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Bioengineered baculoviruses as new class of therapeutics using micro and nanotechnologies: Principles, prospects and challenges ☆

Arghya Paul [a,b,c], Anwarul Hasan [b,c], Laetitia Rodes [a], Mugundhine Sangaralingam [a], Satya Prakash [a,*]

[a] Biomedical Technology and Cell Therapy Research Laboratory, Department of Biomedical Engineering and Artificial Cells and Organs Research Centre, Faculty of Medicine, McGill University, 3775 University Street, Montreal, Québec H3A 2B4, Canada
[b] Harvard-MIT Division of Health Sciences and Technology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA
[c] Center for Biomedical Engineering, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Cambridge, MA 02139, USA

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ABSTRACT

Designing a safe and efficient gene delivery system is required for success of gene therapy trials. Although a wide variety of viral, non-viral and polymeric nanoparticle based careers have been widely studied, the current gene delivery vehicles are limited by their suboptimal, non-specific therapeutic efficacy and acute immunological reactions, leading to unwanted side effects. Recently, there has been a growing interest in insect-cell-originated baculoviruses as gene delivery vehicles for diverse biomedical applications. Specifically, the emergence of diverse types of surface functionalized and bioengineered baculoviruses is posed to edge over currently available gene delivery vehicles. This is primarily because baculoviruses are comparatively non-pathogenic and non-toxic as they cannot replicate in mammalian cells and do not invoke any cytopathic effect. Moreover, emerging advanced studies in this direction have demonstrated that hybridizing the baculovirus surface with different kinds of bioactive therapeutic molecules, cell-specific targeting moieties, protective polymeric grafts and nanomaterials can significantly improve the preclinical efficacy of baculoviruses. This review presents a comprehensive overview of the recent advancements in the field of bioengineering and biotherapeutics to engineer baculovirus hybrids for tailored gene therapy, and articulates in detail the potential and challenges of these strategies for clinical realization. In addition, the article illustrates the rapid evolvement of microfluidic devices as a high throughput platform for optimizing baculovirus production and treatment conditions.

Contents

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* Corresponding author.
E-mail address: satya.prakash@mcgill.ca (S. Prakash).

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1. Introduction to baculovirus

Viruses are naturally available "vectors" for carrying genetic materials inside a cell. Although the ability of viruses to deliver genetic materials into cells makes them a dangerous group of pathogens to a wide variety of hosts, advances in molecular biology and genetic engineering have made it possible to exploit this property for therapeutic applications, particularly gene therapy. Gene therapy is the technique of introducing DNA into the body and making changes at the genetic level as a preventive or therapeutic approach to diseases. Classically, viruses have served as vectors for carrying the therapeutic DNA inside the host cells. Adenovirus, lentivirus, retrovirus, adeno-associated virus, etc. are some of the commonly employed viral vectors for gene therapy application [1,2]. However, these viruses still pose several problems relating to safety, ease of preparation, cloning capacity, stable long term expression of the gene insert and host immunogenicity [3,4]. Several safety issues are involved in using these vectors due to the pre-existing immunity that the human body possess against them and also the resulting toxicity caused by adverse immune responses. Another class of virus originated from insect cells, known as baculoviruses, which are non-pathogenic to humans, have been increasingly tested as they seem to overcome several obstacles posed by the classically used viruses [5,6]. Baculoviruses are a diverse group of enveloped DNA viruses belonging to the class *Baculoviridae*. They are pathogenic to arthropods and known to infect more than 600 insects. The widely exploited *Nucleopolyhedrovirus* genera of *Baculoviridae* family is divided into Type I which encodes the envelope glycoprotein GP64 and Type II that possesses the F protein on the viral envelope [7,8]. Both of these proteins are major mediators of the virus entry and exit through the infecting cell. The Type II genera however are incapable of infecting mammalian cells and hence the Type I genera, also known as *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), have been studied in detail in the context of gene delivery. Success in using recombinant AcMNPV baculoviruses, containing mammalian expression cassettes to direct gene expression into mammalian cells, has encouraged researchers to apply this approach for expression of different essential proteins to study multiprotein complex structures and interactions in healthy and diseased eukaryotic cells including human [9]. The advantages of using this system, commonly referred to as BacMam expression system, include ease of production, reproducible and titratable expression, simultaneous delivery of multiple genes, and potential for transducing a variety of host cells. But the most attractive feature of baculovirus as a gene therapy vector is that they are non-pathogenic and non-replicative in humans but are still capable of transferring the genetic material to human cells. This, combined with the fact that humans do not possess pre-existing antibodies or T cells against baculovirus [10], considerably lowers the risk factors associated with using them as vectors for gene therapy. In fact, BacMam viruses have also been used to express G protein-coupled receptor, ion channel and nuclear receptor target proteins [11].

While these various advantages of baculovirus over other viral vectors have made them an ideal vector for gene delivery applications, the next frontier is to further advance this powerful technology for successful clinical realization. This review discusses in detail the recent understandings on the mechanism for viral cell entry for successful in vivo studies. In addition, the article explores the evolving bioengineering technologies using micro and nano-scale materials and polymeric hybrids to modulate the baculovirus properties with unique features (Fig. 1) for advanced therapeutic applications.

2. Baculovirus–cell interaction: exploring the principal virus entry pathways

During infection, baculovirus exists as two phenotypes — occlusion derived virus (ODV) and budded virus [12]. ODV as the name suggests, is derived from an occluded form of virus that is environmentally stable and is capable of surviving outside the insect, aiding in infecting another host. ODVs cause the primary infection in the midgut of insects. Budded viruses lack the occlusion body and are adapted for survival inside the host, enabling secondary and systemic infection inside the insect [12]. The mode of entry of these viruses into the cell varies depending on the phenotype of the virus, the type of cell and environment conditions. Different mechanisms like adsorptive endocytosis and macropinocytosis direct cell fusion and electrostatic interactions during transduction.

2.1. Endocytosis

Evidence of an endocytosis dependant pathway for the entry of the budded form of AcMNPV was identified as early as 1985 by Volkmann et al. [13]. Since then a number of studies have been conducted to determine the molecules involved in the endocytic pathway and the various steps following endocytic entry into the cell. Sf9 insect cell lines infected with wild type baculovirus were found to fuse with each other at an acidic pH indicating the requirement of acidic conditions for fusion [14]. Treatment of $HepG_2$ cells infected with AcMNPV, with a lysosomotropic agent like Chloroquine showed that endosomal acidification is required for baculovirus mediated gene transfer [15]. In another independent study conducted using Chloroquine on AcMNPV infected Huh7 cells, the transduction and transgene delivery, as measured by the Luciferase reporter gene assay was found to depend on endosomal maturation of these cells [16]. Wang et al. affirmed that endocytosis was the principal pathway by which the budded form of the virus, AcMNPV BV enters Sf21 insect cell line [17]. By studying the binding kinetics of AcMNPV BV on Sf21 cells, they further found the presence of specific binding sites on the cell membrane and that binding to these sites depended on pH, bivalent cations and the composition of the medium [17]. Following these studies, many other researchers also confirmed the dependence of AcMNPV infection on an endocytosis mediated pathway [18–24].

2.2. Involvement of GP64 and F proteins

During cell entry, the virus AcMNPV was indicated in several studies to bind to specific sites and specific receptors [17]. A mathematical model developed by Wickham et al. initially predicted that AcMNPV and insect cell interaction involves greater than 10^5 receptors and there exists a binding affinity of 10^4 to 10^5 M^{-1} between the receptors and the cell surface [25]. Several important proteins on the cell surface were found to be involved in this receptor mediated interaction.

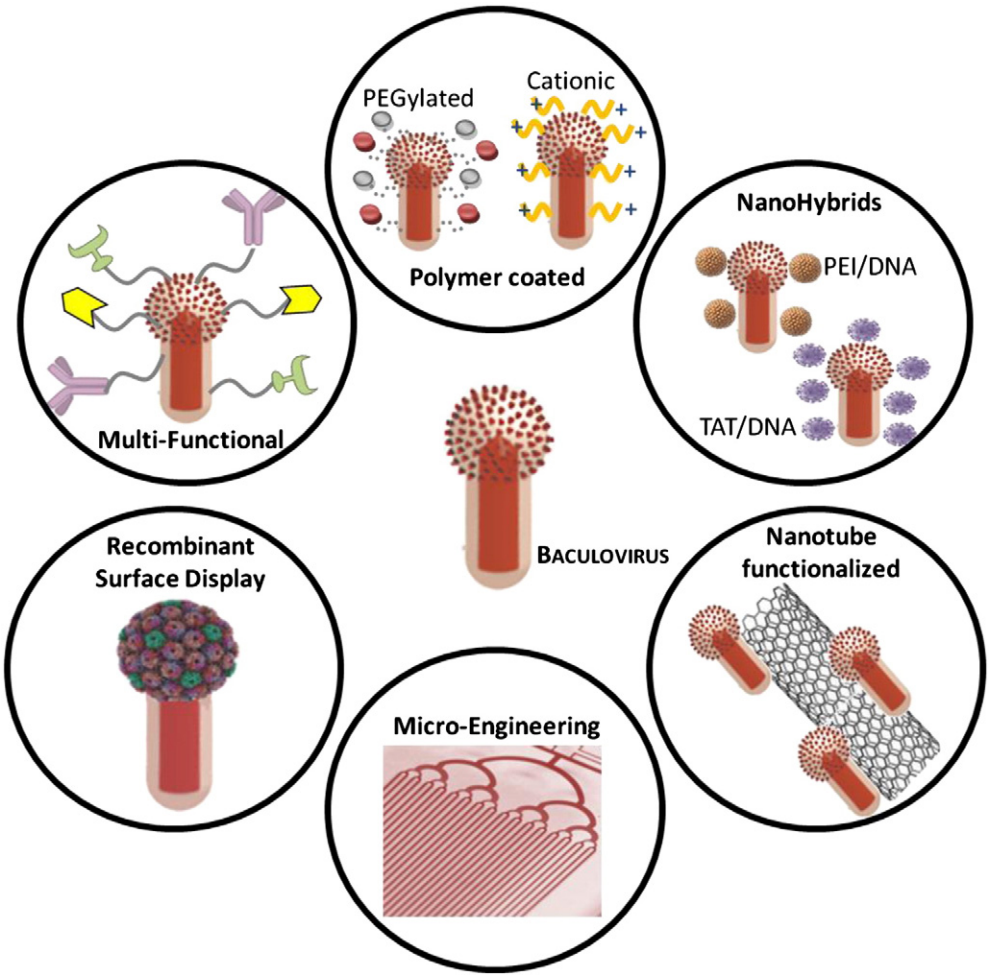

Fig. 1. Different strategies to bioengineer baculovirus gene delivery vectors for efficient biotherapeutic applications with advanced features.

Particularly, the envelope protein GP64 which is unique to Type I NPV baculovirus was found to be an important host cell receptor-binding protein [26]. A soluble form of GP64 protein analogous to the wild type GP64 of AcMNPV was generated which was found to selectively compete with recombinant marker AcMNPV for binding to the host cell, from which it was inferred that GP64 serves as a receptor binding protein [26]. It was also found that the pathogenicity and cell to cell motility of AcMNPV were inhibited by selectively disrupting the GP64 gene in the virus [27]. Their pivotal role in the cell entry ability of the virus was further confirmed by using antibodies to GP64 which showed to inhibit the viral infection [28,29]. Studies performed on other Type I NPV baculovirus like *Orgyia psuedotsugata* found not only GP64 to be important for pH dependent endocytic entry of the virus but also GP64 was sufficient alone to mediate this process [30].

F protein is another envelope protein present in both Type I and Type II NPVs, although it is functional only in Type II NPVs [31]. They were found to be functional analogs of GP64 in Type II NPVs, mediating the endocytic entry of the virus into the host cell [24]. However, GP64 and F protein were found to act differently using different receptors on the host cell. When GP64 null AcMNPV was pseudotyped with F protein, they lost their ability to transduce mammalian cells indicating the absence of receptors for F protein on mammalian cells and hence explaining the inability of Type II NPVs to transduce mammalian cells [24]. Thus due to their importance in the entry of baculovirus into the host cell, viruses pseudotyped with GP64 have been employed to study the entry mechanism in detail.

2.3. Clathrin mediated and caveolae mediated endocytosis

In an attempt to further elucidate the endocytic pathway of entry of Type I NPVs, live imaging of AcMNPV transduction of human hepatoma cells was performed using confocal and electron microscopies. The imaging showed the accumulation and binding of the virus on cell surface pits coated with electron dense clathrin like particles [20]. Following microscopic studies, Long et al. studied the functional importance of these clathrin coated pits in endocytosis [21]. They used inhibitors of various pathways like chlorpromazine, genistein and bafilomycin A in order to identify the exact mode of entry, and confirmed that the entry of AcMNPV is via a clathrin-dependent endocytic pathway. They also found that genistein, an inhibitor of caveolae-mediated endocytosis had some effect on the transduction efficiency at a higher concentration, and hypothesize the possible involvement of a caveolae-mediated endocytic pathway during transduction.

2.4. Alternate modes of entry

Apart from endocytic entry, involvement of alternate modes of entry like macropinocytosis and direct cell fusion have also been indicated during baculovirus infection and transduction of mammalian cells. In the imaging studies performed by Heli Matilainen et al., evidence of the presence of macropinosomes was found during the viral transduction process [20]. The entry of baculovirus was found to be mediated by a cell fusion between the envelope of the virus and the cell

membrane as early as 1986 by Volkmann et al. [32]. Direct cell fusion was also found to be an important mechanism of entry in *Trichoplusia ni* Nuclear Polyhedrosis Virus (TnNPV) [33]. However, this mode of infection was found to be 1000 fold less active than endocytosis [13]. Recently, it was discovered that the virus employs the direct cell fusion pathway for entry into both insect and mammalian cells under low pH conditions [34–36]. Interestingly, such a low pH triggered cell fusion mediated transduction was found to be more effective than the endocytic entry. Thus it would be worthwhile to exploit such properties to increase the efficiency of the virus as a gene therapy vector. Presence of an electrostatic interaction aiding the initial adsorptive binding has also been detected between the virus and the mammalian cell membrane [37]. A brief schematic representation of the two different pathways and cell components involved in cell infection is shown in Fig. 2 [36]. Further detailed mechanistic studies of these cellular pathways will lead us toward better understanding about the infection mechanisms of these viruses and the enhancement of their infection efficiency so as to favor our recombinant protein production.

3. Fate of baculovirus particles after cell entry

Following the entry of the virus into the cell via different mechanisms depending on the conditions of infection and transduction, a sequential process occurs that results in the delivery of genetic material — the virus uncoats its envelope, induces formation of actin filaments that aid in their movement across the cytoplasm and finally enters the nucleus and delivers the genetic material. In an endocytic pathway, the virus was found to fuse its envelope with the endosomal

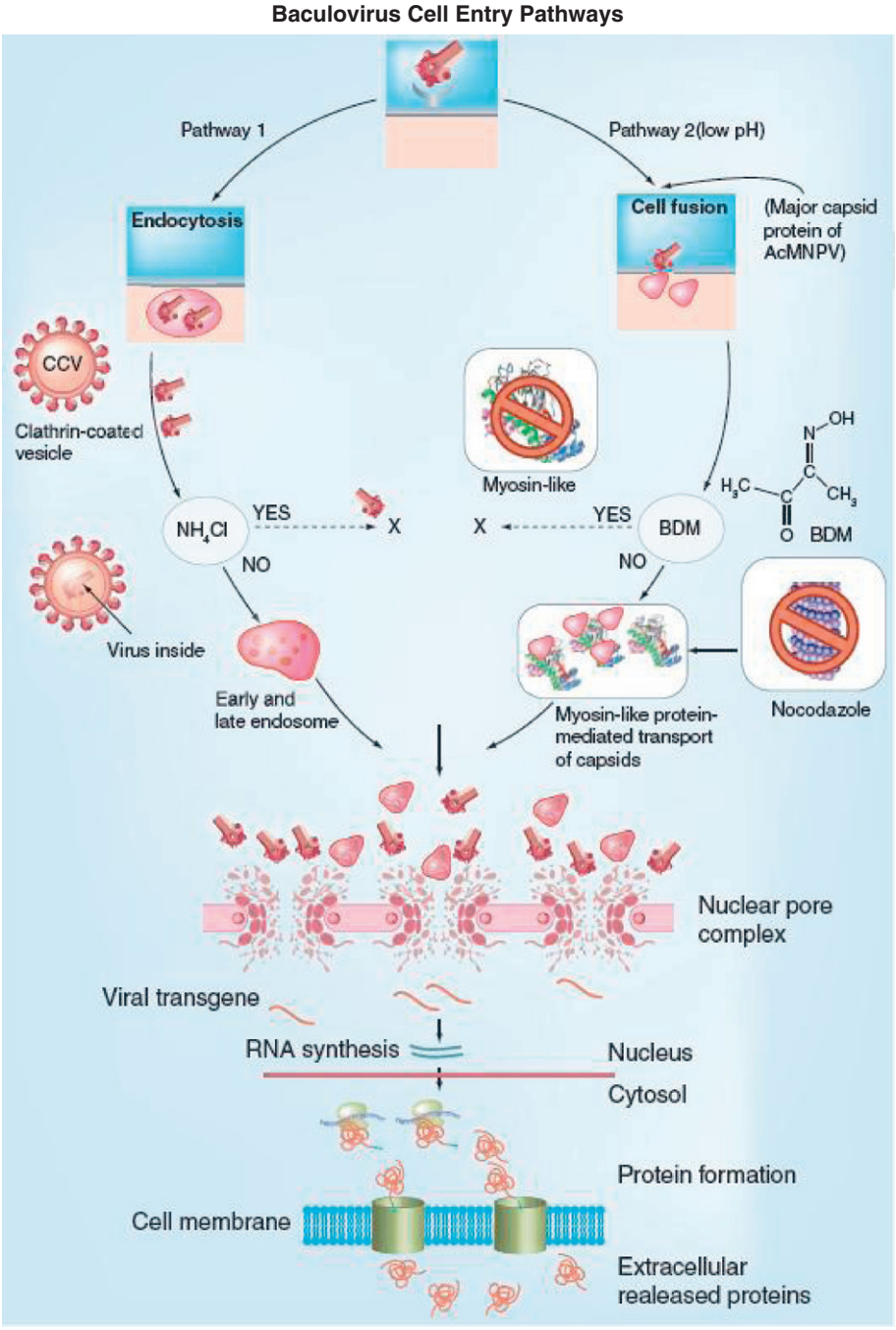

Fig. 2. Endocytosis and cell fusion based cell entry pathways of baculovirus [36].

membrane at a low pH and escape the endosomal membrane as a capsid into the cytoplasm [18,30]. The nucleocapsids of the uncoated virus initiate actin formation in the cytoplasm which helps in their cytoplasmic transit to the nuclear membrane [38,39]. Involvement of actin filaments in the transport of the viral capsid was further demonstrated by the use of Cytochalasin, an inhibitor of actin polymerization. Cytochalasin was found to reduce the transfection efficiency without affecting the cytoplasmic entry of the capsids, thus emphasizing their role in cytoplasmic trafficking [18].

The interaction of the virus with the nucleus was first discovered by Wilson et al. in 1988 when they found the association of capsid with the nuclear matrix in infected cells [40]. This however presented the possibility of entry of the viral capsids into the nucleus during mitosis, when the nuclear membrane disintegrates. This mode of entry of capsids during cell division was later disproved by showing that the virus was able to transduce non-dividing hepatic cells and indicated the presence of a nuclear entry mechanism. Electron microscopy studies further showed the accumulation of viral capsids at the nuclear pores and the simultaneous entry of capsids inside the nucleus, suggesting the transport of capsids via nuclear pores [18].

These discoveries in the mode of entry and interaction of baculovirus into insect and mammalian cells have paved the way for improvements in viral vector design for gene therapy applications by making modifications to the vector and the transduction conditions which will be discussed in the next section.

4. Engineering baculovirus vectors for diverse therapeutic applications

Chemical and genetic modifications have been performed on baculoviruses in order to improve their performance as a gene delivery vector. Although genetic changes have a prolonged and stable effect and less batch to batch variations, it is time consuming and restricted by the properties of the ligands [41]. In contrast chemical modifications are simple and flexible but may not be viable under certain conditions [42]. Thus, each type of modification has its pros and cons and a comprehensive choice has to be made based on the purpose, conditions and application. Several approaches have been taken using either of these modifications in order to achieve specific goals, some of which are listed below.

4.1. Improved cell entry

Classically, an improvement in baculoviral transduction has been achieved by displaying various proteins and peptides that are known to improve viral entry into the cell. GP64, the known envelope protein of Type II NPV baculovirus has been well exploited for expressing genes that would increase transduction. GP64 consists of an N-terminal signal domain and a mature domain that includes the transmembrane and the cytoplasmic regions, and the gene of interest is generally inserted between the signal domain and mature domain, under the control of polyhedrin or p10 promoter [43]. Several fusion proteins have been created in this way to display specific peptides or proteins on the surface of the virus to improve transduction [41,44,45]. In order to improve the rate of transduction in B lymphocytes which are poor targets for baculovirus, a surface display protein of Epstein Barr Virus (EBV) which infects B lymphocytes with ease was fused with GP64 for display [46]. Another protein that is exploited for display of peptides is the major capsid protein VP39. Fusion of enhanced green fluorescent protein with the N-terminal or the C-terminal end of VP39 has been used for tracking the distribution of the inserted gene without affecting the function or titre of the virus [47]. By exploiting the membrane penetrating properties of certain proteins, especially the protein transduction domain (PTD), improved delivery can be achieved. Using this strategy, the cytoplasmic transduction peptide (CTP) and the PTD of human immunodeficiency virus (HIV) TAT protein were fused with GP64 and VP39 for enhanced cell and nuclear entry respectively [48]. Such a design was found to mediate higher transduction efficiency not only of the recombinant virus but also of the co-transfected baculoviruses.

Alternately, expression of several other proteins like the membrane anchoring Vesicular Stomatitis Virus G glycoprotein (VSVG) [49–56], influenza virus neuraminidase [57], *Spodoptera exigua* multiple nucleopolyhedrovirus F protein [58], human endogenous retrovirus envelope protein [59] and single chain antibody fragments [60] has been expressed in baculovirus for improved cell entry. Chemical conjugation of the baculovirus surface with chemicals like cationic polyethylenimine and biocompatible polyethylene glycol has also shown to protect and improve baculovirus entry in mammalian cells [61,62].

4.2. Targeted delivery of biomolecules

Targeted delivery of the transgene to specific cell types has been achieved by expression of tissue specific antibodies or high affinity ligands. One of the most well characterized natural high affinity ligand binding is the avidin–biotin interaction. Exploiting this high affinity interaction, Jani et al. designed a baculovirus, *Baavi* that expresses avidin molecules on its surface by genetic manipulation [63]. They showed that *Baavi* was capable of transducing with a much higher efficiency compared to the wild type virus. By biotinylating the ligand for the specific cell type or the cell itself, transduction can be targeted to distinct cell types. They fabricated *Baavi* to express biotinylated epidermal growth factor (EGF) and demonstrated its targeting to cells expressing EGF. They were also able to achieve magnetic targeting by displaying paramagnetic particles. In another study, baculovirus was genetically altered to express the Vesicular Stomatitis Virus G (VSVG) glycoprotein on the envelope which was previously shown to increase the transduction efficiency by a 10 fold in hepatic cells, along with a short hairpin RNA (shRNA) targeting the genome of a respiratory syndrome virus (PRRSV) [64]. The VSV G gene was under the control of a very late promoter that ensured its expression only during replication in insect cells thus allowing timed targeting. They showed that such a design was able to successfully inhibit the infection of the PRRSV virus by disrupting its replication.

In another study, efficiency and specificity of delivery were simultaneously increased by chemically modifying the baculovirus system to display polyethylene glycol (PEG)–folate on its surface [65]. While PEG increased the efficiency of transduction, folate targeted the virus to folate receptor expressing cells. Since folate receptors are selectively expressed in malignant cells, such a design can be used in drug delivery for cancer [65]. Display of the fragment crystallizable (Fc) region of the antibody on the viral surface has shown to boost the effect of vaccine, since Fc receptors, present on cell surface, mediates phagocytosis and antigen presentation by binding to the Fc portion of antibody [66].

4.3. Prolonged transgene expression

Gene expression in a baculoviral vector system is transient and lasts for less than 14 days [67] due to degradation of the viral genome [68]. In order to prolong the gene expression, Heng-Chuan et al. designed a baculoviral vector that expresses EGFP under the control of the cytomegalovirus immediate–early promoter and transduced myoblast cells [67]. They found their design to successfully express the EGFP gene for around 63 days which is quite high when compared to the normal expression period. Dual baculoviral systems have also been developed wherein one baculovirus codes for FLP recombinase while the other one codes for the gene of interest which is contained in a Frt flanking cassette with oriP/EBNA1 from Epstein Barr virus [69]. When the FLP recombinase, derived from *Saccharomyces cerevisiae*, in the first baculovirus is expressed, it cleaves the Frt flanking region of the second baculovirus. This cleavage results in the formation of a self replicative episome which continuously expresses the transgene without

being degraded for around 48 days [69], thus significantly extending the gene expression period. The other major cause of transient gene expression is the degradation of the virus itself by complement proteins (C5b-9) present in the serum and blood of the patient [70]. Several strategies have been developed with the aim to overcome this problem of complement inactivation which are — (i) expression of inhibitors of complement proteins like soluble component inhibitor I, compstatin, human decay accelerating factor (DAF) or complementary regulatory proteins [71–74], (ii) physical separation of the virus from blood components [75] or (iii) introducing the virus in immunopriviliged sites [76,77].

Apart from chemical and genetic manipulation of the baculoviral vectors, modifying the transduction conditions like epigenetic factors, pH, temperature, surrounding media and incubation time can also result in improved transduction and prolonged gene expression. Ching-Suei Hsu et al. showed that by carrying out the viral transduction at 25°C for 4 h in Dulbecco's phosphate-buffered saline (D-PBS) medium instead of the standard procedure done at 37 °C, for 1 h in Dulbecco's Modified Eagle Medium (DMEM) resulted in more efficient gene delivery in HeLA cells [78]. The temperature was altered to 25 °C since the viruses were found to be degraded faster at 37 °C. The conventional DMEM medium was found to have certain inhibitory factors for the viral transduction and the incubation time was increased to improve the uptake of the virus [78].

4.4. Delivery of microRNA (miRNA)

Recent studies show increasing importance of microRNAs in the regulation of various biological processes in eukaryotes, including host–pathogen interactions. But microRNA delivery remains a hurdle, thus requiring an appropriate vector system for efficient transfer. The group of Hu YC successfully incorporated miRNA into the baculovirus vector to generate recombinant baculovirus miRNA shuttles which effectively repress the overexpression of endogenous inflammatory cytokine TNF-α in arthritic synoviocytes without inducing apoptosis [43]. To induce gene integration for enhanced microRNA expression Sleeping Beauty (SB) transposon was also incorporated into the vector. In a separate study, the group also reported the generation of hybrid SB-baculovirus vector for prolonged antiangiogenic fusion protein expression, consisting of endostatin and angiostatin, for the treatment of angiogenesis-dependent tumors such as prostate and ovarian tumor [79]. Very recently, baculovirus encoded miRNA has been shown to suppress biogenesis of host small-RNA-mediated defense by downregulating the expression of the host GTP-binding nuclear protein Ran, an essential component of nucleocytoplasmic transport machinery involved in small-RNA transport from the nucleus to the cytoplasm [80]. Thus, research in baculovirus gene carrying technology is not only designing different strategies to efficiently deliver genes for prolonged expression, but also opening new insights into the different evasion strategies used by the virus to counter the host defense for its efficient propagation. Table 1 summarizes diverse biotherapeutic applications of recombinant baculoviruses performed in animal models [81–109].

4.5. Vaccine development

Despite the widespread progress in vaccine development, infectious diseases remain the principal cause of death worldwide [110]. Immunogenic antigens of the causative agents of infectious diseases such as, malaria, acquired immunodeficiency syndrome (AIDS) and, H1N1 flue have been already identified. However, vaccine efficacy is often limited as a result of poor stimulation of the protective cellular immune required for the elimination of infected cells. Baculoviruses offer a promising vector vaccine candidate in which the antigens can be (i) expressed by the vector within the host cells, (ii) displayed on the baculovirus surface or (iii) displayed and expressed by the vector [111].

The feasibility of baculovirus as vehicle for vaccination was first explored by Aoki et al. [112] who successfully developed a recombinant baculovirus as a candidate for a pseudorabies virus (PrV) vaccine. The recombinant baculovirus carried the PrV *gB* gene under the control of the CAG promoter. High levels of expression of glycoprotein gB of PrV were observed in recombinant baculovirus transduced mammalian cell lines. Furthermore, intramuscular immunization of recombinant baculovirus induced psuedorabies viral gB protein-specific antibody response in BALB/c mice. Jin and colleagues constructed a recombinant Bombyx mori baculovirus, Bmgp64HA, carrying a synthetic hemagglutinin (HA) fragment of H5N1 virus fused to the portions of the gp64 gene under a control of a polyhedron promoter in order to display highly expressed HA on the surface of the baculovirus envelope [113]. Immunization with the Bmgp64HA virus and an aluminum hydroxide adjuvant protected the experimental cynomolgus monkeys against influenza virus infection. In addition, a randomized controlled clinical trial demonstrated the safety of recombinant baculovirus expressing HA vaccine [114,115]. The vaccine was formulated as a trivalent preparation containing the purified HA from A/New Caledonia/20/99(H1N1), A/Wyoming/3/03(H3N2), and B/Jiangsu/10/03 influenza viruses. The trial further showed preliminary evidence of protection against influenza infection and disease. Yoshida and colleagues constructed a recombinant baculovirus (AcNPV-CSPsurf) that displayed rodent malaria *Plasmodium berghei* circumsporozoite protein (PbCSP) on the virion surface – as a fusion protein with the major baculovirus envelope glycoprotein gp64 – as a vaccine vector for malaria [116]. Immunization of BALB/c mice with the baculovirus displays system elicited specific antibodies and IFN-γ production, and protected 60% of the mice against *P. berghei* challenge infection. The investigators further developed a second-generation AcNPV vaccine (AcNPV–Dual-PbCSP) which combined the baculovirus PbCSP display system and the BacMam system, precisely the single gene cassette that consists of the PbCSP–gp64 fusion gene under control of the CMV-polyhedrin dual promoterexpress PbCSP upon transduction of mammalian cells [117]. The novel vaccine elicited PbCSP-specific humoral and cellular immune responses and consistently conferred complete protection against sporozoite challenge in BALB/c mice. Other researchers have compared the effect of baculovirus vaccine vectors for malaria that (i) displayed the *Plasmodium falciparum* circumsporozoite protein on the baculovirus surface as a fusion with gp64, (ii) expressed the circumsporozoite gene in antigen-presenting cells, and (iii) both displayed and expressed circumsporozoite protein [10]. It was demonstrated in vitro and in BALB/c mice that the vector that both displayed and expressed circumsporozoite protein was superior in inducing anti-circumsporozoite immune responses. Thus, the baculovirus dual expression system is a promising strategy for a novel vaccine delivery platform for infectious diseases. To enhance patient compliance and increase vaccination coverage, current research has focused on developing orally administered recombinant BV based vaccines, thereby promoting a needle-free, painless approach [118].

5. Nanohybrid technologies for advanced baculovirus based therapy

Recent biomedical research has focused on utilizing the timely convergence of two parallel technologies — the decoding of the human genome sequence, which has led to greater understanding of the molecular basis of diseases and therapy, and advancement in the field of nanotechnology, biotherapeutics and molecular medicine. Among the various therapeutic approaches in the field of nanomedicine, nanoparticles, such as polymeric nanoparticles, nanotubes, quantum dots, dendrimers and nanocomplexes play an important role and offer unique features for efficient gene delivery. However, their success is currently limited mainly by suboptimal gene delivery efficiency to target sites, especially with systemic diseases, inability to do stably transfect cells, inconsistent biocompatibility of synthetic nanoparticles and toxic side effects of the degraded products. Merging the viral and non-viral nanodelivery system provides a promising strategy to design the

Table 1
Summary of in vivo investigations of baculoviruses in various therapeutic applications.

Type of application	Objective	Animal model	Description	Results	Ref
Musculo-skeletal	Transduction in skeletal muscles.	Mice	Injected the β-Gal baculovirus and the erythropoietin cDNA baculovirus into skeletal muscles and observed the gene expression.	Transduction efficiency was enhanced and transgene expression duration were increased	[81]
	Promoting cartilage formation	New Zealand White rabbit	P3 passage cells were transduced with baculovirus carrying BMP2 gene and cultured in a rotating-shaft bioreactor	Successfully created cartilage with maximum expression attained around 1047 ng/ml at MOI 75.	[82]
	Augmenting bone repair	Female BALB/c athymic nude mice	Hybrid baculovirus vector carrying BMP2 gene and FLP/Frt was used to genetically modify MSCs to express BMP2 protein.	MSCs transduced with conventional baculovirus vector neither appreciably changed major surface marker expression nor altered the immunological characteristics Gene expression did not sustain as expected	[83]
Eye	Ocular gene therapy.	New Zealand white rabbits	Baculoviruses encoding human VEGF-D and lac-Z, controlled by CMV promoter were injected intravitreally in the eyes.	VEGF-D gene delivery caused enlargement of capillaries. Intravitreal VEGF-D gene transfer caused blood-retina barrier breakdown but not neovessel formation in the rabbit eye.	[84]
	Endothelial-specific gene expression.	Rat eyes	Generated recombinant baculoviruses with the endothelial-specific human flt-1 promoter.	Promoter elicited highest level of transgene expression in the endothelial cell line.	[85]
Neuronal	Neurological diseases, such as Alzheimer	Mouse embryonic fibroblasts from the CF-1 strain of mice.	baculovirus was equipped with the woodchuck hepatitis virus post-transcriptional regulatory element and human embryonic stem cells (HESCs) to generate human neurons.	Transgene expression as early as 1 day high efficiency of expression (up to 80%) Signal easily detectable even after 3 months HES-1 overgrown on feeder cells for 4 weeks	[86]
	Malignant glioma cells in the brain.	Rats' malignant glial cells (C6-luc tumor cells).	Modified recombinant baculovirus to treat glioma cells, then used a GFAP (glial fibrillary acidic protein) to minimize the side effects of the baculovirus.	Greatly increased transduction in glial cells. Halted tumor growth, providing an effective alternative treatment for cancer.	[87]
	Neuron regeneration.	Inbred female BDIX rats ($n = 28$)	-Used AcMNPV baculovirus (a baculovirus that can transduce both dividing and non-dividing cells) to deliver the Lac-Z gene.	Baculoviruses were able to transduce brain cells easily *in viv.*-when injected into the corpus callosum, gene transduction was scarce compared to that of adenoviruses.	[88]
	Resisting gene expression in targeted neural tissue	Striatum of nude mice.	Baculovirus modified with the GFAP protein and three micro-RNA (miRNA) strands to prevent off-target transgene expression.	Incorporating miRNA strands into baculoviral vectors contributes greatly to controlling transgene expression.	[89]
	Increasing neural transgene expression	Female inbred BDIX rat brains	Used 21-amino-acid ectodomain with transmembrane and cytoplasmic tail domains of VSV-G (VSV-GED) in order to increase baculoviral gene expression.	Enhanced baculoviruses were able to carry gene expression with a higher efficiency than the control viruses.	[90]
	Gene delivery for degenerative brain diseases	Adult male nude mice brains.	Modified recombinant baculoviruses with a specific neuron gene promoter in order to increase transgene expression of axons.	Proved that the baculovirus has great potential for neural treatment.	[91]
	Neuronal gene therapy	Adult male Wistar rats.	Modified recombinant baculoviruses with CMV E/PDGF promoters and AAV ITRs.	Proved to be highly promising treatment for neurological diseases.	[92]
	Transgene expression	Male Wistar rats' striatum.	Introduced a GAL4p65 protein to baculoviruses with either the PDGF or SYN promoter. Used mouse NF-kB to create the GAL4p65 proteins.	Proved GAL4p65 to be a potent transactivator in baculoviral gene expression.	[93]
Cardiac	Cardiac tissue regeneration	Male New Zealand White rabbit corticoid arteries	Modified baculovirus with a beta-galactosidase radioactive marker gene. Placed these Lac-Z adenoviruses genes with a CMV promoter around the arteries. Gene transfer done in vitro.	Gene expression remained strong for the first week, but after 14 days, the expression disappeared. The arterial structure of the corticoids remained intact. Upon closer examination, the arteries were inflamed Showed potential for cardiac tissue regeneration.	[94]
	Inducing antiangiogenesis	Female New Zealand White rabbits	Baculoviral genomes created using the BVboost system. Many baculoviral vectors were psuedotyped with the VSV-G protein and/or the woodchuck hepatitis virus	Significant capillary development detected after 6 weeks. Greatly increased gene transfer in the VSV-G baculovirus.	[95]
Cancer	Anti-angiogenesis based cancer therapy.	Mouse prostate cancer cells (TRAMP-C1)	Bac–hEAs that mediated hEA expression were constructed. Then, with the introduction of inverted terminal repeats (ITRs), Bac–ITR-hEAs (express the hEA a little more) were made.	ITR was expected to extend gene transmission, the experiment proved the contrary. Both Bac–hEA and Bac–ITR-hEA began with excellent gene transmission. Treatment of mice cancer cells prolonged life.	[166]
	Transgene expression	Rat brains	Modified baculoviruses with an astrocyte-specific promoter (a hybrid CMV/GFAP promoter) to increase cell transduction. Also used ITR's and AAV's	Greatly increased transgene expression in rat brains and glial cells. (10-100 fold).	[97]
	Gene expression	C6 rat glioma cells	Modified baculoviruses with both the CMV and the RNA Polymerase (Pol) III H1 promoters.	Achieved 95% gene expression in cultured cells, and 82% in rat brains.	[98]
	Cell targeting.	Adult female BALB/c athymic, nude mice	Modified baculoviruses with the herpes simplex virus thymidine kinase (HSVtk) gene driven by the HMGB2 promoter	Killed glioma cells, but did not affect healthy astrocyte cells. Prolonged survival of cancer-afflicted rats.	[99]
	Immune response and tumor metastasis	Female C57BL/6 (B6) mice.	Modified baculoviruses (AcMNPV in particular) with MNPs and NK cells.	AcMNPV efficiently stimulates NK cell-mediated tumor treatment	[100]
	liver cirrhosis	55 female 7-week-old BALB/c mice	Used the AcMNPV baculovirus for cell transduction.	Results show the feasibility of using baculoviruses to treat liver cirrhosis.	[101]

(continued on next page)

Table 1 (*continued*)

Type of application	Objective	Animal model	Description	Results	Ref
Modifying gene transfer	Enhanced gene transfer.	Neonatal rats	Genetically modified the baculovirus so that its viral envelope contains a human decay-accelerating factor protein (DAF) while maintaining the shape of a compliment-resistant baculovirus.	Facilitated enhanced gene transfer into neonatal rats	[102]
	Cell transduction.	Rats' malignant glioma cells and rabbits' aortic smooth muscle	Baculoviruses (Baavi in particular) were modified to express avidin. Avidin, due to its high positive charge in environments with physiological pH, was expected to enhance cell transduction when displayed by baculoviruses.	Avidin-enhanced Baavi baculovirus displayed a 5 fold increase in cell transduction in rat glioma cells (BT4C) Showed a 26 fold increase in rabbit aortic smooth muscle (SMC cells) when compared to wild-type baculovirus.	[103]
	Generation of gonadotropins of goldfish	Goldfish GTH subunit cDNA.	Recombinant hormones, such as cDNAs encoding goldfish GTH subunits were introduced to the silkworm baculovirus. Next, hemolyphs from silkworm larvae containing FSH were collected, and incorporated into the baculovirus.	Goldfish gonadotropins were successfully created. Foretells potential for creation of organs via baculoviruses.	[104]
	Gene expression.	Neural stem cell modified by v-myc transfer	Made a hybrid promoter by fusing CMV and a platelet-derived growth factor (PDGF). Constructed a baculovirus that expresses this hybrid gene.	Transgene expression up to 100x than that of the baculovirus with PDGF alone. Confirmed the possibility of using a tissue-specific promoter in the context of baculovirus vectors for specific gene expression.	[105]
	Library screening in cells.	Female Wistar rats	a tetra-promoter vector system was constructed (pBVboostFG) to facilitate cDNA screening.	The modified baculoviruses solves the problem of protein expression's proximity to its origin.	[106]
	Transfer genes.	Human serum with complement system and liver cells.	Baculovirus was modified for gene expression in the presence of human serum.	The modified viruses exhibited gene expression even in the presence of human serum	[70,107]
	Gene delivery	C57Bl6 mice	Modified baculovoiruses with decay accelerating factor (DAF), factor H (FH)–like protein-1 (FHL-1), C4b-binding proteins (C4BP), and membrane cofactor proteins (MCP) to test protection of baculoviruses.	Increased protection of baculoviruses, as well as efficiency of gene transduction.	[108]
	Gene expression.	Mice	Coupled baculoviruses with PEG (polyethylene glycol) to regulate transduction of GFP (green fluorescent protein).	PEGylated baculoviruses increased transgene expression and its efficiency.	[109]

next generation of gene delivery system because the currently used viral and non-viral nanovectors have complementary strengths, such as the high transduction efficiency of viral vectors, and the high systemic potential and low immunogenicity of non-viral nano-gene delivery systems. It has been reported that replication deficient adenoviral vectors can significantly enhance the transfection efficiency of DNA carrying nanoparticles such as liposomes and poly-lysine/DNA nanocomplexes when coupled together [119,120]. Based on this knowledge, further research is in progress in the field of nanomedicine and biotherapeutics to investigate the scope and advantages of using biologically safe recombinant baculovirus vectors for nanohybrid gene delivery system. This includes tissue and condition specific research on what regulates transgene delivery, cellular processing and, most importantly, protein expression. Some of the recent in vitro and in vivo studies in this direction are being discussed and analyzed in detail in the following section.

5.1. Targeted gene delivery by hybridized baculoviruses

Hybridization of baculovirus surfaces with different DNA carrying nanoparticles provides a unique and simple method to achieve efficient and targeted delivery of genes. This strategy allows the polymeric nanoparticle component to be surface modified with targeting ligands to promote target-specific delivery of the hybrid vector, whereas the baculovirus component can confer greatly improved intracellular uptake of the hybrid nanocomplexes. Towards this direction, Kim et al. constructed a hybrid system composed of baculovirus and non-viral vector components using electrostatic interaction between the positively charged Galactosylated polyethylenimine (GP)/DNA complexes and the negatively charged baculovirus, thus the complementary strengths of individual components [121]. GP was used as a non-viral vector system to provide hepatocyte specificity to the hybrid system via asialoglycoprotein receptor-mediated endocytosis, while baculovirus was used for efficient transportation of the hybrid system into the cells for eventual expression. In addition, GP surface functionalization further shielded the baculovirus from undesired serum inactivation. The hybrid complex showed suitable physicochemical properties and low cytotoxicity in human hepatoblastoma and lung carcinoma cells with enhanced transduction efficiency and cell-specificity.

5.2. Pro-angiogenic potential of nanohybrid recombinant baculoviruses

5.2.1. Use of baculoviruses for direct gene delivery

Direct gene delivery of baculovirus nanohybrids offers an ideal route of administration for in vivo studies, as it reduces the chance of non-specific gene delivery as well as virus inactivation. Recently, we have developed a new approach by hybridizing recombinant baculovirus with endosomolytic Tat/DNA_{Ang1} nanoparticles (Bac–NP_{Ang1}), both carrying proangiogenic Angiopoietin-1 genes, and explore its potential to enhance gene delivery efficiency for myocardial angiogenesis [122]. Tat peptide sequence, obtained from the protein transduction domain of HIV-1 viral proteins capable of penetrating cell membrane, was modified by incorporating histidine and cysteine residues for enhanced Ang1 plasmid DNA transport, efficient cellular internalization, cell vesicle escape and expression [123]. It was postulated that the efficiency of gene delivery can be improved by combining a non-viral vector system, which mediates cell attachment, with baculovirus vector systems, and mediate processes in gene transfer subsequent to binding. Fig. 3 demonstrates the efficient gene delivery potential of the Bac–NP_{LacZ} nanohybrids for myocardial gene delivery, where LacZ was used as the reporter gene. Such a hybrid system took advantage of the unique features associated with the two individual vector systems. Bac–NP_{LacZ} demonstrated higher and sustained LacZ expression in rat myocardial tissue than $Bac_{LacZ\ and}$ NP_{LacZ} individually. Animal study using myocardially infracted rat model also showed increased vasculogenesis, reduced fibrosis and significantly improved cardiac function in Bac–NP_{Ang1} treatment group compared to other groups three weeks post-treatment.

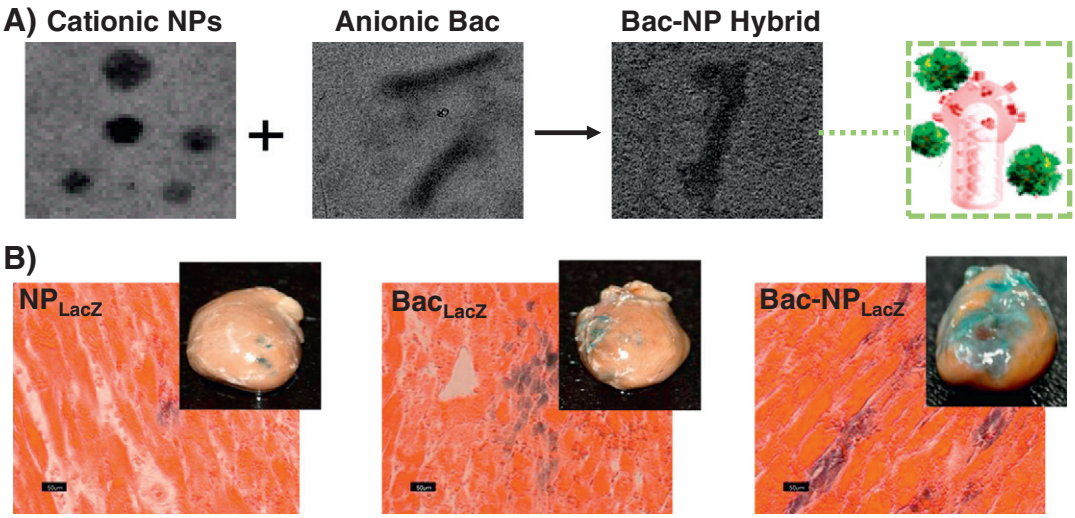

Fig. 3. Myocardial gene delivery using Bac–NP_{LacZ}. (A) Hybridization of the negatively charged baculovirus (Bac) with positively charged nanoparticles (NPs) by ionic interaction to form Bac–NP nanobiohybrids. The prepared nanohybrid complex can be efficiently used for in vitro *and* in vivo gene delivery applications. (B) Detection of LacZ transgene expression (3 days post-injection) in the peri-infarct (site of injection) regions of rat ventricular hearts transduced with NP_{LacZ}, Bac_{LacZ} and Bac–NP_{LacZ}, as observed in microscopic images of histological tissue sections (insets showing the corresponding whole hearts stained blue with LacZ). The samples were treated with X-gal to detect the LacZ gene expressing stained cells (stained in blue) and counterstained with eosin to stain cell cytoplasm (pink-orange). Scale bar indicated 50 μm. Abbreviations: Bac = baculovirus; NP = nanoparticle [122,128].

5.2.2. Use of baculoviruses for stem cell transfection

Recent experimental findings suggest the immense therapeutic potential of stem cell therapy in heart diseases, including acute myocardial infarction [124–127]. Using the above mentioned Ang-1 carrying hybrid baculovirus system, we genetically modified the adipose derived stem cells and demonstrated its favorable angiogenic effect in vitro and

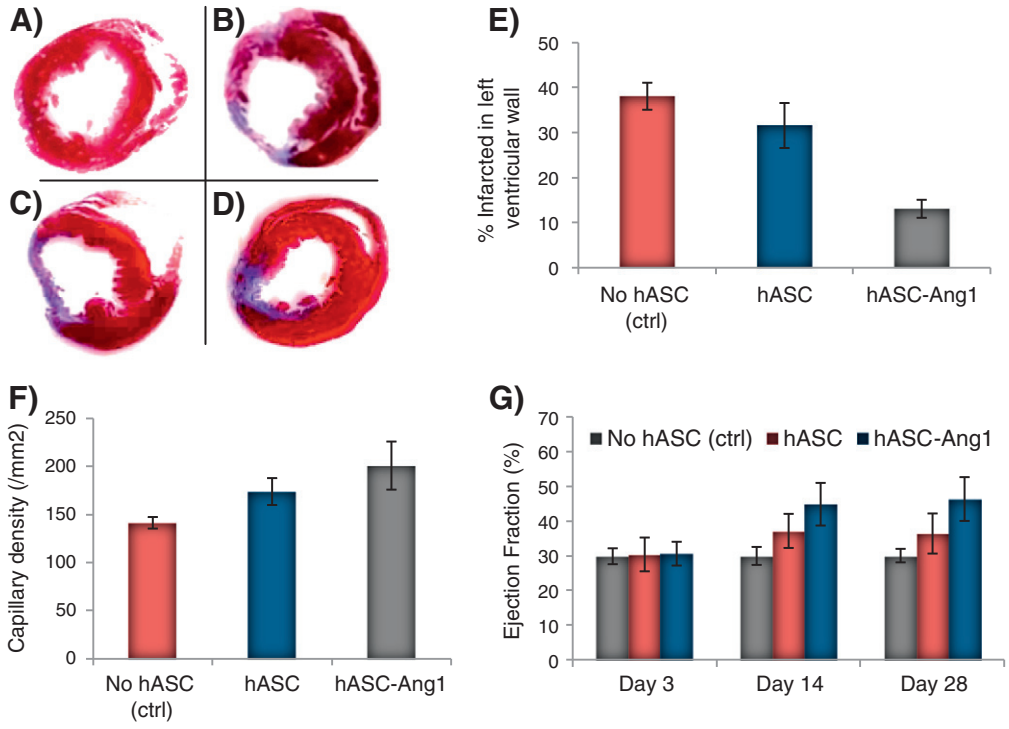

Fig. 4. Combined stem cell-gene based myocardial therapy. To do this, hASCs were genetically modified by Bac–NP_{Ang1} (hASC-Ang1) before transplantation to the heart. (A–D) Histomorphometric analysis of infarct ventricular scar area. Representative images of left ventricle myocardial sections stained with Masson's trichrome show the markedly decreased cardiac fibrosis after hASC and hASC-Ang1 transplantations 28 days post-infarction compared to the control. The blue area represents extracellular matrix deposition in the scar tissue and the red area represents the myocardium. (E) Semi-quantitative analysis of the images of the stained collagen fractions in the infarct region, using Image J software, show that hASC and hASC-Ang1 transplantations significantly decreased cardiac fibrosis compared to untreated group (n = 8) in terms of percent infarction size in left ventricular wall. (F) Angiogenesis in the peri-infarct area. Immunohistological staining of CD31 to detect neovascularization in no hASC, hASC and hASC-Ang1 groups. Quantification of capillary density. (G) Echocardiographic assessment of cardiac function. Effect of hASC-Ang1 transplantation on cardiac function was analyzed over 28 days post-infarction. Notes: Data expressed as mean ± standard deviation with statistical analysis using ANOVA. Statistically significant differences between groups compared to control no hASC are indicated as $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$. Significant difference between hASC and hASC-Ang1 is indicated by $\dagger P < 0.001$. Abbreviations: hASCs = human adipose tissue-derived cell; hASC-Ang1 = Angiopoietin-1-expressing human adipose tissue-derived cell; ctrl = control [128].

in vivo for cardiac therapy (Fig. 4) [128]. In a separate study, we used cationic amino-functional PAMAM dendrimers, commonly used in drug delivery [129], to surface functionalize negatively charged recombinant baculoviruses by highly reactive, polar amine surface groups of dendrimers [130]. This helped in strong binding of the cationic viral particles to the cell membrane, assisting in virus internalization, and thus improving the gene transfer and eventual transgene expression. The formulated PAMAM dendrimer–Bac nanocomplex demonstrated efficient transfer of angiogenic vascular endothelial growth factor (VEGF) gene to stem cells. But survival of transplanted cells after intramyocardial injection is crucial to the efficacy of therapeutic cell transplantation. Hence, genetically modified stem cells were microencapsulated in polymeric membranes to deliver cells in vivo [131,132]. Microencapsulation of stem cells within polymeric microcapsules significantly enhanced the retention of transplanted cells at the site and assists in recovery of cardiac dysfunction and fibrosis.

5.3. Sustained gene delivery features of CNT/baculovirus reinforced hydrogel

Carbon nanotubes (CNTs) have been widely used to tune the biomechanical properties of hydrogels for tissue engineering applications [133,134]. The stem cell containing CNT reinforced 3D hydrogel construct can be modulated for diverse biomedical applications without affecting the biocompatibility and porosity of the hydrogel due to the large aspect ratio of CNTs. Here we demonstrate, for the first time, the application of CNT hybridized baculoviruses for controlled delivery of baculoviruses to rat bone marrow stem cells from hydrogel scaffold. The hydrogel is comprised of denatured collagen matrices, carrying MGFP gene carrying baculoviruses hybridized by ionic interaction to polyacrylic acid (PAA) wrapped single-walled carbon nanotubes. Here, the hydrogel works as a reservoir to carry, protect, and simultaneously deliver the recombinant baculoviruses to the cells in a sustained manner. In vitro results (Fig. 5) demonstrate that the CNTs incorporated in the hydrogel play a major role in extending the gene expression time over a longer period and also helps in improving the mechanical properties of the hydrogel.

6. Application of microscale technologies in baculovirus based therapies

Microscale technologies particularly microfluidics have emerged as a revolutionary platform for a wide range of biomedical applications including biosensing, medical diagnostics, cell based assays, drug screening and disease screening [135–144]. The advantages of microfluidic platforms are: (i) they offer the potential to simulate real tissue microenvironments such as multiple cell types and ECM proteins into a 3D microenvironment, (ii) they use a very small number of cells and small quantities of reagents, typically in the nanoliter to microliter range, (iii) they allow precise control over cell density and cell shape as well as environmental cues such as attachment matrices containing self-assembling proteins, and gel based substances, (iv) they provide the ability of precisely controlling the mechanical properties (e.g. elasticity, rigidity and strain), chemical properties (e.g. ligand density and orientation), and topographic properties (e.g. patterning of surfaces with substances having different cell-substrate affinity), and (v) they allow high throughput analysis and complete automation of the processes. All of these advantages can be used to help in virus based gene therapy and gene delivery systems. Hence microfluidics has great potential to be used as a high throughput research tool for application of Baculoviruses in cancer therapy, Gene therapy, and other therapeutic applications.

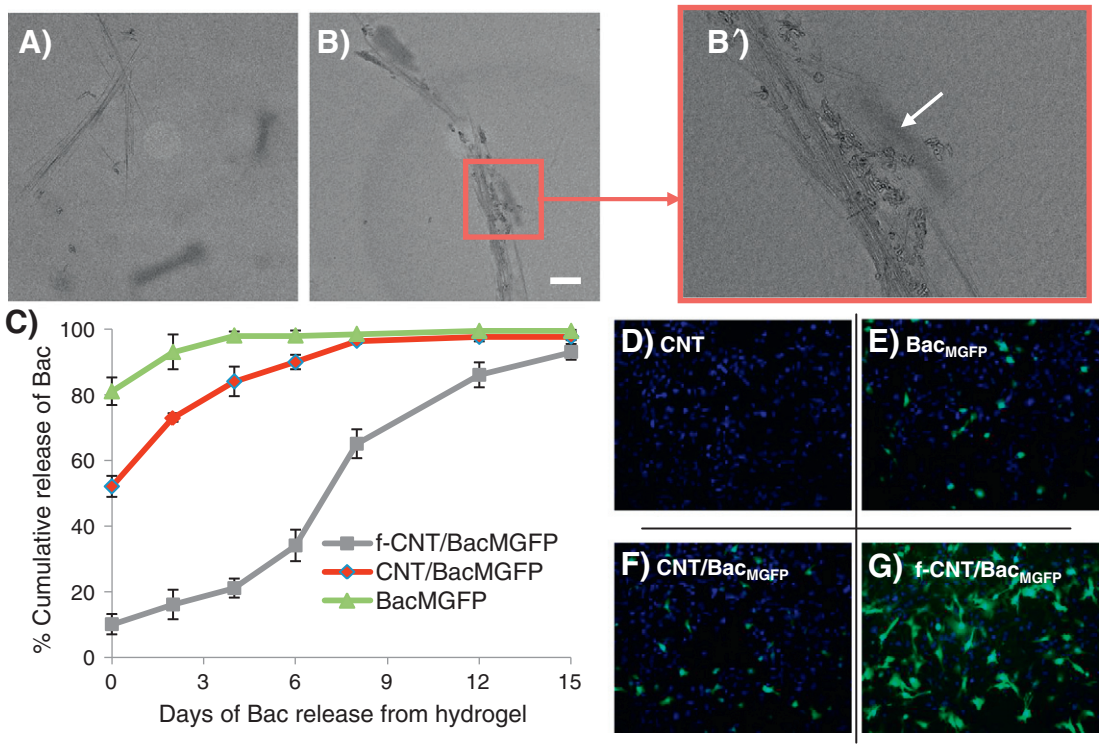

Fig. 5. Controlled release of baculovirus (Bac) using CNT reinforced hydrogel. TEM images of (A) non-functionalized CNT with Bac and (B) CNT functionalized Bac, with arrow showing the baculovirus bound to CNT surface in magnified image (B′). Scale bar indicates 100 nm length. Selfassembled nanocomplex of cationic PAA functionalized CNTs (f-CNT) were hybridized with anionic Bac. (C) CNT helps in sustained release of Bac incorporated in denatured collagen hydrogel (2.5 mg/ml) over time. Cumulative release kinetics of baculovirus from the hydrogel impregnated with Bac–CNT nanocomplex (D–G) rMSCs were overlaid on the collagen hydrogel formulated with CNT (25 μg/ml), Bac_{MGFP}, CNT/Bac_{MGFP} or functionalized CNT (f) f-CNT/Bac_{MGFP}. Fluorescent microscope data obtained after 14 days in CNT/Bac_{MGFP} group show higher number of MGFP expressing cells compared to other groups. This data reconfirms the extended release behavior of BV from f-CNT reinforced hydrogel which resulted in sustained transgene expression in transduced rMSCs. Abbreviations: TEM = transmission electron microscope; CNT = carbon nanotube; rMSCs = rat mesenchymal stem cells; PAA = poly (acrylic acid), MGFP = Monster Green Fluorescent Protein.

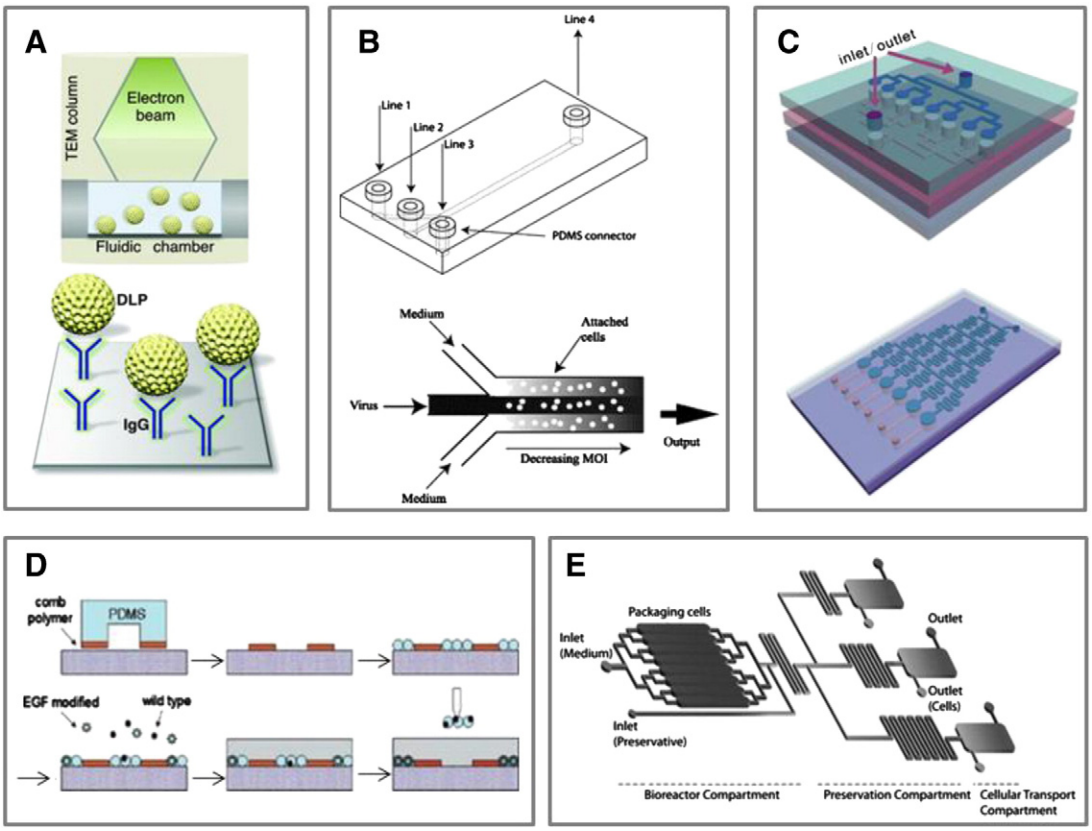

Fig. 6. Microfluidic devices for virus based biotherapeutic applications: (A) a microfluidic chamber for visualizing viral assemblies in a nanoscale biosphere [145]; (B) a microfluidic chip for cell infection using continuous virus gradient [146] where virus concentration ranges from the initial concentration to zero; (C) a three-layer microfluidic chip for virus infection [151]; the top figure shows that the chip comprised of a glass slide as the substrate, a poly dimethyl siloxane (PDMS) middle layer with cell culture chambers (diameter × height: 1 mm × 200 μm) and microfluidic channels (width × height: 200 μm × 40 μm), and a PDMS upper layer with microfluidic channels (width × height: 200 μm × 40 μm). The bottom figure of c shows a schematic diagram of a microfluidic chip for drug inhibition with a concentration gradient generator in the upper layer and six cell culture chambers in the middle layer; (D) fabrication steps of a micro-pattern based microfluidic device for formation and purification of virus plaques [152], (i) micro-patterning non-fouling comb polymer on the surface, (ii) incubating cells, (iii) infecting the cells with viruses, (iv) overlaying agarose on the cell micropatterns and incubating for 5 days, and (v) checking and picking plaques; (E) a microfluidic device for a viral gene carrier production showing different compartments and fluidic channels [153].

A number of researchers have used microfluidic devices and microscale technologies in research involving viruses in general, and the baculoviruses in particular, Fig. 6. One such study involved application of a microfluidic platform for visualizing viral assemblies at high resolution. Gilmore et al. [145] used an affinity capture technique in a microfluidic chamber, whereby the surface of the microfluidic chamber was functionalized with nickel-nitrilotriacetic acid (Ni-NTA), His-tagged protein A and VP 6- specific Guinea pig polycloned antisera for capturing virus particles. The functionalization of the surface enabled capturing and maintaining rotavirus double-layered particles (DLPs) in a liquid environment. The chamber was inserted into a transmission electron microscopy column, and the captured individual virus complexes were imaged, Fig. 6A. The post-processing of the images was performed for 3D reconstruction of the particle images. The technique allowed the authors to observe the structural heterogeneity of individual viral assemblies (double layered particles) in solution and to report that a greater degree of structural heterogeneity exists in viral assemblies in solution compared to those fixed in vitreous ice. This technique can potentially be expanded for imaging the cell-to-virus interaction in 3D environment particularly for future research on interaction of baculoviruses with different cell types.

In another study, the use of a microfluidic device in determining the optimum concentration of baculovirus for infecting *Spondoptera frugiperda* sf9 cells was demonstrated [146]. Determining the optimum concentration of virus is crucial for all virus-based therapeutic applications. In conventional studies separate batches of cells are infected in multi-well plates, and the optimum virus concentration is determined by quantifying proteins collected from each sample. The method is time consuming, requires a large number of cells and agents, which might be difficult to obtain in many cases, and is expensive particularly for expensive cell lines. Walker et al. [146] used a, laminar flow and diffusion mediated, gradient based microfluidic device which allowed them to infect the cells at many different concentrations of virus simultaneously within a single microfluidic channel, Fig. 7B. The microfluidic channel comprised three inlet channels 333 μm wide combining into a larger microchannel, 1 mm wide, the height of all channels being 150 μm, and the length of the main microchannel being 5 cm. The cells were attached to the bottom of the main microchannel. The flow of suspension containing virus particles was connected to the middle channel while cell culture medium was sent through the two side channels. The flow rates in the inlet channels were adjusted until the width of the middle stream was stabilized at 100 μm and location was centered in the main channel. The use of food coloring and fluorescent particles in the flow, and the modification of the Baculovirus to express the green fluorescent protein (GFP) allowed fluorescent imaging of the concentration gradient of the virus and the infection of the cells. The result showed a lower rate of GFP expression than expected, however, the creation of a continuous gradient of virus concentration was successfully achieved and hence infecting the cells at different concentrations of virus was demonstrated. The method, as indicated by the authors, needs further refinement, however, since the laminar flow and diffusion have been used for establishing gradient in many studies [147–150], the issue of reduced fluorescent intensity can be easily tackled.

Xu et al. [151] used a three-layer microfluidic device for in situ monitoring of the infection process of a recombinant pseudorabies virus (GFP-prV) in real time. They also performed drug screening assays on

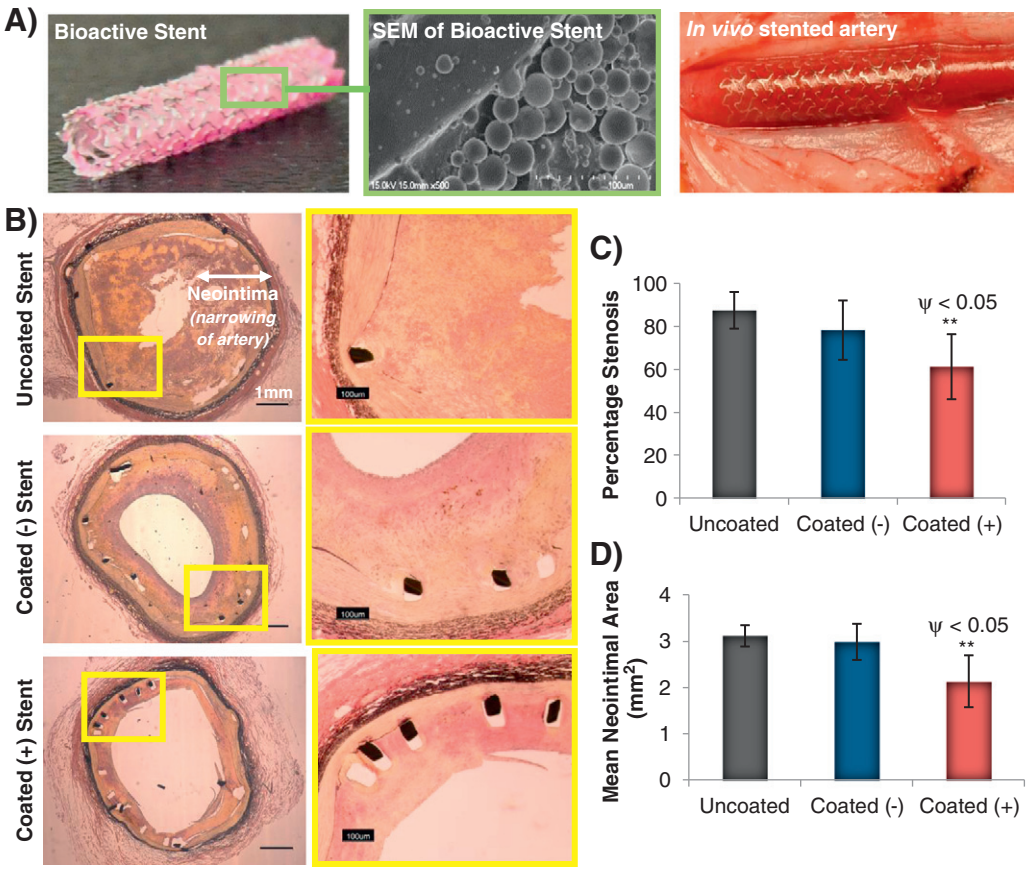

Fig. 7. Baculovirus based bioactive stent (Bac) for stent based in vivo local intra-arterial gene delivery. (A) Bioactive stent containing angiogenic Bac–PAMAM dendrimer nanocomplex entrapped in PLGA microspheres with SEM microphotograph showing the baculovirus encapsulated microspheres loaded on fibrin coated stent. Picture on the right shows the bioactive stent implanted in the balloon denuded dog femoral artery. (B) Assessment of in vivo biofunctionality of the developed bioactive stent. The effect of Bac_{Vegf}–PAMAM delivery on ISR assessed by histomorphometric analysis. Comparison of histomorphometric studies at week 16 after stent deployment in dog femoral arteries. Representative cross-sectional images of elastic Van Gieson stained femoral arteries (magnified images in right panel) with uncoated stents and stents carrying Bac_{Null}–PAMAM and Bac_{Vegf}–PAMAM at week 16 post-deployment. Percentage stenosis (C) and mean neointimal area (D) analysis at the stented regions demonstrated significantly reduced ISR in Coated (+) group compared to Uncoated and Coated (−) groups. The data represent the mean ± SD (n = 8). ANOVA ** = $P < 0.01$; P value on comparing Coated (+) and Coated (−) is denoted by ψ. Abbreviations: Coated (+) = Stent carrying Bac_{vegf}–PAMAM; Coated (−) = Stent carrying Bac_{Null}–PAMAM; Uncoated = bare metal stents; PAMAM = poly (amido amine); PLGA = poly (lactic-*co*-glycolic acid); SEM: scanning electron microscope; ISR = in-stent restenosis [160].

the microfluidic chip with a tree-like concentration gradient generator, Fig. 7C. The results revealed important insights into the infection pathway of GFP-prV in the host cells showing that at least one of the infection pathways of the virus was microtubule-dependent.

Na et al. [152] used soft lithography based technique to create cell adherent and repellant areas on a substrate, thereby depositing cells in desired micropatterns and forming plaques of viruses of controlled size, shape and cell number. They also used the technique for purification of viral plaques from a mixture of multiple types of viruses (wild type and GFP-modified). The described polymer-templated microarrays can be a useful tool in the study of viral genetics for high throughput formation of viral plaques of precise size and characteristics.

Microfluidic platforms have also been used as bioreactors for continuous production of viruses. Vu et al. [153] used a microfluidic device composed of a set of eight parallel channels, and by seeding the PT67-GFP packaging cells in the device, they demonstrated a continuous production of virus at a constant flow rate of 2 μL min^{-1}. The produced viruses were also successfully used in a second microfluidic device for transduction/infection of 3 T3 cells. The authors presented a novel design for an integrated microfluidic bioreactor containing separate compartments for production, preservation and transduction of viruses on a single microfluidic device, Fig. 6C.

Thus microfluidic systems and microscale technologies present novel platforms for production of controlled, automated and reproducible viral vectors as well as bioreactors for transduction/infection of cells, which can increase the efficiency and effectiveness of baculovirus based gene delivery and gene-therapy.

7. Future outlook for clinical translation of bioengineered baculovirus therapeutics

Bioengineered baculovirus has emerged as a promising gene delivery strategy for the repair and regeneration of damaged tissues. In particular, application of this paradigm to stem cell based cardiac therapy [128,154–156], vasculogenesis [122] and bone regeneration [157,158] has shown encouraging results in preclinical studies. Baculovirus has also shown to be effective in genetically modifying induced pluripotent stem cells (iPSCs) without affecting their proliferation and differentiation ability [159]. Very recently, we demonstrated the pre-clinical efficacy of microencapsulated baculovirus for stent based gene delivery in balloon denuded canine femoral artery model (Fig. 7) [160]. The results demonstrate significant attenuation of stenotic area formation due to efficient re-endothelialization of injured artery by angiogenic VEGF gene carrying baculoviruses. Similar therapeutic regimes that more closely mimic the complex spatial and temporal cascade of proteins involved in tissue and bone regeneration can be delivered using multiple gene carrying bioengineered baculoviruses. Application of such temporally regulated gene delivery system encoding a combination of

therapeutic proteins can enhance the clinical outcome in patients compared to single bioactive factor or cell-based approach alone. In addition, such a gene delivery strategy will be superior to direct protein or drug delivery methods, as the later often incurs high cost, frequent side effects, short drug half-life and suboptimal delivery efficiency to the target site. Despite excitement from a large number of animal model studies, including systemic delivery to nonhuman primates, there are a number of hurdles and concerns that must be overcome before baculovirus can be harnessed as a new therapeutic modality under clinical settings. It is unlikely that baculoviruses will be ubiquitously effective for all kinds of biomedical applications. The vectors have to be appropriately tailored and optimized for specific applications. Success of bioengineered baculovirus gene delivery system will depend on proper consideration of the following factors: (i) duration of protein expression required for efficacy (transient or permanent), (ii) target cells (ease of transduction, receptor expression, dividing or non-dividing cells), (iii) route of gene delivery (ex vivo or in vivo), and (iv) threshold value of baculovirus-induced immune response acceptable to the host. Importantly, optimizing route of administration is crucial for baculovirus based in vivo gene delivery as the later tend to get inactivate in presence of serum. The one-step intravenous delivery process is ideal for the doctors to achieve immediate treatment, but they are also associated with significant challenges such as low transduction efficiency, inflammatory responses and nonspecific gene delivery. Although ex vivo strategies are comparatively safer and ideal for efficient baculovirus transduction, clinical application of such methods remains limited by the logistic, economic, and timing issues when harvesting autologous cells from elderly sick patients.

Like all biological discoveries, comprehensive understanding of the mechanism of therapeutic action and underlying safety concerns of the gene delivery system is of paramount importance to successful application of baculoviruses in human diseases. As such, gaining insights into how cells respond to baculovirus transduction is crucial to the safe use of baculovirus transduced cells in vivo. Reports suggest that baculovirus transduction may trigger antiviral effects, activation of natural killer cells, antigen specific $CD4^+$ T cells, and innate immunity, as evidenced by the upregulation of certain cytokines such as interleukin 6 and interferons in immunocompetent animals [161,162]. Recent studies in this direction have confirmed the safety of using mesenchymal stem cell based gene therapy by hybrid baculovirus vectors for preclinical applications [163–166]. In order to establish the safety profile of the developed bioengineered baculovirus nanohybrids for gene delivery and combined cell-gene delivery, in vivo tracking can be useful to (i) understand the mechanism of therapy and (ii) establish the safety profile by assessing the risk associated with non-specific delivery of the injected viruses, (iii) investigate the possibility that viral transgene expression is inactivated in vivo, and (iv) quantify the relative amount of viral clearance due to unwanted cellular responses by host immune system.

Despite the promising in vivo results with pro-angiogenic and osteogenic growth factors carrying baculoviruses [67,122,130,158,162], the clinical feasibility of these growth factor-based gene therapy approaches is still unpredictable because of the complex release kinetics and uncontrolled ectopic bone and vessel formation caused by paracrine signaling to neighboring tissues. Although recent work has shown the feasibility of baculovirus as a gene therapy vector for in vivo cancer therapy by pro-drug approach, further studies on its ability to migrate and infect distal metastases are critical to achieve clinical success [167]. In addition, direct comparative studies are needed to understand in detail the beneficiary effects of baculovirus over other currently used non-viral delivery systems and polymeric nanoparticles for different medical treatments. However, bioengineered baculovirus, with the help of advanced micro- and nano-scale technologies, may revolutionize the treatment of human diseases in the same way that it has revolutionized industrial scale recombinant therapeutic protein production using insect cells.

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