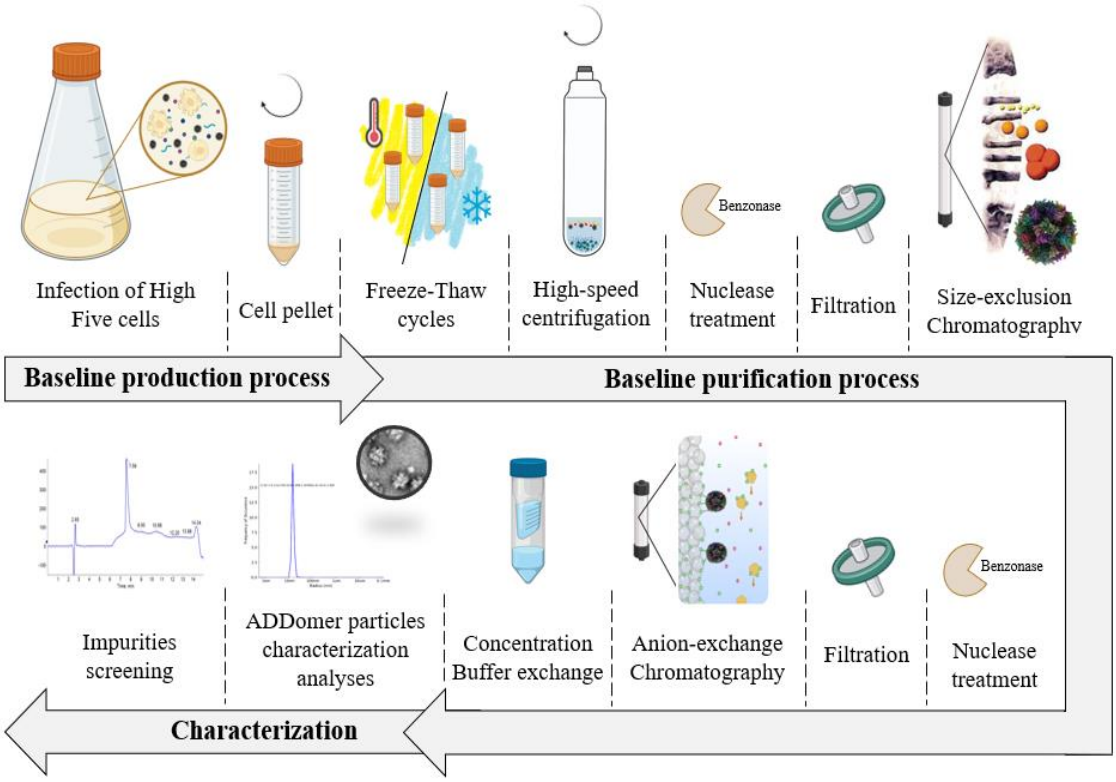


Figure 3.7 *Schematic representation of baseline bioprocess implemented for production and purification of ADDomer particles.*

3.2.1. Production of ADDomer particles

Baseline production of ADDomer particles was performed by infecting High Five cells with rABC-ADDomer-P_2 at a MOI of 1 pfu/cell. The use of this infection condition results in more synchronous infection of all cells [24] and consequently cell growth arrest shortly after infection, when compared with low MOI infection processes (as those previously used for rBAC amplification). The kinetics of cell growth of infected and non-infected High Five cells are illustrated in ***Figure 3.8***, as well as the presence of ADDomer particles in infected cell pellet.

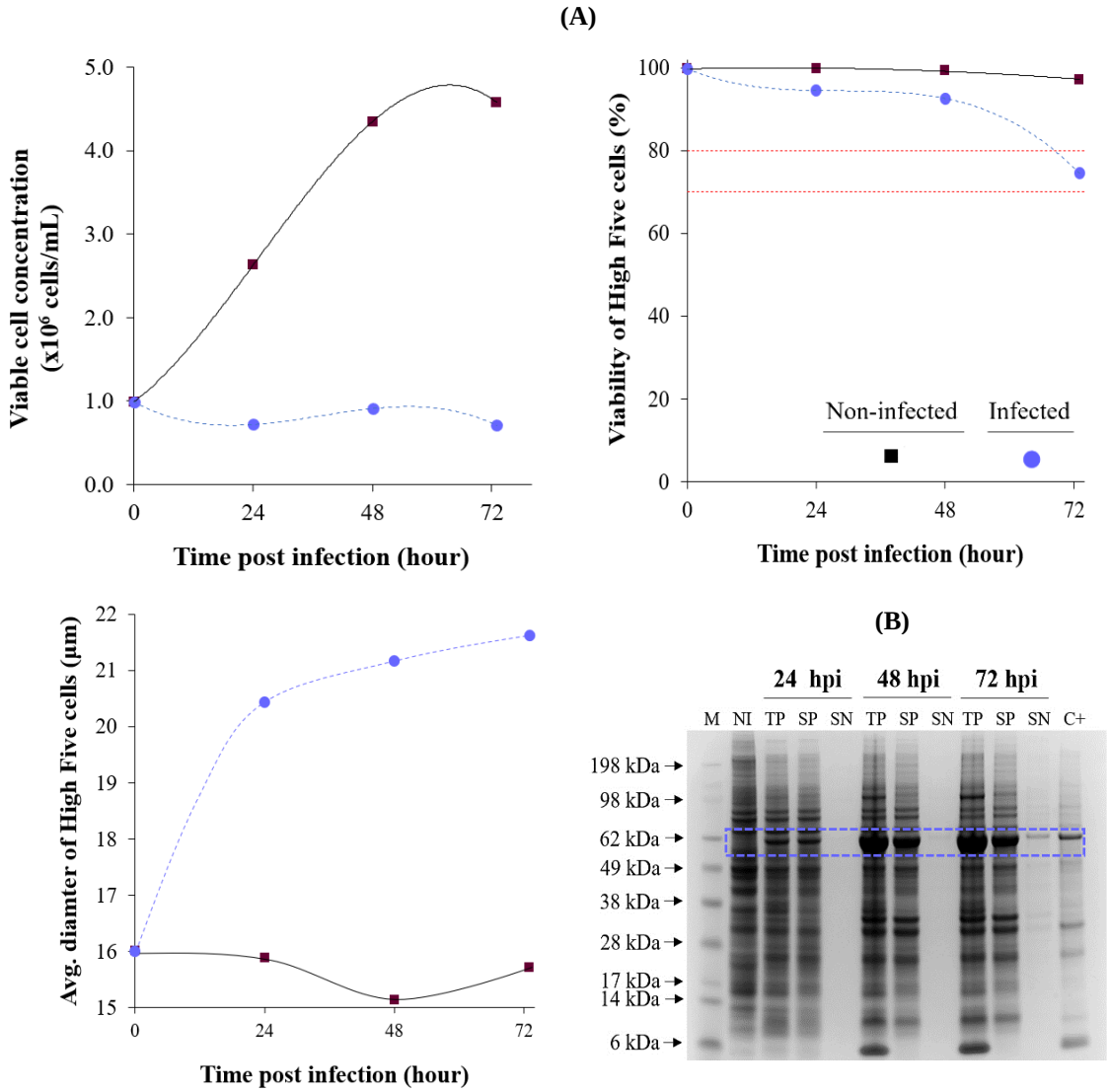


Figure 3.8 *Production of ADDomer particles in High Five cells.* ***(A)*** *Cell growth kinetic of infected and non-infected High Five cells, viable cell concentration, cell viability and average cell diameter from 0 to 72 hpi. Optimal viability range for harvesting is identified as being between the dashed red lines.* ***(B)*** *SDS-Page analysis of pellets, showing the non-infected bulk (NI), total protein (TP), the soluble protein (SP), and the supernatant (SN). The SP of rBAC-ADDomer-P_0 at 120 hpt was used as positive control (C+).*

ADDomer particles production in infected High Five cells was followed by a non-infected High Five cells control with a growth rate of 0.0367 h^{-1} (***Figure 3.8A***). High Five cells growth was arrested at 24 hpi. The decrease in cell viability suggest that rBAC-ADDomer-P_2 required 48 hours to manipule the cells machinery to produce viral proteins, generating a burden to the cells associated with the release of next generation of viruses. This behavior caused a drop in cell viability to the range of 70-80% at 72 hpi, this being the ideal time for cell harvesting. Besides, the decrease on tangential slope of average diameter of infected cells, when compared to a constant slope in previous low MOI infection processes, as visible in ***Figure 3.6***, suggests that most cells were infected simultaneously. By using SDS-Page analysis, it is possible to visualize in ***Figure 3.8B***, the increase in intensity of bands corresponding to ADDomer particles throughout infection. The highest increase in particles concentration occurred between 24 to 48 hpi. At 72

hpi, concentration of particles in the supernatant was higher, as a result of the lytic action of baculoviruses. At 24 and 48 hpi, ADDomer particles was only found intracellularly.

After defined 72 hpi as the ideal TOH, High Five cells were further infected with rBAC-ADDomer-P_2, harvest at 72 hpi and used to performed pellets enriched in intracellular ADDomer particles. Those supplied the baseline purification process.

3.2.2. Three-step purification process

The designed baseline purification process is a three-step procedure relying on robust techniques to recover the ADDomer particles at high purity. The first purification operation unit applied was a high-velocity centrifugation to clarify the cell pellets lysate, disrupted with 3 cycles of freeze-thaw cycles. The second operation unit consisted of a SEC to purify the clarified ADDomer particles and the third technique used was a AEX as a polishing operation unit. Related to chromatography approaches, SEC allowed the separation of ADDomer particles from impurities according to its hydrodynamic size, while AEX intensified the retention of negative charge impurities comparative to ADDomer particles when a charge gradient is applied. Additionally, nuclear digestion by benzonase addition and concentration operation unit were performed before SEC and AEX. Benzonase digestion allowed the degradation of host residual DNA and plasmid in solution, decrease viscosity, and increase the recovery of particles by up to 20% when compared with a process without addition of this enzyme [41]. Therefore, the operation unit herein implemented for the baseline purification process are well studied and characterized, which enables a successful design of the purification process to purify ADDomer particles.

Cell pellets were therefore freeze-thawed to provoke cell lysis and release of ADDomer particles, which were then collected after clarification. Following filtration, ADDomer particles were purified and polishing using SEC and AEX, respectively. Chromatograms evaluation and their analysed fractions are summarized in ***Figure 3.9***.

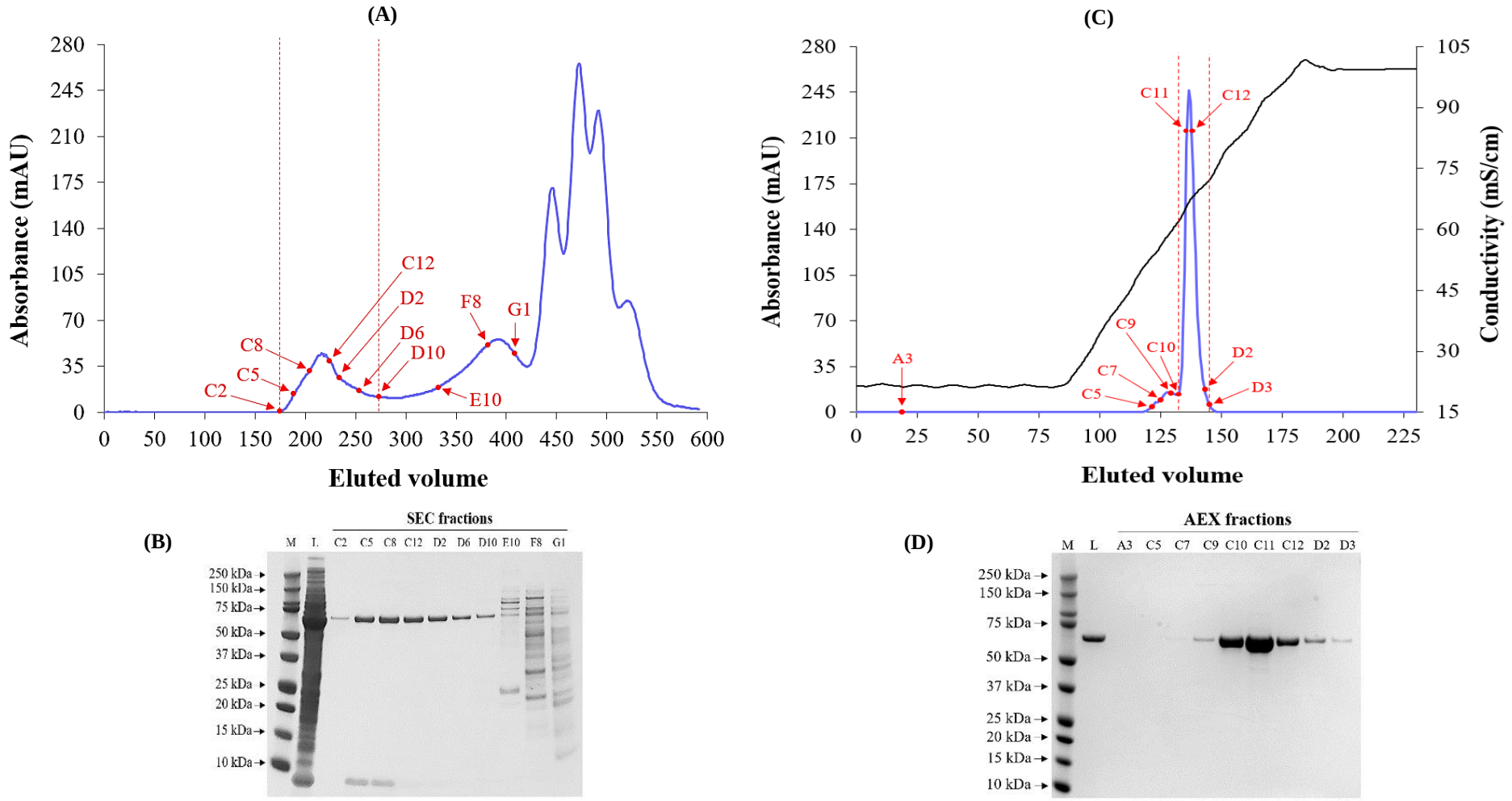


Figure 3.9 *Purification of ADDomer particles.* ***(A)*** *SEC chromatogram using a Work Beads 40/10 000 SEC resin, where the elution of ADDomer particles was monitored by the absorbance at 280 nm along of collected fractions analysed.* ***(B)*** *SDS-Page analysis of eluted fractions of SEC considering the suspension-clarified load sample (L).* ***(C)*** *AEX chromatogram profile using an EconoFit Macro-Prep High Q column, where the elution fractions analysed were recorded on absorbance at 280 nm.* ***(D)*** *SDS-Page analysis of eluted fractions of AEX and the SEC pool loaded sample (L).*

SEC allowed to separate 1 large peak outside of the void volume of the column, followed by 5 grouped peaks in the final stage corresponding to the end of the column (***Figure 3.9A***). It is expected that pore size of shell beads of SEC excluded the target to allow the retention of smaller impurities, and consequently accomplishment a size-separation [20]. As demonstrated in ***Figure 3.9B***, the first peak corresponds to fractions containing only ADDomer particles, whereas the remaining contain smaller host-cell impurities, highlighting the power of this technique. This behaviour demonstrates that Work Beads 40/10 000 SEC resin can successfully separate the ADDomer particles from impurities in solution. Thereby, ADDomer particles whereas collected in 21 fractions (from C2 to D10), that were pooled to a final volume of 105 mL. This pool from SEC was used to load the AEX. The ***Figure 3.9C*** referred that AEX allowed to identify 1 small and large peak followed by 1 bigger defined peak in the middle stage of this polishing process. Knowing that ADDomer particles in its perfect size and shape has a neutral surface without charge at its theoretical pI (5.27), in the pH of elution buffer (7.5), dodecahedral particles express a negative charge which cause attraction events within ADDomer particles and beads, which results in retention of the particles in the AEX column. SDS-Page analysis illustrated in ***Figure 3.9D***, confirmed that the second peak corresponds to fractions containing ADDomer particles. In analysed fractions, it is difficult to verify an improvement on impurities removal since the loaded sample (SEC pooled) was already a high purity. Hereupon, ADDomer particles was eluted in the range of 62-76 mS/cm conductivity, with 6 fractions being collected (from C10 to D3) and pooled to a total volume of 15 mL.

Aiming at understanding the potential of the purification process herein implemented, an overview of impurities removal was assessed and described in ***Figure 3.10***.

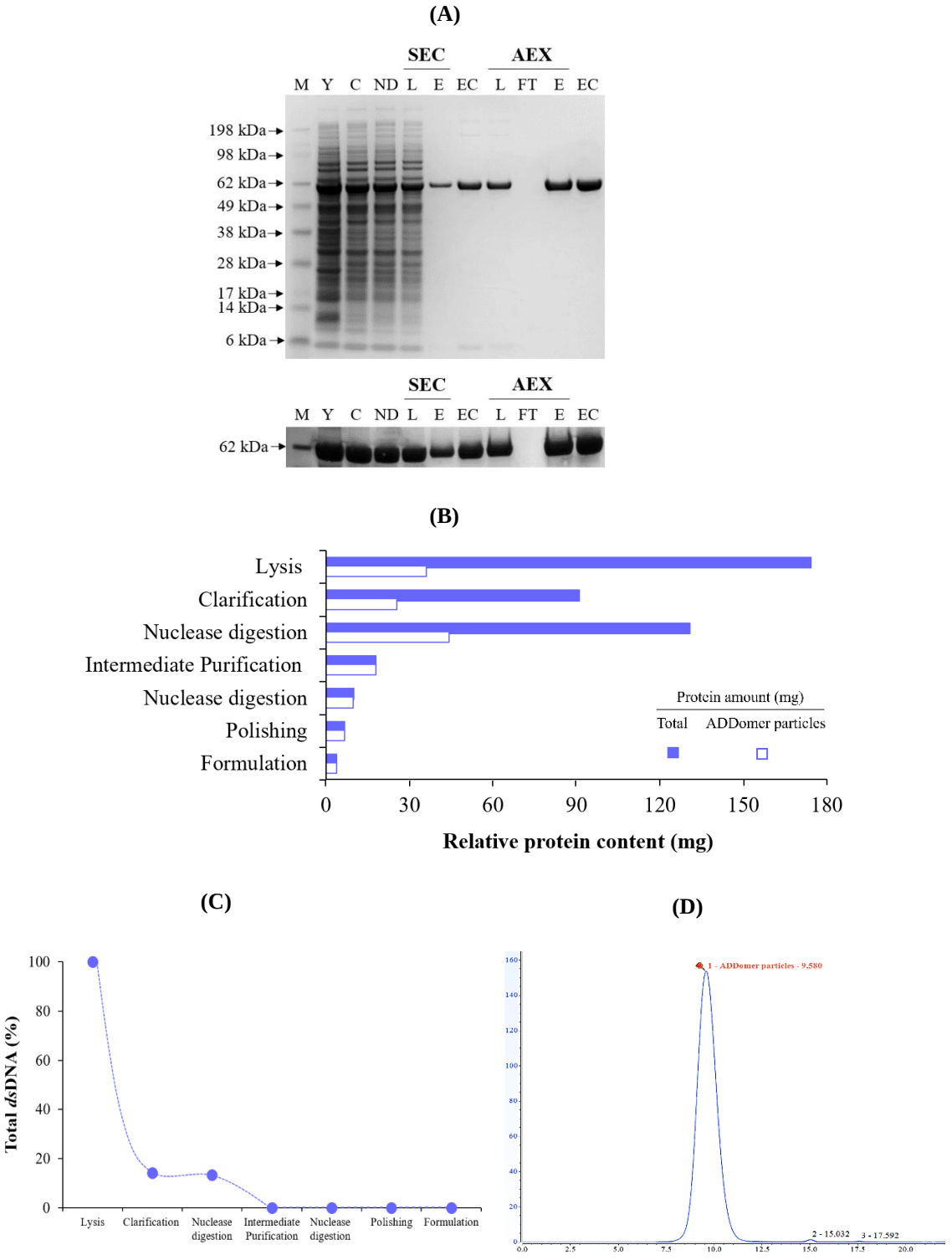


Figure 3.10 *Assessment of impurities removed during baseline purification process.* ***(A)*** *SDS-Page (upper gel) and western blot (lower gel) analyses of ADDomer particles in lysate pellets (Y), clarified lysate pellets (C), after the first nuclease digestion (ND), chromatography sample load (L), chromatography elution (E), concentrated elution (EC) and chromatography flow through (FT) samples.* ***(B)*** *Protein profile of total protein content versus ADDomer particles content, in mg, in each operation units of purification process.* ***(C)*** *DNA profile of percentual dsDNA content throughout each operation unit of the purification process.* ***(D)*** *HPLC-SEC analysis profile of purified ADDomer particles obtained at absorbance of 220 nm.*

As shown in ***Figure 3.10A*** a decrease of host-cell protein impurities was attained along the purification process, also enabling enrichment in ADDomer particles content. In the early stage of this process, the first reduction of impurities, mainly proteins, occours at clarification operation unit and furthermore at SEC. SEC was able to remove the majoraty of impurity proteins, remaining in solution a slight amount of proteins with a high molecular weight eluting with ADDomer particles. Those high molecular weight proteins were removed in AEX, which allowed to achieve a purity > 99% at a concentration of 0.56 mg/mL. As demonstrated in ***Figure 3.10B*** the first centrifugation promotes a ~ 50% decrease in protein content, including ADDomer particles (~ 30%). Even though, the addition of benzonase cause a ~ 20% increase on ADDomer particles amount. A possible explanation for this is that endonuclease releases particles that had been entrapped inside DNA clumps [41]. After SEC, a reduction of ~ 80% in impurity protein content was observed. Based on ***Figure 3.10C***, clarification by centrifugation allowed the highest removal of host cell-DNA impurities followed by SEC. Hence, the capacity of EconoFit Macro-Prep High Q column to remove *ds*DNA was not assessed since the amount of *ds*DNA in the loading solution was already below the limits of quantification of the analytical methods used. In contrast, HPLC-SEC analysis at absorbance of 220 nm at ***Figure 3.10D***, displays a well-defined peak at 9.58 minutes corresponding to ADDomer particles and two small peaks at 15.03 minutes and at 17.59 minutes related to impurities that remained in solution. According to these results, purified ADDomer particles have a purity of 99.6% (assessed by peak areas). Production yield was 19 mg ADDomer particles per L of culture.

Protein identification via MS analysis identified 41 peptides present in solution, where ADDomer particles Uniprot sequence was reported in majority with 533 aminoacids aligned in a cover of 95% confidence. The remaining peptides of *Autographa californica* nuclear polyhedrosis virus (*NPVAC*) and Trichoplusia ni (*T.ni*) species were identified in a residual amount. All peptides have a active function on DNA transcription, and on protein translation and processing. On the other hand, mass intact MS analysis only identified 1 protein eluted at 7.59 minutes, which evidences that these identified molecules in MS analysis probably are not the ones detected in HPLC-SEC analysis. These results reinforced the high purity of ADDomer particles herein produced and purified.

Given the success in obtaining high purity ADDomer particles using this baseline bioprocess, the purified ADDomer particles was used to (i) establish a pure HPLC-SEC calibration curve for ADDomer particles samples (***Figure B*** in ***Appendix B***) and (ii) as internal positive control for further ADDomer particles production, purification and quantification.

3.2.3. ADDomer particles characterization

Purified ADDomer particles were characterized by TEM, DLS and TSA analyses. TEM is a microscopy technique used as an analytic method to screen size and morphology of biological material at high resolutions [42]. Likewise, DLS is a non-matrix assisted method that can assess size distribution of biological material, as well as analyse potential secondary molecular interactions [35]. TSA is an assay performed to determine the melting temperature (T_m) of proteins [43]. Results of TEM and DLS analyses are summarised in ***Figure 3.11***, while TSA result is described in ***Figure 3.12***.

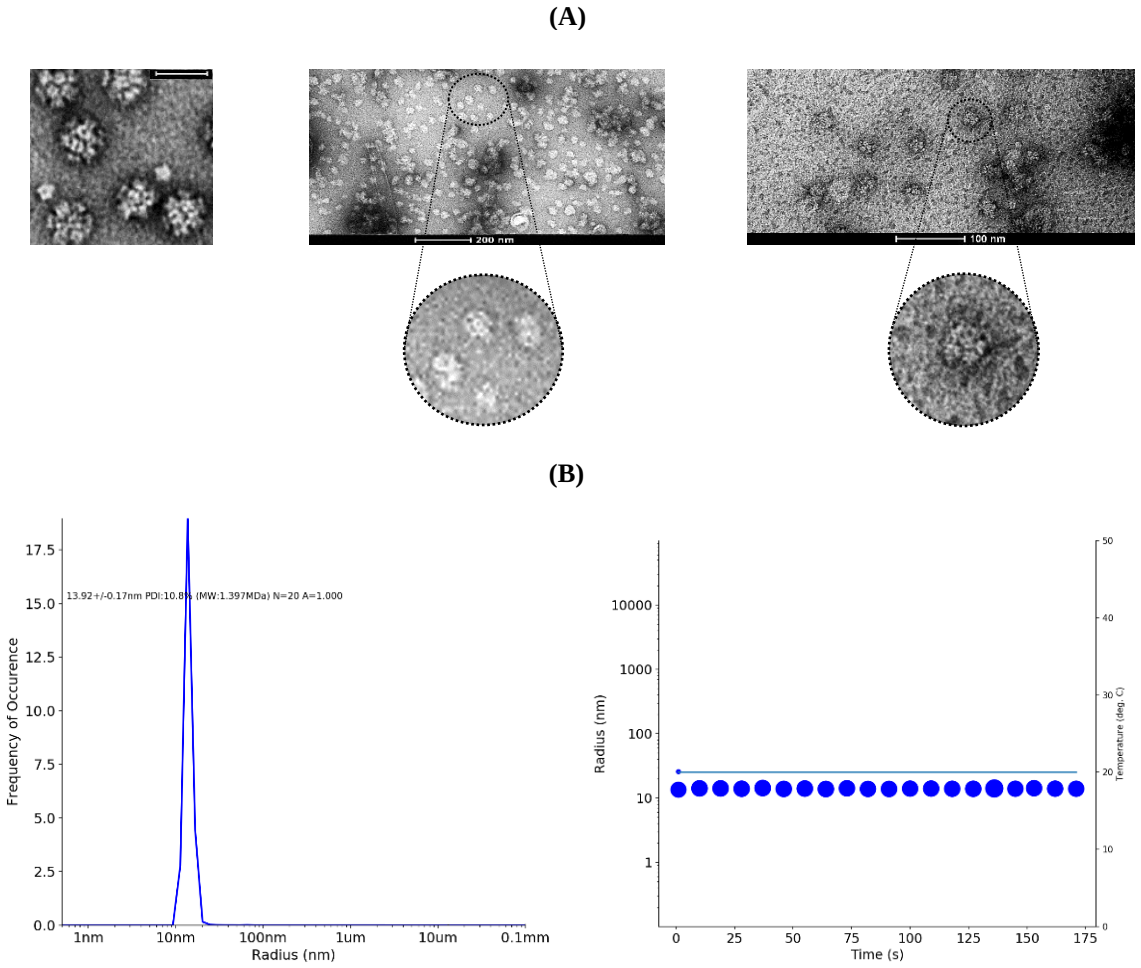


Figure 3.11 *Characterization of purified ADDomer particles.* ***(A)*** *Negative-stained TEM images of purified ADDomer particles. Left panel: Reported representation of ADDomer dodecamers (scale bar 30 nm) on Vragniau et al., (2019) [14]; middle panel: ADDomer particles received from Prof. Dr. Christiane Berger-Schaffitzel (UoB, United Kingdom) (scale bar 200 nm); right panel: ADDomer particles produced in house (scale bar 100 nm).* ***(B)*** *DLS analysis of purified ADDomer particles. Left panel: Particles-size distribution histogram according to hydrodynamic radius (Radius, nm) and its frequency; right panel: Radius-distribution of particles detected in scan analysis.*

The TEM analysis illustrated in ***Figure 3.11A***, compares ADDomer particles produced and purified in-house with the received from *UoB*. These were similar in shape and size, and consequentially, similar to ADDomer dodecamers previously reported, which vindicate that the baseline production/purification process was successful implemented. The dodecahedral format

of particles was efficiently identified, differencing the individual particle's subunits. The multiple freeze-thaw cycles to which the received samples were exposed, and their higher particles concentrations, might be behind any minor differences observed regarding size and aggregation. In ***Figure 3.11B***, a single well-defined peak in DLS analysis showed a population monodispersite (PDI < 12%), constituted of proteins with similar in hydrodynamic radius (~ 30 nm), in agreement with Vragniau *et al.*, (2019) [15].

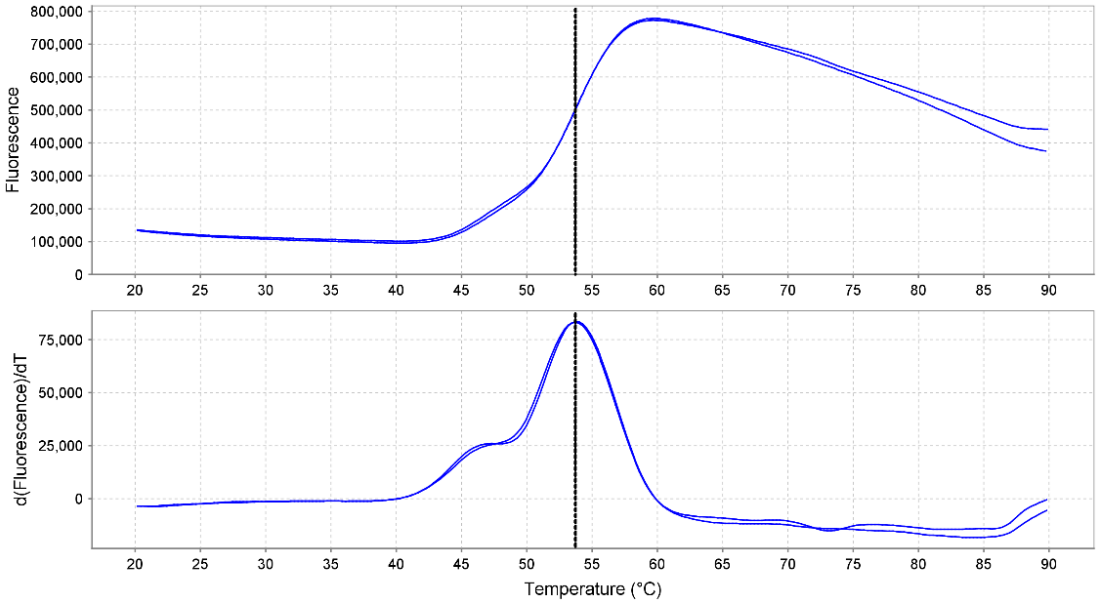


Figure 3.12 *ADDomer particles thermal stability analysis. The Tm was determined having in consideration the average of replicates (R=2).*

TSA analysis, described in ***Figure 3.12***, estimated a T_m for purified ADDomer particles of 53.74℃, similar to the 54℃ value previously reported in Vragniau *et al.*, (2019) [15]. This high value demonstrates that ADDomer particles should be stable at 20℃, requiring a temperature > 45℃ for disintegration of tertiary structure (first peak in ***Figure 3.12***, *bottom panel*) and a temperature > 52℃ for denaturation of monomeric units (second peak in ***Figure 3.12***, bottom panel).

Overall, these results demonstrate the efficiency of the insect cell-based platform for production of ADDomer particles and the efficiency of purification process implement for the removal of impurities without compromising ADDomer particles structure.

3.2.4. ADDomer particles stability study

In order to investigate the possibility of increasing the stability of ADDomer particles, an array of 96 buffers in a commercial 96 well system buffer conditions was explored. Here stability trends according to salt concentration, pH, and buffer type and concentration were analysed. The results are summarised in ***Appendix D - Figure D***. The majority of buffers screened did not seem to improve particles T_m (e.g. sodium acetate, sodium phosphate, ammonium acetate, MES, MOPS,

CHES, bis-tris, tricine and biotin buffers), with imidazole and SPG buffers decreasing ADDomer particles T_m, being defined as additives to avoid in formulation. Sodium phosphate, citrate, and HEPES buffers were the conditions that allowed higher ADDomer particles T_m. Therefore, 100 mM of potassium phosphate with 250 mM of sodium chlorite at pH 7 (Buffer 2), 100 mM of citrate with 250 mM of sodium chlorite at pH 6 (Buffer 3) and 50 mM of HEPES with 1 M of sodium chlorite at pH 7.5 (Buffer 4) were the buffers conditions selected for further assessment of ADDomer particles T_m, using PBS formulation at pH 7.4 (Buffer 1) as a standard buffer control. TSA analyses of buffers selected are registered in ***Figure 3.13***.

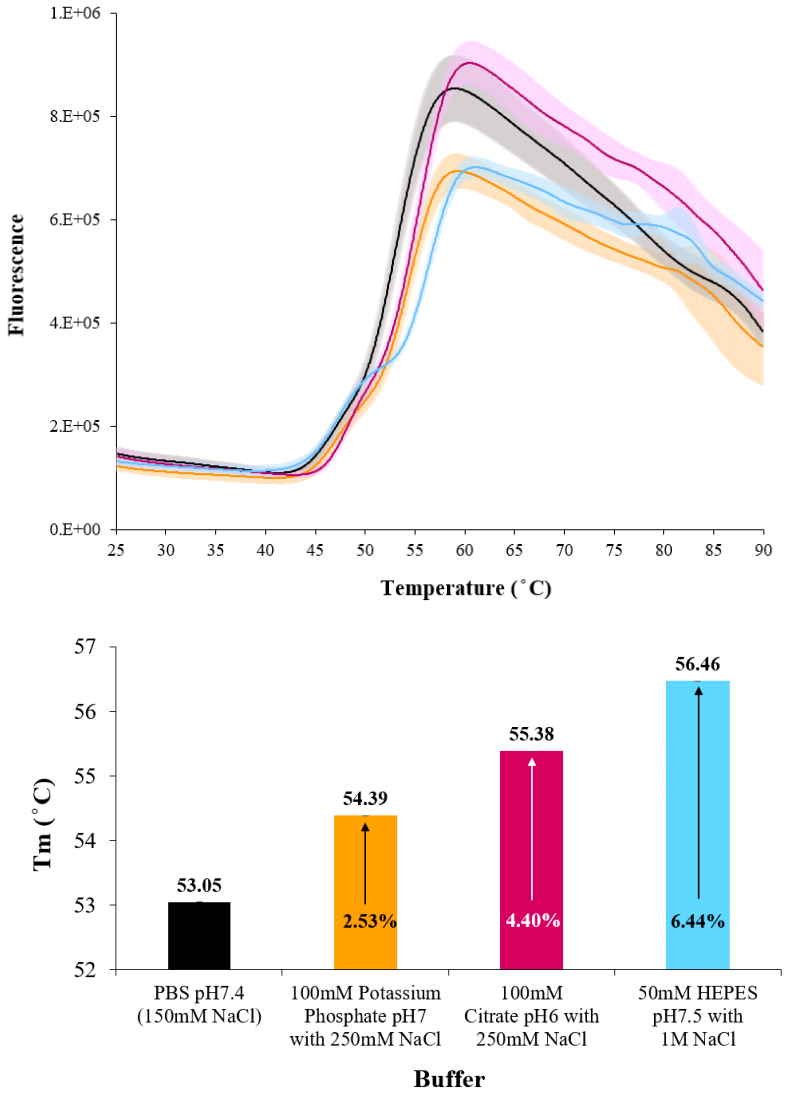


Figure 3.13 *T_m screening of ADDomer particles in 3 different buffers compositions: 100 mM of potassium phosphate buffer with 250 mM of sodium chlorite at pH 7 (in orange), 100 mM of citrate buffer with 250 mM of sodium chlorite at pH 6 (in pink) and 50 mM of HEPES with 1 M of sodium chlorite at pH 7.5 (in blue). Particles formulated in PBS at pH 7.4 were used as control to monitor the increase of T_m. For the bottom panel, the average of three replicates is shown (n=3).*

Thermal kinetic of buffer conditions selected are described in ***Figure 3.13***. According to TSA analysis shown in ***Figure 3.12***, this thermal kinetics display a similar behaviour. The three buffer

conditions herein tested allowed to increase ADDomer particles T_m: 2.53% (Buffer 2), 4.40% (Buffer 3) and 6.44% (Buffer 4). Taking into account FDA regulations related to excipient approved in pharmaceutical products, the active ingredients are ranked in 5 to 100 mM of concentration and pH of 4 to 8, having in attention salt concentration that can ranged from 0 to 300 mM [44]. Although the buffer conditions screened are in the range of excipient concentration and pH allowed, Buffer 4 exceeds salt concentration authorized and thus can no longer be considered as a final formulation buffer. Reflecting about the prices of buffers, Buffer 3 is the most expensive buffer tested costing 217.76 € per 100 mL of solution. Aiming at minimizing costs, the use of this buffer deviates from the objective of this work. For instance, Buffer 1 used in baseline purification process costs 8.80 € per 100 mL of solution. Buffer 2 and Buffer 3 cost 29.73 € and 29.09 € per 100 mL of solution, respectively. According to FDA indications, the maximum amount of potassium phosphate and citrate buffers injected intravenously in the human organism are 68 mg and 235.2 mg, respectively (https://www.accessdata.fda.gov/scripts/cder/iig/index.cfm (accessed on 5th August 2022)). Based on this information, Buffer 3 appears to be a promising buffer formulation to have into account in the future. Notwithstanding, the work of Vajda *et al.*, (2016) [35] created a new perspective about buffer screening into purification process. In this study, SEC was used to purify and formulate virus particles in different buffer conditions, and it revealed that an increase of sodium chloride amount on buffer cause a decrease in particles recover. Furthermore, the amount of particles purified using citrate buffer and phosphate buffer is similar, however it was verified that citrate buffer induces aggregation of virus particles. Aggregation events represent an undesirable outcome, needing to be avoided as they reduce the diffusion of particles and consequentially their therapeutic efficiency [45]. Thus, a long-term stability screening of ADDomer particles in Buffer 1, Buffer 2 and Buffer 3 with 250 mM of NaCl would be an asset to assist future decisions.

3.3. Production and purification of ADDomer particles: optimized process

Aiming at establishing a scalable pipeline for production and purification of ADDomer particles, the baseline bioprocess implemented above was further optimized. This work's pipeline was divided into 2 main parts: (1) optimization of ADDomer particles production and (2) optimization of ADDomer particles purification.

3.3.1. Optimization of ADDomer particles production

Regarding the optimization of ADDomer particles production, performance of High Five cells in two culture medium (*Insect Xpress*™ and *Sf900*™ *II*) was studied using CPP conditions of baseline production, and afterwards the performance of *Sf*-9 and *superSf*-9-2 cell lines was also addressed. High Five cells were infected using rBAC-ADDomer-P_2 at a MOI of 1 pfu/cell and

CCI of 1x10^6 cells/mL. Cell growth kinetics of infect and non-infected High Five cells are summarized in ***Figure 3.14***, alongside of ADDomer particles expression by WB.

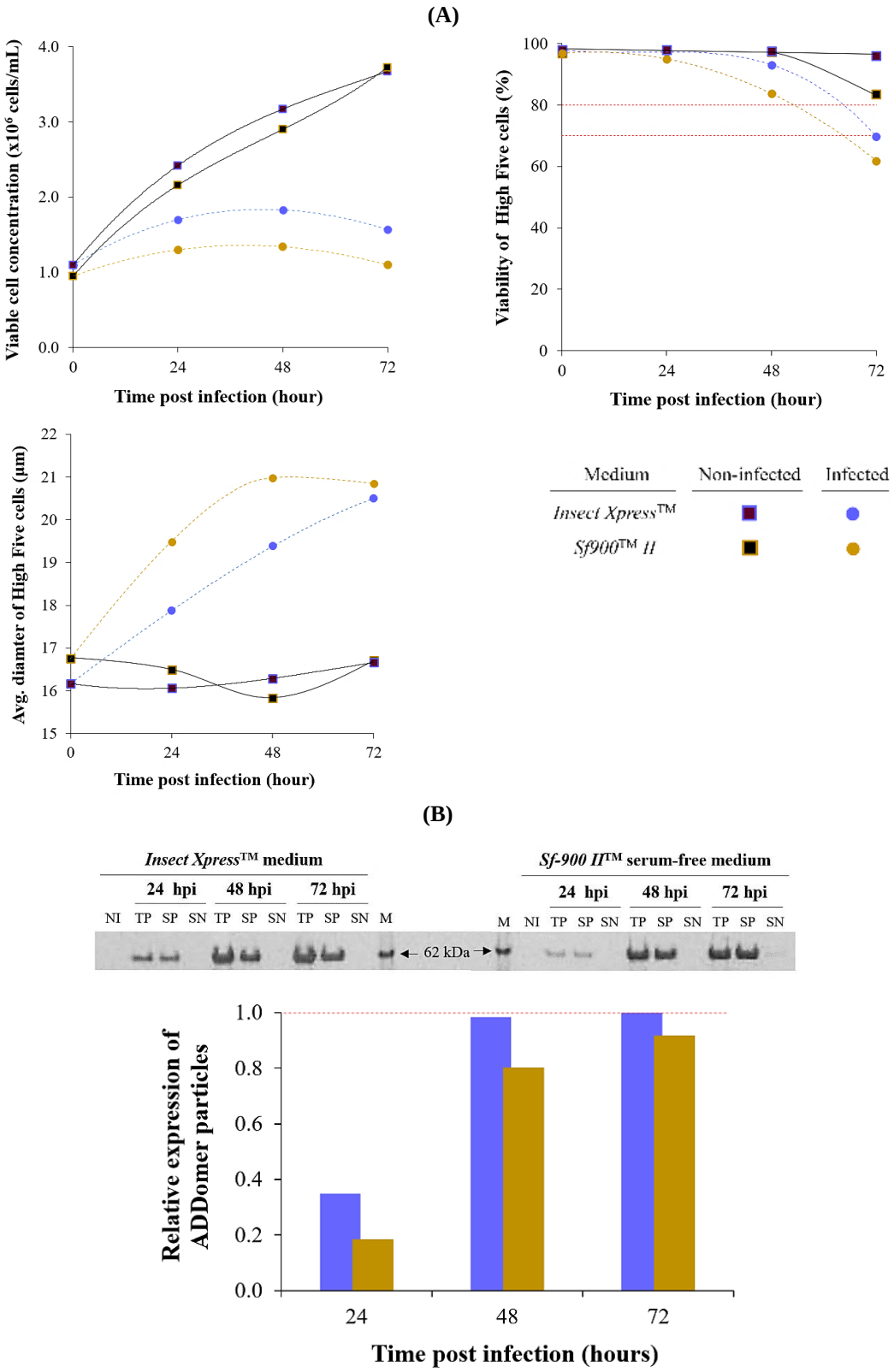


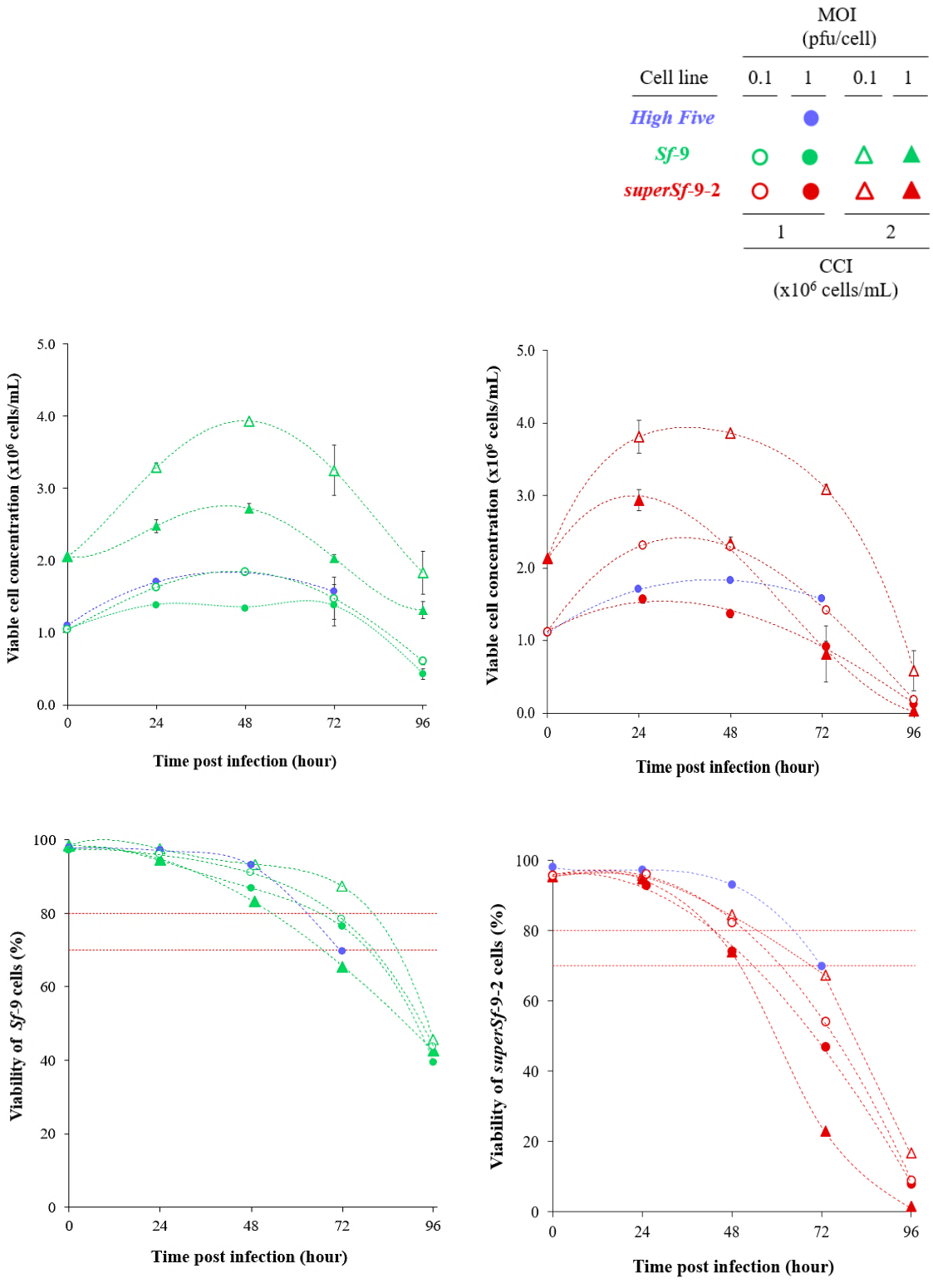
Figure 3.14 *Screening of ADDomer particles production in High Five cells cultured in Insect Xpress™ medium and in Sf900™ II serum-free medium.* ***(A)*** *Cell growth kinetics of infected and non-infected High*

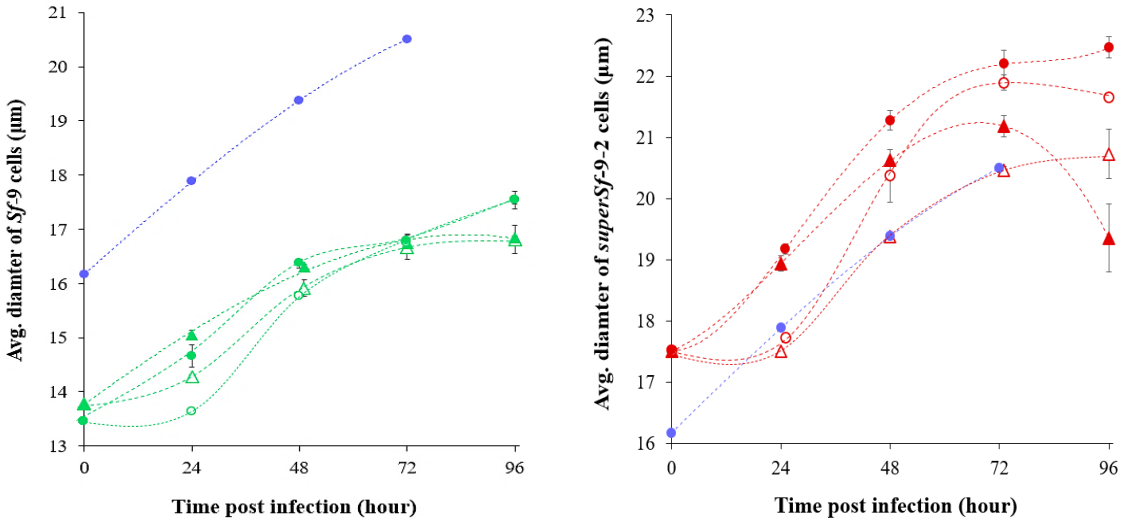
Five cells. ***(B)*** *Western blot analysis of pellets samples, showing the total protein (TP), soluble protein (SP) and supernatant (SN). Bar graph representation of relative expression of ADDomer particles assessed through densitometry analysis performed to the bands (i.e. relative band intensity) corresponding to ADDomer particles in western blot analysis. Data is normalized (at 1) for the amount of protein quantified when using the control condition.*

As ***Figure 3.14A*** demonstrates, non-infected High Five cells cultured in both media, had a similar growth tendency, with comparable growth rates (0.040 h^{-1} and 0.033 h^{-1} for *Insect Xpress™* medium and *Sf900*™ serum-free medium, respectively). However, viability of non-infected cells cultured in *Sf900*™ serum-free medium decreased after 72 hpi before displaying signs of reaching a stationary phase. This may suggest lactate accumulation, a known toxic by-product at later culture stages [19]. Regarding infected cells, viability decreased earlier when using *Sf900*™ *II* medium being under the 70% viability threshold at 72 hpi. Considering the average size of High Five cells, during infection, the culture in *Sf900™ II* serum-free medium was more swollen than the culture in *Insect Xpress™* medium. Cells infected and cultured in *Insect Xpress™* medium expressed higher amount of ADDomer particles than those infected and cultured in *Sf900™ II* serum-free medium as ***Figure 3.14B*** illustrates. However, this difference is not significative at later stages of infection (e.g. 8.35% at 72 hpi). Based on cells viability and variation of ADDomer particles concentration from 48 to 72 hpi, it is not expected that harvesting at 96 hpi would further improve ADDomer particles production. In fact, this consideration would have the added disadvantage of increasing impurities content compromising purification process (due to the decrease in cell viability).

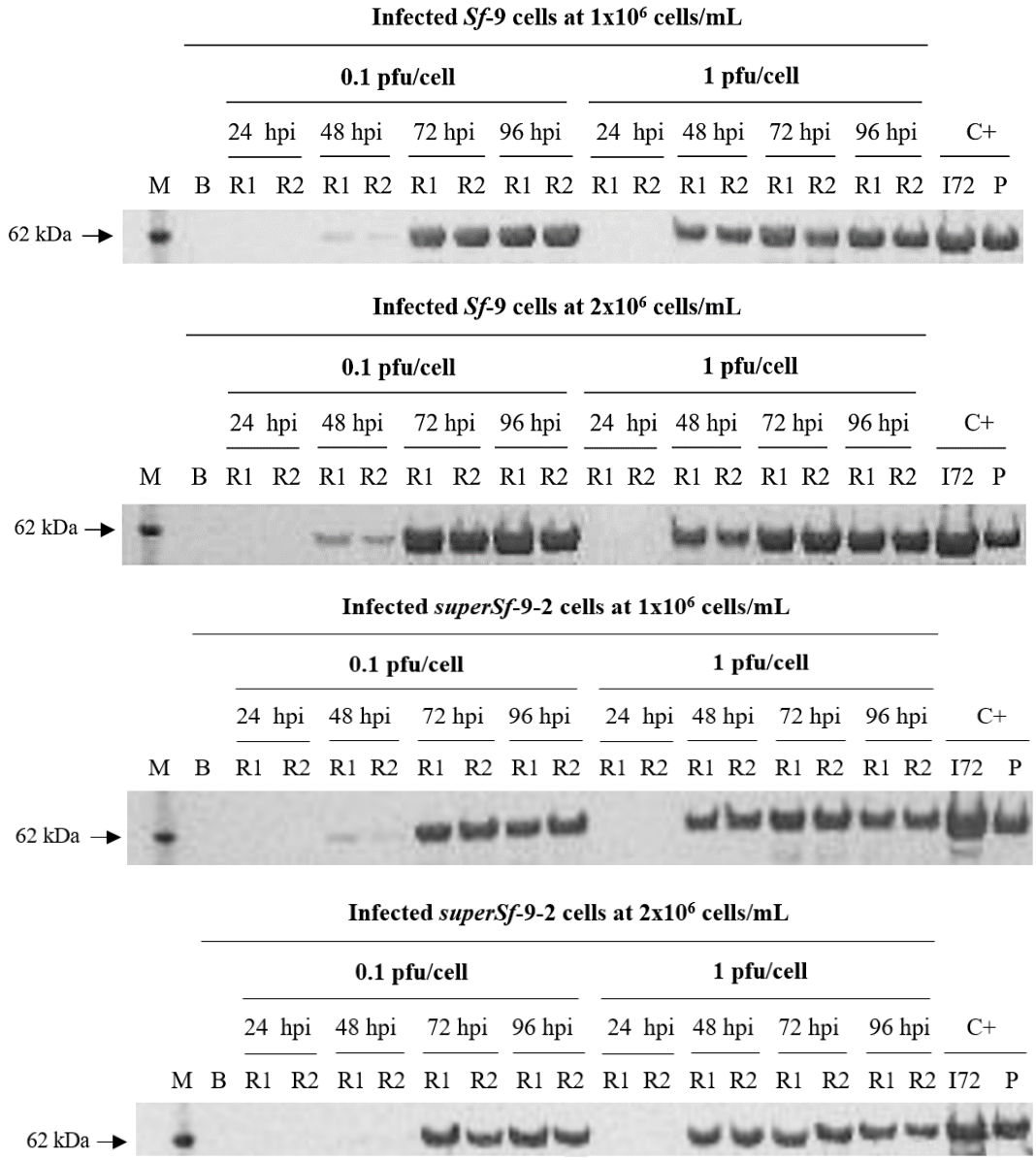
The performance of *Sf*-9 and *superSf*-9-2 cell lines to produce ADDomer particles was assessed and compared to the baseline production process (i.e. High Five cells cultured in *Insect Xpress™* medium and infected with rBAC-ADDomer-P_2 at a MOI of 1 pfu/cell and CCI of $1x10^6$ cells/mL, and optimal TOI of 72 hpi). A screening of CCI ($1x10^6$ and $2x10^6$ cells/mL) and MOI (0.1 and 1 pfu/cell) was performed. The fact that *Sf*-9 cells can reach higher cell concentration than High Five cells could potentially result in higher volumetric productivity [19]. On the other hand, *superSf*-9-2 cells could potentially achieved higher yields of protein that are prone to be degraded [39]. Relative expression of ADDomer particles was assessed by WB analysis of total protein amount of pellets, using purified ADDomer particles as positive control for assessment of relative expression. Cell growth kinetics of *Sf*-9 and *superSf*-9-2 cells are described in ***Figure 3.15***, as well as the screening of ADDomer particles produced.

(A)


MOI
(pfu/cell)
Cell line
0.1
1
0.1
1
High Five
Sf-9
superSf-9-2
1
2
CCI
(x10⁶ cells/mL)
Viable cell concentration (x10⁶ cells/mL)
Time post infection (hour)
Viable cell concentration (x10⁶ cells/mL)
Time post infection (hour)
Viability of Sf-9 cells (%)
Time post infection (hour)
Viability of superSf-9-2 cells (%)
Time post infection (hour)


Avg. diamter of Sf-9 cells (µm)
Time post infection (hour)
Avg. diamter of superSf-9-2 cells (µm)
Time post infection (hour)

(B)


Infected Sf-9 cells at 1x10⁶ cells/mL
0.1 pfu/cell
1 pfu/cell
24 hpi
48 hpi
72 hpi
96 hpi
C+
M B R1 R2 R1 R2 R1 R2 R1 R2 R1 R2 R1 R2 R1 R2 R1 R2 I72 P
62 kDa
Infected Sf-9 cells at 2x10⁶ cells/mL
Infected superSf-9-2 cells at 1x10⁶ cells/mL
Infected superSf-9-2 cells at 2x10⁶ cells/mL

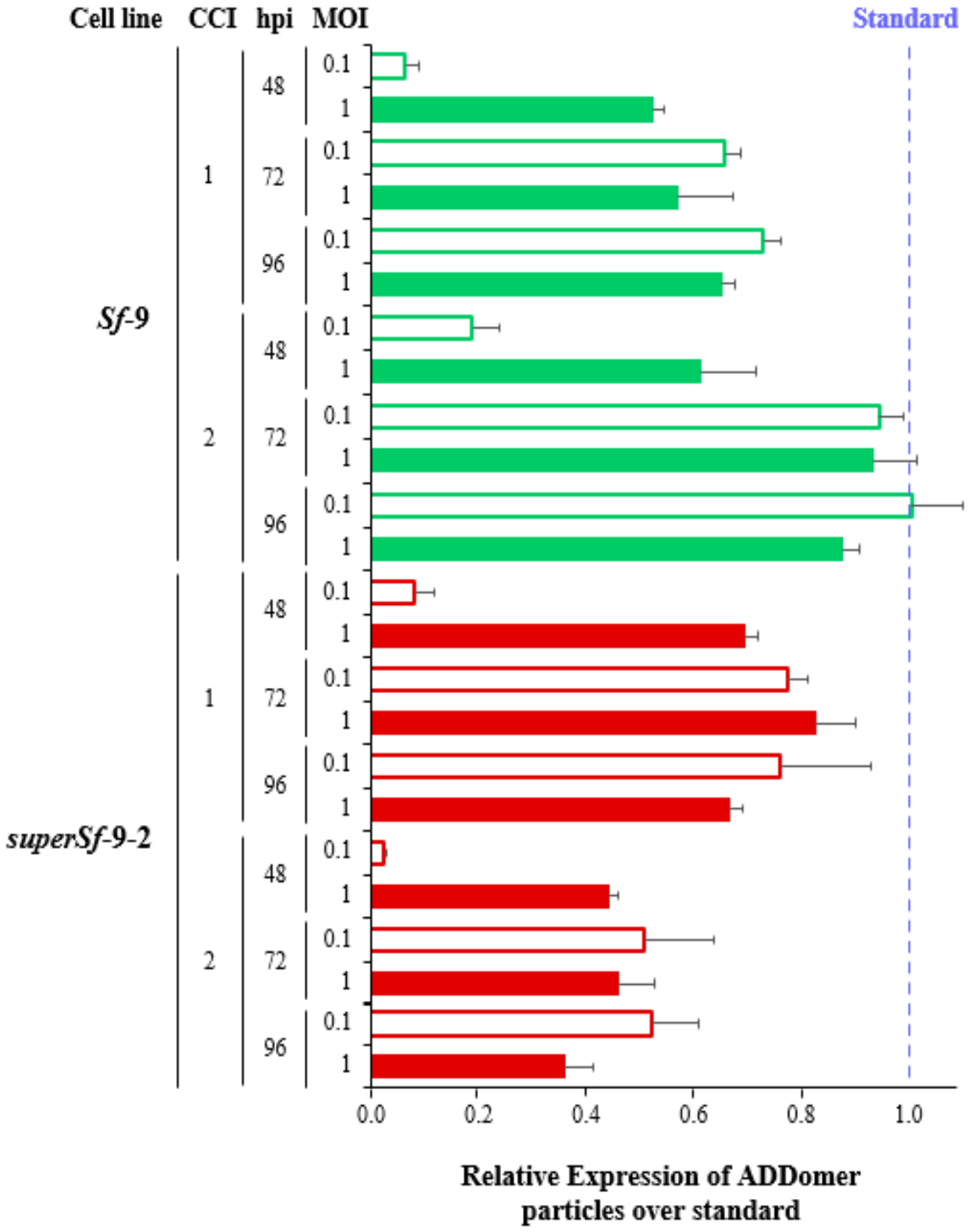


Figure 3.15 *Screening of ADDomer particles production in insect cell cultures.* ***(A)*** *Cell growth kinetics of infected Sf-9 and superSf-9-2 cells performed in duplicates (n=2). High Five cells cultured in Insect Xpress™ medium and infected at CCI of 1x10^6 cells/mL and MOI of 1 pfu/cell was added to the screen.* ***(B)*** *Western blot analysis of pellet samples, considering the total protein of pellets and showing a negative control bulk at 0 hpi (B). Bar graph representation of relative expression of ADDomer particles assessed through densitometry analysis performed to the bands (i.e., relative bands intensity) corresponding to ADDomer particles in western blot analyses. Data is normalized (at 1) for the amount of protein quantified when using the control condition. Positive controls (C+) were added: total protein of the pellet of High Five cells infected according to standard (baseline CPP) and purified ADDomer particles (P) for assessment of relative expression. CCI: Cell concentration at time of infection (cells/mL), hpi: hours post-infection, MOI: multiplicity of infection (pfu/cell), I72: baseline CPP.*

Infections of insect cell lines described in ***Figure 3.15*** were performed simultaneously. Non-infected *Sf*-9 cells had a growth rate of 0.025 h^{-1} and non-infected *superSf*-9-2 cells had a growth rate of 0.026 h^{-1}. Growth profiles of infected *Sf*-9 and *superSf*-9-2 cells in ***Figure 3.15A***, demonstrated typical growth kinetics of infection according to the MOI, where earlier onset of cell viability drop in cultures infected at MOI of 1 pfu/cell than in those infected at MOI of 0.1 pfu/cell. At 72 hpi (the ideal TOH previously identified for infected High Five cells), *Sf*-9 cells infected at $1x10^6$ cells/mL at both MOIs were in the pre-determined viability threshold, *Sf*-9 cells infected at $2x10^6$ cells/mL at 0.1 pfu/cell had a viability > 80%, and *Sf*-9 cells infected at $2x10^6$ cells/mL at 1 pfu/cell and all infected *superSf*-9-2 cells showed a cell viability below the pre-determined threshold. According to Gomez *et al.*, (2013) [39], *superSf*-9-2 cells have a metabolic pathway able to delay the lytic action of rBAC in an infected culture, remaining viable for longer time. However, such behaviour was not observed here for infected *superSf*-9-2 cells. The cell viability could derive from an intensification of ADDomer particles released to suspension. Similar to what had been verified for infected High Five cells, average cell diameter of infected *Sf*-9 and *superSf*-9-2 cells tended to increase throughout infection.

Screening of ADDomer particles produced, illustrated in ***Figure 3.15B***, demonstrated that *Sf*-9 cells are a promising tool to produce ADDomer particles as cells infected at CCI of $2x10^6$ cells/mL and MOI of 0.1 pfu/cell have an expression similar to that achieved from baseline CPP conditions (High Five cells infected at CCI of $1x10^6$ cells/mL and MOI of 1 pfu/cell) with the added benefit of using lower amount of viruses. However, cell viability was 6.1% which translates into a higher loss of ADDomer particles to suspension and impairs further the purification process. Thus, these are non-viable CPP conditions to produce ADDomer particles at large-scale when compared to the baseline CPP. As for infection of *superSf*-9-2 cells, a lower concentration of ADDomer particles was achieved in all tested infection conditions when comparing to production using the baseline CPP conditions. Based on these results, the baseline CPP conditions, i.e. insect High Five cells in *Insect Xpress*^{TM} medium infected at CCI of $1x10^6$ cells/mL and MOI of 1 pfu/mL, and harvested at 72 hpi, continue to be the ones allowing to maximize ADDomer particles production.

3.3.2. Optimization of ADDomer particles purification process

Regarding optimization of ADDomer particles purification process, a scalable procedure for cell lysis and clarification operation unit was assessed. In contrast to the aforementioned baseline bioprocess, cell lysis and clarification operation units were couple together in a 0.005 m^2 BioOptimal^{TM} MF-SL HF. Infected High Five cells were concentrated ~ 10 times and were further lysed using a surfactant solution; cell debris were retained inside HF membranes, replacing freeze-thaw cycles and centrifugation techniques. On top of that, clarified solution was concentrated with a 0.5 m^2 Pellicon XL CAS biomax of 50 kDa and 100 kDa devices to assess a further impurities removal in UF.

3.3.2.1. Performance of surfactant solution

A cell lysis operation unit is essential to extract intracellular VLPs from infected cells at the beginning of purification process [46]. In literature, most processes use a surfacant solution to disrupt cells membrane, conjugated with a centrifugation operation unit. Surfactants induce an extensive and gradual action on cell membranes, act on smaller debris particulates formed that can encapsulate VLPs, do not stimulate a detrimentally effect on VLPs structure (for not being used in excessive higher concentration and time), and are easily used to process larger scales compared with freeze-thaw procedure [46]. Moreover, they can be efficiently removed from the final product after performing the different purification operation units, including chromatography and buffer exchange procedure [47]. Triton X-100 surfactant is the most studied and used for processes derived from its efficiency. However, in January 2021, European Chemicals Agency (ECHA) categorized it as one of the prohibited chemicals to use in processes for confering a risk to human health and the enviroment (https://echa.europa.eu/pt/authorisation-list/-/dislist/details/0b0236e1807df80d (accessed on 28th August 2022). Thereby, other options are available on the market for the same purpose, such as ionic deviron C16 surfactant, zwiterionic zwittergent 3-12 surfactant and nonionic tween 20 surfactants [47].

A cell lysis protocol was developed to assess the disruptive performance of deviron C16, zwittergent 3-12 and tween 20 surfactants; Triton X-100 surfactant performance was also assessed. Intracellular proteins that were released for supernatant were screened by densitometry. An increase in the level of protein in suspension is expected throughout contact times (30 minutes and 1 hour) as result of the action of the surfactant. The supernatant of infected High Five cells bulk was used as a negative control and lysis performed by 3 freeze-thaw cycles was implemented as a positive control. The performance of each surfactant was compared to the cell lysis procedure previously implemented in the baseline purification process. ***Figure 3.16*** describes the release of intracellular proteins to suspension according to surfactant-mediated lysis performance.

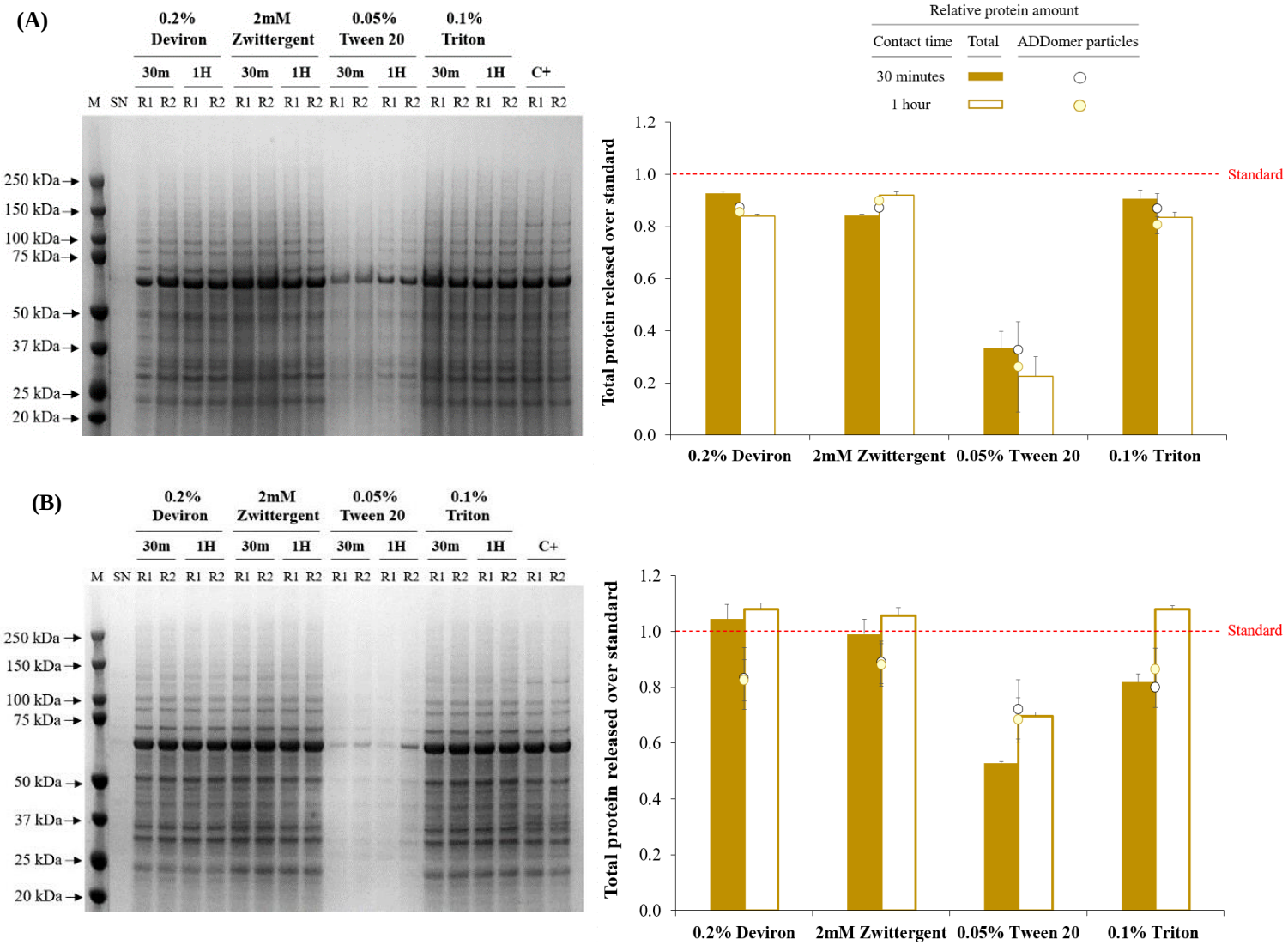


Figure 3.16 *Screening of performance of 0.2% deviron C16, 2mM zwittergent 3-12, 0.05% tween 20 and 0.1% triton X-100 when compared to the control condition (three freeze-thaw cycles (Standard)), for disruption of cells membranes* ***(A)*** *Lysis of infected culture bulk.* ***(B)*** *Lysis of infected cell pellet. Bar graph representation of surfactants performance versus standard (freeze-thaw cycles), assessed through densitometry analysis performed to the bands (i.e., relative band intensity) corresponding to proteins in SDS-Page analysis. Data is normalized (at 1) for the amount of protein quantified when using the control condition. SN: Supernant; 30m: 30 minutes of contact time; 1H: 1 hour of contact time. Duplicate were performed (R).*

The performance of deviron C16, zwittergent 3-12, tween 20 and triton X-100 compared to baseline lysis method is summarized in ***Figure 3.16***. When performing cell lysis directly in the fresh culture bulk, freeze-thaw cycle technique had the highest performance to disrupt cell membranes, releasing the highest amount of soluble intracelullar proteins and ADDomer particles into suspension, as ***Figure 3.16A*** shows. In terms of surfactant action, deviron C16 surfactant (at 30 minutes), and zwittergent 3-12 surfactant (at 1 hour) had the best performance. The use of Tween 20 did not allow to perform efficient extraction. As for lysis performed in fresh cell pellet, referred in ***Figure 3.16B***, the performance of surfactants was better than that of the control condition (freeze-thaw cycles). While deviron C16 surfactant enabled the highest performance to disrupt cell membranes, zwittergent 3-12 surfactant allowed a higher exposure of ADDomer particles (1 fold vs. deviron C16 surfactant). In both contact times, deviron C16 surfactant had the best performance. Similarly to lysis of cell culture bulk, tween 20 surfactant showed the worst performance between all tested conditions.

Considering the cost, buffer A supplemented (with EDTA protease-free inhibitor, 10 U/mL of benzonase, 2mM of chlorite of magnesium, and with surfactant as an additive) has the cost of 13.15 € per 100 mL of solution. Cost it increases by 0.035 € per 100 mL if adding deviron C16 surfactant, 2.37 € per 100 mL if adding zwittergent 3-12 surfactant, and 0.004€ per 100 mL if adding tween 20 surfactant. The use of zwittergent 3-12 surfactant is not ideal due to its higher price and similar results comparable with deviron C16 surfactant. In perspective of development of purification process, it is an advantage to use deviron C16 surfactant, in accordance with its disruptive cell membrane performance in a fresh cell pellet of infected High Five cells, at a lower contact time and with its reduced-implementation cost.

3.3.2.2. Cell lysis, clarification and concentration study

After defining the most suitable surfactant-mediated lysis agent to be used instead of freeze-thaw cycles, the next operation unit of cell lysis (i.e. clarification) was studied. In baseline, high-velocity centrifugation was used to clarify the lysate infected High Five cells; however, this technique is a hardly scalable procedure [20,49]. Flickinger & Drew (1999) [49] characterised MF as a suitable technique to remove cell debris, presenting an alternative to centrifugation for being easier to scale-up. Furthermore, the successful implementation of an UF operation unit after MF was proved, using a CAS device to ultrafilter the clarified product from MF, removing small molecular weight impurities, while concentrate the product of interest and exchange the buffer.

With this in mind, a MF procedure interconnected with the lysis operation unit was carried out in 0.005 m^2 BioOptimal^{TM} MF-SL HF, operating in TFF mode, in a distinct perspective from the ones used so far. The set-up implemented was designed to concentrate the High Five cells, lysate them and recover the intracellular ADDomer particles on the permeate side, as illustrated in

Figure 3.17. Initially, infected High Five cells were concentrated 10 times in HF device. Cells were separated from spent medium, being retained on HF membrane and returned to retentate 1, while spent medium and extracellular impurities passed through the HF membrane, being collected in permeate 1. The cell concentration in retentate 1 was maintained according to the initial cell concentration bulk (10 times concentrated), with a maximum decrease of 27% of cell viability assessed so far; whereas in permeate 1, there were no cells detected. Next, the concentrated infected High Five cells were lysed using buffer A supplemented with 0.2% of deviron surfactant, diluting the cells 7 times in lysis buffer. Retentate 2 suspension was recirculated in closed system for 15 minutes, allowing the surfactant to lyse the High Five cells. Permeate 2 was collected, being the protein enriched-supernatant fraction from which ADDomer particles will be further recovered. Retentate 2 maintained the cell concentration of retentate 1 with 0% of cell viability (7 times diluted) and no cells were observed in permeate 2. Therewithal, cell debris were retained in the HF membranes.

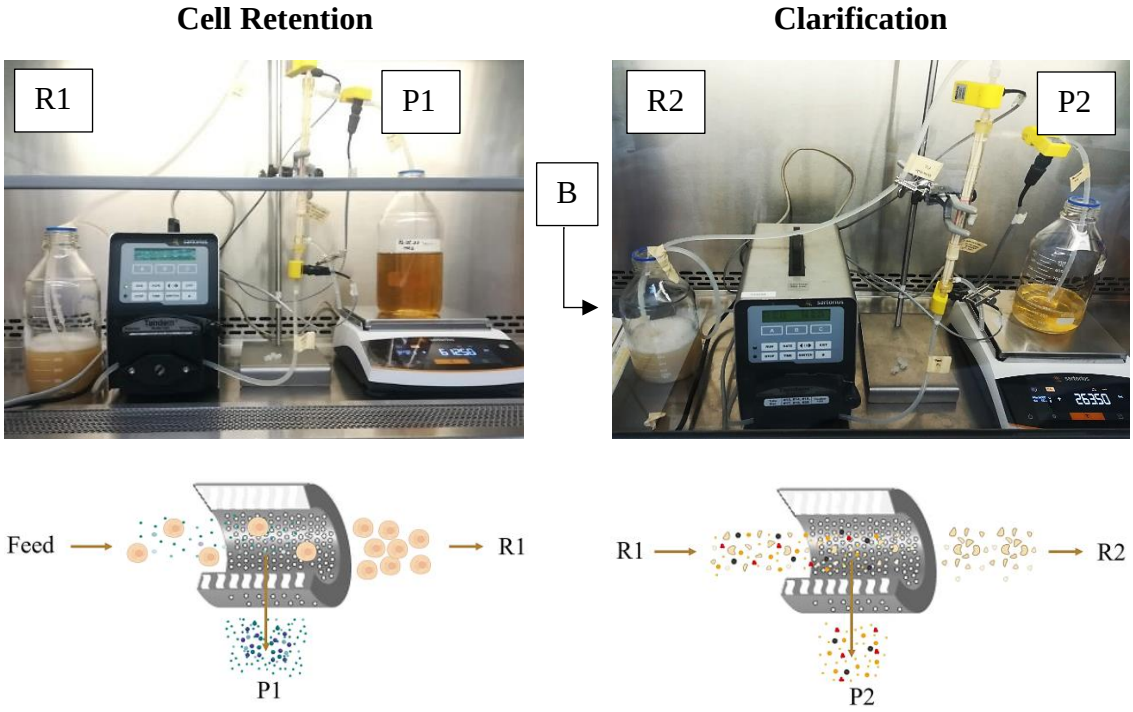


Figure 3.17 *Microfiltration procedure to lysate and clarified infected High Five cells cultured in Sf900™ II serum-free medium using a 0.005 m² BioOptimalTM MF-SL HF operating in tangential flow filtration mode and in sterile conditions. B: Addition of buffer A supplemented with 0.2% of deviron surfactant, R1: Retentate 1, P1: Permeate 1, R2: Retentate 2, P2: Permeate 2.*

Since lysate extract of permeate 2 is in lysis buffer, an additional operation unit to remove the surfactant is required, knowing that intensive concentration and extension action of surfactant can promote detrimental effects on VLPs and causing a decrease in immunogenic activity [50]. To avoid this phenomenon, MF procedure was followed by UF to exchange buffer and to allow impurities removal. According to ADDomer particles molecular weight of 63.13 kDa, 0.5 m^2

Pellicon XL CASs biomax of 50 kDa and 100 kDa were used to concentrate the clarified ADDomer particles. Without compromising the integrity of UF membrane, different CASs cut-offs were evaluated in order to optimize the removal of lower molecular weight proteins. The outcome of these experiments is summarized in ***Table 3.1*** and in ***Figure 3.18***.

Table 3.1 *Global performance of primary recovery of process development centred in the combined action of a 0.005 m^2 BioOptimal™ MF-SL hollow fibber with a 0.5 m^2 Pellicon XL Biomax cassette. CAS: cassette, DF: diafiltration, P: permeate, R: retentate, P_{TMP}: transmembrane pressure.*

Starting material **Infected High Five cells in *Sf900™ II* medium**		
Viable cell concentration ($x10^6$ cells/mL)	1.6	0.9
Cell viability (%)	74	79
Turbidity (FNU)	140.0 ± 1.4	106.5 ± 0.7
Volume (mL)	800	470
Microfiltration **0.005 m^2 BioOptimal™ MF-SL Hollow fibber**		
State of HF device	New	Reused
Cell concentration in R1 ($x10^6$ cells/mL)	16.1	8.3
Cell viability in R1 (%)	66	58
Turbidity in R2 (FNU)	819.0 ± 2.8	-
Turbidity in P2 (FNU)	36.0 ± 0.1	18.7 ± 0.1
P_{TMP}	Increase	Low
Foul of membrane	Yes	No
Operation time (hours)	3.5	1
Ultrafiltration **0.005 m^2 Pellicon XL Biomax**		
	CAS 50 kDa	**CAS 100 kDa**
State of CAS device	New	New
DF number	3	3
P_{TMP}	Increase	Increase
Foul of membrane	Yes	Yes
Operation time (hours)	6	5
Removal of impurities	No	No
Loss of ADDomer particles	No	No

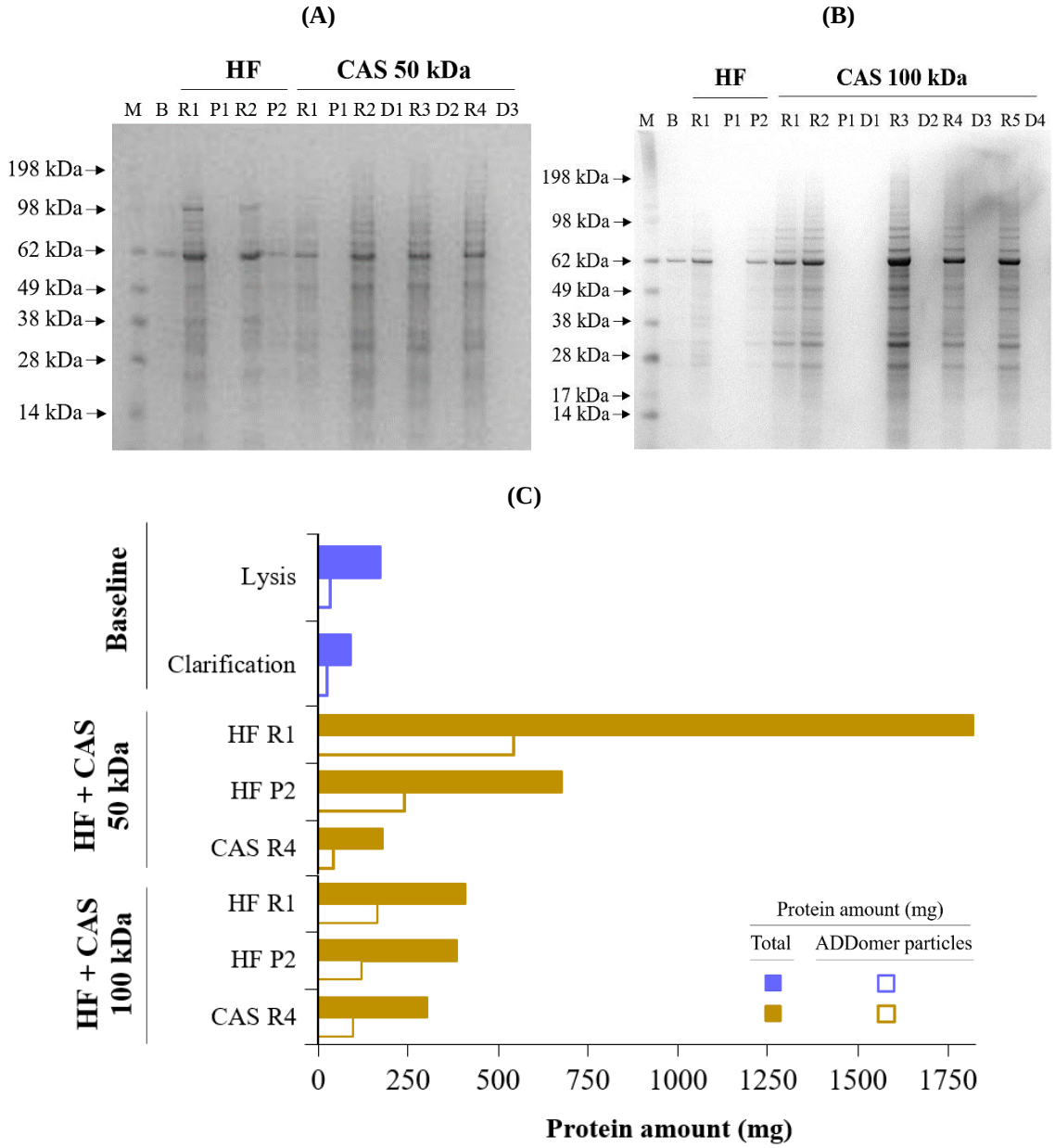


Figure 3.18 *Assessment of protein content during purification process development. Infected High Five cells cultured in Sf900™ II serum-free medium (B), retentate (R) and permeate (P) samples were analysed from hollow fibber (HF) and cassette (CAS) procedures. SDS-Page and western blot analyses of 2 µg of total protein when using of 0.005 m² BioOptimalTM MF-SL HF and of 0.5 m² Pellicon XL biomax* ***(A)*** *cassette 50 kDa and* ***(B)*** *cassette 100 kDa.* ***(C)*** *Protein profile of total protein content versus ADDomer particles content, in mg, in primary recovery process developed.*

The clarification operation unit implemented in 0.005 m² BioOptimal™ MF-SL HF has a suitable processing performance to concentrate and lyse High Five cells, and to retain its cell debris, as described in ***Figure 3.18A*** and ***Figure 3.18B***. BioOptimal™ MF-SL HF was developed to operate at lower P_{TMP} to process higher volumes [51], inducing a lower shear stress to cells. Even through, a slight disturbance was noted after concentrating infected High Five cells (in retentate 1) as shown in ***Table 3.1***. The increase in P_{TMP} indicates that membrane may be compromised by clogging events, which lead to a loss on efficiency and longer operation time.

Relative to CASs efficiency, is possible to observed in ***Figure 3.18A*** and ***Figure 3.18B*** that retentates were concentrated along DFs performed without removal of lower molecular weight impurities. According to range of membranes, CAS of 50 kDa is expected to retain ADDomer particles, while CAS of 100 kDa allow them to pass through to the permeate site. However, ADDomer particles were retained in both CASs, due to the formation of aggregates during initial concentration UF operation unit, and/or the membrane fouling leading to cake formation, pore construction and blockage. This leads to a gradual increase in P_{TMP} (which is characteristic of TFF operation mode), forcing to work at higher pressures to have a permeate flux. The addition of a filtration operation unit before UF could help to prevent the formation of aggregates and remove simultaneously impurities.

Considering the primary recovery of baseline bioprocess and the process here developed, compared in ***Figure 3.18C***, it is possible to observe that HF had a performance similar to combined action of freeze-thaw cycles and high-velocity centrifugation. Importantly, the loss of ADDomer particles is intensified during UF technique, derived from fouling events in CAS membrane. Regarding CAS performances, CAS 100 kDa should be considered to concentrate HF permeate product. With this in mind, a flowthrough chromatography operation unit should be evaluated before performing a UF operation unit.

Although BioOptimal™ MF-SL HF was not developed for retaining insect cells, it proved to be able to retain and lyse High Five cells thus demonstrating the potential of innovative designs for simple and easy scale-up of primary recovery steps.

4

CONCLUSIONS

An insect cell-based platform for production and purification of ADDomer particles was successfully implemented, enabling to obtain ADDomer particles with the expected size and morphology at a production yield of 19 mg ADDomer particles per L of culture and purity > 99%. In addition, the ability of different insect cell lines to produce ADDomer particles was proved, demonstrating the potential of IC-BEVS for ADDomer particles manufacturing. Furthermore, a scalable set-up was implemented for primary recovery steps, aiming at easing the technology transfer to a current Good Manufacturing Practice setting. The work herein developed improves the current ADDomer particles production and represents the basis for the development of an ADDomer-based snakebite therapy.

5

FUTURE WORK

Despite allowing the efficient production and purification of ADDomer particles, the experimental approaches herein used are prone to further improvements. Production of ADDomer particles in stirred-tank bioreactors, exploring different operations modes (e.g. fed-batch, perfusion, continuous), would allow to facilitate future process transfer while potentially improving production yield. Regarding purification process, clarified ADDomer particle pool here developed (surfactant-based cell lysis by MF) should be processed using chromatography approaches. For that, AEX membranes with different specificities should be studied, as well as negative mode chromatography to evaluate performances and defined the best set-up for scale up. In the final stage of process (concentration of chromatography pool and buffer exchange), the use of an UF with a CAS 100 kDa can be explored, followed by an operation unit of sterile filtration. Finally, a long-term stability studies should be carried out at lower temperature (-80℃ and 4℃), 20℃ and higher temperature (50℃) to determine aggregation phenomena which could reduce shelf time and efficiency of ADDomer particles.

In the future, the pipeline developed at iBET will be used to produce and purify ADDomer particle at large scale for the establishment of a snakebite therapy.

BIBLIOGRAFIA

[1] V. Peters, James A.; Wallach, "Snake," *Britannica*. Encyclopaedia Britannica, Inc., 2021, doi: https://www.britannica.com/animal/snake.

[2] D. A. Warrell, "Snake bite," *Lancet*, vol. 375, no. 9708, pp. 77–88, 2010, doi: 10.1016/S0140-6736(09)61754-2.

[3] R. A. Pyron, F. T. Burbrink, and J. J. Wiens, "A phylogeny and revised classification of Squamata, including 4161 species of lizards and snakes," *BMC Evol. Biol.*, vol. 13, no. 93, pp. 1–53, 2013, doi: 10.1186/1471-2148-13-93.

[4] A. Munawar, S. A. Ali, A. Akrem, and C. Betzel, "Snake venom peptides: Tools of biodiscovery," *Toxins (Basel).*, vol. 10, no. 11, pp. 1–29, 2018, doi: 10.3390/toxins10110474.

[5] K. Liblik, J. Byun, C. Saldarriaga, R. Lopez-santi, F. Fsiac, and M. Martinez-sell, "Snakebite Envenomation and Heart: Systematic Review," *Curr. Probl. Cardiol.*, vol. 47, no. 9, pp. 1–21, 2022, doi: 10.1016/j.cpcardiol.2021.100861.

[6] J.-P. Chippaux, "Snakebite in Africa," in *Handbook of Venoms and Toxins of Reptiles*, Second., S. P. Mackessy, Ed. Boca Raton (Florida, USA): CRC Press - Taylor & Francis Group, 2009, pp. 453–473.

[7] J. Gutiérrez, "Snakebite Envenomation as a Neglected Tropical Disease," in *Handbook of Venoms and Toxins of Reptiles*, Second., S. P. Mackessy, Ed. Boca Raton (Florida, USA): CRC Press - Taylor & Francis Group, 2019, pp. 471–483.

[8] M. R. Di *et al.*, "Vipers of Major clinical relevance in Europe : Taxonomy , venom composition , toxicology and clinical management of human bites," *Toxicology*, vol. 453, no. February, p. 152724, 2021, doi: 10.1016/j.tox.2021.152724.

[9] A. Alangode, K. Rajan, and B. G. Nair, "Snake antivenom: Challenges and alternate approaches," *Biochem. Pharmacol.*, vol. 181, no. 114135, pp. 1–8, 2020, doi: 10.1016/j.bcp.2020.114135.

[10] M. Vargas *et al.*, "Current Industrial Production of Snake Antivenoms," in *Handbook of Venoms and Toxins of Reptiles*, Second., S. P. Mackessy, Ed. Boca Raton (Florida, USA): CRC Press - Taylor & Francis Group, 2021, pp. 485–498.

[11] S. N. C. Gimenes and J. W. Fox, "Current Assessment of the State of Snake Venom Toxinological Research with a View to the Future," in *Handbook of Venoms and Toxins of Reptiles*, Second., S. P. Mackessy, Ed. Boca Raton (Florida, USA): CRC Press - Taylor & Francis Group, 2021, pp. 79–96.

[12] R. A. Harrison *et al.*, "Preclinical antivenom-efficacy testing reveals potentially disturbing deficiencies of snakebite treatment capability in East Africa," *PLoS Negl. Trop. Dis.*, vol. 11, no. 10, pp. 1–24, 2017, doi: 10.1371/journal.pntd.0005969.

[13] A. H. Laustsen, "Antivenom in the Age of Recombinant DNA Technology," in *Handbook of Venoms and Toxins of Reptiles*, Second., S. P. Mackessy, Ed. Boca Raton (Florida, USA): CRC Press - Taylor & Francis Group, 2021, pp. 499–510.

[14] C. Vragniau *et al.*, "Synthetic self-assembling ADDomer platform for highly efficient vaccination by genetically encoded multiepitope display," *Sci. Adv.*, vol. 5, no. 9, pp. 1–9, 2019, doi: 10.1126/sciadv.aaw2853.

[15] ADDovenom, "ADDovenom: Novel Snakebite Therapy Platform of Unparalleled Efficacy, Safety and Affordability," *ADDovenom*, 2021. https://addovenom.com/ (accessed Dec. 29, 2021).

[16] LSTM, "ADDovenom," *LSTM*, 2021. https://www.lstmed.ac.uk/research/centres-and-units/centre-for-snakebite-research/csri-programmes/addovenom (accessed Dec. 29, 2021).

[17] L. E. Lansdowne, "Developing Temperature-Stable ADDomer Vaccines for Untreatable," *Technology Networks Biopharma*, 2021. https://www.technologynetworks.com/biopharma/blog/developing-temperature-stable-

addomer-vaccines-for-untreatable-diseases-356788 (accessed Dec. 20, 2021).
[18] S. Au, W. Wu, and N. Panté, “Baculovirus nuclear import: Open, nuclear pore complex (NPC) sesame,” *Viruses*, vol. 5, no. 7, pp. 1885–1900, 2013, doi: 10.3390/v5071885.
[19] J. Drugmand, Y. Schneider, and S. N. Agathos, “Insect cells as factories for biomanufacturing,” *Biotechnol. Adv.*, vol. 30, no. 5, pp. 1140–1157, 2012, doi: 10.1016/j.biotechadv.2011.09.014.
[20] T. Vicente, A. Roldão, C. Peixoto, M. J. T. Carrondo, and P. M. Alves, “Large-scale production and purification of VLP-based vaccines,” *J. Invertebr. Pathol.*, vol. 107, pp. S42–S48, 2011, doi: 10.1016/j.jip.2011.05.004.
[21] S. Haase, L. Ferrelli, M. Luis, and V. Romanowski, “Genetic Engineering of Baculoviruses,” *Current Issues in Molecular Virology - Viral Genetics and Biotechnological Applications*, 2013. https://www.intechopen.com/chapters/45876 (accessed Oct. 20, 2021).
[22] B. Ye *et al.*, “Construction of the Antheraea pernyi (Lepidoptera : Saturniidae) Multicapsid Nucleopolyhedrovirus Bacmid System,” *J. Insect Sci.*, vol. 20, no. 5, pp. 1–10, 2020, doi: 10.1093/jisesa/ieaa088.
[23] G. P. Pijlman *et al.*, “Relocation of the attTn7 Transgene Insertion Site in Bacmid DNA Enhances Baculovirus Genome Stability and Recombinant Protein Expression in Insect Cells,” *Viruses*, vol. 12, no. 1448, pp. 1–17, 2020, doi: 10.3390/v12121448.
[24] A. Roldão, M. J. T. Carrondo, P. M. Alves, and R. Oliveira, “Stochastic simulation of protein expression in the baculovirus/insect cells system,” *Comput. Chem. Eng.*, vol. 32, no. 1–2, pp. 68–77, 2008, doi: 10.1016/j.compchemeng.2007.04.017.
[25] R. S. Felberbaum, “The baculovirus expression vector system : A commercial manufacturing platform for viral vaccines and gene therapy vectors,” *Biotechnol. J.*, vol. 10, no. 1, pp. 702–714, 2015, doi: 10.1002/biot.201400438.
[26] P. Fender, A. Boussaid, P. Mezin, and J. Chroboczek, “Synthesis , cellular localization , and quantification of penton-dodecahedron in serotype 3 adenovirus-infected cells,” *Virology*, vol. 340, no. 41, pp. 167–173, 2005, doi: 10.1016/j.virol.2005.06.030.
[27] S. Hervas-Stubbs, P. Rueda, L. Lopez, and C. Leclerc, “Insect Baculoviruses Strongly Potentiate Adaptive Immune Responses by Inducing Type I IFN,” *J. Immunol.*, vol. 178, no. 4, pp. 2361–2369, 2007, doi: 10.4049/jimmunol.178.4.2361.
[28] P. E. Cruz, C. C. Peixoto, J. L. Moreira, and M. J. T. Carrondo, “Production and quality analysis of Pr55gag particles produced in baculovirus-infected insect cells,” *J. Chem. Technol. Biotechnol.*, vol. 72, no. 2, pp. 149–158, 1998, doi: 10.1002/(sici)1097-4660(199806)72:2<149::aid-jctb886>3.0.co;2-c.
[29] C. Peixoto, M. F. Q. Sousa, A. C. Silva, M. J. T. Carrondo, and P. M. Alves, “Downstream processing of triple layered rotavirus like particles,” *J. Biotecnol.*, vol. 127, no. 3, pp. 452–461, 2007, doi: 10.1016/j.jbiotec.2006.08.002.
[30] R. Chubet, J. Kacmar, and H. Kopf, “Development of a novel platform TFF system for insect cell culture harvest,” *BioPharm Int.*, vol. 19, no. 3, pp. 28–32, 2006, [Online]. Available: https://www.biopharminternational.com/view/development-novel-platform-tff-system-insect-cell-culture-harvest.
[31] K. Saha, Y. C. Lin, and P. K. Y. Wong, “A simple method for obtaining highly viable virus from culture supernatant,” *J. Virol. Methods*, vol. 46, no. 3, pp. 349–352, 1994, doi: 10.1016/0166-0934(94)90005-1.
[32] D. Loewe, T. A. Grein, H. Dieken, T. Weidner, D. Salzig, and P. Czermak, “Tangential flow filtration for the concentration of oncolytic measles virus: The influence of filter properties and the cell culture medium,” *Membranes (Basel).*, vol. 9, no. 12, pp. 1–22, 2019, doi: 10.3390/membranes9120160.
[33] B. Fernandes, R. Correia, P. M. Alves, and A. Roldão, “Intensifying Continuous Production of Gag-HA VLPs at High Cell Density Using Stable Insect Cells Adapted to Low Culture Temperature,” *Front. Bioeng. Biotechnol.*, vol. 10, no. June, pp. 1–9, 2022, doi: 10.3389/fbioe.2022.917746.
[34] L. Norling *et al.*, “Impact of multiple re-use of anion-exchange chromatography media on virus removal,” *J. Chromatogr. A*, vol. 1069, no. 1, pp. 79–89, 2005, doi:

10.1016/j.chroma.2004.09.072.
[35] J. Vajda, D. Weber, D. Brekel, B. Hundt, and E. Müller, “Size distribution analysis of influenza virus particles using size exclusion chromatography,” *J. Chromatogr. A*, vol. 1465, no. 1, pp. 117–125, 2016, doi: 10.1016/j.chroma.2016.08.056.
[36] C. Bieniossek, T. J. Richmond, and I. Berger, “MultiBac: Multigene baculovirus-based eukaryotic protein complex production,” *Curr. Protoc. Protein Sci.*, vol. 5, no. 20, pp. 1–26, 2008, doi: 10.1002/0471140864.ps0520s51.
[37] T. Scientific, “Bac-to-Bac ™ Baculovirus expression system user guide.” Thermofisher, Madrid, pp. 1–72, 2018, [Online]. Available: thermofisher.com/support.
[38] A. Roldão, R. Oliveira, M. J. T. Carrondo, and P. M. Alves, “Error assessment in recombinant baculovirus titration : Evaluation of different methods,” *J. Virol. Methods*, vol. 159, no. 1, pp. 69–80, 2009, doi: 10.1016/j.jviromet.2009.03.007.
[39] M.-G. E. Contreres-Gómez A., Chisti Y., Sánchez-Mirón A., García-Camacho F., “Protein Production Using the Baculovirus-Insect Cell Expression System,” *Biotechnol. Prog.*, vol. 30, no. 1, pp. 1–18, 2014, doi: 10.1002/btpr.1842.
[40] S. G. Mann and L. A. King, “Efficient transfection of insect cells with baculovirus DNA using electroporation,” *J. Gen. Virol.*, vol. 70, no. 12, pp. 3501–3505, 1989, doi: 10.1099/0022-1317-70-12-3501.
[41] J. Transfiguracion, H. Jorio, J. Meghrous, D. Jacob, and A. Kamen, “High yield purification of functional baculovirus vectors by size exclusion chromatography,” *J. Virol. Methods*, vol. 142, no. 1–2, pp. 21–28, 2007, doi: 10.1016/j.jviromet.2007.01.002.
[42] W. Sigle, “Analytical transmission electron microscopy,” *Anal. TEM*, vol. 35, no. 1, pp. 239–314, 2005, doi: 10.1146/annurev.matsci.35.102303.091623.
[43] R. Jafari *et al.*, “The cellular thermal shift assay for evaluating drug target interactions in cells,” *Nat. Protoc.*, vol. 9, no. 9, pp. 2100–2122, 2014, doi: 10.1038/nprot.2014.138.
[44] B. A. Medi B., Chintala R., “Excipient selection in biologics and vaccines formulation development,” *European pharmaceutical*, 2014. https://www.europeanpharmaceuticalreview.com/article/24136/excipient-selection-biologics-vaccines-formulation-development/ (accessed Aug. 15, 2022).
[45] S. Pradhan, A. Varsani, C. Leff, and C. J. Swanson, “Viral Aggregation: The Knowns and Unknowns,” *Viruses*, vol. 14, no. 438, pp. 1–23, 2022, doi: https://doi.org/10.3390/v14020438.
[46] G. S. Kee, N. S. Pujar, and N. J. Titchener-hooker, “Study of Detergent-Mediated Liberation of Hepatitis B Virus-like Particles from S . cere W isiae Homogenate : Identifying a Framework for the Design of Future-Generation Lipoprotein Vaccine Processes,” *Biotechnol. Prog.*, vol. 24, no. 3, pp. 623–631, 2008, doi: 10.1021/bp070472i.
[47] M. G. Moleirinho *et al.*, “Clinical-Grade Oncolytic Adenovirus Purification Using Polysorbate 20 as an Alternative for Cell Lysis,” *Curr. Gene Ther.*, vol. 18, no. 6, pp. 366–374, 2018, doi: 10.2174/1566523218666181109141257.
[48] Y. Hu, “Baculovirus as a highly efficient expression vector in insect and mam- malian cells,” *Acta Pharmacol. Sin.*, vol. 26, no. 4, pp. 405–416, 2005, doi: 10.1111/j.1745-7254.2005.00078.x.
[49] R. R. Bommarius, A. S., Bettina, “Fermentation, Biocatalysis and Bioseparation,” *Biocatalysis*. Wiley-VCH Verlag GmbH & Co. KGaA, pp. 2611–2622, 1999.
[50] F. Gavilanes *et al.*, “Hepatitis B surface antigen,” *Biochem. J.*, vol. 265, no. 3, pp. 857–864, 1990, doi: 10.1042/bj2650857.
[51] BioOptimal MFSL, “BioOptimal MF-SL Hollow Fibber Microfilter.” AsahiKASEI, Tokyo, pp. 1–8, 2010, [Online]. Available: https://planova.ak-bio.com/products_services/clarification-purification/biooptimal/.
[52] T. Bioscience, “SEC Size Exclusion Chromatography.” Tosoh Bioscience, Germany, pp. 1–25, 2016, [Online]. Available: www.toshbioscience.de.

A

APPENDIX

SCREENING OF PRIMARY ANTIBODY FOR WESTERN BLOT APPLICATION

A screen of Pt-Dd sequence was performed using adenovirus as the organism source in *Uniprot database*. According to alignment cover among sequences performed in *Clustal omega* bioinformatic tool, *P12538* was defined as the protein more similar to *Q2Y0H9* (*Uniprot I.D.* of ADDomer particles [14]). *P12538* is the corresponding sequence of *l2* gene in Human adenovirus type C serotype 5. Despite the gaps among alignment, there is a strong probability that primary antibodies developed against adenovirus serotype 5 can recognize ADDomer particles, due to the high number of conserve regions and similar folding. There is no antibody against Pt-Dt for adenovirus serotype 3 and serotype 5, so 2 polyclonal antibodies were tested. One was the primary antibody used in-house to recognize adenovirus serotype 5 ab6982 (abcam) and the other was an anti-adenovirus serotype 3 PA5-22818 (Invitrogen). Primary antibody performance is described in ***Figure A***.

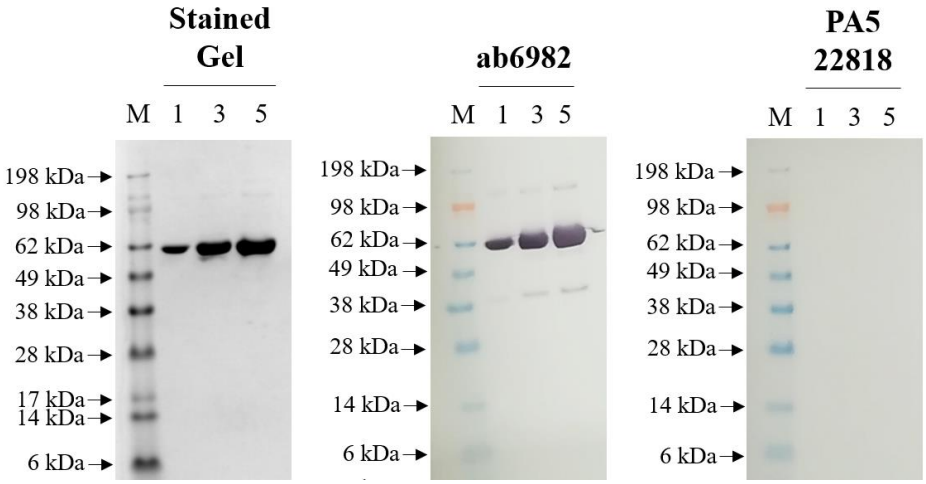


Figure A *Screening of primary antibodies performance using purify ADDomer particles received from UoB (United Kingdom) ranging from 1 to 5 µg. Antibodies ab6982 (made to recognize adenovirus serotype 5) and PA5-22818 (made to recognize adenovirus serotype 3) were study simultaneously. Stained gel was used as a positive control to conform the presence of ADDomer particles.*

The anti-adenovirus serotype 5 recognized ADDomer particles and anti-adenovirus serotype 3 did not, as observed in ***Figure A***. Based on its results, anti-adenovirus serotype 5 ab6982 (abcam) will be used in the following work to analyse the presence of ADDomer particles in solutions.

B

APPENDIX

ADDomer PARTICLES CALIBRATION CURVE

ADDomer particles purified using the baseline bioprocess implemented in-house were used to develop a HPLC-SEC method for quantification of ADDomer particles. TSKgel size exclusion G5000PW$_{XL}$ HPLC column was selected as to its capacity to separate smaller peptides (<1x10^6 g/mol), protein aggregates, DNA fragments and virus particles [52]. A concentration set ranging from 0.050 to 0.560 mg/mL was established to be analysed at absorbance of 220 nm (***Figure B***). Analysis was performed with 95% of confidence and followed Lambert-Beer law.

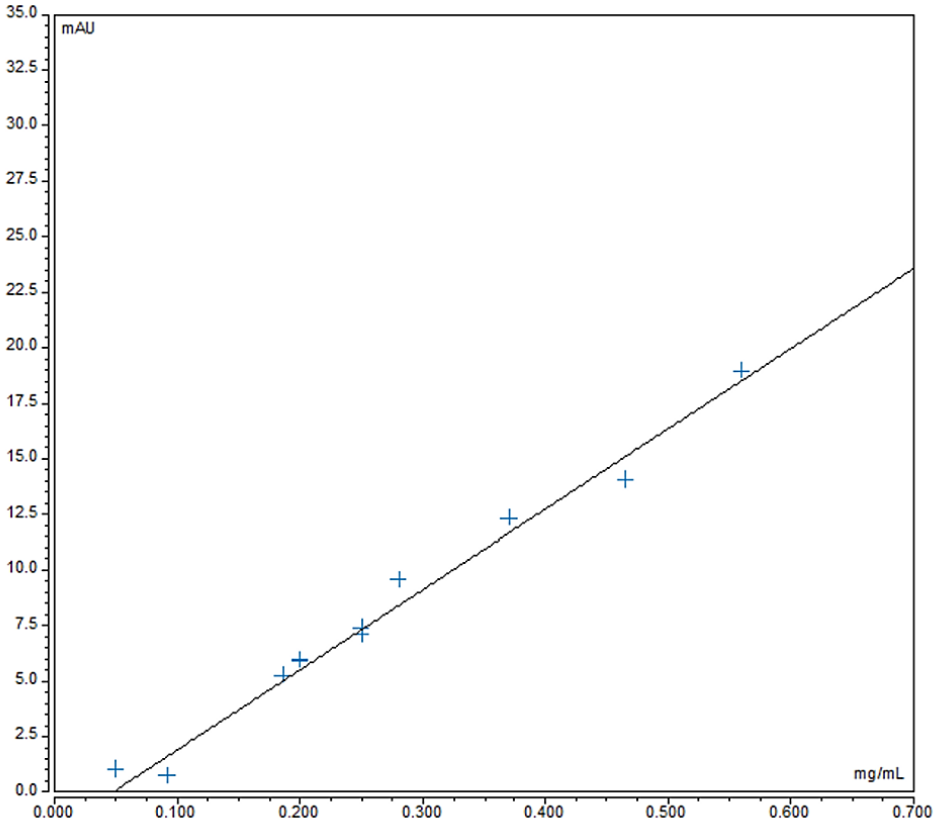


Figure B *HLPC-SEC calibration curve of pure ADDomer particles. Standards were run at 0.3 mL/min on polymethacrylate normal face (30 cm x 7.8 mm I.D., 10 μm particle size, 100 nm pore size), in TSKgel size exclusion G5000PW$_{XL}$ HPLC column* [52]*, monitored at absorbance of 220 nm.*

C

APPENDIX

CONCENTRATION OF BACMID-DNA EXTRACTED FROM WHITE-TRANSFORMED EmBacY COLONIES

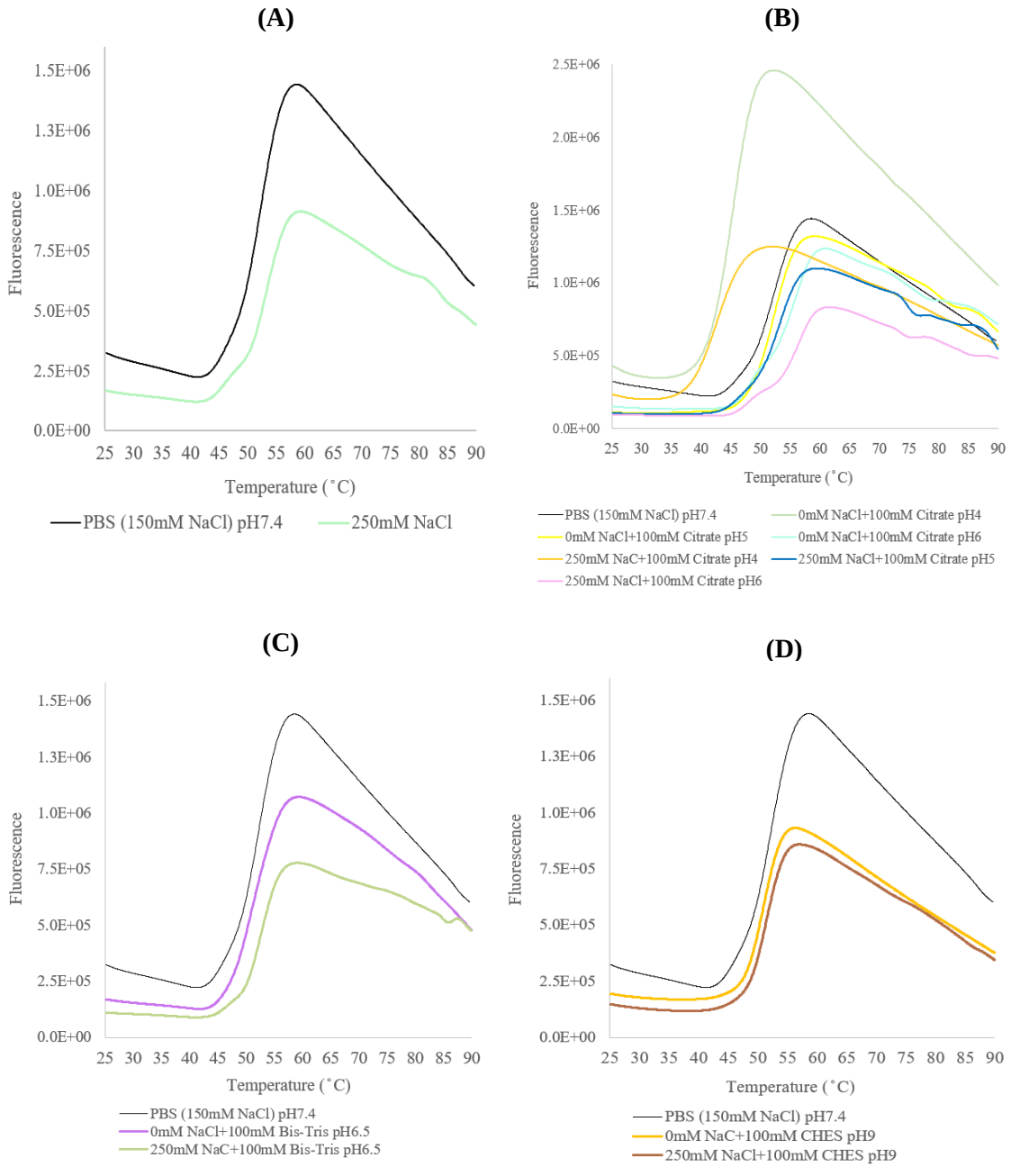
Table C *Purified bacmid-DNA concentration and purity measured by nanodrop (NanoDrop™ Spectrophotometer ND-2000c).*

Colonies selected	**Chemitransformed colony**		**Electrotransformed colony**	
	Concentration (ng bacmid-DNA/mL)	Ratio A_{260}/A_{280}	Concentration (ng bacmid-DNA/mL)	Ratio A_{260}/A_{280}
1	706.4	1.88	142.3	1.85
2	904.2	1.78	50.6	1.70
3	579.3	1.83	67.9	2.07
4	756.0	1.91	49.5	1.80
5			153.6	1.73
6			54.9	1.82
7			21.5	1.86
8			78.4	1.84
9			54.4	1.80
10			481.2	1.84
11			266.8	1.72
12			Blue phenotype	___
13			480.8	1.83
14			169.7	1.88
15			247.9	1.88

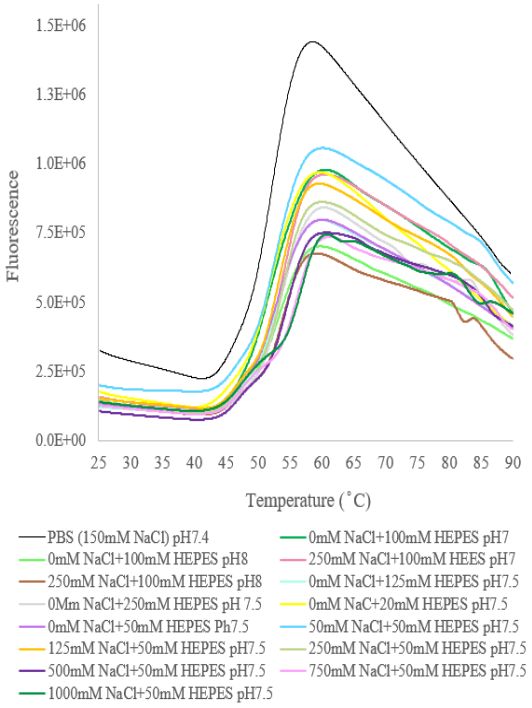
D

APPENDIX

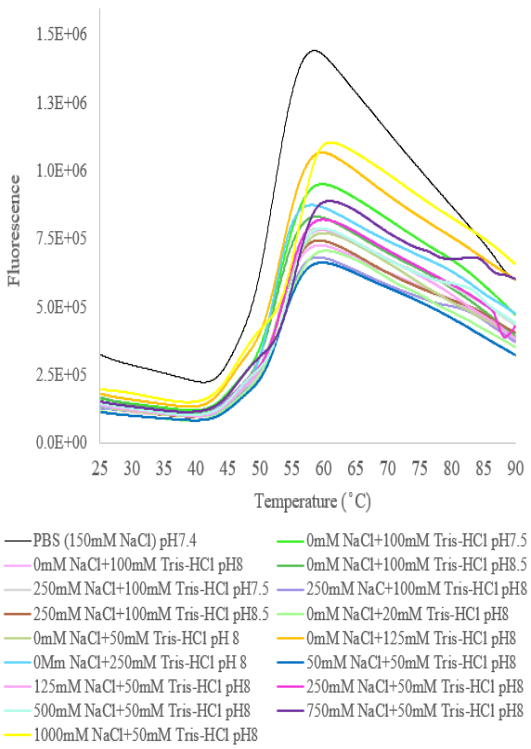
SCREENING OF ADDomer PARTICLES T_m TO ADDRESS TRENDS RELATED TO SALT CONCENTRATION, pH AND BUFFER FORMULATION



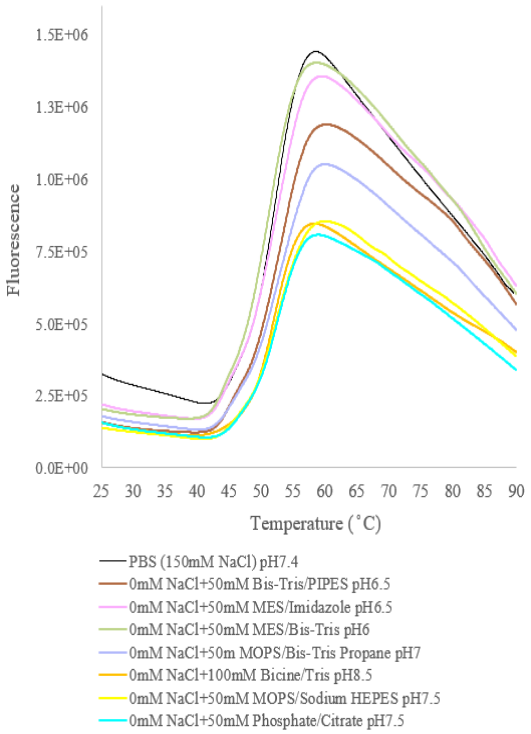
(E)


Fluorescence
1.5E+06
1.3E+06
1.0E+06
7.5E+05
5.0E+05
2.5E+05
0.0E+00
25 30 35 40 45 50 55 60 65 70 75 80 85 90
Temperature (˚C)
PBS (150mM NaCl) pH7.4
0mM NaCl+100mM HEPES pH7
0mM NaCl+100mM HEPES pH8
250mM NaCl+100mM HEES pH7
250mM NaCl+100mM HEPES pH8
0mM NaCl+125mM HEPES pH7.5
0Mm NaCl+250mM HEPES pH 7.5
0mM NaC+20mM HEPES pH7.5
0mM NaCl+50mM HEPES Ph7.5
50mM NaCl+50mM HEPES pH7.5
125mM NaCl+50mM HEPES pH7.5
250mM NaCl+50mM HEPES pH7.5
500mM NaCl+50mM HEPES pH7.5
750mM NaCl+50mM HEPES pH7.5
1000mM NaCl+50mM HEPES pH7.5

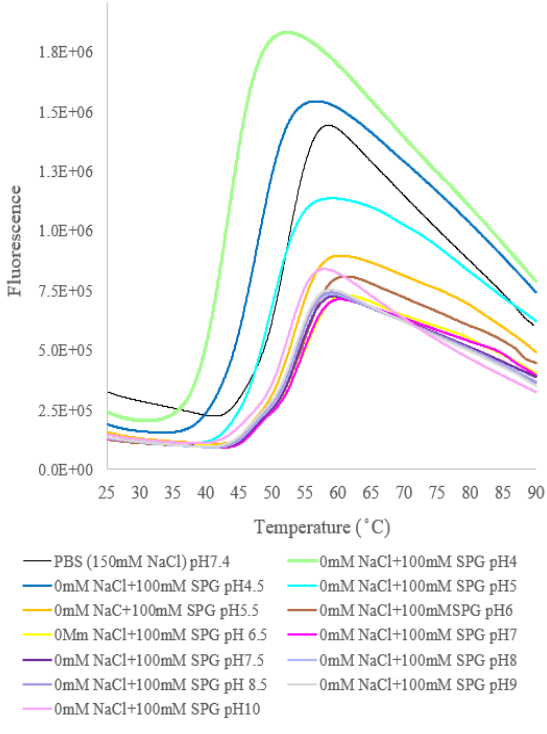
(F)


Fluorescence
1.5E+06
1.3E+06
1.0E+06
7.5E+05
5.0E+05
2.5E+05
0.0E+00
25 30 35 40 45 50 55 60 65 70 75 80 85 90
Temperature (˚C)
PBS (150mM NaCl) pH7.4
0mM NaCl+100mM Tris-HCl pH7.5
0mM NaCl+100mM Tris-HCl pH8
0mM NaCl+100mM Tris-HCl pH8.5
250mM NaCl+100mM Tris-HCl pH7.5
250mM NaC+100mM Tris-HCl pH8
250mM NaCl+100mM Tris-HCl pH8.5
0mM NaCl+20mM Tris-HCl pH8
0mM NaCl+50mM Tris-HCl pH 8
0mM NaCl+125mM Tris-HCl pH8
0Mm NaCl+250mM Tris-HCl pH 8
50mM NaCl+50mM Tris-HCl pH8
125mM NaCl+50mM Tris-HCl pH8
250mM NaCl+50mM Tris-HCl pH8
500mM NaCl+50mM Tris-HCl pH8
750mM NaCl+50mM Tris-HCl pH8
1000mM NaCl+50mM Tris-HCl pH8

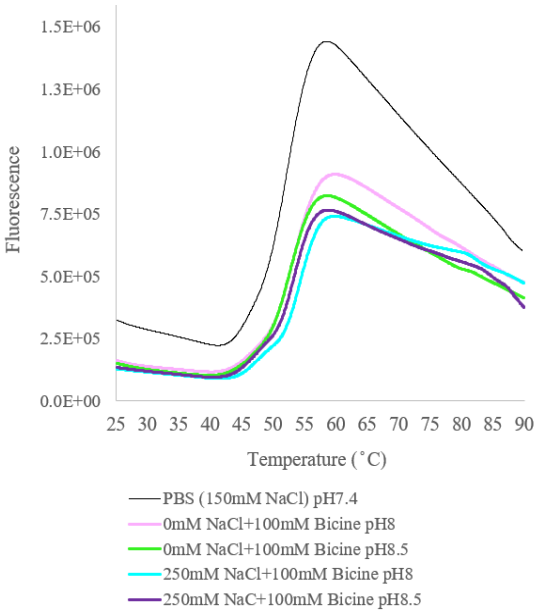
(G)


Fluorescence
1.5E+06
1.3E+06
1.0E+06
7.5E+05
5.0E+05
2.5E+05
0.0E+00
25 30 35 40 45 50 55 60 65 70 75 80 85 90
Temperature (˚C)
PBS (150mM NaCl) pH7.4
0mM NaCl+50mM Bis-Tris/PIPES pH6.5
0mM NaCl+50mM MES/Imidazole pH6.5
0mM NaCl+50mM MES/Bis-Tris pH6
0mM NaCl+50m MOPS/Bis-Tris Propane pH7
0mM NaCl+100mM Bicine/Tris pH8.5
0mM NaCl+50mM MOPS/Sodium HEPES pH7.5
0mM NaCl+50mM Phosphate/Citrate pH7.5

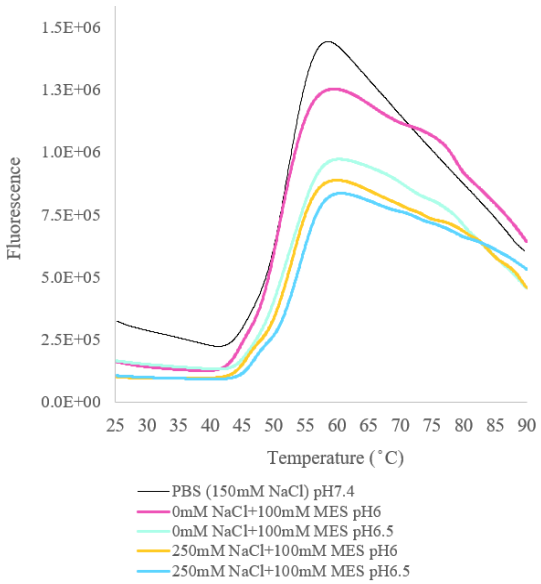
(H)


Fluorescence
1.8E+06
1.5E+06
1.3E+06
1.0E+06
7.5E+05
5.0E+05
2.5E+05
0.0E+00
25 30 35 40 45 50 55 60 65 70 75 80 85 90
Temperature (˚C)
PBS (150mM NaCl) pH7.4
0mM NaCl+100mM SPG pH4
0mM NaCl+100mM SPG pH4.5
0mM NaCl+100mM SPG pH5
0mM NaC+100mM SPG pH5.5
0mM NaCl+100mMSPG pH6
0Mm NaCl+100mM SPG pH 6.5
0mM NaCl+100mM SPG pH7
0mM NaCl+100mM SPG pH7.5
0mM NaCl+100mM SPG pH8
0mM NaCl+100mM SPG pH 8.5
0mM NaCl+100mM SPG pH9
0mM NaCl+100mM SPG pH10

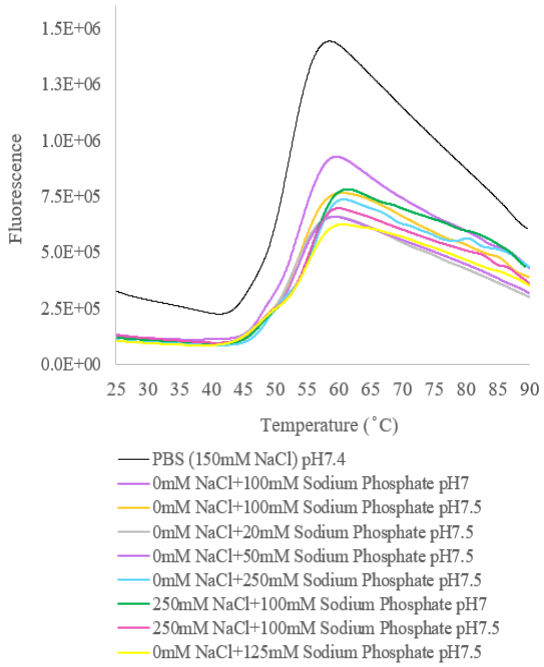
(I)


1.5E+06
1.3E+06
1.0E+06
7.5E+05
5.0E+05
2.5E+05
0.0E+00
Fluorescence
25 30 35 40 45 50 55 60 65 70 75 80 85 90
Temperature (˚C)
PBS (150mM NaCl) pH7.4
0mM NaCl+100mM Bicine pH8
0mM NaCl+100mM Bicine pH8.5
250mM NaCl+100mM Bicine pH8
250mM NaC+100mM Bicine pH8.5

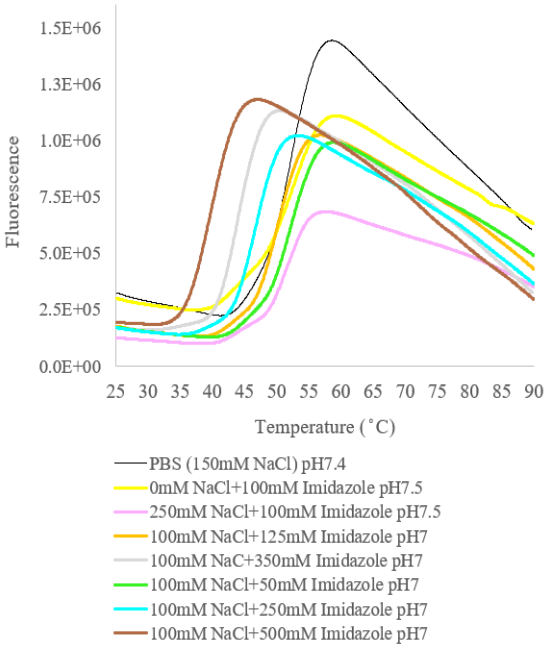
(J)

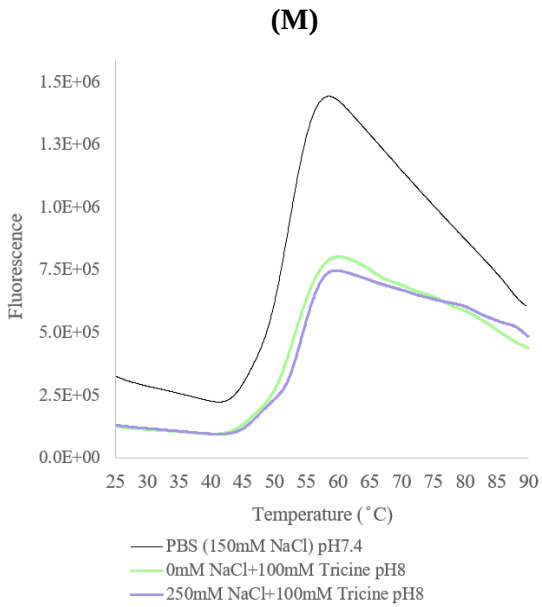

1.5E+06
1.3E+06
1.0E+06
7.5E+05
5.0E+05
2.5E+05
0.0E+00
Fluorescence
25 30 35 40 45 50 55 60 65 70 75 80 85 90
Temperature (˚C)
PBS (150mM NaCl) pH7.4
0mM NaCl+100mM MES pH6
0mM NaCl+100mM MES pH6.5
250mM NaCl+100mM MES pH6
250mM NaCl+100mM MES pH6.5

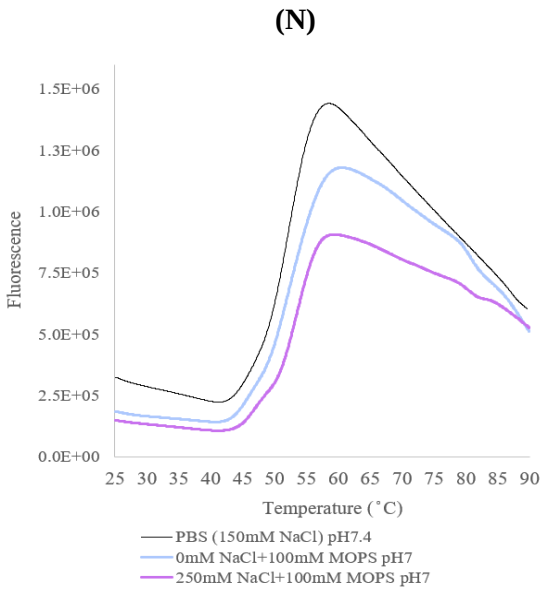
(K)

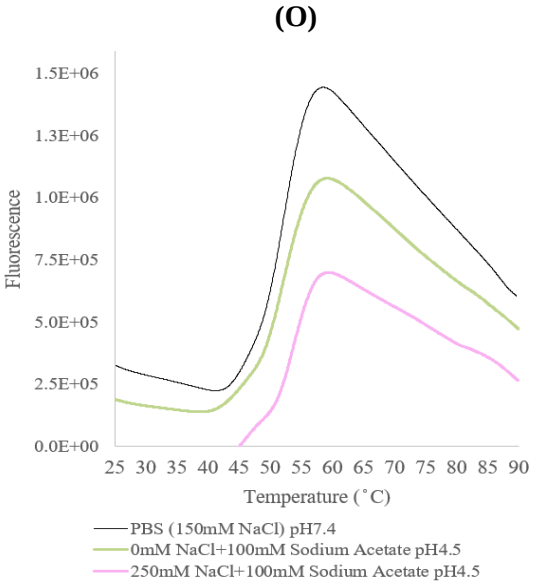

1.5E+06
1.3E+06
1.0E+06
7.5E+05
5.0E+05
2.5E+05
0.0E+00
Fluorescence
25 30 35 40 45 50 55 60 65 70 75 80 85 90
Temperature (˚C)
PBS (150mM NaCl) pH7.4
0mM NaCl+100mM Sodium Phosphate pH7
0mM NaCl+100mM Sodium Phosphate pH7.5
0mM NaCl+20mM Sodium Phosphate pH7.5
0mM NaCl+50mM Sodium Phosphate pH7.5
0mM NaCl+250mM Sodium Phosphate pH7.5
250mM NaCl+100mM Sodium Phosphate pH7
250mM NaCl+100mM Sodium Phosphate pH7.5
0mM NaCl+125mM Sodium Phosphate pH7.5

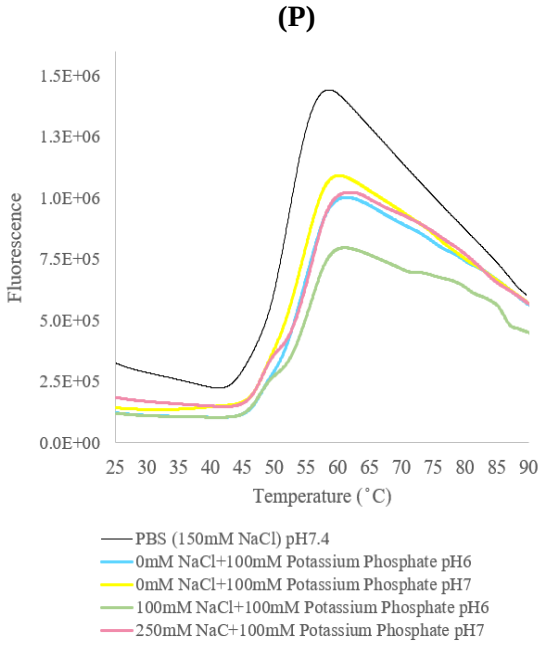
(L)


1.5E+06
1.3E+06
1.0E+06
7.5E+05
5.0E+05
2.5E+05
0.0E+00
Fluorescence
25 30 35 40 45 50 55 60 65 70 75 80 85 90
Temperature (˚C)
PBS (150mM NaCl) pH7.4
0mM NaCl+100mM Imidazole pH7.5
250mM NaCl+100mM Imidazole pH7.5
100mM NaCl+125mM Imidazole pH7
100mM NaC+350mM Imidazole pH7
100mM NaCl+50mM Imidazole pH7
100mM NaCl+250mM Imidazole pH7
100mM NaCl+500mM Imidazole pH7









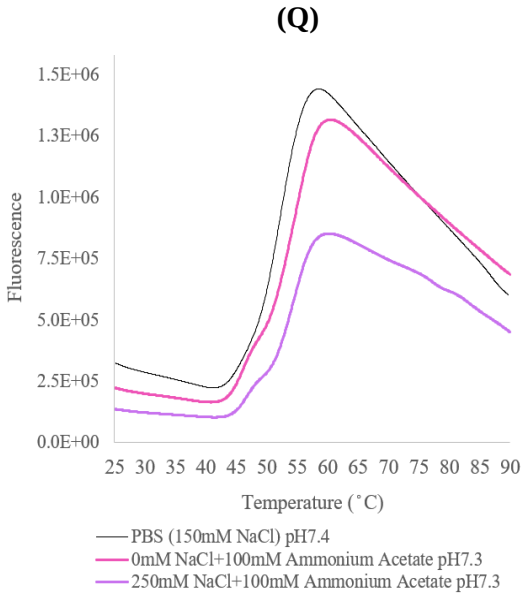


Figure D *Thermal profile of ADDomer particles stability in different buffer formulations. The particles in PBS at pH 7.4 was used as standard profile of Tm determination. Screening of denaturation behaviour of particles according to the action of* ***(A)*** *salt* ***(B)*** *citrate* ***(C)*** *bis-Tris* ***(D)*** *CHES* ***(E)*** *HEPES* ***(F)*** *Tris-HCl* ***(G)*** *buffer ratio* ***(H)*** *SPG* ***(I)*** *bicine* ***(J)*** *MES* ***(K)*** *sodium phosphate* ***(L)*** *imidazole* ***(M)*** *tricine* ***(N)*** *MOPS* ***(O)*** *sodium acetate* ***(P)*** *potassium phosphate* ***(Q)*** *ammonium acetate buffers.*