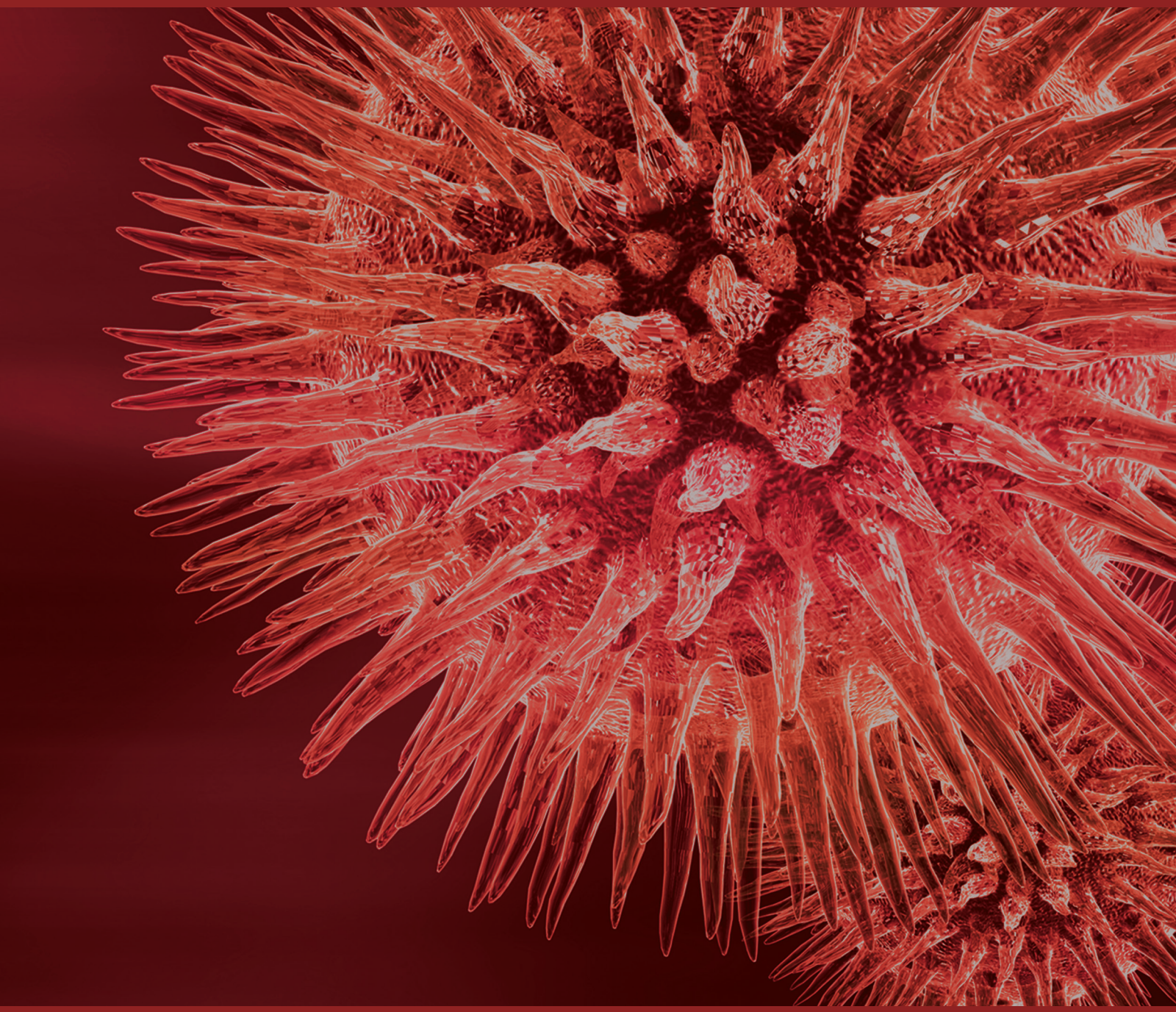
BioMed Research International

Advances in the Development of Biotherapeutics

Guest Editors: Pedro H. Oliveira, Juergen Mairhofer, Paula M. Alves, Alvaro R. Lara, and Cleo Kontoravdi



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Editorial

Advances in the Development of Biotherapeutics

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Biotherapeutics are currently the fastest growing group of pharmaceuticals, being a treatment option for a variety of chronic and sometimes life-threatening conditions. They represent a diverse group of biological products that broadly encompasses nucleic acids, proteins, whole cells, viral particles, and vaccines. In recent years, great strides have been made towards the development of safer and more effective biotherapeutics, as well as in the adoption of cost-effective, more efficient, and streamlined manufacturing processes. This trend is expected to accelerate in the forthcoming years, as emerging fields related to advanced therapies or personalized medicine mature. Despite these progresses, there are still knowledge gaps across all stages of development, as well as technical and regulatory hurdles that can thwart the approval process of these products.

In this special issue, we are pleased to present a collection of papers covering relevant aspects in the field of biotherapeutics.

The work by T. Lin et al. focuses on cell-penetrating peptides (CPPs). These generally correspond to short basic and amphipathic peptides, with a demonstrated capacity of crossing cell membranes, thereby facilitating the delivery of different types of cargoes such as nucleic acids, peptides, proteins, small molecules, and nanoparticles. The authors describe the use of the J-domain of the Heat Shock Protein 40 to enhance the transduction efficiency of arginine-rich CPPs and suggest the internalization of the latter to take place via macropinocytosis followed by endosomal escape.

Q. Chen and H. Lai review the recent progress in the methodology of agroinfiltration as a solution to overcome the challenge of transgene delivery into plant cells for large-scale manufacturing of recombinant proteins. The authors provide a general overview of gene delivery methodologies in plants, followed by a detailed description of agroinfiltration, its applications and scalability, typical vectors used, and how it can be used in different *Nicotiana* and non-*Nicotiana* hosts.

In the work by F. C. Mariz et al., the development of an intellectual property- (IP-) free platform for human papillomavirus (HPV) 16 L1 protein expression based on the constitutive expression of the PGK1 promoter of the methylotrophic yeast *Pichia pastoris* is reported. The authors also claim to have achieved the intracellular assembly of HPV VLPs in yeast cells for the first time.

S. Fujimaki et al. present us with a thorough review of diabetes mellitus-related changes occurring in the central nervous system and skeletal muscle, particularly in terms of dysfunctional neural stem cells and satellite cells. The authors discuss the beneficial effects of exercise in the prevention

and therapy of diabetes, as well as the use of stem cell-based approaches in the context of diabetes treatment.

Due to the complexity of many biologics, the assessment of "equivalence" for products obtained from different manufacturers currently presents a new set of challenges to regulatory agencies, since it is not perfectly clear how similar the biosimilar trastuzumab and its licensed equivalent need to be in terms of physicochemical properties for granting a marketing authorization. To illustrate the complexity of this process C. A. López-Morales et al. used a hierarchical strategy with an orthogonal approach to compare a biosimilar trastuzumab to its reference product in terms of physicochemical and biological attributes.

Finally, J. Zurdo et al. present us with an in-depth review of key aspects that need to be taken into consideration during the development of biotherapeutics, such as those related with the proper definition of a quality by design (QbD) workflow, developability methodologies and risk assessment, and product safety and validation. The authors then illustrate the application of developability tools and strategy described in practical case studies.

Collectively, these papers provide a comprehensive view on the current advances taking place in the broad field of biotherapeutics and on the exciting challenges that lie ahead.

Acknowledgments

We would like to express our appreciation to the authors for their submissions to this special issue and appreciate their efforts and time in improving their papers. We also thank all the anonymous reviewers for carefully checking the submitted papers.

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Research Article

Development of an IP-Free Biotechnology Platform for Constitutive Production of HPV16 L1 Capsid Protein Using the *Pichia pastoris PGK1* Promoter

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The human papillomavirus (HPV) L1 major capsid protein, which forms the basis of the currently available vaccines against cervical cancer, self-assembles into virus-like particles (VLPs) when expressed heterologously. We report the development of a biotechnology platform for HPV16 L1 protein expression based on the constitutive *PGK1* promoter (P_{PGK1}) from the methylotrophic yeast *Pichia pastoris*. The L1 gene was cloned under regulation of P_{PGK1} into pPGKΔ3 expression vector to achieve intracellular expression. In parallel, secretion of the L1 protein was obtained through the use of an alternative vector called pPGKΔ3α, in which a codon optimized α-factor signal sequence was inserted. We devised a work-flow based on the detection of the L1 protein by dot blot, colony blot, and western blot to classify the positive clones. Finally, intracellular HPV VLPs assembly was demonstrated for the first time in yeast cells. This study opens up perspectives for the establishment of an innovative platform for the production of HPV VLPs or other viral antigens for vaccination purposes, based on constitutive expression in *P. pastoris*.

1. Introduction

HPVs are a large family of dsDNA viruses that cause benign warts and malignant tumors. Persistent infection by HPV imposes a huge burden on health services worldwide owing to its links with cancer of the vagina, vulva, penis, anus, tongue, and, in particular, uterine cervix, which is the most serious outcome [1]. About 75% of sexually active people are exposed to HPV during their lifetime [2]. Some of the nearly 120 HPV genotypes reported [3] are encountered in virtually 100% of cervical tumors and can thus be classified as high-risk HPV (hr-HPV) types [4]. Nearly 1.4 million women are affected by cervical cancer all over the world while 520,000 new cases and 274,000 resulting deaths are reported annually, which leads to a mortality rate of 55% [5]. Although HPV16 and HPV18 are responsible for 70% of cervical cancer cases worldwide [5], there are other 12 hr-HPV types whose prevalence is subject to regional variations [6]. In Brazil, HPV16 is the most common genotype, but HPV31 and HPV33 are as prevalent as HPV18, at least in the Northeast and Midwest Regions [7], which illustrates the urgent need to set up vaccination programmes where HPV is prevalent. Apart from the cervical intraepithelial lesions of all grades and warts, it is estimated that 5% of all human cancers are associated with this viral infection [8].

Middle-income developing countries, where more than 80% of the related deaths are found, have failed to establish cervical screening programs in a satisfactory manner [5]. Since 2008, two protective vaccines have been licensed for prophylaxis against HPV infection. Gardasil (Merck) and

Cervarix (GlaxoSmithKline) contain HPV VLPs produced in *Saccharomyces cerevisiae* and baculovirus-infected cells, respectively. Although these vaccines are highly effective and safe [9, 10], their prohibitive costs prevent them from being widely available in developing countries [11]. However, the regional production of prophylactic HPV vaccine could overcome the problem of this price barrier by reducing the cost and also filling the current demand and supply gap [12].

Production of HPV VLPs can be achieved through the expression of recombinant major capsid protein L1 in heterologous systems [14, 15]. These particles preserve the conformational epitopes from native virions and are thus able to induce high titers of neutralizing antibodies [14]. Different expression platforms have been explored for producing HPV VLPs with varying degrees of success [14, 16–20]. Bacterial expression systems are limited to producing economically significant amounts of recombinant HPV VLPs [20, 21] and, among the eukaryotic systems, yeast cells have the greatest potential because of their high expression levels, combined with simple growth requirements and high growth rates.

The expression and characterization of HPV VLPs from the methylotrophic yeast *Pichia pastoris* has been described elsewhere [12, 22–24]. In these studies, the expression of HPV L1 genes was under the control of the promoter from the alcohol oxidase I gene (*AOX1*), which is tightly regulated at the transcription level. Recombinant protein expression under the control of P_{AOX1} relies on a preliminary production of yeast biomass through cultivation on glucose/glycerol, followed by induction of protein production in the presence of methanol as the sole carbon source [25]. Despite the success of the AOX1-based system, the use of methanol as an inducer has drawbacks such as its inflammability, toxicity, and biomass generation requirements prior to the induction phase. This means there is the need for longer time-based protocols, a rigid control of methanol levels during the induction phase, and the use of an inducer compound, which is particularly unsuitable when planning an industrial platform [25]. Furthermore, there is evidence that the culture conditions required for P_{AOX1} induction can compromise the expression levels of other VLPs and this can affect HPV L1 expression [22, 26, 27].

The isolation and molecular characterization of the 3-phosphoglyceratekinase gene (*PGK1*) from *P. pastoris* was reported by de Almeida et al. [28]. In yeast, *PGK1* encodes a glycolytic enzyme which also acts in the gluconeogenic pathway and may represent 5% of the total cellular protein [29]. Secretion of *Bacillus subtilis* α-amylase protein was carried out effectively under the control of the constitutive P_{PGK1} from *P. pastoris* cells grown in glucose, glycerol, or methanol, whereas cells grown in glucose displayed higher expression levels [28]. Unlike the P_{AOX1}-based system, biomass generation and protein production occur simultaneously in medium containing glucose or glycerol. Although a constitutive expression is not recommended when the protein of interest is toxic to the yeast cell [26, 30], this is not the case for HPV L1 protein since its expression has been efficiently achieved for approximately 144 hours [31]. These features make the P_{PGK1} an attractive system for heterologous expression in *P. pastoris*.

In this study, we explored the development of an innovative heterologous expression system for production of HPV16 L1 protein. For this purpose, the P_{PGK1}-driven constitutive expression of the L1 protein was investigated, both through intracellular and secretory pathways. In order to select well-expressing yeast clones, we combined the use of dot blot, colony blot, and western blot techniques in a workflow for the detection of the L1 protein. Additionally, evidence that HPV L1 protein self-assembles into VLPs was observed *in vivo* by transmission electron microscopy. To date, this is the first report of heterologous expression *in P. pastoris* which uses the P_{PGK1} for biopharmaceutical purposes.

2. Materials and Methods

2.1. Strains and Cell Culture Media. *Escherichia coli* DH5α strain [F Φ80*lac*ZΔM15 Δ(*lac*ZYA- *arg*F)U169 *rec*A1 *end*A1 *hsd*R17(rk–, mK+) *pho*A supE44 λ- *thi*-1 *gyr*A96*rel*A1] was routinely used as a host for cloning and plasmid manipulations. This strain was cultured at 37°C in LB medium (0.5% yeast extract, 1% NaCl, 1% tryptone) supplied with appropriate antibiotics.

The *P. pastoris* X-33 strain (wild-type) used in this study was purchased from Invitrogen. The yeast cells were grown at 30°C on YPD medium (1% yeast extract, 2% Bacto-peptone, 2% glucose) and YPDS (1% yeast extract, 2% peptone, 2% glucose, 1 M sorbitol) supplemented with 100 μg/mL zeocin (Invitrogen) when necessary.

All the molecular cloning techniques were carried out as previously described [32]. Restriction enzymes used for cloning were purchased from Promega and used in accordance with manufacturer's recommendations. DNA sequencing analysis was performed on a Genetic Analyser 3500 automatic sequencer (Life Technologies).

2.2. Cloning of the HPV16 L1 and Construction of Expression Vectors. A DNA sequence encoding the HPV16 L1 protein (Gen Bank access number GI: 27752860) was designed with codons optimized for expression in *P. pastoris*. Restriction sites for *Xho*I and *Not*I were added to the flanking regions of the L1 gene so that the cloning could be directed into the expression vector. The gene was synthesized by Epoch Biosciences (TX, USA), and cloned into pBSK plasmid. The resulting construct called pBSK/L1 was amplified in *E. coli* DH5α. L1 gene was released from pBSK after double-digestion with *Xho*I and *Not*I and employed for creating the expression vectors.

Two expression vectors were constructed with the P_{GK1} promoter from *P. pastoris* for constitutive expression of the L1 protein. The original 2 kb P_{PGK1} sequence described by de Almeida et al. [28] was reduced to a minimal ~400 bp sequence after deletion analysis with restriction enzymes [33]. The resulting P_{PGK1} sequence was used to generate the pPGKΔ3 expression vector for intracellular expression. Additionally, a pPGKΔ3α expression vector carries a codon-optimized *S. cerevisiae* α-factor signal sequence (α-MF) cloned downstream to P_{PGK1} to drive recombinant protein secretion. Both P_{PGK1}-based vectors contain the zeocin

resistance gene *Sh ble* for positive selection of *E. coli* and *P. pastoris* recombinants, as well as a C-terminal polyhistidine (6xHis) tag for detection of the fusion protein by immunoblot assays. The L1 gene previously digested with *Xho*I and *Not*I was cloned into pPGKΔ3α and pPGKΔ3 expression vectors digested with the same enzymes, and the resulting vectors were called pPGKΔ3α/L1 and pPGKΔ3/L1, respectively. The construction of the vectors was confirmed by restriction digestion, PCR and DNA sequencing with specific primers for both L1 (5′ TAGGATCCATGTCATTATGGCTTCCA 3′ and 5′ CTGGATCCTTAATGATGATGATGATGATGC-AA 3′ which flank the entire gene while 5′ GGTCAACCT-TTAGGAGTTGG 3′ and 5′ GACGAACATTTGTTCCCT-TCA 3′ amplify an internal L1 fragment of ~400 bp) and P_{PGK1} (5′ TCATAGTTCATCCCTCTCTCC 3′) sequences.

2.3. Electroporation of Yeasts and Selection of P. pastoris Recombinant Strains. Stable integration into the *PGK1* locus of *P. pastoris* was achieved after linearization of the 5 μg P_{PGK1}-based expression vectors with *Sac*I [33]. *P. pastoris* electrocompetent cells were prepared and transformed, as described elsewhere [13]. In this work, yeast clones were either referred as *P. pastoris*/pPGKΔ3α/L1 or *P. pastoris*/pPGKΔ3/L1, depending on the vector that was used.

After selection on agar plates containing YPD supplemented with 100 μg/mL zeocin, the transformants were subjected to a general procedure for the selection of clones containing multiple copies of the expression cassette, called Posttransformational Vector Amplification (PTVA) process [13]. For this purpose, yeast clones were later plated on higher concentrations of zeocin (100, 500, and 1000 μg/mL).

2.4. Screening of Well-Expressing Pichia Clones by Dot Blot and Colony Blot Assays. Multicopy clones resistant to 1000 μg/mL zeocin were subjected to a general screening for selection of recombinant strains expressing high levels of HPV L1 protein. The screening was carried out as follows: (i) *P. pastoris*/pPGKΔ3α/L1 clones were cultivated in agar plates and subsequently subjected to colony blot for detection of HPV L1 protein with the CamVir anti-HPV16 L1 monoclonal antibody (Chemicon, USA); (ii) In contrast, *P. pastoris*/pPGKΔ3/L1 clones were subsequently grown in deep-well plates to detect HPV L1 protein by dot blot with the anti-L1 monoclonal antibody.

In the case of colony blot, the protocol described by Goodnough et al. [34] was modified as follows. After cultivation of *P. pastoris*/pPGKΔ3α/L1 multicopy clones in YPD agar plates for 3 days at 30°C, the PVDF membranes were cut as discs and left standing with the surface colonies for 3 hours at 28°C. Colonies on the master plate could be replicated by placing the PVDF discs on a fresh agar plate when desired. The PVDF discs were washed with tris-buffered saline (TBS) containing 0.05% Tween 20 (TBST) to remove adhering cells. The membranes were blocked by incubation in TBST, supplemented with 5% nonfat milk for 1 hour at room temperature, and then incubated overnight at 4°C with anti-L1 antibody properly diluted (1 : 1000) in the blocking solution. PVDF discs were washed three times with TBST for 10 min and then incubated for 1 hour at room temperature with peroxidase-conjugated goat anti-mouse immunoglobulin (IgG, Sigma-Aldrich) diluted 1 : 3000 in the blocking solution. Chemiluminescence reaction was detected with an ECL kit (GE Healthcare).

For the dot blot assay, *P. pastoris*/pPGKΔ3/L1 multicopy clones were first grown in a deep-well plate containing YPD medium for 3 days at 30°C. The plate was shaken at 300 rpm and *P. pastoris* cells were harvested by centrifugation at 3000 rpm for recovery of the pellets. Preparation of yeast extracts was performed with an "alkaline lysis" procedure as previously described [35]. Briefly, the cell pellets were resuspended in lysis buffer (0.1 M NaOH, 0.05 M EDTA, 2% SDS, 2% β-mercaptoethanol) and heated to 90°C for 10 min and the lysate was brought to neutral pH. To improve solubilization, the lysate was heated again for 10 min and mixed with loading buffer (0.25 M Tris-HCl pH 6.8, 50% glycerol). The PVDF membranes were cut in a proper way and inserted into a dot blotter apparatus. Protein transfer to the membranes was carried out with 100 μL of the lysate for 1 hour and, thereafter, the immunoblot proceeded as already described for the colony blot. The HPV16 L1 protein that was episomally expressed in *P. pastoris* [22] was used as positive control for both dot blot and western blot assays.

2.5. Protein Expression in Shake-Flask Cultures. *P. pastoris* multicopy clones were selected for baffled-flask cultivation in accordance with the highest detected level for the HPV L1 protein. *P. pastoris*/pPGKΔ3α/L1 clones were first preinoculated in 5 mL of YPD to achieve the secretion of the L1 protein and grown at 28–30°C in a shaking incubator (250–300 rpm) until the cultures reached an OD_{600} of ~10 (24 hours). Following this, the cells were inoculated in 20 mL of YPD for 3 days and aliquots were taken for analysis at periodic intervals. These samples were centrifuged at 12000 g for 2 min and the supernatants were stored for further analysis. For the intracellular expression of the L1 protein, *P. pastoris*/pPGKΔ3/L1 clones were subjected to the same protocol but the cell pellets were stored instead of the supernatants.

2.6. Preparation of Intra- and Extracellular Protein Extracts. Aliquots of supernatants were submitted to precipitation with trichloroacetic acid (TCA) (Sigma-Aldrich) to a final concentration of 10% to allow the secretory production of the HPV L1 protein to be analyzed. After concentration, the supernatant was discarded, and the protein pellet was resuspended again in 100% acetone to remove residual TCA. A final volume of SDS-loading buffer (1 M Tris-HCl, pH 6.8, 10% SDS, 0.5% β-mercaptoethanol, 0.1% bromophenol blue) was then added to the washed pellet (corresponding to 100x concentration) and this mix was resolved on 15% SDS PAGE after the samples had been heated for 10 min at 75°C. Polyacrylamide gel was either stained with Coomassie brilliant blue (Pierce, IL, USA) or transferred to PVDF membranes using a V20-SDB semidry protein transfer apparatus (Scie-plas, Cambridge, UK). Immunoblot was performed as described in Section 2.4.

Preparation of cell extracts for intracellular analysis of the L1 expression was carried out with breaking buffer and acid-washed sterile glass beads (0.45 mm in diameter),

according to Cregg et al. [36]. This crude protein sample was mixed in gel loading buffer and prepared for SDS PAGE and western blot (as described above).

2.7. Electron Microscopy. At the end of the induction course, both the cells and culture supernatants were subjected to absorption into carbon-coated grids, as recommended by Falcón et al. [37]. The grids were subjected to examination with a FEI Morgani 268D transmission electron microscope, operated at 100 kV, in order to analyze the VLP formation in cytoplasm and culture medium of recombinant yeast-expressing HPV L1 protein.

3. Results and Discussion

3.1. Construction of Expression Vectors and Generation of Pichia Recombinant Strains. For the production of HPV16 L1 protein, we used a heterologous expression system based on the constitutive *P. pastoris PGK1* promoter which was originally described and employed for secretion of α-amylase from *Bacillus subtilis* [28]. In this work, a variant of the *PGK1* promoter sequence containing ~400 bp was used to allow integration of the expression vector via homologous recombination, as well as a codon-optimized α-factor from *S. cerevisiae* to drive the secretion of recombinant proteins (Figures 1(a) and 1(c)). Since previous studies have analyzed different protocols to optimize VLP production and purification steps in yeast [38–41], we believe that secretion of HPV L1 in the culture media could assist downstream processing. The analysis by PCR, DNA sequencing (data not shown), and restriction digestion showed the successful cloning of a codon-optimized L1 gene into P_{PGK1}-based vectors (Figure 1(b), left panel). *P. pastoris*/pPGKΔ3α-L1H16 and *P. pastoris*/pPGKΔ3-L1H16 recombinant strains were obtained after electroporation of yeast cells with the linearized expression cassettes (Figure 1(b), right panel) and further selection on agar plates containing zeocin.

Although recombination with the expression cassette confers zeocin-resistance to *P. pastoris* cells, there is no way to ensure that a heterologous gene will be expressed at high levels. The selection of clones containing multiple copies of an expression cassette represents an attractive strategy for increasing expression levels [13]. Multicopy clones can be screened by transformants that are resistant to high levels of a selectable marker compound. However, this effective method is still laborious and inefficient, since 50 to 100 transformants usually need to be screened to have a reasonable chance of finding the 1-2% multicopy (>10 copies) clones [42]. Recently, an iterative process termed Posttransformational Vector Amplification (PTVA) has been investigated to generate *P. pastoris* clones containing multiple copies of the entire vector in the genome through the submission of transformants, which were initially selected on a low level of drug and only contained one or a few copies of the vector, to higher levels of zeocin [13]. Molecular details of this process are still unknown, but an analysis of PTVA-selected clones showed a three-to-five-fold increase in the vector copy number, as well as the integration of all the copies into the *P. pastoris* genome in the same *locus* as the original copy. In this work, we tested the generation of multicopy clones by the PTVA process starting with 55 transformants from each cassette. After growth on agar plates with increasing zeocin levels, 50 *P. pastoris*/pPGKΔ3-L1H16 clones and 52 *P. pastoris*/pPGKΔ3α-L1H16 clones showed resistance to 1000 μg/mL zeocin. Zeocin resistance levels can be directly correlated with the copy numbers of the expression cassette integrated in the genome. The clones that only harbor one copy of the integrated expression cassette are resistant to 100 μg/mL zeocin, while integration of 2, 3, and 4 cassette copies causes resistance of up to 500, 1000, and 2000 μg/mL zeocin [26].

3.2. Well-Expressing Pichia Clones Are Rapidly Screened by Dot and Colony Blot. Compared with *E. coli*-based expression systems, the main disadvantage of *P. pastoris* is that it relies on the heterogeneous expression levels of the exogenous gene when the primary transformants are being analyzed [43]. Multicopy screening has the potential to enhance strains expressing increased levels of the heterologous gene, but only a small portion (5%) of highly drug-resistant colonies are generated as a result of an increased gene dosage. Most transformants are resistant to drugs for other (unknown) reasons [13]. Hence, we established a workflow based on the cultivation of recombinant yeasts in deep-well and agar plates and carried out a further analysis by dot and colony blot to identify well-expressing clones. Through this general approach, we were able to access the expression levels of 52 pPGKΔ3α/L1H16 colonies and 25 pPGKΔ3/L1H16 colonies. The multicopy clones generated by the PTVA process displayed a uniform expression signal (Figures 2(a) and 2(b)). The lack of detection by the *P. pastoris* cells that had been transformed by the parental expression vectors (empty PGK-based vectors) together with the detection of the L1 protein expressed episomally in *P. pastoris* cytosol ensured the specificity of the reaction.

It is worth noting that this practical and rapid workflow provides a strategy to screen well-expressing clones through cultivation under nonoptimal conditions—with regard to culture volume, shaking, and aeration, as is the case for deep-well and agar plate cultures—among a considerable high number of colonies. The colony blot assay has an important feature, particularly in the case of PGK-based clones that secrete L1 protein, which is the dispensable use of liquid media. Since *P. pastoris* secretes few proteins into the medium [25, 30, 43], screening strategies based on cultivation in liquid media may be unsuitable and require protein precipitation, for example, which is not the case here.

3.3. HPV16 L1 Expression in Shake Flasks under PGK1 Regulation. Expression of the major capsid protein of HPV16 in *P. pastoris* was first reported by Bazan et al. [22]. According to these authors, the expression level achieved was higher than that obtained in *S. cerevisiae*. Despite being innovative and important means of demonstrating the feasibility of *Pichia* in producing HPV VLPs, the work carried out by Bazan and colleagues, employed a nonintegrative system. As the authors (and other studies) point out, the use of episomal plasmids is not recommended for industrial purposes, since they have

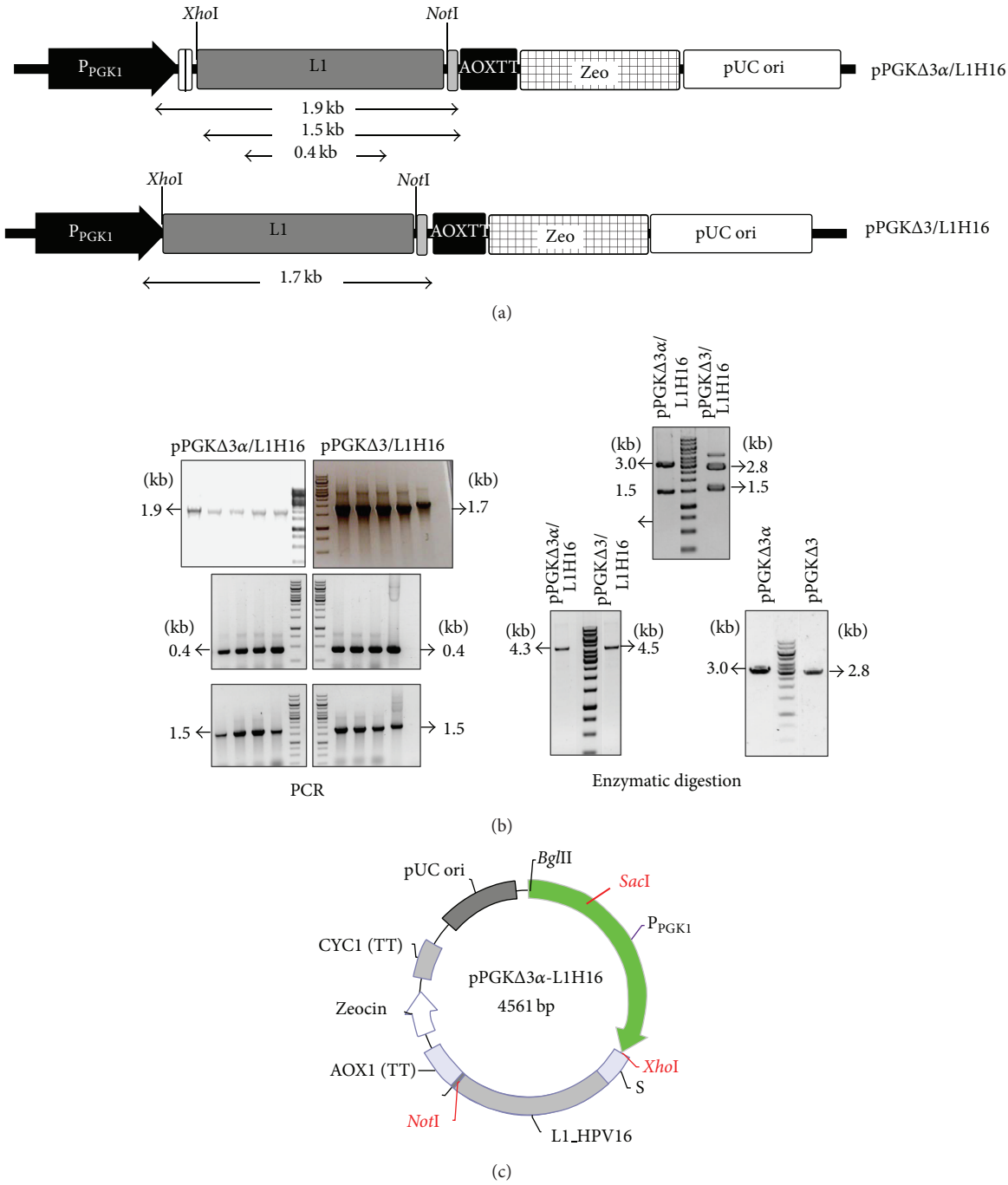

Figure 1: Construction of cassettes for HPV16 L1 gene expression under the control of P_{PGK1}. (a) Schematic illustration of the two expression cassettes in which fragment lengths are highlighted for the confirmation analysis: primers flanking part of the P_{PGK1} and the L1 gene render a 1.9 kb fragment from the pPGKΔ3α/L1H16 vector and a 1.7 kb fragment from the pPGKΔ3/L1H16 vector; primers flanking the L1 gene renders a 1.5 kb fragment from both vectors; internal L1 primers render a 0.4 kb fragment from both vectors. (b, left panel) Expression vectors were extracted from recombinant bacterial strains and subjected to PCR analysis: the three DNA fragments predicted in (a) were amplified from the extracted DNA plasmids. (b, right panel) Extracted DNA plasmids were further confirmed by restriction digestion with *Xho*I and *Not*I enzymes (upper line), through which the release of the L1 gene (1.5 kb) could be observed, along with the presence of pPGKΔ3α (3 kb) and pPGKΔ3 (2.8 kb) vectors. After confirmation by PCR and restriction digestion, the P_{PGK1}-based cassettes were linearized with *Sac*I (lower line) prior to transformation of *P. pastoris*. (c) Map of pPGKΔ3α-L1H16. *PGK1* promoter and *AOX1* transcription terminator regions are flanking the L1 gene at its 5′ and 3′ ends, respectively. Besides the *E. coli* pUC origin and zeocin selection maker, this construct carries a codon-optimized *S. cerevisiae* α-MF (S) downstream from the *PGK1* promoter, which is absent in the pPGKΔ3/L1H16 vector. The positions of *Xho*I, *Not*I, and *Sac*I restriction sites are also highlighted on the map. The map is merely illustrative and there is no correlation between the sizes of the highlighted regions.

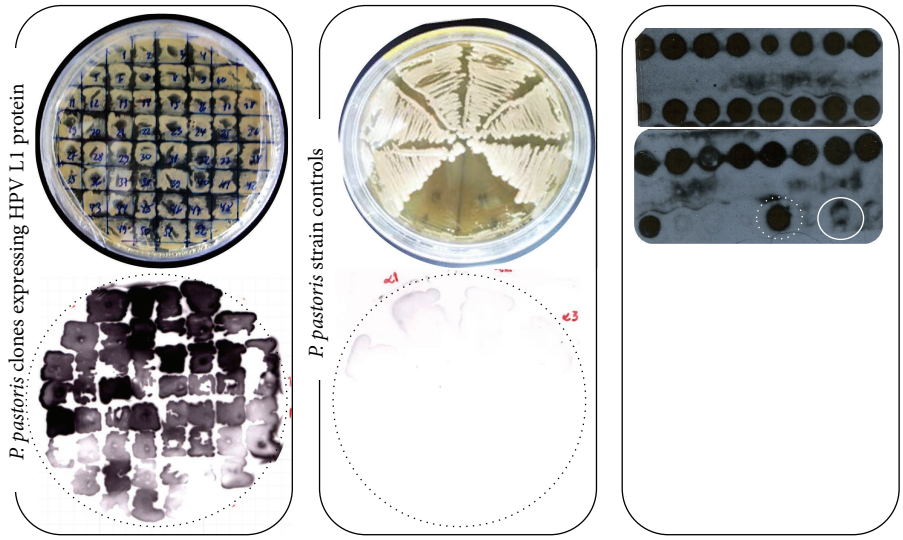

Figure 2: Selection of well-expressing clones. *P. pastoris* transformants were subsequently subjected to higher drug levels (100, 500, and 1000 μg/mL zeocin) for screening of multicopy strains. (a) Secretion of HPV L1 protein from the *P. pastoris*/pPGKΔ3α/L1 clones resistant to 1000 μg/mL zeocin was detected by colony blot. Absence of detection in the *P. pastoris* strains controls (yeasts transformed with the parental vectors) ensured the reliability of the reaction. (b) Intracellular expression of HPV L1 protein was confirmed in *P. pastoris*/pPGKΔ3/L1 clones resistant to 1000 μg/mL zeocin by dot blot. HPV16 L1 protein episomally expressed in *P. pastoris* was used as positive control (dotted circle in white), while *P. pastoris* strain transformed with the empty vector was used as negative control (circle in white).

been associated with the generation of recombinant strains that are genetically unstable and have two undesirable outcomes: (i) instability in the heterologous expression levels; (ii) continuous antibiotic selection which is needed to maintain the expression plasmids [25, 26, 44]. Heterologous L1 protein from other HPV genotypes was also reported in *P. pastoris* by employing AOX1-based integrative vectors [12, 23, 24].

With regard to the metabolization of methanol, *P. pastoris* strains present three distinct phenotypes: (i) the wild-type phenotype designated as methanol utilization plus (Mut^+) and characterized by the presence of a functional copy of the alcohol oxidase 1 gene (AOX1) which is responsible for 85% of the utilization of methanol by the alcohol oxidase enzyme; (ii) methanol utilization slow (Mut^s) phenotype, characterized by the absence of the AOX1 gene and presence of the AOX2 gene, which is about 97% homologous to AOX1 but much less expressed; (iii) methanol utilization minus (Mut^-) phenotype, where both AOX genes are absent. Hence, while Mut^s strains show a poor growth on methanol medium, the Mut^+ strains have a greater growth rate and Mut^- strains, in contrast, are unable to grow on methanol as the sole carbon source. Interestingly, Cregg et al. [27] showed that *P. pastoris* Mut^+strains expressed 10-fold less HBsAg than Mut^s strains, which suggests that consumption of methanol at high levels, coupled with a rapid growth rate, may not lead to efficient HBsAg assembly [26]. In addition, HPV VLPs expressed under AOX1 regulation were described as unstable and inadequately assembled [22], although these features were reversed after the incubation of the VLPs under refolding conditions. In light of this, these findings suggest that cell requirements during P_{AOX1} induction are nonoptimal for the production of HBV and HPV VLPs. Finally, even though recombinant protein expression in *Pichia* Mut^s strain could circumvent the deficiencies observed for the generation of VLPs in Mut^+ strains, the slower methanol utilization phenotype requires long fermentation times to reach peak product concentrations. Once induced, the entire culture cannot be used to start a new culture [26].

In contrast with the reports employing P_{AOX1}-based system, we constitutively expressed the L1 protein through P_{PGK1} in the presence of glucose and by employing an easier cultivation/expression schedule in shake flasks, since L1 expression occurred together with the cell growth and the laborious control of the methanol/inducer levels was dispensable (Figures 3(a) and 3(b)). By using the anti-HPV16 L1 monoclonal antibody, it was demonstrated that a 56 kDa protein was present in the cellular lysate during the cultivation course (Figure 3(b), L1 secretion will be further discussed), while no detection was observed in the control extracts. A continuous culture strategy, which is a cost-effective method for large-scale production, could be achieved through the use of P_{PGK1}-driven expression and would be attractive as it allows an indefinite theoretical production of the heterologous protein [26]. Although there have been a number of different reports showing genetic instability in *P. pastoris* multicopy strains induced with methanol [45–47], we have not observed the same results with the PGK-based clones explored in our work. HPV L1 has been successively detected by western blot after approximately 90 generations, even in the absence of zeocin (Figure 3(b), lower panel). Although more investigations need to be carried out with regard to the structural stability of these clones, these preliminary data suggest there

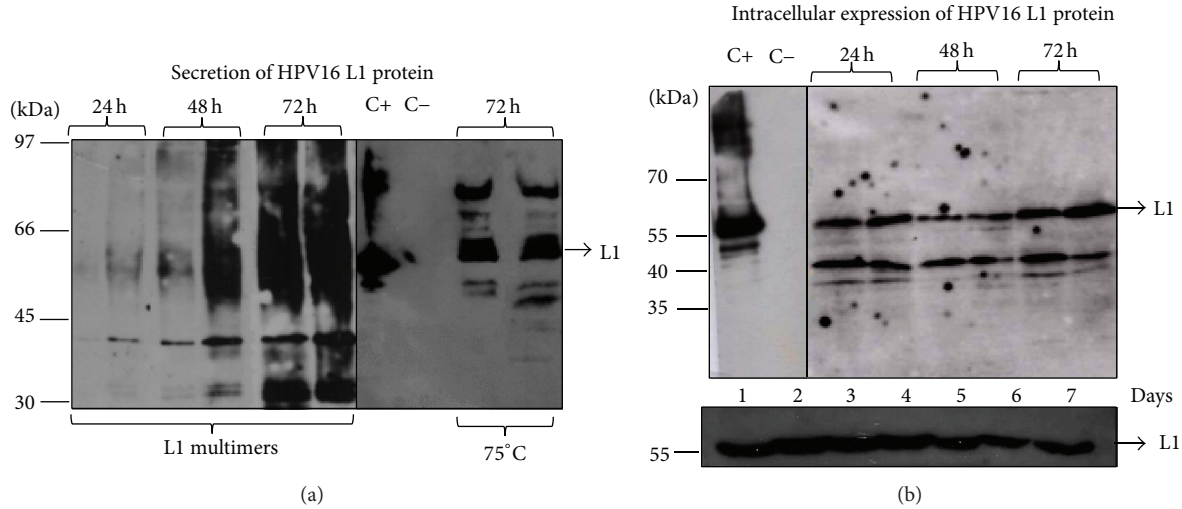

Figure 3: Expression of L1 protein in shake flasks. (a and b) HPV L1 protein was constitutively secreted to the culture media or intracellularly produced after cultivation of four *P. pastoris* clones (two clones for each P_{PGK1}-based vector) with glucose for 72 hours. Upon boiling, multimerization of L1 protein in the protein extract was observed as a protein smear, which was overcome after denaturation at 75°C. Aliquots of HPV16 L1 protein episomally expressed in *P. pastoris* [13] were used as a positive control (C+), while the protein extracts from *P. pastoris* strains transformed with the parental vectors were used as negative control (C−).

is another advantage related to the use of the *P. pastoris PGK1* promoter.

It has been argued that creating optimum conditions for the production and purification of HPV VLPs is a strategy that can reduce the production costs of vaccines [31, 40], since it could require less time and labor in industrial production. In this regard, different procedures have been explored related to VLP purification steps, such as ultracentrifugation, size-exclusion chromatography, and cation-exchange chromatography or even their combination [39–41], as well as the findings about how cell culture conditions can be optimized [31, 38]. We believe that secretion of HPV L1 in the culture media could improve the downstream process. Yeasts such as *S. cerevisiae* and *P. pastoris* have low specificity requirements for signal sequence recognition [25]. The well characterized *S. cerevisiae* α-MF is the most used secretion signal for *P. pastoris* and achieves similar and even higher expression levels than the *Pichia* native signal sequence [30, 48]. In attempting to obtain secretion of L1 protein at high levels in the culture medium, we employed a codon-optimized α-MF along with the PGK1-based vectors. Our initial attempts to detect HPV L1 protein in supernatant of *P. pastoris*/pPGKΔ3α/L1H16 clones showed an unexpected protein smear when the samples were boiled at 95°C, which was not visualized in the extract of negative controls (Figure 3(a)). A similar observation was reported earlier [12], probably due to multimerization of L1 protein in higher structures upon boiling. Although this feature was not pronounced when the cell lysate was analyzed, we proceeded with the protein denaturation at 75°C for all the samples before fractioning in electrophoresis, and this allowed the detection of the L1 protein in its expected molecular weight (56 kDa, Figures 3(a) and 3(b)). Lower bands observed in both media and cell lysate-derived samples are possibly degradation products of the L1 protein, since these species were not seen in the negative controls. Similar degradation patterns were also reported previously [12, 24]. When the identical electrophoretic pattern displayed by the secreted L1 protein was compared with both its nonsecreted L1 version and the L1 episomally expressed version, it was suggested the α-MF was being processed correctly. In addition, this indicates the lack of posttranslational modification in the secreted L1 protein, although previous reports had characterized glycosylation sites in the HPV1n6 L1 protein [49, 50]. More detailed investigations are needed in this area.

3.4. Electron Microscopy Evidence of HPV VLP Assembling within the P. pastoris Cells but Not in the Culture Media. The assembly of HBV and HCV core proteins into VLPs has been previously reported in yeast [37, 51]. However, no clear evidence has so far been provided to demonstrate that the HPV VLP self-assembled inside the yeast cell. Electron microscopic characterizations of VLP formation were achieved after downstream processing and purification from the yeast cell lysate, which is also true for the VLPs employed in the current HPV vaccines. The demonstration of intracellular VLP formation in *P. pastoris* opens up perspectives for the development of a live attenuated vaccine, since this yeast has been granted the GRAS (Generally Recognized as Safe) status by the FDA (Food and Drug Administration) and has been recognized as an efficient vehicle for the delivery of viral antigens when administered by an oral and intramuscular route [52]. Furthermore, immunization of animals with live bacteria strains expressing HPV VLP has proved to be effective in the anti-L1 IgG production [20].

Electron microscopy analysis of *P. pastoris* recombinant strains described here provides evidence of electrodense structures with estimated diameters of 55 nm, as expected

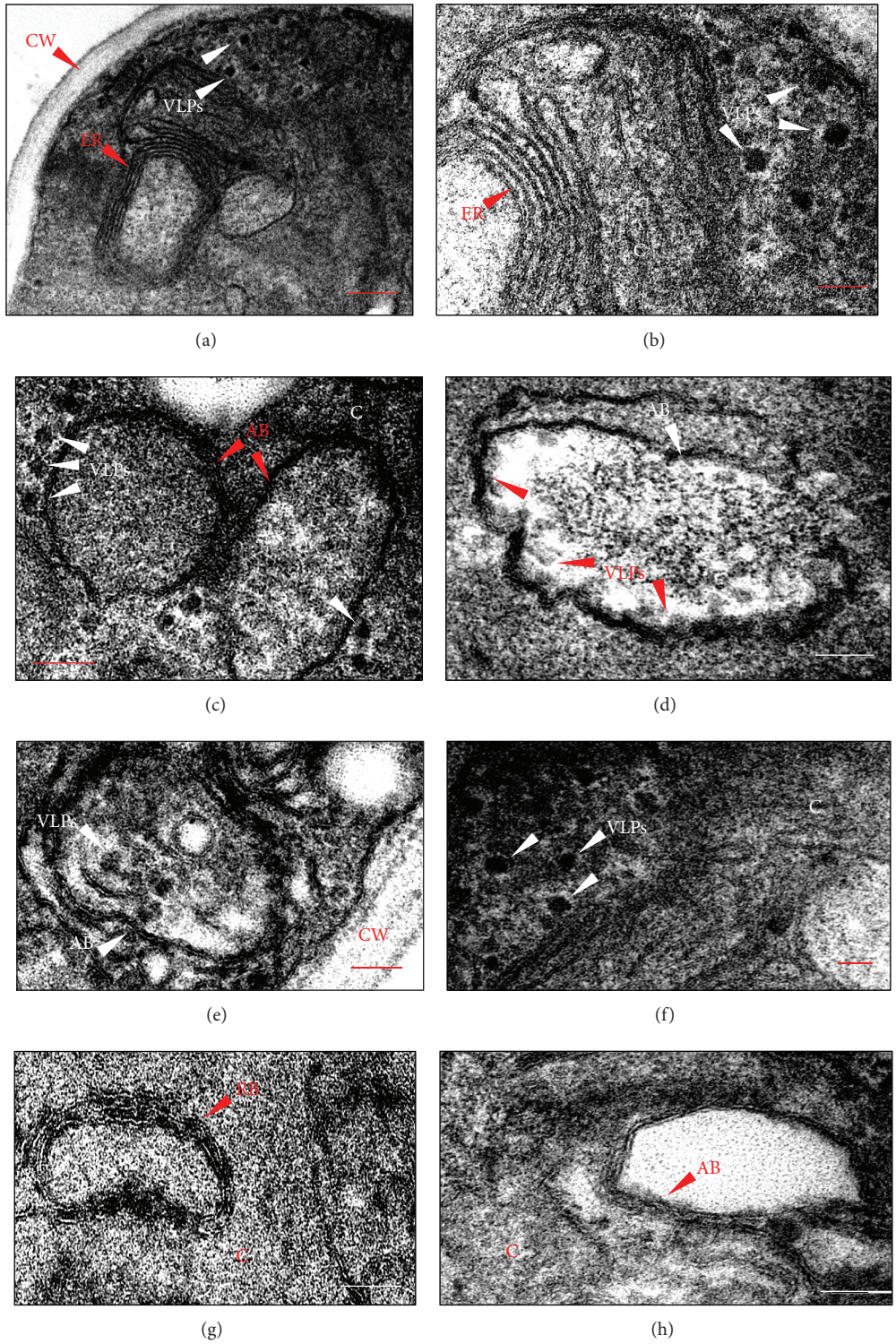

Figure 4: HPV VLPs self-assembly within the *P. pastoris* cells. (a and b) Electrodense structures (VLPs) with estimated diameters of 55 nm were observed near the endoplasmic reticulum (ER) membranes of *P. pastoris* clones expressing L1 protein under regulation of *PGK1* promoter. (c–e) VLPs were visualized both near and inside the autophagic bodies (AB) and were either free from or in contact with the AB membranes. (f) Circumstantially, the VLPs were seen to be transiting in cytoplasm. (g) Close-up of electrodense structures with an estimated size of 20 nm and cluster on ER membranes, which were identified as ribosomes. (h) Similar particles with 55 nm were not observed in the *P. pastoris* cells that were transformed with parental vectors, either in the cytosol or inside AB. (Bar = 200 nm in (a); 80 nm in (b)–(d), (f), and (h); 100 nm in (e); 40 nm in (g)).

for the HPV VLPs (Figures 4(a)–4(f)) and in accordance with the western blot analysis that shows the presence of the L1 protein. These particles were located near the endoplasmic reticulum membranes (Figures 4(a) and 4(b)) and inside the autophagic bodies (Figures 4(d) and 4(e)) (either free or apparently interacting with cellular membranes); however, they were absent from the cytoplasm of *P. pastoris* strains that had been transformed by the parental vectors (Figure 4(h)). We also searched for circular, electron dense cellular structures in the cytosol and likewise clustered on membranes which could be mistaken for VLPs. The structures highlighted in Figure 4(g) were identified as ribosomes mainly owing to their small diameter (~20 nm) and differential disposition when attached to membranes and concomitant presence in cells transformed with empty vectors. An immunocytochemical analysis was conducted with a view to providing a further characterization of VLPs within the yeast cells. However, the VLP detection was not possible by means of the CamVir antibody, which is often used to detect HPV16 capsomeres and VLPs [41, 53]. Previous attempts to detect intracellular HCV VLPs in *P. pastoris* through immunoelectron microscopy were hindered by the retention of cell membrane components in the architecture of the HCV core particles which has an envelope-like structure [37]. According to the authors, neither the anti-HCV core monoclonal antibody nor the core-reacting human sera were able to stain the particles, in spite of their visualization by conventional electron microscopy. This data could explain why we could not detect the HPV VLPs.

We attempted to characterize assembled HPV VLPs in the culture media from *P. pastoris* recombinant strains. It has been shown that assembly of HPV VLPs can be achieved with neutral pH, high ionic strength, and relatively low concentrations of reducing agents [54]. Conversely, high pH, low salt concentration, and the presence of reducing agents disassemble the VLP into capsomeres. As *P. pastoris* growth progresses in unbuffered medium, the pH drops to 3 or below [55]. It can be speculated that under this condition, culture media offers nonoptimal conditions for HPV VLPs assembly, since HPV L1 protein was detected by western blot after concentration. Nevertheless, the absence of VLP assembly under our experimental conditions does not compromise the use of L1 secreted protein as immunogens. Particle assembly protocols have been used for HPV VLP formation even when the L1 protein is expressed intracellularly, either to increase production yields or improve particle immunogenicity [22, 48–51]. Since our main objective was to evaluate the feasibility of the system, we believe that dot blot, colony blot, and western blot data ensure that our goal can be achieved.

4. Conclusion

The data presented in this work demonstrate the functionality of a biotechnology platform based on *P. pastoris PGK1* promoter for the production of HPV16 VLPs. Constitutive expression of the HPV16 L1 capsid protein was efficiently achieved for the first time through an easier production schedule than is the case when an *AOX1* inducible promoter is employed. In addition, an optimized α-MF secretion signal downstream to P_{PGK1} provided a prompt secretion of L1 protein in the culture media. Although further experiments are needed to determine which production strategy is more effective for HPV L1 protein production, the data outlined here underline the efficiency of a PGK1-based platform as a heterologous expression system and support its employment for the production of HPV VLPs for vaccination purposes. Moreover, our preliminary data showing intracellular VLP assembly open up perspectives for the employment of *P. pastoris* cells that can express HPV L1 protein as a live attenuated vaccine. To the best of our knowledge, this is the first time that the expression of HPV L1 protein was achieved in *P. pastoris* through an IP-free biotechnology platform. The discovery of alternatives to biotechnology platforms for producing an efficient and cost-effective vaccine has the potential both to offer greater protection—since not all the HPV genotypes are covered by the current vaccines—and to allow the vaccine to be widely disseminated.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

The J-Domain of Heat Shock Protein 40 Can Enhance the Transduction Efficiency of Arginine-Rich Cell-Penetrating Peptides

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Sense and antisense oligonucleotide pairs encoding cell-penetrating peptides PTD (Tat_{47-57}), DPV3A, E162, pVEC, R11, and TP13 were used to construct two sets of pET22b-CPP-DsRed and pET22b-CPP-J-DsRed vectors for CPP-DsRed and CPP-J-DsRed recombinant proteins expression. PTD-DsRed, DPV3A-DsRed, PTD-J-DsRed, and DPV3A-J-DsRed recombinant proteins were expressed in a soluble form. PTD-J-DsRed and DPV3A-J-DsRed recombinant proteins were able to escape from *E. coli* host cells into the culture medium. The membrane-penetrating activity of PTD-J-DsRed and DPV3A-J-DsRed recombinant proteins to mammalian cells was more effective than that of PTD-DsRed and DPV3A-DsRed. The route of the cellular membrane translocation of these recombinant proteins is suggested via macropinocytosis followed by an endosomal escape pathway.

1. Introduction

1.1. Cell-Penetrating Peptides (CPPs). Since the observation that HIV-1 Tat protein could shuttle between cells and the discovery that purified Tat protein could enter cells and translocate into nuclei [1], the cell-penetrating activity of Tat has been narrowed down gradually from amino acids 36–72 [2], to either amino acids 48–60 [3] or amino acids 47–57 [4]. The protein transduction domain (PTD, specifically indicating the peptide: Tat amino acids 47–57, hereafter) of Tat protein was able to deliver macromolecule, such as 120 kDa β-galactosidase, fused to it *in vivo* [5, 6]. Meanwhile, a 16-amino acid peptide derived from the third helix of the homeodomain of Antennapedia, termed as penetratin, was found to translocate through cell membrane as well [7]. Up to now, a lot of cell-penetrating peptides (CPPs) have been reported (for review, see [8]) and the information has been collected and compiled in a website [9]. CPPs, either protein-derived or chemically synthesized, can be categorized into primary amphipathic, secondary amphipathic, and nonamphipathic [10]. The primary amphipathic CPPs such as transportan [11] and TP10 [12] are usually longer than 20 amino acids with periodically hydrophobic and hydrophilic residues along the primary sequence. In comparison to the primary amphipathic CPPs, the secondary amphipathic CPPs such as penetratin, pVEC [13], and E162 [14] contain less amino acid residues and perform amphipathic structure upon interacting with phospholipid membrane. The third class CPPs, such as R8 [15], DPV3 [16], and PTD [4], are relatively short and contain very high content of arginine.

1.2. Membrane-Penetration Mechanisms of Arginine-Rich CPPs. A metabolic energy-independent, direct plasma membrane translocation mechanism could be detected for the highly positively charged R8 peptide at 4°C at which the receptor-mediated internalization was completely inhibited. However, only a small part of R8 penetration occurred by way

of this pathway at physiological temperature [17]. Pyrenebutyrate can neutralize the positive charge of R8 [15], R9, and Tat_{48-60} [18] and provide a hydrophobic aromatic group to accelerate the direct penetration through the cellular membrane. At 37°C, the translocation of arginine-rich CPPs into cytoplasm is mainly via an endocytic uptake-endosomal escape pathway. The translocation of arginine-rich CPPs is not dependent on both clathrin and caveolin-coated pit-mediated endocytosis but is inhibited by 5-(N-ethyl-N-isopropyl)amiloride (EIPA), an inhibitor of macropinocytosis [19], and cytochalasin D, which prevents actin polymerization [20]. Membrane-associated proteoglycan including heparin sulfate (HSPG) is reported to play a crucial role in the endocytic uptake of arginine-rich CPPs [21]. It could be concluded that the positively charged arginine-rich CPPs associated with negatively charged proteoglycan on the surface of plasma membrane were engulfed by macropinocytosis followed by endosomal escape into the cytoplasm. The endosomal escape step is rate limiting for the CPPs to arrive at the cytosol; however, the mechanism is not well explored.

1.3. PTD-J-Domain. Recently, a vector pET22b-PTD_1J_1 that could be used to highly express recombinant protein fused to PTD-J-domain on its N-terminus was reported. We took advantages of the specific association ability of the J-domain of Hsp40 with the nucleotide binding domain of Hsp70 and the cell membrane-penetrating activity of the protein transduction domain of HIV-1 Tat protein. Higher level and more soluble chicken IGF-I recombinant protein was expressed by the pET22b-PTD_1J_1 vector in comparison to the pET32b vector. An HpNC peptide containing two fragments of human heptoprotein was expressed by the pET22b-PTD_1J_1 vector. The PTD-J-HpNC recombinant polypeptide product could effectively elicit rat antisera specific to subtypes Hp1 and Hp2 heptoproteins in human serum samples, but the counterpart TrxA-HpNC could not [22]. Moreover, overexpression of PTD-J-FMDVepi, where FMDVepi is an assembled T_H and B-epitopes of foot-and-mouth disease virus VP1 capsid protein, is dependent on the combination of PTD and J-domain rather than PTD or J-domain individually. This result suggests that the fused PTD-J polypeptide may possess a special structure that can elicit the immunogenicity of FMDVepi peptide fused with it [23].

In this study, two sets of pET22b-CPP and pET22b-CPP-J expression vectors were constructed. The CPP-DsRed and CPP-J-DsRed recombinant proteins expressed by them were characterized. The cellular membrane-penetrating capabilities of the chosen CPPs were elevated by the J-domain fused to them.

2. Materials and Methods

2.1. Construction of pET22b-CPP-DsRed and pET22b-CPP-J-DsRed Vectors. The coding region of the red fluorescence protein in pDsRed monomer N1 plasmid (Cat. no. 632465, Clontech) was amplified by PCR with forward primer (G AAT TCT CAT ATG ATG GAC AAC ACC GAG GAC GTC ATC) and reverse primer (CTC GAG ACC ACC CTG GGA GCC GGA GTG GCG GGC CT). The amplified DNA fragment was ligated with pGEM TEasy TA-cloning vector (A1360, Promega) to get pGEM TE-DsRed. Then, the cloned DsRed DNA fragment was removed from the cloning vector by EcoRI and XhoI restriction enzyme digestion and subcloned to the pET22b-PTD_1J_1 expression vector [22] which had been treated by the same pair of restriction enzymes to obtain pET22b-PTD-J-DsRed plasmid. The sense and antisense oligonucleotide pairs of PTD, DPV3A (the first amino acid residue R of DPV3 was replaced by A), E162, pVEC, R11, and TP13 (Table 1) were annealed in TEN (10 mM Tris-HCl, pH 8.0/1 mM EDTA, pH 8.0/0.3 M NaCl) at 60°C for 30 min followed by slowly cooling down to room temperature for about 60 min. These annealed primer pairs were inserted between NdeI and EcoRI sites of pET22b to obtain pET22b-PTD, pET22b-DPV3A, pET22b-E162, pET22b-pVEC3, pET22b-R11, and pET22b-TP13. The DsRed DNA fragment described above was inserted between the EcoRI and XhoI sites of pET22b-PTD, pET22b-DPV3A, pET22b-E162, pET22b-pVEC3, pET22b-R11, and pET22b-TP13 to get pET22b-PTD-DsRed, pET22b-DPV3A-DsRed, pET22b-E162-DsRed, pET22b-pVEC3-DsRed, pET22b-R11-DsRed, and pET22b-TP13-DsRed, respectively. The J-DsRed fragment removed from pET22b-PTD-J-DsRed by BamHI and XhoI codigestion was inserted between BamHI and XhoI sites of pET22b-DPV3A, pET22b-E162, pET22b-pVEC3, pET22b-R11, and pET22b-TP13 to get pET22b-DPV3A-J-DsRed, pET22b-E162-J-DsRed, pET22b-pVEC3-J-DsRed, pET22b-R11-J-DsRed, and pET22b-TP13-J-DsRed, respectively.

2.2. Construction of pET22b-DsRed and pET22b-J-DsRed Vectors. The 0.7 kb NdeI-XhoI fragment of pGEM TE-DsRed clone was inserted into the same restriction enzyme sites of pET22b to obtain pET22b-DsRed. The primer pair, CAT ATG GGT AAA GAT TAC TAC CAG ACT CAC GGT and GA ATT CGA ACC ACG TGG AAC TAA ATT CGC ACC ACC AGA, was used to amplify DNA fragment encoding the J-domain and a thrombin cutting site using pET22b- PTD_1J_1 as template. This DNA fragment was utilized to replace the PTD-J fragment which is franked by NdeI and EcoRI sites of the pET22b-PTD_1J_1-DsRed vector to prepare pET22b-J-DsRed.

2.3. Expression and Purification of CPP-DsRed, CPP-J-DsRed, DsRed, and J-DsRed Recombinant Proteins. The *E. coli* Rosetta gamiB(DE3)pLysS host cells transformed by pET22b-CPP-DsRed, pET22b-CPP-J-DsRed, pET22b-DsRed, or pET22b-J-DsRed were grown in 2x YT supplemented with 0.4% glucose, 30 μg/mL chloramphenicol, and 50 μg/mL ampicillin at 37°C. IPTG was adjusted to 1 mM when OD_{600} was 0.6 and cells were cultured for another 4 h. To analyze recombinant proteins released into the medium, cells were centrifuged at 12,000 g for 30 min. The supernatant was concentrated 10-fold using Centricon (Y3, Millipore), and then 30 μL of sample was loaded in each lane of a 12% SDS polyacrylamide gel. To analyze recombinant proteins within cells, the cells were collected by centrifugation at 10,000 g for 10 min. After ultrasonication, protein contents of soluble fraction and insoluble

Table 1: Primers for pET22b-CPP vectors construction.

PTD	Peptide	YGRKK RRQRR R
	Forward primer	**TATG GCT** TAT GGT CGT AAG AAA CGT CGT CAG CGT CGT CGT **GTG GGG ATC CCG**
	Reverse primer	**AATT CGG GAT CCC CAC** ACG ACG ACG CTG ACG ACG TTT CTT ACG ACC ATA **AGC CA**
DPV3A	Peptide	AKKRR RESRK KRRRE S
	Forward primer	**TATG** GCT AAA AAA CGC CGT CGT GAA AGC CGT AAA AAA CGT CGT CGT GAA AGC **GGG ATC CCG**
	Reverse primer	**A ATT CGG GAT CCC** GCA TTC ACG ACG ACG TTT TTT ACG GCT TTC ACG ACG GCG TTT TTT AGC **CA**
E162	Peptide	KTVLL RKLLK LLVRK I
	Forward primer	**TATG** AAA ACC GTG CTG CTG CGT AAA CTG CTG AAA CTG CTG GTG CGT AAA ATC **GGG ATC CCG**
	Reverse primer	**A ATT CGG** GAT CCC GAT TTT ACG CAC CAG CAG TTT CAG CAG TTT ACG CAG CAG CAC GGT TTT **CA**
pVEC	Peptide	LLIIL RRRIR KQAHA HSK
	Forward primer	**TATG** CTG CTG ATT ATT CTG CGT CGT CGC ATT CGT AAA CAG GCC CAT GCC CAT TCT AAA **GGG ATC CCG**
	Reverse primer	**A ATT CGG GAT CCC** TTT AGA ATG GGC ATG GGC CTA TTT ACG AAT GCG ACG ACG CAG AAT AAT CAG **CAG CA**
R11	Peptide	RRRRR RRRRR R
	Forward primer	**TATG** CGC CGT CGT CGT CGC CGT CGT CGC CGT CGT CGT **GGG ATC CCG**
	Reverse primer	**A ATT CGG GAT CCC** ACG ACG ACG GCG ACG ACG GCG ACG ACG ACG GCG **CA**
TP13	Peptide	LNSAG YLLGK ALAAL AKKIL
	Forward primer	**TATG** CTG AAC AGC GCG GGT TAT CTG CTG GGT AAA GCC CTG GCC GCC CTG GCG AAA AAG ATT CTG **GGG ATC CCG**
	Reverse primer	**A ATT CGG GAT CCC** CAG AAT CTT TTT CGC CAG GGC GGC CAG GGC TTT ACC CAG CAG ATA ACC CGC GCT GTT CAG **CA**

fraction corresponding to 0.2 OD_{600} unit of cells were run on a 12% SDS polyacrylamide gel. The gel was stained with Coomassie Blue R250. Soluble forms of CPP-DsRed and CPP-J-DsRed recombinant proteins were purified by Ni-Sepharose 6 Fast Flow affinity column (17-5318-02, GE) in accordance with the manufacture's instruction.

2.4. Protein Transduction. Huh-7 cells were seeded at 1.5×10^5 cells per well in a 24-well plate and cultured in DMEM supplemented with 10% FBS the day before protein transduction experiment. The cells were washed with serum-free medium twice and incubated with various concentrations of recombinant proteins in serum-free medium for various times as indicated. The unpenetrated recombinant proteins were washed off with PBS twice. Then, the penetrated proteins were released from cells using 200 μL of PBS supplemented with 1% Triton X-100. After centrifugation at 10,000 g for 5 min to remove the nonsoluble materials, 100 μL supernatant was transferred to a well of a 96-well plate to measure the amount of recombinant DsRed proteins in the soluble fraction using a fluorometer. The stimulating wave length and emission wave length were set at 557 nm and 585 nm, respectively. Experiments were repeated for four times. The recombinant DsRed proteins penetrated into cells were also detected with a fluorescence microscope. The calibration curves between the fluorescence values of 100 μL sample per well and the concentrations of recombinant proteins in total nontransduced cell lysate were measured as described above. To test the effects of endocytosis inhibitors on the cell-penetration activity of PTD-J-DsRed, cells were pretreated with filipin (5 μg/mL; Sigma, F9765), EIPA (100 μM; Sigma, A3085), or cytochalasin D (10 μM; Sigma, C8273) for 1 hour before the treatment of the PTD-J-DsRed recombinant protein (40 μg/mL) for two hours.

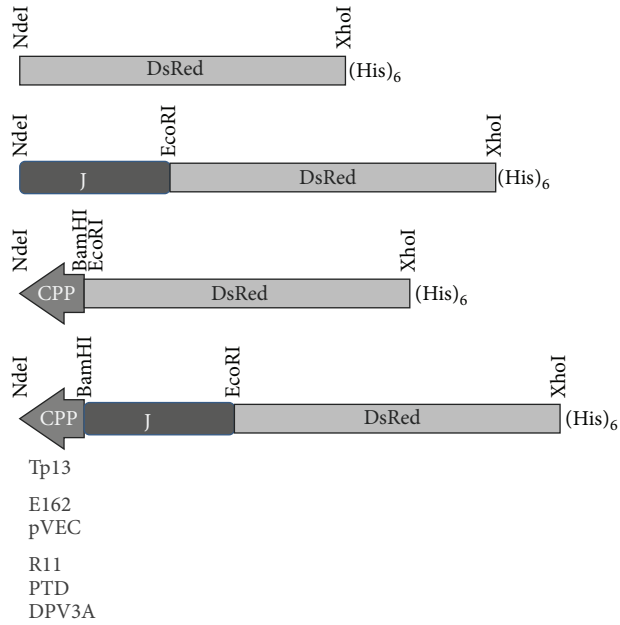

Figure 1: Representative structures of the CPP-DsRed and CPP-J-DsRed. Oligonucleotide pairs encoding DPV3A, E162, pVEC, R11, and TP13 listed in Table 1 were inserted between NdeI and EcoRI sites of pET22b to create pET22b-DPV3A, pET22b-E162, pET22b-pVEC, pET22b-R11, pET22b-PTD, and pET22b-TP13, respectively. The DsRed cDNA fragment cut from pET22b-PTD-J-DsRed by the EcoRI site at 5′-end and the XhoI site at 3′-end was inserted into the above vectors to prepare pET22b-DPV3A-DsRed, pET22b-E162-DsRed, pET22b-pVEC-DsRed, pET22b-R11-DsRed, pET22b-PTD-DsRed, and pET22b-TP13-DsRed. The J-DsRed cDNA fragment cut from pET22b-PTD-J-DsRed by the BamHI site at 5′-end and the XhoI site at 3′-end was inserted into the above vectors to prepare pET22b-DPV3A-J-DsRed, pET22b-E162-J-DsRed, pET22b-pVEC-J-DsRed, pET22b-R11-J-DsRed, and pET22b-TP13-J-DsRed.

3. Results

3.1. Construction of pET22b-CPP-DsRed and pET22b-CPP-J-DsRed Expression Vectors. Published CPPs with better transduction activities were focused and TP13 (primary amphipathic CPP) [24], E162, and pVEC (secondary amphipathic CPPs) [14], as well as R11 [25], PTD [22], and DPV3 [16] (arginine-rich CPPs), were picked up in this study. The pET22b-CPP-DsRed and pET22b-CPP-J-DsRed expression vectors were constructed as described in Section 2 and the representative structures of CPP-DsRed and CPP-J-DsRed accompanied with the DsRed and J-DsRed controls are shown in Figure 1.

3.2. Expression of CPP-DsRed Recombinant Proteins. After transformation with pET22b-CPP-DsRed plasmids, *E. coli* Rosetta gamiB(DE3)pLysS cells were cultured in 2x YT medium supplemented with 0.4% glucose and antibiotics until OD_{600} reached 0.6. Then, IPTG was adjusted to 1 mM and cells were cultured for another 4 h. The expression level of R11-DsRed recombinant protein in the total lysate was nearly undetectable (left panel of Figure 2(a)); therefore, only the other five recombinant proteins were characterized in the following steps. After homogenization by ultrasonication, the cell lysate can be separated into soluble (supernatant) and insoluble (pellet) fractions by centrifugation. PTD- and DPV3A-DsRed recombinant proteins were found in the soluble fraction; on the other hand, TP13-, E162-, and pVEC-DsRed recombinant proteins were found in the insoluble fraction (data not shown). The growth rates of the TP13- and E162-DsRed cultures were slower. After removal of cells by centrifugation and 0.22 μm membrane filtration, the protein contents in medium after IPTG induction were analyzed by SDS-PAGE. As the patterns shown in the left panel of Figure 2(b), large amounts of cellular proteins of *E. coli* were detected in the TP13- and E162-DsRed lanes; however, a 38 kDa protein band was dominantly detected (indicated by an arrow) in the PTD- and DPV3A-DsRed lanes. These results indicated that TP13- and E162-DsRed recombinant proteins might disturb the cell membrane of *E. coli* host cells and cause cell disruption, even if most of them were present in the insoluble form.

3.3. Expression of CPP-J-DsRed Recombinant Proteins. The expression characteristics of PTD-J-, DPV3A-J-, TP13-J-, E162-J-, and pVEC-J-DsRed are similar to those of PTD-,

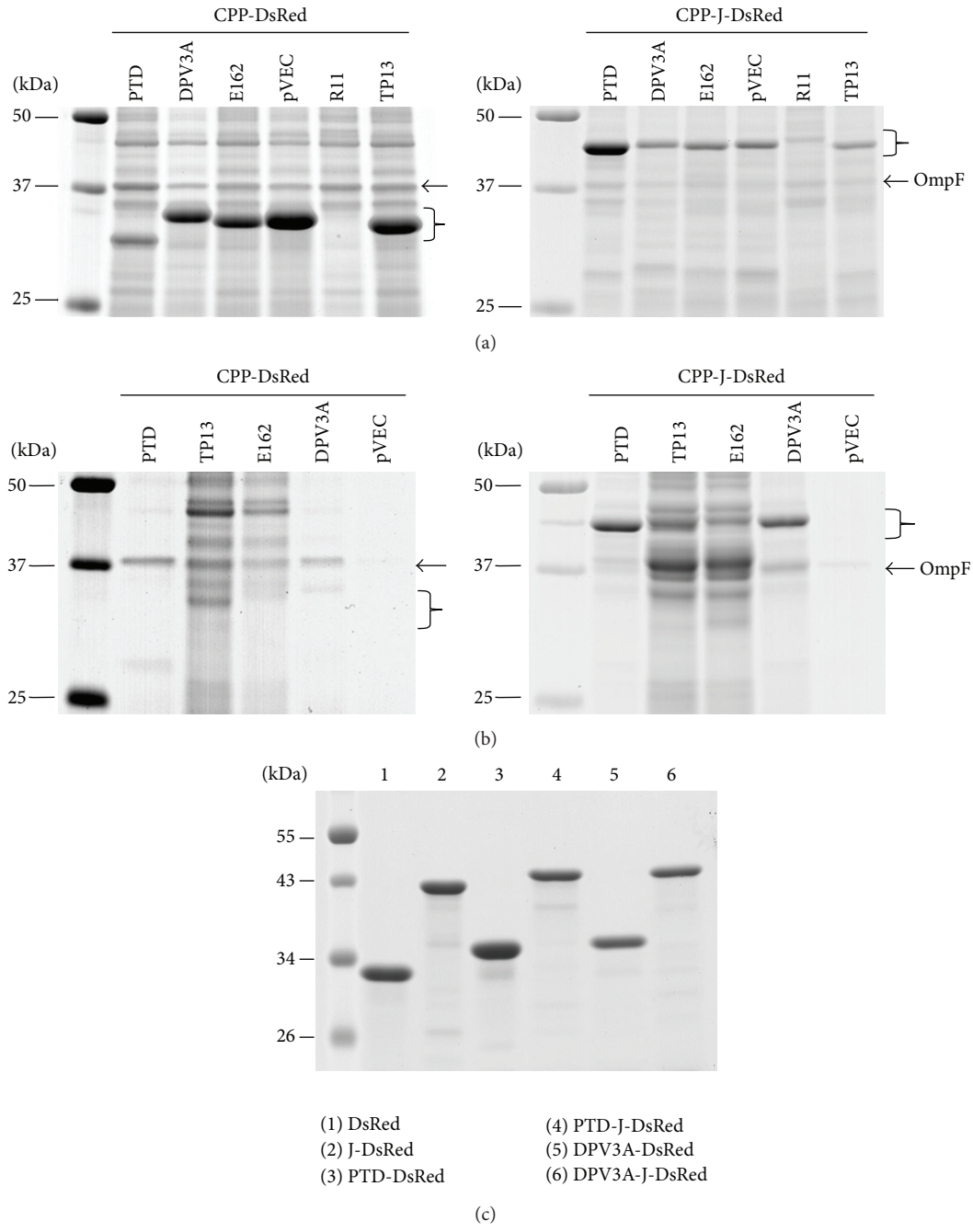

Figure 2: Expression of CPP-DsRed recombinant proteins. (a) Total lysates of *E. coli* expressing PTD-DsRed, DPV3A-DsRed, pET22b-E162-DsRed, pVEC-DsRed, R11-DsRed, and TP13-DsRed recombinant proteins (left panel) as well as those of PTD-J-DsRed, DPV3-J-DsRed, E162-J-DsRed, pVEC-J-DsRed, R11-J-DsRed, and TP13-J-DsRed recombinant proteins (right panel) were analyzed by SDS-PAGE. The recombinant proteins are indicated by braces. (b) The protein contents of medium fractions from *E. coli* expressing PTD-DsRed, DPV3A-DsRed, E162-DsRed, pVEC-DsRed, and TP13-DsRed recombinant proteins (left panel) as well as those of PTD-J-DsRed, DPV3-J-DsRed, E162-J-DsRed, pVEC-J-DsRed, R11-J-DsRed, and TP13-J-DsRed (right panel) were analyzed by SDS-PAGE. A 38 kDa common secreted protein OmpF from host cells is indicated by an arrow as internal standard and the recombinant proteins are indicated by braces. (c) The soluble DsRed, J-DsRed, PTD-DsRed, PTD-J-DsRed, DPV3A-DsRed, and DPV3A-J-DsRed recombinant proteins were purified by Ni-Sepharose 6 Fast Flow affinity column chromatography. Each lane was loaded with 20 μg of purified recombinant proteins.

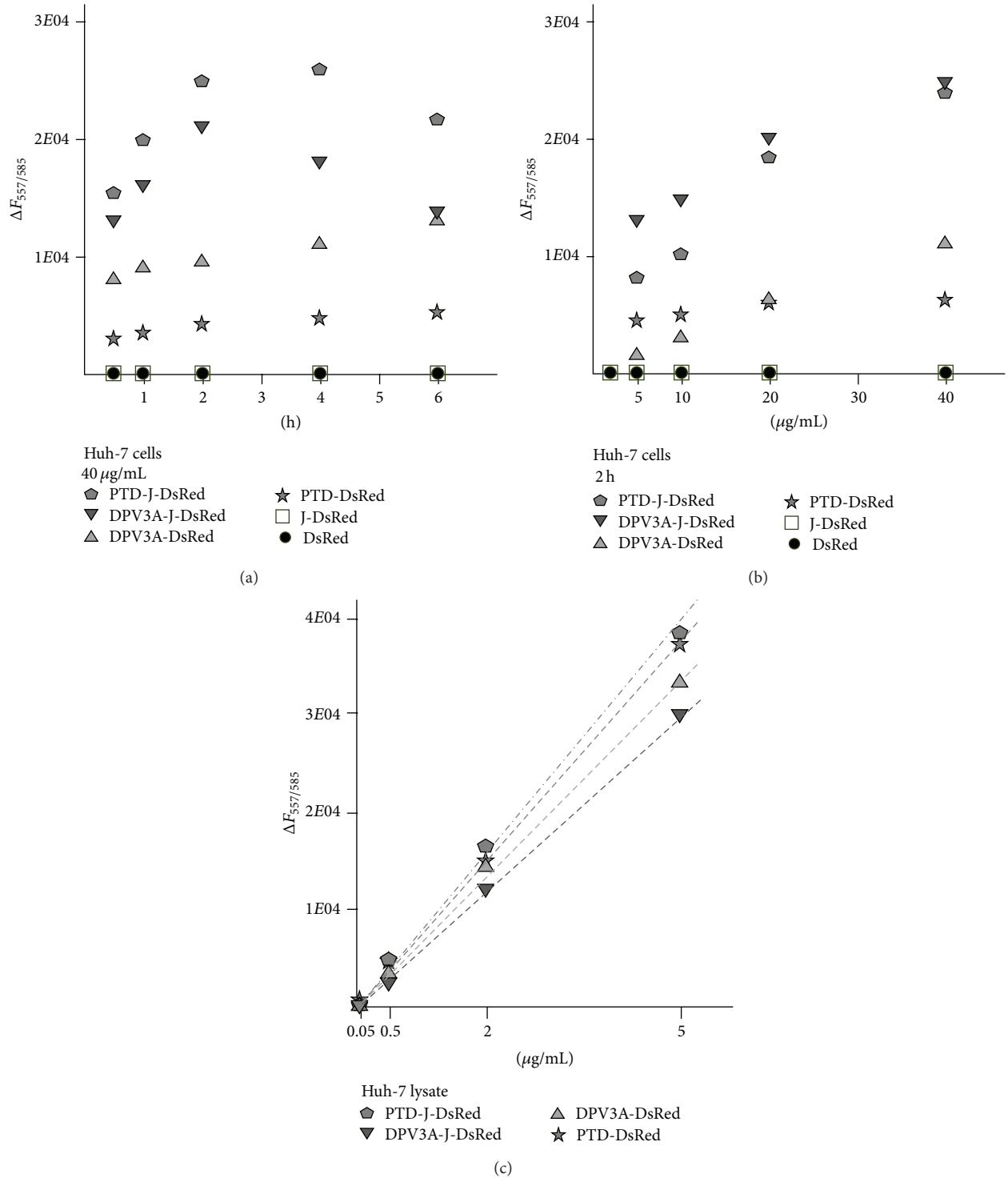

Figure 3: Transduction of PTD-DsRed, DPV3A-DsRed, PTD-J-DsRed, and DPV3A-J-DsRed recombinant proteins. (a) 1.5×10^5 Huh-7 cells in a well of 24-well plate were incubated with 0.2 mL of PTD-DsRed, DPV3A-DsRed, PTD-J-DsRed, or DPV3A-J-DsRed recombinant protein (40 μg/mL) for 0.5, 1, 2, 4, or 6 h. The cells were washed with PBS twice and the recombinant proteins incorporated into cells were released by 0.2 mL of 1% Triton X-100/PBS. After centrifugation to remove cell debris, 0.1 mL of the supernatant was transferred to a well of a 96-well plate and the amounts of recombinant proteins were measured by a fluorometer. The stimulating wave length and emission wave length were set at 557 nm and 585 nm, respectively. (b) 1.5×10^5 Huh-7 cells in a well of 24-well plate were incubated with 0.2 mL of 5, 10, 20, and 40 μg/mL of the PTD-DsRed, DPV3A-DsRed, PTD-J-DsRed, or DPV3A-J-DsRed recombinant protein for 2 hours. The amounts of recombinant proteins were measured as described before. Experiments were done for four times and the average values were shown. All of the standard deviations were less than 1000. (c) The PTD-DsRed, DPV3A-DsRed, PTD-J-DsRed, and DPV3A-J-DsRed recombinant proteins were diluted in Huh-7 cell lysate to 0.05, 0.5, 2, and 5 μg/mL and the fluorescence values were measured as described above.

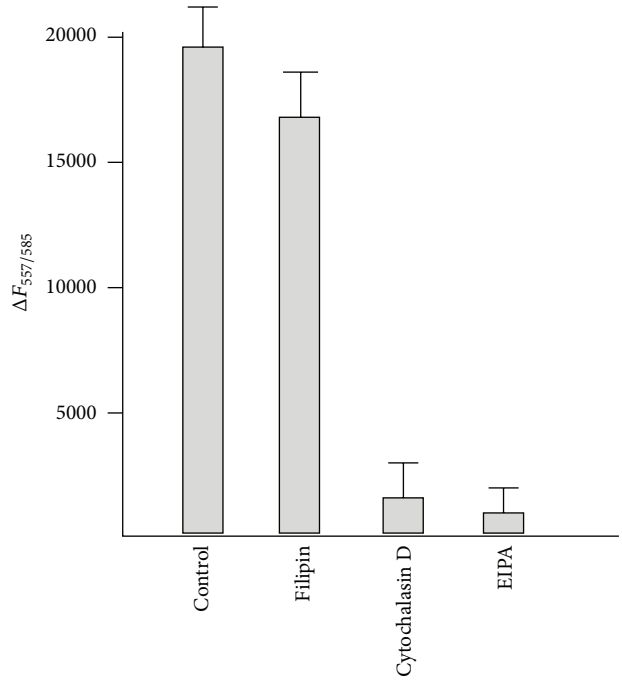

Figure 4: Macropinocytosis is involved in the cell-penetration pathway of PTD-J-DsRed. Huh-7 cells were pretreated with filipin (5 μg/mL), EIPA (100 μM), or cytochalasin D (10 μM) for 1 hour before the treatment of the PTD-J-DsRed recombinant protein (40 μg/mL) for 2 hours. The fluorescence values of 100 μL cleared lysate samples were measured (three experiments were performed and the P values of filipin, cytochalasin D, and EIPA data relative to the control are 0.0257, 0.000812, and 0.000165, resp.).

DPV3A-, TP13-, E162-, and pVEC-DsRed, respectively (right panel of Figure 2(a)). The major difference is that significant amounts of PTD-J-DsRed and DPV3-J-DsRed recombinant proteins were presented in the medium fractions (right panel of Figure 2(b)). These two "secreted" forms of recombinant proteins could be purified by Ni-NTA affinity column and contain N-terminal amino acid sequences the same as those of PTD-J-DsRed and DPV3A-J-DsRed indicating that they have intact N- and C-termini. In comparison with the amount of recombinant proteins that remain within cells, about 10% of these two recombinant proteins were in the secreted fractions.

3.4. Cell-Penetrating Activities of PTD-, DPV3A-, PTD-J-, and DPV3A-J-DsRed Recombinant Proteins. Recombinant DsRed, J-DsRed, PTD-DsRed, PTD-J-DsRed, DPV3A-DsRed, and DPV3A-J-DsRed proteins (Figure 2(c)) were purified from cell lysates by Ni-NTA-Sepharose affinity column chromatography. After elution by 250 mM imidazole, these proteins were dialyzed against 100 volumes of PBS twice, and aliquots were stored at −80°C.

The cell-penetrating activities of the four recombinant proteins were tested. At first, the time course of recombinant protein transduction to Huh-7 cells was measured at 0.5 1, 2, 4, and 6 hours using 40 μg/mL of each recombinant protein in serum-free Opti-MEM medium. Both of the DsRed and J-DsRed could not penetrate into Huh-7 cells. The amount of the PTD-DsRed and DPV3A-DsRed proteins transduced increased with time up to 6 hours. However, the time for optimal amount of PTD-J-DsRed and DPV3A-J-DsRed transduced into cells was around 2 to 4 hours (Figure 3(a)). Then, Huh-7 cells were treated with 5, 10, 20, and 40 μg/mL of each recombinant protein in the same medium for 2 hours. The amounts of recombinant proteins incorporated increased roughly proportional to the amounts of the recombinant proteins added (Figure 3(b)). To estimate the amounts of recombinant proteins transduced into cells, calibration curves of the fluorescence values of 100 μL sample per well relative to the concentrations of recombinant proteins in total cell lysate were illustrated in Figure 3(c). According to the calibration curves, about 2% of PTD-DeRed and DPV3A-DsRed and 7% of PTD-J-DeRed and DPV3A-J-DsRed can transduce into cells when 40 μg/mL of recombinant proteins was used.

It had been suggested that macropinocytosis was involved in the penetration pathway of arginine-rich CPPs [19, 20]. The entrance route of PTD-J-DeRed was analyzed by using inhibitors of different endocytic pathways [26]. The EIPA and cytochalasin D (both are inhibitors on macropinocytosis) and filipin which is an inhibitor of caveolae-mediated endocytosis were tested. As shown in Figure 4, only EIPA and cytochalasin D could reduce the amount of intracellular PTD-J-DsRed indicating that macropinocytosis was involved in its entrance. The same result was obtained for DPV3A-J-DsRed (data not shown). The pattern of PTD-DsRed and PTD-J-DsRed recombinant proteins incorporated into Huh-7 cells was further analyzed using immunofluorescence microscopy. More

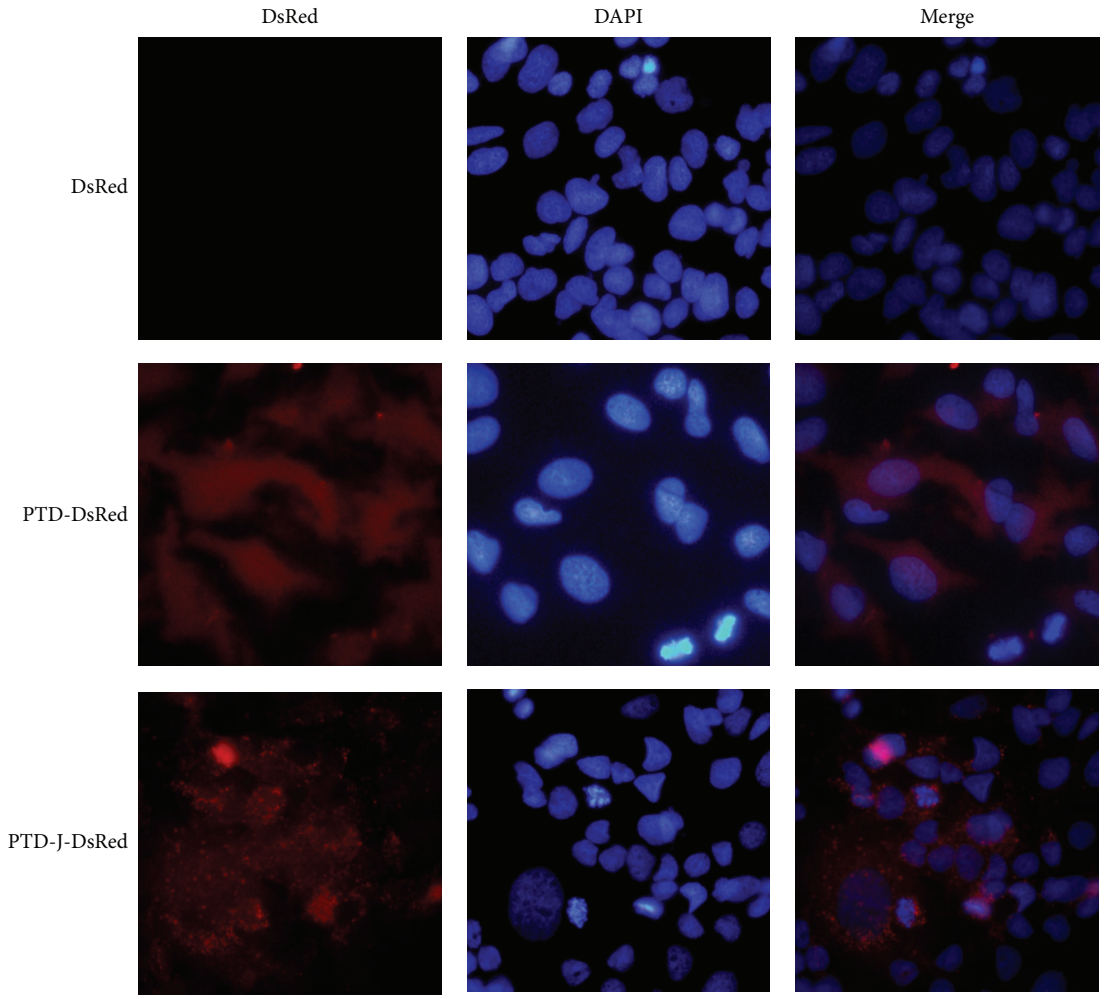

Figure 5: Fluorescence microscopic analysis of the cellular uptake of DsRed, PTD-DsRed, and PTD-J-DsRed recombinant proteins. Huh-7 cells were treated with 40 μg/mL of PTD-DsRed or PTD-J-DsRed recombinant protein for 2 h. After two PBS washes, cells were fixed with 4% paraformaldehyde/PBS for 10 min at room temperature, counterstained with DAPI, and observed under a fluoromicroscope.

granular signals were observed in the PTD-J-DsRed image (Figure 5).

4. Discussion

4.1. PTD-J-DsRed and DPV3A-J-DsRed Recombinant Proteins Were Found in the Medium. During the expression of PTD-J-DsRed and DPV3A-J-DsRed recombinant proteins by *E. coli*, about 10% of total recombinant proteins could be isolated from medium fraction (Figure 2(b)). These "secreted" forms of recombinant proteins were purified by Ni-NTA affinity column and determined with intact N-termini by amino acid sequencing indicating that they are the same as the cellular forms. It is interesting to distinguish how PTD-J-DsRed and DPV3A-J-DsRed recombinant proteins can be released from *E. coli* cells into the medium. Proteins located outside the inner membrane of *E. coli* are usually synthesized with N-terminal signal peptides to target them to either Sec [27, 28] or Tat (twin-arginine translocation) [29, 30] protein export pathway. The major difference between these two export systems is that the Sec apparatus translocates unfolded polypeptides across the membrane, whereas the Tat complex transports already folded proteins. The Tat pathway can transport a heterooligomeric protein complex in which only one subunit possesses a Tat-targeting signal peptide through membrane at once. For example, only the small HybO subunit of HybOC hydrogenase 2 complex has the Tat-targeting signal peptide. The large HybC subunit was transported in complex with the HybO subunit [31]. Another case is the SoxYZ protein complex involved in thiosulfate oxidation. Only SoxY has a Tat-targeting signal peptide and SoxZ is exported in complex with SoxY [29]. Although there is not any datum to support that PTD-J-DsRed or DPV3A-J-DsRed could be carried across cellular membrane by an unknown protein with Tat-targeting signal peptide, it provides a possible pathway to interpret how a protein without signal peptide can be transported across the inner membrane of *E. coli*.

4.2. PTD-J-DsRed and DPV3A-J-DsRed Recombinant Proteins Transduce More Effectively Than Their PTD-DsRed and DPV3A-DsRed Counterparts. When Huh-7 cells were treated with the same concentration (40 μg/mL) of recombinant proteins, the amounts of PTD-DsRed and DPV3A-DsRed incorporated were slightly increased with time after 1 hour. However, the maximal amounts of PTD-J-DsRed and DPV3A-J-DsRed within cells were detected around 2 to 4 hours. Then, the fluorescence values decreased because cells began to be lysed (Figure 3(a)). DsRed and J-DsRed could not penetrate into Huh-7 cells. PTD-J-DsRed and DPV3A-J-DsRed could penetrate into Huh-7 cells more effectively than PTD-DsRed and DPV3A-DsRed, respectively, did. These results indicate that the J-domain itself has no cell-penetrating ability; however, it can enhance the cell-penetrating activity of PTD and DPV3A. When Huh-7 cells were treated with 40 μg/mL of PTD-J-DsRed for 2 hours, about 0.6 μg of recombinant proteins was penetrated into 10^4 cells, corresponding to 10^9 molecules per cell.

The pattern of PTD-DsRed and PTD-J-DsRed recombinant proteins incorporated into Huh-7 cells was further analyzed using immunofluorescence microscopy. More granular signals were observed in the PTD-J-DsRed image (Figure 5). In addition, the penetration of PTD-J-DsRed recombinant protein into Huh-7 cells was inhibited by EIPA and cytochalasin D (Figure 4). These phenomena indicate that endocytic uptake-endosomal escape pathway may be the major route for PTD-J-DsRed to penetrate into Huh-7 cells. In conclusion, the J-domain can assist CPPs and their cargo to penetrate through the cellular membrane of both *E. coli* and Huh-7 cell more effectively.

5. Conclusion

By using red fluorescence protein DsRed as a reporter, the recombinant proteins PTD-J-DsRed and DPV3A-J-DsRed performed higher cell-penetrating activity than PTD-DsRed and DPV3A-DsRed, respectively. Because both DsRed and J-DsRed recombinant proteins could not penetrate into cells, it is suggested that the J-domain could help cell-penetrating peptides PTD and DPV3A as well as their cargo to penetrate through the cellular membrane more effectively.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Gene Delivery into Plant Cells for Recombinant Protein Production

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Recombinant proteins are primarily produced from cultures of mammalian, insect, and bacteria cells. In recent years, the development of deconstructed virus-based vectors has allowed plants to become a viable platform for recombinant protein production, with advantages in versatility, speed, cost, scalability, and safety over the current production paradigms. In this paper, we review the recent progress in the methodology of agroinfiltration, a solution to overcome the challenge of transgene delivery into plant cells for large-scale manufacturing of recombinant proteins. General gene delivery methodologies in plants are first summarized, followed by extensive discussion on the application and scalability of each agroinfiltration method. New development of a spray-based agroinfiltration and its application on field-grown plants is highlighted. The discussion of agroinfiltration vectors focuses on their applications for producing complex and heteromultimeric proteins and is updated with the development of bridge vectors. Progress on agroinfiltration in *Nicotiana* and non-*Nicotiana* plant hosts is subsequently showcased in context of their applications for producing high-value human biologics and low-cost and high-volume industrial enzymes. These new advancements in agroinfiltration greatly enhance the robustness and scalability of transgene delivery in plants, facilitating the adoption of plant transient expression systems for manufacturing recombinant proteins with a broad range of applications.

1. Introduction

The approval of the first plant-derived therapeutic enzyme for Gaucher's disease has demonstrated the promise of plant-based systems for recombinant protein (RP) production [1]. In addition to the traditional advantages in cost, scalability, and safety over current bioreactor-based production platforms, progress in glycoengineering and expression vector discovery has also allowed plants to produce RPs with specific glycoforms to enhance functionality and at unprecedented speed to control potential pandemics and fight bioterrorism [2].

The traditional strategy of producing RPs in plants is to create stable, transgenic lines of plants. The target transgene is integrated into the plant genome and the RP can be produced in successive generations [3]. To eliminate the long time frame of generating transgenic plants, transient expression systems have been developed. In this strategy, the transgene is not integrated into the plant genome but rather quickly directs the production of the RP while residing transiently within the plant cell. In addition to significantly shortening the production timeline, this strategy also enhances RP accumulation level by eliminating the "position effect" of variable expression caused by the random integration of transgene within the genome [4]. Besides its speed and high yield, the transient expression system also offers the versatility for producing personalized RPs, such as therapeutics for patient-specific cancers and vaccines against viruses that have rapid antigenic drift and/or multiple strains with unpredictable outbreaks. This flexibility also provides the "surge" capability to rapidly produce recombinant counteragents in a bioterrorism event. Since no transgenic plant is created, transient expression also addresses regulatory issues and public concerns for genetically modified organisms (GMOs). These advantages demonstrate the vast potential of transient expression as a preferred method for RP production in plants.

However, scale-up of RP production by transient expression poses a bigger challenge than transgenic plants, because no genetically stable seed bank is produced and scale-up is no longer just a matter of increasing acreage to boost yield. To overcome this challenge, a scalable transgene delivery method must be developed for plant transient expression.

2. Methods of Transgene Delivery

The method of choice for introducing transgenes into plants depends on the expression vector and the host plants. These methods include direct delivery by gene gun and indirect delivery through using *Agrobacterium tumefaciens* or plant viruses [5].

2.1. Direct Delivery Methods. DNA or RNA can be directly introduced into plant cells via a so-called microprojectile bombardment method, also known as a gene gun or biolistics. In this method, the transgene is coated onto microgold or tungsten particles and fired into plant cells ballistically [6]. The advantage of this method resides in its versatility and a broad range of susceptible plants. It can be used to deliver transgene to both nuclear and chloroplast genomes. At least in theory, effective transgene delivery by biolistics is vector independent and can be applied to any plant host species [5].

2.2. Indirect Gene Delivery Methods. Indirect transgene delivery exploits the ability of plant virus or certain pathogenic agrobacteria species (e.g., *A. tumefaciens*) that naturally transfer their genome (plant virus) or part of their tumor inducing plasmid (Ti plasmid) DNA (T-DNA) into plant cells (*Agrobacterium*). A transgene can enter plant cells as a by-product of viral infection if cloned into the full viral genome. Infection can be facilitated by rubbing plant tissue with a transgene carrying infectious viral particles or viral nucleic acids [3]. However, this method is only applicable to viruses or plant hosts that are susceptible to mechanical inoculation but not to those that require specialized insects for viral transmission.

Ti plasmids of *Agrobacterium* can be modified into delivery vectors by replacing pathogenic genes in T-DNA with transgenes; transgene transfer from agrobacteria to plant cells is accomplished through the natural interaction between *A. tumefaciens* and its plant hosts [5]. In contrast to biolistics, gene delivery by *A. tumefaciens* requires the cloning of transgene into a modified Ti plasmid and is restricted to dicotyledonous and a limited number of monocotyledonous plants [7]. However, delivery by *Agrobacterium* generally offers better efficiency, transgene expression, and inheritance than biolistics [5]. It is speculated that *Agrobacterium*-based delivery is advantageous because transgene copy numbers and integration into the genome are better controlled. The coevolution of *Agrobacterium* and its plant hosts may favor the integration of transgenes into genomic loci that are transcriptionally active, which leads to its high level of expression [7]. In transient expression, biolistics often cause severe tissue damage and effectively reduce the available biomass for RP production, making indirect delivery by *Agrobacterium* a preferred method. As a result, an *Agrobacterium*-based gene delivery via agroinfiltration has become the favorable gene delivery method for transient expression in plants [7, 8].

3. Agroinfiltration for Expression of Recombinant Proteins

On a per cell basis, the yield of a RP is usually higher in transient expression than that in transgenic plants [4]. The elimination of the "position effect" is responsible for this improvement as the transgene is no longer randomly inserted into genomic areas with variable transcriptional activity [9]. However, earlier gene delivery methods consisted of soaking leaf pieces in *Agrobacterium* culture in which only the cell layer on the edges may receive the transgene. This limits the efficiency and scalability of transient systems.

Agroinfiltration was invented to overcome this challenge. Because up to one-third of the leaf volume is intercellular space, it is possible to replace the air in these cavities with a suspension of *Agrobacterium* [1]. Thus, transgene-carrying agrobacteria are actively delivered into the intercellular space of the leaf tissue by agroinfiltration, allowing for the effective access of agrobacteria to most leaf cells and making the transfer of T-DNA a highly efficient event [8, 10].

3.1. Application and Scalability of Agroinfiltration. The simplest method of agroinfiltration is syringe infiltration. In this method, transgene-carrying *Agrobacterium* in the infiltration medium is injected into the leaf with a needleless syringe (Figure 1(a)). Syringe infiltration offers the flexibility of introducing multiple transgene constructs into different areas, allowing multiple assays to be performed on a single leaf [8]. Thus, it has been used for studying plant-pathogen interactions, abiotic stresses, gene functional analysis, protein localization, and protein-protein interactions [8]. Syringe infiltration also has numerous applications for RP production. For example, it can be used to quickly examine the expression level of a RP under established conditions (Figure 1(b)). If further optimization of expression is necessary, its flexibility permits a quick assessment of various factors' effects on the yield, expression kinetics, and toxicity of the target protein. These factors include concentrations of *Agrobacterium* culture, different expression vectors, organelles favorable for RP accumulation, and the requirement for silencing suppressors. Once optimized, syringe infiltration can also be used to infiltrate several entire plants to rapidly obtain sufficient amounts (milligram level) of RPs for biochemical characterization and preclinical functional studies, as well as for developing purification schemes. Despite these utilities, only a few plant species are naturally amenable to syringe infiltration and the prospect of its scalability is highly limited [11].

A scalable agroinfiltration technology will enhance the competitiveness of transient expression systems with the traditional cell culture-based platforms for RP production. An agroinfiltration method using a vacuum chamber was developed for such purposes [5]. A prototype apparatus routinely used in the laboratory is best illustrative of the vacuum chamber method. First, aerial parts of plants are submerged

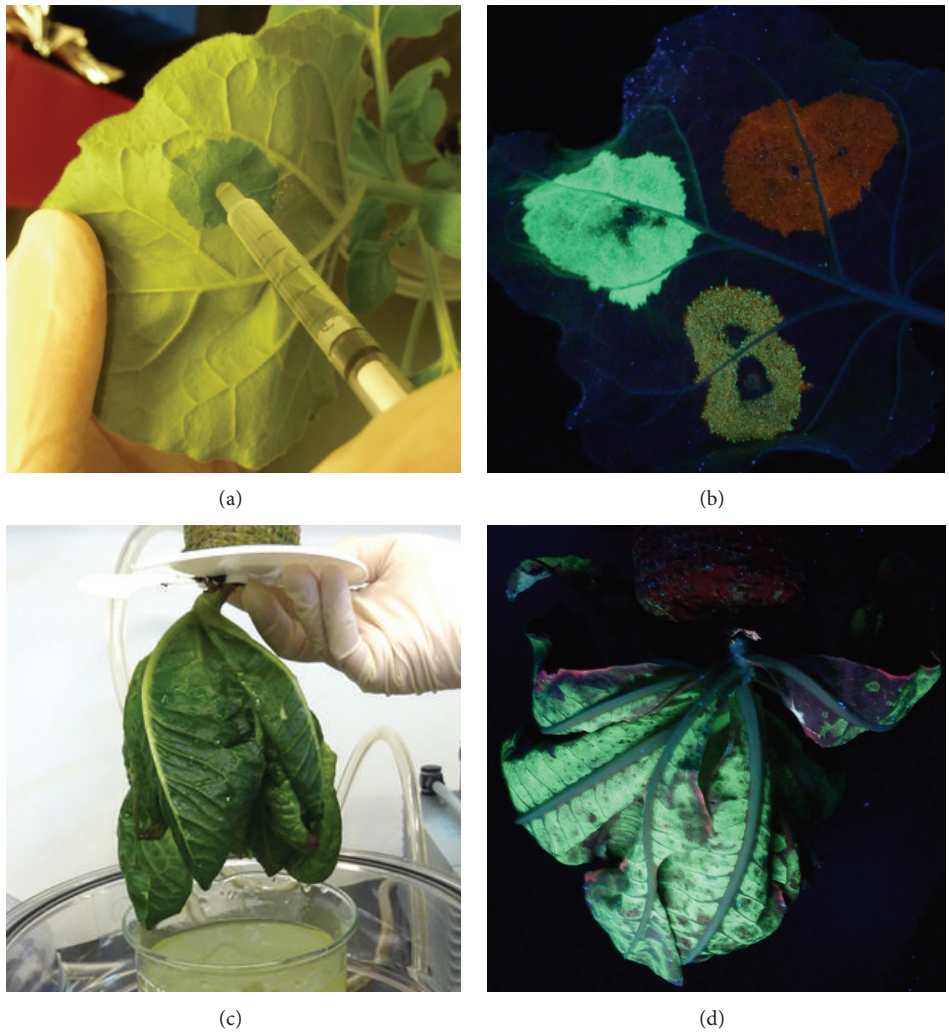

FIGURE 1: Transgene delivery by agroinfiltration into *N. benthamiana* and lettuce plants. Agrobacteria carrying the expression cassette of GFP or DsRed in geminiviral vectors were syringe-infiltrated into a *N. benthamiana* leaf (a) and GFP or DsRed expression was observed 4 days after infiltration under UV light (b). Similarly, *A. tumefaciens* cells harboring the expression cassette of GFP in a geminiviral vector were vacuum infiltrated into a lettuce plant (c) and GFP expression was examined 4 days after infiltration (d). The yellow spot in (b) indicates the leaf area that was coinfiltrated with agrobacteria carrying the expression cassette of GFP and DsRed.

into an *Agrobacterium* suspension. The submerged plants are then transferred into a desiccator that serves as the vacuum chamber. A pump provides the vacuum that exposes the submerged plants to a negative atmospheric pressure and draws the air out of the interstitial space of the leaves. Agroinfiltration is achieved when the vacuum is released, allowing agrobacteria in the medium to enter the intercellular space once occupied by the air (Figure 1(c)).

Vacuum infiltration can efficiently infiltrate plant species that are unamenable to syringe infiltration, effectively expanding the host range of agroinfiltration [12–14]. Furthermore, studies demonstrated that vacuum infiltration resulted in similar yield and temporal expression patterns for several RPs compared to that of syringe infiltration. This suggests that the results from the two agroinfiltration methods are mutually transferable; the simple syringe infiltration can accurately predict the expression pattern of a RP in scale-up settings. Not surprisingly, research has shown the superiority of vacuum infiltration in speed and robustness over syringe infiltration. For example, as the entire plant is subjected to agroinfiltration with vacuum, the expression of the RP can be detected in all leaves of the entire plant (Figure 1(d)). Even at bench scale, the time required for infiltrating a single 6-week-old *N. benthamiana* plant is significantly shortened 30 times by vacuum infiltration [15].

The scalability of vacuum infiltration has been examined for the production of RPs with biomedical applications. To test its scalability beyond the desiccator prototype, we designed a vacuum chamber that is able to accommodate 16 trays of plants per infiltration cycle. Results indicated that vacuum infiltration is highly scalable. Specifically, the accumulation level and the temporal expression patterns of virus-like particles (VLPs) of norovirus and monoclonal antibodies (MAbs) against West Nile virus (WNV) were not affected,

(a) (b)

Figure 2: *N. benthamiana* plant growth (a) and agroinfiltration (b) at commercial production scale at Kentucky Bioprocessing LLC.

regardless of whether they were produced under scale-up conditions or with the desiccator [16–19]. This pilot-scale system has enabled us to produce the norovirus vaccine candidate under current good manufacturing practice (cGMP) regulations which is sufficient both in quality and quantity for a phase I human clinical trial [16, 20, 21]. Biotechnology companies have further explored the scalability of this method. For example, a fully automated vacuum system was developed with the capability to agroinfiltrate up to 1.2 tons of plant biomass per day, allowing for the production of up to 75 g of MAb-based therapeutics per greenhouse lot (Figure 2) [1, 15, 22]. This process can be further scaled up, but its requirement of inverting plants grown in pots or trays may impose limitations on the ways the plants can be cultivated and may confine the use of vacuum infiltration to high-value RPs, such as vaccines and therapeutics.

For even larger scale RP production, especially for low-cost but high-volume RPs, it is desirable to develop new agroinfiltration technologies that allow gene delivery to whole plants without using a vacuum. Fortunately, as only nontransgenic plant material is used in transient expression, biomass can be generated in open fields by conventional agricultural practices without concerns for GMO. This allows the exploration of a spray-based agroinfiltration method to deliver transgene into field-grown plants. Initially, approximately 2% of leaf cells can receive and express the transgene through spray agroinfiltration [1]. New developments in this methodology include the use of surfactant and/or abrasives in the *Agrobacterium* suspension to enhance transfection and new *Agrobacterium* strains with super transfectivity [1, 23]. When improved spray agroinfiltration is combined with an expression vector that can generate a replicon with cell-to-cell movement capability, up to 90% of leaf cells can receive the transgene and express the target protein at high levels of 50% total soluble protein (TSP) [1]. This provides a simple and indefinitely scalable process of transgene delivery into field-grown plants, allowing transient expression on a large agricultural scale. The demonstration of large-scale agroinfiltration under the US Food and Drug Administration's (FDA) cGMP guidelines supports the regulatory compliance of this technology and extends its application to manufacture RPs with human biomedical interests. Collectively, these studies demonstrate that the value of vacuum and spray agroinfiltration lies in their enormous scalability potential, facilitating the adoption of plant transient expression-based systems for commercial manufacturing of RPs.

3.2. Vectors for Agroinfiltration. Agroinfiltration is versatile and can be performed with any vectors as long as they can replicate in *A. tumefaciens* and initiate T-DNA transfer with the help of virulent genes on chromosomes and/or another plasmid [8]. The earliest vectors used for agroinfiltration were transcriptional vectors such as pBIN19 or pCAMBIA, driven by nopaline synthase (pnos) or cauliflower mosaic virus (CaMV) 35S promoters (CaMV35S). While these transcriptional vectors are not as robust as later developed plant virus-based vectors, they do have a broad host range and can be used in almost all plant species. It is these vectors that demonstrated the superiority of transient expression in regard to the speed and RP yield over the traditional protein expression in transgenic plants [20, 24, 25].

The robust replication and/or transcription of plant viruses has led to the development of viral vectors for enhancing the RP yield [25]. Each type of plant virus offers its unique advantages and limitations as an agroinfiltration vector and may be useful for the production of a specific type of RP. For example, double-stranded DNA plant viruses such as CaMV are useful only for producing small RPs, because they have limited packaging capacity and can lose their genome functions when a fraction of their genomes are removed or substituted [25]. In contrast, single-stranded RNA viruses (e.g., tomato busy stunt virus (TBSV), tobacco mosaic virus (TMV), and potato virus X (PVX)) offer vectors for expressing large RPs, because they are more tolerant to large gene substitutions and insertions [25]. However, these RNA-based vectors have to be generated by an unscalable *in vitro* process.

"Deconstructed" viral vectors represent a new generation of vectors that combine the robustness of full viral vectors and the versatility of nonviral vectors. The elimination of unnecessary or unbeneficial genomic components during viral deconstruction significantly reduces the size of the replicon, allowing the insertion of larger transgenes while maintaining the robustness of viral replication and transcription. Deconstructed RNA viral vectors can be delivered in the form of

DNA which will be transcribed and spliced into autonomous replicons in plant cells [25]. This not only effectively eliminates the need for generating RNA vectors by an *in vitro* process but also allows for all deconstructed viral vectors to be delivered by agroinfiltration. Since agroinfiltration can deliver vectors to most of the cells on the entire plant [15], the viral systemic spreading function is no longer needed. This alleviates the concern for transgene loss during systemic spreading and allows the deletion of coat protein to accommodate larger transgene insertion. Agroinfiltration also broadens the range of plant species susceptible to viral vector delivery beyond the natural virus hosts and allows for the delivery of vectors that are not mechanically transmissible in nature. Therefore, the development and application of deconstructed viral vectors not only overcome the limitations of full viral vectors but further enhance their versatility and transgene expression in transient systems.

The most commonly used deconstructed vectors rely on the MagnICON system, derived from TMV [26]. This system can be used either in the modular or the fully assembled form, depending on the application. The modular system facilitates simple cloning of the transgene because it is located in a separated plasmid of reasonable size. Furthermore, the transgene in the 3′ module can be paired with a suite of 5′ modules that contains different promoters and/or targeting sequences to various organelles. As a result, the modular MagnICON system provides the flexibility to test the expression of a transgene with different promoters and in different organelles by simply mixing different *A. tumefaciens* strains that carry various modules. Thus, it is best suited for the optimization of transgene expression and small scale RP production. In contrast, the fully assembled system sacrifices the flexibility to gain robustness for industrial scale production. It requires only one vector and one *A. tumefaciens* culture for agroinfiltration, greatly simplifying the upstream processing and reducing the overall cost of good. The MagnICON system has been tested at various production scales, from a few plants in a laboratory to 1.2 tons of plant material/day in industry. Collectively, they demonstrated that very high level accumulation of RPs (up to 5 mg per g leaf fresh weight (LFW)) can be achieved within 7–10 days after infiltration (dpi). They include RPs of all sizes and complexity, ranging from small subunit vaccines to large tetravalent antibodies [16, 17, 26–29].

In spite of the success, the current MagnICON system cannot produce RPs with more than two heterosubunits. This problem is associated with the phenomenon called "competing replicons," as codelivery of viral vectors built on the same viral backbone often results in early segregation and subsequent preferential amplification of only one of the vectors in a single cell [25]. For example, TMV and PVX are both competing viruses, but not with each other. If the heavy (HC) and light chain (LC) genes of a MAb are both cloned into the TMV or PVX vector, only one of the chains will be produced in a single cell and assembled MAb will not be produced. However, if the HC and LC gene are built on the TMV and PVX backbone, respectively, both chains can be expressed in the same cell, permitting their proper assembly into a functional MAb. [26]. This allows the MagnICON system to produce MAbs. However, identifying additional

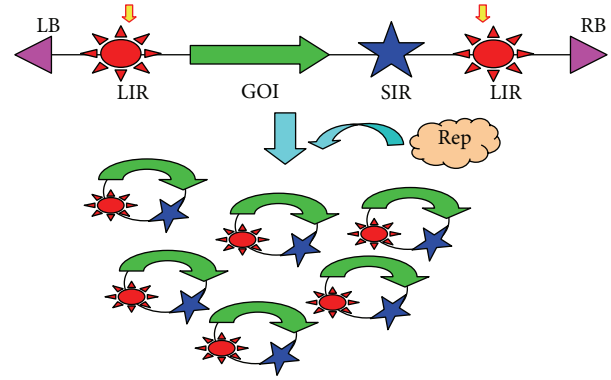

Figure 3: Geminiviral BeYDV vector for expression of recombinant proteins. The left (LB, pink triangle) and right border (RB, pink triangle) delineate the T-DNA construct that will be transferred into plant cells by *Agrobacterium*. Upon delivery into plant cells, expression of Rep gene produces the Rep protein (brown cloud) that nicks the LIRs (red stars) in the T-DNA to release a single-stranded DNA molecule (between the two yellow arrows). This DNA molecule recircularizes and is copied to make double-stranded DNAs that can replicate by the rolling circle mechanism to produce very high copy numbers of DNA templates (circles) and, in turn, abundant mRNAs of gene of interest (GOI) for the translation of the recombinant protein. Blue star: SIR; pink triangle: LB and RB of the T-DNA; red stars: LIRs; green arrow: gene of interest; brown cloud: Rep protein.

viruses that are noncompeting with both TMV and PVX for expressing proteins with three or more distinct subunits is a very difficult if not impossible task [20, 30]. We have circumvented this problem by developing a noncompeting vector system based on bean yellow dwarf virus (BeYDV), a monopartite virus in the Geminiviridae family [31]. Upon infection of plant cells, very high copy numbers of BeYDV genome are produced by rolling circle replication, which requires only one single viral protein (replication associated proteins (Rep)) (Figure 3) [31]. In the first generation of geminiviral vectors, the transgene and the Rep protein are supplied in two separate modules [19, 31]. Coagroinfiltration of the two modules resulted in high-level accumulation of RPs, which can be further increased by including a third module carrying a suppressor of gene silencing from TBSV (P19) [19]. For example, we showed that inclusion of the third P19 module increased the accumulation of hepatitis B core antigen (HBcAg) in *N. benthamiana* >4-fold [21]. Southern and Northern blot analyses indicated that codelivery of P19 only marginally increased the replicon copy number but greatly enhanced the accumulation of HBcAg-specific mRNA [21]. These results indicate that P19 indeed can increase target mRNA and protein accumulation, most likely by suppressing posttranscriptional silencing of the transgene. We then integrated the transgene, Rep, and P19 modules into a single vector system and demonstrated that the geminiviral system is noncompeting and permits the efficient expression and assembly of MAbs [32]. For large-scale manufacturing, we developed a single vector system that contains multiple replicon cassettes with each encoding for a distinct protein. Upon

Table 1: Examples of recombinant proteins produced in plants by agroinfiltration.

Plant host	Vector	Biologic target	Development stage	References
N. benthamiana	Nonviral vector	Influenza A H5N1 HA VLP vaccine	Phase I/II human trial	[38, 39]
N. benthamiana	TMV	NHL personalized vaccine	Phase I human trial	[37, 40]
N. benthamiana	CPMV	BTV 4-component VLP vaccine	Preclinical	[30]
N. benthamiana (WT, ΔXF)	TMV/PVX	Tetravalent antibody WNV therapeutic	Preclinical	[28, 41]
N. benthamiana	TMV	Cellulases for ethanol production	Early development	[1, 42]
N. benthamiana	TMV/PVX	Ebola immune complex-based vaccine	Preclinical	[27, 43]
N. benthamiana	TMV, BeYDV, CPMV	HBcAg nonenveloped VLP vaccine	Preclinical	[19, 44]
Lettuce, N. benthamiana	TMV/PVX, BeYDV	Ebola therapeutics based on MAb	Preclinical	[18, 32, 34, 45]
Lettuce, N. benthamiana	TMV, BeYDV	Norovirus NVCP VLP vaccine	Preclinical	[19, 34, 46]
Lettuce, N. benthamiana	TMV/PVX, BeYDV	WNV therapeutics based on MAb	Preclinical	[17, 28, 34]
Lettuce, N. benthamiana (WT, ΔXF)	TMV, BeYDV	WNV DIII vaccine	Preclinical	[31, 34, 47]

HA: hemagglutinin; VLP: virus-like particle; NHL: non-Hodgkin's lymphoma; BTV: bluetongue virus; WT: wild-type; ΔXF: plants with double knockdown of β-1,2-xylose and core α-1,3-fucose; WNV: West Nile virus; HBcAg: hepatitis B core antigen; MAb: monoclonal antibody; NVCP: Norwalk virus capsid protein; DIII: domain III of envelope protein.

agroinfiltration into plant cells, each cassette was shown to assemble into an independent replicon and produces high levels of the protein/subunit it codes for, without competing with the replication of other replicons or the production of other proteins [32]. This single vector system obviates the need to generate several vector modules and manufacture multiple inocula of *A. tumefaciens* strains, further reducing capital and operational cost. Recently, a different geminiviral vector based on the mild strain of BeYDV-m has been developed and has shown its robustness in expressing two vaccine candidates [33]. These geminiviral systems may have broader plant host ranges than the MagnICON system [18, 19, 32, 34]. Overall, the geminiviral replicon system overcomes the difficulty of producing multiple heterosubunit proteins.

Other examples of deconstructed viral vectors include systems based on 5′ and 3′ untranslated regions (UTRs) of cowpea mosaic virus (CPMV) RNA-2 and tobacco yellow dwarf *Mastrevirus* (TYDV) [35, 36]. One version of the CPMV-based vectors is replication independent and, therefore, has great promise for agroinfiltration in plant hosts that are not compatible with replication-dependent vector systems. Excitingly, the CPMV-based vector has allowed the expression and assembly of Bluetongue VLPs in *N. benthamiana* that requires coexpression of four different protein components [30]. These plant-produced VLPs were shown to be immunogenic and provide protective immunity in sheep against a challenge of a Bluetongue virus field isolate, demonstrating the utility of this vector in producing complex and heteromultimeric proteins [30]. The TYDV-based vector system represents "bridge" vectors that allow the stable inheritance of the transgene and a robust yet controlled transient expression of a RP upon the induction with a specific chemical signal [36]. Plants are allowed to accumulate biomass in the growth phase while the integrated transgene remains silent and replicon amplification will be triggered upon induction for RP production. This type of bridge vector system effectively combines the strengths of both the stable and transient expression systems and potentially offers a complete platform for the rapid assessment of RP candidates and their transition to a large-scale commercial production.

3.3. Plant Hosts for Agroinfiltration. A prerequisite for a plant species to be successfully agroinfiltrated is its susceptibility to *A. tumefaciens* infection. Among susceptible plants, however, the amenability of different species to agroinfiltration varies significantly due to leaf structural differences in the cuticle, the density of stomata on the epidermis, and the compactness of mesophyll cells. Due to technological improvements, a rapidly expanding spectrum of plant species is now amenable for transgene delivery by agroinfiltration. Since transient expression systems for RP production do not generate transgenic plants, it does not have the risk of contaminating food crops or unintended transgene escape. This further expands plant hosts infected via agroinfiltration as an acceptable technology to the public and regulatory agencies. The choice of a particular plant host for protein expression is determined by its compatibility with available expression vectors, the nature of the target RP, and the scale of production.

3.3.1. Nicotiana Hosts. The most popular host plant for agroinfiltration is *N. benthamiana* and related *Nicotiana* plants including tobacco. Besides being most amenable to agroinfiltration, these plants can produce large amount of biomass rapidly and are prolific seed producers for the industrial scale-up of production [20]. In addition, they are permissive to the replication of a variety of replicon-based vectors. The FDA and other regulatory agencies are familiar with clinical trial materials from these plant hosts, thus facilitating their acceptance in regulation-compliant processes [29, 37]. As a result, numerous RPs of various natures, sizes, and applications have been produced in *Nicotiana* hosts (Table 1). The examples below demonstrate the advantages and versatility of utilizing these plant hosts for agroinfiltration.

In a clinical trial, *Nicotiana* host-produced biologics were used to treat Non-Hodgkin's lymphoma (NHL). NHL is

a group of blood cancers that is estimated to result in over 70,800 new cases in the USA alone in 2014. In NHL, each malignant B cell clone expresses a unique cell surface immunoglobulin (Ig) as the tumor-specific marker, making standard treatments ineffective. The variable nature of NHL calls for patient-specific cancer treatments that require an expression system with the flexibility to rapidly produce patient-specific vaccines. One of these vaccines consists of MAbs derived from each patient's own tumor. The cell culture-based production platforms do not have the speed and flexibility to produce these personalized vaccines. In contrast, plant production systems based on agroinfiltration can provide the optimal platform to meet this demand. Results showed that 20 patient-specific MAbs were produced at high levels in *N. benthamiana* leaves within two weeks of agroinfiltration [29]. The manufacturing process is robust, requiring only two weeks for MAb-based vaccine expression and purification and less than 12 weeks from biopsy to vaccination [29]. To test the safety and immunogenicity of the plant-expressed vaccine candidates, a phase I human clinical trial was initiated with 12 patients. Results indicated that the vaccine was well-tolerated without major side effects and 73% of the patients developed a tumor-specific immune response [37, 40]. This study demonstrated the rapidness and versatility of the agroinfiltration-based transient system in generating multiple patient-specific cancer vaccines and showcased the capacity of *N. benthamiana* in producing vaccines that are safe to administer and effective in the treatment NHL patients.

Nicotiana plant hosts were also utilized for commercial scale enzyme production. In the production of ethanol as a fuel extender, large quantities of cellulase are needed to saccharify cellulosic feedstocks. For more than 30 years, the high cost of cellulase from fungal fermentation has been a major impediment to the economic viability of cellulosic ethanol programs [48]. To reduce the cost of cellulase, *N. benthamiana* was used as a host to produce four cellulases for cell wall degradation via agroinfiltration [42]. Results showed that all four cellulases were expressed at high levels, up to 75% TSP [42]. Further analysis indicated that plant-produced cellulases are functional, efficiently converting cellulose to glucose [42]. Similar results were obtained between using syringe and spray agroinfiltration, indicating the scalability of the upstream process [42]. Importantly, the necessity of purification and costs associated therewith are avoided in the downstream processing, as the cellulases can be simply preserved at room temperature for up to four months in dehydrated *N. benthamiana* biomass as silage [42]. Technoeconomic analysis of a similar cellulase production system based on transgenic *N. tabacum* concludes that the plant-based system may offer a >30% reduction in unit production costs and an 85% reduction in the required capital investment compared with the current fungal-based fermentation system [49]. We speculate that the system based on spray agroinfiltration may result in a similar cost-saving benefit, presenting a system of cellulase production with unprecedented efficiency and cost-effectiveness. This process can find broad applications for production of other cost-sensitive RPs.

N. benthamiana hosts also offer opportunities to produce RPs with enhanced functionalities (biobetters). For example, *N. benthamiana* with "humanized" glycosylation pathways have been developed to enhance the safety and efficacy of plant-produced MAbs [50]. The difference in N-glycosylation between plant and mammalian-produced MAbs may alter the stability and/or efficacy of plant-produced MAbs or cause potential adverse effects through immune complex formation. To overcome this challenge, a double knockdown (ΔXF) *N. benthamiana* line was created to suppress the production of the two plant-specific glycans: β-1,2-xylose and core α-1,3-fucose [51]. Results indicated that anti-Ebola MAbs produced in the ΔXF plant line had no plant-specific N-glycans but contained the highly homogenous (90%) mammalian glycoform GnGn [52]. The lack of fucose and the high homogeneity of plant-derived MAbs have led to their higher affinity to the Fc receptor (FcgRIII) and their enhanced potency against Ebola virus over the mammalian cell-produced MAbs [52]. The superior potency of plant-produced MAbs was further demonstrated in a challenge study with nonhuman primates, in which plant-produced MAbs were far more protective against a lethal Ebola challenge than those produced in mammalian cells [45]. In a remarkable and exciting development, these plant-made MAbs were recently used to treat two American Ebola patients and showed promising results [53]. Similarly, our studies showed that ΔXF plant-derived anti-WNV MAbs displayed enhanced viral neutralization in comparison to their mammalian counterparts [28]. These new *N. benthamiana* hosts are being applied to produce biobetter RPs beyond the realm of MAbs.

3.3.2. Non-Nicotiana Hosts. Certain RPs require a non-*Nicotiana* plant host for their optimal expression. Fortunately, improvements in technologies have allowed the application of agroinfiltration to many plant species beyond *Nicotiana* plants, including lettuce, tomato, alfalfa, petunia, potato, cotton, grapevine, switchgrass, radish, pea, lupine, flax, citrus, lentil, sunflower, and *Arabidopsis* [11]. Agroinfiltration methods have also been applied to woody trees including aspen, poplar, birch, eucalyptus, pines, and spruces [13]. In addition to leaf tissue, petals of tobacco, petunia, *Antirrhinum majus*, *Gerbera jamesonii*, several species of *Dendrobium* flowers, and the fruits of tomatoes and strawberries have also been successfully agroinfiltrated with transgene constructs [54].

Among these options, lettuce is a prime example to demonstrate the special utility of non-*Nicotiana* hosts in producing RPs. Despite the aforementioned advantages of *Nicotiana* hosts, they do produce unusually higher levels of phenolics and alkaloids than other plant species. These compounds foul purification resins and are difficult to remove from the target RP in downstream processing, adding to production resources and costs [55, 56]. This is especially problematic for RPs with pharmaceutical applications, as they need to be free of these plant compounds to meet regulations of the FDA. Thus, there is a need to identify plant hosts that produce lower levels of phenolics and alkaloids yet retain the robustness of RP production. Lettuce (*Lactuca sativa*) is already cultivated commercially in large scales and its yield and speed of biomass generation can easily match those of *Nicotiana* plants. Lettuce produces negligible quantities of phenolics

and alkaloids and thus would overcome the challenge of their removal during downstream processing. Agroinfiltration with nonviral vectors indicated that lettuce expresses a variety of functional RPs, albeit the expression levels were low [57, 58]. To further demonstrate the potential of lettuce as a host for RP production, we explored the use of deconstructed viral vectors to express pharmaceutical proteins in lettuce (Table 1). We first examined the expression of a VLP vaccine candidate for norovirus based on the capsid protein of Norwalk virus (NVCP) with geminiviral vectors. NVCP was expressed at levels which are comparable with that in *N. benthamiana*, at the highest expression levels ever reported for a vaccine in lettuce [34]. Furthermore, lettuce-produced NVCP efficiently assembled into VLPs with a diameter typical of native NVCP VLPs [21, 34]. Moreover, these VLPs are fully functional and can induce potent immune response in mice [21]. These studies demonstrated that lettuce is as robust as *Nicotiana* plants for RP production with agroinfiltration. Beyond that, this study also demonstrated the superiority of lettuce over *Nicotiana* plants for expressing VLPs. Owing to their structural resemblance to native viruses but lacking infectious viral genomes, VLPs have been shown to have tremendous potential in immunogenicity, multivalency and safety as vaccines against many diseases [20]. However, the porous and dynamic nature of the VLP structure also makes it susceptible to trap contaminant molecules inside. As a result, it is a very difficult task to remove plant secondary metabolites from the feedstream of *Nicotiana* plant-produced VLPs [16]. If not resolved, this problem will diminish the vast potential of VLPs and plant transient expression technology. Due to the low level of secondary metabolites in lettuce tissue, NVCP VLPs can be purified to high purity from lettuce extracts without the extra need for elimination of phenolics and alkaloids [34].

The advantage of lettuce has also been showcased for the production of MAbs, another group of proteins with high pharmaceutical relevance. MAbs can be efficiently produced in several plant hosts. For example, MAbs against Ebola virus and WNV can be expressed at very high levels in *N. benthamiana* with MagnICON and geminiviral vectors [17, 32]. These plant-produced MAbs are fully functional in protecting animals from lethal challenge of viral infections [17]. In addition to the native MAbs, large MAb variants such as tetravalent antibodies and recombinant immune complexes have been successfully produced in *N. benthamiana* [27, 28, 41, 43]. The most popular and efficient method of purifying MAbs is Protein A affinity chromatography. As a RP itself, Protein A is costly and can be easily damaged by a cleaning reagent. Unfortunately, phenolics and alkaloids in *N. benthamiana* feedstream foul Protein A resins and are hard to remove from the target MAb. Consequently, extra purification steps are required to remove these secondary metabolites from the feedstream before loading to Protein A resin [17]. Moreover, frequent cleaning of resins with harsh reagents is necessary to prevent fouling [59]. These extra measures complicate downstream processing, shorten the life span of protein A, and add extra capital and operational cost for MAb production. Our results indicate that the ammonium sulfate precipitation step for MAb purification, which is partially responsible for removing secondary metabolites from tobacco extract, can be bypassed due to the negligible amounts of plant compounds in the lettuce feedstream [18, 34]. Thus, lettuce extracts containing MAbs can be directly loaded onto the Protein A column, avoiding resin fouling concerns [18, 34]. As a result, the life cycle of Protein A resin is prolonged and the overall production cost of MAbs is reduced.

Our success in producing functional VLPs and MAbs with commercially produced lettuce demonstrated another advantage of lettuce as a host for large-scale agroinfiltration. Since agricultural and food industries have already established the infrastructure and technology needed for large-scale lettuce growing and processing, they can be rapidly adapted for the production of RPs. This suggests that biomass production could be subcontracted to existing commercial growers. This will forego the need for capital investment of purpose-built biomass facilities but allows access to potentially unlimited quantities of inexpensive plant material for large-scale manufacturing of RPs. Besides lettuce, other plant species are being explored as hosts for agroinfiltration to allow for the production of RPs with unique properties.

4. Conclusions

The development of deconstructed viral vectors has reinvigorated the field of plant-made RPs and provided a production platform with superior protein yield, speed, scalability, versatility, safety, and cost-saving benefits. A recent breakthrough in plant glycoengineering allows plants to produce RPs with tailor-made N-glycans and expands the utility of plants in developing biobetters with superior functional and safety profiles. The lack of a scalable technology to deliver transgene into plant cells was one of the remaining hurdles for the commercial application of plant transient systems. As discussed in this review, this challenge has been overcome effectively by various agroinfiltration technologies. We believe that further optimization of agroinfiltration technologies will expedite the acceptance of plant transient expression systems for the commercial production of a broad range of RPs.

Abbreviations

RP: Recombinant protein
GMO: Genetically modified organism
cGMP: current good manufacturing practice
TSP: Total soluble protein
LFW: Leaf fresh weight
dpi: Days postinfiltration
BeYDV: Bean yellow dwarf virus
TMV: Tobacco mosaic virus
PVX: Potato virus X
TBSV: Tomato busy stunt virus
VLP: Virus-like particle
NHL: Non-Hodgkin's lymphoma
WNV: West Nile virus
BTV: Bluetongue virus
MAb: Monoclonal antibody.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Diabetes and Stem Cell Function

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Diabetes mellitus is one of the most common serious metabolic diseases that results in hyperglycemia due to defects of insulin secretion or insulin action or both. The present review focuses on the alterations to the diabetic neuronal tissues and skeletal muscle, including stem cells in both tissues, and the preventive effects of physical activity on diabetes. Diabetes is associated with various nervous disorders, such as cognitive deficits, depression, and Alzheimer's disease, and that may be caused by neural stem cell dysfunction. Additionally, diabetes induces skeletal muscle atrophy, the impairment of energy metabolism, and muscle weakness. Similar to neural stem cells, the proliferation and differentiation are attenuated in skeletal muscle stem cells, termed satellite cells. However, physical activity is very useful for preventing the diabetic alteration to the neuronal tissues and skeletal muscle. Physical activity improves neurogenic capacity of neural stem cells and the proliferative and differentiative abilities of satellite cells. The present review proposes physical activity as a useful measure for the patients in diabetes to improve the physiological functions and to maintain their quality of life. It further discusses the use of stem cell-based approaches in the context of diabetes treatment.

1. Introduction

Diabetes mellitus (DM) is one of the most common serious metabolic diseases that has spread all over the world, and the number of people with diabetes has continued to grow in recent years. The patients with DM represent a hyperglycemic state induced by impairments in insulin secretion (type 1), insulin action (type 2), or both. Type 1 diabetes mellitus (T1DM), which accounts for less than 10% of patients with diabetes, is characterized by an immune-mediated destruction of pancreatic β cells in the pancreatic islets of Langerhans, leading to insulin deficiency [1]. It is well known that T1DM is developed in childhood and can lead to severe long-term complications such as retinopathy, neuropathy, and nephropathy, as well as macrovascular diseases, including cerebral, coronary, or peripheral vascular systems [2]. On the other hand, type 2 diabetes mellitus (T2DM), which accounts for over 90% of patients with diabetes, occurs through mechanisms such as insulin resistance in peripheral tissues and increased blood glucose levels induced by overnutrition associated with the deficiency of insulin secretion [3, 4]. DM is often associated with secondary complications that affect multiple organs such as the eyes, kidneys, heart, brain, and skeletal muscle [5].

The central nervous system is notably affected by diabetes. DM has been reported to induce pathological alterations in the nervous system, resulting in the onset of cognitive deficits and an increased risk for vascular complications in the brain [6]. Furthermore, it has been demonstrated that DM is associated with vascular dementia, depression, and Alzheimer's disease (AD) [7–11]. These disorders may be caused by morphological changes, such as white matter leukoaraiosis, as well as hippocampal, cortical, and amygdala atrophies, in the brains of the patients with DM [12, 13]. Additionally, the skeletal muscle is critically influenced by diabetes. It has been reported that DM induces skeletal muscle atrophy [14–16] and fiber-type transition from oxidative to glycolytic [17, 18]. Moreover, the impairment of energy metabolism has been observed in diabetic skeletal muscles [19, 20]. These alterations lead to skeletal muscle dysfunctions, such

as muscle weakness and exercise intolerance [16, 21]. Among the multiple factors that can cause the disturbances to the central nervous system and skeletal muscle function, one of the candidates is stem cell dysfunction in DM. Neural stem cells (NSCs) are self-renewing multipotent cells that generate neurons, astrocytes, and oligodendrocytes in the nervous system [22]. It has been reported that the proliferative abilities of NSCs are declined in the hippocampus of T1DM model animals [23, 24]. NeuroD1 is a basic helix-loop-helix transcription factor that promotes neurogenesis [25]. The neurogenesis of NSCs is impaired through the inhibition of the NeuroD1 transcription factor expression in DM [26]. Similar to NSCs, the proliferation and differentiation of skeletal muscle stem cells, termed satellite cells, are attenuated in diabetic muscles [27, 28]. The stem cell dysfunction may induce the impairment of cell turnover, resulting in the disturbed functions of the brain and skeletal muscle in DM.

This review will focus on the alterations to the central nervous system and skeletal muscle in diabetes, including the function of NSCs and satellite cells. Furthermore, we will attempt to clarify the effects of exercise as diabetes prevention and therapy on the brain and skeletal muscle in diabetes.

2. The Alteration of Neurogenesis in Diabetes

2.1. The Central Nervous System in Diabetes. In both human and animal models, DM is associated with pathological changes in the nervous system that lead to cognitive deficits and to an increased risk for vascular complications in the brain [6]. Patients with diabetes, especially older adults, apparently face a greater risk of vascular dementia. However, according to large population studies, DM is also associated with depression and AD [7–11]. Numerous studies have reported cognitive deficits in both T1DM and T2DM patients, who show slowing of information processing speeds and worsening psychomotor efficiencies and deficits in vocabulary, attention, and memory [5, 29, 30].

Morphological changes have been identified in patients with both T1DM and T2DM diabetes [12, 31–34]. These include global subcortical and cortical atrophies and white matter leukoaraiosis (local white matter intensity changes observed in MRI images). Diabetic patients are more prone to developing extensive and earlier leukoaraiosis [12]. People with more advanced leukoaraiosis are at an increased risk for cognitive impairment and dementia [32, 33]. Also, leukoaraiosis is usually found in the brain scans of elderly people, especially those over 80 years old; therefore, diabetes has been considered to accelerate the aging process. Magnetic resonance imaging (MRI) has also demonstrated that T2DM patients have hippocampal and amygdala atrophies when compared with control subjects [13]. The hippocampus, where the atrophy is found in AD, is responsible for learning and memory functions, suggesting that diabetes is a risk factor for AD.

2.2. Adult Neurogenesis. Neurogenesis in the mammalian brain is a multistep process that includes the proliferation of neural progenitor cells, fate determination, migration, neuronal maturation, and the functional integration of newborn cells into the existing neuronal circuitry (Figure 1). Although neurogenesis almost completely ceases after birth, recent studies have demonstrated that it still takes place constitutively at low levels in the adult brain of mammals including rodents, primates, and humans [35–40]. NSCs are present and continuously generate functional neurons specifically in the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus. NSCs born in the SVZ become proliferating neuroblasts and migrate through the rostral migratory stream (RMS) into the olfactory bulb. Neuroblasts then become immature neurons and integrate into local interneurons. In the DG of the hippocampus, the proliferating neuroblast cells become immature neurons and project their axons into the CA3 region. The neurons eventually differentiate into mature neurons and are integrated into the preexisting hippocampal circuitry as functional granule cells. Recent studies show that newly formed neurons are incorporated into the functional networks of both the OB and the DG, which suggests significant effects of adult neurogenesis on brain functions associated with learning, memory processing, and odor discrimination [41–45].

In the SVZ and SGZ neurogenic niche, there are astrocytes, endothelial cells, astrocytes, ependymal cells, oligodendrocytes, and mature neurons. A transplantation study indicated the importance of the neurogenic niche in determining the fate of adult NSCs. Neural stem cells derived from the adult hippocampus or spinal cord can give rise to neurons after grafting into the DG, but not into the spinal cord [46]. Moreover, recent work by Song et al. [47] has suggested that specified microenvironments provide the unique neurogenic niche for adult neurogenesis. In this study, astrocytes from the hippocampus provided signals that instructed the adult NSCs to differentiate into neurons, but the astrocytes from the adult spinal cord could not. This evidence suggests that the local environment dictates the fate of adult NSCs, and that astrocyte-derived soluble and membrane-bound factors promote neurogenesis. Other components of the neurogenic niche have been also been identified as supporting neurogenesis, such as endothelial cells [48], microglia [49], and the vascular system [50]. Particularly astrocytes regulate neurogenesis by secreting factors such as Wnt3 and a major proinflammatory cytokine, interleukin-1b. Therefore, the microenvironments of the SVZ and SGZ, but not other brain regions, are thought to possess specific factors that allow the differentiation and integration of new neurons. A variety of molecules serve as niche signals to regulate the maintenance, activation, and fate choice of adult NSCs, including Notch, BMPs, Shh, and Noggin, growth and neurotrophic factors, and Wnts [51]. Through intrinsic and extrinsic factors, adult neurogenesis is tightly regulated in order to allow for the maintenance and self-renewal of the stem cell pool and the generation of fully functional neurons.

2.3. The Role of Wnt and Insulin/IGFs Signals in Adult Neurogenesis. Several studies have demonstrated the role of Wnt signaling in adult neurogenesis. For example, Wnt3 is strongly expressed in the astrocytes of the neurogenic niche. NSCs

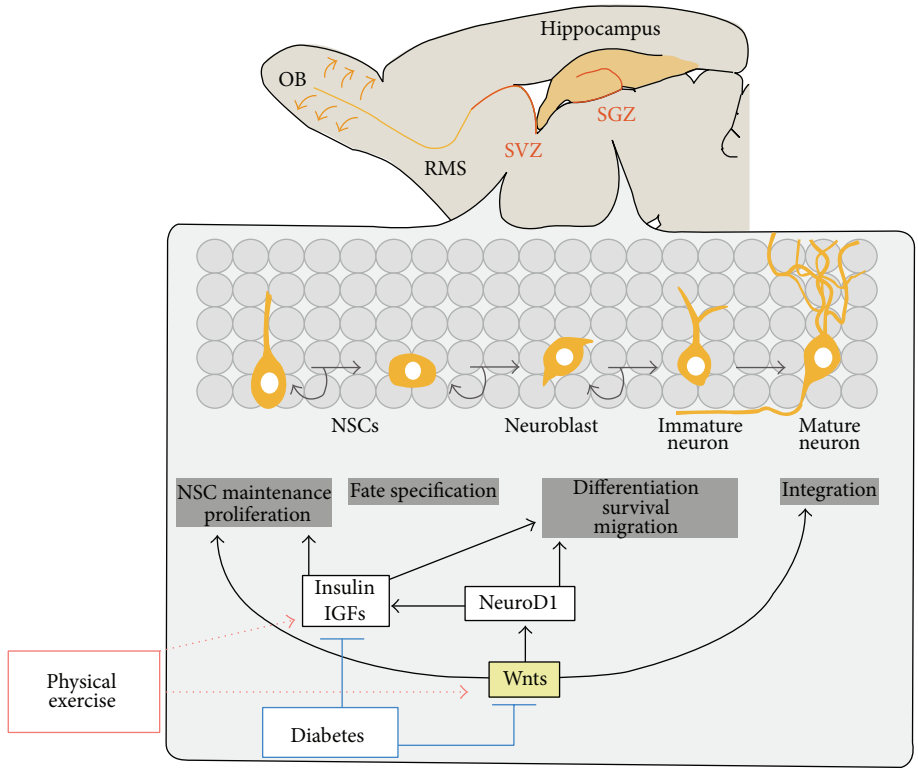

Figure 1: The schematic diagram of regulation of adult neurogenesis by insulin/IGFs and Wnt signals. Adult neural stem cells (NSCs) are primarily located in two distinct regions of the brain: the SVZ of the lateral ventricles and the SGZ of hippocampal dentate gyrus. In the SGZ, adult NSCs undergo proliferation, fate specification, maturation, migration, and eventual integration into the preexisting neural circuitry. In the SVZ, adult NSCs give rise to neuroblasts, which migrate into the olfactory bulb through rostral migratory stream (RMS) and differentiate into mature local interneurons. The progression of NSCs to mature neurons in adult SVZ and SGZ is multistep process with distinct stages and is controlled by insulin/IGFs and Wnts. Diabetes inhibits insulin/IGFs and Wnts signaling in adult neurogenesis, which lead to the decline of adult neurogenesis, while physical exercise may recover diabetes-induced inactivation of Insulin/IGF and Wnt signaling. NSCs, neural stem cells, SVZ, subventricular zone, and SGZ, subgranular zone.

expressed major components of the canonical Wnt/β-catenin pathway [52, 53]. Therefore, NSCs could receive Wnt signals and stimulate the canonical Wnt/β-catenin pathway. The coculture study of NSCs with the hippocampus astrocytes also showed that astrocyte-derived Wnts stimulated neuroblast proliferation and neuronal differentiation in adult hippocampal NSCs through the Wnt/β-catenin pathway [52]. In addition to the astrocyte-derived Wnts, there is an autocrine Wnt signaling in hippocampal NSCs as several Wnts are expressed in the hippocampal NSCs [53]. Interestingly, the inhibition of the autocrine Wnt stimulation promotes neurogenesis and reduces the multipotent progenitors, which indicates that the Wnt autocrine pathway promotes differentiation into neurons, but it also helps in the maintenance of the stem cell pool. The injection of a lentivirus vector expressing Wnt3 or a dominant negative Wnt into the DGs of mice also showed that the activation of Wnt signaling increased the adult neurogenesis, while the inhibition of the Wnt signaling reduced neurogenesis significantly. Furthermore, the inhibition of the Wnt signaling in the DGs of adult rats also demonstrated the impairment of spatial memory and object recognition [54]. These results indicate the profound role of Wnt signaling in adult neurogenesis and its involvement in the cognitive function.

Interestingly, NeuroD1, one of the major targets of Wnts, is selectively expressed in dividing neural progenitors and in immature granule neurons in the adult DG, but not in Sox2-expressing hippocampal NSCs. Furthermore, Kuwabara et al. demonstrated that the NeuroD1 promoter could bind to Sox2 and TCF/LEF, the major downstream transcription factor of the Wnt/β-catenin pathway thorough *in silico* analyses of NeuroD1 promoter. According to the study, it is suggested that NeuroD1 transcription is activated by Wnts through TCF/LEF in NSCs, which allow neurogenesis to proceed, while its transcription is silenced by Sox2 that inhibited neurogenesis [25]. In using NeuroD1 conditional knockout (KO) mice, Gao et al. demonstrated that NeuroD1 is required for neurogenesis in the adult hippocampus *in vivo* and *in vitro* [55]. Therefore, Wnt-mediated neurogenesis requires NeuroD1 in adult hippocampal neural progenitor cells. According to these studies, the activation of the canonical Wnt pathway accumulates β-catenin, which induces the transcription of

NeuroD1 in the NSCs and therefore induces neuronal differentiation. On the other hand, Sox2+ multipotent progenitor cells silence NeuroD1 transcription and maintain undifferentiated NSCs. Our recent study also demonstrated that insulin is synthesized in adult hippocampal neurons, but also in adult NSCs derived from the hippocampus and OB by the induction of neuronal differentiation [56]. According to the study, NeuroD1 is shown to activate the insulin gene expression directly in NSCs from the adult hippocampus and the OB. Thus, under the Wnt3 activation, NeuroD1 promotes neurogenesis but also induces insulin production in adult OB and hippocampal NSCs.

In addition to Wnt3, recent studies provided the evidence that other Wnt factors are involved in the adult neurogenesis. Erickson et al. investigated that Wnt7a has a function to promote the proliferation and self-renewal of adult NSCs through the canonical Wnt signaling pathway in neurogenic regions of the adult brain [57]. Overexpression of Wnt3a and Wnt5a in the adult SVZ was shown to promote the proliferation and neuronal differentiation of adult neural progenitor cells *in vitro* [58]. The stabilization of β-catenin by retrovirus-mediated expression was also shown to promote the proliferation of neural progenitor cells in the SVZ *in vivo*, resulting in increased neurogenesis in the OBs [59]. Thus, the regulation of Wnt signaling proves to be essential for adult neurogenesis (Figure 1).

Several studies have reported that insulin/IGF signaling has important role in controlling differentiation of NSCs [56, 60–63]. The activation of insulin/IGF signaling stimulates the proliferation of neural stem cells in the undifferentiated state, induces the differentiation of oligodendrocytes, and increases the survival of neurons and oligodendrocytes [60]. Additionally, IGF-1 signaling is necessary for neuroblast migration from the SVZ, as a result of neuroblast accumulation in the SVZ and improper migration to the OB in IGF-1 KO mice [64]. Therefore, insulin/IGF signaling is necessary for the maintenance of NSCs, cell fate specification, and migration and survival of neurons.

2.4. Impairment of Adult Neurogenesis in Diabetes. The hippocampal formation is clearly recognized as being involved in learning and memory. Increasing evidence has shown that diabetes may be associated with deficits in learning and memory. In a pharmacologically induced model of T1DM diabetes, streptozotocin- (STZ-) induced diabetes consistently decreased hippocampal cell proliferation in rodents [23, 24, 65–69]. Through the incorporation of BrdU, immature neurons were demonstrated to decrease significantly in STZ-induced animals [23, 24], which indicates that neuronal differentiation is downregulated in STZ-induced diabetic rats. In addition, the proportion of mature neurons after STZ-induced diabetes in rats was shown to be either decreased [24] or unchanged [67]. In summary, STZ-induced T1DM models consistently decreased hippocampal cell proliferation and survival, and in some investigations the models were also negatively affected by neuronal differentiation. Nonobese diabetic (NOD) mice are another model of T1DM diabetes, which spontaneously develop T1DM through the autoimmune destruction of the pancreatic β cells [70]. Similar to the STZ-induced T1DM model, NOD mice showed decreased hippocampal cell proliferation [71, 72] and reductions in neuronal differentiation [72]. Diabetic NOD mice, as well as NOD mice that did not develop diabetes, showed significantly lower levels of cell survival than the controls [71]. Interestingly, neuronal survival was also more increased in the NOD mice that did not become diabetic than in NOD mice that became diabetic at 15 weeks of age, but the rate of cells becoming neurons did not differ between the nondiabetic and diabetic NOD mouse groups. This suggests that the impairment of hippocampal neurogenesis is expected in the NOD mice long before the diabetic features are apparent, and the mice that eventually develop diabetes will exhibit greater reductions in neurogenesis.

Hippocampal neurogenesis has been studied in a number of animal models of T2DM including genetic models in mice and rats, such as the *db*/*db* mouse, the Zucker diabetic fatty (ZDF) rat, the Goto-Kakizaki (GK) rat, and an environmental model of high-fat diet-induced obesity. Both the *db*/*db* mouse and ZDF rat are leptin-receptor deficient and are used as models of obesity complicated by diabetes. The GK rat is a polygenic model with elevated blood glucose, peripheral insulin resistance, a nonobese phenotype, and the exhibition of many degenerative changes observed in human T2DM. The reduction in adult neurogenesis in these models has been demonstrated in many studies [73]. For example, ZDF rats have decreased hippocampal cell proliferation and neuronal differentiation compared to their lean, nondiabetic controls as measured by Ki67 or doublecortin immunoreactivity [74]. Similarly, *db*/*db* mice also demonstrated decreased hippocampal cell proliferation in the diabetic mice compared to the control group [67, 75]. According to these studies, the hippocampal neurogenesis is severely impaired in T2DM.

Diabetic mice with decreased hippocampal proliferation also showed cognitive deficits, as demonstrated by various hippocampus-mediated behavioral tests, including the Morris water maze, novel object recognition, and novel object placement [65, 67, 69]. STZ-induced T1DM models showed deficits involving learning and memory by measuring the Morris water maze and novel object recognition tests [67]. The STZ-induced diabetic rats spent less time identifying the novel object than the control group in novel object recognition, which implies that the STZ-induced diabetic rats incorrectly identified a novel object as familiar. The STZ rats also showed significant deficits in learning the location of the hidden platform in the Morris water maze. Similar deficits have also been observed in *db*/*db* mice [67]. The *db*/*db* mice showed impairments in novelty discrimination when compared to the control, as well as a significantly impaired performance in the Morris water maze. Altogether, the impairment of hippocampal neurogenesis in diabetes accompanied with cognitive deficits in animal models is similar to diabetic patients.

Our previous study provided evidence that the Wnt signaling pathway and NeuroD1 expression are both inhibited in the hippocampus and OBs of STZ-induced diabetic rats [26]. Immature neurons migrate from the SVZ to OBs and become mature neurons. The inhibition of Wnt signaling and

NeuroD1 in both neurogenic niches of STZ-induced diabetic animals suggests that the impairment of neurogenesis in diabetes is the result of the inhibition of Wnt signaling (Figure 1). In addition, the inhibition of the Wnt signaling in the DG of adult rats leads to the impairment of spatial memory and object recognition [54]. The inhibition of Wnt signaling may also be attributed to the cognitive deficits in diabetes.

2.5. Neurodegenerative Diseases and Diabetes. Neurodegenerative diseases are typically progressive late-onset disorders that lead to impairments in cognition and/or motor function. These diseases share similar features including an abnormal accumulation of protein, including plaques and tangles in AD, Lewy bodies in Parkinson's disease, and nuclear and cytoplasmic accumulations in polyQ diseases like Huntington's disease (HD). Diabetes has been identified as a risk factor for neurodegenerative diseases such as AD and HD.

T2DM has been specifically identified as a risk factor for AD, which is most likely linked to an impairment of insulin signaling in the brain. AD is an age-related neurodegenerative disease associated with the increased production and aggregation of amyloid-β (Aβ) peptides and intracellular neurofibrillary tangles of the hyperphosphorylated tau protein in the brain [76]. Recent studies provided the evidence that the insulin receptor and its signaling activities are reduced in the brain with AD [77, 78]. In addition to the impairment of insulin signaling, the brain/neuron-specific insulin-receptor KO mice exhibited a substantial increase in the phosphorylation of the microtubule-associated protein tau, a hallmark of neurodegenerative diseases [79]. In addition, the treatment with antidiabetic agents, including glucagon-like peptide-1 (GLP-1) receptor agonists, exendin-4, and liraglutide, which are approved for the treatment of T2DM, has been shown to facilitate insulin signaling and sequential reductions in the endogenous levels of Aβ in the brain and prevent hippocampal neuronal death [80]. The GLP-1 has been shown to enhance cognitive performance in rodents [81], thus suggesting a protection from neuronal loss and cognitive deficits in AD. Based on these investigations, insulin resistance in AD may contribute to the disease pathophysiology.

HD is an autosomal, dominantly inherited, neurodegenerative disorder characterized by neurological, cognitive, and psychiatric symptoms. Diabetes frequently develops in HD patients [82] and in transgenic mouse models of HD such as the R6/2, HD-N171-82Q mice [83, 84]. The R6/2 mice exhibited cognitive impairments [85] and deficits in the replication of β cells and insulin secretion [86], which indicates that HD is expressed not only in neurons but also in the pancreatic islets. Recent studies have demonstrated that hippocampal NeuroD1 expressions were impaired in R6/2 mice [87] and a significant reduction in hippocampal cell proliferation has been observed in R6/2 mice and another HD model mice, R6/1 [88], suggesting the impaired neurogenesis-induced cognitive deficits.

Interestingly, NeuroD1 is known to be expressed in pancreatic β cells and to play an essential role in endocrine pancreatic development, as NeuroD1 KO mice exhibited severe diabetes and died perinatally [89]. Furthermore, our previous study provided the evidence that Wnt3 is expressed in pancreatic α and its expression is decreased in STZ-induced diabetic rats [56]. Because Wnt3-induced NeuroD1 is a critical factor for neurogenesis and pancreatic development, it is suggested that the cognitive deficit and impairment of insulin secretion in diabetes, AD, and HD are due to the impairment of Wnt3-induced NeruoD1 activity. In summary, recent studies suggest that insulin accelerates AD-related pathologies through its effects on the Aβ metabolism and tau phosphorylation. In addition, HD exhibits the similar diabetic features of insulin secretion deficiency and cognitive deficits. Insulin regulates peripheral energy homeostasis but it also helps the proliferation and differentiation of neuronal precursor cells in the brain. As there are many functions of insulin, the impairment of insulin function leads to multiple deficits in both the brain and the peripheral tissues that could be associated with other neurodegenerative diseases.

3. The Alteration to the Satellite Cell Function by Diabetes

3.1. The Response and Adaptation of the Skeletal Muscle to Diabetes. The skeletal muscle is the most important organ for insulin action; therefore, the impairment of insulin action can induce various changes in the former including structural, functional changes. The main structural change in skeletal muscles induced by diabetes is muscle atrophy. It is well known that the diabetic muscles lead to the loss of muscle mass. For example, the myofiber diameters of the soleus and extensor digitorum longus in STZ-induced diabetic rats, which is the model of T1DM, decreased by about 30% compared to the control rats [90]. Further studies demonstrated that diabetes induces skeletal muscle atrophy [14–16]. Furthermore, there is increased protein degradation along with decreased protein synthesis in the skeletal muscles in STZ-induced diabetic rats [91], which may be responsible for the loss of muscle mass in diabetes, and muscle atrophy is observed in the T2DM patients. Huang et al. evaluated the skeletal muscle masses of T2DM patients using MRI and showed the reductions in the muscle masses of DM patients [92]. Pedersen et al. also demonstrated that the weight of the appendicular skeletal muscle is significantly lower in DM patients than in the controls [93]. These results suggest that T2DM leads to muscle atrophy, as with T1DM.

Additionally, diabetes induces a muscle fiber-type transition in the skeletal muscle. The skeletal muscle is composed of muscle fibers, which are roughly classified into three types: type I, type IIa, and type IIb [94]. A type I fiber is a slow-twitched oxidative fiber, a type IIb fiber is a fast-twitched glycolytic fiber, and a type IIa fiber is an intermediate fast-twitched oxidative glycolytic fiber [94]. In general, the muscle fiber type shifts from a fast fiber to a slow fiber with increased activity of the skeletal muscle, such as electrostimulation and exercise, whereas inactivity of the skeletal muscle, such as casting and denervation, leads to the transition from a slow fiber to a fast fiber [94]. In the diabetic condition, the shift between muscle fiber types from slow to fast is due to

an inactive condition. Hickey et al. demonstrated that DM patients have a significantly lower percentage of type I muscle fibers compared with the control subjects [17]. Oberbach et al. also showed a 16% reduction in slow-fiber fraction and a 49% increase in fast-fiber fraction in diabetic patients [18]. These results may indicate that the oxidative capacity is reduced in diabetic skeletal muscles. In addition to the fiber-type transition, diabetes induced a variety of alterations to the physiological systems in the skeletal muscle, such as vascular changes [95–98], neuropathy [99, 100], and ultrastructural changes [101].

These alterations to the skeletal muscle's structure by diabetes are often associated with reductions in muscle function. Previous studies have demonstrated that diabetes induces muscle weakness and a decline of exercise performance. Regensteiner et al. evaluated exercise performance using a graded treadmill protocol, concluding that the exercise times of the DM patients were significantly lower than those of the sedentary subjects [21]. Kamei et al. demonstrated that FOXO1 transgenic mice, which have insulin resistance, also have a lower exercise tolerance compared to the controls [16]. The diabetes-induced muscle weakness was caused not only by structural changes but also by functional changes in the skeletal muscle. Insulin-stimulated glucose uptake is important for muscle contraction. Thus, diabetes-induced impairment of insulin functions inhibits glucose uptake into the skeletal muscle, resulting in the disturbances to muscle contractions. Cameron et al. reported that diabetic muscles have 15–29% reductions in sciatic motor and sensory saphenous nerve conduction velocities [102]. In addition, DM also leads to a decline in muscle strength. Andersen et al. demonstrated that diabetic patients had 17% and 14% reductions in ankle flexor and ankle extensor strengths, respectively [103]. Sayer et al. also reported that the grip strengths of diabetic patients were significantly lower than those of the control subjects [104]. Moreover, DM induced reductions in endurance capacities. A reduced time to exercise exhaustion was shown in T1DM or T2DM patients compared with nondiabetic subjects [105, 106]. Altogether, diabetes-induced alterations to the skeletal muscle structure and function can lead to muscle weakness and a decline in exercise performance.

The skeletal muscle is the biggest organ to produce energy for biological activity. It is also well known that diabetes alters the energy metabolism in the skeletal muscle. It was observed that diet-induced thermogenesis, which is expressed as a percentage of energy intake, is reduced in DM patients [19], and the maximal oxygen consumption is reduced [20]. The reduced capacity of oxygen consumption may be one of the early phenomena of diabetes-induced energy metabolism disturbances [107]. The impairment of the energy metabolism in DM patient can be caused by multiple factors, and one of the candidates is the functional capacity of mitochondria, which produces adenosine triphosphate (ATP) through respiration and regulates cell metabolism in the skeletal muscle. The mitochondria protein synthesis rate and mitochondria enzyme activities are reduced in DM patients [108]. Furthermore, it was reported that the storage of glycogen in the diabetic skeletal muscle was significantly reduced compared with the controls [109], which is caused by the impairment of glycogen synthase activity. There is a report that glycogen synthase I activity and the content of glucose 6-phosphate, which is an intermediate metabolite produced by hexokinase in the first step of glucose uptake, were significantly increased after insulin injection into the skeletal muscles of nondiabetic rats, whereas a reduced reaction was observed in those of diabetic rats [110]. These results suggest that diabetes induces a disturbance to the glucose metabolism in the skeletal muscle. In addition to glucose metabolism, diabetes leads to impaired lipid oxidation and protein degradation (negative protein balance) [111, 112], resulting in the impairment of energy metabolism.

3.2. Skeletal Muscle Stem Cells: Satellite Cells. The skeletal muscle is an abundant source of adult stem cells. Skeletal muscle-specific stem cells, termed satellite cells, contribute to the postnatal maintenance, growth, repair, and regeneration of the skeletal muscle [113]. Satellite cells are characterized anatomically by their location between basal lamina and plasma membranes of muscle fibers and functionally by their myogenic differentiation [114]. In adult skeletal muscles, satellite cells are in a quiescent state under normal physiological conditions. However, in response to muscle injury or exercise, satellite cells are activated and then can proliferate, undergo self-renewal, and differentiate into mature new fibers [115]. A previous study investigated the contribution of satellite cells to skeletal muscle regeneration using satellite cell-depleted mice, and then the satellite cell-depleted skeletal muscle could not be repaired after injury [116]. Thus, satellite cells are essential for the regeneration after a muscle injury.

Satellite cells demonstrate two states in a skeletal muscle turnover: a quiescent state and an activated state (Figure 2). Both quiescent and activated satellite cells express a characteristic marker, Pax7 [117], whereas only activated satellite cells also express Myf5 and MyoD, which are key transcription factors of myogenic lineage progression and differentiation [117]. Although most activated satellite cells proliferate and differentiate through downregulation of Pax7, others withdraw from the cell cycle and return to a quiescent state [118]. The transcription factor Pax7 upregulates the Myf5 expression through the recruitment of the histone methyltransferase (HMT) complex, and then the complex directly methylates histone H3 lysine 4 (H3K4) on the promoter of the Myf5 locus [119]. Pax7(+)/Myf5(+) coexpressing satellite cells upregulates the MyoD expression [120], and then MyoD initiates the transcription of myogenin and other muscle-specific genes [121]. MyoD may be a master regulator of myogenesis to induce the upregulation of muscle-specific gene transcription.

3.3. Impairment of Satellite Cell Function in Diabetes. Previous studies have shown that diabetes induces the impairment of satellite cell function. First, T1DM leads to an impairment of the regenerative capacity of the skeletal muscle caused by a decline in satellite cell function. Jeong et al. reported that satellite cells derived from STZ-induced diabetic mice fail in their abilities to form myotubes, resulting in an impairment in regeneration following a cardiotoxin-induced muscle

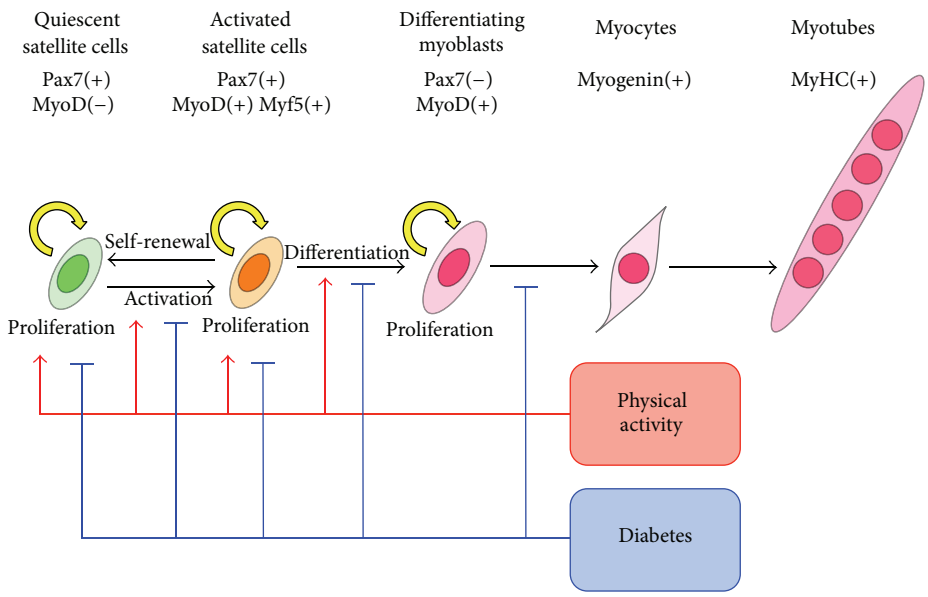

FIGURE 2: The schematic diagram of regulation of satellite cell activation and differentiation into myotubes. Adult skeletal muscle stem cells (satellite cells) are located between the basal lamina and the myofiber plasma membrane. Although satellite cells are mainly in a quiescent state, they are activated in response to muscle injury or exercise. Activated satellite cells can proliferate, undergo self-renewal, and differentiate into myoblasts and then to myocytes. Myocytes can mutually fuse and generate myotubes. The phases of satellite cell are determined by the expression of marker genes. Quiescent satellite cells express Pax7 (a stem cell-specific transcription factor) alone, whereas activated satellite cells coexpress Pax7, Myf5, and MyoD, which are key transcription factors for myogenic differentiation. Diabetes impairs satellite cell proliferation and activation, resulting in the inhibition of terminal differentiation. However, physical activity (exercise) induces satellite cell activation and improves its proliferative ability. Therefore, physical activity may recover the impairment of satellite cell function in diabetic skeletal muscle.

injury [122]. Aragno et al. showed that the MyoD and myogenin expressions are reduced in the gastrocnemius muscles of STZ-induced diabetic rats [27]. Furthermore, diabetic Akita mice demonstrated attenuated muscle regenerations following injury through an impairment of macrophage infiltration and satellite cell recruitment into degenerative fibers [123]. Although the investigations into satellite cells in T1DM remain poor, some evidences indicate that insulin action is important for maintaining satellite cell function. A better understanding of the alterations to the satellite cell population and function in T1DM are needed for the development of clinical therapeutics in muscle health.

Second, although the reports investigating satellite cell function in T2DM are limited, there are some investigations into the alterations to satellite cell function in the conditions of hyperglycemia and lipotoxicity. Hu et al. reported that insufficient muscle regeneration was observed after cardiotoxin injuries in mice fed high-fat diets for 8 months [124]. Woo et al. also demonstrated that 3 weeks of high-fat diet feeding induced a reduction in the number of satellite cells, as well as the impairment of muscle regeneration [125]. Furthermore, an *in vitro* study reported that satellite cells cultivated in a growth medium with a high glucose content tended to differentiate into adipocytes [126], suggesting that the myogenic capacities of satellite cells may be influenced by diabetes. Additionally, ZDF rats (typically used as model of metabolic syndrome) showed the reductions in satellite cell proliferations with no changed proportions of quiescent satellite cells [127]. This study also indicated that MyoD and myogenin protein levels decreased in the plantaris muscles of ZDF rats compared with the control lean Zucker rats [127]. Similarly, transgenic *ob*/*ob* and *db*/*db* mice, which are common mouse models of T2DM, displayed impaired satellite cell proliferation and muscle regeneration [28]. In addition to the myogenic potential, satellite cells derived from DM retain diabetic phenotypes, such as increased expressions of inflammatory cytokines [128], reduced lipid oxidation [129], disturbed glucose uptake [130], and insulin resistance [131]. These results suggest that T2DM and obesity promote various impairments of satellite cell function such as proliferation and differentiation.

Although it is clear that diabetes induces the impairment of satellite cell function, understandings of the molecular mechanisms remain insufficient. One of the candidates of the satellite cell dysfunction in DM is oxidative stress. In both T1DM and T2DM, oxidative stress in the skeletal muscle is elevated in association with glucose concentration [27, 132, 133]. Evans et al. reported that a redox imbalance is induced in the pathogenesis of diabetes and its complications [134]. Additionally, an *in vitro* study demonstrated that a culture of human satellite cells with ROS-inducing hydrogen peroxide (H_2O_2) reduced cell lifespans and proliferative capacities [135]. These results suggest that oxidative stress contributes to the impairment of myogenesis in diabetes. In addition to the oxidative stress, there are inflammatory factors as other candidates of the satellite cell dysfunction in DM. In T1DM and T2DM patients, the level of circulating interleukin-6 (IL-6), which is a proinflammatory cytokine playing an important

role in skeletal muscle metabolism through its receptor, is increased [136, 137]. A transient elevation of IL-6 leads to satellite cell proliferation, whereas a chronic elevation of IL-6 induces the impairment of satellite cell function [137]. Therefore, chronically elevated IL-6 in DM may be responsible for diabetic satellite cell dysfunction. In this manner, there are various factors of diabetic satellite cell dysfunction, whereas further investigation is needed for a better understanding.

4. Improved Function of Stem Cells by Exercise

4.1. Recovery of Adult Neurogenesis by Exercise. A number of studies have shown that physical exercise enhances the adult neurogenesis [138–144]. According to Van Praag et al., the voluntary physical exercises of young adult (3-month-old) mice promoted cell proliferation, cell survival, and neurogenesis within the DG [138]. Other studies also demonstrated the exercise-mediated increases in neurogenesis in the DGs of the hippocampus in young, adult, and aged animals [139–144]. Moreover, running has been shown to improve the cognitive functions in aged mice and humans [145–147]. Thus, it is suggested that exercise enhances adult neurogenesis, which may contribute to cognitive functions.

Although few studies have examined the effectiveness of exercise in adult neurogenesis in diabetes, these studies demonstrated that exercise has a positive effect on adult neurogenesis in STZ rats [148, 149]. Physical exercise had no particular effects on the blood glucose levels and body weights of diabetic rats. The reduction of hippocampal cell proliferation observed in STZ-induced diabetic rats has increased significantly through treadmill exercise as well [148]. In addition, physical exercise recovered the cognitive deficits in STZ-induced diabetes by measuring the novel object recognition task [149]. These studies provide evidence that physical exercise improves neurogenesis and cognitive deficits in diabetes, which suggests that physical exercise helps the recovery of diabetic complications in the central nervous system.

Exercise has recently been shown to modulate the expressions of genes involved in the Wnt signaling [150]. Moreover, running was found to increase the expression of Wnt3 in the astrocytes of the DGs significantly and to increase the population of Wnt3-expressing cells in young and aged mice [151]. Altogether, it is suggested that physical exercise in diabetes may promote neurogenesis through the activation of Wnt3 and Wnt signaling even though the role of Wnt3 in exercise-induced increases in neurogenesis in diabetes has not been studied yet (Figure 1).

4.2. The Response of the Diabetic Muscle to Exercise. The skeletal muscle has a high plasticity and it is affected by various external stimuli. Among a variety of external stimuli, exercise is the best measure to prevent muscle atrophy, because it brings about large effects and it can be performed relatively easily by many people. Many studies demonstrated that exercise causes skeletal muscle hypertrophy [152, 153], and the effects of exercise on the prevention of muscle atrophy have been reported [154, 155]. Fluckey et al. reported that resistance exercises inhibit the loss of skeletal muscle masses in rodents subjected to tail suspension, which is a model of muscle atrophy in which rodents have no grounding stimulation to their hindlimbs [154]. Fujino et al. also showed that low-intensity treadmill running has a protective effect on tail-suspension-induced muscle atrophy [155].

In addition to the preventive effects of muscle atrophy, exercise has some effects on diabetes prevention and therapy. It is well known that exercise increases the insulin sensitivity of the skeletal muscle in patients with T2DM [156]. Furthermore, Meex et al. reported the improvements in insulin sensitivity following exercise accompanied by improved mitochondrial function [157]. Consistent with these results, a number of studies demonstrated that endurance training improves mitochondrial function, maximal oxygen uptake, and insulin sensitivity in patients with T2DM [158–160]. However, it has been reported that the response to exercise is attenuated in the insulin-resistant patients. de Filippis et al. have shown that the exercise-induced upregulation of PGC-1α, which is a major regulator for mitochondria biogenesis, is significantly lower in obese subjects than in control subjects [161]. Similarly, some studies have reported the lack of training effects in patients with T2DM [162, 163]. Additionally, high-intensity training has similar effects on endurance training in the improvement of maximal oxygen uptake and mitochondrial function in a healthy skeletal muscle [164, 165]. In the patients with T2DM, two weeks of high-intensity training increased mitochondrial function and blood glucose profiles [166]. From the above, exercise may be a very useful measure to prevent alterations to skeletal muscle mass and functions in DM.

4.3. Recovery of Satellite Cells Function by Exercise. Although there are few studies about the effects of exercise on the satellite cells in diabetic skeletal muscles, it is well known that exercise has positive effects on satellite cells. Several studies demonstrated that the number of satellite cells increases after acute or chronic exercises [167, 168]. This increase in satellite cell numbers is also observed in human skeletal muscle [169]. The long-term effects of exercise on satellite cells are apparent in the skeletal muscle of well-trained power lifters, who have 70% more satellite cells than the control subjects [170]. The increased number of satellite cells after training gradually decreases during detraining [169], suggesting that a continuation of exercise is required for maintaining an abundant pool of satellite cells in the skeletal muscle. There is a report indicating that the intensity, rather than duration, of exercise is important for the accretion of the satellite cell pool [171]. As for the exercise style, the most effective method for increasing or maintaining the pool of satellite cells is still being investigated [172]. These results suggest that exercise may contribute to recovering the reduction of satellite cell numbers in DM.

It is reported that exercise is useful not only to the increase in satellite cell numbers but also to the satellite cell activation. Fujimaki et al. demonstrated that 4 weeks of voluntary wheel running induces the upregulation of Wnt signaling, which contributes to facilitating myogenesis and the activation of

satellite cells in the skeletal muscles of mice [173]. Consistent with this study, Aschenbach et al. reported that acute treadmill running induces the upregulation of β-catenin, which is a key transcription coactivator in Wnt signaling, and this is done through the downregulation of GSK-3β [174]. Armstrong and Esser suggested that functional overload, which is a model of resistance training and induced muscle hypertrophy, has revealed activated β-catenin in the plantaris muscle [175]. Using immunoprecipitation assay, Fujimaki et al. also showed that the exercise-induced upregulation of Wnt signaling directly modulates the chromatin structures of both the *Myf5* and *MyoD* genes and accelerates their transcription in adult satellite cells, resulting in increases in the mRNA expressions of *Myf5* and *MyoD* and the activation of satellite cells [173]. These results suggest that exercise may inhibit the disturbance of satellite cell function by DM, such as proliferation and differentiation, whereas the effects of exercise on satellite cells in diabetic muscles remain unclear.

5. Stem Cell Therapy for Diabetes

Embryonic stem cells are potential sources for insulin producing β cell replacement and these transplantations have been proposed as a potential treatment for diabetes. A previous study by D'Amour et al. showed preliminary success at β cell differentiation from human embryonic stem cells (hESCs) [176]. However, these cells were not functionally matured so that they coexpressed glucagon and insulin and failed to increase insulin secretion in response to high ambient glucose levels. Interestingly, implantation of these immature cells into mice promoted the generation of mature β cells and prevented STZ-induced hyperglycemia [177]. In addition, recent findings demonstrated that the generation *in vitro* of more mature β cells from stem cells is possible before transplantation. Using a complex multistep method, hESCs could generate β cells that are very similar to the cells in the human pancreas [178, 179]. Contrary to β cells from earlier studies, these differentiated β cells express insulin, but not other pancreatic hormones, and contain mature insulin granules. Although the latter were not functionally the same as the cells in the human pancreas, they were able to respond to repeated glucose stimulation with increased insulin secretion. Furthermore, transplantation of these cells into T1DM mice maintained normal blood glucose levels for several weeks. Recent progress in hESC research suggests the use of these cells as a potential approach for diabetic treatment in the future, but further studies are still required for their clinical use.

Additionally, it was shown that the transplantation of various stem cells into skeletal muscle may be useful for alleviating diabetic symptoms. Himeno et al. reported that transplantation of mesenchymal stem cell- (MSC-) like cells derived from mouse induced pluripotent stem cells (iPSCs) into hindlimb muscles inhibits the decrease in the capillary number in the transplanted hindlimb muscles of STZ-induced diabetic mice [180]. Okawa et al. showed that transplantation of neural crest-like cells derived from iPSCs into hindlimb muscles ameliorates the reduction of nerve conduction velocity, intraepidermal nerve fiber density, sensitivity to thermal stimuli, sciatic nerve blood flow, plantar skin blood flow, and capillary number-to-muscle fiber ratio by STZ-induced diabetes [181]. This study also reported that the engrafted cells produce growth factors: nerve growth factor, vascular endothelial growth factor, and basic fibroblast growth factor. Furthermore, Naruse et al. demonstrated that the intramuscular injection of endothelial progenitor cells derived from cord blood mononuclear cells into hindlimb skeletal muscles significantly inhibited the impairment of nerve and vascular function in the transplanted limb muscle of diabetic mice [182]. This group also reported that bone marrow-derived MSCs improved the diabetic polyneuropathy in skeletal muscle [183]. In addition, several studies showed that transplantation of human skeletal myoblasts (hSkMs) into hindlimb muscles improves insulin sensitivity and attenuates hyperglycaemia in KK mice, which is a model of T2DM [184, 185]. In conclusion, stem cell transplantation has the potential to become a useful method for treatment of DM, although more investigation is still needed.

6. Conclusion

The present review described the diabetes-related changes in various tissues as well as adult stem cell functions. In particular, the central nervous system and skeletal muscle are most affected by DM. In neuronal tissue, neurogenesis is attenuated in diabetes by the downregulation of Wnt signaling, and diabetes is associated with a number of neurodegenerative diseases. In the skeletal muscle, diabetes leads to a variety of structural, functional, and metabolic changes, such as muscle atrophy, muscle weakness, and the reduction in energy turnover. Satellite cell dysfunction is also induced by diabetes, resulting in a disturbance to myogenesis. Exercise is very useful for preventing in diabetes-related alterations to the neuronal tissue and skeletal muscle. The effects of exercise implicate the upregulation of Wnt signaling, resulting in the activation of neurogenesis in adult neuronal tissue and myogenesis in mature skeletal muscles. Although more investigation is required for a thorough understanding of diabetes-related changes and their biological mechanisms in a variety of tissues, this review proposes exercise as a useful measure for DM patients to prevent the negative effects of diabetes and to maintain their quality of life.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Early Implementation of QbD in Biopharmaceutical Development: A Practical Example

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In drug development, the "onus" of the low R&D efficiency has been put traditionally onto the drug discovery process (i.e., finding the right target or "binding" functionality). Here, we show that manufacturing is not only a central component of product success, but also that, by integrating manufacturing and discovery activities in a "holistic" interpretation of QbD methodologies, we could expect to increase the efficiency of the drug discovery process as a whole. In this new context, early risk assessment, using developability methodologies and computational methods in particular, can assist in reducing risks during development in a cost-effective way. We define specific areas of risk and how they can impact product quality in a broad sense, including essential aspects such as product efficacy and patient safety. Emerging industry practices around developability are introduced, including some specific examples of applications to biotherapeutics. Furthermore, we suggest some potential workflows to illustrate how developability strategies can be introduced in practical terms during early drug development in order to mitigate risks, reduce drug attrition and ultimately increase the robustness of the biopharmaceutical supply chain. Finally, we also discuss how the implementation of such methodologies could accelerate the access of new therapeutic treatments to patients in the clinic.

1. Introduction

Failure of new therapeutic candidates during development is unfortunately a very common occurrence. Recent estimates show that, on average, pharmaceutical companies seem to spend between four and eleven billion US dollars for every new therapeutic treatment that is eventually commercialised (Forbes—the truly staggering cost of inventing new drugs. http://goo.gl/C2KSB). The main reason for this is fundamentally the extraordinarily high rate of failure observed during drug development. Approximately 90% of drug candidates will fail during clinical development; maybe over 99% if preclinical stages of development are also included (PhRMA. http://phrma.org/) [1]. This level of failure is further compounded with increasing expectations from payers in terms of therapeutic outcomes and value for money. As a result, there is a growing interest in maximising the return on the investment made in the development of new therapeutic candidates, avoiding whenever possible late and expensive failures. However, the true reasons behind drug failure during development remain a highly debated and poorly understood issue for many, primarily due to the lack of detailed and up-to-date data on the subject. This occurs either because of lack of public data on the reasons behind development discontinuation, or because a combination of different elements often play a role in the demise of a particular drug candidate, making it difficult to identify specific contributing factors. Kola and Landis [2] and other analyses published since [3] have shed some light on the subject, suggesting a collection of different causes behind drug attrition.

Inadequate efficacy is perhaps "the" major single reason behind clinical failure, but other relevant causes include bioavailability and pharmacology shortcomings, safety and toxicology problems, or even stability and quality issues. Furthermore, strategic and commercial plans are scrutinised ever more closely as health care providers in many countries demand more value for their money. Discontinuation can, therefore, not only hint to problems with the design but also cost of goods, insufficient demand at the required pricing/available reimbursement, lack of competitive advantage over other products in the market or under development, and even insufficient available investment to complete development. Whereas the failure of new drug candidates during late clinical development and registration is primarily linked to inadequate biological activity and efficacy or pharmacology and dosage issues [4], attrition during early clinical development fundamentally relates to problems with safety (immunogenic reactions or hypersensitivity in biopharmaceuticals) and, less often, pharmacology. Preclinical drug attrition is a very complex area to survey, but manufacturing and quality issues related to product stability and even productivity are common problems observed.

The tragic consequence of the current fragmented approach to drug development is that key design elements that are essential for the success of new therapeutics can inadvertently be left out during discovery and early development stages of new drug candidates. Whilst strides have indeed been made in recent years to address at least some of these risk aspects early on in development, the methodologies employed are still far from being robust and efficient and many gaps do still exist [5]. Such gaps can often cause significant problems that are only discovered quite late in development. Severe delays can ensue, requiring additional investments or, in some cases, trigger the discontinuation of an entire drug development programme. Indeed, delays in development, reworkings, failed batches, or deviations are all frequent and costly issues observed during development and manufacturing of biological products and, in many occasions, can ultimately be traced back to poor design of the product candidate and/or manufacturing process.

From a biopharmaceutical development perspective, a significant financial commitment is made for the development of a qualified manufacturing process well before the product has even been cleared for its assessment in clinical trials. In fact, almost fully commercially defined processes are usually developed for "prototypes" (drug candidates) that, in a majority of cases, will fail at some point during development. Such investment is obviously at risk, subject to success at various preclinical and clinical development stages. In addition, potential manufacturing or safety concerns can also have a major financial impact in other ways:

(i) extend already long development timelines (reducing market exclusivity period);

(ii) require additional investment in process development, repeated work, or implementation of corrective measures;

(iii) prevent a programme from entering or progressing towards later stages of clinical development;

(iv) cause the failure of a programme during clinical trials, requiring a repeat of the trials or stop or delay final commercial approval due to quality or safety concerns;

(v) require considerable investment in process redesign and adaptation, reformulation, or even resolving product recalls.

These issues can be worth many millions in lost opportunities or investments lacking a return. In this context, it is desirable to select or design a successful candidate early on by asking the right questions.

2. Developability as an Intrinsic Part of QbD

The original definition of quality by design (QbD) by Juran and others [6, 7] established the importance of understanding customers' needs (customers defined very broadly) and designing product features and performance to satisfy those needs, as well as processes able to produce those features, not only in terms of manufacture, but also storage and distribution, utilisation, reimbursement, and so forth. As recognised by several authors, the implementation of QbD methodologies to the development of new therapeutics requires the definition of a quality target product profile (QTPP) as a basis for performance, and the identification of those quality attributes that are critical (CQA) and need to be controlled carefully to maintain product integrity and efficacy [8]. However, current QbD implementation, as defined by current ICH Q8(R2) and subsequent guidelines [9] (http://www.ich.org/) is primarily limited to manufacturing process understanding, but does not integrate product knowledge aspects, such as product design and product specifications for intended use.

In this context, "developability" can, in fact, be considered as an extension of QbD guidance, providing a bridge between "product knowledge" and "process understanding," addressing the influence of product characteristics in manufacturing and clinical outcome, and helping expand the design space for a drug candidate. We show how developability can be applied to early derisking and how it can be seamlessly integrated with both discovery and process development activities.

Any new therapeutic candidate needs to answer the following questions: can it be made (at the right cost)? Is it stable? Can it be formulated for the intended route of administration? Is it safe for patients? Can it access the target tissue/organ at the required dose and during an adequate time window? Will it produce the intended biological activity and show sufficient effectiveness in patients?

Even before a lead candidate is found, such requirements can be summarised in an intended performance profile, and from that profile one can derive the required characteristics that will help ensure the development of a high quality therapeutic candidate. In this context, developability addresses more than simply "purity" or "stability" aspects of the manufactured product. It also provides a platform to incorporate early on a solid basis for "product knowledge" and defines, right from the outset, a robust QTPP that would

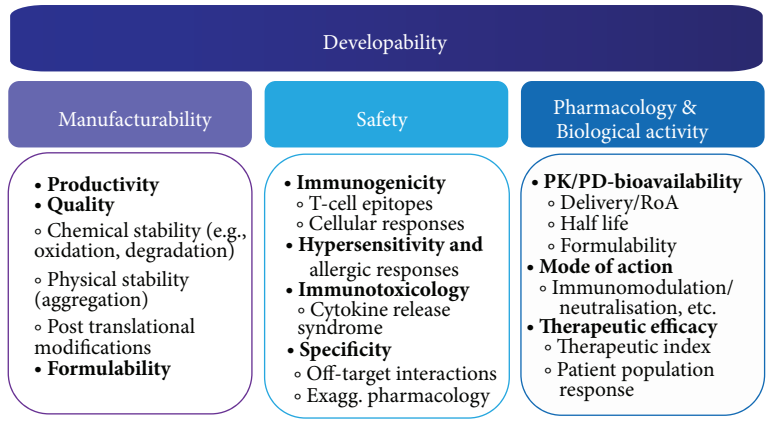

Figure 1: Developability rests in three main "Quality areas" or "pillars": Manufacturability, Safety-Toxicity, and Pharmacology & Biological Activity. Abbreviations: RoA: route of administration; PK/PD: pharmacokinetics/pharmacodynamics. Adapted from [5, 10].

greatly increase the odds of a successful, safe, and efficacious drug product (see ICH Q8(R2) for a definition of QTPP).

The subject of developability has been covered quite thoroughly in other publications [5, 10]. In short, the developability profile of a given new drug candidate is sustained by three "quality areas" or "pillars" that ultimately define its performance (Figure 1).

(i) *Manufacturability*: the main purpose of this type of assessment is to evaluate whether a given product can be manufactured with the expected quality characteristics, stability, and purity, at an assumable cost and able to be formulated for the intended route of administration.

(ii) *Safety*: biologics usually lack the toxic effects seen in small molecules, for example, associated to their metabolism, and so forth. However, immunogenic and hypersensitive reactions are a growing area of concern, as we will see later. Also, events associated with lack of specificity (off-target) or "exaggerated pharmacology" (on-target) can potentially compromise the therapeutic window for a particular product.

(iii) *Pharmacology & Biological Activity*: the third pillar consists of longstanding critical issues, which are becoming an important aspect for many biologics. For example, half-life, compatibility with specific formulations (i.e., sustained release) and routes of administration and "effective" concentration at target tissue are very important aspects that can influence the efficacy of a treatment. Also, early assessment of mode of action (particularly in immunomodulatory products) and patient segmentation and dosing can provide useful information that could potentially help designing clinical trials and increase likelihood of success.

These categories are also interrelated. Low stability can cause aggregation and thus safety issues (immunogenicity). Also, the ability of a product to be formulated for a specific route of administration can impact the bioavailability and pharmacology (and hence the efficacy) of a given candidate. Along with these "quality pillars," the QTPP will ultimately define what the requirements for a given product are, so it can be considered to be "fit-for-purpose" aligning with each of the areas just defined:

(i) *Fit for process*. It can be manufactured at the required scale using standard processes. It is sufficiently robust to endure process excursions without impacting significantly CQAs. It is stable enough to endure process and formulation requirements (Manufacturability).

(ii) *Fit for patient*. It achieves desired therapeutic outcome without compromising patient safety. Does not introduce potentially dangerous side-effects (Safety).

(iii) *Fit for indication*. It is suitable for required disease condition, dosing regime, patient population, route of administration, and circulating half-life (Mode of Action & Pharmacology).

3. Beginning with the End in Mind

3.1. Defining a QbD Workflow. The implementation of a developability risk assessment requires a good understanding of the intended product properties and performance. Figure 2 provides an illustration of how an ideal development workflow could be structured and how developability risk assessment sits at its core. One important aspect to notice is that, for this workflow to operate successfully, an adequate Quality Target Product Profile (QTPP) needs to be defined right at the outset of the drug development process (See ICH Q8(R2) for a definition of QTPP). This should be done during early discovery stages (product design) in order to formulate in the highest possible detail the intended performance, safety, and economic target profile, which will ultimately determine the product characteristics to aim for during the drug development process.

From this starting point, CQAs can be derived and a suitable developability risk assessment implemented to

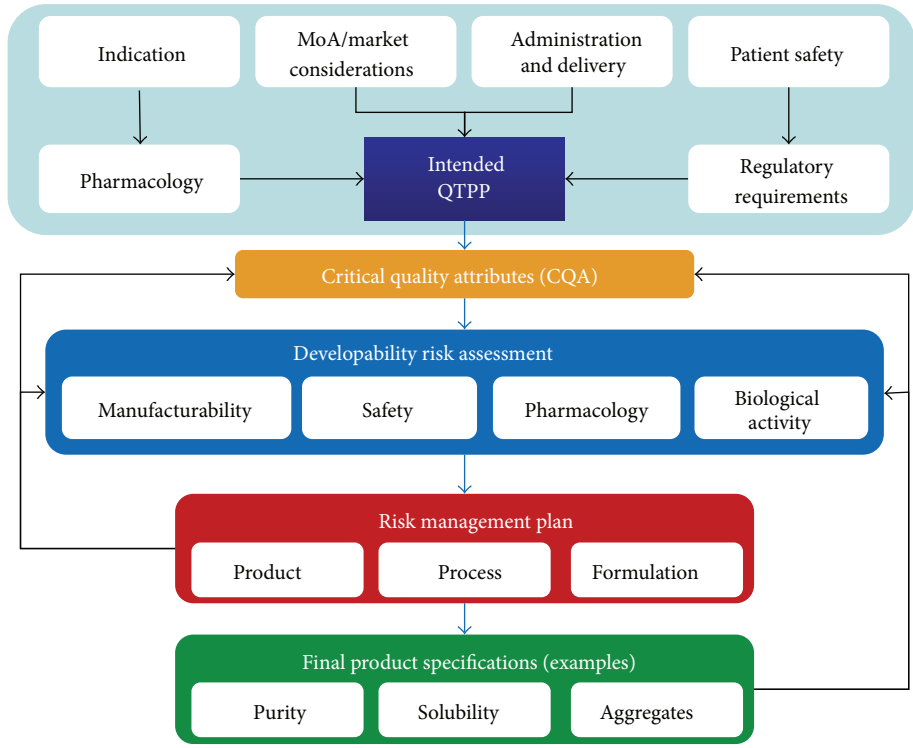

Figure 2: Developability flowchart. The flowchart illustrates how a development plan could be articulated to integrate effectively developability risk assessment tools. Setting an intended performance profile (QTPP) based on indication, pharmacology, mode of action, market, and delivery, amongst other considerations, allows the developer to determine the CQAs against which the lead candidate and process should be evaluated. A developability risk assessment would help identify specific risks impacting those CQAs and design and implement a risk-mitigation plan. This might involve modification in the selection or design of a lead candidate, potential reengineering (product), designing specific elements of the manufacturing process aimed to minimise or control risk, or perhaps some specific formulation requirements. All these steps will define the final product specifications in terms of measurable and controllable characteristics.

either derive optimal candidates matching the required CQAs or redesigning lead candidates that are able to match the target profile. Indeed, during the design stage these attributes should be mapped out and introduced or selected in the lead candidates but, of course, they should also be part of the design and optimization of the manufacturing process, so that such attributes can be properly controlled in an effective way. One might expect that the derisking methodologies introduced early on during candidate design and selection will, in turn, increase the robustness of the manufacturing processes, making it easier to control specific CQAs and minimising the incidence of deviations or out-of-specification (OOS) excursions.

The definition of a relevant QTPP is not a simple task. It does require the involvement of technical experts from multiple disciplines and areas of development (discovery, manufacturing, and clinical development), supply chain, distribution, and so forth. Most importantly, it should also incorporate key input needs and requirements from end-users or what in QbD nomenclature is known as the "voice of the customer." It is important to note that "end-users" or stakeholders should be defined in a broad sense to ensure success (in concordance with the definition of "Big Q" by Juran) [7] and should include patients, clinicians, payers, and health care provision agencies, as well as input from discovery, manufacturing, regulatory, supply chain and commercial functions.

QTPP characteristics will relate to the desired indication, patient population, drug target, and dosing regime; the route and method of delivery; the target indication and market; the manufacturing platform; the specific molecular format to be used; and the inherent properties of the product. It is important that the QTPP arises from consideration of the whole life cycle of the drug, from design and manufacturing to distribution and patient administration and, even very importantly, its potential utilisation in additional indications in the future that could incorporate very different specific requirements for the product.

3.2. Developability: A Three-Stage Process. A developability assessment programme basically consists of three different stages.

3.2.1. Risk Assessment. The simplest and most cost-effective way of assessing risk is by implementing computational approaches able to predict specific developability features by using the sequence of the biopharmaceutical candidates as a single input. These methodologies can have an extraordinarily high throughput and are relatively simple to implement.

As we will see later on, suitable proxy analytics (high throughput) can also be utilised for this type of assessment.

3.2.2. Implementation of a Risk-Mitigation Strategy. Depending on where in the process the risk assessment has been performed, different courses of action can be considered. In the case where process development (i.e., cell-line development) has not been initiated, two different routes can be explored: (a) selecting alternative candidates with a better risk profile and (b) redesigning a candidate to correct issues highlighted by the risk assessment [11]. If, by contrast, the product has already been taken into process development or in cases where reengineering is not an option, process-related interventions can help mitigate some of these potential problems. These could potentially include the screening larger numbers of clones during cell-line development or the utilisation of alternative downstream processes amongst others.

3.2.3. Validation of Course of Action. The developability risk-mitigation cycle is completed by introducing appropriate validation studies. For example, in the case of immunogenicity of biopharmaceuticals, candidates can be reengineered to eliminate the occurrence of specific T-cell epitopes in the sequence and then tested using relevant cell-based assays that make use of blood samples from human donors.

3.3. Developability Methodologies. There are a number of different methodologies that can be used to assess different developability aspects relevant to biopharmaceutical products [5]. Two main approaches involve the use of computational methodologies alongside suitable *in vitro* assays.

3.3.1. Computational Tools. The use of computational tools in early development is experiencing a growing attention due to their relative simplicity of implementation and flexibility, providing considerable benefits in terms of high throughput, low cost, and relatively short time of analysis. They can also be applied at any given point in time, given that they are usually not limited by material availability or assay constraints. These methodologies make it possible to begin building product understanding as soon as the sequence of a candidate is known. They offer a window onto properties that would otherwise not be available until much later in the manufacturing or clinical development process, and can help build quality into the product by selecting or designing lead candidates with favourable characteristics. Currently, there are a number of computational methods available for the prediction of immunogenicity (Safety) and physical and chemical stability (Manufacturability) of biopharmaceuticals, amongst other properties [5]. And we expect that in the near future, computational methods will also be able to assist in the design of purification protocols or formulation compatibility [12, 13].

3.3.2. Surrogate/Proxy Analytical Tools. Standard process analytics are often not "fit for purpose" in an early developability assessment context, primarily because of limitations in throughput, assay time, resource, or material requirements. Therefore, there is a drive towards methodologies that could potentially reduce material requirements by as much as 10^3-10^4 fold as well as increasing sample throughput by 10^2-10^3 fold. Obviously, these methods cannot provide the same level of information than that is achieved by standard analytical technologies. In many cases this will mean that a "surrogate" or "proxy" assay is sufficient to assess a given property for a product candidate. The analytical methods used in early-stage development are undergoing a rapid development towards miniaturisation and high-throughput analysis [11, 14–21] and their integration with early, rapid, and low-cost analytical and computational methods lie at the heart of the concept of "Developability." We have reviewed examples of such methodologies elsewhere [5].

3.4. Key Areas for Developability Risk Assessment

3.4.1. Protein Aggregation and Chemical Stability. Aggregation and degradation are two particularly important issues that can appear at various stages of biopharmaceutical development. They can affect negatively the yield and economics of the manufacturing process but also can impact the performance of the product and, ultimately, patient safety [22, 23]. From a manufacturing perspective, tackling aggregation and chemical degradation through process design can be complex and costly. In the clinic, the presence of aggregates in biopharmaceutical preparations can be harmful to patients [24], and also can increase immunogenic reactions in patients [25, 26]. Formulation and container-closure interactions with product can also enhance aggregation, with potentially devastating effects in patients [27–29]. Furthermore, besides aggregation, the incidence of chemical degradation or posttranslational modifications (PTM) can also have a negative impact on the immunogenicity and safety of biological therapeutics [30]. For example, some specific PTMs, such as abnormal (non-human) glycosylation, can increase the incidence of anaphylactic reactions to biopharmaceuticals [31].

Over the years, a number of different models have been developed to predict the intrinsic aggregation propensity of proteins, and many of them have been reviewed elsewhere [5, 32–35]. Aggregation prediction algorithms are generally useful when comparing the aggregation propensity of highly similar candidates (i.e., sequence variants of a parental molecule) and also for detecting and disrupting aggregation hot-spots through protein-engineering methods. However, it is still challenging to assess the aggregation risk of a given biotherapeutic in the absence of a reference protein of similar nature for which experimental aggregation properties are known.

We have recently developed an antibody-specific algorithm to predict aggregation, based on experimental data obtained by expressing several hundred of antibodies in a CHO-GS mammalian expression system and further validated in a collection of 50 unrelated antibodies with good predictability results [36]. This tool can be used to assign molecules to two different classes (*Low* and *High* aggregation risk), using sequence and structural descriptors as input. This classification uses a pre-defined cut-off calibrated experimentally as an indicator of relative process risks linked to

aggregation events during process development and manufacturing. Methods such as this are an important step in implementing high-throughput and inexpensive aggregation assessments that can be incorporated into a simple and actionable manufacturability risk.

Modifications in the chemical composition of biopharmaceutical products, whether due to cellular processes, enzymatic or chemical and degradation reactions, can result in a complex level of product microheterogeneity. It is estimated that up to 10^8 different species could be found in a single vial of a biopharmaceutical product [9]. An in-depth review of different types of chemical instabilities and PTMs can be found elsewhere [37, 38]. These include degradation pathways such as deamidation, oxidation, and isomerisation, as well as undesired glycosylation.

Many of these modifications are sequence-specific and can be predicted using computational approaches. However, generally speaking, not all the instances of chemical degradation or PTMs are equally relevant to the performance of a given biopharmaceutical product. For example, their proximity to the active site of the molecule or potential role in significant product degradation might increase their potential risk. For example, Asparagine deamidation and Aspartate isomerisation, two of the most commonly found chemical instabilities in antibodies, can either have very little impact on stability and functionality of the molecule or, in severe cases, can potentially cause loss of activity, high product heterogeneity, and promote aggregation and fragmentation. The incidence of these modifications is influenced by pH, temperature, sequence, and solvent accessibility [39]. These types of instabilities can potentially be managed by process control and formulation [40–42], but they may also, in some instances, require protein engineering due to their high impact on product quality [43].

Product heterogeneity and instability can also be the result of cellular processes such as glycosylation. Proper glycosylation is important not only to confer specific biological characteristics to a given biopharmaceutical, including its potency and pharmacological properties [44, 45], but it also can be a determinant factor in the adequate folding and assembly of a product. It also often defines other key attributes, such as stability, solubility, and immunogenicity [31, 46]. Undesired glycosylation can, in occasions, interfere with the biological activity of a biopharmaceutical. Furthermore, it is also important to mention that the presence of nonhuman glycans in a product is a known risk for hypersensitivity and anaphylactic reactions to biopharmaceutical products [47]. In addition, finally, chemical glycation during bioprocessing, due to reaction with sugars present in culture media, can potentially introduce product heterogeneity resulting in aggregation, stability and potentially immunogenicity issues. Susceptible sites for glycation can be predicted. However, forced glycation studies could be more useful in confirming not only susceptible positions, but also in helping to define the magnitude of the problem as well as determine the conditions that promote or prevent its occurrence.

3.4.2. Productivity and Yield. There is one important aspect not often recognised in biomanufacturing, and it is the relationship between productivity and product stability (primarily aggregation). As we have described before [48], protein aggregation and stability do, in fact, have many different "faces." Aggregation can also appear in the form of intracellular inclusions, low cell/culture viability, or low levels of productivity. This is primarily due to the fact that biological systems have developed an array of tools and systems specially tailored to prevent misfolding and aggregation. However, industrial requirements are often not properly matched to the capabilities of the biological platforms used in biomanufacturing. For example, in the case of mammalian cell hosts, upon the occurrence of a misfolding event, proteins are held in the endoplasmic reticulum and either pushed towards a refolding or a degradation pathway. Therefore, unstable products would naturally have a lower chance to be secreted. We have observed such behaviour particularly in mammalian systems, linking high-aggregation propensity with low productivity. Clonal selection can, occasionally, offset the intrinsic challenges contained within the polypeptide chain to be expressed. However, we typically observe a high degree of correlation between productivity and aggregation.

Figure 3 shows one example of such correlation in three different antibody families that were built from three different parental monoclonal antibodies by incorporating single and double mutations. Similar patterns have also been described by our group in instances where biopharmaceuticals had been reengineered to reduce their aggregation levels. We therefore believe that aggregation prediction could be potentially utilised as a surrogate for productivity levels, particularly in biopharmaceuticals expressed in mammalian systems. Furthermore, we and others have found a correlation between the amino-acid composition of specific areas of the antibody molecule and the productivity observed in mammalian systems. These observations open the door to the development of predictive platforms that could be used to assess product expression by means of computational tools [49, 50].

3.4.3. The Importance of Formulation: Formulability Assessment. Formulation and its impact in the delivery of biopharmaceuticals are gaining increased attention in the industry. Formulation can influence the pharmacology of the product and its efficacy, as discussed earlier, but also can have an important impact on other vital product attributes that are linked to patient compliance and even costs associated with a given treatment. For example, in some extreme cases, the costs associated with the infusion of a biopharmaceutical product, in a hospital and under specialised supervision, could surpass the cost of the product dose itself [51]. Therefore, there is a growing interest in formulations and delivery methods that could facilitate self-administration as well as increase patient compliance and reduce the total cost of treatment [52, 53]. Subcutaneous delivery presents a number of advantages compared to traditional infusion approaches. It is simpler, less invasive, reduces patients' discomfort, and can modulate the product pharmacology by facilitating a gradual/sustained release of the product. However, the delivery of a sufficient dose typically requires high product concentrations (100–200 mg/mL for a monoclonal antibody). The use of such high-concentration formulations introduces

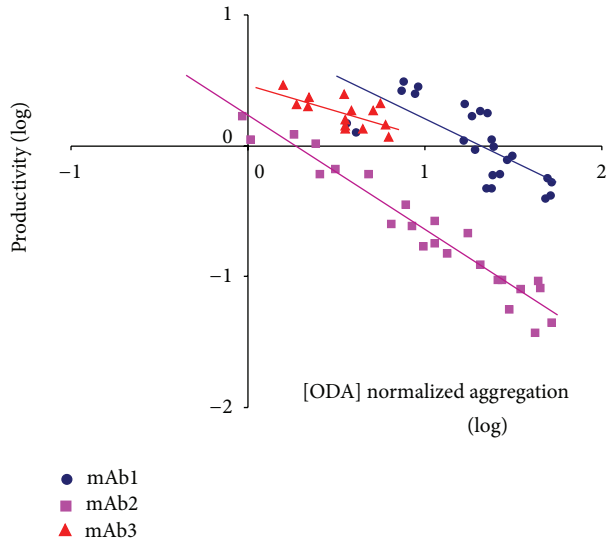

Figure 3: Correlation between antibody productivity and aggregation in three different antibody families: mAb1, mAb2, and mAb3. Variants were derived from three different parental antibody molecules by incorporating single and double sequence modifications. All different antibodies were expressed transiently under identical conditions to minimise any clonal variability in expression. Relative aggregation was assessed using Lonza's Oligomer Detection Assay, or ODA [11].

new challenges in the form of solubility constraints, high viscosity, aggregates, and phase separation that could make subcutaneous delivery unsuitable for a given product [21, 54].

Therefore, a good understanding of the suitability of a given product for a required formulation and RoA ("formulability") can be crucial very early on in product development. Moreover, "formulability" is also very important in other areas of development. For example, product losses are unfortunately common in cases where products are not stable in a given buffer or do not tolerate a specific pH range. This is also the case where products need to be concentrated during the manufacturing process. Drug-substance storage during downstream processing can require concentrations ranging from 25–200 g/L, because of limitations in volumes that a plant can store at a given time. This problem has been exacerbated by the increase in product titre that can be achieved in today's manufacturing platforms [55].

Formulability assessment is still a nascent area and we still lack simple platforms to assess the suitability of a given product candidate to be formulated at high concentrations (i.e., for subcutaneous administration) or compatibility with basic solution and process conditions. Formulation screening can be informed by computational methods in terms of aggregation propensity, long-term stability [56], and selection of excipients [57]. However, a number of high-throughput strategies have been proposed to assess protein stability at high concentrations as well as viscosity [15, 18–20, 58]. Furthermore, the use of computational methods can help make the formulation screening process more manageable. One interesting approach involves combination of machine-learning computational tools with high-throughput analytical tools, allowing the design of biopharmaceutical formulations with very limited product availability and early on in the development process [14]. Furthermore, computational methods are also useful in integrating measurements from different orthogonal analytical methods, potentially allowing the analysis of large data sets. Examples of this type of approach include Chernoff faces, star charts, and Empirical Phase Diagrams [59].

3.4.4. Safety in Biopharmaceuticals: Immunogenicity and Immunotoxicology. Biopharmaceuticals are generally considered to be relatively safe to patients when compared to small molecule therapeutics. However, their administration to patients can cause a number of undesirable side effects, usually related to pharmacology issues, mechanism of action or, more commonly, immunogenic reactions [60, 61]. Immunogenicity is often considered to be one of the principal safety concerns for biotherapeutics and one of the primary causes for attrition during early clinical development.

Current clinical data suggests that the majority of therapeutic proteins are to a variable extent immunogenic [62]. The generation of an unwanted immune response can negatively influence both the efficacy and safety of the therapeutic protein. Therefore, the incorporation of an immunogenicity assessment early on during preclinical drug development can significantly reduce the risk of generating an unwanted immune response in the clinic, which could potentially modify the pharmacology of the product or render it completely inefficacious. In extreme circumstances, biopharmaceuticals can also cause severe hypersensitivity, anaphylactic or immunotoxicology reactions that can put a patient's life at risk [31, 63–65].

In general, immune responses to therapeutic proteins are assessed in the clinic by monitoring the generation of antibodies raised against the protein. However, regulatory bodies encourage innovators to explore the use of preclinical methodologies that could give an early indication of immunogenicity risks to patients, including both *in silico* and *in vitro* methodologies [66–68].

Immunogenic responses to biopharmaceuticals (humoral or not cell-mediated) can be either T cell dependent or independent. T cell independent antibody responses are generated when B cells are able to recognise and bind to epitopes in the protein, but in the absence of T cell help these are generally low affinity, transient IgM antibodies. When a T cell response is also induced by the therapeutic protein then the antibody response can lead to high-affinity, long-lived IgG antibodies, which are much more likely to affect the safety and efficacy of the therapeutic protein in the clinic. Due to the importance of the T cell response in the development of long-lived, high-affinity antibodies, there is much focus on the identification and removal of T cell epitopes during the development of therapeutic proteins to reduce their potential immunogenicity risk.

During the last two decades a number of computational methodologies have been developed for the prediction of immunogenicity. Most of these tools assess the T cell epitope

content in proteins by predicting the binding specificities of peptide fragments from the protein of interest to HLA class II receptors. Such tools are reviewed elsewhere [69, 70]. *In silico* T cell epitope profiling tools can be efficiently applied during the lead selection and optimisation stages in three ways: (a) to rank protein leads based on their relative immunogenicity risk, (b) to identify specific peptides within a protein sequence with high immunogenicity risk, and (c) to guide protein reengineering by helping remove T cell epitopes, a process known as deimmunisation. The efficacy of many of these computational approaches has been validated in the lab using, amongst others, HLA binding assays or *ex-vivo* T-cell activation assays that we describe below. However, one common question often asked is whether such computational platforms are effective at predicting immunogenicity in a clinical setting. On one hand, most of such tools use HLA binding as the "main trigger" for immunogenic reactions; however, as we discuss in this paper, immune responses involve multiple cellular and humoral components and are subject to the influence of many different elements that would be impossible to encode in an algorithm in a simple way, including genetic and disease-related patient variability. On the other, validation of the efficacy of such algorithms would require testing many different protein molecules with controlled variations in sequence (and potential T-cell epitopes), standardised formulations, route of administration, aggregation content, and so forth in a sufficiently large number of patients providing a good coverage of different HLA halotypes and controlling any potential disease-related influence. Besides being ethically inadmissible by any regulatory agency, such trials would be extremely expensive for any standards. However this does not mean that some degree of validation is not achievable. For example there are studies confirming the clinical safety of biopharmaceuticals that were previously assessed using such computational methods [71, 72].

In vitro and *ex-vivo* cell-based assays have the advantage of being able to evaluate and characterize the immune response to a therapeutic protein in a fully human system, thus providing important information on the safety of the protein prior to first-in-man trials. Human *ex-vivo* cell-based assay platforms have the additional advantage of being able to assess much more than just the potential T cell epitope content of the primary amino-acid sequence. These assays can also include the analysis of any conformational epitopes (e.g., B cell epitopes), impurities (e.g., aggregates or particles), and contaminants (e.g., host cell protein, endotoxin) present in the protein sample. A number of fully human *ex-vivo* assay platforms, including T-cell activation assays, and so forth are currently being used to assess immunogenicity risk, and have been reviewed in more detail elsewhere [5, 70].

In all these assays, the source and quality of the human primary cells used for the *ex-vivo* assays are of critical importance. Donors should be selected to match the intended target population (e.g., a global population that would closely represent a Phase I clinical trial). Moreover, blood samples can be sourced from patients suffering from a specific disease indication or with a given genetic or ethnic background that could be relevant for the therapeutic agent being developed. For example, PBMCs can be sourced from patients suffering from rheumatoid arthritis to assess their response to a therapeutic protein being developed to treat this condition, thus taking into account both the immune status and genetic background (i.e., HLA allotype makeup) of the intended patient population. The use of PBMCs taken from patients with the targeted disease indication may ultimately be more representative of the type of immune responses that could be observed in subsequent clinical trials.

T cell assays are frequently used as a key indicator of the potential immunogenicity of a given product. T cell activation can be assessed by means of intracellular cytokine expression or cytokine secretion as well as cell surface activation marker and proliferation [73, 74]. In the case of T cell assays, the format of assay is very important, and a number of product-related factors should be considered when selecting the most suitable approach. These include the nature of the protein (e.g., peptides, antibodies, antibody fragments, novel protein scaffolds, fusion proteins, and recombinant proteins), mode of action of the protein (e.g., toxic or immunomodulatory proteins can interfere with some assays), and the purity of the protein (e.g., some assay formats are more sensitive to endotoxin and aggregates). Often an optimisation of the intended assay format is required to ensure that the most suitable assay format is being used for the therapeutic protein. Optimisation parameters often include protein dose, kinetics of the assay, and interference in the assay (e.g., coculture with a positive control to identify any inhibitory effects of the test protein).

There is increasing concern about the prevalence of preexisting antibodies to many of the novel protein therapeutics that are currently being developed. Many novel protein scaffolds and small antibody fragments are being modified to extend the half-life of the molecules. One such half-life extension technology is PEGylation, and there are recent reports showing that up to 20% of the healthy general population has detectable pre-existing antibodies to PEG [75, 76]. Some novel antibody scaffolds have also reported problems with preexisting antibodies in the clinic [77], leading to significant delays and increased costs associated with identifying B cell epitopes and reengineering the molecule. The prevalence of preexisting B cell responses against a given therapeutic protein can be assessed in PBMC samples from human donors to determine the production of antibodies that could cross-react with the therapeutic protein being assessed [78].

3.4.5. Aggregation and Immunogenicity. There is a well-documented link between the incidence of aggregation in biopharmaceuticals and observed immunogenicity in the clinic [26]. However, the majority of therapeutic proteins contain at least a low level of aggregates, and it is not currently known what type and amount of aggregation can pose a risk for increased immunogenicity [79]. Some examples of the relevance of aggregation in immunogenicity include erythropoietin or interferon. Eprex is a human erythropoietin (EPO) which underwent a formulation change that was subsequently linked to increased antibody formation to the endogenous form of EPO. This increased immunogenicity

was associated with the development of pure red cell aplasia (PRCA) in patients treated with this product. There have been multiple explanations of the reasons behind this immune response, but one of the most prevalent views seems to associate the incidence of PRCA with the increase of product aggregates upon changes in formulation and enclosure systems utilised in the manufacture of the product [28, 29]. Another example is IFNβ1a, prescribed for the treatment of Multiple Sclerosis (MS) in the clinic. Out of the two products currently registered for clinical use, Avonex and Rebif, the former seems to induce low levels of immunogenicity in patients (approximately in 2% of patients), whereas Rebif seems to be highly immunogenic (with approximately 25% of patients developing antibodies against the drug) in MS patients. In this particular case, the observed rates of immunogenicity can be linked to levels of aggregates found in each of the products, with Rebif exhibiting higher levels of aggregation than Avonex [80]. There is also recent data suggesting that the aggregation of monoclonal antibodies can lead to a significant change in the presentation of potential T cell epitopes by dendritic cells *in vitro* [81].

3.4.6. Preclinical Immunotoxicology and Hypersensitivity. A large proportion of therapeutic proteins both in commercial use and in development have a mechanism of action reliant, at least in part, on immunomodulatory activities. This also raises the risk of overstimulating the immune system and potentially increasing the chances of an immunotoxic response to the therapeutic protein. This was clearly seen during first-in-man trials for the anti-CD28 agonistic monoclonal antibody TGN1412, where a severe inflammatory response was induced in treated patients. This response included cytokine release syndrome (CRS, or "cytokine storm") and multiple organ failure. Subsequent studies have indicated that it was the CD28 agonistic activity rather than any sample contamination or errors in the manufacturing, formulation, dilution or administration of TGN1412 that led to the CRS response [63, 82–86]. In this particular case, preclinical studies both *in vitro* and *in vivo* failed to predict the induction of CRS, mainly due to suboptimal conditions using human PBMC *in vitro* and differences in the immune system between humans and primates *in vivo* [87]. More recently, a number of new *in vitro/ex-vivo* assays are currently being developed that seem to be able to detect CRS responses and, therefore, have been proposed as a new tool to assess this type of risk during preclinical development of therapeutic proteins [88].

Hypersensitivity and anaphylactic reactions to biopharmaceuticals can negatively affect patient safety and the development of new treatments. In some cases, such adverse reactions to some biopharmaceuticals could be linked to preexisting antibodies (IgA, IgM, IgG, or IgE) that recognise nonhuman epitopes present in the product, such as nonhuman glycoepitopes. Interestingly, it has been proposed that at least some cases of hypersensitivity to biotherapeutics could be associated with the presence, before the start of the therapy, of IgE antibodies able to react with the product [47]. All this suggests that this type of assay could perhaps help avert hypersensitivity or anaphylactic reactions before entering the clinic.

4. Targeting QTPP: Developability Applications

Developability methodologies can indeed have an important beneficial potential if applied during early stages of discovery, ensuring that the right quality attributes are designed into the chosen candidates for development. As indicated above, this requires the combination of both predictive computational tools and adequate surrogate or proxy *in vitro* methodologies. As we have discussed, there are areas where new developments are needed to produce better predictive approaches and ultimately a balance needs to be achieved between both approaches to maximise outcomes. We have discussed elsewhere how such a balance could be articulated at different stages of development and, more importantly how different decision-making tools could be defined to achieve effective solutions that would improve success during preclinical and clinical development [89].

We would like to illustrate, however, that reliance on different approaches could evolve during the different stages of development for a new therapeutic product. For example, during early stages of development of a biopharmaceutical (typically an antibody), large numbers of candidates are evaluated (e.g., binding candidates out of display libraries) and consequently a lot of data is generated. At these stages, the most effective strategy for a developability risk assessment would largely rely on the use of computational tools to categorise or flag (ideally automatically) high-risk candidates to help guide the selection of more favourable molecules to be taken into later stages of development. For example, the aggregation prediction tools described earlier could be used in combination with the assessment of potential chemical instabilities and immunogenicity risk scores (using T cell epitope prediction). This approach would integrate early on both physical and chemical stability together with immunogenicity risks in a simple, fast and inexpensive manner. Furthermore, as fewer and fewer different candidates move into successive stages of development, novel assessment approaches (*in vitro* and *in vivo*) become feasible, and as a result the amount of data generated using such approaches increases steadily (Figure 4). In this way, as the project moves further into preclinical development, computational assessments gradually transition towards experimentally verified data to assist in the selection of lead candidates to move into later stages of development.

Practical Case Studies. Below we describe the application of some of the developability tools described in this article to the selection and engineering of biopharmaceutical candidates with enhanced properties. We have chosen two different case studies to illustrate their implementation in different areas of risk. In both cases we comment on the application of the respective risk assessment, risk mitigation and validation steps and how both *in silico* and suitable surrogate or proxy *in vitro* methodologies can be combined in a unified workflow.

Table 1: QTPP, CQA, and design criteria derived for case study 1. In this particular case, previously known product shortcomings in terms of stability have been used to focus criteria around quality requirements for a hypothetical final product.

QTPP	CQA	Design criteria (Developability)
Optimise manufacturing and development costs Safety to patient	Aggregation Productivity	Increase product stability Reduce aggregation Maintain/increase product titre
Adequate product efficacy	Biological activity	Retain affinity to target within acceptable levels

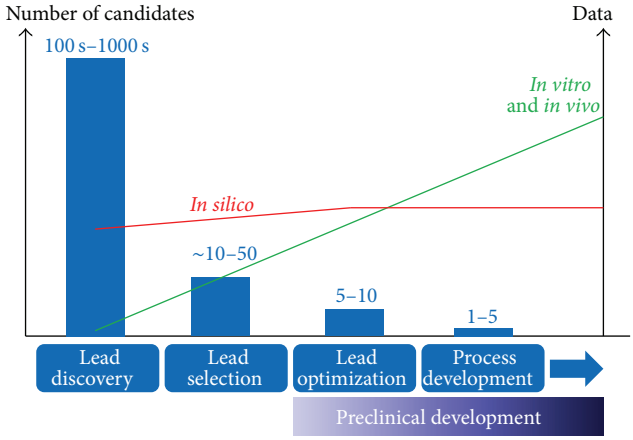

Figure 4: Implementation of developability methodologies in different stages of discovery and development and its relationship with number of lead candidates and available data. As the number of potential candidates converge into a smaller number, the amount of available experimental data increases. *In silico* computational methods can, by comparison, yield a lot of information at an early stage. As the product candidates progress in development, the introduction of *in vitro* analytics becomes feasible and an important element to help the decision-making process.

4.1. Engineering Antibodies with Improved Manufacturing Properties That Retain Biological Activity. The following case describes how a developability assessment and remediation programme can be utilised to ensure that manufacturing, safety, and efficacy requirements are included in the product specifications. As we indicated above, the definition of a relevant QTPP early on in the development of a new drug can be useful in identifying potential areas of risk and designing adequate (and inexpensive) remediation strategies. Table 1 reflects some of the requirements for this particular product. One of the key criteria, often determinant in process development, is to achieve acceptable manufacturing costs and ensure patient safety by achieving a high product quality (linked to stability) and adequate efficacy, which will ultimately define to a great extent the performance of a product. As we have seen earlier, product instabilities in the form of aggregate, impurities or degradation are often responsible for the incidence of immunogenic responses in patients. These criteria therefore are often closely linked to specific CQAs, namely, aggregation levels (as well as other product impurities), product yields and, of course, biological activity. From these CQAs then a number of design criteria can be used to define a suitable developability programme. This is, however, not a trivial matter, given the fact that, often, stability problems in molecules such as antibodies colocate with biologically active regions of the molecule. This colocation is primarily due to the highly variable character of complementarity determining regions (CDRs) in the molecule. However, recent studies suggest that because of the nature of interactions involved, antibody-target binding regions are likely to be enriched in aggregation-prone regions [90].

For the purpose of this study we used a model monoclonal antibody with potential therapeutic applications. We selected a humanised anti-IFNγ antibody previously described in the literature [91]. In this particular case, the parental molecule (humanised antibody) exhibits significant aggregation problems both under native conditions (after capture step using protein A chromatography) as well as in accelerated stability studies (i.e., incubation at high temperature). Aggregate content was determined by gel-permeation HPLC methods (GP-HPLC), and, in some cases, monomer recovery (quantified by GP-HPLC) was used as a more precise way to assess protein loss due to aggregation and other factors.

With this case in mind, the design criteria chosen included the increase of product stability, reduction of aggregation, maintaining an adequate productivity and achieving all these requirements whilst maintaining acceptable affinity to target. The design plan, as described earlier, included a risk assessment, mapping areas of the molecule potentially responsible for the observed behaviour, and introduction of a mitigation plan that would involve the substitution of key residues in the molecule to improve the required parameters. Finally, the resulting product candidates would be assessed using relevant experimental techniques to determine whether the remediation plans satisfied the requirements for the product.

To this aim, three-dimensional structural homology models were built for the Fv regions of the humanised anti-IFNγ antibody. The molecule's sequence and structural properties were analysed using the latest version of Lonza's proprietary Aggresolve *in silico* platform to identify potential aggregation hotspots or "weak" regions that could justify the stability and aggregation issues observed in the molecule, as well as assessing the relative impact of specific amino acid modifications in the aggregation propensity of the molecule. Description of early aggregation-predicting algorithms and examples of their application to specific biopharmaceuticals can be found elsewhere [11, 92–95]. These analyses highlighted several potential aggregation hot-spots on the humanized antibody when compared to reference sets of monoclonal antibodies of known behaviour. After this

analysis was completed, we selected a library of different sequence variants that targeted those potential aggregation hotspots as well as potential structural liabilities or "weak-points" that could influence the behaviour of the molecule. We further used structural information on the molecule and original murine antibody to refine this library and discard unsuitable modifications that could have a negative impact on the stability and structural integrity of the molecule or that could potentially impact its biological activity, for example, because of their physicochemical characteristics or proximity to key residues in the binding interface. After this secondary screening was completed a reduced number of variants were selected for further characterisation in relevant *in vitro* assays. (Homology three-dimensional models of variable domains of antibodies were built using standard commercial software (Accelrys's Discovery Studio—Biovia). Structural liabilities can be assessed by computing a variety of different parameters, such as structural alterations to key regions of the molecule (i.e., residues in close proximity to domain interfaces) or by assessing alterations in domain-domain interactions (i.e., energies of interaction or changes alterations in hydrogen bonds). In some cases, molecular dynamics simulations (Gromacs) can be utilised to assess changes in local flexibility that could affect the stability of the complex.)

Relative productivity of variants and parental molecules was assessed in suspension cultures of CHOK1SV cells, using small-scale transient transfections in 96 well plates. From these initial screenings two final variants were selected and expressed again transiently in suspension cultures using 200 mL flasks. These cultures generated sufficient material to perform confirmatory protein stability (aggregation) and activity studies. The main rational for using transient expression for this type of assessment resides in the fact that it eliminates any potential contribution of clonal selection in the observed product quality characteristics. Alternatively, pooled stable transfections can also be used successfully for this purpose.

After expression in culture, the two reengineered antibodies displayed significantly improved properties when compared to the parental humanised anti-IFNɣ, validating the re-design approach taken. Specifically, GP-HPLC analysis showed an almost complete elimination of aggregation for the two selected candidates (Figure 5(a)). The same type of analysis after an accelerated stability study, in which the antibodies were incubated at 60°C for 2 hours, also showed positive results, with a significant reduction of monomer loss for both reengineered variants, compared to the parental molecule. Remarkably in one of the variants monomer loss was virtually undetectable after incubation at high temperature (Figure 5(b)). Furthermore, the observed yield of the reengineered variants increased up to three-fold when compared to the parental molecule (Figure 5(c)), in line with earlier observations in our group, linking antibody stability and aggregation to productivity. Also, very importantly, the reengineered variants also retained biological activity to similar or even better levels to those of the parental molecule (Figure 5(d)).

There is a growing concern about the presence of sub-visible particles in biopharmaceutical preparations because of their potential impact on immunogenicity risk [96]. To address this all variants were analysed using Micro Flow Imaging (MFI). MFI is able to quantify a distribution of sub-visible particles in a given protein solution based on their size. In our tests, the two reengineered antibody variants showed a significant reduction in particles across the spectrum when compared to the parental molecule (Figure 6).

These results, therefore, highlight how, the application of computational and adequate analytical tools during the initial stages of drug development can lead to a significant improvement in developability of a drug candidate. It also exemplifies the implementation of reengineering to control or improve essential design criteria that can have a significant impact in product quality attributes, thus decreasing the likelihood of quality and safety issues that could creep in during later stages of preclinical and clinical development.

4.2. Selecting Half-Life Extension Products with Reduced Risk of Immunogenicity Risk. This second case concerns with the design of strategies aimed to extend the half-life of biotherapeutic molecules, particularly small proteins, whilst controlling the potential incidence of potential immunogenic reactions in patients.

Why extended half-life and why immunogenicity? As we discussed earlier, the pharmacological properties of a biotherapeutic candidate can have a dramatic impact not only on the biological activity and performance of a product, but also can have a knock-on effect on healthcare costs (linked to administration of drug) and patient compliance. A product that requires daily administration is likely to face a substantial level of resistance by patients and payers, both in terms of costs and convenience. This can be exacerbated by the cost of producing the active molecule itself. For example, antibodies and other binding scaffolds usually need to be administered at relatively high concentrations in order to generate a suitable response in patients. As we have discussed earlier, this requires typically high product concentrations that also maintain relatively long circulating half-life in serum.

Antibodies are privileged molecules in this regard and they have been engineered by nature to remain circulating in the bloodstream for extended periods of time (typical half-life for an IgG is around 2-3 weeks). This is achieved through the interaction of the Fc portion of the antibody with the neonatal Fc receptor (FcRn) present in endothelial cells, which rescues antibodies destined for intracellular degradation, and reintroduce them in the bloodstream. Albumin also maintains long circulating half-life by a similar binding to the FcRn. As a result, a number of approaches have been proposed to extend the circulating half-life of biopharmaceuticals, including fusion or conjugation to Fc fragments, albumin, lipids (able to bind albumin) or albumin-binding proteins [97]. This is particularly important in the case of new "scaffold" molecules or alternatives to antibodies, which often are designed as small protein domains to increase their tissue penetration properties, particularly for the treatment of solid tumours.

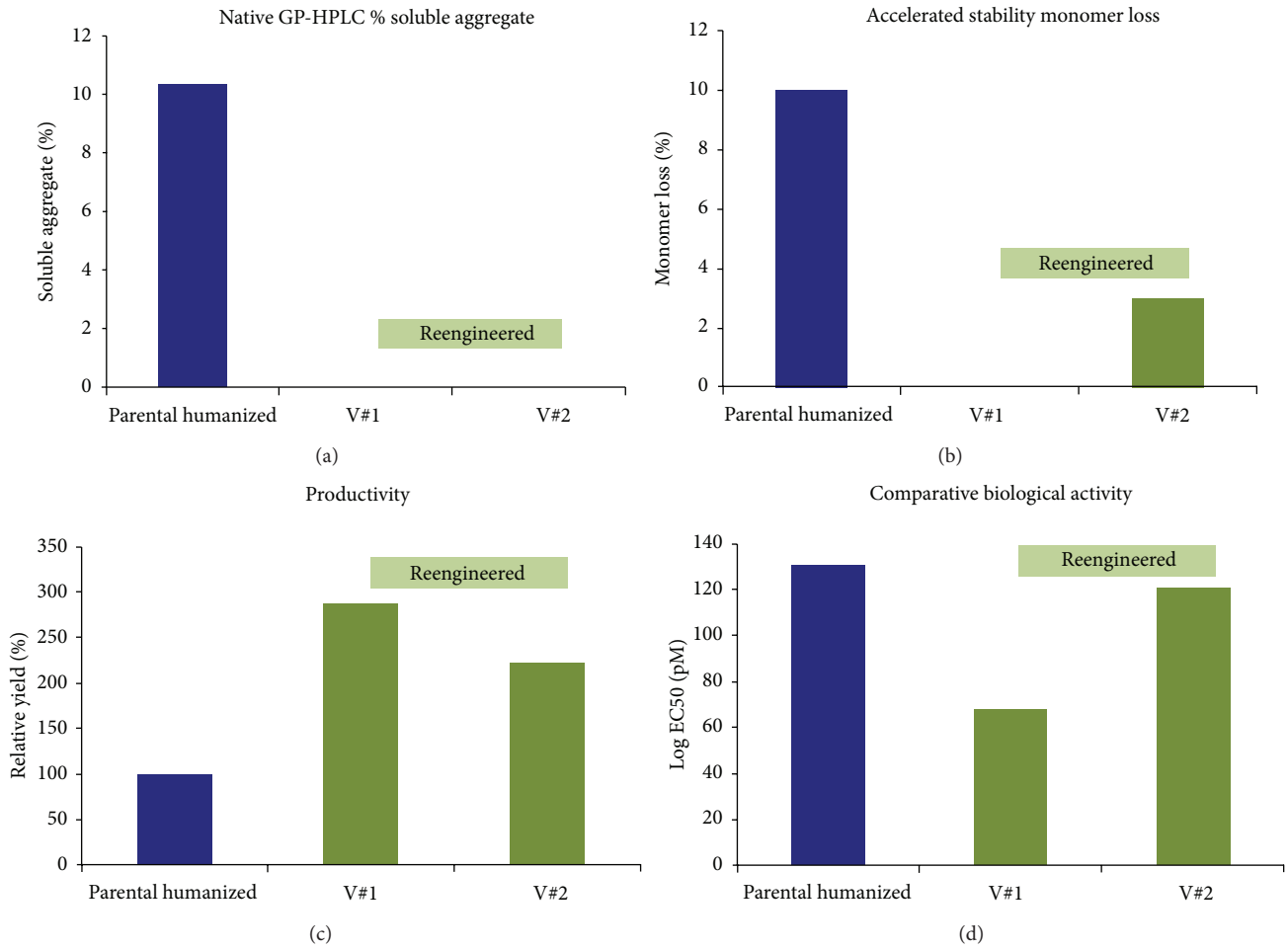

Figure 5: Reengineered antibodies display improved developability properties. Panel (a) shows the virtual absence of aggregation for both reengineered variants under native conditions when assessed by GP-HPLC. Panel (b) shows that the percentage of monomer loss after incubation 2 h at 60°C is significantly reduced in both reengineered variants, and virtually eliminated in V#1, indicating improved stability upon reengineering. Panel (c) shows that the productivity increases more than 2-fold in reengineered variants. Panel (d) shows that biological activity is not negatively impacted upon reengineering, with one of the variants V#1 showing increased affinity for the ligand.

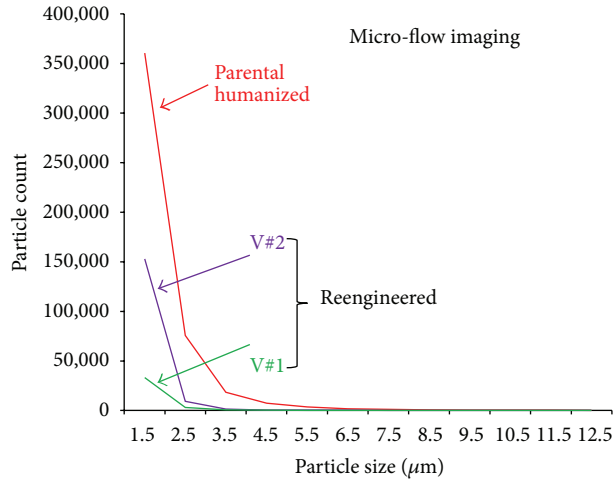

Figure 6: The amount of subvisible particles, including protein aggregates, in the humanized anti IFNϒ and both reengineered variants was characterized using Micro-Flow Imaging (MFI). V#1 and V#2 variants display a 5- to 10-fold reduction in the number of particles below 3.5 μm compared to the humanized anti IFNϒ. Both reengineered variants contain no detectable particles over 3.5 μm.

Table 2: QTPP, CQA, and design criteria derived for case study 2. The main criteria considered in this particular example revolve around pharmacology and safety requirements for the final product.

QTPP	CQA	Design criteria (Developability)
Increase therapeutic index Reduce dosage	Extended half life	Increase product size (passive) Fuse/conjugate to "carrier"
Safety to patient Minimise resistance to drug	Low immunogenicity	Eliminate T cell epitopes Minimise aggregation

Small molecules and proteins below the threshold for renal clearance (around 70 kDa) are rapidly cleared from circulation, in contrast to large proteins that have longer circulating half-lives. It is therefore no surprise that early strategies to extend the circulating half-life of therapeutic molecules involved the conjugation to polymers, such as polyethylene glycol (PEG), that are able to increase the apparent hydrodynamic radius of the product and hence reduce its clearance from the bloodstream through the kidney. However, recent developments are questioning the utilisation of PEG as an adequate approach to half-life extension. According to a long-held view, besides its impact in circulating half-life, PEG could also reduce aggregation of the product by hiding hydrophobic patches beneath a highly hydrated polymer shell, but also reduce immunogenicity by hiding potential T-cell epitopes present in the molecule [86]. Although this is indeed the case, a growing number of observations report an increase in the observed immunogenicity of biological drugs when linked to PEG. This is perhaps associated to the fact that many patients, in fact, possess pre-existing antibodies against PEG, likely due to earlier exposure to the agent from processed food, pharmaceuticals, and cosmetic products [75, 76]. Also there is the possibility that new epitopes could potentially be created upon conjugation of PEG to a protein of interest. Independently of its immunogenicity potential, the very high stability of PEG in the body is an issue. For example, there is growing suspicion of potential safety risks associated with the chronic administration of PEG, primarily linked to its accumulation in renal cells and the subsequent risk of renal failure [98].

It is therefore no surprise that alternatives to the use of PEG are being explored. In addition to increasing safety concerns referred above, the considerable increment in manufacturing costs associated with conjugated products are contributing to this interest. Many of these alternatives involve fusions to poly-amino acids that increase the apparent size of the molecule [99] or to moieties that take advantage of the recycling mechanism mediated by FcRn, described above.

With this background, the definition of a QTPP and associated CQAs and design criteria follow a similar approach to that described in the previous example, as reflected in Table 2. In this particular case, the main pharmacological drivers aiming to increase therapeutic index and reduce dosage can be achieved by extending the half-life of the product. As stated above, this can be done by increasing product size (passive mechanism) or by fusing the product to a "carrier" molecule that actively extends product half-life by using the "FcRn recycling" mechanism. In this particular case, the latter approach was utilised in the form of an albumin-binding domain. However, as we have seen above, it is important that the methodology employed to extend the product half-life also addresses safety concerns, primarily potential immunogenic reactions that could negatively impact the usability of the product. In this way it is important that methodologies aimed to reduce or eliminate T-cell epitopes are utilised as well as other potential contributors to immunogenicity (i.e., aggregation, degradation, or impurities).

In the case described here the chosen strategy for half-life extension of therapeutic proteins is to take advantage of the long circulatory half-life of human serum albumin (HSA) in plasma [100, 101]. The Albumod technology developed by Affibody is a proprietary albumin binding technology and is based on a small Albumin Binding Domain (ABD). This domain consists of a 5 kDa protein that has been engineered to bind HSA with high affinity and is designed to enhance the efficacy of biopharmaceuticals by extending their circulatory half-life in patients. The original ABD domain (ABD001, ABD3) was isolated from a bacterial protein, streptococcal protein G (SpG), which has the capacity to bind serum albumin. ABD001 had undergone affinity maturation, and one of the resulting engineered mutants, ABD035 demonstrated excellent stability along with an increased affinity for serum albumin of several species, including femtomolar affinity for human serum albumin [102]. ABD035 also retained an experimentally confirmed T cell epitope from ABD001 [103] and was therefore subjected to deimmunisation via protein engineering. A number of variants were designed to remove/reduce the number of T and B cell epitopes whilst maintaining thermal stability, solubility, expression yield, and affinity to HSA. The protein engineering stages were guided by B and T cell epitope prediction programs and available literature on ABD, and included iterative rounds of protein expression and analytical characterisation [104].

In order to mitigate potential immunogenicity risks we made use of the Epibase *in silico* immunogenicity prediction platform to select variants of the willd-type ABD001 with reduced immunogenicity potential. This platform has previously been used successfully in a number of biopharmaceuticals [105, 106]. The wild-type ABD001 and a total of 133 different engineered variants were subsequently screened for immunogenicity using the Epibase *in silico* platform, with profiling performed for the Caucasian population using 42 HLA class II allotypes. The variants were ranked based on their immunogenicity score incorporating DRB and DQ allotypes. Three variants, ABD088, ABD094, and ABD095, were then selected from the collection of variants based on their sequence, HSA affinity, thermal stability, solubility, and predicted lower immunogenicity risk. Figure 7(a) shows

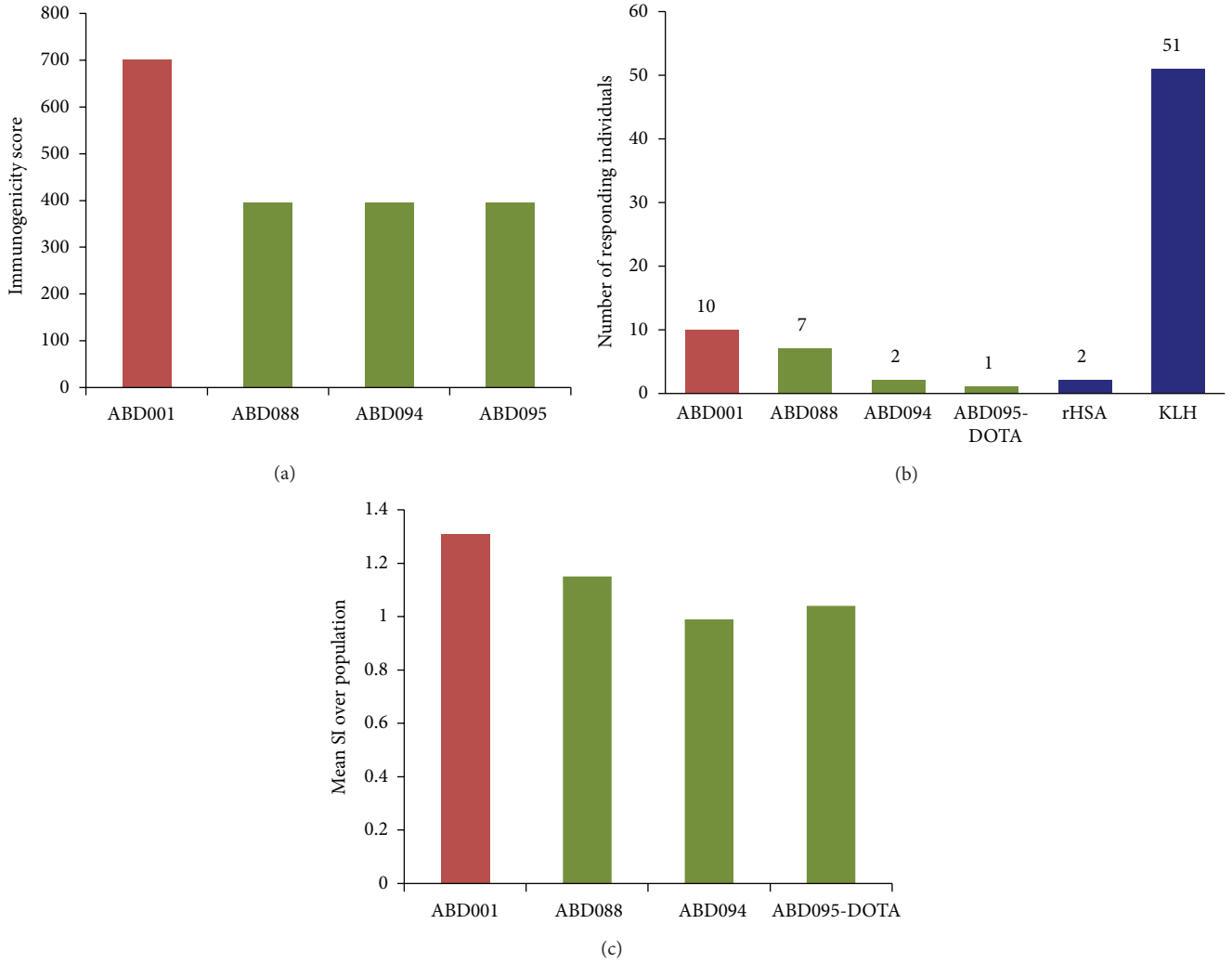

Figure 7: Immunogenicity assessment of ABD variants. (a) Predicted immunogenicity scores for three ABD variants and parental sequence ABD001. (b) Relative CD4+ T cell proliferation responses to ABD variants in a cohort of 52 donors, expressed as number of donors with proliferative responses to each of the ABD variants compared to negative (rHSA) and positive (KLH) controls. (c) CD4+ T cell proliferation responses to ABD variants in a cohort of 52 donors expressed as mean stimulation indices (SI) over the population. rHSA is used as a reference (SI = 1).

the predicted immunogenicity scores for these variants and their comparison to the parental ABD001. Deimmunised variants were predicted to have a reduction of approximately 40% in their immunogenicity risk when compared to the parental molecule ABD001.

The wild-type ABD001 and 3 deimmunised variants, ABD088 and ABD094, and the conjugate ABD095-DOTA (DOTA—chelator for divalent metal ions) were further assessed *in vitro* for their ability to activate CD4+ T cells. During the *in vitro* immunogenicity assessment, proliferation of CD4+ T cells was used to monitor T cell activation response induced by the ABD variants. CD4+ T cell responses were assessed in PBMCs from 52 healthy donors representing the Caucasian population (frequencies based on HLA-DRB1 allotype distribution). Keyhole Limpet Hemocyanin (KLH) was used as a highly immunogenic benchmark protein and recombinant human albumin (rHSA) as a control reference. Data analysis included identifying the number of individual donors eliciting a significant CD4+ T cell response to each ABD variant and a measure of the mean CD4+ T cell response over the whole 52 donor population. Figure 7(b) shows the number of donors with statistically significant proliferative responses using a blank control as reference. When compared to a blank control, only 2 out of 52 individuals responded to ABD094 and ABD095-DOTA, versus 10 donors responding to the wild-type ABD001. On the other hand, a total of 51 donors out of 52 responded to the KLH positive control and only 2 donors responded to rHSA. Figure 7(c) shows the mean stimulation index (SI) over the population using rHSA as a reference for the four ABD variants (the Stimulation Index (SI) is calculated by dividing the number of proliferating $CD3^+CD4^+$ cells in the test condition by the number of proliferating $CD3^+CD4^+$ cells in the blank condition. The criteria for a statistically significant $CD4^+$ T cell response

was set at an SI value > 2 with an associated P value < 0.05. A cumulative count of the individual donor responses to each test protein over the 52 donor population was used to compare the test protein immunogenicity at a single donor level. The magnitude of the T cell response induced by each test protein was also calculated over the entire 52 donor population. To compare the population response to each test protein, the (geometric) mean SI value (with associated P value) was calculated compared to the reference (blank) condition and individual test proteins directly compared).

All three deimmunised variants show a reduction in T cell proliferation in comparison with the wild-type ABD001. The mean population response for ABD001 and ABD088 is statistically different ($P < 0.05$) from that for rHSA (mean SI of 1.31 and 1.15, resp.). The SI for ABD094 and ABD095-DOTA variants was not significantly different over the test population (mean SI of 0.99 and 1.04, resp.).

No significant *in vitro* CD4+ T cell response was detected against the lead candidate ABD094, indicating that the removal of T cell epitopes via engineering was successful in reducing the immunogenicity of the molecule. As a result of these studies, the candidate ABD094 was selected to progress into development and future clinical trials.

This project demonstrates the successful use of a combination of *in silico* predictions and *in vitro* immunogenicity assessment tools as suitable platforms to guide protein reengineering to remove T cell epitopes and to enable lead selection based on the relative immunogenicity risk of different candidates.

5. Conclusions

As drug attrition during development remains a critical hurdle, underpinning the scarcity of new therapeutic treatments that are both effective and affordable, a true, holistic implementation of QbD and Big Quality principles, as defined by Juran and Godfrey [7], is desperately needed in the industry. Existing ICH guidelines concerning the application of QbD to drug development provide mainly a structured framework for process understanding and characterisation. However, they do not emphasise adequately the relevance of product knowledge and design and their true impact on product quality as well as manufacturing and clinical outcomes. Despite of what it might seem obvious to most people, the industry still lacks the implementation of "true QbD" methodologies that start with the design of the product itself. We argue that the definition of a meaningful quality target product profile (QTPP) right at the inception of a new product, as well as the early determination of relevant CQAs and effective risk-management strategies, can facilitate this process. Indeed, having such clear sets of design requirements at the very beginning can help driving effectively the development of an appropriate manufacturing process with a higher probability of success.

Unfortunately, current standard practice in biopharmaceutical development usually makes use of highly fragmented and siloed processes. In our experience, this often means that many important product properties are not properly addressed during the design and lead selection stages and are left to be managed during manufacturing. This "traditional approach" can increase considerably risks for the product and can have negative consequences in product viability and development costs. We propose an alternative workflow that moves away from the classical linear-hierarchical development model into one that is more integrated and where adequate early risk-assessment tools can help controlling CQAs at a very early stage. This introduces a change in emphasis, by defining QTPP right at the outset and with a larger number of criteria that will ultimately determine the success of a given product (mode of action, target patient population, delivery requirements, etc.). Secondly, it does involve the introduction of additional derisking tools that increase the stringency of candidate selection, in order to meet the required QTPP, and that properly control CQAs in the product from the beginning of development. Ultimately we believe that such early risk-assessment paradigms can not only be financially beneficial in reducing development costs and the costs of "poor quality" (deviations, recalls, failed batches, or clinical inefficacy), but could also potentially accelerate the development of new product candidates, for example, by speeding up their transition from preclinical to clinical development.

The two examples chosen try to illustrate, in a very simple and succinct way, how this "early QbD" process could be articulated. We use a simplified and somewhat limited description of a target QTPP to show how CQAs can be subsequently derived and risk assessment and mitigation strategies can be rolled out. Furthermore, we describe how developability can in fact emerge as a "bridge" between discovery and development functions, raising CQAs awareness very early on as well as aligning the objectives of different stakeholders towards developing better, safer, and more cost-effective products. Indeed, developability assessment approaches are emerging as an important tool to expand on the current, still-limited implementation of QbD in the pharmaceutical industry, but as we describe, there are still many gaps that require further attention, such as early formulability assessment or more comprehensive safety profiling. We are also aware that, in our examples, many other aspects relevant to biopharmaceutical success have not been fully addressed, primarily due to limitations of space and scope of this manuscript.

As it happens with the introduction of QbD in commercial drug manufacturing, this "early QbD" approach still requires the definition of suitable tools to integrate information from different risk areas (relevant to CQAs) and "objective" approaches to decision making that are relevant to the intended performance of a given drug. For example, the evaluation of the relative importance of different risks during lead selection and their potential impact in product quality and clinical success remains a mayor challenge that needs to be tackled. We have started to address some of these issues elsewhere [89], but anticipate that alongside the introduction of predictive algorithms and surrogate analytics discussed here, the implementation of better data integration, and the development of objective decision-making tools will facilitate a more effective application of the methodologies reviewed

in this article. This will, very likely, not only involve the development of new technical solutions, like computational models and tools, but also some degree of coordination and alignment across different industry stakeholders, from innovators to contractors to regulatory bodies to healthcare providers. We humbly hope that this review can help in fostering further discussions in the industry around this topic, ultimately biopharmaceutical quality and efficacy, which we believe will be essential for the long term success and sustainability of our industry.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Physicochemical and Biological Characterization of a Biosimilar Trastuzumab

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According to the World Health Organization, the incidence of malignant neoplasms and endocrine, blood, and immune disorders will increase in the upcoming decades along with the demand of affordable treatments. In response to this need, the development of biosimilar drugs is increasing worldwide. The approval of biosimilars relies on the compliance with international guidelines, starting with the demonstration of similarity in their physicochemical and functional properties against the reference product. Subsequent clinical studies are performed to demonstrate similar pharmacological behavior and to diminish the uncertainty related to their safety and efficacy. Herein we present a comparability exercise between a biosimilar trastuzumab and its reference product, by using a hierarchical strategy with an orthogonal approach, to assess the physicochemical and biological attributes with potential impact on its pharmacokinetics, pharmacodynamics, and immunogenicity. Our results showed that the high degree of similarity in the physicochemical attributes of the biosimilar trastuzumab with respect to the reference product resulted in comparable biological activity, demonstrating that a controlled process is able to provide consistently the expected product. These results also constitute the basis for the design of subsequent delimited pharmacological studies, as they diminish the uncertainty of exhibiting different profiles.

1. Introduction

Biopharmaceutical products containing chimeric, humanized, or fully human monoclonal antibodies (mAbs) are among the most successful and demanded therapies due to their highly specific mechanisms of action that result in an improvement of the patients' conditions and an increase in the survival rate, while minimizing the adverse side-effects when compared to other treatments [1]. Consequently, new manufacturing sites, process scale-ups as well as process improvements contribute to the well-known heterogeneity, naturally present in biotherapeutic products. For this purpose, the ICH Q5 E guideline provides the principles for assessing comparability of licensed biotechnological products subject to process changes throughout their life cycle [2].

In this sense, the approval of biosimilar products, which have been recognized not only as an alternative but as a necessity to increase health coverage and improve the quality of life of patients, follows a similar comparability scheme. International guidelines on biosimilarity [3–5] outline that the approval of biosimilars must rely on the demonstration of comparability towards the reference product, starting with an exhaustive physicochemical and biological characterization whose results will provide evidence to support the extent of additional clinical evaluation [6–8].

For this purpose, the proper identification of critical quality attributes (CQAs) that may impact on the pharmacokinetics, pharmacodynamics, and immunogenicity can be achieved through a deep knowledge of the chemical composition and the higher order structure of the active pharmaceutical ingredient (API) contained in the reference product, as well as the known relationships between specific attributes and biological functionality, anticipated by the biotechnological industry and the scientific community [9–17]. Furthermore, the ICH Q9 guideline highlights the need of evaluating the quality of a biopharmaceutical product based on a risk analysis that considers relevant attributes to the drug's safety and efficacy [18].

In this work we present a comparability study between a biosimilar trastuzumab and its reference product. Trastuzumab is a humanized monoclonal antibody targeted against the extracellular portion of the human epidermal growth factor receptor (HER2, p185), which is overexpressed in approximately 15 to 30% of the invasive breast cancer cases [19–22]. The chemical, physical, and functional properties closely related to its pharmacological behavior were identified through a risk analysis; then those CQAs were evaluated using several analytical techniques in an orthogonal approach that increases the reliability of the results obtained.

2. Materials and Methods

2.1. Materials. Biosimilar trastuzumab (440 mg powder for concentrate for solution for infusion) from Probiomed S.A. de C.V. (Mexico City, Mexico) and Herceptin (440 mg powder for concentrate for solution for infusion) from F. Hoffmann, La Roche Ltd. (Basel, Switzerland), were used for the comparability study.

2.2. Methods

2.2.1. Physicochemical Properties. Primary sequences, verified from the whole-molecule exact masses and tryptic peptide mappings, were analyzed by reverse phase ultra-performance-liquid-chromatography coupled to a tandem quadrupole/time-of-flight mass spectrometer (RP-UPLC-MS/MS). Higher order structure was evaluated by differential scanning calorimetry (DSC), circular dichroism (CD), and fluorescence lifetime using the time correlated single photon counting technique (TCSPC). Charge heterogeneity of the whole, carboxypeptidase-digested, and papain-digested molecule was assessed either by capillary isoelectrofocusing (cIEF) or by cation exchange ultra-performance-liquid-chromatography (CEX-UPLC). Purity was determined by capillary gel electrophoresis under reducing (CGE-R) and nonreducing (CGE-NR) conditions and size exclusion ultra-performance-liquid-chromatography (SE-UPLC). Sample treatment and analysis conditions were performed as previously described for RP-UPLC-MS/MS, DSC, CD, CEX, CGE-R, and CGE-NR by Flores-Ortiz et al., 2014 [23]; TCSPC by Pérez Medina Martínez et al., 2014 [24]; cIEF by Espinosa-de la Garza et al. [25].

N-linked glycans were released from trastuzumab by enzymatic hydrolysis using PNGase F from New England Biolabs Inc. (Ipswich, MA) and then were labeled with 8-aminopyrene-1,3,6-trisulfonic acid (APTS) and analyzed by capillary zone electrophoresis (CZE) [26]. The electrophoretic separation was carried out in a PA 800 plus Analysis System from Beckman Coulter Inc. (Brea, CA) using an amine coated capillary of 50 μm I.D. × 50.2 cm total length, with 40 cm effective length at 20°C. Laser induced fluorescence (LIF) detection was used at an excitation wavelength of 488 nm and emission band-pass filter of 520 nm. An orthogonal analysis was performed by hydrophilic interaction ultra-performance-liquid-chromatography (HILI-UPLC) after labeling with 2-aminobenzoic acid (2-AB) following a previously reported methodology [27].

2.3. Functional Properties

2.3.1. FcγRIIIa Affinity by Isothermal Titration Calorimetry (ITC). Affinity constants under equilibrium (K_a) were obtained from a Nano ITC instrument (TA Instruments Inc.; New Castle, DE). 300 μL of FcγRIIIa solutions at 5.0 μM in PBS at pH 7.2 was titrated with continuous injections of 1.9 μL trastuzumab solutions at 50 μM in PBS at pH 7.2 until saturation at 25°C. NanoAnalyze Software v2.4.1 (TA Instruments Inc.; New Castle, DE) was used for the integration of heat signals and nonlinear regression analysis of the data.

2.3.2. FcRn Affinity by BLI. Binding kinetics of trastuzumab to FcRn were determined using a Bio-Layer Interferometry (BLI) instrument, Octet QK384, from Pall ForteBio Corp. (Menlo Park, California). Biotinylated FcRn was immobilized to biosensors coated with streptavidin. Binding profiles were displayed by sensograms. Global kinetic analyses were determined using a 2 : 1 heterogeneous ligand model fit using R-linked analysis.

2.3.3. HER2 Affinity Assay. HER2 expressing cells SK-BR-3 (ATCC HTB-30) were incubated in the presence of different concentrations of trastuzumab in McCoy-5A medium with 10% FBS for 2 h at 37°C. HRP-conjugated goat anti-human IgG was added to detect the trastuzumab–SK-BR-3 complex after 1 h of incubation at 37°C, using TMB as substrate for 30 min at room temperature. Absorption was measured at 450 nm. Test results were expressed as the relative percentage of the EC_{50} from the dose-response curve of the biosimilar trastuzumab with respect to the reference product.

2.3.4. Antiproliferation Assay. BT-474 cells (ATCC HTB-20) were seeded in DMEM media with 10% FBS, 1% nonessential amino acids, and incubated at 37°C. Different concentrations of trastuzumab were added with further incubation for 8 days. Crystal violet was added to stain the cells for 15 min at room temperature followed by fixation with formaldehyde and water rising. Acetic acid aqueous solution (33% v/v) was added to remove the dye excess; absorbance was measured at 540 nm. Test results were expressed as the relative percentage

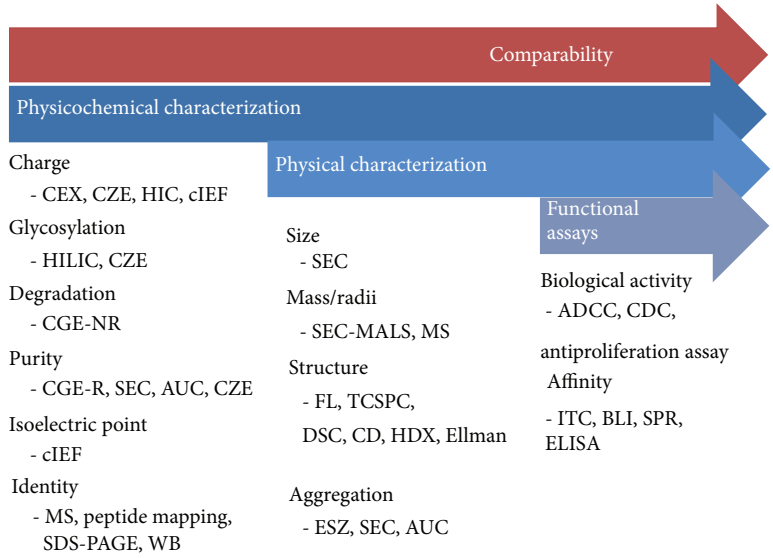

Figure 1: Characterization strategy performed for Trastuzumab-Probiomed.

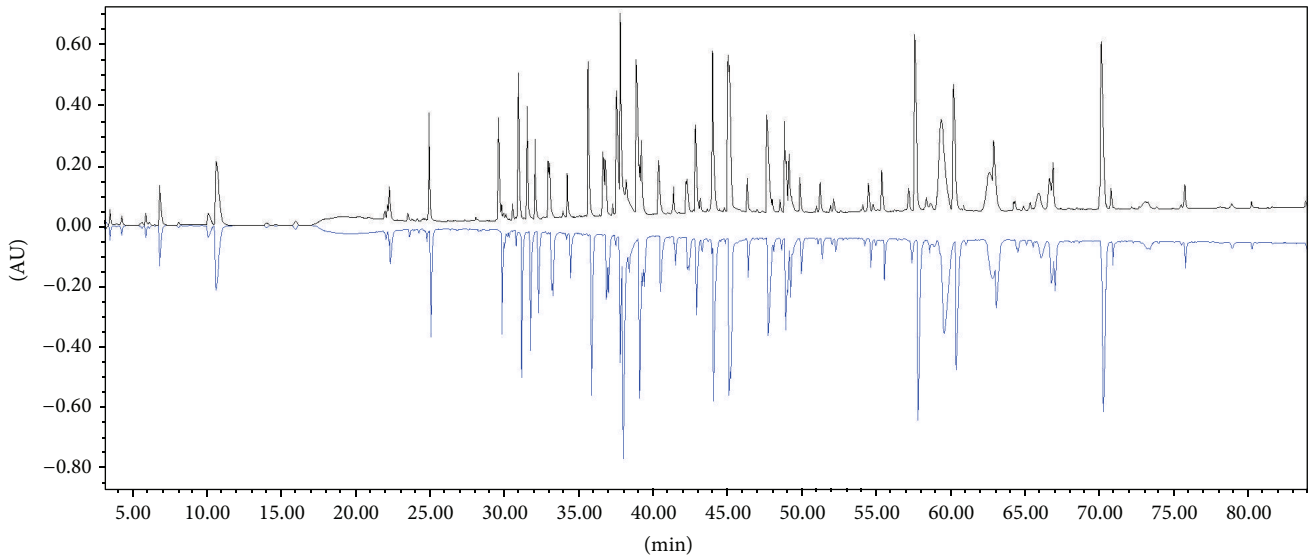

Figure 2: Mirror plot of peptide mapping chromatograms obtained from RP-UPLC-UV for Trastuzumab-Probiomed (upper) and the reference product (lower).

of the EC_{50} from the dose-response curve of the biosimilar trastuzumab with respect to the reference product.

3. Results and Discussion

Our characterization strategy (Figure 1) comprised a set of state-of-the-art analytical techniques planned for a hierarchical study of a biosimilar trastuzumab using an orthogonal approach. CQAs were identified using a risk analysis, considering each of the physicochemical and functional properties that may have an impact on efficacy (pharmacokinetics and pharmacodynamics) and safety (immunogenicity) of trastuzumab (Table 1) [9–17]. In this work, only certain methodologies were selected to depict a global overview of the characterization study. Hereafter, CQAs were classified by their physicochemical, physical, or biological nature and analyzed comparatively for a biosimilar trastuzumab (Trastuzumab-Probiomed) and its reference product.

3.1. Physicochemical Properties. The identity of Trastuzumab-Probiomed towards the reference product was determined by the correspondence of their tryptic peptide mappings (Figure 2). MS/MS analysis verified the amino acid sequence of both products against the theoretical stated on the invention patent of trastuzumab [28], unveiling a sequence matching of 99.8% and 99.3% for the heavy chain and 99.5% and 99.5% for the light chain, for both Trastuzumab-Probiomed and the reference product, respectively (Figures 3 and 4).

Table 1: Impact of CQAs on safety and efficacy.

Attribute	Pharmacodynamics	Pharmacokinetics	Immunogenicity
Sequence	Nonspecific	Nonspecific	Determined by the sequence variation against endogenous domains [9] Differential response due to sequence modifications for distinct batches or processes
Higher order structure	Nonspecific	Nonspecific	Determined by molecular weight and structure complexity [9]
Glycosylation profile	Fucosylated, highly mannosylated, and sialylated variants could alter *in vivo* efficacy [10–12]	Highly mannosylated variants show higher clearance Sialylated variants show lower clearance [10–12]	Sialic acid residues can hide antigenic determinants [9, 10] Highly mannosylated, hybrid, and nonglycosylated variants are prone to elicit immunogenicity
Charge heterogeneity	Effector functions altered if pI differences are >1 unit [10, 14, 15]	Major differences alter volume of distribution and clearance [10, 14, 15]	Acidic variants are prone to elicit immunogenicity [9]
Aggregates	Lower biological activity [11]	Less subcutaneous absorption and lower bioavailability [11]	ADAs presence [10]
FcγRI affinity FcγRII affinity FcγRIII affinity	Affects endocytosis, antigen presentation ADCC, phagocytosis [17] Higher affinity to specific variants [11, 12] Affects endocytosis, antigen presentation, ADCC, phagocytosis [17]	Not determined	Not determined
FcRn affinity	Not determined	Lower affinity to acidic variants Lower affinity for oxidized methionine Not expected measurable differences in variants with 3- to 4-fold changes in FcRn affinity [16]	Not determined

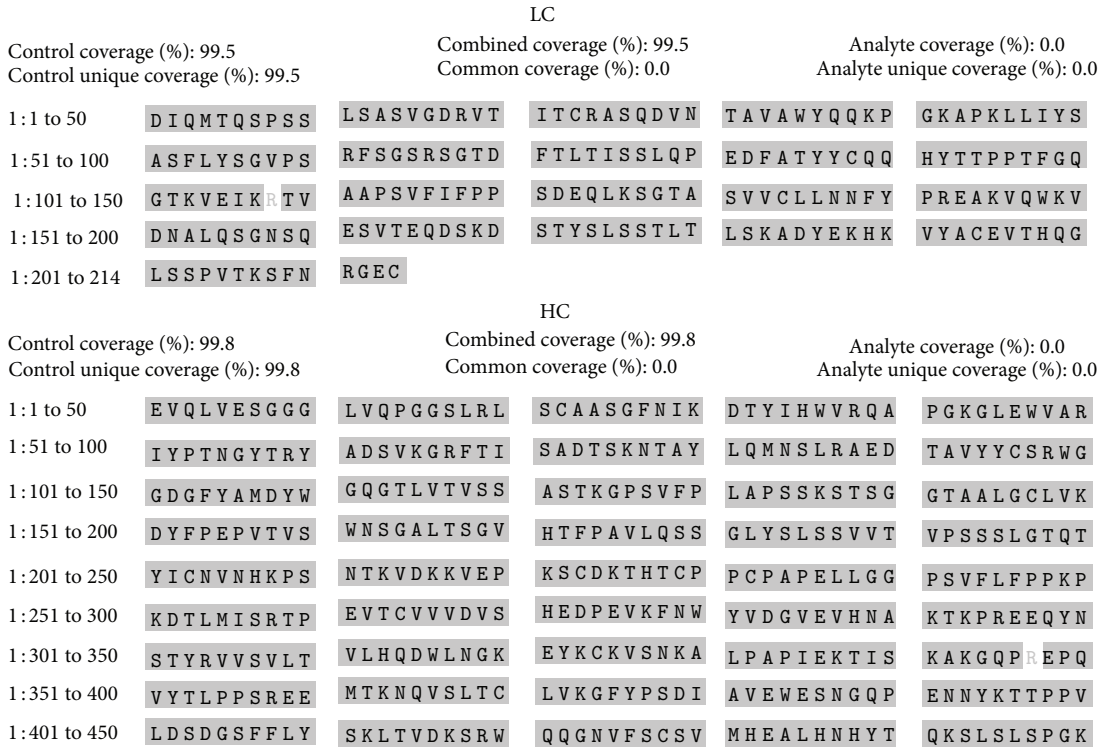

Figure 3: Sequence coverage of the heavy and light chains of Trastuzumab-Probiomed obtained from the MS/MS analysis.

This correspondence was further confirmed by the analyses of the exact masses against the theoretical mass [28, 29] for both whole and deglycosylated molecules (Tables 2 and 3). The sequences coverage confirms that the amino acid sequence of Trastuzumab-Probiomed is identical to the reference product, while the <25 Da observed differences in intact masses for the whole molecule, below the expected width of the isotopic pattern distribution of a mAb, show in advance a comparable degree of heterogeneity, due to posttranslational modifications, in both products, ultimately producing an equivalent immunogenic response.

Regarding glycan microheterogeneity, which is known to contribute to the correct folding and stability of a mAb, it was analyzed by CZE and HILI-UPLC. Particularly, highly mannosylated and sialylated glycoforms are reported to alter a mAb half-life in blood and are linked to potential immunogenic responses; moreover effector functions can be altered due to the presence of highly mannosylated, bisected, and fucosylated glycoforms, as a consequence of charge or steric hindrances [10–12].

CZE analyses revealed that the glycan patterns of Trastuzumab-Probiomed and the reference product are comprised of the same principal glycoforms (Figure 5(a)), showing a mean relative abundance of galactosylated variants of 66.01% and 49.57% ± 6.18 (CI 95%) for Trastuzumab-Probiomed and the reference product, respectively, which is not expected to have an impact on the functional properties, since galactosylation has not been reported to alter the mechanisms of action of mAbs, as confirmed by the affinities and biological potency analyses discussed below. Further analysis by HILI-UPLC of the glycoforms identified as critical for PK, PD, or immunogenicity (Table 1) revealed comparable relative abundances of highly mannosylated variants, being 2.00 ± 0.10 (CI 95%) and 3.96 ± 0.45 (CI 95%) for Trastuzumab-Probiomed and the reference product, respectively, whereas the mean abundance for hybrid and sialylated variants was 4.75 ± 0.19 (CI 95%) and 0.27 ± 0.08 (CI 95%) for the reference product and 2.95 ± 0.15 (CI 95%) and 1.06 ± 0.14 (CI 95%) for Trastuzumab-Probiomed, respectively. These results confirm similarity of the critical glycoforms between Trastuzumab-Probiomed and the reference product; thus similar PK and PD profiles and no differential immunogenicity response are expected.

On the other hand, charge heterogeneity evaluated through cIEF analysis revealed that isoelectric points (pI) for the main isoform were 8.69 ± 0.00 (CI 95%) for Trastuzumab-Probiomed and 8.70 ± 0.01 (CI 95%) for the reference product, in accordance with the expected pI variations during manufacturing, no larger than 0.2 units [15, 16]. The observed isoform-abundance-weighted pI values confirmed similarity of charge heterogeneity among products, being 8.60 ± 0.01 (CI 95%) for Trastuzumab-Probiomed and 8.61 ± 0.01 (CI 95%) for the reference product. It has been reported that only changes in one pI unit can significantly alter the therapeutic activity of a mAb; thus the observed variation is not expected to affect the clinical behavior of Trastuzumab-Probiomed with respect to the reference product.

An orthogonal analytical technique for the evaluation of charge heterogeneity was CEX-UPLC, which revealed that the averaged abundances of the main, acidic, and basic

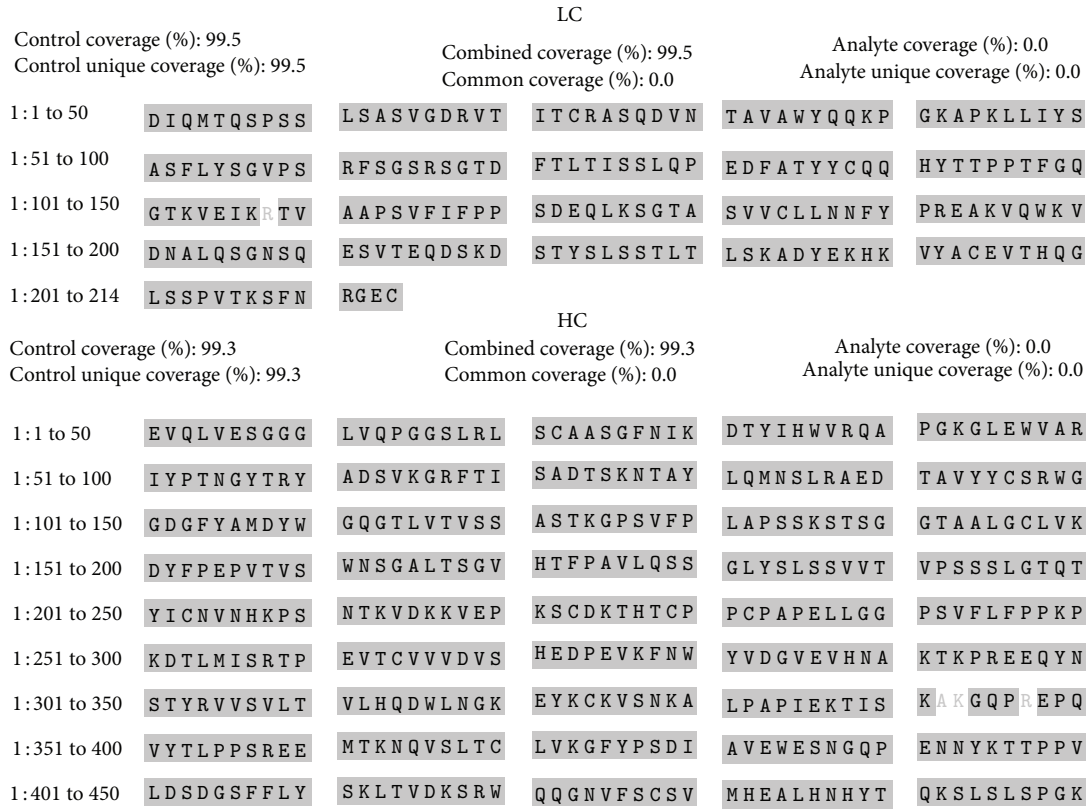

Figure 4: Sequence coverage of the heavy and light chains of the reference product obtained from the MS/MS analysis.

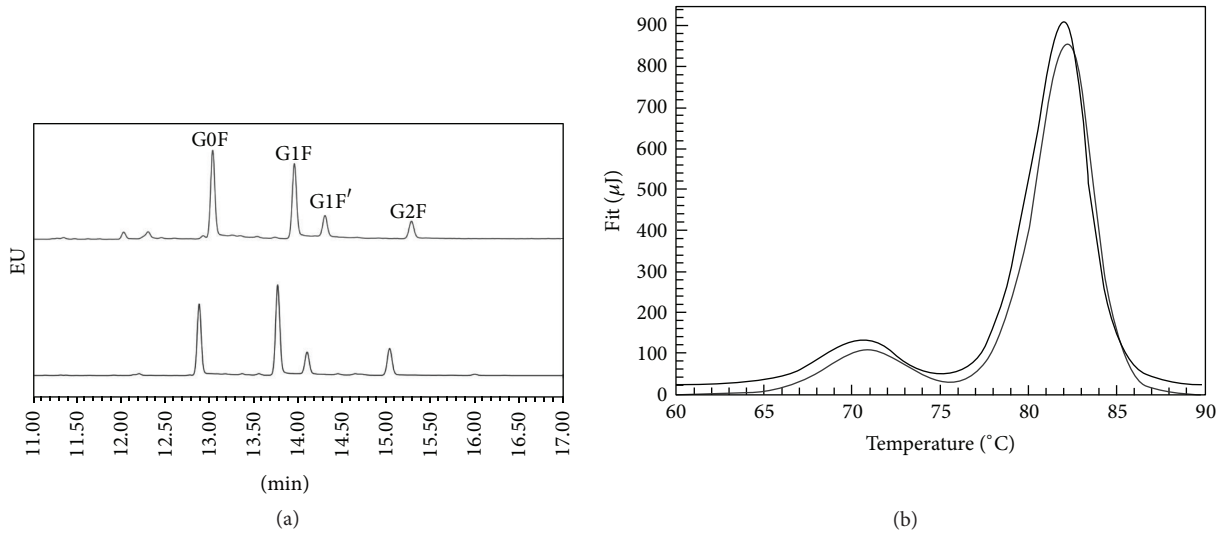

Figure 5: (a) Glycan profile for the reference product (upper) and Trastuzumab-Probiomed (lower). (b) Thermostability by DSC for the reference product (lower) and Trastuzumab-Probiomed (upper).

isoforms were within the same order of magnitude for both products, being the mean values of 57.0%, 33.2%, and 9.8% ($n = 3$) for Trastuzumab-Probiomed and 62.5%, 27.3%, and 10.3% ($n = 3$) for the reference product, respectively. Furthermore, the results obtained after digestion with carboxypeptidase B showed also a comparable content of basic, acidic, and main isoforms among the two products, with a main relative content of 16.4%, 30.6%, and 53.0% ($n = 3$) for the reference product and 8.8%, 37.8%, and 53.4% ($n = 3$) for Trastuzumab-Probiomed, respectively.

After papain digestion, the mean abundance of basic isoforms in the reference product ($n = 3$) was 3.8% for

Table 2: Whole-molecule exact masses by MS.

Product	Batch	G0/G0F	G0F/G0F	G0F/G1F	G1F/G1F	G1F/G2F	G2F/G2F
Averaged theoretical	—	147911.76	148057.91	148220.05	148382.19	148544.33	148706.46
Reference product	B3417B010	147907.81	148061.92	148220.20	148378.84	148536.86	148692.48
	B3433B010	147897.68	148058.03	148218.21	148377.11	148534.93	148690.07
	N3477B021	147899.82	148058.00	148218.10	148377.32	148535.39	148690.95
Trastuzumab-Probiomed	TZPP12001	147901.19	148057.88	148218.18	148378.54	148537.97	148695.60
	TZPP12002	147900.45	148057.89	148217.99	148378.23	148537.59	148694.92
	TZPP12003	147898.55	148057.58	148217.49	148377.84	148537.14	148694.42

Table 3: Deglycosylated molecule exact masses by MS.

Product	Batch	Mass (Da)
Theoretical	—	145167.36
Reference product	B3417B010	145167.47
	B3433B010	145167.36
	N3477B021	145167.16
Trastuzumab-Probiomed	TZPP12002	145167.53
	TZPP12001	145167.08
	TZPP12003	145167.69

Table 4: Monomer content of trastuzumab by SE-UPLC and CGE-NR. Variation is presented as confidence intervals at 95% ($n = 3$).

Product	Batch	SE-UPLC (%)	CGE-NR (%)
Trastuzumab-Probiomed	TZPP11002	99.6 ± 0.0	92.3 ± 0.3
	TZPP12001	98.9 ± 0.0	90.8 ± 1.1
	TZPP11001	99.4 ± 0.0	96.6 ± 0.4
Reference product	N3597B013	98.9 ± 0.1	92.8 ± 0.6
	N35973	99.7 ± 0.0	93.5 ± 0.7
	B34310	99.5 ± 0.0	93.1 ± 0.4

the Fc fragment and 4.9% for the Fab fragment, whereas for Trastuzumab-Probiomed ($n = 3$) it was 4.2% for the Fc fragment and 6.5% for the Fab fragment. Regarding acidic isoforms, the mean abundance was 3.3% for the Fc fragment and 16.7% for the Fab fragment of the reference product, while for Trastuzumab-Probiomed it was 3.7% for the Fc fragment and 16.1% for the Fab fragment. Finally, the abundance of the Fc and Fab fragments was 26.7% and 44.6%, respectively, for the reference product, and for Trastuzumab-Probiomed the abundance of the Fc and Fab fragments was 25.7% and 43.8%, respectively.

Overall the results from cIEF and CEX-UPLC show that both products exhibit comparable charge heterogeneities, either as a whole molecule or as the fragments responsible for the recognition and effector functions of trastuzumab; thus no differences in functional activity should be expected.

CGE-NR and SE-UPLC results demonstrated that both products have a similar degree of purity (Tables 4 and 5) based on the relative content of monomer with respect to the presence of aggregates and other degraded or truncated isoforms. It is known that protein aggregation can induce immunogenicity; although a small amount of aggregates is expected, this amount is likely to increase due to stress conditions that a mAb may undergo during its manufacture, purification, formulation, and shelf-life [9, 30]. Aggregation may reveal new epitopes that potentially could stimulate the production of anti-drug antibodies (ADAs) resulting in the loss of activity, immunogenic reactions, or adverse effects during administration. Likewise, the presence of fragments or truncated forms coming from hydrolysis reactions could negatively impact on the safety and therapeutic effect of a mAb [31, 32]. The content of aggregates and truncated forms of Trastuzumab-Probiomed were lower than the limits established by the USP [29] and were comparable to the reference product; thus the risk of developing a different immunogenic response (differential immunogenicity) is diminished.

3.2. Physical Properties. Since the functionality of trastuzumab is affected by its three-dimensional structure, which results from its primary sequence and posttranslational modifications that alter its size, mass, folding, and stability [8], we performed analyses to assess the spatial configuration of Trastuzumab-Probiomed compared to its reference product. Time correlated single photon counting analysis (TCSPC) was employed to evaluate the fluorescence lifetime (τ), which depends on the exposure of aromatic amino acids within the protein, thus demonstrating similarity when the results are obtained from comparative analyses [33–36]. TCSPC results showed that the averaged τ of Trastuzumab-Probiomed was $3.43E^{-09} \pm 1.39E^{-10}$ s (CI 95%), while the averaged τ for the reference product was $3.49E^{-09} \pm 1.69E^{-11}$ s (CI 95%). Regarding CD, the obtained spectrograms were superimposable in both near- and far-UV regions (Figure 6) suggesting that alpha helix, beta sheets, random coil, disulfide bonds, and aromatic amino acids are distributed in a comparable spatial arrangement. Finally, transition temperatures (T_m) of Trastuzumab-Probiomed ($n = 3$) by DSC (Figure 5(b)) were 70.4°C, 79.1°C, 81.0°C, and 82.5°C, whereas for the reference product ($n = 3$) they were 70.5°C, 79.6°C, 81.2°C, and 82.7°C; for both products the CI at 95% was <0.02°C for all the temperatures. Collectively TCSPC, CD, and DSC determined that thermostability and secondary and tertiary structures of Trastuzumab-Probiomed were comparable to the reference product. In particular, thermostability results are indicative of a proper protein folding of both products in their respective

Table 5: Relative abundance of trastuzumab subunits by CGE-R. Variation is presented as confidence interval at 95% ($n = 3$).

Product	Batch	HC %	NGHC %	LC %
Trastuzumab-Probiomed	TZPP12001	66.18 ± 0.16	0.57 ± 0.06	32.89 ± 0.19
	TZPP12002	64.46 ± 0.47	0.58 ± 0.02	34.59 ± 0.32
	TZPP12003	65.45 ± 0.97	0.50 ± 0.05	33.53 ± 1.08
Reference product	B3393B019	65.14 ± 0.25	0.57 ± 0.03	33.93 ± 0.13
	B3417B010	66.02 ± 0.27	0.52 ± 0.09	33.04 ± 0.25
	B3430	66.40 ± 0.25	0.63 ± 0.01	32.46 ± 0.16

HC: heavy chain, NGHC: nonglycosylated heavy chain, and LC: light chain.

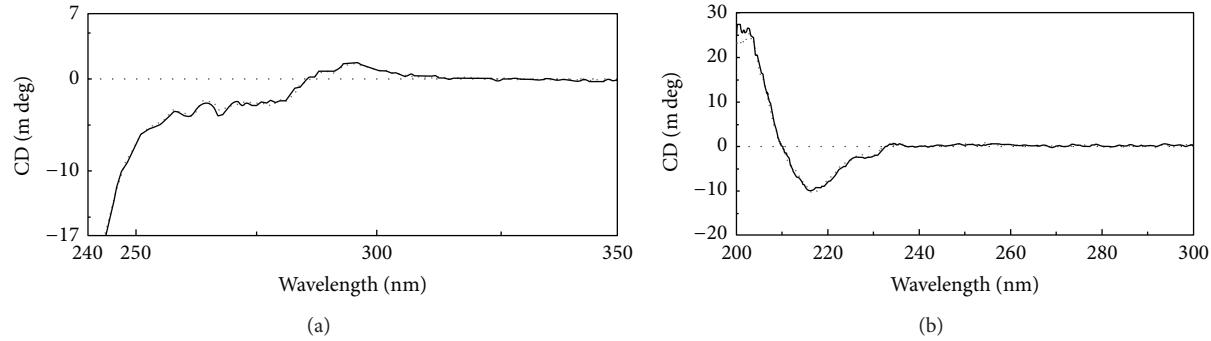

Figure 6: Analysis of the three-dimensional structure of trastuzumab by CD of Trastuzumab-Probiomed (solid line) and the reference product (dotted line) in both near-UV region (a) and far-UV region (b).

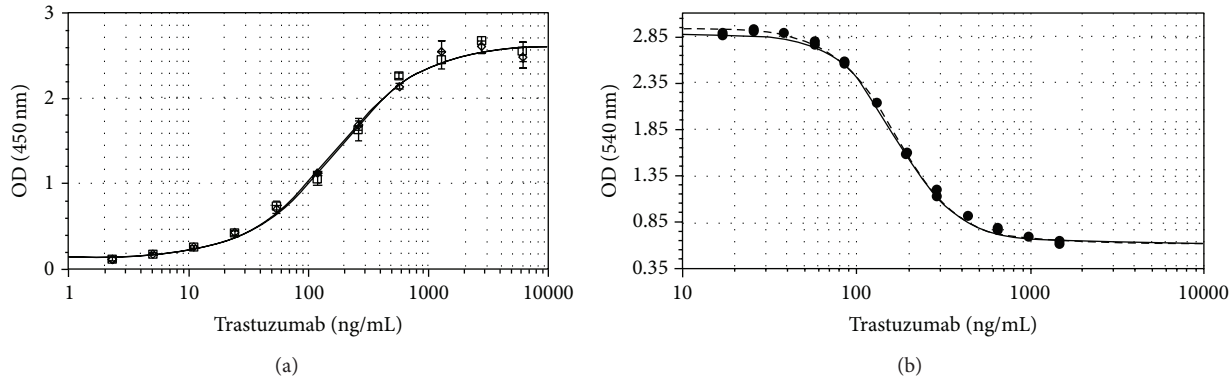

Figure 7: Comparison of *in vitro* activity between Trastuzumab-Probiomed and the reference product. (a) Curve of binding affinity to HER2; (b) potency curve obtained from the antiproliferation assay; the solid line corresponds to Trastuzumab-Probiomed, while the dashed line corresponds to the reference product.

formulation. This physicochemical and physical similarity is the major contributor to equivalent biological and functional responses.

3.3. Functional Properties. The relative affinity of Trastuzumab-Probiomed towards its target molecule, HER2 (Figure 7(a) and Table 7), was evaluated with respect to the reference product, resulting in an averaged relative affinity of 97.7%. Thus, it is expected that Trastuzumab-Probiomed can exert its activity through the reported mechanisms of action, including HER2 downregulation, prevention of the heterodimer formation, initiation of Gl arrest, induction of p27, and prevention of HER2 cleavage [37].

The main mechanisms of action rely on the affinity of the Fc fragment of trastuzumab towards Fcγ receptors. For instance, FcγRIIIa present on effector cells such as macrophages, monocytes, and natural killer cells activates and induces ADCC mechanism against HER2-positive cells [37, 38]. Binding affinities towards FcγRIIIa were evaluated by ITC, being the averaged affinity constants (K_a) of $2.61 \pm 0.54E^{+06}$ M^{-1} for Trastuzumab-Probiomed and $2.48 \pm 0.30E^{+06}$ M^{-1} for the reference product (Table 6). Likewise, the mean dissociation constants (K_D) to FcRn, which regulates IgG catabolism, were determined by BLI as $2.58E^{-07}$ M $\pm$ $1.02E^{-07}$ M (CI 95%) for Trastuzumab-Probiomed with a relative binding affinity of 114.3% ($n = 3$) with respect to

Table 6: Affinity of trastuzumab to FcγRIIIa.

Product	Batch	Affinity constant (K_a) to FcγRIIIa (M^{-1})
Trastuzumab-Probiomed	TZPP14001	$2.71E+06$
	TZPP12002	$2.86E+06$
	TZPP12003	$2.25E+06$
Reference product	N35893	$2.66E+06$
	N35812	$2.48E+06$
	N36003	$2.31E+06$

Table 7: Binding affinity of trastuzumab to the epidermal growth factor receptor (HER2).

Product	Batch	Relative affinity (%)
Trastuzumab-Probiomed	TZPP11001	98
	TZPP12004	98
	TZPP12003	97
Reference product	N3654	119
	N36263	111
	N36443	112

the reference product. Based on these results no differences in the half-life in blood are expected.

The overall *in vitro* activity was tested between Trastuzumab-Probiomed and the reference product with an antiproliferation assay (Figure 7(b)), which demonstrated that both products have the same potency to deplete HER2-positive cells, being the mean relative potencies towards the reference product of 105%, 103%, and 110% for three different batches of Trastuzumab-Probiomed, demonstrating that similarity on physicochemical and physical critical quality attributes results in a comparable biological potency.

4. Conclusions

During the development of a biosimilar, an extended characterization of its physicochemical and functional properties is required to gain a strong knowledge of its CQAs. This allows the establishment of in-process control strategies and quality specifications to ensure batch-to-batch consistency in order to obtain the desired product, despite the fact that it has been produced using a different manufacturing process with respect to the reference product. In addition, the use of orthogonal methods during a comparability study provides a global overview of the molecule and confirms the observed results on relevant modifications. Here, it was demonstrated that similarity between the critical physicochemical attributes resulted in comparable biological properties.

The observed physicochemical and functional similarity between products, as part of the totality-of-the-evidence scheme, will determine the extent of upcoming nonclinical and clinical studies, considering that it diminishes the uncertainty of exhibiting different pharmacological profiles.

Conflict of Interests

Carlos A. López-Morales, Mariana P. Miranda-Hernández, L. Carmina Juárez-Bayardo, Nancy D. Ramírez-Ibáñez, Alexis J. Romero-Díaz, Nelly Piña-Lara, Néstor O. Pérez, Luis F. Flores-Ortiz, and Emilio Medina-Rivero are employees of Probiomed S.A. de C.V., which is developing, manufacturing, and marketing biosimilar products. Víctor R. Campos-García declared no conflict of interests.

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