3 Chapter Three: Material and methods (clone creation, upstream and downstream process)

3.1 Model proteins and CHO cell cultures

Two recombinant produced, CHO-cell-derived model-glycoproteins, a less glycosylated monoclonal antibody (3% (m/m) glycosylated) and a heavily glycosylated enzyme-type protein (20% (m/m) glycosylated), were chosen for the examinations. These two glycoproteins should be representative for the majority of mammalian cell-derived products today.

The first model protein, an IgG1-type antibody, was named CHO-Mab. The second model protein was a recombinant glycoprotein, denoted as CHO-GP.

3.2 Clone creation

3.2.1 CHO cell stock preparation for CHO-Mab

CHO-Mab was produced by a CHO-K1-cell line. Clone selection was performed by using glutamin-synthetase (GS) positive mutants in glutamin-free medium [137, 138]. For inhibition of the CHO-cell-own GS-activity, MSX (methionin sulphoximin) was used, so that only clones with additional glutamin synthetase by transfection could survive.

For CHO-Mab-production, only one clone was created.

3.2.2 CHO cell stock preparation for CHO-GP

CHO-GP was produced by a CHO-dhfr(-)-cell line derived in 1978 from CHO-K1 (DXB11) [139]. Four different clones were created for the examination of the influence of process parameters on glycosylation. The clones emerged from different transfection experiments. They were named clone 1-4.

Clone 1 was transfected with a dhfr-CHO-GP-gene-construct by electroporation. To amplify this gene-construct, methotrexate was added to a

nutrition medium, containing guanidine, hypoxanthine and thymidine (GHT). Only transfected clones could survive under these conditions.

Clone 2 - 4 were created by recombinase mediated cassette exchange (RMCE). A functionalised cell line was created at first. A reporter construct containing a recombinant *human Leptin-IgG4-Fc* fusion protein (*hobFc* = *human-obesity-Fc*) as a typical test gene, flanked by three heterospecific *frt* sites was stably transfected into the *CHO-DXB11* cells. The construct contained a conventional selection marker (hyg) and the *dhfr* gene in order to allow gene amplification at a later stage. In addition, it contained an incomplete *neo* gene lacking a promoter. The cassette to be exchanged was flanked by heterospecific *frt* sites. A top producer was selected from 1500 analysed individual clones. The functionalised cell line was the starting point for a fast clone creation. To generate producer clones, the test gene was exchanged for the gene of interest (CHO-GP-gene) via recombination at the *frt* sites mediated by cotransfection of an flp-expressing plasmid. Recombination reconstituted a functional neo gene. This procedure ensured rapid and reliable insertion into an optimised locus (Figure 10).

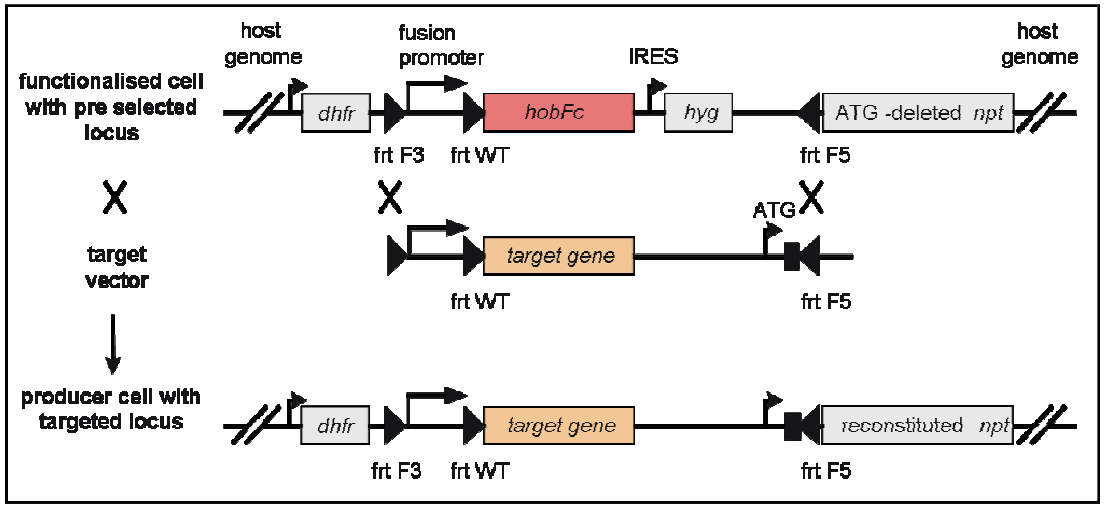

Figure 10: FLP (RMCE)-strategy for generation of CHO-GP-clones 2 - 4[7]

[7] *frt = FLP-recombinated target site, hyg = hygromycin gene, dhfr = dehydro-folate-reductase gene, npt = neomycin phosphotransferase gene, hobFc = human-obesity-Fc-fusion-protein gene, WT = wild type, IRES = internal ribosomal entry site*

Clone selection took place by neomycin addition. Neomycin resistent clones survived. Clones 2, 3 and 4 resulted from different clone picking procedures to get single-cell cell cultures.

3.3 CHO cell culture (upstream process)

3.3.1 Production of CHO-Mab

The CHO cells expressing the mab were expanded in CELLine (BD Biosciences) culture systems. After that, the cells were transferred into two different hollow fibre bioreactor systems, first an ACUSYST MAXIMIZER (ASM), BioVest Int. Minneapolis and second an ACUSYST X-CELL (ASX), BioVest Int. Minneapolis. The ASX (Figure 11) contained 6 hollow fibre cartridges. The ASM was a smaller version of the ASX-system which contained only 2 hollow fibre cartridges (Figure 12).

Figure 11: ACUSYST X-CELL (BioVest Int.)

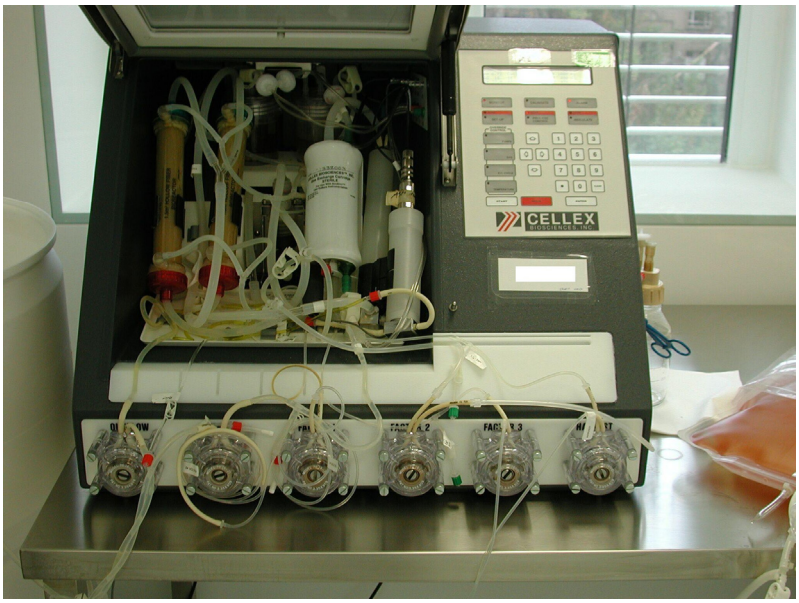

Figure 12: ACUSYST MAXIMIZER (BioVest Int.)

Hollow fibre bioreactors represent a special kind of continuous perfusion bioreactor systems, characterized by an independent medium and harvest stream. The system does not only allow cell but also product retention in the cell culture chamber. The separation is realized by using hollow fibre cartridges with an intra- and an extracapillary space. The system consists of one cycling pathway in which fresh medium is transported through the intracapillary space of the hollow fibres, an oxygenator (GEX = gas exchange) and an inoculation and harvest pathway that runs through the extracapillary space. The mass transfer through the membrane is diffusion controlled. The cells grow in the extracapillary space (Figure 13).

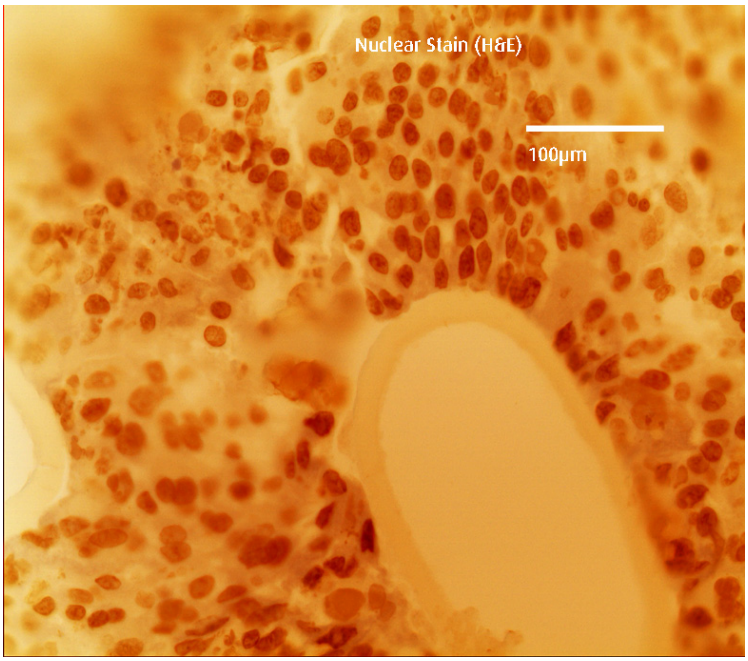

Figure 13: Picture of cells growing in high density in the extracapillary space of the hollow fibre cartridge

These older types of bioreactors have problems regarding oxygen supply, membrane fouling, homogeneous product harvest and removal of dead cells and debris. Therefore a specific generation of systems was developed. They have an expansion chamber for each pathway, on each chamber the system can selectively add gas pressure (up to 100 mm Hg) and thus lead to a controlled transmembrane pressure that leads to a mass transfer much larger than that reached via diffusion. Another positive effect is the mixing that happens as this transmembrane pressure is reversed and the flow goes in the other direction. ASM- as well as ASX-bioreactors belonged to the newer generation of HFBRs with transmembrane flows set as high as six times the cell culture volume per hour (Figure 14).

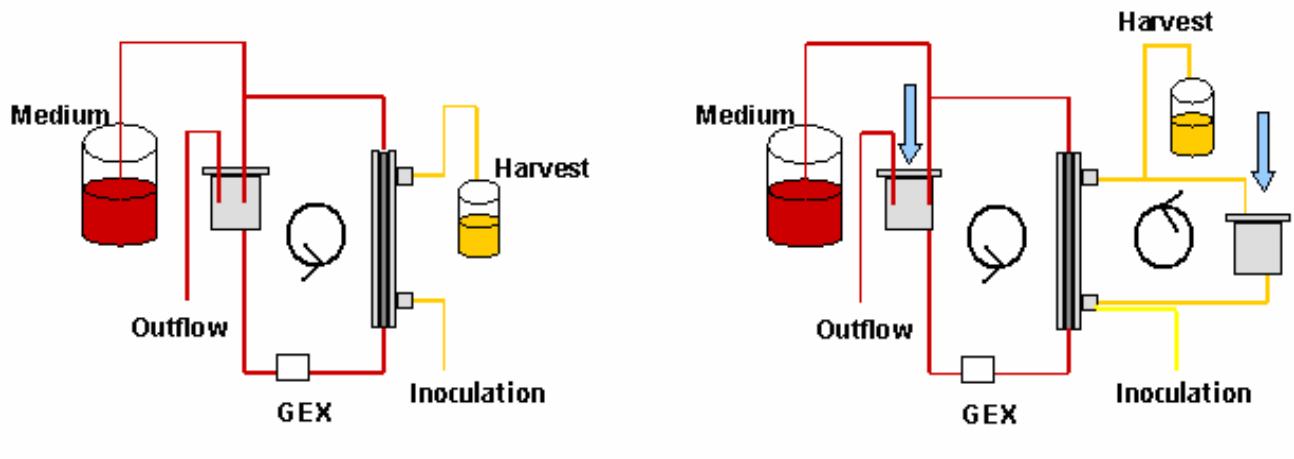

Figure 14: Schematic flowchart of older (left) and newer (right) hollow fibre bioreactor systems[8]

Harvest bulks were individually purified during these continuous production processes (Figure 15) and analyzed afterwards.

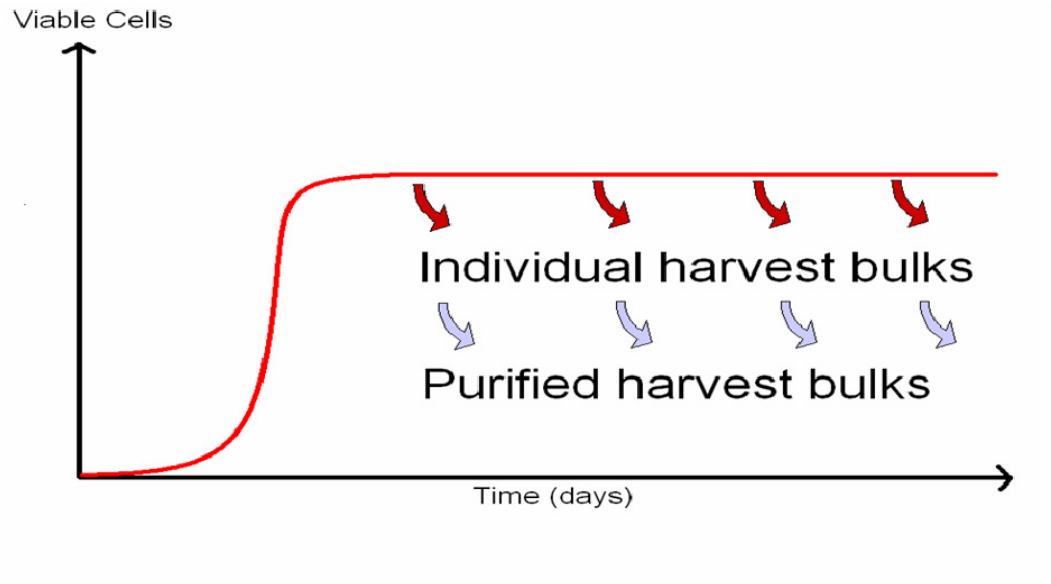

Figure 15: Purification scheme of a continuous production process

The first production of CHO-Mab was a development run and was performed in the smaller ASM-bioreactor. In the second production run an upscaling to the ASX-bioreactor was performed.

[8] *GEX = gas exchange, black circles = cycling pathways, blue arrows = pressure*

3.3.2 Production of CHO-GP

For the production of CHO-GP the following systems were used:

- SuperSpinner (Sartorius) (Figure 16) - The SuperSpinner is a lab scale cultivation system with 0.5 L or 1 L volume to process shear stress sensitive cells. Main feature is a membrane stirrer which allows controlled mixing and aeration and which avoids foam generation. Cells are only able to grow in low cell density.

Figure 16: SuperSpinner (Sartorius)

- ASM (ACUSYST MAXIMIZER - BioVest Int.) - Cells can be cultivated continuously over a long time at high cell densities.

Both production systems could be used to examine the influence of different cell densities on the glycosylation pattern of CHO-GP.

3.4 Protein chemistry (downstream process)

Depending on the total volumes of the individual harvest bulks, different downstream devices were used.

For small harvest bulks, the Äkta Purifier or Äkta Explorer system (Amersham Biosciences) were used for the chromatography steps (Figure 17). Both systems only differ in the fact that the Äkta Explorer keeps ready some more hardware- as well as software-features for the method development, whereas the Äkta Purifier is mainly arranged for standardized purification procedures.

Figure 17: Äkta Purifier system (Amersham Biosciences)

Larger harvest bulks were purified with the K-Prime-II-system (Millipore) (Figure 18).

Figure 18: K-Prime-II-system (Millipore)

3.4.1 CHO-Mab downstream process

The monoclonal antibody CHO-Mab was purified by a generic downstream process containing five steps. At first Protein A affinity chromatography was performed, virus inactivation by lowering the pH to 4 followed. After that, cation exchange chromatography was processed. The last chromatography step was a gel filtration. A membrane filtration under aseptic conditions was performed at the end to be able to store the product under sterile conditions (Figure 19).

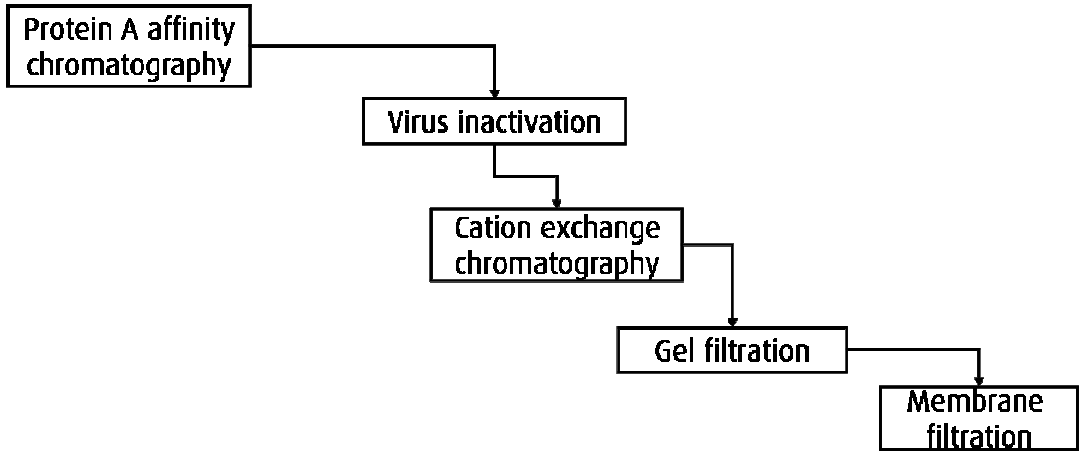

Figure 19: Scheme of the generic downstream process of CHO-Mab

3.4.2 CHO-GP downstream process

CHO-GP was purified with six purification steps including dia-/ultrafiltration, hydroxyapatite-chromatography, virus inactivation, anion exchange chromatography, size exclusion chromatography and sterile filtration (Figure 20).

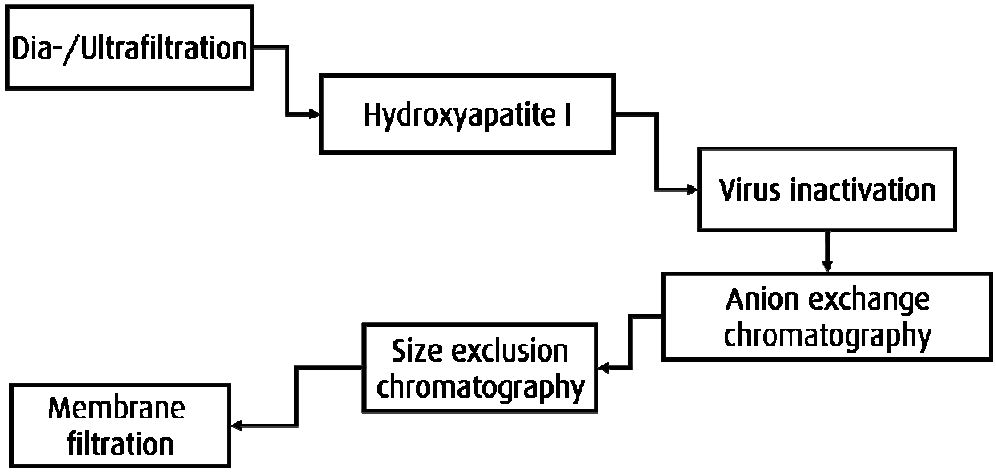

Figure 20: Scheme of the CHO-GP-downstream procedure